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Somatic embryogenesis of some *Daucus* species influenced by ABA

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Summary

A comparison of different species and subspecies of *Daucus* showed that the capacity of their petioles for somatic embryogenesis correlates broadly with the endogenous ABA concentration before culture. Here the competence for this process decreases with lower endogenous ABA concentrations. In some cases somatic embryogenesis could be improved or promoted by an application of ABA to the nutrient medium.

Whereas for differentiation and further development of somatic embryos the concentration of ABA seems to be essential, the induction of somatic embryogenesis seems to depend also on the concentration ratio of endogenous ABA/auxin.

Introduction

For several plant species it was shown that the process of somatic embryogenesis is promoted by ABA (NICKLE and YEUNG, 1994; CENTENEO et al., 1997; KLIMASZEWSKA et al., 1997; DONG et al., 1997; HESS and CARMAN, 1998). Also malformations of somatic embryos are suppressed by ABA (AMMIRATO, 1977) and it was reported that a synchronization of cell suspensions is possible by application of ABA to the nutrient medium (NADEL et al., 1990). Further, the desiccation tolerance of somatic embryos can be increased by an application of ABA (TETTEROO et al., 1996). ABA seems to influence the metabolism and the synthesis of proteins (DONG et al., 1997) and the phytohormone seems to be involved in the regulation of specific genes related to somatic embryogenesis (DUNN et al., 1990; VIVEKANADA et al., 1992). All these results indicate an often neglected central role of this phytohormone in the process of somatic embryogenesis. An application of ABA to cell cultures of *Daucus carota sativus*, i.e. the garden carrot, however, in some experiments had negative influences on somatic embryogenesis (unpublished results of our laboratory).

A recent paper reported genetic influences on the ability to somatic embryogenesis using several *Daucus* species (IMANI et al., 2001). Out of 12 species and subspecies of *Daucus* only 8 were able to produce somatic embryos to various extend. As reported earlier for a domestic variety, in a petiole system able to produce somatic embryos readily a high concentration of ABA, exceeding those of IAA or cytokinins, was detected in excised petiole explants before culture initiation (GRIEB et al., 1997). Therefore in the present study the concentration of ABA in petiole explants of some of these *Daucus* species before culture was determined to check whether a relationship between high ABA concentrations in these excised petiole explants before culture initiation and their capacity to produce somatic embryos exists. Further, responses of cultured petiole explants to exogenous ABA supplied to the nutrient medium were followed in some experiments.

Abbreviations: ABA: abscisic acid
IAA: indole-acetic acid
2,4-D: 2,4-dichlorphenoxy-acetic acid

Material and methods

Plant material and tissue culture

Seeds from *Daucus carota sativus* L. var. Rotin were purchased from a local trader, seeds from the other *Daucus* species and subspecies were obtained from the Bundesanstalt für Züchtungsforschung an Kulturpflanzen (BAZ), Quedlinburg, Germany.

The seeds were germinated and grown in pots in soil under natural conditions.

For cultures, petioles of 6-8cm length were surface sterilized for 1 min with 70% ethanol and 20min with a sodium hypochlorite solution containing 5% active chlorine. After rinsing three times with sterile distilled water, 1-cm-long explants were cultured in a modified B5-medium (GAMBORG et al., 1968; SCHÄFER et al., 1988). Continuous illumination at $21 Wm^{-2}$ was provided by TLD 18 W/840 lamps (Philips, Germany), and the temperature was maintained at 28°C. The explants were cultured at first in a B5-medium containing $2.26*10^{-6}$M 2,4-D for 14 days (induction phase) before they were transferred into an auxin free B5-medium in order to enable the differentiation of somatic embryos (realization phase, SCHÄFER et al., 1988; NEUMANN and GRIEB, 1992; NEUMANN, 1995).

In one experiment petiole explants were cultured in a NL2-medium (NEUMANN, 1966, 1995) with $1.14*10^{-5}$M IAA.

ABA and fluridon were filter sterilized before adding to the nutrient media as indicated.

Determination of abscisic acid

For the extraction of ABA a modified method described earlier (GRIEB et al., 1997) was employed. Petioles of 6cm length were used for the extraction. Samples of 4g freshweight were frozen in liquid nitrogen, lyophilized, and powdered. The extraction was carried out in the dark at 4°C. Samples were extracted three times (overnight, 2 X 1h) with cold 80% methanol (10ml/g fw) supplemented with 10mg/l butylated-hydroxy-toluene (BHT) as an antioxidant. The extracts were centrifuged (3500rpm), the supernatants collected in brown-stained flasks and reduced in vacuo to 1ml. The reduced extracts were transferred to centrifuge tubes and the flasks were washed with 5ml 70% methanol. To remove lipophilic substances like chlorophyll, the crude tissue extracts were passed through a spe C-18 column (Baker bound,spe) equilibrated with 10ml 70% methanol. The extracts were dried under nitrogen at 35°C to a 0.5ml aqueous phase. The tissue extracts from the first purification step were loaded on a C-18 cartridge (Baker bound, spe) which was equilibrated with 10ml methanol and 10ml 50mM TEEA-buffer (pH 3.5). After washing three times with TEEA-buffer the hormones retained on the cartridge were eluted with 5ml 60% methanol and dried under nitrogen at 35°C. The separation and further purification of the ABA-fraction was performed by HPLC. The samples were redissolved in 100µl 40mM TEEA-buffer (pH 3.35-3.4) and passed through a small filter (Millipore Ultrafree) to remove insoluble material. Chromatography was performed on a Merck Lichrosphere Rp-18 column. ABA concentrations were determined by calculating

the integrals of the peaks (TRAN THI, 2000). The investigations were carried out with three replicates.

Results and discussion

Using a modified B5-medium (SCHÄFER et al., 1988) with $2.26*10^{-6}$M 2,4-D as auxin the competence of petiole explants for somatic embryogenesis later on in a auxin free medium decreases as follows:

Daucus carota sativus L. var. Rotin > *Daucus carota ssp. halophilus* Brot. > *Daucus carota ssp. maximus* (Desf.) Ball > *Daucus carota ssp. maritimus* (Lam) Batt. This coincides with an increase of time of culture required before first somatic embryos could be observed (Tab. 1).

Petiole explants of *Daucus muricatus* (L.), *Daucus pusillus* (Michx), *Daucus montevidensis* Link ex Sprengel did not differentiate somatic embryos under these culture conditions. Occasionally only some callus was produced. However, staining with phenosafranine revealed that these explants were still vital (data not shown).

The data in Tab. 1 indicate that the competence for somatic embryogenesis seems to be correlated with the endogenous ABA concentration of the petioles before culture initiation. Petioles of *Daucus carota sativus* L. var. Rotin showed the highest concentration of ABA. This domestic variety also produced within the shortest time of culture somatic embryos (Tab. 1). Petioles of *Daucus muricatus*, *Daucus pusillus*, and *Daucus montevidensis* which failed to produce somatic embryos showed the lowest concentration of endogenous ABA. Also the ABA concentration of petioles of *Daucus carota ssp. maritimus* was comparable to that of *Daucus muricatus*.

In the following it was tried to induce the non-embryogenic species to acquire the competence for somatic embryogenesis by an exogenous supply of ABA. In preliminary experiments the optimum concentration of ABA in the nutrient medium was determined at 10^{-7}M. Therefore 10^{-7}M ABA were added to the nutrient medium at culture initiation (t_0) and at t_{14} (change to the auxin free medium for further 31 days). This concentration of ABA improved somatic embryogenesis of the embryogenic species (estimated increase of number of embryos per vessel) considerable. Still non of the species with low ABA concentrations in original petioles and which were unable to produce somatic embryos (*Daucus muricatus*, *Daucus pusillus*, and *Daucus montevidensis*) became embryogenic after an ABA supplement to the nutrient medium.

As a follow up to these experiments the same ABA treatments were applied to NL2-medium in which instead of 2,4D IAA is used as the auxin and within 3 days of culture in the light all of this phytohormon is removed from the nutrient medium by photooxydation (BENDER and NEUMANN, 1978). To define further on the role of ABA for somatic embryogenesis, here the phytohormone was supplied to the medium at different days after the start of the experiment. As before *Daucus carota sativus* L. var. Rotin, *D. c. ssp halophilus* and *D. c. ssp. maximus* produced somatic embryos (Tab. 2). But an application of ABA at t_0 and t_3 inhibited the production of somatic embryos in *Daucus carota ssp. sativus* L. var. Rotin and only with a supplement of the phytohormone at the end of the first week of culture embryogenesis is observed. Further, petiole explants of *Daucus carota ssp. maritimus* did not produce somatic embryos unless ABA is supplied at t_0 or at t_{10}. Considering the high ABA concentration in *Daucus carota ssp. sativus* L. var. Rotin in the petiole tissue before culture initiation after application of the exogenous ABA the concentration of this phytohormon is now too high to allow the induction of somatic embryogenesis. As shown earlier during the first week of culture the concentration of endogenous ABA is reduced and hence now low enough to permit the initiation of somatic embryos (GRIEB et al., 1997). On the other hand the application of ABA to cultures of *Daucus carota ssp. maritimus* at t_0 initiates the development of somatic embryos otherwise not possible in this *Daucus* subspecies with a low endogenous ABA concentration in the original petiole explants if culture occurs in NL2-medium. This indicates a requirement for a specific level of ABA/IAA during the initial phase of culture for the induction of somatic embryogenesis, which can not be further interpreted at present.

At any rate as was observed earlier for cv.Rotin the culture of up to 48 h in the B5-medium is sufficient to promote the differentiation of somatic embryos from the 12. day of culture onward. Auxin is involved in this process as shown also by many others without being able to specify its function in more detail.

As shown by molecular analysis, also the genome of *Daucus carota ssp. maritimus* contains two DNA stretches characteristic for the embryogenic species used in our study (IMANI et al., 2001). Consequently, petiole explants of this subspecies should basically be able

Tab. 1: ABA concentrations of petioles of some *Daucus* species and subspecies at explantation and the capability of these petiole explants to somatic embryogenesis

species/subspecies	origin	ABA [ng/g fw] at explantation	somatic embryogenesis	first observation of somatic embryos [days] after t_0
Daucus carota sativus L. var. Rotin	Germany	34.35[a]	yes	16.-17.
Daucus carota ssp. halophilus Brot	Mediterranean	16.90[b]	yes	18.-19.
Daucus carota ssp. maximus (Desf.) Ball	Spain, Algeria, Cyprus, Lebanon	13.49[c]	yes	20.-21.
Daucus carota ssp. maritimus (Lam.) Batt.	Mediterranean, Spain, Turkey	7.75[c]	yes	21.-23.
Daucus muricatus (L.)	Spain, Portugal, Italy, Algeria	7.53[c]	no	/
Daucus pusillus (Michx.)	USA, Mexico, Chile	3.15[d]	no	/
Daucus montevidensis Link ex Sprengel	Uruguay, Chile, Argentine	2.17[d]	no	/

a, b, c, d: significant differences

Petiole explants were cultured in a modified B5-medium containing $2.26*10^{-6}$M 2,4-D for 14 days (induction phase) before they were transferred into an auxin free B5-medium for further 21 days.

Tab. 2: Influences of 10^{-7}M ABA on somatic embryogenesis of *Daucus carota sativus* L. var. Rotin, *Daucus carota ssp. maritimus*, *Daucus carota ssp. halophilus* and *Daucus carota ssp. maximus*

subspecies	control	t_0	t_3	t_7	t_{10}	t_{14}
Daucus carota sativus L. var. Rotin	all stages of embryos	/	/	all stages of embryos, but only few embryos	all stages of embryos, but only few embryos	all stages of embryos, high number of embryos
Daucus carota ssp. maritimus (Lam.) Batt.	/	only few embryos	/	/	only few embryos	/
Daucus carota ssp. halophilus Brot	all stages of embryos	all stages of embryos	all stages of embryos, higher number of embryos as at control	all stages of embryos, higher number of embryos as at control	all stages of embryos	all stages of embryos
Daucus carota ssp. maximus (Desf.) Ball	all stages of embryos, but only few embryos	all stages of embryos, but only few embryos	all stages of embryos, higher number of embryos as at control and t_0	all stages of embryos, higher number of embryos as t_3	all stages of embryos, but less embryos as at t_7	all stages of embryos, mainly globular stages

Petiole explants were cultured in a NL2-medium with $1.14*10^{-5}$M IAA for 47 days.
t_0, t_3, t_7, t_{10}, t_{14}: day of application of 10^{-7}M ABA respectively of Aqua dest. for control

to produce somatic embryos and as shown above this is the case. Cultured petiole explants of *Daucus muricatus*, *Daucus pusillus*, and *Daucus montevidensis* failed to be induced to undergo somatic embryogenesis even after ABA application. The genomes of these three species did not show these two specific DNA sequences (IMANI et al., 2001). The simultaneous absence of these two DNA-sequences, the low ABA concentration in the original petiole explants, and the missing competence for somatic embryogenesis of these three *Daucus* species could be mere coincidence, still some more investigations on these species could be rewarding.

NICKLE and YEUNG (1994) demonstrated for carrot somatic embryos that ABA seems to affect the capacity of these embryos to develop a functional shoot meristem. Through application of the ABA biosynthesis inhibitor fluridon a rapid vacuolation of cells in the apical bilayer occured. This vacuolation was concurrent with a decline in conversion of these embryos. It could be partially reversed by application of ABA. If in our system fluridon was supplied in a concentration of 0.05mg/l to the modified B5-medium at t_0 (induction phase) as well as subsequential to the auxin free medium at transfer (realization phase), petiole explants of *Daucus carota sativus* L. var. Rotin did not produce somatic embryos. If, however, the inhibitor was only supplied to the induction phase explants differentiated embryos. Therefore, a de novo synthesis of ABA seems to be not required during the induction phase. Apparently the original endogenous ABA concentration of the petiole explants is sufficient. However, a de novo synthesis of ABA is essential for the differentiation and further development of somatic embryos in the auxin free medium.
Since an ABA supplement to the non-embryogenic species does not induce somatic embryos apparently other factors essential for somatic embryogenesis (besides ABA) are different in these species.

SENGER et al. (2001) showed that after application of 1µM fluridon to the medium of *Nicotiana plumbaginifolia* cell cultures morphological changes like loosening of cell boundaries and large unorganized cell clusters could be observed. These effects were most pronounced if the inhibitor was supplied at day 10 of culture when pre-globular embryos were formed. These effects were reversible by addition of ABA to the medium. This agrees with the observation above to induce somatic embryos by an ABA application at t_0 and at t_{10} to *Daucus carota ssp. maritimus*, at a time (t_{10}) when globular embryos are formed in *Daucus carota sativus* L. var. Rotin petiole cultures. Here also a small peak of endogenous IAA occurs and the concentration of endogenous cytokinins is raised (GRIEB et al., 1997).

It becomes more and more obvious that sugars are signaling compounds which have profound effects at all stages of the plant's life cycle. Particularly hexoses seem to be involved in the induction and realization of somatic embryogenesis in petiole explants of carrot (PLESCHKA, 1995; PLESCHKA et al., 2001). Sugar signaling pathways seem to be part of cellular regulatory networks. Interactions between sugars, light, and phytohormones probably exist (SMEEKENS, 2000). ARENAS-HUERTERO et al. (2000) suggested that ABA could play a central role in the glucose signaling network governing plant growth and development in mediating the glucose responses. ROOK et al. (2001) presented an alternative model in which ABA is not directly involved in the sugar signaling chain. Instead the phytohormone regulates the way in which tissues respond to sugar signals. It remains to be elucidated if ABA influences somatic embryogenesis through a sugar signaling network either as being a direct part of it or by changing the way in which specific cells respond to the hexose signal.

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