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VASCULARIZATION OF ZYGOTIC AND SOMATIC EMBRYOS OF *ARABIDOPSIS THALIANA*

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Somatic embryos were induced from immature zygotic embryos of *Arabidopsis thaliana* cultured on Gamborg basal medium (B5) supplemented with dichlorophenoxyacetic acid (2,4–D) at 5 μM l^{-1}. The somatic embryos were of unicellular origin. The sequence of divisions and the orientation of newly formed walls resembled the Onagrad pattern of early embryogenesis. Histological studies revealed no connection between the somatic embryos and explant tissue. In contrast to zygotic embryos, in late heart-shaped somatic embryos both sieve and tracheary elements were present. The sieve elements that formed in somatic embryos were characterized by larger plates than normal sieve elements observed in seedlings. Typical features of the tracheary elements in somatic embryos were irregular shape and thickening of the secondary walls.

Key words: *Arabidopsis thaliana*, zygotic embryos, somatic embryogenesis, vascularization.

INTRODUCTION

Somatic embryogenesis is a regeneration system that leads to the formation of bipolar structures resembling zygotic embryos from somatic cells. During this process, induced under in vitro conditions, somatic cells acquire embryogenic potential. Moreover, somatic embryos pass through the subsequent stages characteristic for zygotic embryogenesis (von Arnold et al., 2002). In contrast to zygotic embryogenesis, somatic embryogenesis can easily be observed in vitro. Additionally, plant regeneration via direct somatic embryogenesis limits somaclonal variation, especially when somatic embryos are of unicellular origin (Karp, 1988).

Arabidopsis thaliana is a well-known model plant for classical and molecular genetics, plant development and experimental embryology. Somatic embryogenesis has been achieved in *Arabidopsis thaliana* from protoplasts (O'Neill and Mathias, 1993) as well as from mesophyll cells (Luo and Koop, 1997). Zygotic embryos showed the greatest ability to undergo somatic embryogenesis (Wu et al., 1992; Pillon et al., 1996). An efficient system for direct somatic embryogenesis has been developed (Gaj, 2001a). Systems for *Arabidopsis* somatic embryogenesis have become an attractive model for detailed analyses of the cellular and genetic basis of somatic embryogenesis (Ikeda-Iwai et al., 2002; Gaj et al., 2005; Kurczyńska et al., 2007). Several authors have emphasized differences in morphology and histology between zygotic and somatic embryos at corresponding stages of development (Čellárová et al., 1992; Erdalská and Sýkorová, 1997).

This paper compares the vascularization patterns of zygotic and somatic embryos of *Arabidopsis thaliana*.

MATERIALS AND METHODS

ZYGOTIC EMBRYOGENESIS

Zygotic embryos at different stages of development were isolated from the seeds of diploid plants of *Arabidopsis thaliana* (L) Heynh. ecotype Columbia. For study of the pattern and timing of embryo vascularization, ~100 seeds were inoculated in Petri dishes on moist blotting paper in three replicates. Material for histological analysis was collected every hour for three days.

SOMATIC EMBRYOGENESIS

Plants of *Arabidopsis thaliana* were cultured in test tubes on MS medium (Murashige and Skoog, 1962) containing 3% sucrose and 0.8% agar. Sterile plants

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were cultured in order to establish an embryonic culture from zygotic embryos. Immature embryos at the bent-cotyledon stage were isolated from sterile green siliques under sterile conditions and used as an explant source. Five explants were cultured in each Petri dish (60 mm diam.) filled with basal B5 medium (Gamborg et al., 1968) containing 5 μM l^{-1} 2,4-D and 20 g/l sucrose, solidified with 2 g/l Phytagel (Gaj, 2001); 100 explants were cultured in three replicates. All cultures were kept under stable conditions with a 16 h photoperiod at 21°C and 70% humidity. After 21 days of culture, explants undergoing somatic embryogenesis were transferred to MS medium supplemented with 20 g/l sucrose, 100 mg/l inositol, vitamins and 2g/l Phytagel. The ability of immature embryos to undergo somatic embryogenesis was estimated as the percentage of zygotic embryos producing at least one somatic embryo.

HISTOLOGY

Zygotic embryos, somatic embryos and germinated seeds at different developmental stages were fixed in 70% ethanol and clarified in 50% lactic acid for 90 min for histological analysis. At least 20 of each of the above were studied. Only somatic embryos showing the greatest similarity to zygotic embryos at the corresponding stages were taken for histological analysis. To visualize callose in sieve plates, the material was stained with 5% fuchsin and methyl green for light microscopy, and with 0.005% solution of aniline blue for fluorescence microscopy. The presence of the secondary cell wall in tracheary elements was confirmed by staining with either phloroglucin with 48% sulphuric acid or the fluorescent dye DAPI. The natural fluorescence of lignified secondary walls was detected by means of fluorescence microscopy.

Zygotic and somatic embryos at corresponding stages were fixed in 3% solution of glutaraldehyde in sodium phosphate buffer (pH 7.2) for 4 h, rinsed three times in 0.1 M phosphate buffer, dehydrated in a series of alcohol and propylene oxide, and embedded in Epon resin. Semi-thin sections (~1 μm) were cut with a glass knife on an ultramicrotome (Tesla), and some of these were stained with a 1:1 mixture of azure B and methylene blue. To detect callose, semi-thin sections were treated with 0.5% periodic acid, washed in running water for 10 min and air-dried. After staining in Schiff reagent for 60–75 min, slides were rinsed in tap water for 10 min and stained with toluidine blue for 4–5 min. Following dehydration through an ethanol series (20–100%) the slides were rinsed in isopropanol and mounted in DPX. The sections were analyzed under a light microscope (Leica).

RESULTS

VASCULARIZATION OF ZYGOTIC EMBRYOS

The first provascular cells were visible in late globular embryos. Distinct layers were observed in globular embryos consisting of more than 10 cells (Fig. 1a). The outer monolayer protoderm was composed of small flattened cells. Under the protoderm, cells were arranged in two clearly visible tiers. The lower tier, adjoining the suspensor, consisted of cells elongated on their apical-basal axis; these were precursors of procambial cells. In heart-stage embryos with a cotyledon primordial, these cells marked the axis of the embryo (Fig. 2a). The procambial cells increased in number and formed a strand in the torpedo-stage embryo that extended from root to hypocotyl (Figs. 1b, 2b). The procambial strand in the embryo root was built of 5 or 6 cells, and the one in the hypocotyl of 9 cells. In the bent-cotyledon stage of the embryo the procambial strand spread to 2/3 the length of the cotyledon (Fig. 2c). The width of this strand differed in various parts of the embryo: widest in the hypocotyl and built of 10 cells, and consisting of 7 to 9 cells in the embryo root (Fig. 1c). In the basal part of the cotyledon there were 4 procambial cells, and in the distal part only one cell. The mature embryo was characterized by the presence of procambial tissue in all organs (Fig. 2d). In the cotyledon the procambial strand branched into primary and secondary ramifications. The main strand consisted of 4–6 cells. Vascularization processes in the mature zygotic embryo were arrested in the procambial state.

VASCULARIZATION IN GERMINATED EMBRYOS

Three distinct stages were distinguished during germination: (1) the appearance of roots (Fig. 2e), (2) the formation of root hairs (Fig. 2f) and (3) the appearance of released seedlings (Fig. 2g). The first roots protruded from the seed coat 15 h after the beginning of germination. At 39 h of germination the roots produced macroscopically visible root hairs, and the first free seedlings became apparent after 42 h. Those three visible events of germination were strictly correlated with the successive stages of vascularization. The first vascular bundle elements detected were protophloem elements, observed when free roots appeared (Fig. 2f). At this stage, sieve tube elements forming a single thread were found in the root, and sparse phloem elements in the upper part of the hypocotyl. When root hairs were visible, two opposite-running strands of protophloem usually developed in the root and hypocotyl (Fig. 2f). The sieve-tube members were connected to each other by sieve plates with various depositions of callose (Fig. 1d). The first phloem elements also appeared in the basal part of

the cotyledon. At this stage of germination, the first-formed xylem elements with annular wall thickenings were initiated in some roots. Their wall was not lignified at this stage. After 43 h of germination, xylem elements with annular wall thickenings formed two parallel threads in the root, hypocotyl and cotyledon, and the processes of lignin incrustation started. After 48 h, additional threads of xylem and some tracheary elements with helical wall thickening were observed in the hypocotyl (Fig. 1e). Regular vascular bundles consisting of phloem and xylem were observed in free seedlings after 63 h of germination (Fig. 2g).

SOMATIC EMBRYOGENESIS

Immature zygotic embryos showed higher than 70% capability of somatic embryogenesis. During the first four days of culture, only enlargement of explants was observed. Swelling occurred on day 6 of culture, especially in the shoot apical meristem region and hypocotyl (Fig. 1f). Additionally, in the region of the shoot apical meristem, unicellular structures resembling trichomes appeared, and short, irregularly shaped tracheary elements were present below the cotyledonary node of explants (Fig. 1g). The next changes in the appearance of explants were observed after 9 days of culture. Some cells of the hypocotyl and cotyledon started to dedifferentiate into callus, resulting in the splitting and deformation of these parts of the embryo (Fig. 1h). Groups of meristematic cells were visible among these callus cells. At the same time, brilliant globular structures and scant callus tissue were observed on shoot meristems. The PAS reaction revealed the presence of callose on the thick wall, enveloping these structures and thus isolating them from the surrounding cells. The cell arrangement in these structures strictly reflected the cell pattern in the early globular stage of an embryo of the Onagrad type (Fig. 1i). Starch grains were deposited in the adjacent cells. On day 15 of culture, structures resembling heart-stage embryos were present, but they were larger than their zygotic counterparts (Figs. 1j, 2h). Simultaneously, regenerated roots, shoot and structures resembling globular-shaped zygotic embryos formed from the callus in hypocotyls and on the abaxial side of cotyledons. These somatic embryos had no connection to the remaining part of the callus tissue and consisted of randomly arranged cells. On day 19 of culture, somatic embryos at the torpedo stage with well-developed cotyledons were observed in the shoot apical meristem region (Fig. 2i). Somatic embryos originating from hypocotyls and cotyledons developed later than those induced in the apical meristem region. Interestingly, ~80% of the regenerated somatic embryos had cotyledons atypical in number and shape. The number of cotyledons varied from one to two or more. Cotyledons were completely fused and were fan- and cornet-shaped (Fig. 1k, l, m).

VASCULARIZATION OF SOMATIC EMBRYOS

During early somatic embryogenesis, that is, at the early globular stage, the arrangement of cells was well-defined and resembled the arrangement observed in zygotic embryos at the same stage. Somatic embryos at the heart stage were bigger than zygotic embryos at the same stage (Fig. 2h). In the central part of these embryos, a few cells differed from the neighboring ones and became slightly enlarged. Their shapes were typical of procambial cells. In heart-shaped somatic embryos before day 15 of culture, neither sieve nor tracheary elements were present. The first sieve elements appeared in embryos at the torpedo-shaped stage on day 15 of culture (Fig. 2i). On day 18 of culture, all regenerated somatic embryos showed the presence of sieve elements, though only 1% of them had tracheary elements. One day later, both types of vascular elements were formed in all somatic embryos (Figs. 1n, o, 2j). The sieve elements were characterized by sieve plates arranged at different inclinations and were larger than in zygotic embryos (Fig. 1n, o). Tracheary elements formed continuous threads and differed in diameter in various places (Fig. 1p). Thus the shapes of these elements were rather similar to the tracheary elements developing in the callus. The pattern of the secondary wall of the tracheary elements was either annular or helical, usually transforming continuously from one to the other (Fig. 1p). These elements showed a positive reaction to phloroglucin with concentrated sulphuric acid, indicating that their secondary walls were lignified.

Somatic embryos showing morphologically atypical cotyledons also tended to form additional tracheary elements in the distal part of the hypocotyl. These elements were parallel to the vascular bundles, differed in length, and were all completely lignified. In some cases the vascular strands were interrupted by procambial cells.

DISCUSSION

The first precursors of sparse procambial cells are present in the zygotic embryo of *Arabidopsis thaliana* at the late globular stage and are composed of one tier. Simultaneously with changes in the shape of the zygotic embryo, the procambial system is established. Within mature zygotic embryos, a continuous procambial system extended the length of the hypocotyl-root axis and the cotyledons, where the procambial strand branched into two lateral strands. Mature sieve tube elements or vascular elements were not present in zygotic *Arabidopsis* embryos. Our findings are in line with those of Busse and Evert (1999). Sundberg (1983), on the other hand, found evidence of the presence of pro-

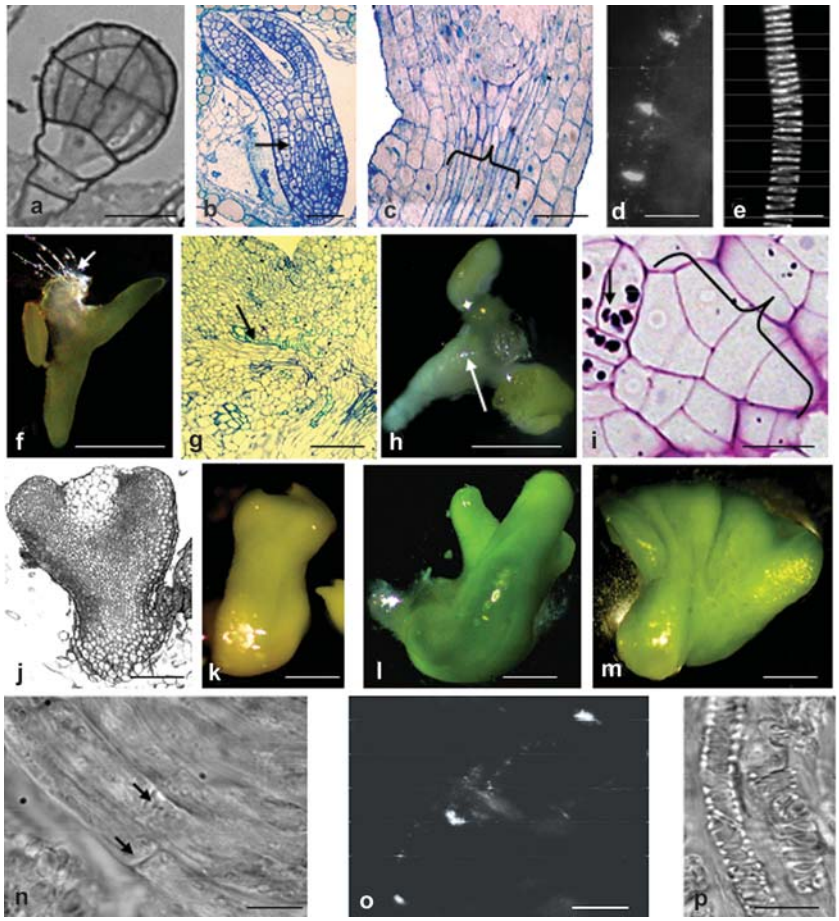

Fig. 1. Zygotic embryos of *Arabidopsis thaliana* (**a–d**) and somatic embryos (**f–p**). (**a**) Globular embryos with two tiers. Bar = 5 μm, (**b**) Torpedo-shaped embryo with visible procambial strand (arrow). Bar =5 μm, (**c**) Procambial strand in cotyledon node of bent-cotyledon embryos. Bar = 5 μm, (**d**) Sieve-tube members with sieve plates in root of embryo after 15 h of germination. Bar = 5 μm, (**e**) Tracheary element with lignified annular thickenings in root of embryo after 39 h of germination. Bar = 5 μm, (**f**) Enlargement of zygotic embryos after 6 days of culture, trichome-like structures (arrow) visible in apical region. Bar = 0.5 mm, (**g**) Tracheary elements (arrow) originated in cotyledonary node of zygotic embryos after 6 days of culture, (**h**) Originated callus in cotyledon node region of explant after 9 days of culture. Bar = 0.5 mm, (**i**) Early globular stage of somatic embryo observed after 9 days of culture, in adjacent cell starch grains visible (arrow). Bar = 5 μm, (**j**) Somatic embryo at heart-shaped stage. Bar = 5 μm, (**k–m**) Atypical number and morphology of cotyledons of somatic embryos, Bar = 0.5 mm, (**k**) Somatic embryo with fused cotyledon, (**l**) Somatic embryo with three cotyledons, (**m**) Somatic embryos with fan-shaped cotyledons, (**n**) First sieve elements (arrows – sieve plates) in somatic embryo after 18 days of culture – light microscopy. Bar = 5 μm. (**o**) Sieve plate in somatic embryo after 19 days of culture – fluorescence microscopy. Bar = 5 μm. (**p**) Tracheary elements with irregular wall thickenings in somatic embryo after 19 days of culture. Bar = 5 μm. a, b, c, g, j – semi-thin sections stained in 1:1 mixture of azure B and methylene blue; I – semi-thin section stained by PAS method; d, o – aniline blue staining for fluorescence microscopy; n – aniline blue staining for light microscopy, e – natural fluorescence of lignified secondary wall of tracheary element; p – clarified somatic embryo stained in phloroglucin with 48% sulphuric acid, short tracheary elements of different diameter visible along length.

tophloem elements in zygotic embryos of *Populus deltoides*.

A well-defined vascular system developed in zygotic embryos of *Arabidopsis thaliana* during seed germination. The first sieve tube elements were present 15 h after the beginning of germination, when the root protruded from the seed coat. The first tracheary elements developed 39 h after germination, coinciding with the appearance of root hairs. Busse and Evert (1999) described a similar vascularization process in the *Arabidopsis thaliana* embryo, and emphasized that the formation of the roots and the first sieve tube elements was synchronized in germinated embryos.

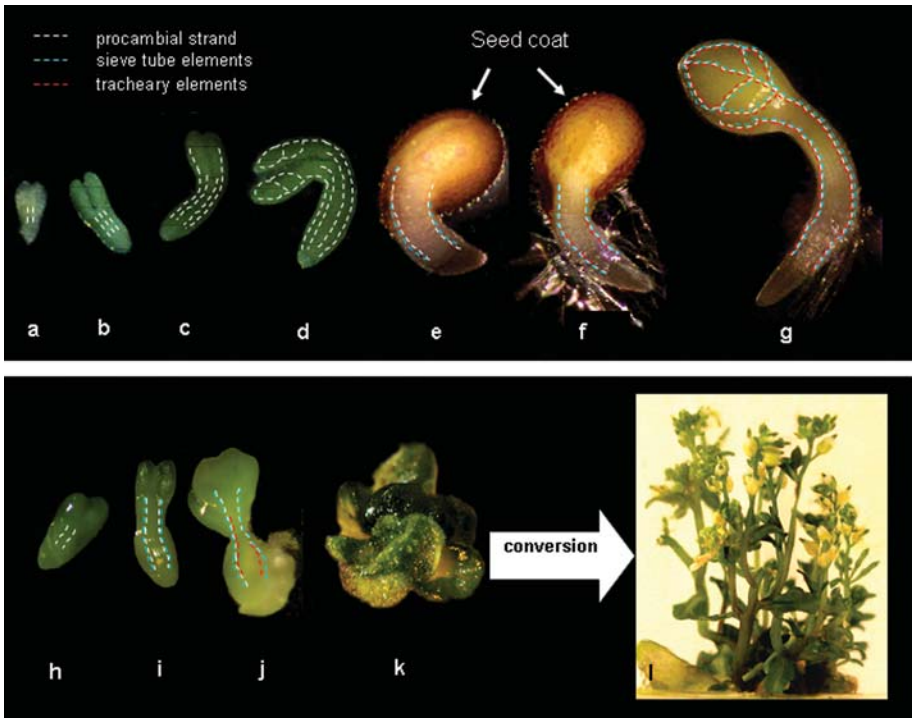

Fig. 2. Vascularization in zygotic embryos during their development (**a**–**g**) and in somatic embryos (**h-l**).

Our experiment showed somatic embryogenesis to be a continuous and asynchronous process. New somatic embryos originated de novo during the whole culture process. All obtained somatic embryos were bipolar structures, some of which originated directly from shoot meristem and from the adaxial side of the cotyledon on day 9 of culture. These somatic embryos were formed with about 80% frequency. They appeared as a group of 3–4 cells covered by a thick common wall. The orientation of cell walls displayed a special arrangement resembling that observed in their zygotic counterparts during early embryogenesis. Such an organization suggests the unicellular origin of these structures. Some groups of cells formed in callus were separated from each other by a thick common wall, but they did not display such organization. The cell wall orientation was random. Thus they were probably of multicellular origin. Several authors have described the unicellular origin of somatic embryos of various species (Barciela and Vietez, 1993; Quiroz-Figueroa et al., 2002; Mandal and Dutta Gupta; 2003). In a study of somatic embryogenesis in *Arabidopsis thaliana*, Gaj (2001) concluded that the origin of most of the somatic embryos was unicellular; Kurczyńska et al. (2007) suggested both uni- and multicellular origins. Loiseau et al., (1998) and Griga (2002) found the origin of somatic embryos to be multicellular in *Pisum sativum*.

The somatic embryos at late globular stage we obtained here were similar to zygotic embryos at the same stage. The differences in size, morphology and anatomy between somatic and zygotic embryos were pronounced in more advanced stages of somatic embryos. More than 80% of all somatic embryos exhibited abnormalities, mainly involving cotyledons, confirming earlier observations (Gaj, 2001a). Different morphological aberrations have been observed in somatic embryos of *Cicer arietinum* (Suhasini et al., 1997), *Pisum sativum* (Griga, 2002), *Glicyne max* (Fernando et al., 2002) *Schlumbergera trunctata* (Al-Dein Al-Ramamneh et al., 2006) and *Arabidopsis thaliana* (Luo and Koop, 1997). In those studies the somatic embryos were usually much larger than the zygotic ones.

A typical feature of zygotic embryos was the presence of a vascular system in the form of procambial strands during the whole course of embryogenesis. This was not the case in somatic embryos, in which vascular tissue first occurred at the late heart-shaped stage. Kärkönen (2000) observed the spontaneous formation of tracheary elements in somatic embryos at the cotyledonary stage and the lack of such elements in zygotic embryos. Histological analyses of other species have shown differentiated vascular systems in precocious somatic embryos (Quiroz-Figueroa et al., 2002; Al-Dein Al-Ramamneh et al.,

2006). This rapid vascularization of somatic embryos may have resulted from the presence of exogenous auxin in the medium. Confirmation of this suggestion might be found in the appearance of tracheary elements in immature zygotic explants after six days of incubation on medium supplemented with 2,4-D in this experiment. The composition of the medium is crucial for inducing embryogenesis. Kärkönen (2000) noted that the presence of abscisic acid in the medium inhibited induction of tracheary elements.

Several authors have reported that the morphological and physiological characteristics and developmental stages of somatic embryos are similar to those of zygotic embryos (Kärkönen, 2000; von Arnold et al., 2002; Griga, 2002; Ikeda and Kamada, 2005). However, they observed some differences in origin, development and morphology between somatic and zygotic embryos. These may have been due to the conditions of culture in vitro, and genetic changes in the plant material cannot be ruled out, especially when the somatic embryos develop with an intervening callus phase.

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