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Storage protein accumulation patterns in somatic embryos of cotton (*Gossypium hirsutum* L.)

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Summary

The storage protein content of somatic embryos of Gossypium hirsutum L. cv. Coker 201 was determined using extinction level, antigen/antibody association detection methods. Mature storage protein was first detected in early globular-stage somatic embryos at a total concentration of 0.36% of the embryo protein mass. Tulip-stage and mature somatic embryos were comprised of 3.0% and 1.3% mature storage protein, respectively. Maximum storage protein synthesis was found to occur during early globular- and early heart-stages. During this period of development, significant levels of protein precursors were found also to accumulate. The pattern of storage protein synthesis, processing and accumulation paralleled the pattern that has been reported for the zygotic system, although somatic embryos accumulate storage protein at much earlier stages and to a lesser degree. The possibility of using complex biochemical pathways to monitor embryogenic systems in vitro is discussed.

Key Words: Cotton - Gossypium hirsutum L. - Somatic embryogenesis - storage proteins.

Introduction

For many species, a gross morphological comparison of somatic and zygotic embryos reveals striking similarities between the stages that can be identified during development. In 1969, Nitsch proposed that biochemical investigations of zygotic development in angiosperms could be modelled using in vitro somatic systems. However, since then, although many studies have been conducted to characterize and compare the morphological development of zygotic and somatic embryos, only a few attempts have been made to establish the extent of similarity between in vivo and in vitro biochemical processes within these systems.

In a previous report, we have characterized the morphological and histological development of cotton during in vitro somatic embryogenesis (Shoemaker et al. 1986). In addressing the relationship between the molecular events found within zygotic and somatic embryogenesis, we turned our attention to the major seed storage proteins that have been characterized in cotton. An intricate relationship exists between seed storage protein synthesis and accumulation and normal embryo development within several dicotyledonous species (Crouch and Sussex 1981; Crouch 1982; Murray 1984). In comparisons of storage protein accumulation in zygotic embryos and in primary microspore-derived somatic embryos of Brassica napus L., it has been found that secondary embryos begin to accumulate storage protein at an earlier stage than do zygotic embryos, but accumulate to much lower amounts (Crouch and Sussex 1981; Crouch 1982). In cotton, extensive studies have been conducted on the developmental regulation, synthesis, and accumulation of storage protein during zygotic embryo development (Dure et al. 1983, and ref. cited therein. Dure et al. 1981). The principle storage proteins that accumulate in the cotyledons of developing cottonseed are comprised of 48-kD and 52-kD protein sets (Dure and Chlan 1981). These proteins are initially sythesized as precursors and undergo extensive processing before being deposited in mature form (Dure and Galau 1981). A good measure of the fidelity and efficacy of an in vitro cotton embryogenesis system may be the recapitulation of a relatively complex processing scheme such as demonstrated with cotton seed storage proteins. This, coupled to the ease with which the relatively-abundant seed storage proteins can be manipulated at the molecular level plus the availability of a reproducible system for the induction of somatic embryogenesis from true somatic tissue (Shoemaker et al. 1986), suggested to us that seed storage proteins could comprise the ideal vehicle by which to compare molecular events during somatic and zygotic embryo development in cotton.

The objectives of this study were therefore: (a) to determine whether correctly-processed storage proteins accumulate within somatically-derived cotton embryos, and (b) to determine the level and pattern of storage protein accumulation.

Abbreviations: BSA = bovine serum albumen; NAA = 1-naphthyleneacetic acid; PBS = phosphate-buffered saline; SDS = sodium dodecyl sulfate; TCA = trichloroacetic acid; HRP = horseradish peroxidase

Materials and Methods

Callus culture: Seed of cotton cultivar Coker 201 was obtained from Gro-Agri Seeds, Lubbock, Texas. Seeds were germinated aseptically and callus was initiated from longitudinal half-sections of the hypocotyl. Callus initiation medium comprised the salts of MS medium (Murashige and Skoog 1962), B5 vitamins (Gamborg 1975), with 2 mg/L NAA, 1 mg/L kinetin, and 3% glucose, solidified with 0.3% Gel-rite, pH 5.8. After approximately 30 days on the initiation medium, embryogenic callus was induced by substituting 3% sucrose for glucose (Shoemaker et al. 1986).

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Embryos were allowed to develop without further media transfers.

Total protein extraction: Callus, somatic embryos, and zygotically-derived embryos were homogenized by grinding in a pre-chilled mortar for 5-10 min, using an equal amount (w/v) of an extraction buffer containing 2% SDS, 1% 2-mercaptoethanol, and 50 mM tris-HCL, pH 8.3 (Dure and Galau 1981). The crude homogenate was centrifuged at 15,000 g for 10 min. One-half volume of extraction buffer was added to the pellet, and the process of homogenization and centrifugation was repeated. The two supernatant fractions were pooled and were clarified by further centrifugation at 15,000 g for an additional 20 min. The protein content of the supernatants were determined by a modification of the Folin assay (Peterson 1977).

Protein purification: Total seed protein was subjected to preparative SDS gel electrophoresis according to the method of Laemmli (1970). Gels were stained for 30 sec in Coomassie Brilliant Blue R-250 and then were rinsed in distilled water. Bands corresponding in molecular mass to the mature storage proteins were cut from the gel and placed in dialysis tubing filled with 0.1 M tris-HCl, 0.05 M tricine, pH 8.6. The tubes were sealed and were taped to the tray of a conventional horizontal gel electrophoresis apparatus (B.R.L. Model H5). Sufficient tris/tricine buffer was added to submerge the dialysis bags, which were oriented orthogonally to the path of the current. The proteins were electroeluted from the polyacrylamide gel strips for a period of 15-18 hr at 30 V, with constant circulation of buffer. The proteins were precipitated by the addition of 10% (v/v) of 0.15% (w/v) deoxycholate and 10% (v/v) of a solution of 72% (w/v) TCA. After precipitation, the pellet was extracted in ether to remove traces of TCA. The pellet was then resuspended in extraction buffer and the proteins were further purified by another cycle of preparative SDS polyacrylamide gel electrophoresis. After the second ether extraction, the proteins were resuspended in extraction buffer and were precipitated by bringing the solution to 80% (v/v) in acetone. This precipitation step served to remove the final traces of the Coomassie dye that had been associated with the protein.

Preparation of antisera and Western blotting

Approximately 0.5 mg of purified protein in tris-buffered saline was injected subcutaneously into rabbits at two-week intervals. Animals were bled at two-week intervals, beginning one month after the initiation of the series of injections. The gamma globulin fraction of the serum was isolated by ammonium sulfate precipitation (Garvy et al. 1977). After extensive dialysis against tris-buffered saline, the antisera were titered, using a simple dot-blot procedure which involved the hybridization and detection protocol outlined below.

Total embryo or callus protein, separated by SDS-polyacrylamide gel electrophoresis, was electrophoretically transferred to nitrocellulose. Ligand-protein associations were detected according to the procedures of Towbin et al. (1979), with minor modifications: incubation buffers with primary antisera and enzyme-conjugated antisera were adjusted to contain 1% BSA (Fraction V, Sigma Chemical Company) and 0.1% Triton X-100 in place of gelatin and NP40, respectively. Washing buffer consisted of PBS, 0.1% Triton X-100, 0.5% SDS, and 0.1% BSA, pH 7.8. HRP-protein ligand associations were detected as recommended by the manufacturer (Bio-Rad).

Scanning electron microscopy: Initial fixation of the tissues was achieved by overnight incubation at 20 C in 0.1 M phosphate buffer (pH 7.2) containing 2% (v/v) glutaraldehyde. After rinsing briefly in 0.1 M phosphate buffer, the tissues were post-fixed for 60 min in 0.1 M phosphate buffer containing 1% (w/v) osmium tetroxide. Dehydration of the fixed samples was carried out by immersion in ethanol solutions, graded from 0% to 100%, before critical point drying with CO_2. The specimen were sputter-coated with 200 A-thick layer of gold and were examined using a Cambridge S4-10 Stereoscan operated at 20 kV.

Results

Scanning electron microscopy reveals the patterns of development of embryos emerging from callus derived from somatic tissues (Figure 1). The earliest recognizable stage (termed globular) consists of a series of smooth-surfaced, essentially isodiametric, tissue masses emerging from irregularly-organized, extremely friable callus tissue (Figure 1A). The somatic embryos during subsequent enlargement reveal a well-defined suspensor and an apical indentation (Figure 1B), this stage being termed heart-stage. The apical indentation represents the initiation of cotyledon development, which in the next stage (termed tulip-stage) results in the emergence of well-defined, green cotyledons (Figure 1C). Maturation of the somatic embryos involves elongation of the embryonic axis without further cotyledonary growth, to the point that the entire embryo can no longer be encompassed within the viewing field of the SEM (Figure 1D). Finally, root elongation is observed with emergence of true leaves.

During this process of somatic embryogenesis, we were able to demonstrate the accumulation of cotton seed storage proteins. We were routinely able to detect mature storage protein in early-globular stage embryos. We occasionally detected low levels of mature storage protein in embryonic callus. This could be the result of actual storage protein synthesis and accumulation in embryonic callus, or it could be the result of the presence of globular embryos hidden within the callus mass and not detected during tissue preparation.

Quantitative estimates were made using serial dilutions of total protein samples (unknown) and purified storage proteins (known). By comparing the amount of protein at which a hybridization signal was no longer detectable, we were able to estimate the percentage of total protein comprising mature storage protein. Using this procedure we estimated that 29.8% of cotton seed total protein is comprised of storage protein in mature form. This corresponds closely to a previous estimate of 27% (Dure and Chlan 1981), which confirms the accuracy of this method of quantitation.

Table 1. Characterization of somatic embryos of Gossypium hirsutum.

Embryo stage	Embryo Dimensions Mass (mg)	Length (mm)	Width (mm)	Mature storage protein amounts
Globular	1.61	0.95±0.11	0.69±0.06	0.36
Heart	4.45	1.46±0.11	1.03±0.09	2.40
Tulip	9.13	1.96±0.11	1.10±0.64	3.00
Mature	24.66	2.77±0.26	2.05±0.20	1.30

Means ± S.E. for n = 10.

Fresh weight of early globular-stage embryos comprised approximately 0.36% mature storage protein; heart-stage, 2.4% mature storage protein; tulip-stage embryos, 3.0% mature storage protein, and; mature somatic embryos, 1.3% mature storage protein (Table 1). Bands corresponding to the estimated molecular weights of storage protein precursors also were evident on Western blots containing total protein of vigorously growing embryos (Fig. 2). Because antisera was raised only against the mature form of the storage protein we cannot assume that hybridization to the pre-processed forms is equivalent to the processed mature proteins. Thus, no estimate of the quantity of precursors was made.

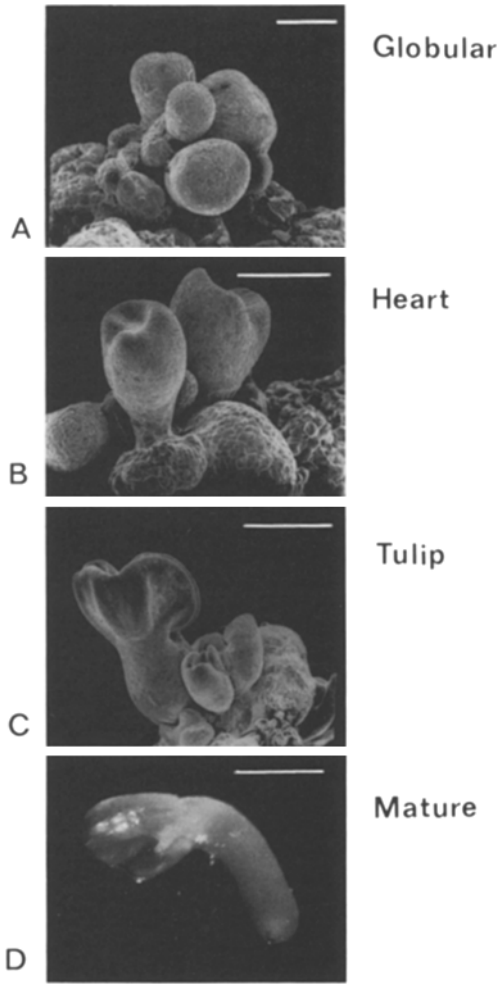

Fig. 1A-D. Stages of G. hirsutum L. somatic embryos. A-C are scanning electron photomicrographs and D is a light photomicrograph.
Bar = 1mm.

On the basis of intensity of hybridization (Fig. 2), the 48 kD protein set accumulates more rapidly than the 52 kD protein set, as has been previously established during zygotic embryogeny (Dure and Chlan 1981). During the early globular-stages an apparent precursor of about 60 kD has begun to accumulate and is present in greater concentration than either of the mature proteins (Fig. 2). Shortly afterwards, during late globular-stage/early heart-stage, the amount of mature protein has increased greatly, and apparent precursors of 67-70 kD have begun to accumulate (Fig. 2). By tulip-stage, the 60 kD precursor has virtually disappeared, leaving only mature proteins and traces of a precursor of about 70 kD.

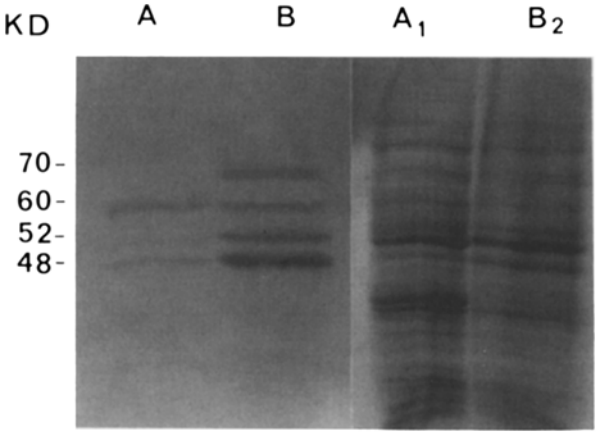

Fig. 2A-B. Left panel: A,B = Western blots of total protein (130 ug) of early globular- and early heart stage somatic embryos, respectively, challenged with antibodies raised against mature storage protein of cotton seed. Right panel: A,B = Coomassie stained SDS-PAGE gel containing total protein (130 ug) of early globular- and early heart-stage somatic embryos. Approximate molecular masses of the mature proteins and the storage protein precursors are shown in kilodaltons.

Discussion

The morphological patterns of embryogeny observed in cultured somatic tissues of cotton in many ways parallel that observed during seed development. There are, however, some important differences. During seed development in dicotyledonous plants, the endosperm acts as a source of nutrients, being gradually digested as development proceeds. In many systems, somatic embryos accumulate nutrients from the medium and therefore have no direct equivalent of the endosperm. We find that the earliest indications of somatic embryogenesis within the cotton system occur as organized zones within friable callus (Shoemaker et al. 1986). As development proceeds, the embryogenic callus increases in friability, with an accompanying disintegration strongly reminiscent of that occurring within the endosperm during zygotic embrogeny.

In the cotton system, obvious morphological differences can be seen during cotyledon development. During zygotic embrogeny, the cotyledons adapt a coil form, in the process elongating to many times the length of the embryonic axis (Pundir 1972). In somatic embrogeny, the cotyledons of the developing embryos are very small, i.e. we see a cotyledon length: axis length ratio of only 1.0 in late tulip-stage and about 0.5 in mature stage embryos. This may be a consequence of the premature initiation of embryo germination, through elongation of the axis, prior to completion of the developmental program that would give rise to more-completely formed cotyledons.

In examining the metabolic pathways that are operating during somatic and zygotic embrogeny, it is clear that there are broad overall similarities as well as specific differences. Storage protein accumulation patterns in somatic embryos closely follow the patterns seen in the zygotic system. However, accumulation in our somatic system occurs

somewhat earlier and to a much lesser amount. Dure et al (1983) detected storage protein mRNA in embryos as small as 5 mg, and though no concentrations were reported, low levels of storage protein accumulation was observed in 10 mg embryos. These embryo weights correspond to embryos in the globular and early heart-stage, respectively (Reeves 1935). Globular-stage somatic embryos show readily detectable levels of storage protein (0.36% fresh weight). Zygotic embryos of 50 mg, 70 mg, and 90 mg fresh weight comprised 16%, 21%, and 24% storage protein, respectively (Dure and Chlan 1981). These embryo weights correspond to cotyledon-stage embryos (Reeves 1935) and can be compared morphologically to our tulip-stage somatic embryos which comprise only 3% storage protein.

Our results establish that cotton somatic embryos carry out a complex pattern of synthesis, processing and accumulation of seed storage protein similar to that proposed to occur in the zygotic system (Dure and Galau 1981), though accumulation occurs to a lesser degree in somatic embryos. It should be emphasized that this occurs in somatic embryos produced from true somatic tissues, thus representing the metabolic manifestation of the initiation of novel developmental programs within previously undifferentiated tissues. However, the final level of accumulation of mature seed storage proteins are accumulated within cotyledons, the substantially decreased levels of storage protein within cotton somatic embryos could be simply explained on the basis of cotyledon size. A similar reduction in seed storage protein has been reported for Brassica napus L. (Crouch 1982). Although we expected to see maximum storage protein accumulation in mature embryos, we actually observed an approximate 57% decrease in percent storage protein between tulip-stage and mature embryos. This can probably be explained by the axis elongation of mature embryos, not accompanied by concurrent cotyledonary growth. Therefore even though the total amount of storage protein in mature embryos is greater than in tulip-stage embryos, the relative mass of the storage proteins as a function of the total embryo mass is decreased (Table 1).

Because storage protein accumulation is intricately correlated to normal embryo development (Crouch and Sussex 1981; Murray 1984) it is possible that abnormal embryo development could be reflected in abnormal patterns of storage protein processing and accumulation. Though not detailed in this study, we have observed that non-vigorous or abnormal embryos, although accumulating detectable levels of mature storage protein, do not exhibit the short period of rapid synthesis resulting in detectable levels of precursors. It should be possible to optimize regeneration protocols be fine-tuning easily identifiable developmentally regulated biochemical pathways. This could be done through early testing of embryos from any given culture condition. These types of assays could conceivably save the weeks of time usually necessary to optimize a protocol by nursing embryos through maturity, germination, and plantlet stages.

Acknowledgments

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