Protoplasma (2000) 211: 217–224

PROTOPLASMA

Structure and development of somatic embryos formed in *Arabidopsis thaliana pt* mutant callus cultures derived from seedlings

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Received July 8, 1999
Accepted October 25, 1999

Summary. Seeds of the *Arabidopsis thaliana* mutant primordia timing (*pt*) were germinated in 2,4-dichlorophenoxyacetic acid-containing liquid medium. The seedlings formed somatic embryos and nonembryogenic and embryogenic callus in vitro in a time period of approximately two to three weeks. Embryogenesis and callus formation were monitored with respect to origin, structure, and development. Ten days after germination globular structures appeared in close vicinity of and on the shoot apical meristem (SAM). Somatic embryos formed either directly on the SAM region of the seedling or indirectly on embryogenic callus that developed at the SAM zone. Globular structures developed along the vascular tissue of the cotyledons as well, but only incidentally they formed embryos. Upon deterioration, the cotyledons formed callus. Regular subculture of the embryogenic callus gave rise to high numbers of somatic embryos. Such primary somatic embryos, grown on callus, originated from meristematic cell clusters located under the surface of the callus. Embryos at the globular and heart-shape stage were mostly hidden within the callus. Embryos at torpedo stage appeared at the surface of the callus because their axis elongated. Secondary somatic embryos frequently formed directly on primary ones. They preferentially emerged from the SAM region of the primary somatic embryos, from the edge of the cotyledons, and from the hypocotyl. We conclude that the strong regeneration capacity of the *pt* mutant is based on both recurrent and indirect embryogenesis.

Keywords: *Arabidopsis thaliana*; Primordia timing; Somatic embryogenesis; In vitro culture; Structure; Microscopy.

Abbreviations: 2,4-D 2,4-dichlorophenoxyacetic acid; DIC days in culture; SAM shoot apical meristem.

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Introduction

The formation of embryos on in vitro cultured somatic plant tissues has been studied for years and has become a powerful tool in plant breeding and in the study of embryogenesis (Zimmermann 1993). Somatic embryos can be obtained under controlled conditions, at defined stages, and in large numbers, which facilitates manipulation and biochemical and molecular analysis. In addition, somatic embryogenesis has become an important model system to study somatic cells acquiring new fates with respect to a change in identity and embryogenic potential (for a review, see, Mordhorst et al. 1997).

Although carrot has served as a model plant for a long time (de Vries et al. 1988, Choi and Sung 1989), in recent years attention has also been paid to establish embryogenic in vitro cultures from *Arabidopsis thaliana*. This small weed from the mustard family has been studied with classical genetics for over fifty years, because of its small genome size, but last decennium it has become widely used as a model organism in plant molecular genetics and developmental biology (for reviews, see Meyerowitz 1989, 1998). Regenerating cultures of *A. thaliana* were obtained from various sources such as leaves and protoplasts (O'Neill and Mathias 1993, Luo and Koop 1997). However, organogenesis occurred frequently in these cultures, while the yield of somatic embryos was low. The use of immature plant tissues, i.e., zygotic embryos, improved the regeneration capacity of the in vitro cultures (Sangwan et al. 1992, Wu et al. 1992, Huang and Yeoman 1995, Pillon et al. 1996, Luo and Koop 1997).

The embryogenic potential of the cultures has been further improved by the use of mutants that exhibit enhanced regeneration in vitro such as *amp1* (Chaudhury et al. 1993) or *pt*, which has been described recently by Mordhorst et al. (1998). The *pt* mutation is recessive and allelic to häuptling (*hpt*) (Jürgens et al. 1991), constitutive photomorphogenic2 (*cop2*) (Hou et al. 1993), and altered meristem programming1 (*amp1*) (Chaudhury et al. 1993). It was originally identified in genetic screens for the formation of shoot apical meristems (SAMs). Mutations in the *PRIMORDIA TIMING* gene result in a pleiotropic phenotype, which is characterized by a broader SAM at the embryonic and seedling stages, by polycotyly, and by a higher number of rosette leaves as compared to the wild type (Conway and Poethig 1997, Mordhorst et al. 1998). In vitro cultures of immature embryos obtained from the *pt* mutant were more efficient and reproducible in the formation of somatic embryos than those obtained from at least 11 other ecotypes. In addition, in vitro cultures from seedlings were embryogenic only with the *pt* mutant (Mordhorst et al. 1998). It was found that changes in the SAM, leading to increased numbers of noncommitted embryogenic cells, are responsible for the facilitated establishment of somatic embryogenesis in the *pt* mutant. However, the increased embryogenic capacity might also be caused by a higher cytokinin content, as was observed earlier (Chaudhury et al. 1993).

In order to study in more detail the induction and developmental processes occurring during the early stages of somatic embryogenesis in this system, we decided to monitor carefully embryo initiation and development by morphological and histological means, using light as well as scanning electron microscopy. It worked out that next to direct embryogenesis also the process of secondary or recurrent embryogenesis, in which new embryos are formed on other embryos (see, e.g., Puigderrajols et al. 1996, Parra and Amo-Marco 1998, das Neves et al. 1999), is an important factor adding to the high regeneration efficiency of the *pt* cell line in in vitro culture, as was already suggested by Mordhorst et al. (1998).

Material and methods

Initiation of embryogenic callus cultures

Plants of the *Arabidopsis thaliana* Hey. *pt* mutant were grown in a phytotron at 25 °C with 16 h light and 8 h darkness periods under illumination with TL 050 W184 HF super 80 lamps (Philips), and dry seeds were collected (Medford 1992, Mordhorst et al. 1998). Seeds were first surface sterilized with ethanol and bleach, rinsed, and then imbibed in a liquid nutrient medium at 4 °C for 2 days in order to break dormancy. The nutrient medium contained the basal salts and vitamins according to Murashige and Skoog (1962) supplemented with 2% sucrose and 10 mM 2-morpholinoethanesulfonic acid (Merck) and 1 mg of 2,4-dichlorophenoxyacetic acid (2,4-D) (initiation medium) per ml. Seeds were germinated and seedlings were obtained by culture in liquid initiation medium on a rotary shaker at 100 rpm with 16 h light and 8 h darkness periods at 25 °C, essentially according to Mordhorst et al. (1998). Two weeks after the onset of the culture the medium was refreshed. Somatic embryos and callus aggregates appeared on seedlings two to three weeks after the initiation of the culture.

Selection of embryogenic callus in liquid medium

Three weeks after the initiation of the cultures, callus was selectively subcultured. Green embryogenic and white nonembryogenic parts of the callus were separated from each other by breaking the tissue with a pipette (volume, 5 ml) without a tip (Sarstedt, Nürmbrecht, Federal Republic of Germany). The callus pieces were transferred to fresh liquid initiation medium. In this way both embryogenic and nonembryogenic callus culture lines were established.

The embryogenic callus culture was maintained by weekly subculture for more than two years, provided that the proportion of green parts to white tissue did not decrease below 80%. The nonembryogenic culture line was maintained without selection. The white callus was just regularly diminished in size every fortnight and the medium was refreshed weekly.

Embryo formation and germination

To study the outgrowth of somatic embryos on embryogenic callus, small pieces of callus were placed on nutrient medium without 2,4-D, either liquid or solidified with 1% technical agar (Difco). On both media the existing embryos further developed and germinated, but in the liquid medium the development was arrested after the root had formed and the material had to be transferred to solid medium for further growth.

Procedures for morphological obser ation

The development and germination of somatic embryos on solid medium were daily monitored with a dissecting microscope (MZ8, Leica). Images were recorded with a 3-CCD camera (WV-E550, Panasonic), processed with Adobe Photoshop, and printed with a Kodak XLS 6800 PS printer.

In addition, scanning electron microscopy (SEM) was used. Somatic embryos were fixed in 2.5% glutaraldehyde in 0.05 M cacodylate buffer (pH 7.2) for 2 h at room temperature, dehydrated in an ethanol series ranging from 10% to 100% during 3 h, dried in a critical-point drier over CO_2 (Balzers Union CPD 020), and sputter-coated with gold and palladium with a Polaron 5100 coating unit. Observations were carried out with a JSM-5200 SEM (JEOL) at 10 kV. SEM micrographs were recorded on Ilford FP4 Plus film.

Roots and the delimitation of the radicle and hypocotyl were visualized by staining somatic embryos with 1% Procian Blue MX-R (Serva, Heidelberg, Federal Republic of Germany) in distilled water according to Dubrovsky and Tykarska (1995).

Procedures for histological obser ation

For histological observations, aggregates of embryogenic callus were sampled at various developmental stages. The clusters were fixed

like the samples prepared for the SEM observations, but after dehydration in the ethanol series they were embedded in Technovit 7100 (Heraeus Kulzer GmbH, Wehrheim/Taunus, Federal Republic of Germany). Semithin sections (3 μm) were cut with a rotary microtome (HM 340, Microm), mounted, air dried, and stained with 0.5% toluidine blue in 1% borax at room temperature. Sections were examined with a light microscope (Nikon, Optiphot), and images were recorded with a 3-CCD camera (DXC-950P, Sony). Pictures were printed with a Kodak printer XLS 6800 PS.

Results

Initiation culture in liquid medium with 2,4-D

In vitro cultures were started from imbibed *pt* mutant seeds. The seedlings grown on liquid initiation medium formed embryogenic and nonembryogenic callus and somatic embryos within 3 weeks. The initiation and development of both embryogenic callus and somatic embryos are summarized in Figs. 1 and 2.

Seedlings changed their original shape after 5–8 days in culture (DIC). Transparent protrusions became visible (Fig. 1 a). The hypocotyl and abaxial sides of the cotyledons became swollen (Fig. 1 b; see also Fig. 2 a). Strong cell proliferation and the formation of green protuberances indicated a high growth activity in the outer ring of the SAM (Fig. 2 a). Concomitantly with the above mentioned phenomena, elongated cells were released into the medium in increasing rates between 5 and 10 DIC (Fig. 1 d). The longitudinal shape of these cells and their highly vacuolated state point to their callogenic origin. Swelling of the seedling hypocotyl became more prominent in this period. The green cotyledons formed white, unorganized tissue patches. The cotyledons etiolated, degenerated and formed callus with time (Fig. 1 d, f), but their tips remained green. Although deterioration of the seedlings continuously became apparent from 6 DIC onwards, their shape was still recognizable after 12 days (Fig. 1 e). The toluidine blue-stained Technovit sections showed that globular meristematic structures appeared in or on various organs such as the disorganized cotyledons, the SAM region, and, sporadically, the hypocotyl. The globular cell clusters on the cotyledons were lined along the vascular tissue and seemed to originate from the meristele. The root of the seedling remained unchanged.

The changes in the SAM region of the seedling, cultured on initiation medium, did not resemble the normal pattern of primordia formation observed when seedlings developed on paper moistened with water. First, the SAM region of the seedling enlarged fast, became dome-shaped, and consisted of actively proliferating cells which sometimes formed globular structures instead of leaf primordia at 14 DIC (Fig. 2 a). In vivo, the first leaf primordia domes are formed in planes alternating with those of the cotyledons, but on the initiation medium, protuberances and cell domes were formed in a random fashion (Fig. 2 a). Alternatively, some seedlings initiated one or two leaf rosettes, but not in the phyllotactic spiral order. Cotyledons degenerated and the first leaves stopped development, whereas groups of somatic embryos and/or clusters of embryogenic callus arose from the central zone of the SAM (Fig. 1 d, f). It was also often observed that the outer zone of the seedling SAM proliferated in such a way that a fused collar structure was formed. In some cases embryos were formed on the collar.

From 14 DIC onwards the original shape of the seedlings was hardly recognizable. Figure 1c shows such a seedling culture at 14 DIC. A stalklike structure arose from the central zone of the SAM carrying embryogenic callus with somatic embryos. At 16 DIC, seedlings had changed into structures carrying somatic embryos and callus (Fig. 1 f). Some regions of this callus formed somatic embryos after further culture.

From these observations we conclude that both direct and indirect embryogenesis occurs in the SAM region.

Maintenance culture in liquid medium with 2,4-D

At 21 DIC, the seedlings consisted of clusters of nonembryogenic white parts, potentially embryogenic green parts, parts that were a mixture of both, and somatic embryos directly formed on the SAM. At this stage, the embryogenic and nonembryogenic parts were selected and weekly subcultured. The embryogenic capacity and fate of the calli depended on the proportion of white and green tissue within a callus. Embryonic callus tissue was characterized by faster growth compared to nonembryonic callus. Nonembryogenic callus aggregates were distinguished from potentially embryogenic callus tissue by the following characteristics: a friable structure, lack of organization, hairs protruding on the surface, and whitish to slightly transparent and elongated cells. In contrast, embryogenic callus tissue was characterized by a more tight tissue, more organized cell growth without trichomes, a green color, faster growth compared to nonembryogenic callus, and, eventually, the presence of somatic embryos. A highly embryogenic callus culture

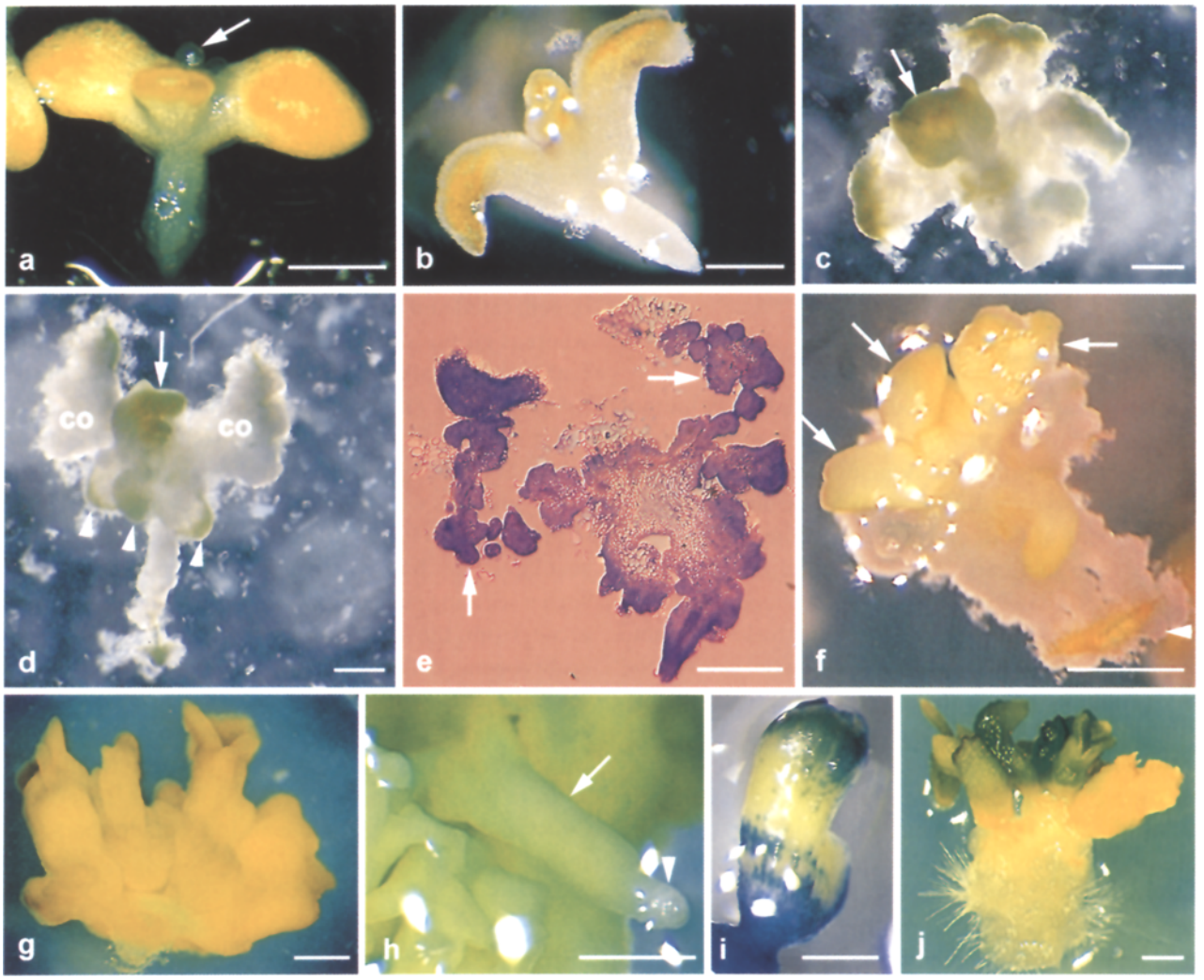

Fig. 1. **a** Tricot *pt* mutant seedling 5 DIC showing a protrusion (arrow) in the SAM region. **b** Seedling after 6 DIC with swollen hypocotyl and cotyledons. **c** Seedling after 14 DIC showing terminal embryo formation (arrow) in the inner zone of the SAM. The inner zone is surrounded by a green fused collar region (arrowhead). Note the longitudinal, callogenic cells and cell clusters which are suspended into the medium. **d** Deformed seedling at 10 DIC showing the three first leaves (arrowheads), somatic embryos (arrow) on a stalk emerging from the SAM and many white callogenic tissue patches along the cotyledons (*co*). **e** Toluidine blue-stained semithin Technovit section of a seedling after 12 DIC showing callus formation and zones of regeneration (arrows). The original form of the seedling is still recognizable, i.e., root points downward and cotyledons to upper right and left. **f** Deformed seedling with newly formed somatic embryos (arrows) in the SAM region at 16 DIC. The arrowhead points to the tip of a cotyledon where callus formation has started. **g** Mature cluster of partially fused, somatic torpedo-shaped embryos after 26 days in liquid medium. **h** Callus with an inverted somatic embryo (arrow). 8 days after transfer to liquid medium without 2,4-D, root formation occurred (arrowhead). **i** Somatic embryo stained with Procian Blue to visualize the border between the unstained hypocotyl and the blue-stained root. Also some staining is visible on the edges of the fused cotyledons and on the callus tissue. **j** Range of embryo phenotypes with varying colors, shapes, surfaces and degrees of cotyledon fusion, 5 days after transfer to solid medium. Bars: a–d and f–j, 500 µm; e, 50 µm

was maintained by subculture for more than two years.

Histological analysis of the embryogenic callus showed that the callus formed clusters of meristematic cells which were mostly formed inside the callus (Fig. 2e). These clusters probably originated from single cells, as could be deduced from their shape and the pattern of the common cell walls. Some meristematic clusters were shaped like globular embryos, sometimes with a suspensor-like structure resembling those from zygotic embryos. Late heart-shaped stages and young torpedo-shaped embryos broke through the outer callus cell layers and appeared at the surface of the callus aggregates. See also Fig. 2b, showing a well developed somatic embryo, the cotyledons of which arise above the surface of the callus. Somatic

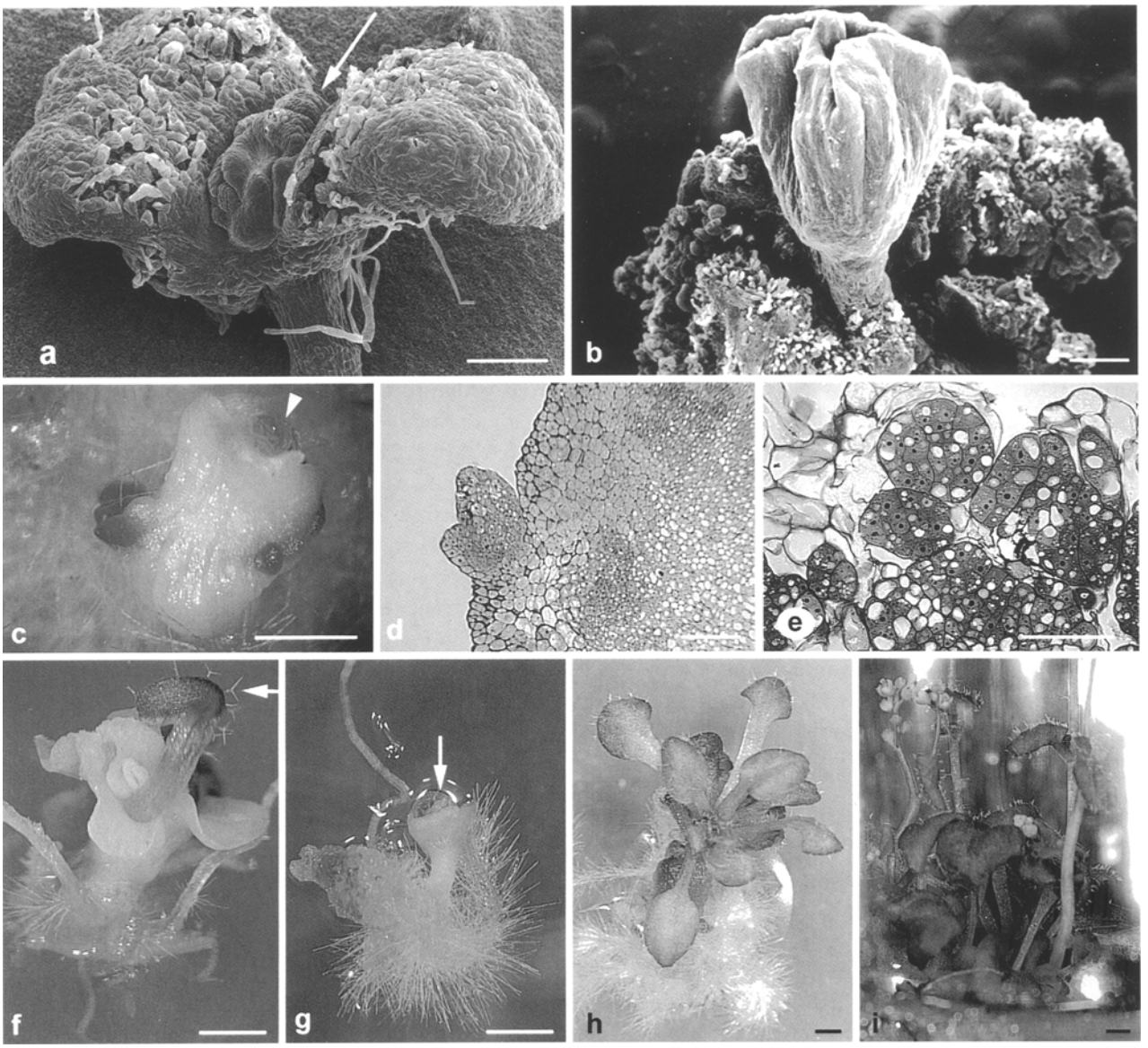

Fig. 2. **a** SEM image of a seedling 14 DIC showing the first signs of proliferating cotyledon cells separating from the seedling, and a highly proliferating SAM region (arrow) with many, randomly distributed cell domes. **b** Somatic embryo at the stage of final maturation showing three well developed and partially fused cotyledons. The embryo axis emerges from the inside of the callus. The callus was carefully moved aside manually to show that hypocotyl and radicle were hidden in the inner part of the callus. **c** Primary somatic embryo bearing secondary embryos at the globular to heart-shaped stage which emerged from the SAM region (arrowhead) and from the hypocotyl-cotyledon region of the primary somatic embryo, 7 days after transfer to solid medium. **d** Semithin section through a heart-shaped secondary somatic embryo arising from the subepidermal layers of a cotyledon tip of a primary somatic embryo. **e** Semithin section through embryogenic callus showing several globular embryos covered with callus cells. **f** Tricot somatic embryo with lateral roots and one leaf emerging from the SAM, 15 days after transfer to solid medium. Note the tripod-shaped trichomes on the leaf blade (arrow). **g** Somatic embryo with roots and fused cup-shaped cotyledons surrounding an enlarged SAM region (arrow) 20 days after transfer to solid medium. **h** Plantlet with many lateral roots and a shoot with various leaves in a rosette configuration observed nine days later than the embryo in **g**. **i** Flowering somatic plant derived from the stage shown in **h**, now on day 38 after transfer to solid medium. Note the bushy morphology of the plant, an indication for reduced apical dominance during plant development. Bars: a, b, and f–i, 250 μm; c, 500 μm; d and e, 50 μm

embryos on callus mainly extended with their cotyledon side from the callus surface, but in about 20% of the cases the embryos had a reversed position: the tips of the cotyledons were attached to the callus and the hypocotyl extended into the medium (Fig. 1 h).

Somatic embryos were frequently found in clusters. Such clusters (Fig. 1 g) consisted of embryos which were either totally separated or partially fused. Fusion occurred along the hypocotyl axis or along the hypocotyl and root. Many somatic embryos within a

cluster had aberrant shapes. They were squeezed or deformed, probably due to limitation in space. The number of cotyledons varied from one to three, and they could be distinct or partially or totally fused (Figs. 1g and 2b).

Because of the variable shape of the somatic embryos, the radicle and the hypocotyl territories within the developing embryo axis were often difficult to identify. The boundary between hypocotyl and radicle could, however, be visualized with the dye Procian Blue MX-R, due to the presence of a cuticle on the hypocotyl surface and its absence on the radicle surface (Fig. 1i). Staining was also observed on the cotyledon edges and the callus, indicating that there neither was a cuticle.

When somatic embryos were subcultured on initiation medium, their development became arrested in the torpedo-shaped stage (Fig. 1g). Upon transfer to liquid medium without 2,4-D they formed a root after 8 days (Fig. 1h) with a length up to several centimeters. Shoot formation was limited to the development of a stalklike structure or a tiny rosette. Eventually, after prolonged culture in the same medium with or without subculture for several weeks, the plantlets, however, etiolated, became hyperhydric, and deteriorated.

Regeneration culture on solid medium without 2,4-D

When embryogenic callus or isolated somatic embryos were transferred onto solid medium without 2,4-D, embryos either germinated and formed plantlets or they formed numerous secondary embryos which germinated and then degenerated. Generally, whenever embryos retained the ability to germinate, they formed no or only a limited number of secondary embryos. The appearance of primary embryos on callus and secondary somatic embryos on the primary ones, and their different phenotypes and development are summarized in Figs. 1j and 2c, d.

Globular and heart-shaped somatic embryos were rarely found in the callus cultures, but torpedo-shaped embryos were abundant. Figure 1j shows a typical callus grown in liquid medium and transferred to solid medium. The lower part of the callus is nonembryogenic, whereas the upper, white-yellowish part formed multiple somatic embryos. Embryo cotyledons were distinct, but they could also be partly or totally fused. In young torpedo-shaped embryos they were often pressed against each other. Cotyledon lengths increased as the embryos matured (Fig. 1j).

Callus pieces which had been selected on the basis of their green appearance, and thus their embryogenic capacity, always turned yellow to whitish during subculture on solid medium. The arising embryos, however, varied in color from green to yellow or white (Fig. 1j). They had either smooth or rough surfaces, could be slender or swollen, and might have 2, 3, or even 4 cotyledons, commonly with different sizes.

Simultaneously with the development of primary somatic embryos, secondary embryos arose on the primary ones, having similar morphological features. Several secondary embryos, ranging in development from young to mature torpedo stage, were visible on top of a primary embryo. They frequently emerged on the SAM (Fig. 2c) and on the tips of the cotyledons of primary ones. Also near the border between cotyledon and hypocotyl, secondary embryos arose (Fig. 2c). Histological analysis revealed that the secondary embryos originated from the subepidermal cell layers (Fig. 2d). The globular and heart stage secondary embryos in Fig. 2c emerged at the boundary between the hypocotyl and the fused cotyledons, and were visible 7 days after transfer to solid medium. They developed to the torpedo stage within 11 days, whereas the primary embryo hyperhydrated and degenerated concomitantly.

Somatic embryos did not always form secondary somatic embryos but also could germinate when transferred to solid medium without 2,4-D. The frequency of this event did vary between experiments. Root formation was followed by shoot formation within 2–4 weeks. Usually, the roots were covered with many root hairs (Fig. 2g, h). Frequently, the SAM formed a single leaf first (Fig. 2f). Later, more leaves were formed (Fig. 2h). Alternatively, the shoot apex formed many leaves about simultaneously at the base of a broad shoot apical meristem and with a rosette configuration, as is also known for *pt* mutant seedlings. The first leaves that were formed were characterized by the presence of many trichomes, with a typical inverted tripod shape (Fig. 2f, h). Regenerated somatic plants exhibited the typical bushy Pt phenotype (Fig. 2h, i).

Discussion

The observations described in this paper are in close connection with those reported earlier by Mordhorst et al. (1998). These authors suggested that facilitated somatic embryogenesis occurred frequently in the

pt mutant in vitro cultures under the conditions described. The results, shown here, indicate that somatic embryos preferentially emerged from three sites, i.e., from embryogenic callus, from the SAM region of cultured seedlings, and from the SAM region of somatic embryos. The hypocotyl of zygotic and somatic embryos, and to a lesser extent the edges of the cotyledons of somatic embryos can also be a site for embryo initiation. The process of recurrent secondary embryogenesis indeed adds to the high embryogenic regeneration capacity in this in vitro culture system.

Concerning the origin of the embryogenic callus that developed on cultured seedlings, it was found that it mainly originated from the SAM region of the seedling. In contrast, nonembryogenic callus could arise from other parts of the seedlings but not from roots, which lacked callus formation. The primary somatic embryos either originated directly without a callus phase, or indirectly via a callus phase, from this broad SAM region.

The SAM region as main source for somatic embryogenesis undergoes drastic morphological changes as shown in this study. In vivo, the SAM consists of a small group of undifferentiated cells which are surrounded by the leaf primordia and give rise to all aerial parts of the plant except the hypocotyl and the cotyledons (Cutter 1965, Sussex 1989). There are functionally distinct zones according to a widely accepted model (Steeves and Sussex 1989, Medford 1992, Clark 1997, Kerstetter and Hake 1997); the central zone provides cell transition to the peripheral zone and to the leaf primordia which are arranged in a strict phyllotactic pattern. However, 2,4-D-induced cell proliferation leads to the simultaneous and random initiation of many cell domes in the *pt* mutant instead of the sequential initiation of two or three cell domes in a phyllotactic order observed in vivo. A less severe form was also observed, in which the production of the first two or three primordia was disturbed; they appeared but not in the right position. In that case, the first leaves degenerated soon and formed callus, analogous to the proliferation of the cotyledons and the SAM as described above.

Detailed histological analysis of the origin of indirect embryogenesis gave more insight into the striking observation that globular and heart-shaped embryos were found rarely, while torpedo-shaped embryos were present in high quantities in both the liquid and solid cultures. The low frequency of proembryos and early globular and heart-shaped somatic embryos could not be explained sufficiently by their small size and their fast development, in contrast to the bigger size of the torpedo-shaped embryos and the longer duration of their maturation phase. Semithin sections of the embryogenic callus, however, clearly revealed that proembryos and globular embryos were embedded inside the embryogenic callus tissue. For that reason, only the very tips of the heart-shaped embryos were visible on the surface of the callus. Upon expansion of the bilateral axis and longitudinal growth, the heart- to torpedo-shaped somatic primary embryos broke through the callus layers and appeared on the surface. The scanning electron micrographs give supporting evidence for this view.

The high frequency of torpedo-shaped embryos in the cultures could also be caused by a developmental arrest. This phenomenon might be analogous to the events occurring in carrot embryogenic cultures with high osmolarity and addition of abscisic acid (Tetteroo et al. 1995). In case of the Arabidopsis cultures the growth regulator 2,4-D might be responsible for the growth arrest of the somatic embryos in the torpedo stage, because by subculture in liquid medium without 2,4-D somatic-embryo development continued and root formation became apparent within 5–7 days. However, this development was limited to root formation and shoot outgrowth, and further plant development could only take place after transfer to hormone-free solid medium.

Somatic embryos often appeared in clusters both in the indirect way and the direct way of embryo formation. This might well be caused by their position on a common group of cells that gives rise to somatic embryos. The common site of formation is reflected in their morphology on a callus piece. Neighboring embryos were either totally separated, or partially fused along the hypocotyl, or even shared a common axis. Fusion and the aberrant and squeezed shapes thus seem to originate from a lack of space and the close distance between the embryos, due to simultaneous initiation.

The finding that the embryogenic capacity of the callus coincided with the presence of the green color indicates a possible role of photosynthesis metabolism during early embryogenesis. It has, however, to be recalled that embryos might form on white callus and also under dark-culture conditions (Mordhorst et al. 1998). Rodkiewicz et al. (1994) observed chlorophyll fluorescence in developing zygotic embryos of *A.*

thaliana. Also, the effect of light quality on somatic embryogenesis has been mentioned before; red and far-red light stimulated the proliferation of embryogenic callus of *Fresia refricta* (A. Bach pers. commun.). Further, the presence of amyloplasts has been claimed to be a possible indication of morphogenetic potential in protoplasts of *Helianthus annuus* (Laparra et al. 1997). In sections stained with periodic acid-Schiff's reagent, we also found clear gradients of starch distribution in globular embryos and the underlying embryogenic callus or in globular embryos which originated on primary ones, respectively. We observed that secondary globular *pt* mutant embryos were devoid of starch grains, whereas strong starch accumulation was visible in their basal part and the underlying cotyledon tissue of the primary embryo (data not shown). This once more stresses the importance of defined light conditions for embryogenic cultures, a factor which is still neglected in the setup of many culturing protocols.

Acknowledgments

The authors thank Sacco de Vries for valuable discussions and critical reading of the manuscript and Sybout Massalt and Allex Haasdijk for the artwork. I.v.R., A.I., and A.M. were supported by grants from the EC-program European Plant Embryogenesis Network, contract nr. ERBBIO4CT 96089.

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