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Polyembryony in *Citrus*

Accumulation of Seed Storage Proteins in Seeds and in Embryos Cultured in Vitro

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Citrus **exhibits polyembryonic seed development, an apomictic process in which many maternally derived embryos arise from the nucellus surrounding the developing zygotic embryo.** ***Citrus*** **seed storage proteins were used as markers to compare embryogenesis in developing seeds and somatic embryogenesis in vitro. The salt-soluble, globulin protein fraction (designated citrin) was purified from** ***Citrus sinensis*** **cv Valencia seeds. Citrins separated into two subunits averaging 22 and 33 kD under denaturing sodium dodecyl sulfate-polyacrylamide gel electrophoresis. A cDNA clone was isolated representing a citrin gene expressed in seeds when the majority of embryos were at the early globular stage of embryo development. The predicted protein sequence was most related to the globulin seed storage proteins of pumpkin and cotton. Accumulation of 33-kD polypeptides was first detected in polyembryonic Valencia seeds when the majority of embryos were at the globular stage of development. Somatic** ***Citrus*** **embryos cultured in vivo were observed to initiate 33-kD polypeptide accumulation later in embryo development but accumulated these peptides at only 10 to 20% of the level observed in polyembryonic seeds. Therefore, factors within the seed environment must influence the higher quantitative levels of citrin accumulation in nucellar embryos developing in vivo, even though nucellar embryos, like somatic embryos, are not derived from fertilization events.**

In most angiosperms, a single embryo usually develops per seed. However, in some cultivars of *Citrus*, multiple embryos can be found in an individual seed that is described as polyembryonic. Polyembryonic seed formation in *Citrus* is one of many apomictic processes that have been described to occur in the ovules of angiosperm species (Koltunow, 1993). In polyembryonic seed formation, many nonzygotic, nucellar embryos are initiated directly from the maternal, nucellar cells surrounding the embryo sac containing a developing zygotic embryo. During embryo sac expansion, embryogenic nucellar cells obtain access to endosperm and develop into embryos alongside the zygotic embryo that may or may not complete development. Nucellar embryos give rise to seedlings that are of the same genotype as the female parent.

The mechanisms that stimulate the initiation of nucellar embryogenesis in the ovule are not known. Given that fertilization is not necessary to stimulate nucellar embryogenesis, the process has been likened to somatic embryogenesis in vitro. However, somatic embryogenesis occurs external to the developmental influences of the ovule. Furthermore, molecular comparisons between developing nucellar embryos and somatic *Citrus* embryos in vitro have not been made. For example, nothing is known about the spatial and temporal accumulation of seed proteins during multiple embryo formation in a polyembryonic *Citrus* seed or during *Citrus* somatic embryogenesis in vitro. Study of the expression of seed-storage protein genes might begin to address the question of the degree of similarity between the processes of nucellar embryogenesis in vivo and somatic *Citrus* embryogenesis in vitro.

Seed storage proteins and the genes that encode them have been isolated and studied in many plant species (Shotwell and Larkins, 1989; Morton et al., 1995). These proteins are classified into four groups according to their solubility properties. Albumins are water soluble; globulins are salt soluble; glutelins are soluble in acid, alkali, ionic detergent, and urea-containing solutions; and the prolamins are alcohol soluble. The globulins are usually the most prevalent class in legumes and oats, and the glutelins and prolamins are the major forms of cereal storage proteins (Shotwell and Larkins, 1989).

Seed storage protein accumulation is tissue specific. It occurs primarily in specialized storage cells in the embryonic axis, cotyledons, and the endosperm of developing seeds, but never in mature vegetative organs (Thomas, 1993). Seed storage protein genes are regulated temporally and spatially during seed development (Goldberg et al., 1989; Perez-Grau and Goldberg, 1989; Thomas, 1993; Morton et al., 1995). The seed-specific location of seed protein gene expression and the high abundance of seed protein mRNAs have made the genes encoding these proteins attractive for the study of the molecular mechanisms regulating tissue-specific gene expression. Seed storage protein genes are also useful marker genes for understanding seed developmental processes (Goldberg et al., 1989; Perez-Grau and Goldberg, 1989; Thomas, 1993).

Globulins have been reported to form the major seed storage protein fraction in the monoembryonic *Citrus limon* (Garcia-Agustin and Primo-Millo, 1989) but have not been studied intensively. The globulins most studied in terms of

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protein structure have been those found in leguminous species. They are oligomeric proteins in which each subunit of the protein consists of a heavy, acidic (A) chain and a light, basic (B) chain, which are processed from a precursor peptide possessing a hydrophobic amino-terminal leader peptide that is absent in the mature protein. The leader is thought to aid in directing the translocation of the nascent polypeptide into the RER and is then removed. A single disulfide bond is formed between the linked A- and B-chain regions of the resulting proglobulin. Trimers are assembled in the ER and these are transported to the vacuole via the Golgi. In the vacuole, proteolytic processing occurs to cleave the protein into acidic and basic polypeptides, which remain linked by the disulfide bond. These are then assembled into hexamers. As the proteins accumulate, the vacuole subdivides to form spherical protein bodies containing a protein matrix surrounded by a membrane. These specialized protein bodies are degraded during germination to provide carbon and nitrogen for the growing seedling (Shotwell and Larkins, 1989).

In this paper we describe the isolation of globulin seed storage proteins from *Citrus*, which we have named citrins, and investigate whether seed protein accumulation is comparable during multiple embryo formation in polyembryonic *Citrus* seeds and in somatic embryos in vitro. We found that citrins are first detected at the globular stage of *Citrus* embryo development in vivo and that citrins are also present at much lower levels during embryogenic regeneration of somatic *Citrus* embryos in vitro. The polyclonal citrin antibodies and the citrin cDNA clone isolated provide valuable tools for further molecular analysis of gene expression patterns during nucellar embryony in *Citrus* species.

MATERIALS AND METHODS

Plant Material

Tissue from several cultivated *Citrus* species was harvested from either field-grown trees or trees and cuttings maintained in a greenhouse. Depending on the tissue and the season, material was harvested and used immediately for protein extraction or stored at −70°C after rapid freezing in liquid nitrogen. Tissue for DNA and RNA extraction was collected, frozen rapidly with liquid nitrogen, and stored at −70°C until ready for use.

Tissue Extraction

Tissues were homogenized in 6 volumes of buffer (20 mM bis-tris-propane, 10 mM DTT, pH 7.0), filtered through Miracloth (Calbiochem), and centrifuged for 20 min at 40,000*g*. The supernatant was retained as the water-soluble fraction. The pellet was resuspended in the same buffer containing 1 M NaCl (2 mL/g of original tissue weight), vortexed, and centrifuged for 30 min at 40,000*g*. This second supernatant was retained as the globulin protein fraction. Protein was determined by the Coomassie dye-binding assay of Bradford (1976) using bovine gamma globulin as a standard. Samples were denatured and separated by SDS-PAGE using the Tricine gel system of Schägger and Von Jagow (1987). In some instances (see Figs. 4 and 5B), total protein was extracted by grinding tissues in the buffer containing 1 M NaCl, centrifuged as above, and the supernatant was retained.

Protein Purification

Embryos (5 g) extracted from seeds taken from mature Valencia fruit (Bain, 1958) were frozen in liquid nitrogen and ground to a fine powder in a coffee grinder. The frozen powder was added to 30 mL of grinding medium (20 mM bis-tris-propane, pH 7.0, 10 mM DTT, 1 mM PMSF, 1 mM *p*-aminobenzamidine, and 10 μM leupeptin) and blended with a Polytron (Kinematica, Luzern, Switzerland) homogenizer for 1 min on ice. The homogenate was centrifuged for 20 min at 40,000*g*, and the supernatant was retained as the water-soluble protein fraction. The pellet was resuspended in 20 mL of grinding medium and centrifuged again. The washed pellet was resuspended in 8 mL of grinding medium containing 1 M NaCl, vortexed, and then centrifuged for 30 min at 40,000*g*. The supernatant was retained and the pellet was extracted again with 4 mL of grinding medium containing 1 M NaCl and centrifuged. The two supernatants from the salt extraction were combined and filtered through a 0.22-μm filter, and this was retained as the globulin protein fraction.

The globulin fraction was desalted on a 170-mL column of Sephadex G-25 equilibrated with 20 mM Tris, pH 7.0, 5 mM DTT, and 1 M NaCl and then frozen. After thawing, the globulin fraction was centrifuged for 20 min at 40,000*g* and the supernatant was concentrated to 10 mL with an Amicon (Beverly, MA) YM30 ultrafiltration membrane before loading onto a 2.6 × 90 cm column of Sephacryl S300 equilibrated with the same buffer. Fractions eluted from this column were analyzed by SDS-PAGE and those containing the 33- and 22-kD globulin peptides were pooled and concentrated with an Amicon YM30 membrane.

The two globulin peptides were further purified by preparative electrophoresis and recovered separately by electroelution. The extract was fully denatured by heating it for 3 min at 100°C in buffer containing 225 mM Tris, pH 8.45, 10% (v/v) glycerol, 2% (w/v) SDS, 100 mM DTT, and then it was loaded onto SDS-PAGE gels using the Tricine gel system described by Schägger and Von Jagow (1987) with 10 mM reduced glutathione included in all buffers. After electrophoresis the protein bands were visualized in the gel by staining with KCl (Prussak et al., 1989) and excised with a scalpel. Proteins were electroeluted from the gel in a buffer containing 25 mM Tris, 192 mM Gly, 2 mM DTT, 0.1% SDS, pH 8.3, then diluted with 50 mM Tris, 150 mM NaCl, 2 mM DTT, pH 7.5, and concentrated with a Centricon 10 (Amicon) ultrafiltration cartridge.

Protein Gel Blot Analysis

Separate polyclonal antibodies were prepared to the purified 33- and 22-kD globulin peptides. Rabbits were given three successive injections, 100 μg of purified protein each, over 10 weeks. The rabbits were bled and the IgG fraction

was collected. *Citrus* samples were separated by SDS-PAGE using the Tricine gel system described by Schägger and Von Jagow (1987), and proteins were transferred onto a polyvinylidene difluoride membrane in 25 mM Tris, 192 mM Gly, 20% (v/v) methanol, pH 8.3, using a Bio-Rad Mini Transblot apparatus. To detect globulin peptides, the membranes were rinsed in water and washed in Tris-buffered saline (20 mM Tris, pH 7.5, 500 mM NaCl) for 15 min. The membranes were blocked for 45 min in Tris-buffered saline containing 3% gelatin and washed for 15 min (three changes of 5 min each) in Tris-buffered saline containing 0.05% Tween 20. Membranes were incubated with primary antibody, diluted 1:2000 in Tris-buffered saline with 0.05% Tween 20 containing 1% gelatin and 0.02% sodium azide for 1 h. The membranes were then washed for 15 min (three changes of 5 min each) in Tris-buffered saline containing 0.05% Tween 20. The secondary antibody, alkaline phosphatase conjugated with goat anti-rabbit IgG, was diluted 1:2000 in the same solution used for the primary antibody and incubated with the membranes for 1 h. Membranes were washed in Tris-buffered saline and then incubated with 0.65% nitroblue tetrazolium and 0.325% 5-bromo-4-chloro-3-indolyl phosphate (alkaline phosphatase substrates; Promega) in alkaline phosphatase buffer (0.1 M Tris, pH 9.5., 0.1 M NaCl, 5 mM $MgCl_2$) for color development. The reaction was stopped by rinsing in water.

Regeneration of *Citrus* Embryos from Embryogenic Callus

Embryogenic callus cultures of the polyembryonic tangor variety, Murcott, were initiated from anthesis-stage ovules, maintained on Suc-kinetin medium, and stimulated to initiate embryogenesis on Gal-sorbitol medium as described by Hidaka and Omura (1989). Samples were taken at different stages of embryogenesis for protein extraction.

Polysomal mRNA Isolation

Polysomal poly(A) mRNAs from *Citrus* vegetative and floral organ systems were isolated according to the procedures described by Cox and Goldberg (1988).

DNA Isolation

Citrus leaf nuclear DNAs were extracted from newly flushed unexpanded leaves that were no greater than 3 cm in length. Genomic DNA was purified as described by Jofuku and Goldberg (1988).

DNA and RNA Gel Blot Analysis

DNA and RNA gel blot studies were carried out according to previously published procedures (Jofuku and Goldberg, 1988).

Construction and Screening of Valencia Polyembryonic Seed cDNA Library

Polysomal poly(A) mRNA (5 μg) extracted from polyembryonic Valencia seeds removed from mature fruit was used to construct a cDNA library using the Lambda Zap XR directional vector cDNA synthesis kit (Stratagene) according to the instructions of the supplier. A total of 2.3×10^6 independent phage isolates were plated and amplified to form a permanent library and greater than 99% of these phage were recombinants. The rabbit IgG fraction made to the 33-kD citrin peptide was used to screen 2×10^5 plaque-forming units for *Citrus* seed storage protein cDNA clones. In total, 12 primary plaque plugs were picked, and these were rescreened twice. Finally, for each isolate, a purified plaque was isolated and the Lambda Zap phagemid containing the cDNA insert was excised according to the protocol provided by Stratagene.

Citrin cDNA Clone Characterization

All 12 clones isolated from the library were partially sequenced from both ends of the excised phagemid (a Bluescript KS[−] derivative) using T7 and T3 primers on an Applied Biosystems automated sequencing machine. The *Citrus* seed protein cDNA clone, D3, was fully sequenced in both directions from subclones in Bluescript SK(+) (Stratagene) using the appropriate T7 or T3 primer. D3 was not full length and lacked 5′ sequence information; therefore, the 5′-Amplifinder Race Kit (Clontech, Palo Alto, CA) was used to isolate the remaining 5′ sequence from poly(A) mRNA isolated from mature polyembryonic seeds. The instructions of the supplier were followed and the first-strand synthesis primer was 5′-GGGCGTTGAGGTTCTG-GATATTGCAC-3′. Amplification reactions were carried out using the anchor primer supplied in the kit and the oligonucleotide 5′-GCCGGAATTCTGGGTTTGGAAGCG-TTGCTGCCG-3′. Both the anchor primer and the amplification oligonucleotide contained *Eco*RI sites and the amplified fragment was cloned into the *Eco*RI site of Bluescript SK(+) for sequencing.

Comparison to DNA and Protein Data Banks

Data base searches and sequence alignments were carried out using the GCG software package of the Genetics Computer Group (University of Wisconsin, Madison).

Tissue Embedding and Sectioning

Embryos extracted from mature Valencia polyembryonic seeds were separated into three size classes: small, medium, and large. Cotyledon samples from each size class were cut into small pieces (2 mm^2) and fixed and embedded in resin as described by McFadden et al. (1988). Sections were cut at 2 μm using a Reichert-Jung (Vienna, Austria) microtome and stained with Coomassie blue as described by O'Brien and McCully (1981).

Immunolocalization in Frozen Tissue Sections

The largest embryo was removed from mature Valencia seeds, cut into small pieces (3 mm × 3 mm), and fixed in a solution of 4% paraformaldehyde, 0.25% glutaraldehyde, 0.1 M Suc in PBS at pH 7.0 for 12 h at 4°C. The tissue was then transferred to a similar solution but with a Suc concentration of 0.9 M, and the tissue was incubated for 5 h at 4°C. For long-term storage the tissue was kept in PBS containing 0.9 M Suc without fixative. The tissue was embedded in Tissue-Tek OCT compound (Miles, Inc., Elkhart, IN) frozen in liquid

nitrogen, and sliced into 10-μm sections using a cryostat. The sections were mounted onto glass microscope slides and air dried. The solutions used for immunolocalization were similar to those used in the protein gel blot procedure described above. Primary antibody to the 33-kD citrin peptide was applied at a dilution of 1:1000. The secondary antibody was fluoroscein isothiocyanate-conjugated goat anti-rabbit IgG (40 μg/mL [Sigma]). After the final wash in Tris-buffered saline, the sections were mounted in fluorescent mount medium (1.5% *n*-propyl gallate, 50% glycerol, 50% PBS) and viewed using a fluoroscein isothiocyanate filter set on a Zeiss Axioplan microscope. Control sections did not receive primary antibody but were instead incubated with rabbit preimmune serum at a dilution of 1:1000.

RESULTS

Purification and Distribution of Storage Proteins in Seeds of *Citrus* Cultivars

Citrus contains ex-albuminous seeds in which endosperm is present transiently during the development of the seed and seed storage protein reserves accumulate in the cotyledons of the mature embryo. The developmental events of polyembryonic seed formation have been described histologically in Valencia (Koltunow et al., 1995). Nucellar embryo initial cells are specified from nucellar cells all around the embryo sac, although only those in the nucellus of the micropylar one-third of the developing seed undergo embryogenesis. The process is initially relatively synchronous in developing seeds until both the zygote and some nucellar initial cells become early globular embryos. Embryo size and shape varies in later stages of seed development (Koltunow et al., 1995). In a seed extracted from a mature, edible Valencia fruit (Bain, 1958), one large nucellar embryo typically occupies the chalazal one-third of the seed and embryos of decreasing size and shape crowd toward the micropylar end of the polyembryonic seed.

Protein extracts were initially prepared from all of the embryos extracted from mature seeds of ripe Valencia fruits. These proteins were separated by SDS-PAGE under denaturing conditions. The water-soluble albumin fraction, which yielded 24 mg of protein per g fresh weight, contained a wide range of proteins, although there were two prominent bands of apparent molecular mass of 40 and 42 kD (Fig. 1, lane 1). The globulin fraction was extracted with 1 M NaCl and yielded 47 mg of protein per g fresh weight. With denaturing SDS-PAGE there were two major bands, of 33 and 22 kD apparent molecular mass, present in equal amounts in the globulin fraction (Fig. 1, lane 2). Lower protein loadings, such as those shown in Figure 2, revealed that each of these two bands comprised two or three bands of slightly differing mobility. When the globulin fraction extracts were electrophoresed without denaturation, the amounts of the 33- and 22-kD bands were decreased and an additional band at 57 kD was observed (data not shown). These observations are consistent with the major seed storage protein being a salt-soluble globulin, composed of 33- and 22-kD subunits, joined by disulfide bonds. We propose that the *Citrus* globulins be named citrins.

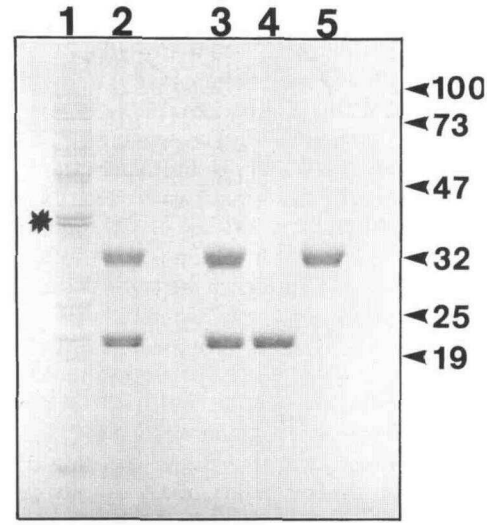

Figure 1. Purification of *Citrus* seed storage proteins from polyembryonic Valencia seeds. Coomassie blue-stained SDS-PAGE gel of seed storage proteins extracted from mature Valencia seeds as described in "Materials and Methods." Lane 1, Water-soluble albumin fraction (40 μg) from a small-scale purification. Two prominent bands at 40 and 42 kD are designated by the asterisk. Lane 2, Thirty micrograms of salt-soluble globulin protein fraction. Lane 3, Thirty micrograms of globulin fraction following purification on Sephacryl S300. Lane 4, Fifteen micrograms of 22-kD peptides purified by preparative electrophoresis. Lane 5, Fifteen micrograms of 33-kD peptides purified by preparative electrophoresis.

A larger-scale preparation of the globulin fraction was made from Valencia seeds and further purified by chromatography on Sephacryl S300. A single major peak of protein eluted that contained the 33- and 22-kD peptides (Fig. 1, lane 3). The peptides were separated by preparative SDS-PAGE and electroeluted to give pure preparations of each (Fig. 1, lanes 4 and 5).

The water-soluble albumin fraction and salt-soluble globulin protein fractions were extracted from seeds obtained from mature fruit of a range of polyembryonic cultivars and a monoembryonic cultivar. The ratio of globulin to albumin protein was determined (Table I). Neither the albumin nor the globulin content was constant and the globulin:albumin ratio varied from 0.7 to 2.8, showing that seed storage protein content varies among *Citrus* species. Globulins were generally the most abundant of the storage proteins in seeds of the various cultivars analyzed (Table I). The globulin protein fraction from seeds of a range of cultivars was separated by SDS-PAGE (Fig. 2). Two major subunits containing peptides averaging 33 and 22 kD in size were present in all of the cultivars. There were, however, differences in the number of peptide bands and their intensities, suggesting that, as in other species, there are several different genes encoding these proteins in *Citrus*.

Accumulation of Citrins during Embryogenesis in Vivo

Seeds extracted from mature Valencia fruit contained two to eight embryos, misshapen and of various sizes, with distinguishable cotyledons. Often, a mass of small, globu-

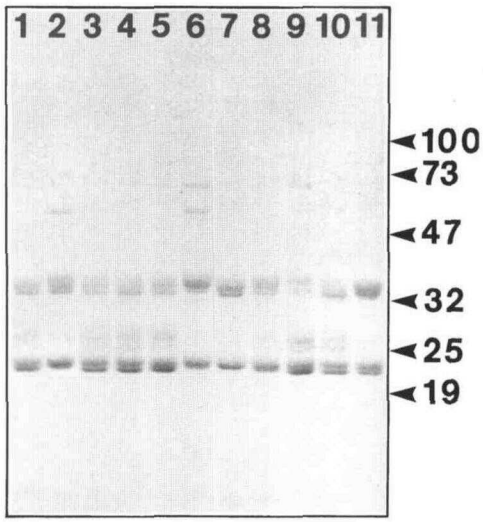

Figure 2. Citrins purified from a variety of *Citrus* cultivars. Coomassie blue-stained SDS-PAGE gel showing citrin peptide species extracted from mature seeds of a number of *Citrus* cultivars as described in "Materials and Methods." All lanes contain 20 μg of globulin fraction protein. M, Monoembryonic; P, polyembryonic. Lane 1, *C. sinensis* L. cv Valencia (P). Lane 2, *C. limon* cv Lisbon (P). Lane 3, *C. paradisi* cv Thompson (P). Lane 4, *C. paradisi* cv Marsh (P). Lane 5, *C. sinensis* × *C. reticulata* cv Murcott (P). Lane 6, *C. aurantifolia* cv West Indian (P). Lane 7, *Fortunella hindsii* (P). Lane 8, *Fortunella margarita* (P). Lane 9, *C. reticulata* cv Kara (P). Lane 10, *P. trifoliata* × *C. sinensis* cv Carrizo (P). Lane 11, *C. reticulata* × *C. sinensis* cv Ellendale (M).

lar-shaped embryos was also present at the micropylar end of the seed (Koltunow et al., 1995). Samples of Valencia embryos were sectioned to determine if the different-sized embryos represented different developmental stages or if they were mature embryos restricted in size because of physical restrictions on growth. The development of seed protein bodies was used as the morphological marker to aid assessment of the developmental age of the embryo.

Embryos were extracted from mature Valencia seeds and grouped randomly into three size classes: small (less than 3 mm in length), medium (3–6 mm in length), and large (greater than 6 mm in length). Representative sections from these size classes are shown in Figure 3. Staining of sections with Coomassie blue showed that protein bodies were not easily detectable in the sections of cotyledons of the small embryo class at the light microscopy level (Fig. 3A). Protein bodies were observable in the medium embryo class (Fig. 3B), and highly prevalent in the large embryo class (Fig. 3C). Therefore, the micrographs indicate that the different-size embryos are at different developmental stages with respect to protein body formation.

To monitor seed protein accumulation during polyembryonic seed development, polyclonal antibodies were raised to the purified 33- and 22-kD peptides. The antibody to the 33-kD peptide allowed detection of 2 to 5 ng of 33-kD peptide in protein dot blots. No signal was observed with preimmune serum and the antibody showed minimal cross-reaction with the 22-kD peptide when dilutions of purified 22-kD protein peptide (Fig. 1) were used in preliminary experiments (data not shown). The ability of 33-kD antibody to detect seed proteins in situ was determined by immunolocalization. Figure 3, D and E, shows the results of immunolocalization experiments in sections of the large-embryo class dissected from mature polyembryonic seeds. The antibody to the 33-kD peptide clearly detected citrin proteins localized in most of the protein bodies in the storage parenchyma cells of the large-embryo class (Fig. 3E). Cells containing storage protein bodies resembled those described for the monoembryonic variety, *C. limon*, in cell size, shape, and numbers of protein bodies per cell (Garcia-Agustin and Primo-Millo, 1988).

The amount of 33-kD polypeptide species in small-, medium-, and large-embryo classes extracted from seeds obtained from mature fruit was determined by protein gel blots using the antibody to the 33-kD peptide species. Total protein was extracted from each embryo class. Figure 4A, lane 1, shows that the antibody to the 33-kD peptide detected low levels of 33-kD species in the small-embryo class (globular to 3 mm in length) and that greater amounts of 33-kD peptide were present in the medium-embryo class (Fig. 4A, lane 2), where the embryos ranged from 3 to 6 mm in length. However, the medium (Fig. 4A, lane 2) and large (Fig. 4A, lane 3) embryos had approximately the same proportion of 33-kD protein present in the 1-μg sample of total protein loaded. Although there is more 33-kD polypeptide per embryo in the larger embryos, the abundance of the 33-kD polypeptide relative to other proteins appears to be similar in the two largest-size classes.

The identity of the species at around 26 kD that cross-reacts with the antibody was not determined. The fact that citrin could be detected in protein extracts of the small-embryo class, even though protein bodies were not clearly discernible at the light microscopy level, reflects the sensitivity of the protein blot procedure. Taken together, these observations show that the different-sized embryos found in polyembryonic Valencia seeds extracted from mature fruit are at different developmental stages and that only a small proportion of the embryos are mature.

The antibody to the 33-kD subunit was used to detect citrins in whole seeds extracted from immature developing Valencia fruits to determine when citrin protein could first be detected in Valencia seed development. In Coomassie blue-stained denaturing protein gels, bands migrating at 22 and 33 kD were first detected above a background smear of bands in seed proteins extracted from 40-mm-diameter fruit (data not shown). However, Figure 4B shows that the 33-kD peptide species was detected by protein gel blots in whole Valencia seeds extracted from fruits with a diameter of 35 mm (Fig. 4B, lane 4). Such Valencia seeds have increased in size 5-fold from the ovule size observed at anthesis, endosperm development has gotten underway, and most of the embryos within the micropylar one-third of the polyembryonic seed have reached an early globular stage of development (Koltunow et al., 1995). The 33-kD peptide band could not be detected using the loading conditions described in seeds extracted from fruits with a diameter of 15 mm (Fig. 4B, lane 3) where embryos were at a preglobular stage.

Table I. *Globulin and albumin content of monoembryonic and polyembryonic seeds of various Citrus cultivars*

Sample[a]	Seed Type[b]	Albumins	Globulins	Ratio (globulin:albumin)
		mg/g fresh weight	*mg/g fresh weight*	
Valencia orange	P	23	47	2
Lisbon lemon	P	16	45	2.8
Thompson grapefruit	P	21	38	1.8
Marsh grapefruit	P	22	49	2.2
Murcott tangor	P	28	54	1.9
West Indian lime	P	33	80	2.4
Fortunella hindisii	P	18	21	1.2
Kara mandarin	P	31	21	0.7
Carrizo citrange	P	26	40	1.5
Ellendale mandarin	M	41	30	0.7
Fortunella margarita	P	13	10	0.8

[a] Species names for samples are given in the legend to Figure 2. [b] M represents a monoembryonic seed and P represents a polyembryonic seed.

The level of 33-kD protein subunit in whole seeds extracted from immature, 33-mm fruit (Fig. 4B, lane 4), where embryos are at the early globular stage, was similar to that found in the small-embryo class (globular to less than 3 mm in length) extracted from polyembryonic seeds of mature fruit, 65 mm in diameter (Fig. 4A, lane 1). Furthermore, whole seeds extracted from 40-mm fruit containing predominantly globular-stage embryos had 33-kD polypeptides in amounts comparable to those observed in similar loadings of protein extracted from medium-sized embryos (3–6 mm in length) from mature seeds. Excluding the possibility of staging differences during seed collection, it is likely that the 33-kD protein accumulates in the globular embryos. However, accumulation may also occur in other parts of the seed structure (e.g. endosperm) to account for such high levels of protein.

The 33-kD peptide was not detectable in fruit tissues or other *Citrus* plant organs when these were extracted and examined by protein gel blots (Fig. 5A). There appeared to be cross-reaction of the antibody with a protein species of approximately 57 kD in both flowers and roots (Fig. 5A). This 57-kD band may represent the uncleaved citrin protein precursor, since it is unlikely that the antibody would recognize an unrelated protein of exactly the same size as the precursor. However, seed protein accumulation has not been observed in nonseed organs of other species (Morton et al., 1995). The protein at 57 kD in flowers and roots requires further investigation because if it is the uncleaved citrin precursor, it suggests that processing of the precursor may be a tissue-specific event in *Citrus*.

Citrin Accumulation during Somatic Embryogenesis in Vitro

Somatic *Citrus* embryos were regenerated from nucellar callus in vitro according to the method of Hidaka and Omura (1989). Valencia embryogenic callus proved difficult to initiate and maintain; therefore, nucellar callus derived from Murcott tangor ovules was placed onto medium containing Gal and sorbitol to stimulate embryo formation. Murcott callus was selected for these experiments because Murcott and Valencia are both polyembryonic seeds and contain similar amounts of citrin in mature seeds (Table I) with similar protein subunit bands in denaturing gels (Fig. 2). Tissue was assayed at different stages of somatic embryogenesis for the presence of the 33-kD peptide. Figure 5B shows that the 33-kD species were first detected when the somatic embryos were 2 to 4 mm in length and had attained a torpedo-like structure with expanded cotyledons. The 33-kD species were not detectable at the callus-to-embryo transition phase, where the surface of the callus was pale yellow and sparsely covered in very small globular embryos and an occasional, very small heart-stage embryo.

The quantitative level of 33-kD peptide detected during somatic embryogenesis (Fig. 5B) was significantly lower than that observed during seed development in vivo (Fig. 4). Comparison of protein loading in the experiments represented in Figures 4A (lane 2) and 5B (lane 2) indicated that the level of 33-kD peptide present during somatic embryogenesis was only 10 to 20% of that observed in embryos during seed development in vivo. The inability to detect the 33-kD peptide at the earliest stage of somatic embryogenesis investigated (Fig. 5B, lane 1) may relate to a deficiency of factors that stimulate quantitative accumulation of citrins at this stage in vitro.

Citrin cDNA Isolation and Characterization

Poly(A) mRNA was isolated from polyembryonic Valencia seeds extracted from mature fruit and used to construct a cDNA library in a Lambda Zap vector. Seeds from mature fruit were chosen because we had shown that such seeds contain a variety of developmental stages that would facilitate rapid collection of material for RNA extraction. The antibody prepared to the 33-kD peptide was used to screen the library. The number of positive plaques in the initial screening was very high and only 12 purified plaques were chosen for further analysis. Insert sizes ranged from 0.95 to 1.6 kb. Partial sequencing of the 12 purified clones and restriction mapping showed that these clones could be divided into two distinct groups with respect to insert sequence. Group-specific probes were

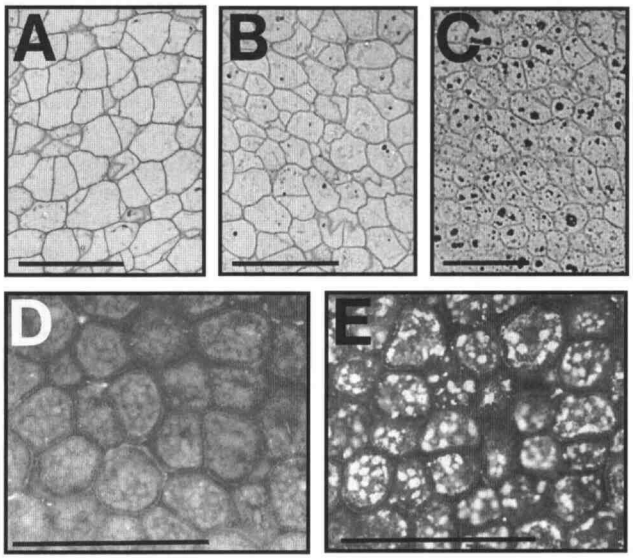

Figure 3. Histological observation of seed storage protein body accumulation in embryos extracted from mature Valencia seeds. A to C, Detection of protein bodies by light microscopy in Coomassie blue-stained, 2-μm sections of embryos embedded in LR Gold (London Resin Co., London, UK). Embryos extracted from mature Valencia seeds were grouped randomly into three size classes (small [globular and less than 3 mm in length], medium [3–6 mm in length], and large [greater than 6 mm in length]) prior to fixation and embedding. A, Section from a cotyledonous small embryo. Protein bodies were rare and difficult to discern in cotyledon cells at the level of light microscopy. B, Cotyledon section from a medium-sized embryo. A number of protein bodies are obvious per cotyledon cell. C, Cotyledon section from a large embryo. Numerous large protein bodies are evident in most cotyledon cells. D and E, Ten-micrometer-thick cryostat sections from a large cotyledonous embryo. Tissue was prepared for immunolocalization as described in "Materials and Methods." D shows an overexposed view of control cells treated only with preimmune serum and secondary antibody. E shows a view of cells treated with primary antibody to the 33-kD citrin peptide species and with fluoroscein isothiocyanate-conjugated secondary antibody as described in "Materials and Methods." Fluorescent signal was specifically localized to the seed storage protein bodies within the cotyledon cells. All bars represent 100 μm.

identified by cross-hybridization analysis of the different restricted clones (data not shown). The complexity of the citrin gene family was not further determined.

RNA gel blot analysis with selected, labeled restriction fragments showed that one clone, D3, represented a gene expressed at the globular stage of embryogenesis in *Citrus* when the average size of the seed was approximately 3.5 mm in length and 2 mm in width (Fig. 6). D3 transcripts were not detected in leaf or pistil tissue (Fig. 6) and were found at high levels in seeds extracted from mature fruit containing developing embryos of different stages (Fig. 6).

The D3 clone was sequenced and found to be less than full length. The missing 5′ sequences were obtained by a 5′ rapid amplification of cDNA ends procedure described in "Materials and Methods." The complete sequence of the citrin cDNA clone, with its deduced amino acid sequence, is shown in Figure 7. The cDNA sequence is 1727 bp in length and contains a 5′ noncoding region of 64 nucleotides and a 3′ noncoding region of 186 nucleotides containing an additional 18 adenosine residues, remnants of the poly(A) tail. The open reading frame contains 1458 bp encoding a protein coding region of 486 amino acids. The sequences surrounding the initiating Met ATG at position 65 are CAAAATGGC and these are similar to the consensus sequence surrounding the ATG initiation codon for plants, AACAATGGC (Lutcke et al., 1987).

The amino terminus of the predicted polypeptide contains a hydrophobic region with characteristics similar to a signal peptide. We have assigned the cleavage site between Ala^{22} and Glu^{23} (Fig. 7) using the rules for determining the cleavage sites between signal peptides and the remaining peptide that were described by Von Heijne (1983). This predicts a precursor with a molecular mass of approximately 61.7 kD, co-translationally processed to 58.9 kD. The second cleavage site to generate an A-chain polypeptide of 35.2 kD and a B-chain polypeptide of 23.7 kD probably occurs between Asn^{299} and Gly^{300} (Fig. 7), which begin a region of homology (underlined in Fig. 7) observed at the precursor cleavage site of several legumin proteins (Borroto and Dure, 1987). There are five Cys residues in the citrin A chain and one in the B chain; without further investigation, it is not possible to identify which of the Cys residues in the A chain is used to link the acidic and basic peptides. The sequence does not contain the tripeptide Asn-X-Ser/Thr that serves as a signal for amino-linked oligosaccharide attachment.

The deduced amino acid sequence for the citrin cDNA clone was compared to sequences in the GenPeptide data

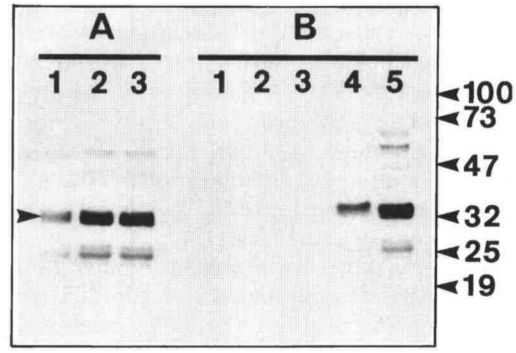

Figure 4. Detection of citrins in embryos from immature and mature Valencia seeds. The polyclonal antibody to the 33-kD citrin subunit was used to detect citrins in proteins extracted from embryos removed from mature seeds at the end of fruit development (A) and also in whole developing seeds removed from immature fruit (B). In all lanes, 1 μg of total protein was loaded and citrin was detected by protein gel blotting as described in "Materials and Methods." A, Citrins in small (lane 1), medium (lane 2), and large (lane 3) embryos extracted from mature Valencia seeds described in Figure 3. B, Citrin detection in developing seeds of Valencia. Seeds were extracted from immature fruit of different diameters. Lane 1, Seeds from 5- to 7-mm-diameter fruit. Lane 2, Seeds from 10-mm-diameter fruit. Lane 3, Seeds from 15-mm-diameter fruit. Lane 4, Seeds from 35-mm-diameter fruit (embryos at early globular stages). Lane 5, Seeds from 40-mm-diamater fruit (embryos at globular stage). A mature fruit has a diameter of approximately 65 mm.

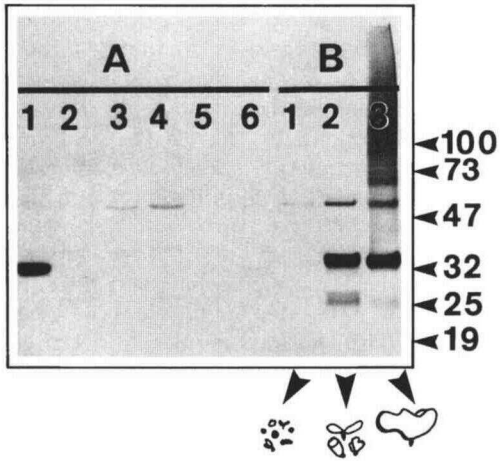

Figure 5. Detection of citrins in other *Citrus* tissues and in somatic embryos in vitro. The antibody to the 33-kD protein was used to detect citrin proteins. A, Analysis of citrin accumulation in nonseed tissues of Valencia. In lanes 2 to 6, 5 μg of protein from the globulin fraction of each tissue was loaded. Lane 1, 0.2 μg of purified 33-kD protein. Lane 2, Leaf. Lane 3, Root. Lane 4, Flowers at anthesis. Lane 5, Rind of fruit (40 mm diameter). Lane 6, Flesh of fruit (40 mm diameter). B, Citrin accumulation in Murcott somatic embryos in vitro. Somatic embryos were initiated as described in "Materials and Methods." Diagrams at the bottom of each lane indicate morphology of selected embryos and their relative sizes. In each lane, 5 μg of total protein was loaded. Lane 1, Early globular embryos and some small-heart stages, all less than 1 mm in size, collected with some callus tissue. Lane 2, Variable morphologies, expanded cotyledons, tubular embryos, flared hearts, all around 2 to 4 mm in size. Lane 3, Fully grown embryos with very large, expanded, flared cotyledons and ready for root induction.

base in GenBank using the FASTA program of Pearson and Lipman (1988). Citrin was most similar to a cotton β-globulin B storage protein and shared 60% identity over a 202-amino acid overlap primarily located in the 22-kD region of both proteins. The citrin protein sequence was also similar to a pumpkin globulin protein with 42% identity over almost the entire peptide sequence of both proteins (overlap of 479 amino acids).

The relatedness of the citrin protein to other globulins is depicted in the dendrogram shown in Figure 8. The dendrogram was compiled using the 35 peptide sequences found to be most similar to citrin in the GenPeptide data base using the FASTA search. Although these proteins perform a similar function in seeds and are related to a certain degree, several trends are obvious from the dendrogram (Fig. 8). Citrin is located in a group of proteins including pumpkin and cotton. These are related to oat and rice globulins and then to a group of globulins isolated from crucifers and *Prunus* species. Globulins isolated from legumes and sunflower form the least-related groups.

DISCUSSION

Citrus Seed Storage Proteins: Historical Perspective and Nomenclature

Purified proteins with the character of globulins were originally called pomelins when they were isolated from mono- and polyembryonic *Citrus* by Rotha and Saunders (1932) and Bass and Saunders (1933). Garcia-Agustin and Primo-Millo (1988, 1989, 1990) studied changes in the protein content of monoembryonic *Citrus* (*C. limon*) seeds during seed germination at the ultrastructural and biochemical levels. The specific cultivar utilized was not stated. In this study we confirmed that the structural appearance of mature cotyledon cells accumulating seed storage proteins in nucellar embryos of polyembryonic Valencia is similar to that seen in zygotic, monoembryonic embryos of *C. limon* (Garcia-Agustin and Primo-Millo, 1988). Our study also extends those of Garcia-Agustin and Primo-Millo (1988, 1989, 1990) in that the globulin protein fraction was purified from polyembryonic *Citrus* varieties during seed development, and one member of the expressed gene family was isolated and sequenced. We have elected to designate the globulin proteins isolated from *Citrus* as citrins to eliminate the confusion with apples that the original term pomelin may cause.

Protein Composition of Mono- and Polyembryonic *Citrus* Seeds

Garcia-Agustin and Primo-Millo (1988) showed that the main reserve proteins in the mature seed of monoembryonic *C. limon* were the globulins, which constitute 26% of the total protein, whereas albumins, glutelins, and prolamins contributed 8, 7, and 3% of the total protein, respectively. According to that study, the ratio of globulin to albumin was 3.2:1. In our study, the ratio of globulin to albumin ranged from 0.7 to 2.8. This may reflect differences in extraction procedures between the two studies and possibly cultivar differences.

Irrespective of whether the cultivar was mono- or polyembryonic, the globulin fraction could be separated into two distinct subunit sizes averaging 22 and 33 kD in denaturing gels. The differences in the minor band pattern of these subunits did not correlate with either the mono- or

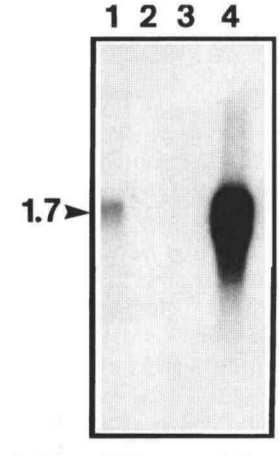

Figure 6. Analysis of citrin mRNA accumulation in various tissues of Valencia. In each track, 1 μg of polysomal poly(A^+) mRNA was loaded, blotted, and probed with a D3 cDNA clone probe and then washed at high stringency. Lane 1, mRNA from seeds extracted from 30-mm-diameter fruit. Lane 2, Leaf mRNA. Lane 3, mRNA from pistils extracted from flowers at anthesis. Lane 4, mRNA from seeds extracted from mature fruit. The size marker is in kb.

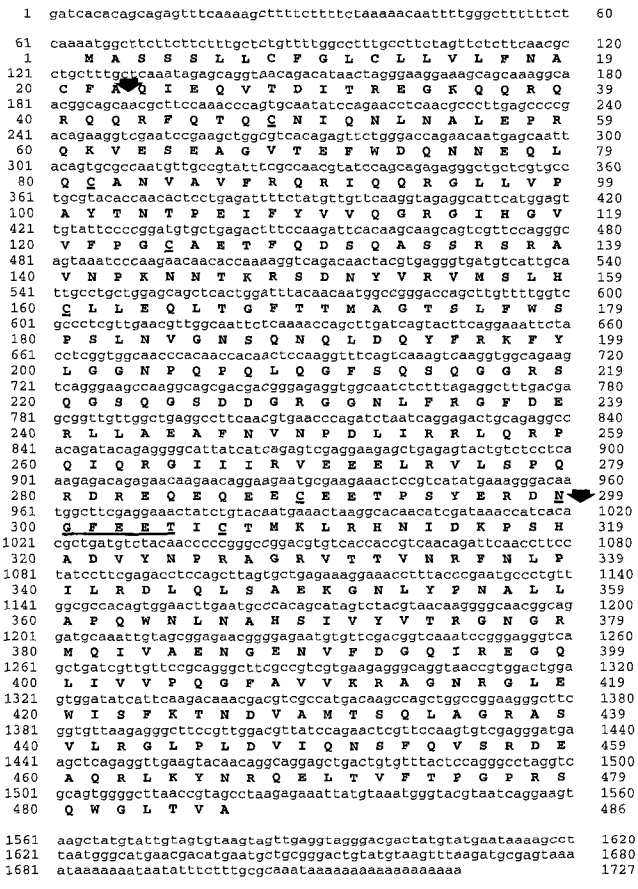

Figure 7. Nucleotide and predicted amino acid sequence of a full-length citrin cDNA clone. Arrows show predicted cleavage sites that split the primary protein into a 22-amino acid leader sequence, a 35.2-kD peptide, and 23.7-kD peptide. The underlined stretch of six amino acids are at the junction of the two peptides that have some conservation with those at the peptide cleavage site of other globulin storage protein genes. Cys residues are underlined. Apart from the single Cys residue in the 23.7-kD peptide, an additional five are indicated in the 35.2-kD peptide. It is not known which of these participates in disulfide bridge formation in the processed protein.

polyembryonic phenotype (Fig. 2). However, the variation in subunit bands suggested that the complexity of gene family members may vary between cultivars.

Citrin Accumulation during Seed Development in Valencia, a Polyembryonic Cultivar

In this study we showed that misshapen embryos of varying size in seeds extracted from mature fruit are arrested at different developmental stages. Thus, at fruit maturity the polyembryonic *Citrus* seed contains a large proportion of immature embryos that have no space for further growth. This initial observation suggested that nucellar embryos develop independently of each other within a polyembryonic seed.

Citrin proteins were first detected in small quantities by western blot in whole seeds extracted from 35-mm fruit in which embryo development was shown to be relatively synchronous and most embryos were at the globular stage (Koltunow et al., 1995). In general, seed storage protein accumulation in dicotyledonous species is undetectable during the first phase of embryo growth, when cells are dividing and the plant organ systems, axis, and cotyledons are not yet organized from the three primary tissues: protoderm, ground meristem, and procambium (Goldberg et al., 1989; Steeves and Sussex, 1989). Usually, seed storage proteins accumulate primarily in the cell-expansion phase of embryo development (Goldberg et al., 1989). Therefore, the accumulation of citrin protein in seeds at the globular stage of embryo development is early compared with some other species. It is possible that some of the seeds collected from 35-mm-diameter fruits in this study may have contained embryos at stages later than the globular stage of embryo development. However, accumulation of these proteins may also occur in the endosperm. Determination of the spatial patterns of protein accumulation during the early stages of *Citrus* seed development by immunolocalization is necessary to identify whether the proteins specifically accumulate in the embryo and/or other seed compartments.

It was not possible for us to separate the zygotic embryo from the nucellar embryos in this study. Nucellar embryos outnumber the zygotic embryo. The detection of citrins at

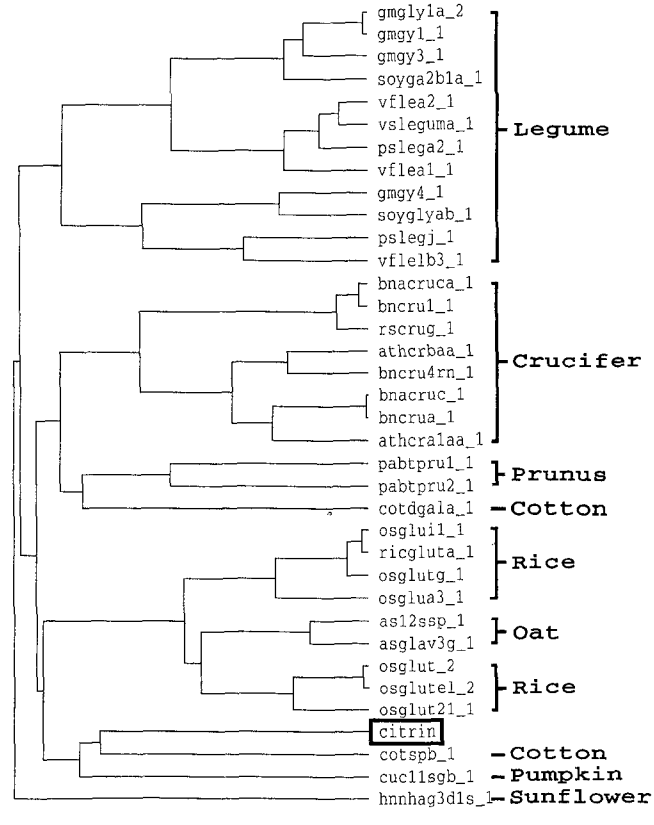

Figure 8. Dendrogram showing relatedness of citrin to other globulin seed storage proteins. The dendrogram was compiled using PILEUP in the Genetics Computer Group (University of Wisconsin, Madison) suite of programs. Thirty-five globulin sequences most similar to the predicted D3 amino acid sequence were selected from a FASTA search of the GenPeptide data base. For purposes of gene identification, in all cases except citrin, the nomenclature in the dendrogram represents the GenPeptide code.

the globular stage of embryo development may even be specific to nucellar embryos and may not reflect the true timing of protein accumulation in the zygotic embryo. Protein accumulation also needs to be observed in a monoembryonic seed containing a single, zygotic embryo to fully address this question of timing.

Citrin mRNA Accumulation

The citrin cDNA clone sequenced represents a gene that is not expressed in leaves or pistils and is detected in poly(A^+) mRNA isolated from whole polyembryonic Valencia seeds, where the embryos are at the early globular stage of development. Soon after this stage, 33-kD polypeptides were detected using the polyclonal citrin antibody. In other plant species, the patterns of seed protein accumulation have also been observed to correlate closely with mRNA accumulation (Morton et al., 1995).

Citrin genes are expressed early in Valencia polyembryonic seed development. Whether the D3 citrin message identified in this study specifically accumulates in the embryo or also in other compartments of the seed at the globular stage of development remains to be determined by in situ hybridization. However, the message must accumulate in the embryo during development because it was isolated from a cDNA library derived from mature polyembryonic seeds containing embryos of different stages of development but lacking in endosperm.

Citrin Accumulation in Somatic Embryos in Vitro

Nonzygotic *Citrus* embryos in culture accumulate citrin proteins to only 10 to 20% of that observed for nucellar embryos in vivo. Processed 33-kD polypeptides were first detected when the morphology of the in vitro embryos was at the torpedo stage, where the embryos exhibited expanded cotyledons. The timing of accumulation of citrins in somatic embryos is therefore delayed relative to that observed in polyembryonic seeds in vivo.

In celery, seed protein accumulation is not detected during somatic embryogenesis in vitro. However, seed proteins have also been observed at low levels in in vitro cultures of alfalfa (Stuart et al., 1985) and zygotic and somatic *Brassica* embryos in culture (Crouch, 1982). In those studies the levels of seed proteins accumulated in vitro were one-tenth of the level observed in vivo. Levels of seed protein accumulation in cultured embryos of *Brassica* and alfalfa (ex-albuminous seeds) were observed to be influenced by 2,4-D concentration (Stuart et al., 1985), ABA, and osmoticum (Crouch and Sussex, 1981; Crouch, 1982). In *Citrus*, a change in the carbon source from Suc to Gal and sorbitol is necessary to induce and maintain efficient embryogenesis from nucellar callus in vitro (Hidaka and Omura, 1989). Different sugar combinations affect the levels of *Citrus* somatic embryogenesis in vitro (Hidaka and Omura, 1989). It is also possible that the change in osmoticum may influence the level of seed protein accumulation in *Citrus* embryogenic culture in vitro.

We conclude from our in vitro embryo studies that the current embryogenic regeneration conditions for *Citrus* from nucellar callus are not sufficient to maintain the levels of seed protein accumulation that are observed in embryos developing in vivo. This may relate, in part, to differences in the temporal initiation of seed protein accumulation in vivo and in vitro.

How Similar Are Seed Protein Gene Expression Patterns in Vivo and in Vitro?

Perez-Grau and Goldberg (1989) showed that the soybean β-conglycinin and KTi1, KTi2, and KTi3 mRNAs accumulate in a wave-like pattern from the outer margins to the inner margins of the cotyledons and recede in the reverse direction during seed maturation. A similar wave-like pattern of seed storage protein mRNA accumulation has been observed in embryos during *Brassica* seed development by Fernandez et al. (1991), who have suggested that this progressive accumulation is determined by an internal clock that is set in response to factors that exist in a gradient centered on the apex of the developing embryo.

The wave-like pattern of KTi3 mRNA accumulation was also observed by Perez-Grau and Goldberg (1989) in somatic soybean embryos forming in vitro. They suggested that maternal structures are not necessary to establish the pattern of seed protein accumulation in soybean and that embryo-specific events set in motion the timing and the pattern of seed protein message accumulation.

Our study in *Citrus* shows that citrin mRNA accumulation correlates closely with protein accumulation in polyembryonic seeds. Accumulation of citrin mRNA begins at the early stages of seed development, when most embryos are at the globular stage. Each embryo in a polyembryonic seed develops independently of its neighbors, suggesting that seed protein accumulation in individual nucellar embryos may be linked to the timing of embryo-specific morphogenic events as described for KTi3 mRNA accumulation in soybean (Perez-Grau and Goldberg, 1989). However, high levels of citrin proteins are not accumulated in somatic *Citrus* embryos in vitro in the absence of seed structures. Therefore, factors or mechanisms in the *Citrus* seed function to stimulate these higher levels of citrin accumulation in nucellar embryos even though they are not derived from fertilization events.

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