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ORIGINAL ARTICLE

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An auxin surge following fertilization in carrots: a mechanism for regulating plant totipotency

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Abstract All plants exhibit the property of cellular totipotency, whereby individual cells can regenerate into an entire organism. However little is known about the underlying mechanisms regulating totipotency. Using a preparative microtechnique, we report an 80-fold surge in the concentration of free auxin that is correlated with the initial stages of zygotic embryogenesis in carrots. The concentration of free IAA increases from a basal level of ca. 25 ng/g FW in unfertilized ovules to ca. 2,000 ng/g FW in the late globular and early heart stages, then back to the basal level in the torpedo stage. This initial increase in IAA levels is diagnostic of the activity of the tryptophan-mediated pathway for IAA biosynthesis, while the maintenance of the basal levels is attributed to the tryptophan-independent pathway for IAA biosynthesis. Our observations are consistent with the hypothesis that the sequential activation of alternative IAA biosynthetic pathways is a critical mechanism for regulating carrot (*Daucus carota* L. cv. Danvers 126) embryogenesis and other instances of plant totipotency.

Keywords *Daucus* (totipotency) · Embryogenesis Indole-3-acetic acid (totipotency) · Phytohormone analysis · Reproduction (carrot) · Totipotency

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Abbreviations: *2,4-D:* 2,4-dichlorophenoxyacetic acid · *IAA:* indole-3-acetic acid

Introduction

Multicellular eukaryotic organisms can be distinguished by the ability of individual cells to regenerate into an entire organism (Buss 1987; Kaplan and Hagemann 1991). For instance, in animals, reproductive cells are typically segregated from the somatic body during early embryogenesis, with the consequence that somatic cells in young animal embryos acquire restricted developmental fates. By contrast, plants undergo very late segregation of the reproductive cells; thus, all living cells in plants must retain their totipotency, i.e., the potential for an individual cell to develop into an entire organism. Plant totipotency is thus expressed as a normal process during the formation of zygotic embryos from fertilized egg cells. Totipotency is also expressed during the regeneration of mature reproductive plants from isolated rootstocks, bare twigs, and single leaves (Hartmann and Kester 1983). In some plants, vegetative leaf cells can develop into foliar embryos that are virtually identical to zygotic embryos (Yarbrough 1932). Nevertheless, despite the central role that totipotency plays in plant development, very little is known about the underlying regulation of plant totipotency.

The first hints about how plants might regulate totipotency came from studies on carrot cell cultures, which can produce large quantities of somatic embryos that are easily fractionated into the various stages of embryo development (Raghavan 1986). Pretreatment with a synthetic auxin (e.g., 2,4-dichlorophenoxyacetic acid (2,4-D) or naphthaleneacetic acid (NAA)) is essential for inducing the embryogenic potential of callus cells such that the subsequent removal of auxin leads to the formation of somatic embryos (Steward et al. 1958; Reinhert 1959). Analytical studies have recently demonstrated that, irrespective of the particular auxin pretreatment, high levels of free native

auxin, indole-3-acetic acid (IAA), and/or synthetic auxins are invariably associated with early stages of carrot somatic embryogenesis, whereas low levels of free IAA are found during the later stages (Michalczuk et al. 1992a; Ribnicky et al. 1996). Finally, exogenous auxin antagonists (e.g., 2,4,6-trichlorophenoxyacetic acid) inhibit young somatic embryos undergoing isodiametric growth; subsequently, inhibitors of polar auxin transport (e.g., naphthylphthalamic acid) disrupt the polarized growth of older somatic embryos (Schiavone and Cooke 1987; Cooke et al. 1993).

Somatic and zygotic embryos exhibit similar morphological stages (Borthwick 1931; Halperin 1966; Schiavone and Cooke 1985), which suggests that high IAA levels may also regulate zygotic embryogenesis. However, young zygotic embryos are difficult to isolate in sufficient quantities for using conventional gas chromatography-mass spectrometry (GC-MS) to quantify their IAA levels. Here we report the use of a microscale technique that permits the GC-MS measurements of the levels of free and conjugated IAA present in zygotic embryos at different stages. Such analyses show that IAA in zygotic embryos increases to very high levels following fertilization and returns to basal levels for development at the early torpedo stage and beyond; this pattern is notably similar to that previously observed during somatic embryogenesis in carrot (Michalczuk et al. 1992a).

Materials and methods

Sample collection

Plants of *Daucus carota* L. cv. Danvers 126 were grown in standard greenhouse conditions, and the flowers were pollinated by indigenous insects (Ribnicky 1996). Microscapels were used to dissect individual ovules from unfertilized ovaries with short epidermal trichomes and from fertilized ovaries, using the initiation of trichome elongation as the indicator of fertilization (compare a and b in Fig. 1). Similarly, individual embryos were hand-dissected from expanded ovules using petal abscission, nectary appearance, and ovary color as external indicators of embryo stage. Each sample weighing between 0.1 and 4.0 mg was placed in a pre-weighed microcentrifuge tube and frozen in liquid nitrogen.

Sample preparation

The protocol for the microtechnique used for IAA analysis was described by Ribnicky et al. (1998). Twenty microliters of 65% isopropanol/35% 200 mM imidazole buffer (pH 7), containing 500 pg [$^{13}C_6$]IAA, was added to the frozen sample in the microcentrifuge tube and ground using a plastic pestle. The sample was kept at 4 °C for 90 min to allow for full extraction and isotopic equilibration (Cohen et al. 1986). 200 µl of water was then used to rinse the pestle and dilute the buffer. Insoluble materials were removed by a 30-s centrifugation at 12,000 *g*; the sample was divided into two fractions for the determination of free IAA (IAA obtained from the tissue without hydrolysis) and conjugated IAA (IAA liberated by hydrolysis of ester- and amide-conjugated forms). Total IAA is the sum of free and conjugated IAA levels.

Hydrolysis

After centrifugation, one fraction of the sample was removed from the microcentrifuge tube for hydrolysis and determination of the total IAA (Cohen et al. 1986). The sample was placed in a 300-µl Teflon vial with an equal volume of 14 N NaOH, hydrolyzed for 3 h at 100 °C, and titrated to approximately pH 2.5 using a 1:10 solution of phosphoric acid:6 N HCl.

Sample purification

Both fractions were partially purified prior to HPLC using small columns made from 200-µl pipette tips. Free IAA was analyzed using a small column containing DEAE-Sephadex A25. The hydrolyzed fraction was desalted after titration on a small column containing C-18 Sepralyte. Further purification was performed by microbore HPLC on a C-18 Phenomenex UltraCarb (30) column (100 mm long, 1 mm i.d.) using a solvent of 25% acetonitrile/1% acetic acid at 50 µl/min. The sample was then taken to near dryness on a rotary evaporator under high vacuum at 25 °C and then dissolved in 5 µl of ethyl acetate. The sample was methylated using a stream of diazomethane gas, resuspended in 5 µl of ethyl acetate, and analyzed by gas chromatography-mass spectrometry-selected ion monitoring (GC-MS-SIM).

GC-MS-SIM

A Hewlett-Packard 5890 GC/5971A MS equipped with a DB-1701 fused silica capillary column (15 m long; 0.237 mm i.d ; J & W Scientific, Folsom, Calif., USA) was used. Chromatographic parameters were: injector temperature 250 °C, and initial oven

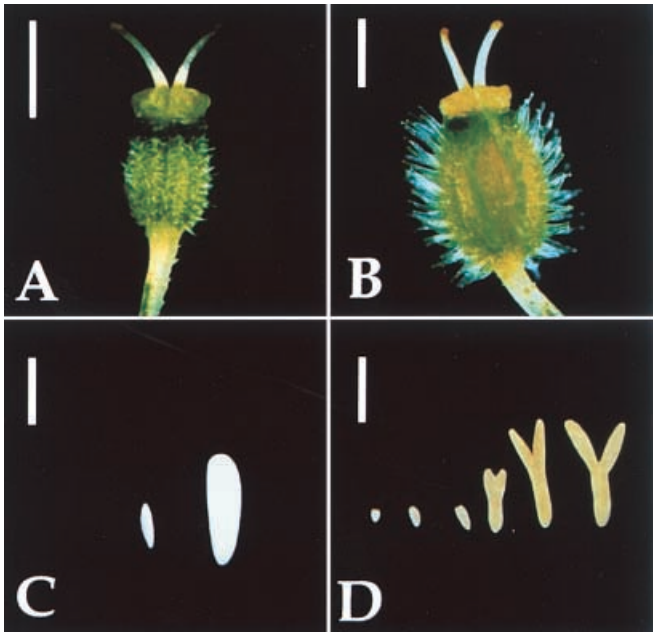

Fig. 1a–d Different structures from carrot (*Daucus carota*) flowers analyzed for IAA concentrations. **a** Unfertilized carrot flower with its two-part style, nectary, and basal ovary covered with short trichomes. **b** Fertilized carrot flower with expanded ovary covered with elongated trichomes. **c** Unfertilized ovule (*left*) dissected from an ovary identical to one depicted in **a** and fertilized ovule (*right*) dissected from an ovary identical to the one depicted in **b**. **d** Various stages of zygotic embryos dissected from their ovules (*left to right*): globular, early heart, late heart, early torpedo, late torpedo, and mature embryos. Bars = 1 mm

temperature 140 °C for 1 min followed by a temperature program at 20 °C/min to 280 °C. The monitored ions were m/z 130 and 136 (quinolinium ions from native IAA and $^{13}C_6$-labeled IAA internal standard, respectively), and 189 and 195 (molecular ion and $m^+ + 6$). All samples were injected in the splitless mode (0.7 min).

Results and discussion

IAA levels in carrot zygotic embryos

During the formation of zygotic embryos in plants, the embryos and surrounding floral structures undergo dramatic size and shape changes. In carrots, the bicarpellate ovary of an unfertilized flower produced numerous short epidermal trichomes and one small ovule in each carpel (Fig. 1). Immediately after fertilization, these trichomes underwent a 7-fold increase in their length. The ovary and the enclosed ovules started to expand in order to accommodate subsequent embryo development. Subsequently, like most dicot embryos (Kaplan and Cooke 1997), carrot zygotic embryos proceeded through a characteristic growth sequence, i.e., spherical growth during the globular stage, cotyledon formation during the heart stage, and axial elongation during the torpedo stage (Fig. 1).

The levels of free and conjugated auxin in ovaries, ovules, and zygotic embryos from carrot flowers were measured by GC-SIM-MS. In unfertilized carrot ovaries, the level of free IAA was 15 ± 4 ng/g FW; by contrast, the level of conjugated IAA exceeded 2,000 ng/g FW in unfertilized ovaries (Table 1). The ovules had levels of free and conjugated IAA that were similar to those found in the ovaries (Table 1).

After fertilization, an increase in the levels of both free and conjugated IAA was measured in carrot ovaries (Table 1). The level of free IAA in ovaries increased 25-fold to 600 ng/g FW, which is comparable to the free auxin levels reported from cell culture systems supplemented with exogenous auxin (Michalczuk et al. 1992a). The total IAA concentration in the ovary exceeded 5,500 ng/g FW, which established that an increase in IAA levels did not result from the hydrolysis of preformed conjugates, and thus must have resulted from increased biosynthesis, decreased degradation, and/or altered transport. Also, the fertilized ovaries accumulated 4-fold higher levels of conjugated IAA than the ovules arising inside those ovaries (Table 1).

High levels of native auxin have been predicted to be essential for normal fruit development because synthetic auxins promote the parthenocarpic development of unfertilized fruits (Gustafson 1936; Nitsch 1950). Large immature seeds and seed-fruit composites, such as pine seeds (Sandberg et al. 1987), bean seeds (Bialek and Cohen 1989), pea pods (Magnus et al. 1997), maize kernels (Jensen and Bandurski 1994), and peach endocarps with enclosed seeds (Miller et al. 1987), have been reported to have total IAA levels of more than 1,000 ng/g FW. However, because of the limitations of prior methods, it was impossible to determine IAA levels in the young ovules and developing zygotic embryos.

The microscale preparative technique coupled to GC-MS as described here has made it possible to determine the concentrations of free and total IAA in small samples composed of limited numbers of fertilized ovules and of zygotic embryos. An increase in the free IAA levels to over 500 ng/g FW was also measured in fertilized ovules containing globular embryos 75–125 μm in length (Table 1). No significant difference in the total IAA levels was seen in unfertilized vs. fertilized ovules. The smallest embryos that could be reliably hand-dissected from carrot ovules were late globular and early heart embryos that ranged from 125 to 225 μm in length. In these embryos, the amounts of free and total IAA reached even higher peak levels of over 2,000 and 9,500 ng/g FW (1.1 and 5.5×10^{-5} M), respectively (Table 1). Subsequently, the early and late torpedo embryos exhibited reduced levels of ca. 25 ng/g FW of free IAA and ca. 800 ng/g of conjugated IAA. These free IAA levels are similar to the basal levels reported in seedlings (Bandurski and Schulze 1977; Ribnicky et al. 1996).

What is the significance of the IAA surge during the initial stages of zygotic embryogenesis? The initial stages of carrot somatic embryogenesis have high levels of the free forms of IAA and 2,4-D, which decline to basal free auxin levels in the torpedo embryos (Michalczuk et al. 1992a). Inhibitor studies suggest that these changes in auxin concentration are causally related to somatic

Table 1 The concentrations of free, conjugated and total IAA measured in the ovaries, ovules, and embryos from carrot (*Daucus carota*) flowers using gas chromatography-mass spectrometry

Structure	Embryo lengths (μm)	Structures/replicate	IAA concentrations (ng/g FW)[a]		
			Free	Conjugated	Total
Unfertilized ovaries	–	2	15 ± 4	2,257 ± 424	2,272 ± 413
Unfertilized ovules	–	60–100	28 ± 5	1,962 ± 563	1,990 ± 483
Fertilized ovaries	75–125	2	608 ± 69	4,986 ± 596	5,594 ± 582
Fertilized ovules	75–125	2–4	504 ± 52	1,063 ± 482	1,567 ± 463
Late globular/early heart embryos	125–225	50–200	2,060 ± 695	7,490 ± 3,440	9,550 ± 4,126
Early torpedo embryos	400–500	50–100	24 ± 5	824 ± 386	848 ± 392
Late torpedo embryos	900–1,100	25–40	27 ± 4	796 ± 292	823 ± 289

[a]The data are presented as the mean ± SE of 4–8 replicates

embryo development (Cooke et al. 1993). In particular, several anti-auxins, which function as competitive inhibitors of native IAA, act as specific inhibitors of early embryogenesis (Schiavone and Cooke 1985). Subsequently, polar-auxin-transport inhibitors, which interfere with auxin efflux in polar auxin transport, block polarized growth in late embryogenesis (Schiavone and Cooke 1985). Strong auxins, which are presumed to overwhelm the resulting auxin gradient, have an effect similar to that caused by transport inhibitors (Borkird et al. 1986). Because comparable changes in free auxin concentrations occur during both somatic and zygotic embryogenesis (Table 1 and Michalczuk et al. 1992a), we hypothesize that an auxin surge is a critical mechanism regulating plant embryogenesis.

One difference between carrot somatic embryogenesis and zygotic embryogenesis is that the decrease in free auxin levels occurred during the globular stage in somatic embryos, which was earlier than it occurred in zygotic embryos (Table 1 and Michalczuk et al. 1992a). This difference can account for the observation that somatic embryos initiated axial elongation prior to cotyledon emergence, whereas the opposite sequence occurred in zygotic embryos (Schiavone and Cooke 1985). Thus, the cotyledons of somatic embryos are usually shorter than cotyledons of zygotic embryos.

Stable-isotope labeling studies have recently resolved that two different pathways for IAA biosynthesis operate during somatic embryogenesis (Michalczuk et al. 1992b; Normanly et al. 1995). Proliferating carrot cultures grown on 2,4-D medium maintain high levels of free and total IAA (Michalczuk et al. 1992a), using the pathway that involves degradation of tryptophan via sequential decarboxylation and deamination to form the IAA (Michalczuk et al. 1992b; Normanly et al. 1995). These IAA levels are consistent with the large pool of the precursor tryptophan available and with the apparent absence of any significant feedback inhibition of IAA biosynthesis (Michalczuk et al. 1992b). By contrast, postglobular embryos maintain much lower IAA levels (Michalczuk et al. 1992a) via the pathway that directly utilizes indole rather than tryptophan to provide the indole skeleton (Michalczuk et al. 1992b; Normanly et al. 1995). Since exogenous IAA appears to suppress IAA biosynthesis via the tryptophan-independent pathway in carrot hypocotyls (Ribnicky et al. 1996), it appears that feedback inhibition of this pathway, in conjunction with IAA conjugation reactions, regulates the basal IAA levels measured in postglobular embryos. The results presented in Table 1, however, do not directly confirm the sequential activity of the two IAA biosynthetic pathways during zygotic embryogenesis. Nevertheless, the 80-fold increase in free IAA levels measured in young zygotic embryos is diagnostic of IAA biosynthesis via the tryptophan-mediated pathway in carrot somatic embryos as well as other plants (Michalczuk et al. 1992b; Normanly et al. 1995). Similarly, the measured return to basal IAA levels in older embryos is a characteristic of regulated biosynthesis via the tryptophan-independent pathway (Normanly et al. 1995). An increasing body of information from our group on the pathways utilized during fruit growth, cold stress and wounding (Cohen and Slovin 1999; Sztein 1999) in plant species other than carrot is also consistent with the general concept of the tryptophan-independent pathway being responsible for maintenance of basal auxin levels. In addition, the necessary precursors, as shown by labeling studies using both [^{14}C]anthranilate and [^{3}H]tryptophan, are both rapidly taken up by growing zygotic embryos when the compounds are supplied through the vascular system (data not shown). Thus, it is our hypothesis that the tryptophan-independent pathway is primarily operative in differentiating cells in organized plant structures where it serves to maintain basal free IAA concentrations that are homeostatically controlled via feedback inhibition and conjugation reactions (Tam et al. 1998).

The presence of levels of high free IAA in fertilized carrot ovules may also explain the occurrence of adventitious embryony whereby differentiated diploid cells in the nucellus and/or the integument of the ovule start growing into the embryo sac and develop into somatic embryos resembling the nearby zygotic embryo (Naumova 1993). If our observations of IAA levels measured in carrot ovules are representative of angiosperm ovules in general, then it appears that these high levels are capable of triggering cell proliferation in differentiated ovular tissues. Thus, adventitious embryony in angiosperm ovules may be the unavoidable consequence of the free IAA levels associated with the initiation of zygotic embryos.

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