REVIEW ARTICLE

Can carrot and *Arabidopsis* serve as model systems to study the molecular biology of somatic embryogenesis?

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Following the discovery of somatic embryogenesis in cell cultures of carrot nearly 50 years ago, carrot has served as the primary experimental system to study the molecular biology of this embryogenic episode. Although several genes activated or differentially expressed during somatic embryogenesis in carrot have been identified, the list does not include those critical genes whose regulatory mechanisms control the embryogenic transformation of somatic cells. Consequently, research on the molecular biology of somatic embryogenesis in carrot seems to face an uncertain future. Much work on somatic embryogenesis in the model plant *Arabidopsis thaliana* has been focused on culture of zygotic embryos of wild-type plants and germinating seeds of mutants with enlarged shoot apical meristem for increasing the yield of somatic embryos for biochemical and molecular studies from their single-celled beginning. The compact nature of the callus formed on cultured wild-type embryos, the failure of the callus to dissociate into single cells and cell clusters, and the probable involvement of the same progenitor cells in the formation of leaves and somatic embryos on cultured zygotic embryos as revealed by tissue-culture approaches, have raised questions on the limitations of *Arabidopsis* as a model system for molecular studies of somatic embryogenesis.

Keywords: *Arabidopsis thaliana*, carrot, leaf formation, somatic embryogenesis, zygotic embryogenesis.

THE formation of embryo-like structures by experimental treatments is an alternative way of life for somatic cells of angiosperms and gymnosperms and goes by the name somatic embryogenesis. Starting with the very first accounts of somatic embryogenesis in carrot (*Daucus carota*) in the late 1950s, the number of reports on embryogenic induction from somatic cells of plants has steadily increased, initially confined to members of the carrot family (Umbelliferae or Apiaceae), but rapidly spreading to members of a number of angiosperm and gymnosperm families. No consolidated listing of these plants is currently available, but separate listings of herbaceous eudicots[1], herbaceous monocots[2], woody angiosperms and gymnosperms[3], and angiosperms in general[4] have been published.

This article highlights the history of discovery of somatic embryogenesis in carrot using tissue-culture based approaches. Coming fast on the heels of establishing protocols for obtaining potentially embryogenic cells reproducibly and in large quantities, key biochemical and molecular investigations on the carrot system are reviewed to show that biologically compelling and functionally important genes that control the ability of undifferentiated somatic cells to lapse into an embryogenic mode of development remain yet to be identified. Although the high-profile *Arabidopsis thaliana* (hereafter referred to as *Arabidopsis*) offers some advantages over carrot for cytological investigations of somatic embryogenesis, recent studies reviewed here show that this plant may not serve as a paradigm model to decipher the fundamental molecular basis for embryogenic transformation of somatic cells. The exploitation of innovative genetic and molecular techniques, combined with traditional tissue-culture approaches appears necessary to provide new insights into the mechanism underlying the embryo developmental programme of somatic cells of *Arabidopsis*.

Unconventional tissue-culture approaches lead to the discovery of somatic embryogenesis

The discovery of somatic embryogenesis in flowering plants is usually traced to a prophecy by Haberlandt[5] that it would be possible to grow facsimiles of embryos from vegetative cells of plants. Implied in this prophecy is the dictum of totipotency, namely, all plant cells, except perhaps those that have undergone irreversible differentiation, are capable of regenerating whole new plants in full multicellularity, sexuality and structure. Although Haberlandt failed to confirm the prediction, the stage was set to demonstrate totipotency in plant cells beginning in the 1930s with the formulation of methods for growing organs, tissues and cells of plants under aseptic conditions. Using cultured secondary phloem of carrot, Steward *et al.*[6,7] initiated pioneering experiments that led to the demonstration of totipotency. These studies showed that the culture of carrot explants on a solid medium containing inorganic

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salts, vitamins and organic nutrients supplemented with the liquid endosperm of coconut (coconut water), typically gave rise to a proliferating callus of parenchymatous cells. Transfer of the callus to a liquid medium of the same composition with gentle agitation resulted in a suspension of single cells and cell clusters, the latter originating by repeated division of cells dissociated from the callus. Continued growth of the cell suspension in this medium caused lignification of inner cells of the clump, formation of cambium-like cells and appearance of lateral root primordia. Transfer of the rooted aggregate to a solid medium with coconut water finally led to the regeneration of a normal carrot plant in the culture flask. Although these observations did not unequivocally prove that a single cell was transformed into a plant, Vasil and Hildebrandt[8] showed that a single cell originating from a hybrid tobacco (*Nicotiana glutinosa* × *N. tabacum*) pith callus grown in isolation in a drop of a defined culture medium in a microculture chamber, divided repeatedly to form a callus that was subsequently induced to form an organized plant complete with roots and shoots on a solid medium.

Steward[9] called attention to the remarkable resemblance between certain cell aggregates in the carrot cell suspension culture and stages of zygotic embryogenesis in typical eudicots; yet, evidence that single cells present in the suspension, through aggregation, precisely duplicated the pathway of embryogenesis normally followed by a zygote was found wanting. This challenge was partially met by Reinert[10], who first showed that a callus originating from a strain of carrot root cultured for a long period in a medium containing indoleacetic acid (IAA) and coconut water differentiated into bipolar embryo-like structures upon transfer to a medium enriched with an elaborate mixture of amino acids, amides, vitamins, the purine hypoxanthin, and IAA or 2,4-dichlorophenoxyacetic acid (2,4-D). What was lacking in this work was proof that embryo-like structures that surfaced in the culture had their origin in single somatic cells. Maheshwari and Baldev[11] provided this proof by showing that cultured embryos of the common parasitic angiosperm, *Cuscuta reflexa* differentiated numerous adventive embryos directly from their superficial cells or from a callus formed on their radicular end. After establishing by histological methods, the single-celled origin of adventive embryos from the radicle of the cultured embryo, it was found that the adventive outgrowths passed through several stages typical of zygotic embryogenesis, before appearing outside as macroscopic bipolar structures. Besides dispelling the notion that the zygote is unique in its capacity to form an embryo, this work can be considered to have provided definite credence to the Haberlandt prophecy.

Several follow-up investigations on carrot placed the phenomenon of somatic embryogenesis on a sound footing and established its uniqueness within the angiosperms. This can be attributed to the efforts of Steward[12], and Wetherell and Halperin[13], who reported that under certain experimental conditions, cultured cells and cell clusters of carrot are restructured in an embryogenic pathway, and regenerate an enormous number of replicas of zygotic embryos. Steward's group[12,14] showed that when free cells sloughed off from immature embryos of carrot grown in a mineral salt medium supplemented with coconut water are plated on a solidified nutrient medium of the same composition, virtually every cell of the suspension yielded an embryo-like structure, faithfully recapitulating stages of zygotic embryogenesis. Following an initial demonstration that cells originating from a callus obtained from the root tissue of wild carrot nurtured in a medium containing coconut water formed somatic embryos[13], later studies showed that it was possible to dispense with coconut water as an ingredient of the medium to promote somatic embryogenesis in carrot. These latter investigations[15,16] highlighted the fact that various vegetative organs of carrot such as the root, peduncle and petiole readily form a callus when cultured in a medium containing moderately high level of 2,4-D and that embryogenic development of somatic cells is triggered by the simple expedient of transferring the callus to a medium containing reduced level of the auxin. This new observation illuminated the emerging field of somatic embryogenesis for a period of time with lively disagreements in the literature on the role of coconut water in the embryogenic transformation of somatic cells of carrot[17]. However, use of a defined medium and a single-step transfer of cells from a medium containing high concentration of 2,4-D to one containing reduced amount of the auxin or none at all, was adopted as the standard protocol to study the physiology, biochemistry and molecular biology of somatic embryogenesis in carrot in later years and became popular in inducing embryogenic episodes from somatic cells of other plants. The convergence of these observations was finally capped by the demonstration that a single cell from a carrot root callus grown in isolation in a microculture chamber initially formed a mass of cells from which a somatic embryo emerged, thus reinforcing the conclusion that embryo-like structures observed in suspension cultures had their origin in single cells[18]. The work on carrot that highlighted somatic embryogenesis has spawned reports of embryogenic induction from somatic cells of numerous herbaceous eudicots[1], monocots[2], and woody angiosperms and gymnosperms[3]; these reports have included important additions such as *Medicago sativa*, *M. truncatula* (Fabaceae), *Cichorium* (Asteraceae), *Santalum album* (Santalaceae), and *Dactylis glomerata* and other members of the grass family (Poaceae) to the repertoire of somatic embryogenesis models.

As a popular model, the carrot system has been streamlined in later years to show that synchronously developing single cells or cell clusters known as proembryogenic masses, which are competent to form embryos can be selected from a heterogeneous population of callus cells by sieving the culture and/or by density gradient fractionation[19,20]. Somatic embryos pass through the same sequence

of stages of embryogenesis as their zygotic counterparts, such as the globular, heart-shaped, torpedo-shaped and mature stage embryos synchronously in the medium. Moreover, there are molecular similarities between zygotic and somatic embryos of carrot in the patterns of expression of embryogenesis-related mRNAs[21–24] and proteins[25,26]. These observations suggest that the same molecular mechanisms may be operating in both types of embryogenic events. As is well-known, division of the zygote in flowering plants occurs within the privileged location of the embryo sac, which itself is buried within several layers of the nucellus and integuments of the ovule and consequently, study of the molecular developmental biology of early embryogenesis has understandably fallen low on the agenda of investigators. In contrast, because of their easy accessibility, potentially embryogenic somatic cells offer an attractive system to circumvent this problem, besides providing a fascinating view of plant embryogenesis outside the embryo sac.

Carrot leads the way up to a point

The carrot system has been widely used to monitor the cytological, physiological, biochemical and molecular changes associated with transformation of somatic cells into embryos. A video cell-tracking system has identified both cytoplasmic single cells and vacuolated single cells in a carrot cell suspension that can develop into somatic embryos, thus reinforcing their single-celled origin[27]. In hypocotyl segments of carrot seedlings exposed to 2,4-D, it was possible to trace the origin of embryogenic single cells to the provascular (procambial) cells, the only cells in the explant which divide in the presence of auxin to generate the proembryogenic masses of cells. Cytohistological analysis of the cultured explant showed that from the thousands of cells produced by the activated procambium, only a small number of elongate, oval to triangular cells actually become embryogenic. These cells are undoubtedly released from the proliferating explant and apart from their ability to produce proembryogenic masses, they are indistinguishable from the majority of isodiametric cells formed from the procambium[28,29]. Evidently, embryogenic commitment is not an intrinsic property of all cells of the cultured explant, but is acquired by certain meristematic cells exposed to 2,4-D.

Since the arsenal of available chemical or molecular markers is limited, the problem of identifying potentially embryogenic cells in a suspension culture consisting of several different cell types and cell clusters with ill-defined morphology is quite vexing. Pennell *et al.*[30] suggested the use of monoclonal antibody JIM8, which reacts with cell-wall arabinogalactan proteins or with arabinogalactans (glycoproteins with high levels of arabinosyl and galactosyl residues as well as of alanine, serine, threonine and hydroxyproline residues), as a molecular marker for an early stage in the developmental pathway of cells in a carrot cell suspension culture to somatic embryos. However, the usefulness of this marker to reliably predict whether single cells thus identified will develop into somatic embryos, became doubtful when the video cell-tracking method showed poor correlation between antibody-reacting cells and somatic embryo formation[31]. Cell suspension culture of a transgenic carrot line transformed with a fragment of the *A. thaliana LIPID TRANSFER PROTEIN1* gene promoter fused to the firefly luciferase coding sequence, has met with some success in tracking cell clusters that develop into somatic embryos[32]. A breakthrough in identifying embryogenic cells occurred when it was shown that expression of the *SOMATIC EMBRYOGENESIS RECEPTOR KINASE* (*SERK*) gene isolated from carrot cells, coincides with the acquisition of embryogenic competence by cells. The protein product of this gene which contains the signature motif of a leucine-rich transmembrane receptor-like kinase probably functions in a signal transduction pathway. It was shown by cell-tracking that only the transgene-expressing cells in a carrot cell suspension culture transformed with a *SERK* promoter-luciferase construct develop into somatic embryos[33]. Using a whole mount *in situ* hybridization method, the carrot *SERK* gene was also found to be a good molecular marker of cells competent to form somatic embryos directly on cultured leaf explants of *Dactylis glomerata*[34]. Thus, the closest we have come to identifying with some degree of precision, a cell type endowed with embryogenic competence is through the use of this marker.

As pointed out earlier, a sequence of transfer of cells from a medium containing 2,4-D to an auxin-free medium essentially defines the protocol for inducing somatic embryogenesis in carrot. A key question relating to the molecular function of auxin in carrot somatic embryogenesis is whether cells are programmed for embryogenesis before they encounter auxin in the medium. Although it has been difficult to dissociate the role of auxin in promoting callus growth from its role in conferring embryogenic competence on cells, the demonstration that epidermal cells of carrot hypocotyl acquire the capacity to form embryos only after exposure to auxin for at least 12–24 h, engenders the notion that auxin treatment is necessary to bestow embryogenic competence on cells[35].

There is a long history of interest in the possibility that transfer of carrot cells from a medium containing 2,4-D to an auxin-free medium modulates embryogenesis by the synthesis of new mRNA and proteins[36,37]. Yet, it is not clear whether gene activity for embryogenic induction in cells is initiated upon their transfer to an auxin-free medium. The issue is whether proteins synthesized in cells grown in the auxin-free medium are encoded on newly formed mRNAs or on mRNAs transcribed when cells are bathed in the auxin-containing medium. Wilde *et al.*[38] found striking similarities between the *in vitro* translation products of mRNA populations of proembryogenic masses and

torpedo-shaped somatic embryos of carrot, to invoke a model in which gene expression programme for somatic embryogenesis is initiated when the cells are grown in the auxin-containing medium. Based on a comparison of the spectrum of proteins synthesized by carrot cells grown for 12 days in the presence or absence of 2,4-D in the medium, Sung and Okimoto[39] found no pronounced differences in the 200 or so polypeptides spotted on gels, except for the appearance of two additional proteins, E1 and E2, designated as embryonic proteins, in embryogenic cells grown in the absence of auxin. The surprising finding is that regardless of the presence or absence of 2,4-D in the medium, these two proteins are synthesized by cells as early as 4 h of growth in the fresh medium, but in the presence of auxin, they gradually diminish and disappear. Synthesis of embryonic proteins appears to be an early event of somatic embryo induction triggered by 2,4-D; because auxin also inhibits the continued synthesis of these proteins and the execution of embryogenic programme by cells, the role of embryonic proteins in the embryo induction process represents a biochemical puzzle. Another factor complicating a direct analysis of the proteins and mRNAs activated during somatic embryogenesis, is the excretion by cells of high molecular weight compounds into the medium, which has been identified as a biochemical hallmark of somatic embryogenesis in carrot and other systems[40].

The carrot system has been used by several investigators to identify genes associated with the commitment of somatic cells to an embryogenic fate, and with progressive differentiation of the committed cells into somatic embryos[41–43]. Given the overlap between the *in vivo* synthesized proteins of nonembryogenic and embryogenically-induced cells, it appeared likely that only minor changes in the gene expression programme accompany embryogenic induction, and that understanding the molecular regulation of somatic embryogenesis in carrot may hinge on some rare class of genes. In an attempt to identify genes that have a role in the initiation of embryogenic development in somatic cells, Aleith and Richter[44] using a traditional approach of differential screening, found that transcripts of several clones isolated from carrot cells cultured in an auxin-free medium accumulate transiently in cells from three days up to 16 days after transfer, coinciding with the development of globular or heart-shaped somatic embryos. Involvement of one of the clones in somatic embryogenesis was confirmed by the activity expressed by its promoter sequences in somatic embryos produced in transgenic carrot[45]. A gene isolated by Sato *et al.*[46] by subtractive differential screening was expressed as early as one day after transfer of embryogenic cell clusters to an auxin-free medium. Response to the earliest signal of embryogenic induction can be attributed to a gene designated as *CARROT EARLY SOMATIC EMBRYOGENESIS1*, as it is expressed in embryogenic cells within 8 h of their transfer to an auxin-free medium[47]. Despite the fact that the deduced protein products of these genes show some resemblance to certain cell-wall proteins, their relevance to the potentiation of embryogenic development is remarkably difficult to pin-down in view of the lack of a clear inductive function. Genes expressed at high levels during late stages of somatic embryogenesis in carrot include those encoding LATE EMBRYOGENESIS ABUNDANT proteins[23,48] and the eukaryotic translation elongation factor 1α[49], homeobox genes[50,51], and the carrot homologue of the *Arabidopsis LEAFY COTYLEDON1* (*LEC1*) gene[24]. Comparative studies of gene expression patterns in nonembryogenic and embryogenic cells of other systems have demonstrated up-regulation of genes associated with cell-cycle activity in *Medicago sativa*[52], MADS-box genes in *Cucumis sativus*[53] and maize[54], genes involved in nuclear regulatory functions in *Dactylis glomerata*[55], and genes encoding 'germin-like' oxalate oxidase in wheat[56]. Recent use of a high-resolution proteomic analysis has ascribed a modest role to the proteins, thioredoxin H and 1-cysteine peroxiredoxin, in the embryogenic transformation of somatic cells of *M. truncatula*[57]. Although these studies provide validation of the occurrence of substantial reprogramming of the gene expression pattern during the developmental switch of somatic cells to embryos, the great mystery of somatic embryogenesis in carrot is that genes whose protein products are dedicated to embryogenic transformation appear to be in the extreme minority and their identification continues to be elusive.

As one way around this conundrum, it has been proposed that the transition of somatic cells to proembryogenic masses or somatic embryos might not involve changes in the most abundant proteins or mRNAs, but is rather programmed by the down-regulation of some genes expressed in the somatic cells. In support of this view, characterization of the expression and regulation of a collection of 38 different genes isolated by subtraction-probe strategy using mRNA from carrot seedlings to screen embryo-enhanced genes from somatic embryos has shown that most of the genes are not only expressed in the callus, but some are even expressed at higher levels in the callus than in somatic embryos[58]. Although the list included several previously identified embryo-enhanced genes and other new and known genes whose relevance to embryogenesis is not established, this work did not identify any rare genes whose down-regulation modulates the transition of somatic cells to embryos. Current speculation on the molecular mechanism of somatic embryogenesis does not end here, as the role of other tractable changes such as DNA methylation, which inhibits transcription by blocking the binding of transcription factors, is now being investigated[59]. It thus seems that, although some genes which respond to signals of embryogenic development have been isolated from cell-suspension cultures of carrot, a glaring absentee is the putative signal that might trigger and maintain cells in the embryogenic pathway or whose expression might induce somatic embryogenesis in a transgenic setting. Thus, for all intents and purposes, the arduous task of decipher-

ing the fundamental molecular mechanisms involving genes and proteins controlling the transformation of undifferentiated somatic cells to embryos in this well-studied system can be considered to be at an impasse.

Arabidopsis appears on the scene

The successful use of *Arabidopsis* as a model system to study the genetics and molecular biology of numerous problems in plant development was the impetus for investigations on somatic embryogenesis in this plant, motivated also by its potential as a model for zygotic embryogenesis. In particular, establishment of protocols to obtain somatic embryos in *Arabidopsis* from their single-celled beginning, would allow entry into the powerful mutant-based molecular and genetic approaches employed to identify developmentally important genes active during zygotic embryogenesis[37,60,61]. Synchronously developing somatic embryos offer opportunities to investigate pattern formation involved in establishing distinct functional domains in wild-type and mutant embryos in the absence of maternal tissues, identify nutritional mutants by embryo rescue, and study biochemical embryogenesis starting with single-celled progenitors[62]. The role of auxin in pattern formation in *Arabidopsis* embryos has been brought into focus by the analysis of mutants such as *monopteros*[63,64], *bodenlos*[65] and *pinoid*[66], perturbed in auxin signalling and by following the dynamics of auxin distribution in individual cells of early-stage embryos using a fluorescent reporter gene construct and transgenic approaches[67]. As described below, in a way reminiscent of carrot, 2,4-D is involved in inducing the formation of early-stage somatic embryos in *Arabidopsis*, raising the prospects for a direct analysis of the newly discovered connection between auxin signalling and early embryogenesis, beginning with single-celled embryogenically competent cells.

In *Arabidopsis*, embryo-like structures have been infrequently obtained from callus cultures originating from seedling hypocotyls or roots[68–70] and from cell colonies reformed from cultured protoplasts[71–73]. Somatic embryos are now routinely induced on cultured zygotic embryos of *Arabidopsis* by the use of 2,4-D alone or in combination with other hormones[69,73–75]. In a method introduced by Pillon *et al.*[76], heart-shaped to walking-stick-shaped embryos are cultured for 21 days in a liquid medium containing 4.5 μM 2,4-D as the only hormone, followed by their transfer to an auxin-free medium for plantlet formation. This study, as well as a later one by Ikeda-Iwai *et al.*[77] showed that it was possible to obtain cell lines with continued embryogenic potential, if cultured zygotic embryos with early-stage somatic embryos are maintained on a solid medium with an increased concentration of the auxin. In another investigation, Mordhorst *et al.*[78] cultured bent-cotyledon stage embryos of wild-type *Arabidopsis* in a liquid medium containing 2,4-D for 21 days, followed by subculture of specially selected, green embryogenic cell clusters in an auxin-free solid medium for the development of somatic embryos. The focus in all of the above-mentioned investigations was in increasing the yield of somatic embryos for biochemical and molecular studies. This has also resulted in the successful demonstration of enhanced somatic embryogenesis in seedling cultures of mutants such as *primordial timing*, *clavata1* (*clv1*) and *clv3* with enlarged shoot apical meristem, leading to the suggestion of an unexpected relationship between increased somatic embryogenesis and the presence of noncommitted cells of the enlarged shoot apical meristem[78,79]. However, the role of an active shoot apical meristem in the production of embryogenic cells and somatic embryos in *Arabidopsis* is not entirely clear, as embryos isolated from mutants defective in the formation of shoot apical meristem such as *shoot meristemless*, *wuschel* (*wus*), and *zwille/pinhead* also readily form somatic embryos in the same medium that favours somatic embryogenesis in wild type *Arabidopsis*. To explain this, somatic embryos are believed to arise from cells confined to the axils of the cotyledons of mutant embryos[80].

Whole-mount and histological observations of bent-cotyledon stage zygotic embryos of a transgenic stock of *Arabidopsis* harbouring a cylin1 *Arabidopsis thaliana* (*At*): β-glucuronidase (GUS) reporter gene construct cultured in a medium containing 2,4-D, have implicated the cotyledons in a central role in initiating divisions that lead to somatic embryogenesis. Following a brief period of growth by cell expansion, divisions are initiated in the procambial cells facing the adaxial side at the base of the cotyledons causing this region to bulge (Figure 1 *a*, *b*). Cell-division activity later spreads to the mesophyll and epidermal cells in both adaxial and abaxial sides at the base of the cotyledons and eventually to almost the entire length of the cotyledons to form a callus on which globular and heart-shaped somatic embryos are formed. Similar morphogenetic changes were observed somewhat slowly when cotyledons were extirpated from the embryo axis and cultured, but culture of the root–hypocotyl–shoot axis, including the shoot apical meristem and cells in the axils of cotyledons failed to produce a callus from any part of the cultured explant. When embryos growing in the medium containing 2,4-D for ten days are transferred to an auxin-free medium, tubular structures similar to torpedo-shaped zygotic embryos are formed from the cotyledonary callus. Without going through the bent-cotyledon stage, these embryos project outside the callus as mature-stage somatic embryos during growth in the hormone-free medium[81]. These experiments show that in wild-type zygotic embryos of *Arabidopsis*, somatic embryos have their origin in the cotyledons and not in the shoot apical meristem or cells in the axils of the cotyledons. The compact nature of the callus formed on the cotyledons and failure of the callus to dissociate into single cells and cell clusters place serious limitations on

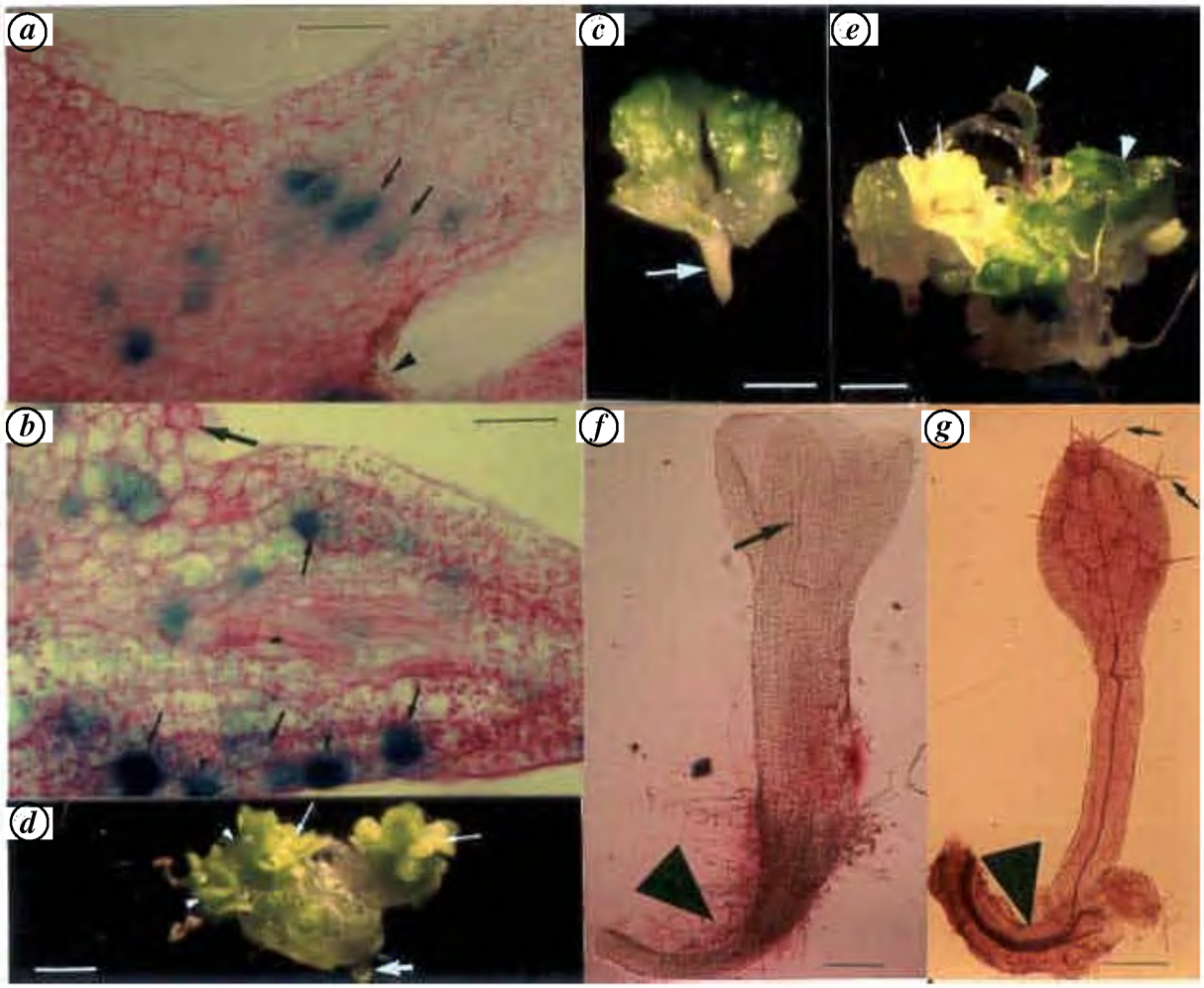

Figure 1. Somatic embryogenesis in cultured bent-cotyledon stage zygotic embryos of *Arabidopsis*. ***a***, Section through basal region of cotyledon of an embryo two days after culture in a liquid medium containing 2,4-D, showing GUS-expressing cells in the procambium (arrows). Arrowhead shows location of shoot apical meristem. ***b***, Section through basal region of cotyledon of an embryo four days after culture in a liquid medium containing 2,4-D, showing additional GUS-expressing cells in the mesophyll (small arrows) and abaxial epidermis (arrowheads). The adaxial side at the basal region of the cotyledon has bulged, indicating beginning of callus growth. Large arrow indicates location of shoot apical meristem. Scale bar = 50 µm. ***c***, Embryo initially grown in a liquid medium containing 2,4-D for seven days and subsequently in an auxin-free solidified medium for five days in light, showing formation of bifid, tubular structures on the callus formed on the cotyledons. Arrow points to embryonic root. Scale bar = 1.8 mm. ***d***, Embryo initially grown in a liquid medium containing 2,4-D for seven days and subsequently in an auxin-free solidified medium in light for eight days, showing further growth of bifid structures into somatic embryos (arrows) or leaves (arrowheads). The large arrow points to embryonic root. Scale bar = 7 mm. ***e***, Embryo initially grown in a liquid medium containing 2,4-D for seven days and subsequently in an auxin-free solidified medium in light for 14 days, showing further development of somatic embryos (arrows) and leaves (arrowheads) on the callus. The leaf at left has formed trichomes. Scale bar = 10 mm. ***f***, A cleared somatic embryo formed on a zygotic embryo initially grown in a liquid medium containing 2,4-D for ten days and subsequently in an auxin-free solidified medium in light for 14 days; the embryo was stained with basic fuchsin to reveal lignified elements (arrow). Arrowhead points to the root. Scale bar = 500 µm. ***g***, A cleared leaf dissected from the callus formed on cotyledons of a zygotic embryo initially grown in a liquid medium containing 2,4-D for seven days and subsequently in an auxin-free solidified medium in light for 14 days; the leaf was stained with basic fuchsin to reveal the venation pattern. Arrows point to the trichomes. Arrowhead points to the root. This leaf originated as a bifid structure like a somatic embryo. Scale bar = 1 mm.

the development of a suspension culture in which one can follow the development of single cells into somatic embryos and thus use it as a model system to study the biochemistry and molecular biology of zygotic embryogenesis.

In contrast to the formation mature-stage somatic embryos on zygotic embryos grown in the auxin-free medium after pretreatment with 2,4-D for ten days, embryos pretreated for shorter periods with 2,4-D are found to form in a transitional order normal seedlings, abnormal seedlings consisting of a short stem with ovate, spoon-shaped and bifid or lobed leaves and adventitious leafy shoots, callus of cotyledonary origin bearing a mixture of leaves and tubular somatic embryos with fused, bifid or lobed cotyledons, and finally, callus with mature-stage tubular somatic embryos with fused, bifid or lobed cotyledons[82]. Zygotic embryos grown in the basal medium after pretreatment with 2,4-D for 7–8 days are of particular interest because of the formation of a mixture of leaves and somatic embryos. Daily examination of the callus during growth in the basal medium showed that these morphogenetic changes are initiated as

early as 2–3 days by the appearance of bifid tubular structures. Dissection of the callus showed that these structures had their origin in small callus masses with roots. During further growth, some of the bifid tubular structures became fused, bifid or lobed somatic embryos with roots, whereas others were transformed into leaves with roots (Figure 1 *c–e*). These leaves rarely displayed their original bifid nature during further growth, but had trichomes and a venation pattern, similar to that of normal leaves, consisting of a midvein and one or two secondary veins, which connected at the periphery dividing the tissue into 3–4 compartments. In contrast, cotyledonary lobes of somatic embryos had a venation pattern consisting of isolated strands which did not connect with each other during the experimental period (Figure 1 *f*, *g*). Somatic embryos in which cotyledons are transformed into leaves are reminiscent of the *lec* mutant of *Arabidopsis*[83], and their occurrence on zygotic embryos cultured in an auxin-free medium following growth in a medium containing 2,4-D for a suboptimal period, might indicate that a defect in the synthesis or maintenance of the local concentration of auxin during the late stage of embryogenesis causes this phenotype.

Carrot and *Arabidopsis*

In cell-suspension cultures of carrot, somatic embryogenesis appears to be an all-or-none phenomenon in which 2,4-D induces the formation of proembryogenic masses, a few cells of which form somatic embryos when transferred to a hormone-free medium. In contrast, somatic embryogenesis in cultured zygotic embryos of *Arabidopsis* can be considered to occur in two distinct stages. The first stage which is labile, occurs after suboptimal periods of exposure of embryos to auxin when cells of the callus formed on the cotyledons become potentially embryogenic and form tubular somatic embryos with bifid or lobed cotyledons, which revert to leaves during a subsequent period of growth in an auxin-free medium. The second stage is the maturation of somatic embryos formed on the callus after a long period of exposure of embryos to auxin. A key feature of the role of 2,4-D in somatic embryogenesis in *Arabidopsis* is that the embryogenic identity of the callus cells initiated by suboptimal periods of auxin action is stabilized only after an optimum period of exposure to 2,4-D at the same time as these cells seem to lose their leaf-like identity. Thus, in cultured zygotic embryos, a close correlation seems to exist between the development of the unique morphology of leaves and somatic embryos and the duration of treatment with auxin necessary to implement these morphologies during a period of growth in the absence of auxin. The competing activities of the two different genomes, one concerned with leaf formation and the other with the formation of somatic embryos have to be kept in mind in attempts to isolate genes that induce somatic embryogenesis in *Arabidopsis* and might constitute an aspect of *Arabidopsis* biology that detracts from its use as an ideal model plant to study the molecular biology of somatic embryogenesis.

This does not mean that all is lost in the quest for genes that induce somatic embryogenesis in *Arabidopsis* and that its status as a readily accessible model system for somatic embryogenesis is in jeopardy. For example, ectopic expression of the *LEC1* and *LEC2* genes of *Arabidopsis* leads to the production of morphologically abnormal plants and somatic embryos on them[84,85]. It has been found that overexpression of a *WUS*-type gene designated as *PLANT GROWTH ACTIVATOR6* causes high frequency somatic embryogenesis from vegetative tissues and zygotic embryos of *Arabidopsis* even in the absence of hormone in the medium[86]. Expression of the MADS-box gene designated as *AGAMOUS-Like15*, enhances the production of somatic embryos from zygotic embryos of transgenic *Arabidopsis* cultured in a hormone-free medium[87]. It is possible that protein products of these genes modulate somatic embryogenesis by promoting the embryogenic transition of somatic cells and/or by maintaining their embryogenic identity in the same way as a long treatment with 2,4-D.

Conclusion

From this survey, it appears that neither carrot nor *Arabidopsis* has provided the complementary insights needed to crack the secrets of the signalling cascades triggered by 2,4-D during somatic embryogenesis. In carrot, the problem is that there is an overlap between genes that are expressed during growth of somatic cells in the callus mode and during their embryogenic transformation and those critical genes whose up-regulation or down-regulation is essential for embryogenesis have not been identified. In *Arabidopsis*, tissue-culture approaches have identified the probable involvement of the same progenitor cells in the formation of leaves and somatic embryos and that this overlap between leaf-forming genes and genes involved in somatic embryogenesis is likely to make fishing out the genes specifically involved in somatic embryogenesis a difficult expedition.

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