AN ANATOMICAL STUDY OF SECONDARY EMBRYOGENESIS IN CAMELLIA RETICULATA

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SUMMARY

An anatomical study was carried out during the sequences of events which lead to the differentiation of secondary embryos of Camellia reticulata cv. ‘Mouchang.’ Secondary embryogenesis can be induced by culturing somatic embryos on a modified Murashige and Skoog medium supplemented with 0.5 mg liter\(^{-1}\) 6-benzylaminopurine and 0.1 mg liter\(^{-1}\) indole-3-butyric acid. After about 12 days of culture, globular-shaped secondary embryos became apparent, and by 18 to 20 days of culture cotyledonary stages were formed. Embryos developed mainly on the hypocotyl of primary embryos without an intermediate callus. Histologic monitoring revealed that secondary embryos apparently had a multicellular origin from embryogenic areas originating in both epidermal and subepidermal layers of the hypocotyl region. This morphogenetic competence is related to the presence, at the time of culture, of relatively undifferentiated cells in superficial layers of the primary embryo hypocotyl. Microcomputer image analysis was applied for quantifying cytological events associated with somatic embryogenesis. This method showed an increasing gradient in the nucleus-to-cell area ratio from differentiated cells passing through preembryogenic cells to embryogenic cells. The formation of embryogenic areas was preceded by accumulation of starch in the surrounding cortical cells. The cells underlying globular secondary embryos still contained abundant starch, but it declined as the secondary embryos developed.

Key words: Camellia reticulata; secondary embryogenesis; histology; starch accumulation; image analysis; tissue culture.

INTRODUCTION

Secondary embryogenesis can be an efficient method of plant micropropagation (31,37). Somatic embryos may be suitable material for biochemical or genetic manipulation studies (28), for encapsulation as artificial seeds (25), and for cold storage to preserve germplasm (33). Maintenance of embryogenic competence during repetitive or secondary embryogenesis has been reported for several woody species, including Juglans regia (35), Robinia pseudacacia (14), Eucalyptus citriodora (17), and Prunus persica (24). In a previous article (20) we reported the expression of somatic embryogenesis from immature zygotic embryos of Camellia reticulata and the maintenance of embryogenic competence by secondary embryogenesis. We emphasized the potential of this propagation system for obtaining hybrids when the embryos produced by cross fertilization do not survive to maturity.

Several authors (2,15,16,21,30) have described the developmental anatomy of somatic embryogenesis in initial explants, but there seems to have been little histologic or histochemical work on the formation of secondary embryos. According to the criteria of Thorpe (32) our working species, C. reticulata, would be a suitable experimental system for such studies because it requires only simple culture conditions, it has high frequency of expression, and the site at which differentiation will occur is predictable.

The objectives of this work were to determine the origin of secondary embryos and the developmental events occurring during secondary embryogenesis in C. reticulata. In pursuing these objectives, we applied microcomputer image analysis techniques to quantify changes associated with the embryogenic process, e.g., changes in starch accumulation and the nucleus-to-cell ratio.

MATERIALS AND METHODS

The C. reticulata cv. ‘Mouchang’ embryos studied were taken from a culture line originally established from immature zygotic embryos (20) and maintained for 18 mo. by subculturing successive generations of secondary embryos every 6 to 7 wk. Isolated somatic embryos 7 to 10 mm long were cultured five to ajar in 300-ml glass jars containing 50 ml of Murashige and Skoog’s (18) mineral salts supplemented with 1 mg liter\(^{-1}\) thiamine, 0.1 mg liter\(^{-1}\) nicotinic acid, 0.1 mg liter\(^{-1}\) pyridoxine, 100 mg liter\(^{-1}\) myoinositol, 0.5 mg liter\(^{-1}\) 6-benzylaminopurine, 0.1 mg liter\(^{-1}\) indole-3-butyric acid, 30 g liter\(^{-1}\) sucrose and 6 g liter\(^{-1}\) Difco agar. The cultures were kept in a growth chamber under 30 #mol. m\(^{-2}\). s\(^{-1}\) delivered during a 16-h day by cool white fluorescent lamps, with day and night temperatures of 26° and 20° C, respectively.

Histologic processing was performed on 10 freshly isolated embryos (Day 0) and on lots of 5 embryos collected each alternate day over 3 wk of culture. The embryos were fixed in formalin-glacial acetic-etanol 50% (5:5:90 v/v/v) (FAA) (except those used for DNA location, which were fixed in Carnoy’s fluid), dehydrated through a graded n-butanol series and embedded in paraffin wax. Transverse or longitudinal 8- to 10-mm sections were cut with a microtome; inasmuch as secondary embryos form mainly on the hypocotyl of the primary embryo, we concentrated on this region. For general examination, sections were stained with safranin-alecan blue and safranin-fast green (7). DNA was stained by Feulgen’s method, starch and other insoluble polysaccharides by the periodic acid-Schiff (PAS) reaction (7), and total proteins by mercuric bromophenol blue (12). Control preparations carried out for Feulgen and PAS tests were omission of CHL and periodic acid treatments, respectively; in neither case did the controls stain.

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PLATA ET AL.

The areas of cells and nuclei were measured using a computerized image analyzer (MIP 1.4, MICROM). A Zeiss light microscope was connected via a C mount to the video camera of the image analyzer, and digitalized images of sections were displayed on a monitor screen. For each of the cell types distinguished in Fig. 1, 200 cells and their nuclei were measured. Data were expressed as percentage of nucleus-to-cell area ratio. (Safranin-fast green staining method for all figures except where stated otherwise).

The accumulation of starch and other insoluble polysaccharides was quantified at successive stages of embryogenesis as PAS density. This term is defined as the stained proportion of 100 × 100-μm frame containing 15 to 20 subepidermal cortex cells, expressed as a percentage; a screen pixel was deemed to represent stained tissue if its color lay in the range defined by stained starch grains. For each stage of embryogenesis considered (Fig. 2), at least 15 frames of different sections were analyzed in this way.

**Results**

**Morphologic observations.** Primary embryos undergo no morphologic changes other than a slight increase in size until Day 12, when the newly formed secondary embryos become visible. Secondary embryos develop mainly in groups on the hypocotyl of the mother embryo, with no intermediate callus formation. They pass through typical globular, heart, and torpedo stages before reaching the cotyledonary stage, which for the first secondary embryos to be formed occurs after 3 wk culture of the mother embryo. After 6 to 7 wk culture, 70% of the primary embryos produce secondary embryos, at an average rate of 10 per productive embryo. Secondary embryos over 7 mm long can be isolated and used for a further embryogenic cycle (Fig. 3 A) or transferred to germination medium to develop into plantlets (20). Smaller secondary embryos have much less embryogenic capacity, and tend to suffer necrosis in both embryogenic and germination media.

Anatomical observations. At the time of culture (Day 0), somatic embryos possessed well-defined closed root and shoot axes with vascular systems connected via the hypocotyl. Their cotyledons were thin, composed of epidermis and cotyledonary parenchyma penetrated by vascular bundles, and unlike those of zygotic embryos (unpublished results) had no protein reserves and scant starch deposits limited to the apical region. The hypocotyl consisted of medulla, a vascular system whose most mature region contained xylem cells with spiral thickenings, a 12- to 14-layer cortex and the epidermis (Fig. 3 B). The cortex was made up of isodiametric parenchymatous cells (the differentiated subepidermal cells of Fig. 1) with a large central vacuole and a thin peripheral cytoplasm with low protein content; their nucleus-to-cell area ratio was 11.1%. Differentiated epidermal cells were smaller (Fig. 1), with a nucleus-to-cell ratio of 14.1%. Both epidermal and subepidermal layers contained a number of cells with larger nuclei and dense cytoplasm which we assume were relatively undifferentiated (Fig. 3 B); they were most numerous near the root pole.

After 2 days of culture, the number of relatively undifferentiated cells had increased in the epidermis and first and second layers of the cortex (the subepidermis). The mean nucleus-to-cell ratio of these cells was 24.8% in the epidermis and 23.3% in the subepidermis (Fig. 1); as well as relatively dense cytoplasm and a large nucleus with prominent nucleoli, they also contained small vacuoles and often exhibited mitosis. These cells were considered as being involved in the embryogenic pathway, and were referred to as “preembryogenic cells” (Fig. 3 C). In the epidermis, most observed divisions were anticlinal (though cell patterns suggested that periclinal divisions also occurred) (Fig. 3 D). The majority of subepidermal cells divide periclinally although various planes of division have also been observed. The size of the dedifferentiating regions, increasing with time, differed from one mother embryo to another, being restricted to the vicinity of the root pole in some and stretching the whole length of the hypocotyl in others.

After 8 to 10 days of culture, repeated divisions and dedifferentiation of preembryogenic cells had given rise to what were considered as true embryogenic cells that contained a large, densely Feulgen-stained nucleus (Fig. 3 E) with two to three large nucleoli, and densely stained protein-rich cytoplasm (Fig. 3 F) with small starch grains. The nucleus-to-cell ratio in both epidermis and subepidermis was about 31%, a figure similar to that observed in typical shoot meristem cells belonging to the primary embryo (Fig. 1). The embryogenic cells developed into a superficial meristem that, after repeated cell division, formed a pattern of indentations and protruberances of embryonic tissue (Fig. 4 A, B). After about 12 days of...