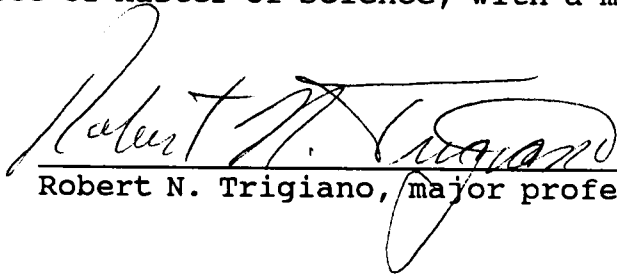
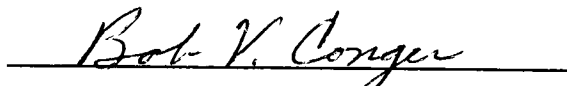
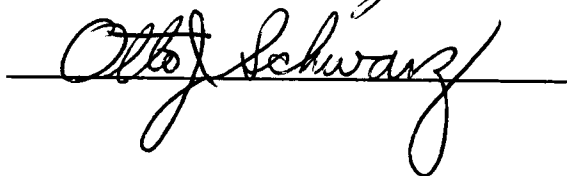


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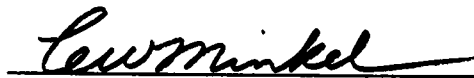
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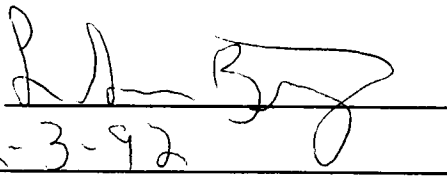

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Relationship of ovule developmental parameters to
somatic embryogenesis in Cercis canadensis

A Thesis

Presented for the

Master of Science

Degree

The University of Tennessee, Knoxville

L. Graham Buckley

December, 1992

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ABSTRACT

Ovules of Cercis canadensis L. (Eastern Redbud) were only competent to produce somatic embryos during a discrete period of their development. This interval was characterized by relating the somatic embryogenic potential of ovules with ovule development and changes in ovule protein profiles over time. Redbud pods were collected once or twice weekly from 82-159 days post anthesis (dpa). Ovules were removed from pods, cultured on a modified SH medium, and evaluated six weeks later for the development of somatic embryos. Additional ovules were analyzed for zygotic embryo length and developmental stage, and ovule fresh weight. Somatic embryogenesis occurred when ovules were in the torpedo and cotyledonary stages of development. Zygotic embryo length was associated with somatic embryogenesis during the exponential phase of elongation; ovule fresh weight was less effective in predicting embryogenesis.

In a second study, proteins were extracted from redbud ovules, and analyzed by SDS-PAGE gel electrophoresis. Changes in intensity of six protein bands were associated with changes in the somatic embryogenic potential of the ovules over time. Protein bands weighing 32 and 36 kDa decreased significantly in intensity when ovules became competent to produce somatic embryos. The four remaining bands increased in intensity from the middle to the end of the sampling period, and this was

associated with a decrease in the somatic embryogenic potential of the ovules.

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INTRODUCTION

Cercis canadensis L. (eastern redbud) is an leguminous tree indigenous to the eastern deciduous forests of the United States. It is five to ten meters in height; the trunk usually divides near the ground and the ascending branches form a spreading crown. It has simple, alternate leaves and perfect flowers, often produced in small clusters. Redbud is a popular ornamental that is grown individually and as groups along avenues.

Redbud is an obligate outcrosser, and the wild type is grown from seed. Cultivars are usually propagated by grafting and budding; cuttings are difficult to root. Axillary bud multiplication in tissue culture has been achieved, and recently, redbud has been propagated by somatic embryogenesis. This method of propagation is potentially more efficient than any production scheme now used. It utilizes zygotic embryos or ovules collected from immature seed pods as explants. The developmental stage of the explant critically influences the ability to induce embryogenesis, yet the relationship between developmental parameters and somatic embryogenesis is poorly understood. Protein profiles, anatomical studies and mRNA analysis have been used to characterize the period during which explants are embryogenically competent.

In order to better understand when redbud ovules were competent to produce somatic embryos, ovule development was studied over time. Development was characterized by zygotic

embryo length and developmental stage, ovule weight, and ovule protein profiles. These parameters were related to the embryogenic response of ovules in culture in order to define the period of embryogenic competency.

The results of this research are presented herein as two parts and each is formatted for publication in Annals of Botany. The first part explores the relationship between ovule anatomical development and somatic embryogenesis in redbud. Part two relates ovule protein profiles to somatic embryogenesis.

PART 1: Association of anatomical development of ovules
with somatic embryogenesis in Cercis canadensis

ABSTRACT

Zygotic embryos within ovules of Cercis canadensis L. (eastern redbud) were competent to produce somatic embryos only during a discrete period during development. This period was characterized by comparing the ability to produce somatic embryos with zygotic embryo development. Zygotic embryo ontogeny was studied during a single growing season, from samples collected once or twice weekly between 82 and 159 days post anthesis, using standard paraffin methods. Additional ovules were cultured on a modified SH medium and evaluated six weeks later for development of somatic embryos. The incidence of somatic embryogenesis was related to zygotic embryo developmental stage, length, and ovule fresh weight. Somatic embryogenesis occurred when zygotic embryos were in the cordate and cotyledonary stages of development. The length of zygotic embryos was associated with embryogenesis during the exponential phase of elongation and ovule fresh weight was related to somatic embryogenesis when ovules weighed between 11.5 and 29.5 mg.

INTRODUCTION

Cercis canadensis L. (eastern redbud) is a leguminous tree, 5 to 10 meters in height and indigenous to the eastern deciduous forests of the United States. Redbud is used extensively as an ornamental species and has traditionally been propagated from seeds. Conventional methods of vegetative propagation, such as rooted cuttings and grafting are difficult and expensive. Axillary bud multiplication of redbud has been achieved, and this method of propagation has been commercialized by several laboratories (Bennett, 1987; Geneve, personal communication). Axillary multiplication and other tissue culture techniques offer the potential for commercial propagation of superior cultivars at reasonable costs.

Recently, redbud has been propagated by somatic embryogenesis (Geneve and Kester, 1990; Trigiano et al., 1988). Although this procedure is currently limited to research, it is potentially more efficient than other production schemes now used. Somatic embryogenesis utilizes zygotic embryos or ovules harvested from immature seed pods as explants. The developmental stage of the explant critically influenced the ability to induce somatic embryogenesis in redbud (Trigiano et al., 1988) and in many other culture systems, including other legumes (Maheswaran and Williams, 1984; Hammat and Davey, 1987; Weaver and Trigiano, 1991) and other woody species (Merkle et al., 1987; Trigiano et al.,

1989). The period of embryogenic competency likely varies with climate and geographic conditions and has been variously characterized by anatomical studies (Trigiano et al., 1989; Eapen and George, 1990), protein profiles (Sotak et al., 1991; Roberts et al., 1989), and mRNA analyses (Sharp, 1980; Pechan et al., 1991).

Ovule development was studied in order to understand when redbud ovules were competent to produce somatic embryos. Objectives of this study were to define developmental parameters for ovules and zygotic embryos of C. canadensis and to use these parameters to characterize the time period during which ovules were competent to produce somatic embryos. Ovule fresh weight, and zygotic embryo length and developmental stage were compared with the embryogenic response of ovules in culture in order to define the period of embryogenic competency.

MATERIALS AND METHODS

Immature redbud fruits were collected from a tree on the University of Tennessee campus. Pods were harvested on a weekly basis from 82 to 159 dpa (days post anthesis) with a second midweek sampling during the estimated time of somatic embryogenic competency (Trigiano et al., 1988), 107 to 145 dpa. Anthesis, which was estimated to have occurred March 2, 1991, was considered to be the date when one-half the flower buds had opened and anthers were exposed.

Harvested pods were surface sterilized in 50% (v/v) commercial bleach (2.62% NaOCl) and 0.1% (w/v) Triton X-100 for five minutes with constant, vigorous agitation and then rinsed three times in sterile, distilled water. Ovules were removed from the pods; the integuments were scored longitudinally on one side with a scalpel, then placed with the cut surface in contact with the culture medium. The basal medium consisted of SH salts (Schenk and Hildebrandt, 1972) amended with 100 mg/L myo-inositol, 1 mg/L thiamine, 30 g/L sucrose, 8g/L Phytagar (Gibco) and 9 μ M 2,4-D. Ammonium sulfate was added to yield a final ammonium ion concentration of 7.6 mM. The pH of the medium was adjusted to 5.7 with KOH prior to the addition of the Phytagar and before sterilization. A minimum of 40 ovules were explanted per sampling date and the experimental unit consisted of two ovules per 60 mm petri dish. Dishes were sealed with Parafilm (American National Can) and incubated at 22C with a 16 hour

photoperiod under cool white fluorescent lights ($25 \mu\text{mol m}^{-2} \text{sec}^{-1}$). Six weeks after plating, the cultures from each sampling date were evaluated for the presence or absence of somatic embryos; some of the embryogenic cultures were then photographed. The percentage of dishes in which somatic embryos were formed for each sampling date and the number of somatic embryos per dish were determined. Twenty additional ovules were collected and weighed per sampling date, and the average fresh weight per ovule calculated.

Ovules and/or zygotic embryos from each sampling date and individual and clusters of somatic embryos that formed from explants were prepared for histological examination. Ovules were fixed initially in the pods due to their small size; later they were removed from the pods and fixed individually. The distal end of the ovules was excised to facilitate infiltration of the fixative, dehydration solutions and paraffin. All tissues were fixed at least 24 hours (Appendix 1) in a solution of formaldehyde, glacial acetic acid and ethanol (FAA). Samples were then dehydrated in a graded ethanol-tertiary butyl alcohol series (Appendix 1). Specimens remained in each alcohol solution for a minimum of 30 minutes. Samples were infiltrated with Paraplast Plus (Sherwood Medical Industries, St. Louis Mo.) by placing them in an oven at 60°C , adding paraffin flakes, and evaporating the alcohol overnight. Samples were embedded in 5 ml plastic microbeakers, the blocks trimmed and mounted onto paraffin filled molds. Specimens

were cut on a rotary microtome into 12 μm sections, mounted onto glass slides and stained in a modified Fleming's triple stain (Johansen, 1940) consisting of safranin, crystal violet and fast green. The slides then were coverslipped with permount and photographed on a light microscope.

Three to seven zygotic embryos from each sampling date and their suspensors, if present, were measured with an ocular micrometer and the average length computed. Embryo growth was assessed visually, then assigned to the preglobular, globular, cordate, torpedo, or cotyledonary stage of development. Sections of somatic embryos were analyzed for size and morphology, and compared with those of zygotic embryos at similar developmental stages.

RESULTS

Somatic embryos were not formed from ovules collected prior to 96 dpa; instead, only a light green crystalline callus proliferated from the immature integuments. Although only one (3%) of the experimental units (two ovules per dish) produced somatic embryos 96 dpa, the six embryos produced were the highest of any sample period (Table one). Fifty-three percent of dishes containing ovules cultured 107 dpa produced somatic embryos; this was the highest response of any sampling date. The embryogenic response oscillated between 27-33% over the next eight sampling periods, although it declined to 17% twice during this time. The last sample that produced somatic embryos was cultured at 139 dpa. The number of somatic embryos per embryogenic experimental unit ranged from 1.8-6.0 and most experimental units formed 2.5-3.5 somatic embryos.

Somatic embryos often developed singly or in small groups (Plate 1, Figures 1-3). Many appeared to follow normal developmental stages; however, some variation did occur, such as fasciated cotyledons. Embryos usually formed from epicotyls (Plate 1, Figure 1), and along the margins of cotyledons (Plate 1, Figures 2 and 3). Clusters of somatic embryos also developed asynchronously, often from the cotyledons (Plate 2, Figures 4 and 5).

Ovule weight at 82 dpa was not recorded; therefore, 5.0 mg per ovule at 89 dpa was the initial weight measured. The mean weight of ovules increased 44% during the next week, then

Table I. Characterization of zygotic embryo development and somatic embryogenesis from ovules of Cercis canadensis.

DPA ^a	Zygotic embryo stage ^b	Mean length of embryo proper (μm) ^c	Fresh weight of ovule (mg) ^d	Somatic embryogenesis (%) ^e	Mean No of somatic embryos ^f
82	PG	30 $\pm$ 5	-	0	0
89	GL	49 $\pm$ 3	5.0	0	0
96	CR	150 $\pm$ 22	7.2	3	6.0 ^g
103	CR	498 $\pm$ 176	8.5	7	2.5 $\pm$ 0.7
107	TO	875 $\pm$ 316	11.5	53	3.6 $\pm$ 3.3
111	TO	2160 $\pm$ 330	14.0	27	3.9 $\pm$ 3.1
115	CT	3135 $\pm$ 340	16.0	17	3.0 $\pm$ 3.5
118	CT	3080 $\pm$ 215	18.5	33	2.8 $\pm$ 1.6
122	CT	4035 $\pm$ 125	22.5	30	3.6 $\pm$ 5.9
125	CT	3320 $\pm$ 565	22.5	33	4.6 $\pm$ 3.7
129	CT	3680 $\pm$ 315	28.0	30	2.8 $\pm$ 1.9
132	CT	3960 $\pm$ 215	29.0	17	2.0 $\pm$ 1.2
136	CT	4830 $\pm$ 230	29.5	30	2.7 $\pm$ 1.7

Table I. continued

139	CT	4900 ± 185	36.0	13	1.8 ± 1.0
145	CT	4975 ± 195	41.0	0	0
152	CT	5030 ± 45	45.0	0	0
159	ME	-	23.0	0	0

^a DPA = Days postanthesis - Anthesis March 2, 1991

^b PG = preglobular; GL = globular; CR = cordate; TO = torpedo; CT = cotyledonary; ME = mature

^c Mean length of 3-7 embryo proper ± standard deviation

^d 20 ovules weighed and weight per ovule calculated

^e Experimental unit was 2 ovules/dish

^f Mean number of somatic embryos per embryogenic experimental unit ± standard deviation

^g Only 1 experimental unit responded

Plate 1, Figures 1-3. Somatic embryos of Cercis canadensis

Figure 1. A somatic embryo (SE) with fused cotyledons growing from the epicotyl (E) of a zygotic embryo.

Bar=1000 μm

Figure 2. Two somatic embryos (SE) growing from the margin of a zygotic embryo cotyledon (ZC). Bar=1000 μm

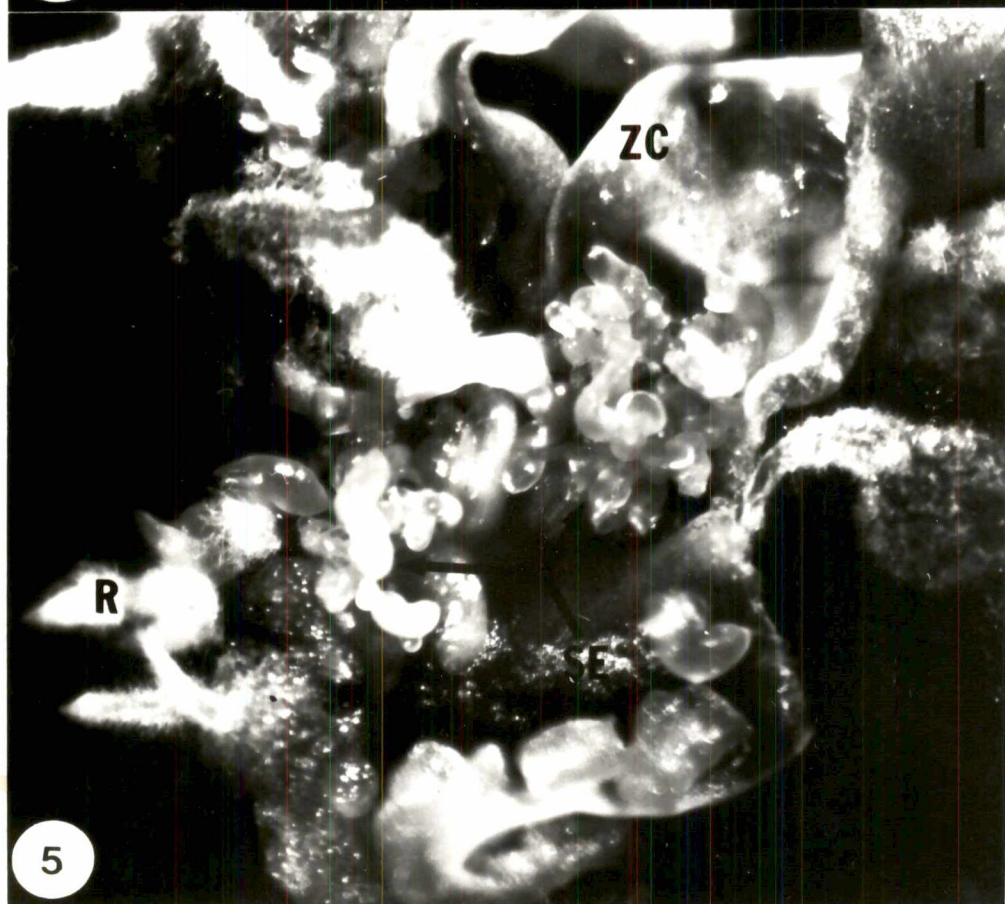
Figure 3. Somatic embryos with well developed cotyledons (SC) growing from the margin of a zygotic embryo cotyledon (ZC). Bar=1000 μm



Plate 2, Figures 4 and 5. Somatic embryo clusters of Cercis canadensis.

Figure 4. A small cluster of somatic embryos (SE) growing from a cotyledon (ZC) of a zygotic embryo. Note the asynchronous development of the somatic embryos. Bar=1000 μm

Figure 5. A large cluster of somatic embryos (SE) growing from a zygotic embryo cotyledon (ZC). Note the adventitious roots (R) also growing from the zygotic cotyledon. Bar=1000 μm



increased an additional 35% between samples collected 103 and 107 dpa. Although the relative increase in weight was greatest during early development, the largest absolute gain occurred between ovules collected 136 and 139 dpa, when mean ovule weight increased 6.5 g. Weight increased linearly over the sampling period, until 159 dpa when ovule fresh weight showed a dramatic decline, losing nearly 50% in one week (Figure 6).

Histological examination of developing ovules of C. canadensis revealed a cellular type of endosperm (Plate 3, Figure 7). Zygotic embryos, which were in the preglobular stage of development, were first detected in ovules collected 82 dpa. These preglobular embryos displayed a multiseriate suspensor 65 μm in length which was more than twice the length of the embryo (Plate 3, Figure 8). The outer cell layer of these embryos formed a single layer of presumptive protoderm in globular stage embryos, collected 89 dpa (Plate 3, Figure 9). Multiseriate suspenders, which had increased in length to an average of 74 μm , were distinctly visible and haustoria were present at the proximal end.

Zygotic embryos collected at 96 dpa had developed to the cordate stage (Plate 3, Figure 10) and displayed flattened apices and cotyledonary primordia. The mean length of the embryo more than tripled, whereas the suspensor had apparently decreased in size to a mean length of 55 μm . Embryos 103 dpa

Figure 6. Fresh weight of individual ovules of Cercis canadensis collected from 89-159 days post anthesis (anthesis occurred March 2, 1991). Points represent data collected for each date, whereas the curve is a best fit through these points.

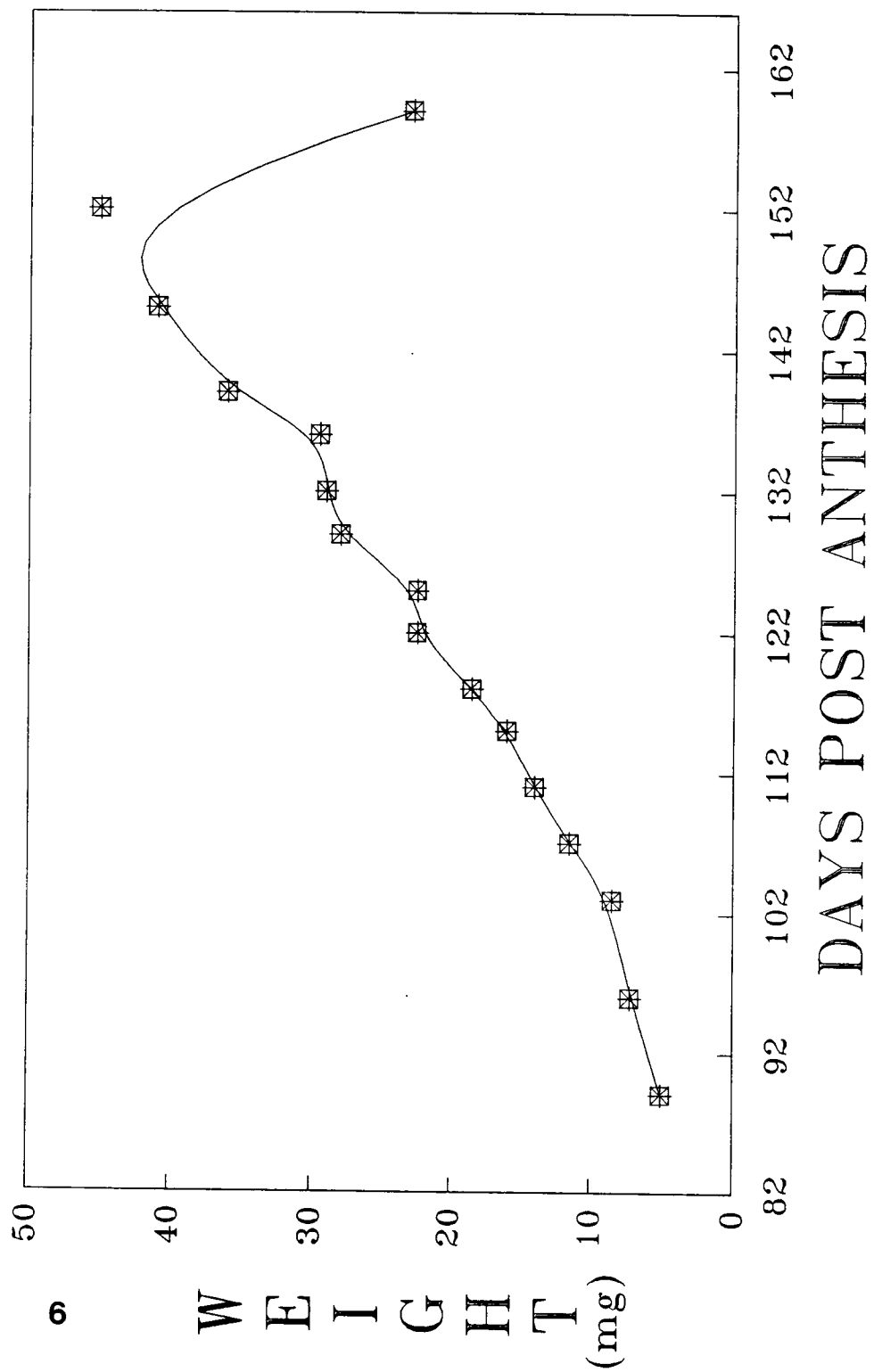


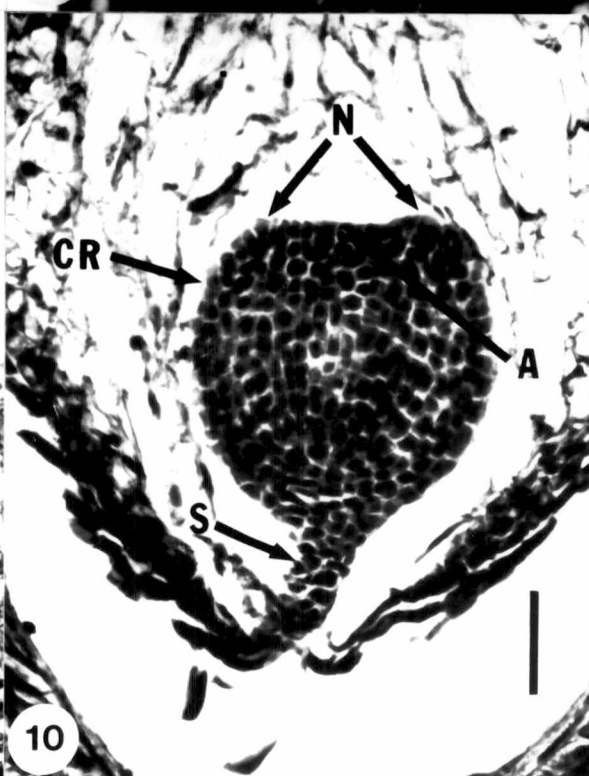
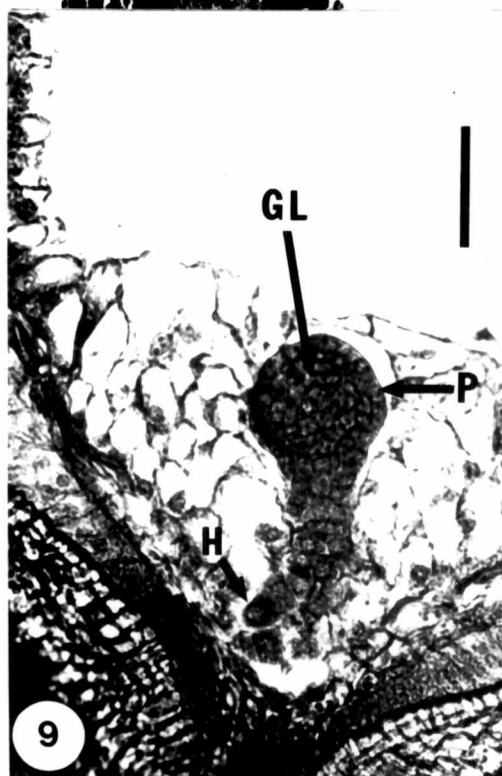
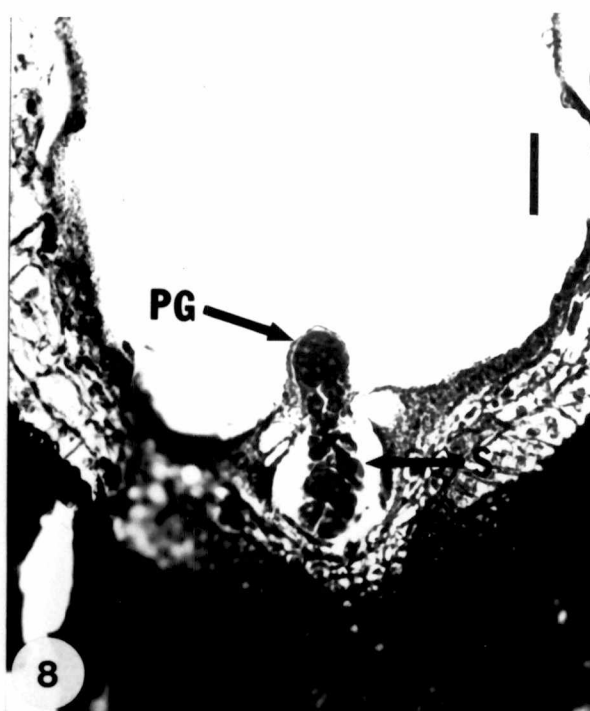
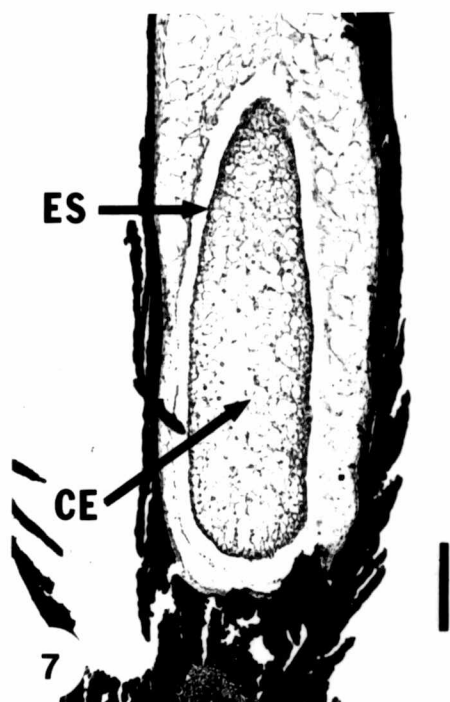
Plate 3, Figures 7-10. Early zygotic embryo development of
Cercis canadensis.

Figure 7. Embryo sac (ES) containing cellular endosperm (CE). Bar=200 μm

Figure 8. Preglobular zygotic embryo (PG) including a multiseriate suspensor (S). Bar= 40 μm

Figure 9. Globular stage zygotic embryo (GL) in which a single layer of presumptive protoderm (P) has differentiated. Note haustoria (H) at proximal end of the suspensor. Bar=50 μm

Figure 10. Early cordate stage zygotic embryo (CR) exhibiting a flattened apex (A), a well defined suspensor (S), and cotyledonary primordia (N) beginning to differentiate. Bar=50 μm



were in the late cordate stage and measured 498 μm in length whereas the suspensor size had decreased to only 41 μm . The densely staining cells of the future shoot apex and those of the cotyledonary tips were visible and procambial tissue became conspicuous at this stage of development (Plate 4, Figure 11).

The mean length of the suspensor continued to decrease, and measured only 35 μm at 107 dpa, whereas the mean length of the torpedo stage embryo proper was 875 μm (Plate 4, Figure 12). The cotyledons constituted nearly one-half the length of the embryo at this stage of development, and vascular traces could be distinguished clearly.

Embryos 111 dpa were in the late torpedo stage of development (Plate 4, Figure 13). The increase of nearly 1300 μm in embryo length during these four days was the largest recorded over the sampling period, and much of this growth occurred in the length of the cotyledons. This was accompanied by an apparent loss of the suspensor at this and later stages of development. The cotyledonary notch, which is characteristic of C. canadensis, was noticeable at this phase of development (Plate 4, Figure 13). The densely staining, rounded apical dome, which contained the apical meristem, became apparent between 111 and 118 dpa (Plate 5, Figure 14).

The rapid increase in embryo size continued into the cotyledonary stage, where embryos 118 dpa measured 3080 μm in length. Embryos developed through this stage and became

Plate 4, Figures 11-13. Cordate and torpedo stages of zygotic embryo development of Cercis canadensis

Figure 11. Cordate stage zygotic embryo (CR) with suspensor (S). The cotyledons (C) have differentiated and procambial tissue (PT) is visible. Bar=100 μm

Figure 12. Early torpedo stage zygotic embryo (TO) with well defined vascular traces (VT) and suspensor (S). Bar=100 μm

Figure 13. Late torpedo stage zygotic embryo (TO) with the cotyledons exhibiting a notch (CN) typical of C.canadensis zygotic embryos. Bar=250 μm

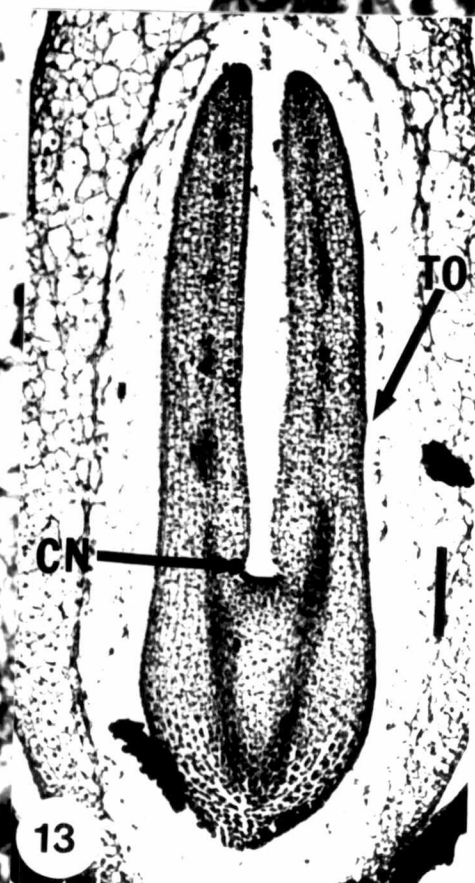
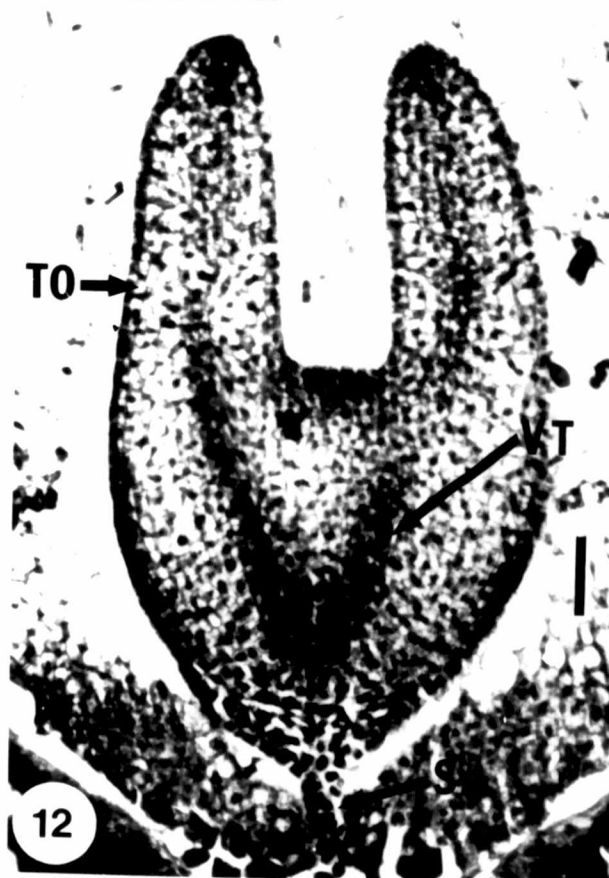
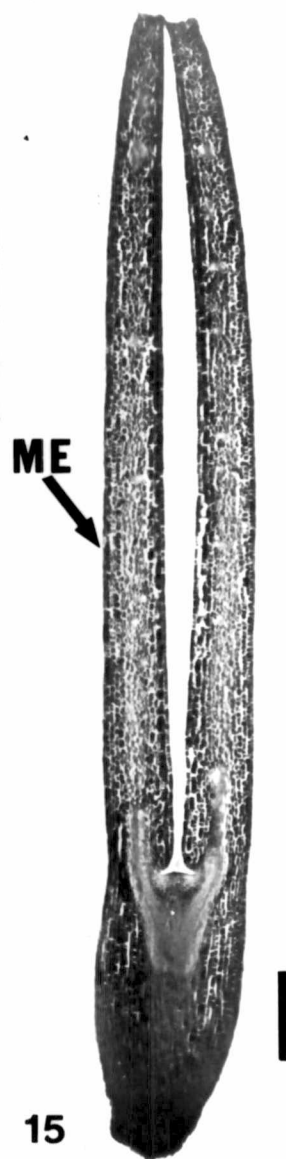


Plate 5, Figures 14 and 15. Zygotic embryos of Cercis canadensis in late stages of development.

Figure 14. Early cotyledonary stage zygotic embryo (CT) and surrounding embryo sac (ES). The distinctive cotyledonary notch (CN), and densely staining apical dome (AD) are apparent. Bar= 275 μ m

Figure 15. Mature zygotic embryo (ME) removed from the ovule. The tips of the cotyledons were removed before histological processing. Bar=380 μ m



morphologically mature as they increased in length to 5030 μm (Plate 5, Figures 14 and 15). These later stage embryos displayed a flattened disklike shape when removed from the developing seed (Figure 16).

Histological examination revealed that somatic embryos formed singly or in small groups (Plate 6, Figure 17), or asynchronously in clusters (Plate 6, Figure 18). These embryos displayed many of the same developmental stages observed for the zygotic embryos.

Figure 16. The mean length of zygotic embryos of Cercis canadensis collected from 82-152 days post anthesis (anthesis occurred March 2, 1991). Points represent the mean of data collected for each date, whereas the line is a best fit through these points.

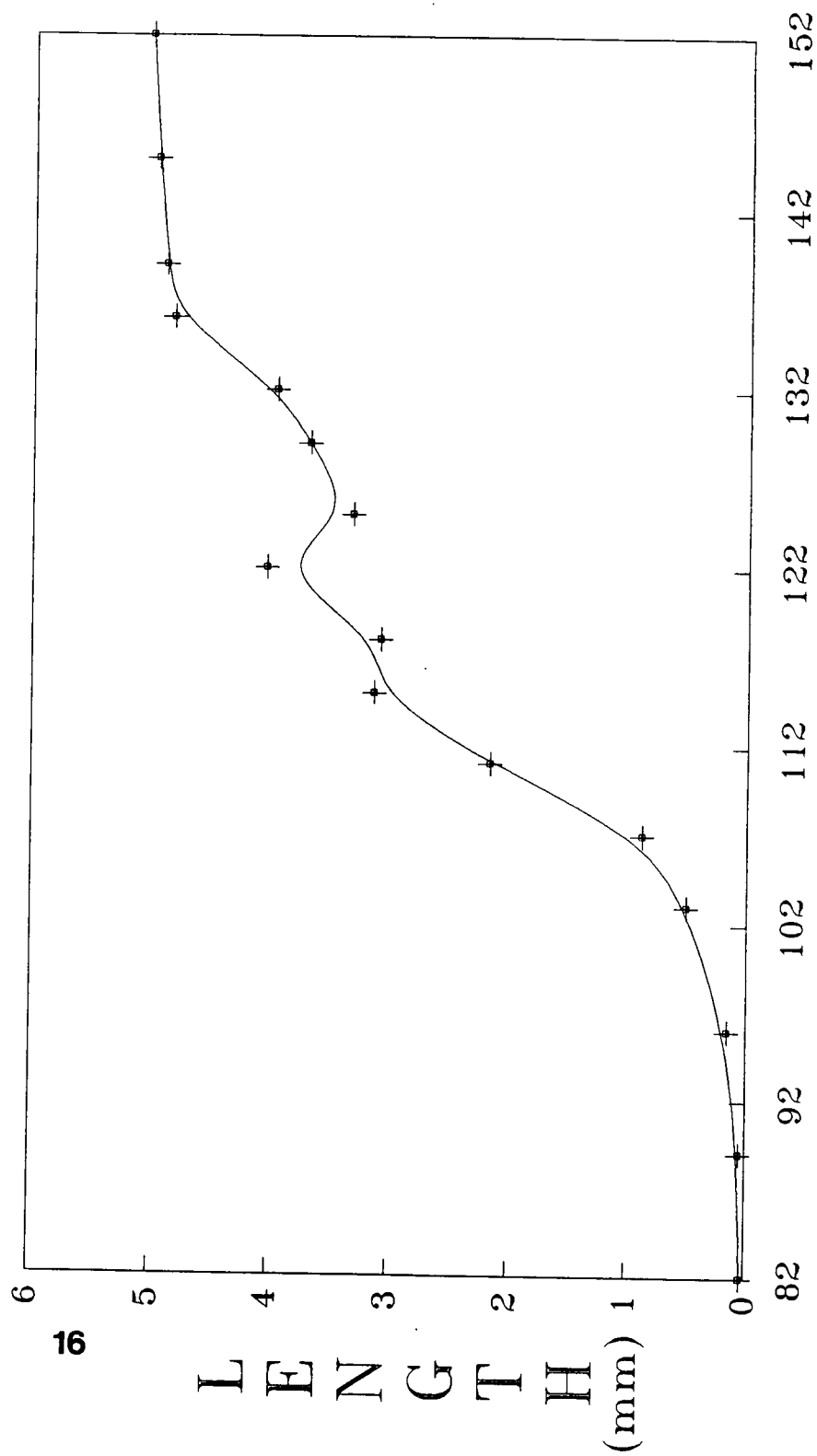
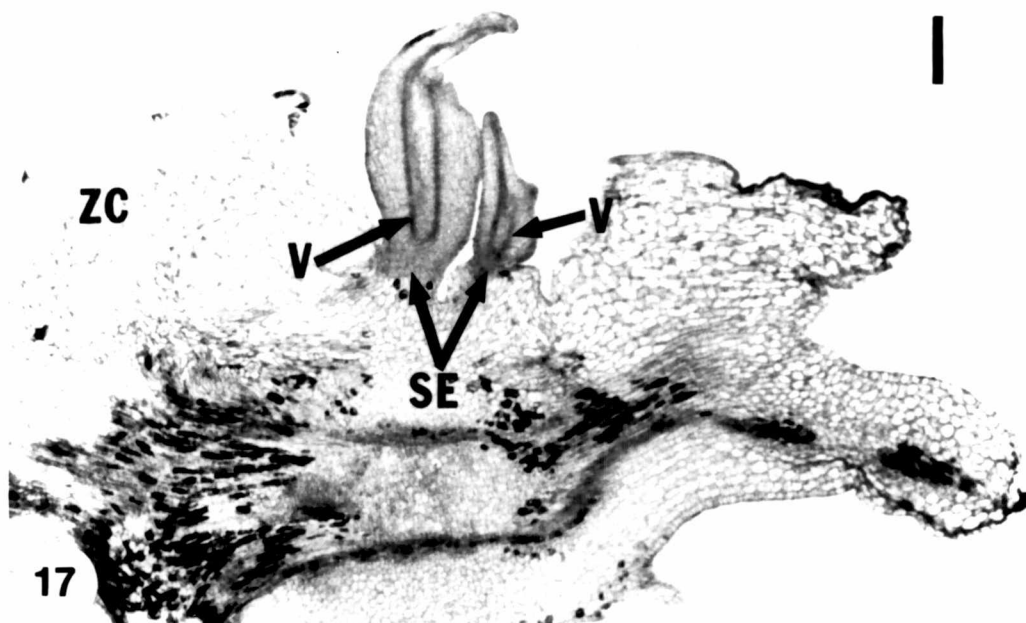


Plate 6, Figures 17 and 18. Histological examination of somatic embryos of Cercis canadensis.

Figure 17. Two somatic embryos (SE) originating from a zygotic cotyledon (ZC). The smaller embryo has only one cotyledon, whereas the second cotyledon of the larger embryo is not visible in this section. Note the closed vascular system (V) of both embryos. Bar=200 μm

Figure 18. Somatic embryo cluster (SE) displaying asynchronous development. Bar=250 μm



DISCUSSION

Cercis is considered to be one of the most archaic genera within the family Leguminosae (Polhill et al., 1981), and is classified in the tribe Cercideae, within the subfamily Caesalpinioideae (Gunn, 1991; Cowan, 1981). The ovules of the Caesalpinioideae are anatropous to campylotropous, bitegmic and crassinucellar. The embryo sac is of the polygonum type (Davis, 1966). Endosperm development is reported to be free nuclear with later tissue becoming cellular at the micropylar end of the embryo sac, whereas the chalazal end remains free-nuclear (Davis, 1966). The evidence presented herein contradicts published reports since histological examination of C. canadensis ovules 82 dpa revealed a cellular type of endosperm. However, it is possible that additional sections, especially of the more distal region of younger ovules, would reveal free nuclear endosperm.

Embryo development in Cercis is reported to follow the Onograd (Crucifer) model (Chubirko, 1982). This form of embryo classification is based on the respective contribution of the two cells of the proembryo to the formation of the embryo and suspensor, and by the type of division undergone by the terminal cell. In Onograd development, the apical cell derivatives produce a major portion of the embryo, including the hypocotyl, cotyledons, and stem tip, whereas the basal cell is responsible for generating the suspensor, and contributes little or nothing to the embryo (Maheshwari,

1950). In the Onagrad developmental type, exemplified by Capsella bursa-pastoris L. (Raghaven, 1986; Maheshwari, 1950), the zygote divides transversely, forming a basal cell and a terminal cell. The terminal cell divides longitudinally producing the upper two cells of the four cell proembryo. While this is occurring, the basal cell divides transversely, producing a two cell suspensor (Maheshwari, 1950).

The suspensor of Capsella and other genera often grow faster than the embryo proper during earlier stages of embryogeny and may initially be larger (Raghaven, 1986). The suspensor has been reported to reach full development during the proembryo stage (Johri, 1984) and degenerates later in embryo development (Johri, 1984; Cooper, 1938). In contrast, Crotalaria juncea L. forms a massive suspensor that reaches full development before the cotyledons differentiate, and remains intact until germination (Samal, 1936).

The Leguminosae display a wide range of suspensor morphology (Johri, 1984; Lersten, 1983; Davis, 1966) but species of the Caesalpinioideae reportedly lack a well developed suspensor; a rudimentary or small suspensor maybe present (Lersten, 1983). The suspensor of C. siliquastrum L. was reported to develop at the same time as the embryo proper, but reached maximum size earlier in development (Goursat, 1963). This suspensor was described as small, not sharply delimited from the proembryo, and composed of cells larger than those in the proembryo, (Lersten, 1983). The suspensor

in C. canadensis was multiseriate and reached maximum length at the globular stage of embryo development. At both this and the preglobular stage, the suspensor was longer than the embryo; the preglobular suspensor also appeared to contain more cells than the embryo at this stage of development.

The basal cell of the C. siliquastrum two cell pro-embryo was the progenitor of the suspensor. Unlike C. bursa-pastoris, the first division of the basal cell was longitudinal, and subsequent divisions produced a multiseriate structure. Goursat (1963) concluded that the suspensor developed solely from derivatives of the basal cell. Cercis chinensis Bunge also has a multicellular suspensor that forms entirely from basal cell derivatives (Kostrikova, 1986). The embryos of the above three species of Cercis and C. griffithii Boiss. develop similar to Trifolium repens f. minus L. (Gibelli and Belli ex. Bald) (Chubirko, 1982). In this Trifolium species, the basal cell of the two cell proembryo contributes only to the formation of the suspensor and the apical cell derivatives form the embryo (Anantaswamy, 1951). In addition, cells of the Cercis suspensor have been reported to divide in random planes to produce a multicellular suspensor that is not distinct from the embryo (Chubirko, 1982). The lack of distinction between the suspensor and embryo was noted in C. canadensis during the preglobular and globular stages. However, later stages depict a suspensor clearly delineated from the embryo. One cell at the

micropylar end of the suspensor enlarged significantly and become haustorial (Chubirko, 1982).

The development of the embryo of C. canadensis followed the basic developmental sequence of dicotyledonous embryos. This sequence is divided into five stages and begins with the preglobular/globular stage and then progresses through the cordate and into the torpedo stages (Street, 1974; Sharp, 1980). A cotyledonary stage of maturation is sometimes included next (Ammirato, 1985; Maheswaran and Williams, 1985), with the developmental sequence terminating with a mature zygotic embryo. These five developmental stages have been anatomically verified in this study for C. canadensis.

Embryo development in Cercis followed the Onagrad model (Chubirko, 1982). However this model only describes embryo development to the four cell stage. Embryo development in species of Cercis has been compared to T. minus (Chubirko, 1982), or implied by the extended description of C. bursa-pastoris ontogeny (Maheshwari, 1950). However, the exact sequence of cell divisions from the four cell stage of development to the preglobular embryo has not been reported, and embryos were not discovered by histological examination prior to the preglobular stage in this study.

The preglobular embryo appeared to be a mass of undifferentiated cells, whereas the globular stage embryo displayed a single layer of presumptive protoderm. This is usually the first primary meristem to develop (Raghaven, 1986)

and likely arises by periclinal division of the outer cell layer of the preglobular embryo, as reported in C. bursa-pastoris (Maheshwari, 1950).

A week after detection of globular stage embryos, ovule samples revealed embryos in the cordate stage of development. This stage was identified by the flattened apical region of the embryo that resulted from increased cell divisions in the lateral region of the terminal pole. This gave rise to the cotyledonary primordia and altered embryo morphology from radial to bilateral symmetry. During cotyledon initiation, a group of rapidly dividing cells, which constitute the future shoot apex, became organized in the depression between the developing cotyledons (Raghaven, 1976). The cells of the future root apex also became organized at this time. The formation of these two apical regions allowed the somatic embryos to simultaneously develop roots and shoots, as in zygotic embryo development (Sharp, 1980).

The process of cell differentiation in the cotyledons and hypocotyl is accompanied by elongation of these regions resulting in the formation of the torpedo shaped embryo (Raghaven, 1976). The densely staining tips of the two cotyledons and the presumptive shoot apex indicate that cell divisions continued in these regions. Hence, 107 dpa embryos, which displayed cotyledons that accounted for 50% of their length, gave rise four days later to late stage torpedo embryos with cotyledons extending nearly two-thirds their

length. As embryos matured through the cotyledonary stage, much of the growth continued to be in the cotyledons, which represented 75-80% of the embryo length at this stage of development. In addition, the relative thickness of the cotyledons appeared greater than in earlier stages.

Ovule weight (Geneve and Kester, 1990) and zygotic embryo size (Wang et al., 1984; Hazra et al., 1989) have been used to characterize the time period when zygotic embryos were competent to produce somatic embryos. The fresh weight of ovules in this study increased linearly over time until the last sampling date. However, relative water content decreased as embryos developed (Trigiano, unpublished). Thus, the increase in weight resulted more from the increase in dry matter. At 159 dpa, the weight of the mature ovule decreased by nearly 50%, and this decrease was accompanied by a larger decline in water content (Trigiano, unpublished). Perhaps the mature ovule was being prepared for dormancy at this stage of its development.

Ovules must apparently attain a minimum fresh weight (7.2 mg at 96 dpa) in order for somatic embryogenesis to occur. As weight increased, the embryogenic potential rose from 3% to 53%, then plateaued at approximately 30% for four weeks, and then declined to zero. Ovules weighing between 11.5 and 29.5 mg were associated with an embryogenic response of 30% or higher. As weight increased above 29.5 mg, the embryogenic competency fell to zero. Although ovule weight increased

linearly, the increase in weight of the zygotic embryo was not known. Zygotic embryo weight may prove to be a better indicator of embryogenic potential than ovule weight. However, determination of embryo weight is difficult and time consuming, as it requires removing intact embryos from ovules, removing all ancillary tissue, and maintaining embryo water status before weighing.

The length and perhaps the morphology of the zygotic embryo may be a better indicator of the somatic embryogenic potential of the ovule than ovule weight. The increase in length of the embryo over time followed a sigmoidal curve. Embryos from the first two sampling dates were in the preglobular (82 dpa) and globular (89 dpa) stages and both were in the lag phase of the growth curve. Ovules that contained cordate stage embryos measuring 150 μm were the first to be capable of producing somatic embryos, and this appears to coincide with the transition from lag to exponential growth of the embryos. Cordate embryos sampled one week later were in the exponential phase of their growth curve and the number of experimental units producing somatic embryos doubled during this time.

The most dramatic growth and the greatest somatic embryogenic potential of zygotic embryos occurred during the torpedo stage of development. Zygotic embryos 107 dpa showed the greatest somatic embryogenic potential (53%), whereas the absolute size increase between torpedo stage embryos 107 and

111 dpa (nearly 1300 μm) was the largest of any developmental stage and may represent the inflection point in the growth curve. The rapid increase in size continued as cotyledonary embryos 115 dpa increased nearly 50% in length. The cause of the large decrease in relative somatic embryogenesis for this date, and for cotyledonary embryos 132 dpa, is not known. This decrease may have resulted from sampling error, temporary environmental stress experienced by the ovules in vivo, variance in the culture regime or from other unknown factors.

Cotyledonary stage zygotic embryos from 118 thru 136 dpa were also in the exponential phase of growth, and supported relatively high levels of somatic embryogenesis (approximately 30%). Embryos 139 dpa appeared to be at the transition of exponential to stationary growth and the potential for embryogenesis was significantly reduced. After embryo size plateaued the somatic embryogenic potential was lost. Thus, zygotic embryos appeared to be capable of forming somatic embryos only when zygotic embryo length was in the exponential phase of growth. After zygotic embryos entered into the stationary phase of growth, embryo cells may have lost the potential for somatic embryogenesis and became committed to a particular developmental fate (Williams and Maheswaran, 1986).

This study has defined developmental parameters for ovules and zygotic embryos of C. canadensis, and related them to the somatic embryogenic potential of ovules in culture. Somatic embryogenesis was associated with zygotic embryos in

the exponential phase of growth, and occurred when zygotic embryos were in the torpedo and cotyledonary stages of development. Ovule fresh weight, which increased linearly over time, usually supported levels of somatic embryogenesis at 30% or higher, when ovule weight was between 11.5 and 29.5 mg.

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PART 2: Changes in ovule protein profiles associated with
somatic embryogenesis in Cercis canadensis

ABSTRACT

Ovules of Cercis canadensis L. (eastern redbud) were only competent to produce somatic embryos during a discrete period of their development. The embryogenic period was characterized by comparing the somatic embryogenic potential of ovules with changes in their protein profiles over time. Redbud pods were collected once or twice weekly from 82-159 days post anthesis (dpa). Ovules were removed from the pods, cultured on a modified SH medium, and evaluated six weeks later for the development of somatic embryos. These ovules were embryogenic from 96 to 139 dpa, with the maximum embryogenic response occurring 107 dpa. Proteins were extracted from additional ovules and analyzed by SDS-PAGE gel electrophoresis. Changes in intensity of six protein bands were associated with changes in the somatic embryogenic potential. The intensity of bands containing proteins weighing 32 and 36 kDa decreased when ovules became competent to produce somatic embryos. The four remaining bands, including two that contained putative storage proteins, increased in intensity from the middle to the end of the sampling period. This increase in intensity was associated with the loss of the somatic embryogenic potential of the ovules.

INTRODUCTION

Cercis canadensis L. (eastern redbud) is a leguminous tree, 5 to 10 meters in height and indigenous to the eastern deciduous forests of the United States. Redbud is used extensively as an ornamental species and has traditionally been propagated from seeds. Although conventional methods of vegetative propagation are difficult and expensive, axillary bud multiplication of redbud in tissue culture has been achieved. Recently, redbud has been propagated by somatic embryogenesis (Geneve and Kester, 1990). Although this technique is currently limited to research it is potentially more efficient than schemes now used for propagation.

Propagation of redbud by somatic embryogenesis utilized ovules harvested from immature seed pods as explants. The developmental stage of the explant critically influenced the ability to induce somatic embryogenesis in redbud (Trigiano et al., 1988) and in many other culture systems, including other legumes (Maheswaran and Williams, 1984; Hammat and Davey, 1987; Weaver and Trigiano, 1991) and woody species (Merkle et al., 1987; Trigiano et al., 1989). The period of embryogenic competency likely varies with climate and geographic conditions, and has been variously characterized by anatomical studies (Eapen and George, 1990; Trigiano et al., 1989), mRNA analyses (Pechan et al., 1991; Sharp, 1980), and protein profiles (Sotak et al., 1991; Roberts et al., 1989)

Until recently, anatomical studies have been the

predominant means of identifying when tissues were competent for somatic embryogenesis (Roberts et al., 1989). The utilization of proteins as biochemical indicators of competency more closely reflects the state of developmental regulation of the explant. Understanding which proteins influence somatic embryogenesis will permit selection of appropriate protein markers of somatic embryogenic competency. In order to understand when redbud ovules were competent to produce somatic embryos, ovule protein content was studied. The objective of this study was to compare the embryogenic potential of ovules cultured during different developmental stages to differences in ovule protein profiles over time.

MATERIALS AND METHODS

TISSUE CULTURE

Immature redbud pods were collected from a single tree on the University of Tennessee campus. Fruits were harvested on a weekly basis from 82 to 159 dpa (days post anthesis) with a second midweek sampling during the estimated time of somatic embryogenic competency, 107 to 145 dpa (Trigiano et al., 1988). Anthesis, which was estimated to have occurred March 2, 1991, was considered to be the date when one-half the flower buds had opened and anthers were exposed.

The embryogenic potential of ovules was assessed using the method of Trigiano et al. (1988). Pods were surface sterilized in 50% (v/v) commercial bleach (2.62% NaOCl) and 0.1% (w/v) Triton X-100 for five minutes with constant, vigorous agitation and then rinsed three times in sterile, distilled water. Ovules were removed from the pods, the integuments scored longitudinally on one side with a scalpel, then placed with the cut surface in contact with the culture medium. The basal medium consisted of SH salts (Schenk and Hildebrandt, 1972) amended with 100 mg/L myo-inositol, 1 mg/L thiamine, 30 g/L sucrose, 8g/L Phytagar (Gibco) and 9 μ M 2,4-D. Ammonium sulfate was added to yield a final ammonium ion concentration of 7.6 mM. The pH of the medium was adjusted to 5.7 with KOH prior to the addition of the Phytagar and before sterilization. A minimum of 40 ovules were explanted per sampling date, and the experimental unit consisted of two

ovules per 60 mm petri dish. Dishes were sealed with Parafilm (American National Can) and incubated at 22C with a 16 hour photoperiod under cool white florescent lights ($25 \mu\text{mol m}^{-2}\text{sec}^{-1}$). Six weeks after plating, the cultures from each sampling date were evaluated for the presence or absence of somatic embryos. The percentage of dishes in which somatic embryos were formed for each sampling date and the number of somatic embryos per dish were determined.

PROTEIN ELECTROPHORESES

A minimum of 9 g (fresh weight) of redbud ovules were collected per sampling date. Ovules were lyophilized and frozen at -80C until needed. Protein extractions were made from samples collected from 82-152 dpa, with at least one sample extracted from ovules collected each week during this time. A minimum of 2 g dried sample per sampling date were ground with a mortar and pestle in liquid nitrogen. Polyvinylpyrrolidone (PVPP) was added 2:1 (w/w) to the sample and then reground to a fine powder in liquid nitrogen. A 100 mM Tris buffer, pH 8.5 (Mayer et al., 1987) was added at a 4:1 (v/w) buffer/tissue ratio, liquid nitrogen added and the mixture finely ground again. The preparation remained at room temperature (22-25C) for a minimum of one hour, and then 4-6 ml (v/w) of additional buffer was added. The mixture was poured into 50 ml Sarstedt tubes and centrifuged at $2500 \times g$ for 10 minutes. The supernatant was poured into 15 ml Sarstedt tubes, 1-2 mg of protamine sulfate added per ml of

supernatant, and the solution incubated for 10 minutes while on a rotary shaker. The sample was then centrifuged for 10 minutes at 2500 x g, the supernatant collected, diluted 1:2 with a 2x Laemmli buffer (Laemmli, 1970) containing bromophenol blue as a tracking dye, and heated for four minutes at 95C. Extracts were then frozen at -80C until needed.

Protein concentration was determined for each extraction from 0.5-1.0 ml of supernatant without Laemmli buffer. Proteins were precipitated with acetone (1:3 v/v protein supernatant: acetone), and then resuspended in water (Appendix 2). Protein levels were determined spectrophotometrically at 595 nm utilizing a Biorad protein assay dye (Bio-Rad Laboratories, Richmond, California). Protein concentrations were then calculated from a standard curve prepared from bovine serum albumen (BSA), assayed at concentrations of 300, 600, 900, and 1200 μ g/ml of water.

Four μ g of protein in 8.9-42.3 μ L of extract were loaded per lane for electrophoresis. Samples were aligned in a 4% stacking gel (pH 6.8) then separated on a 9%-18% polyacrylamide gradient gel (pH 8.8) containing sucrose. Both stacking and resolving gels were 1.5 mm in thickness (Appendix 2). The 9% and 18% acrylamide solutions were degassed under vacuum, the gradient poured utilizing a peristaltic pump, and the gels loaded onto a Protean II Slab Cell (Bio-Rad Laboratories, Richmond, California). A discontinuous Tris

running buffer (pH 8.3) was used and a Pharmacia LKB-EPS 500/400 power supply provided a constant current of 25 mA per gel in the stacking gel, and 35 mA per gel in the separating gel. Running time varied between 4 and 4.5 hours.

Gels were fixed for a minimum of 18 hours in a methanol-formaldehyde solution (Appendix 2) and stained with silver (Wray et al., 1981). Gels were electronically analyzed with a computer integrated laser densitometer (Ultrosan XL; LKB Produkter AB Bromma, Sweden) and scans evaluated using Gelscan XL version 2.1 software (Pharmacia LKB Biotechnology, Uppsala, Sweden). Silver stain SDS-PAGE molecular weight standards (Bio-Rad Laboratories, Richmond, California) weighing 97.4, 31.0, and 14.4 kDa were used during scanning to calculate the molecular weight of individual protein bands and the relative amount of protein accumulated in each band.

RESULTS

Somatic embryos were not formed from ovules collected prior to 96 dpa; instead only a light green callus proliferated from the immature integuments. Although only one (3%) of the experimental units (two ovules per dish) produced somatic embryos 96 dpa, the six embryos produced were the highest of any sample period (Table 1). Fifty-three percent of dishes containing ovules cultured at 107 dpa produced somatic embryos; this was the highest response of any sampling date. The embryogenic response oscillated between 27-33% over the next eight sampling periods, although the response declined to 17% twice during this time. The last sample that produced somatic embryos was cultured at 139 dpa. The number of somatic embryos per embryogenic experimental unit ranged from 1.8-6.0 with most responding dishes containing 2.5-3.5 somatic embryos.

SDS-PAGE analyses were made from proteins extracted from ovules collected over ten sampling dates that represented the sampling period (Figure 1). These protein profiles revealed numerous bands; differences in staining intensity of six bands were associated with changes in the somatic embryogenic potential of the ovules. A second series of gels contained proteins extracted from three samples collected before (82 dpa), during (122 dpa), and after (152 dpa) ovules were competent to produce somatic embryos (Figure 2). These gels

Table I. Somatic embryogenesis from ovules of
Cercis canadensis

DPA ^a	Somatic embryogenesis (%) ^b	Mean No of somatic embryos ^c
82	0	0
89	0	0
96	3	6.0 ^d
103	7	2.5 ± 0.7
107	53	3.6 ± 3.3
111	27	3.9 ± 3.1
115	17	3.0 ± 3.5
118	33	2.8 ± 1.6
122	30	3.6 ± 5.9
125	33	4.6 ± 3.7
129	30	2.8 ± 1.9
132	17	2.0 ± 1.2
136	30	2.7 ± 1.7
139	13	1.8 ± 1.0
145	0	0
152	0	0
159	0	0

^a DPA = Days postanthesis - Anthesis March 2, 1991

^b Experimental unit was 2 ovules/dish

^c Mean number of somatic embryos per embryogenic
experimental unit ± standard deviation

^d Only 1 experimental unit responded

Figure 1. A SDS-PAGE gel of 10 protein extracts prepared from ovules of Cercis canadensis, and stained with silver. The molecular weight protein markers are in lane 1, and lanes 2-11 contain proteins extracted from ovules collected on the following dpa (days post anthesis): Lane 2, 82 dpa; lane 3, 89 dpa; lane 4, 111 dpa; lane 5, 115 dpa; lane 6, 122 dpa; lane 7, 129 dpa; lane 8, 136 dpa; lane 9, 139 dpa; lane 10, 145 dpa; and lane 11, 152 dpa.

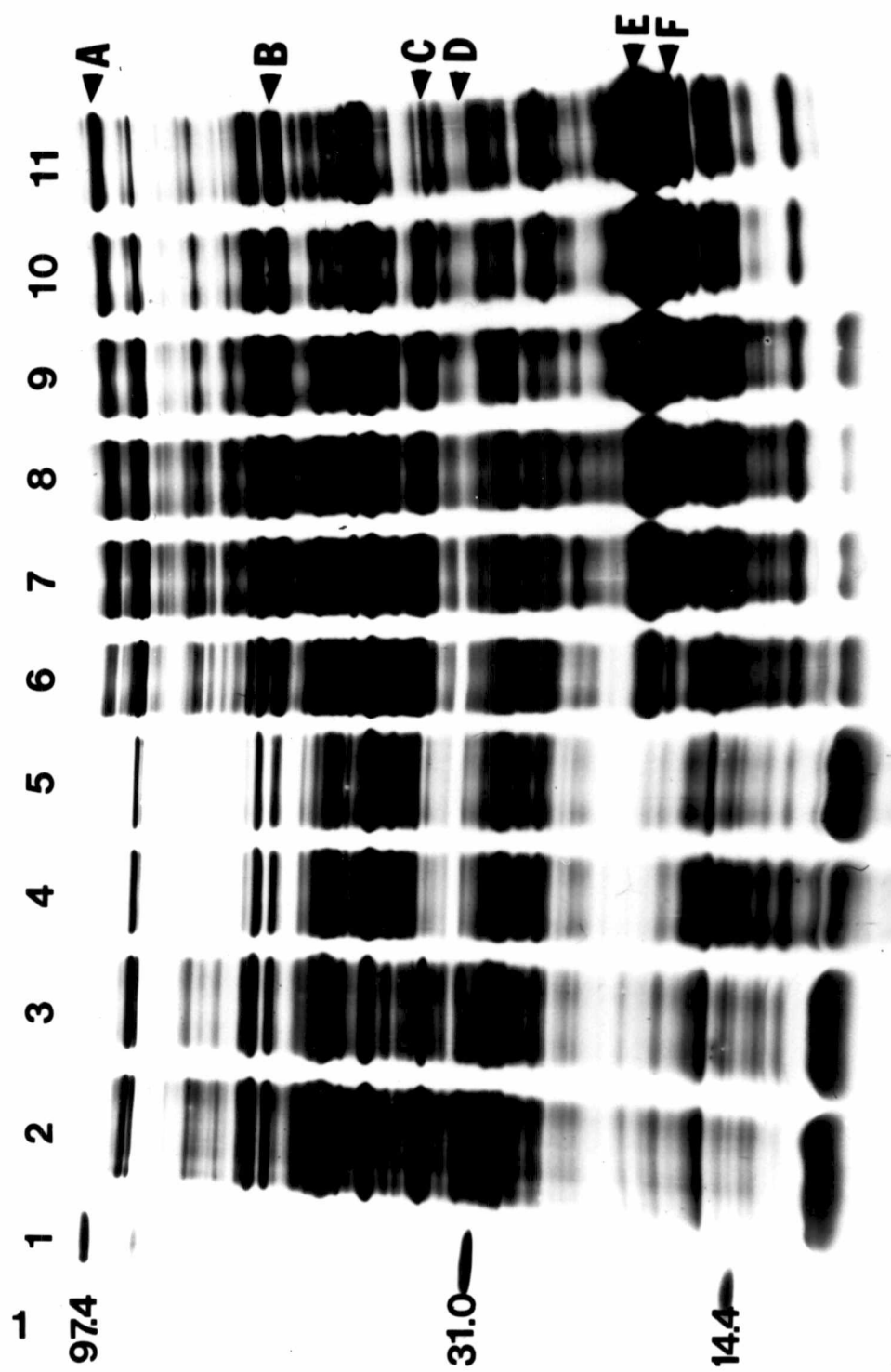
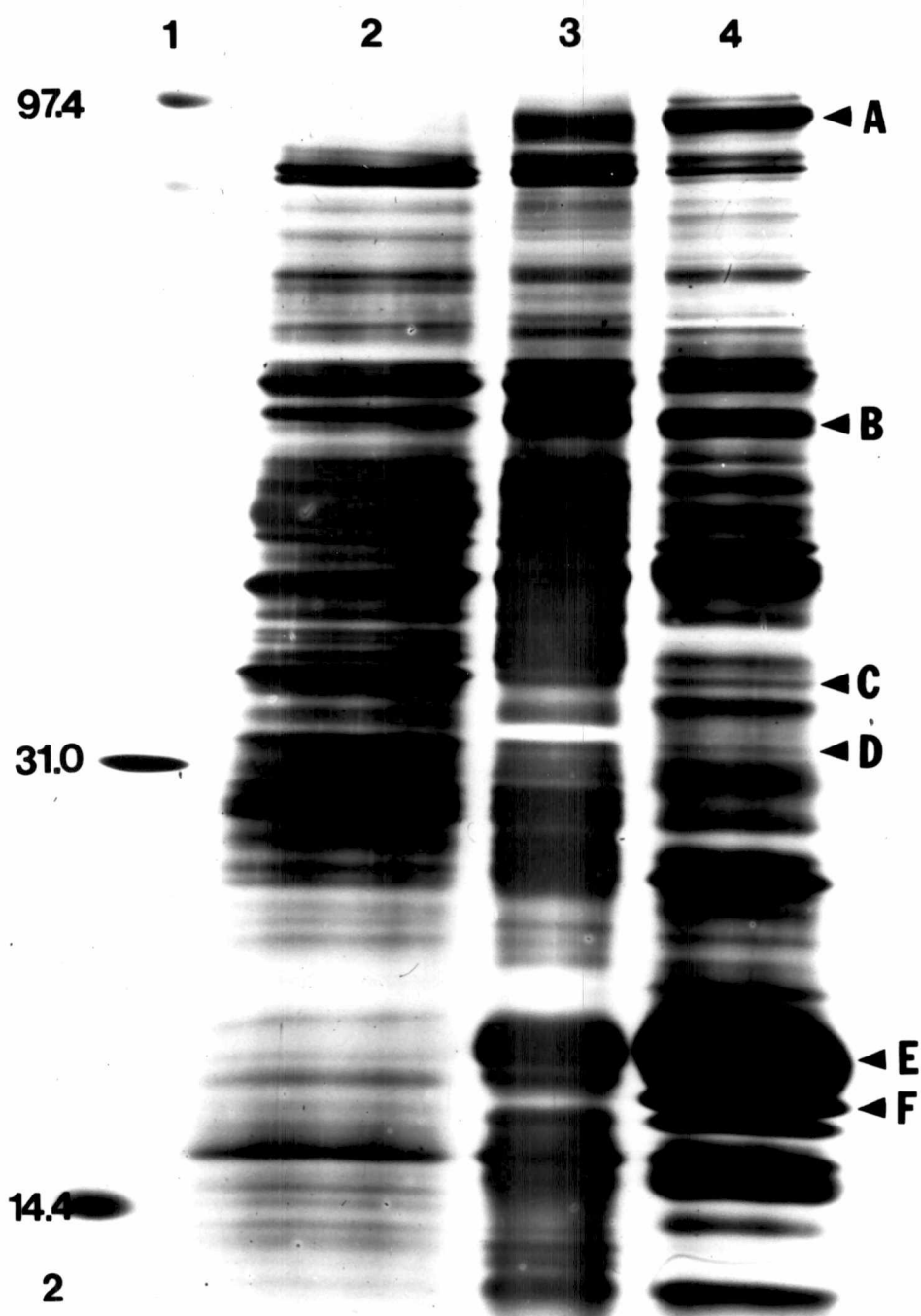


Figure 2. A SDS-PAGE gel of three protein extracts prepared from ovules of Cercis canadensis, and stained with silver. The molecular weight markers are in lane 1, and lanes 2-4 contain proteins extracted from ovules collected on the following dpa (days post anthesis): Lane 2, 82 dpa; lane 3, 122 dpa; and lane 4, 152 dpa.



were analyzed on a densitometer (Figure 3), and weight of the bands and relative (%) protein computed for each sampling date (Table 2). Densitometric analyses and visual inspection of the gels suggested grouping these protein bands into three categories. The first group consisted of bands A, E and F (containing proteins weighing 94 kDa, 19 kDa, and 18 kDa) that appeared faintly or not at all at 82 dpa, then appeared very strongly at 122 dpa, and continued to increase in intensity at 152 dpa. The second group consisted of a single band, B (56 kDa), which was distinctly visible at 82 dpa, and increased in intensity at 122 and 152 dpa. Group three consisted of protein bands C (36 kDa) and D (32 kDa) that appeared strongly at 82 dpa, decreased in intensity at 122 dpa, then stabilized or decreased further at 152 dpa. The largest change in protein levels occurred in bands E and F, which collectively accounted for 1% of total protein at 82 dpa, and 15% at 152 dpa.

Figure 3. Densitometric analyses of protein extracts from ovules of Cercis canadensis collected before (82 dpa), during (122 dpa) and after (152 dpa) the time during which the ovules were competent to form somatic embryos. M= Molecular weight markers of 97.4, 31.0 and 14.4 kDa. A-F represent significant protein bands of the following weights: A, 94 kDa; B, 56 kDa; C, 36 kDa; D, 32 kDa; E, 19 kDa, and F, 18 kDa.

Table 2. Densitometric calculation of protein weight and percent of total protein for ovule extracts of Cercis canadensis ovules collected before, during, and after the ovules were competent to form somatic embryos.

Band	Protein weight (kDa)		
	82 dpa	122 dpa	152 dpa
A	-	94 2% ^a	94 3%
B	56 4%	56 5%	56 7%
C	36 8%	36 2%	36 2%
D	32 4%	-	-
E	-	19 7%	17 ^b 15%
F	18 1%	18 3%	-

^a Percent of total protein for sampling date

^b No band resolution, therefore value reported for E & F at 152 dpa

- No value given

DISCUSSION

Ovules of C. canadensis were competent to produce somatic embryos between 96 and 139 dpa. Protein extracts from samples collected 82 dpa, 122 dpa, and 152 dpa were selected to represent protein profiles of ovules collected before, during or after the embryogenic period. Juxtaposition of these three extracts during electrophoresis facilitated visual comparison of differences in protein banding patterns that appeared to be associated with changes in the somatic embryogenic potential. These differences had previously been followed over time by analyzing gels containing proteins extracted from ovules collected on ten sampling dates representative of the sampling period.

Studies of callus formation (Kiyosue et al., 1991; Stirn and Jacobsen, 1987), zygotic embryogenesis (Sotak et al., 1991; Roberts et al., 1989), and somatic embryogenesis (Dahmer et al., 1992; Schnall et al., 1991), have utilized PAGE to identify proteins potentially involved in somatic embryo development. Five seed storage protein subunits of soybean were analyzed in situ and during development of cultured zygotic and somatic embryos. These proteins had reported molecular weights of 83, 78, 53, 38, and 20 to 22 kDa (Dahmer et al., 1992). Three of these proteins corresponded in size and/or behavior in situ to protein bands found significant in this study. These include the 83 kDa and 53 kDa proteins, which maybe comparable to band A (94 kDa) and band B (56 kDa)

proteins respectively, and the 20-22 kDa protein which may correspond with band E (19 kDa) and/or band F (18 kDa).

Bands E and F were faintly visible from 82 dpa extracts, became more abundant at 122 dpa, where they collectively contained 10% of the total protein, and increased further at 152 dpa, containing 15% of total protein. The increase in protein levels between 122 and 152 dpa was associated with a decline in the embryogenic potential of the zygotic embryo of C. canadensis. The increase in abundance of the 20-22 kDa protein studied by Dahmer et al., which began when the zygotic embryo was in the cotyledonary stage of development, was also associated with a decrease in somatic embryogenesis in soybean. (Dahmer et al., 1992). Other research reported that zygotic embryos were most competent to produce somatic embryos before a significant amount of storage proteins had accumulated (Roberts et al., 1989). The similarity in size and behavior between bands E and F, and the 20 to 22 kDa storage protein used by Dahmer et al. (1992) suggest that these two bands may contain storage proteins.

The 53 kDa protein subunit of soybean studied by Dahmer et al. (1992) was detected at a later time than the 56 kDa protein band in this study. The 53 kDa protein in soybean was not visualized until the end of the cotyledonary stage of zygotic embryo development. The 56 kDa band was present before somatic embryogenesis had been induced in C. canadensis. This difference may have occurred because the 56

kDa protein band consisted of several proteins of the same molecular weight. The collective presence of these proteins may have masked the actual appearance of a single protein. The level of the 56 kDa protein band over time was consistent with a 55 kDa protein band in yellow poplar thought to be a storage protein (Sotak et al., 1991). The 55 kDa band was present at 70 days post pollination and increased in amount with the maturation of the zygotic embryo. This protein was associated with a decrease in the somatic embryogenic potential of the zygotic embryo of yellow poplar. The similarity in size to known and putative storage proteins, and the increase in amount during later development suggest that the 56 kDa protein in this study maybe a storage protein.

The 83 kDa storage protein studied by Dahmer et al. (1992) was smaller then the 94 kDa protein in this study. However, both proteins are similar in size and relative abundance. The 94 kDa band appeared faintly during early development of the ovule, increased in intensity over time, and reached maximum intensity at the last sampling date, as did the 83 kDa protein. The similarity to a known storage protein, suggest a possible function for 94 kDa protein.

Group three protein bands (C and D) were present at 82 dpa. The amount of protein in band C declined at 122 dpa, and appeared to maintain this level at 152 dpa. Band D was not detectable by densitometry on these two later dates. The decrease in band intensity between 82 and 122 dpa coincided

with the onset of somatic embryogenesis. Although reports were not found that discussed the role of a 36 kDa protein in somatic embryo induction, a 31 kDa protein was present at much higher levels in carrot embryogenic cell clusters than in later stage somatic embryos (Kiyosue et al., 1991). In another study, a 32 kDa protein was isolated by 2-Dimensional electrophoresis from petiole segments of Trifolium rubens and T. pratens after five days in culture, and remained throughout the induction period. This protein was thought to be an embryogenic specific protein (McGee et al., 1989).

Changes in the levels of the six protein bands analyzed in this study were associated with changes in the somatic embryogenic potential of redbud zygotic embryos. Proteins weighing 94, 56, 19, and 18 kDa maybe storage proteins; when they (individually or synergistically) reached an as yet undefined concentration, the somatic embryogenic potential began to decline, and eventually was lost. Proteins weighing 36 and 32 kDa were most abundant before somatic embryogenesis occurred, and a decline in the level of these two proteins was associated with the induction of somatic embryogenesis. Isolation and identification of individual proteins within these bands is required to further understand their role(s) in somatic embryogenesis.

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APPENDICES

APPENDIX 1

5% formalin (containing 37% formaldehyde)
5% glacial acetic acid
40% double distilled water
50% ethanol (95% grade)

<u>% total alcohol</u>	<u>50</u>	<u>70</u>	<u>85</u>	<u>95</u>	<u>100</u>
dd water	250	150	75	--	--
95% ethanol	200	250	250	225	--
t-butanol	50	100	175	275	375
100% ethanol	--	--	--	--	125
Total mls	500	500	500	500	500

1)	Crystal Violet	1 g crystal violet in 100 ml of water
2)	Safranin	4 g safranin 100 ml of 95% ethanol 100 ml of water 4 g sodium acetate 8 ml 37% formaldehyde

3) Fast Green	1.2 g fast green
	80 ml methyl cellusolve
	80 ml methyl salicylate
	80 ml absolute ethanol

STAINING SERIES: time (in minutes unless otherwise specified)
following in brackets.

Microclear (5)
Microclear (5)
Microclear (5)
50% absolute ethanol + 50% microclear (5)
Absolute ethanol (5)
95% ethanol (5)
70% ethanol (5)
50% ethanol (5)
Safranin (overnight)
Water (rinse and change until water is clear)
Crystal violet (45 seconds)
Water (rinse and change until water is clear)
50% ethanol (5)
70% ethanol (5)
95% ethanol (5)
Absolute ethanol (5)
Fast green (45-90 seconds)
Absolute ethanol (1 minute - removes excess stain)
Absolute ethanol (5)

50% absolute ethanol + 50% microclear (5)

Microclear (5)

Microclear (5)

Microclear (5)

APPENDIX 2

SOLUTIONS AND PROCEDURES FOR PROTEIN EXTRACTION, SDS-PAGE ELECTROPHORESIS AND SILVER STAINING

100 mM TRIS EXTRACTION BUFFER (100 ml)

100 mM Trizma base pH 8.5	1.21 g
0.25 M sucrose	8.56 g
1 mM EDTA	0.034 g
1 mM EGTA	0.038 g
10 mM MgCl ₂	0.203 g
2% Triton X-100	2.0 ml
100 mM DTT	1.54 g
10 µg/ml leupeptin	1.0 mg

2x LAEMMLI BUFFER

Distilled water	4.0 ml
0.5 M Tris-HCl pH 6.8	2.0 ml
Glycerol	1.6 ml
10% SDS (w/v)	3.2 ml
2 β-mercaptoethanol	0.8 ml
0.05% (w/v) bromophenol blue	0.4 ml

PROTEIN PRECIPITATION

- 1) Chill acetone in ice for one hour
- 2) Add acetone to supernatant containing protein at 3-5:1
(v/v)

- 3) Chill for one hour in ice
- 4) Centrifuge at 16000 x g for 10 minutes
- 5) Slowly decant acetone
- 6) Add acetone to pellet in the same volume used in step #2
- 7) Centrifuge at 16000 x g for 10 minutes
- 8) Decant acetone and let pellet dry by evaporation
- 9) Add distilled water to pellet (100-1000 μ L)
- 10) Resuspend pellet in water for 30 minutes
- 11) Centrifuge 5 minutes at 16000 x g and collect supernatant

POLYACRYLAMIDE GEL COMPONENTS (for one 9%-18% resolving gel)

	9%	18%
Distilled water	6.50 ml	1.90 ml
1.5 M Tris-HCl pH 8.8	3.70 ml	3.70 ml
Acrylamide 29.2% / Bis 0.8 (w/v; 30% stock)	4.50 ml	9.00 ml
10% SDS (w/v)	0.15 ml	0.15 ml
10% Ammonium persulfate(w/v)	0.10 ml	0.10 ml
TEMED	0.01 ml	0.01 ml
Sucrose	0.15 g	0.75 g

POLYACRYLAMIDE GEL COMPONENTS (Stacking gel)

Distilled water	12.20 ml
0.5 M Tris-HCl pH 6.8	5.00 ml
10% SDS (w/v)	0.20 ml
Acrylamide (30% stock)	2.60 ml
10% Ammonium Persulfate (w/v)	0.10 ml
TEMED	0.02 ml

ELECTRODE (RUNNING) BUFFER pH 8.3 (5X)

Tris base	15 g/L
Glycine	72 g/L
SDS	5 g/L

SILVER STAIN (Wray et al 1981)

- 1) Fix overnight in solution containing 50% methanol, 0.1% of 37% formaldehyde and dd (double distilled) water
- 2) Rinse gel in dd water, then wash in 500 ml dd water for 30 minutes with gentle agitation
- 3) Soak gel in 150 ml of the following ammonical silver nitrate solution for 30 minutes

Ammonical Silver Nitrate

3.15 ml of 0.9M NaOH

2.1 ml of 58% NH₄OH

0.8 g AgNO₃ in 6.75 ml dd H₂O

138 ml dd H₂O

4) Rinse gel with dd water, then wash in dd water for 30 minutes with gentle agitation

5) Soak gel in developer until proteins become visible

Developer

500 ml dd water

2.5 ml 1% citric acid

0.25 ml formaldehyde (37%)

6) Stop development by soaking in solution containing 45% methanol and 10% acetic acid in dd water

VITA

L. Graham Buckley was born on August 20, 1951 in Seattle, Washington. He attended Bellevue High School, and graduated in June 1969. In 1970, he entered Seattle University, graduating in 1973 with a degree in economics. He later returned to school, and obtained a Bachelor of Science degree in biology from the University of Washington in 1989.

In August, 1990, Graham entered the Graduate School at the University of Tennessee-Knoxville. He was employed as a graduate research assistant in the Department of Ornamental Horticulture and Landscape Design, while he pursued an interdepartmental Master of Science degree in Life Sciences. He was granted the degree in December, 1992.