ULTRASTRUCTURAL CHANGES DURING EARLY DEVELOPMENTAL STAGES OF SOMATIC EMBRYOS IN LOBLOLLY PINE (PINUS TAEDA L.)

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Abstract:--Ultrastructural changes of somatic embryo at early developmental stages were studied in loblolly pine (Pinus taeda L.). Proliferation and maturation media of Gupta and Pullman (1991) have been used to obtain embryogenic tissues and somatic embryos, respectively. Some degenerating and declined suspensor cells were observed in well-maintained embryogenic tissues. This may explain why the embryogenic cultures have to be subcultured frequently and sometimes lose embryogenic ability. In stage 1 embryos, starch granules started to accumulate in plastids, but lipid bodies showed no significant increase. In comparison to the stage 1 embryos, embryos at stage 2 contained increased number of lipid bodies. Cytoplasm of embryonic cells was also richer in free ribosomes, which may be an early sign of storage protein synthesis. Vacuoles in stage 1 and 2 embryos were larger and/or more numerous than that of embryonic cells in maintained embryogenic tissue. Lack of osmoticum in maturation medium was suspected as one of the potential causes of vacuolated embryonic cells with no protein accumulation.

Keywords: lipid body, somatic embryo, storage protein, suspensor cells

INTRODUCTION

Somatic embryogenesis in conifers has been extensively studied (Becwar et al. 1995; Tautorus et al. 1991). However, the main effort in this area has primarily focused on mass and rapid production of somatic embryos due to its high commercial value. In conifers, detailed ultrastructure of developing zygotic embryos has not been studied (Tautorus et al. 1991) except in Douglas fir, Pseudotsuga menziesii (Owens et al. 1993). Also, the ultrastructure of somatic embryos has been studied only in white spruce, Picea glauca (Fowke et al. 1990; Hakman et al. 1987) and European larch, Larix decidua (Rohr et al. 1989). In contrast to the success in Picea and Larix species, difficulties still remain in efficiently producing mature somatic embryos in loblolly pine (Pinus taeda L.). The objective of the present work is to study the ultrastructural changes during early development of somatic embryos. This research will provide cellular information of somatic embryo development, which may help to improve embryo maturation in loblolly pine.

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MATERIALS AND METHODS

Plant material. Seed cones of loblolly pine were collected at Hope, Arkansas, on June 30, 1993. Seeds were sterilized by the method described by Li and Huang (1996). Intact megagametopyhtes containing immature zygotic embryos were excised from seeds with a sharp scalpel and used as explants for initiation of embryogenic tissue. The explants were laid on the surface of culture medium in petri dishes. One of initiated embryogenic cell lines, named as H_{10}, was a clonal product from an explant. This cell line had been maintained for about one year and subcultured at least 20 times before it was used in this ultrastructral study.

Initiation and proliferation of embryogenic tissue. The composition of basal medium used in this experiment was described by Gupta and Pullman (1991) except that the concentration of boric acid was doubled to 31 mg/liter (Li et al. 1996). The initiation medium (BM_1) was supplemented with 50 μM 2,4-Dichlorophenoxy acetic acid (2,4-D), 20 μM kinetin, 20 μM 6-Benzylaminopurine (BA) (Gupta and Pullman 1991). The initiated embryogenic cultures were then transferred to BM_3 medium for proliferation, the basal medium plus 5 μM-2-4-D, 2 kinetin, 2 μM BA, and 9,000 mg/liter myo-inositol (Gupta and Pullman 1991). Translucent and mucilaginous embryogenic tissues containing both embryonic and suspensor cells were excised from the surface of culture and used for the ultrastructural study. The embryogenic tissues were healthy and vigorously growing, and had potential to show all the developmental stages, from the cell masses to mature somatic embryos, which were dependent on the composition of culture medium and duration of culturing time.

Stage 1 and 2 somatic embryos. The staging system for somatic embryo development followed the work of von Arnold and Hakman (1988). Stage 1 and 2 embryos are defined as:

Stage 1 embryos are small embryos consisting of an embryonic region subtended by an elongated suspensor.

Stage 2 embryos are bullet-shaped embryos with a clear prominent embryonic region that is more opaque than stage 1 embryos.

To induce stage 1 and 2 embryos, embryogenic tissues were transferred to maturation medium (BM_4), the basal medium supplemented with ABA at 80 mg/liter and activated charcoal at 1 mg/liter (Gupta and Pullman 1991). After culturing about a month on the maturation medium, stage 1 somatic embryos were grown about 0.5 to 1 mm long. Somatic embryos at stage 2 with a length of about 2 mm were formed in about 2 months. Somatic embryos at both stages were studied under a transmission electron microscope. The samples for the electron microscopy were selected from cultures raised on solid media.

Electron microscopy. Small pieces of embryogenic tissue and somatic embryos at different developmental stages were fixed in a modified Kamovsky's fixative consisting of 2% glutaraldehyde and 2% paraformaldehyde in 0.2 M cacodylate buffer at pH 7.4 for 2 hr in a

weak vacuum and then washed with 0.05 M cacodylate buffer at pH 7.2 twice. This procedure was followed by post-fixation in 1% OsO_4 in the same buffer for 2 hr. The tissues were then prestained with 0.5% aqueous uranyl acetate at 4° C overnight, dehydrated stepwise in a graded ethanol series (30 to 100%) and propylene oxide two times to ensure complete dehydration. The specimens were then infiltrated with the mixture of 50% to 50% propylene oxide-Spurr's embedding medium (Spurr 1969) for 2 hr. The specimens were finally embedded in 100% Spurr's medium and polymerized in an oven at 70 ° C overnight. Ultra-thin sections cut with a LKB ultramicrotome were stained with 2% uranyl acetate for 4 min, followed by lead citrate at pH 12.0 for 2 min (Reynolds 1963).

RESULTS

Embryogenic tissue. Embryogenic tissue contained two types of cells, smaller embryonic cells and long suspensor cells. The smaller embryonic cells at this stage were tightly attached together. Nucleus generally occupied a large area in the center of embryonic cell and contained two nucleoli, but nucleoplasm was clear. Plasmodesmata occurred between embryonic cells. Some very small vacuoles were observed in the cytoplasm. Mitochondria were abundant and varied in shape. Golgi bodies were scattered in the cytoplasm and were primitive. Plastids usually contained starch granules and had only a few thylakoids, if any. Endoplasmic reticulum (ER) was mostly of a rough variety. A few lipid bodies were scattered in the cytoplasm while no typical protein bodies and only a few starch granules in plastids were observed in embryonic cells at this stage.

The long and large suspensor cells were characterized by having a large central vacuole, which reduced cytoplasm to a thin parietal layer. The suspensor cells were loosely attached each other, and no intercellular connections or plasmodesmata were observed. Cytoplasm also contained abundant mitochondria. Plastids had varied profiles. The ERs were mostly of a rough variety. The cytoplasm of suspensor cells was as dense as that of embryonic cells, and Golgi bodies were also very primitive. One striking phenomenon of embryogenic tissue was that a considerable number of suspensor cells showed various signs of degeneration. The cytoplasm first became electron-lucent due to the loss of matrix substance; Golgi cristae became dilated and contained numerous small membranous vesicles while some of the other organelles, such as mitochondria and plastids, appeared still intact. In some cells, the plastids and mitochondria collapsed and the nuclei were disintegrating.

Stage 1 somatic embryos. Embryonic cells at this stage were smaller but had denser cytoplasm than that in the maintained embryogenic tissue. The cells in embryonal apical dome showed the outer cell wall with a cuticle. Cells along the outside had thicker cell wall than those positioned in the center. Embryonic cells were more closely associated with each other at this stage. There were numerous plasmodesmata between the thin cell walls. Many of the nuclei contained two to four nucleoli and clumps of chromatin were much more abundant throughout the nucleoplasm in comparison to that of the embryonic cells at the previous stage. More numerous and larger vacuoles in the embryonic cells at this stage were formed in the cytoplasm compared to the embryonic cells in embryogenic tissue. Starch

synthesis appeared to be very active as indicated by the fact that a large number of starch granules were accumulated in plastids. The plastids showed early differentiation, indicated by the appearance of thylakoids. Ribosomes were also more abundant at this stage than at the previous stage. However, no storage protein accumulation was observed in the cytoplasm. The number of lipid bodies had no obvious change.

Stage 2 somatic embryos. The most significant phenomenon at this stage was an obvious accumulation of lipid bodies in embryonic cells. Starch granules were also abundant and accumulated in plastids. The accumulation of storage reserves suggested that the embryos were on the way toward maturation. Free ribosomes were also richer in embryonic cells at this stage than at the previous stages, indicating active protein synthesis, although the storage protein or protein body was not observed. Microtubules were also observed in the cells. However, small vacuoles had fused together and formed bigger ones.

DISCUSSION

Embryogenic tissue of loblolly pine was whitish to translucent in color and resembled embryogenic tissue of other conifers, but showed no embryo development on the proliferation medium. The embryogenic tissue was also called embryonal-suspensor cell mass and contained numerous very early stage embryos (Gupta and Durzan 1987). Each embryo had elongated cells at one end (suspensor) and smaller cells with large nuclei and dense cytoplasm at the other (embryonal) end. The little differentiated plastids in embryonic cells with dense stroma but very few thylakoids if any indicated that the tissues possessed embryogenic ability. Non-embryogenic calli generally contained chloroplasts, typically with numerous grana and large grains of starch; differentiated plastids in embryonic cells in embryogenic tissues indicated that the tissues had lost or were losing embryogenic ability (Rohr et al. 1989; Becwar et al. 1988).

The structurally unhealthy and degenerating suspensor cells found in embryogenic tissue were another striking phenomenon. It was not surprising, however, because a previous study showed that suspensor cells can be generally stained with Evan's Blue, while embryonic cell can be stained with acetocarmine (Gupta and Durzan 1987). This so-called double-staining technique has been used to identify embryonic and suspensor cells in embryogenic tissue in coniferous species (Gupta 1995). However, Evan's Blue is often used to determine cell or tissue viability (Gaff and Okang 1971). The cells stained by Evan's Blue are generally considered that they are dead or, at least, under degeneration. This study clearly showed that there were a considerable number of degenerating suspensor cells in the embryogenic tissue.

It has been demonstrated in Larix decidua that suspensor cells showed the advanced signs of senescence after the embryonal apical dome had been formed during somatic embryo development (Rohr et al. 1989). *In vivo,* suspensors started to degenerate as soon as zygotic embryos were extruded from archegonium by the support of elongated suspensor. However, embryos in embryogenic tissue were still at very initial development stage and suspensors

had not elongated yet. Suspensor cells should not start to degenerate at this stage, and various signs of senescence in suspensor cells may be abnormal. Degenerating and declined suspensor cells in embryogenic tissues may explain why the tissues needed to be subcultured frequently and sometimes lost embryogenic ability.

It was evident that starch among storage reserves first started accumulation in somatic embryo and this accumulation was followed by development of lipid bodies. No protein body was observed in somatic embryos at early stages. Presence of protein body in somatic embryos may not occur until the late cotyledonary stage (Smith, personal communication). This order in accumulation of storage reserves seems to be different from that of zygotic embryos. In Douglas fir, protein bodies were first observed in zygotic embryos although they were small and only a few (Owens et al. 1993). In comparison to stage 1 somatic embryos, no lipid bodies and starch were found in zygotic embryos at the similar stage. Later, accumulation of lipid bodies and starch started almost simultaneously. Protein bodies had not significant increased until very late developmental stage, but they were less abundant in zygotic embryos than in megagametophyte (Owens et al. 1993). Ultrastructural study of zygotic embryos in loblolly pine is lacking.

Highly vacuolated embryonic cells and a lack of protein bodies were consistently observed in stage 1 and 2 somatic embryos. They may be not an independent phenomenon but appeared to be associated with each other. Absence of osmoticum in maturation medium resulted in highly vacuolated embryonic cells, non-uniform embryo maturation and smaller cotyledon region in white spruce (Attree and Fowke 1993). High vacuole profile was also accompanied with lower storage protein and lipid contents in mature embryos of white spruce. Lack of osmoticum in the maturation medium, therefore, was suspected to be one of potential reasons for vacuolated embryonic cells with no protein accumulation; in turn, the early stage somatic embryos failed to develop to the cotyledonary stage in loblolly pine.

Seed storage proteins play an exceedingly important role in the reproduction and survival of angiosperm (Bewley and Black 1994). The storage protein has been observed during somatic embryo maturation in other conifer genera, such as *Picea* and *Larix* (Attree and Fowke 1993; Rohr et al. 1989). A richness of free ribosomes and a rough ER profile in stage 2 embryonic cells may indicate the onset of deposition of storage protein. Gene expression of storage protein has been demonstrated to be activated by ABA treatment (Shiver and Mundy 1990). However, both osmotic pressure and ABA controlled the gene expression of storage protein in angiosperm (Bewley and Black, 1994). In white spruce, it was evident that ABA initiated synthesis of storage protein, but osmoticum was needed to regulate storage protein synthesis at the post-transcriptional level (Attree and Fowke 1993). Osmoticum may also have an important role in regulating synthesis of storage protein in loblolly pine. In this experiment, the high osmolarity induced by myo-inositol was present only in the proliferation medium but was absent in the maturation medium. Cell line H_{10} has not produced cotyledonary embryos on this maturation medium with 2 to 80 mg/liter ABA in our lab. Increasing osmolarity of culture medium may reduce the extent of vacuolation of somatic embryonic cell and enhance protein accumulation; in turn, somatic embryo maturation will be improved. Later studies demonstrated that the addition of 3 to 10%

polyethylene glycol (MW 3,350) as an osmoticum induced cotyledonary somatic embryos for H_{10} and another cell line (Li et al. 1997).

CONCLUSIONS

Degenerating and declined suspensor cells found in well-maintained embryogenic tissues may explain why embryogenic cultures needed to be subcultured frequently and sometimes lost embryogenic ability. The order in accumulation of storage reserves in somatic embryos *seems to be different from that of zygotic embryos. Although* no protein body was observed, richness of free ribosomes and a rough ER profile in stage 2 embryo may indicate that storage protein synthesis had been activated. However, the stage 2 embryos have failed to mature further on the medium with only ABA. Lack of osmoticum may be one of reasons resulting in vacuolated embryonic cell without storage protein depositions. Since osmoticum has been evident having an important role in regulating storage protein synthesis and somatic embryo maturation, it deserves to be investigated for improving the maturation of somatic embryos in loblolly pine.

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