Desiccation tolerance of somatic embryoids

Uitdroogtolerantie van somatische embryoiden

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BIBLIOTHEEK
LANDBOUWUNIVERSITEIT
WAGENINGEN

1110820, 2098

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Desiccation tolerance of somatic embryoids

Proefschrift
ter verkrijging van de graad van doctor
in de landbouw- en milieuwetenschappen
op gezag van de rector magnificus
dr C.M. Karssen
in het openbaar te verdedigen
op vrijdag 7 juni 1996
des namiddags te vier uur in de aula
van de Landbouwuniversiteit te Wageningen

925724

The work presented in this thesis was performed at the Wageningen Agricultural University, Department of Plant Physiology, Arboretumlaan 4, 6703 BD Wageningen, The Netherlands.

CIP-DATA KONINKLIJKE BIBLIOTHEEK, DEN HAAG

Tetteroo, F.A.A.

Desiccation tolerance of somatic embryoids / F.A.A. Tetteroo.
-[S.l. : s.n.]. -Ill.
Thesis Landbouwuniversiteit Wageningen.
- With ref. - With summary in Dutch.
ISBN 90-5485-517-7
Subject headings: somatic embryogenesis / desiccation tolerance / carbohydrates

mi0870, 2012

Stellingen behorende bij het proefschrift getiteld "Desiccation tolerance of somatic embryoids" door F.A.A Tetteroo.

1. Somatische embryoiden van plantensoorten met orthodoxe zaden zijn potentieel uitdroogtolerant. (dit proefschrift)

2. Umbelliferose en saccharose zijn inhoudsstoffen van *Daucus carota* embryoiden die essentieel zijn voor het overleven van volledig uitdrogen. (dit proefschrift)

3. De belangrijkste oorzaak voor uitdrooggevoeligheid van *Daucus carota* embryoiden is het optreden van omvangrijke membraanschade, waardoor de permeabiliteit irreversibel toeneemt. (dit proefschrift)

4. Het gebruik van de term somatische embryoiden voor embryoiden die ontstaan zijn uit microsporen is onjuist.

5. De inductie van somatische embryogenese is nog altijd gekoppeld aan de aanwezigheid van een auxine-analoog in het medium; het is daarom onzeker of het auxine-analoog dan wel het embryogenese proces zelf de somaclonale variatie veroorzaakt.

6. Het onderzoek naar uitdroogtolerantie is gevoelig voor het functioneren van de geldkraan.

7. Het schrijven van een proefschrift is eenvoudiger dan het verzorgen en opvoeden van kinderen.

8. De commotie die ontstond bij de oprichting van het niet door iedereen te ontvangen sportkanaal geeft eens te meer aan dat de romeinse keizers exact begrepen wat onontbeerlijk was voor het volk, n.l. brood en spelen.

9. Het, ten opzichte van Creutzfeldt-Jacob ziekte, vele male grotere aantal doden op de weg, zou het afschaffen van autoverkeer logischer maken dan het op grote schaal afslachten van Engelse runderen.

10. De bezuinigingen in het wetenschappelijk onderzoek hebben een even "kennis vernietigende" werking als het afschaffen van de vliegtuigindustrie; het valt alleen minder op.

11. Het is een vorm van inhumane discriminatie dat wel hondenbelasting maar geen kattenbelasting wordt geheven.

12. Dit boekje bestaat niet alleen uit vulling.

Wageningen, 7 juni 1996

Content

Voorwoord

Het schrijven van het voorwoord van een proefschrift is in vele gevallen het begin van het einde van een promotie-onderzoek. Ook ik ben aan het einde gekomen van een leerzame en vermoeiende promotieperiode en ik tracht met mijn laatste creativiteit een vriendelijk voorwoord te schrijven (lees typen). Als ik terug denk aan de afgelopen periode dan herinner ik een aantal hoogtepunten: trouwerij, vaderschap, verkrijgen van een nieuwe baan, en natuurlijk deze promotie, maar evenzeer denken aan ik een aantal dieptepunten: werkeloos worden en zijn, opname in het ziekenhuis en overlijden van dierbaren. Niettemin kijk ik met veel genoegen terug op deze periode, vooral omdat ik door vele mensen gesteund ben en daardoor het doorzettingsvermogen behouden heb om dit proefschrift te voltooien. Een aantal van deze mensen wil ik daarom speciaal bedanken.

In eerste instantie wil ik de initiatiefnemers Cees Karssen (promotor en rector magnificus) en Jan de Boer (toenmalig directeur Royal Sluis) bedanken. Zonder jullie was dit onderzoek nooit verricht. Helaas zijn jullie inhoudelijke bijdragen tot dit project tot een minimum beperkt gebleven, vanwege jullie drukke werkzaamheden op een veel hoger niveau. Dit gebrek aan inhoudelijke sturing is ruimschoots gecompenseerd door de copromotor Folkert Hoekstra. Speciaal op de momenten (na zes uur), waarop jij niet door anderen kon worden afgeleid, was het heerlijk om met jouw te discussiëren over de lopende experimenten en de nieuwe ideeën. Folkert jouw creativiteit is ongenaakbaar en zonder jouw zou dit project niet zo voorspoedig zijn afgelopen en daarvoor mijn hartelijke dank.

Naast de inhoudelijke steun is ook praktische hulp onontbeerlijk om een promotie met succes af te ronden. Het produceren van grote aantallen embryoiden in weefselkweek is niet eenvoudig en vereist een zekere mate van groene vingers. Daarom moet ik allereerst Birgit bedanken. Toen jij bij mij als analiste kwam werken, wist jij niet of je wel die groene vingers bezat, omdat jij totaal geen ervaring bezat met weefselkweek van planten. Toch ben jij erin geslaagd door jouw onuitputtelijke enthousiasme om in zeer korte tijd de techniek onder de knie te krijgen en vele uitdroogtolerante wortel embryoiden te regenereren. Jouw ervaring met HPLC was zeer welkom en heeft mij

verder geholpen met de suikeranalyses. Gelijktijdig moet ik Tineke van Roekel bedanken. Voor jouw was de afgelopen periode heel moeilijk gezien de gezondheidsproblemen van Willem. Desondanks heb je mij altijd weer kunnen helpen met die lastige fosfolipide extracties en de vetzuurketen bepalingen op de gaschromatograaf. Adriaan van Aelst ben ik dank verschuldigd vanwege zijn praktische medewerking met alle elektronen-microscoopstudies. Vooral het samenwerken met jouw op de FESEM zal ik niet gauw vergeten, vanwege jouw enthousiasme, de eenvoud van de techniek en de mooie plaatjes die wij hebben gemaakt waarvan er een is gebruikt op de voorkant van dit proefschrift.

Voor het uitvoeren van een groot aantal experimenten heb ik het geluk gehad dat een aantal goede enthousiaste studenten hun doctoraal vak of stage bij mij hebben verrichten. Emannuel, Sander, Monique, Anette, Rob, Adrie en Claude bedankt voor de bijdragen die jullie geleverd hebben aan de totstandkoming van dit proefschrift. Ik heb met veel voldoening en enthousiasme met jullie samengewerkt. Ik hoop dat jullie de verworven ervaringen met die lastige wortel embryoiden nog van pas zullen komen. Tevens moet ik Ite, Marianne en dr Bryan McKersie bedanken. Hoewel ik niet met jullie heb mogen samenwerken hebben jullie toch ook een bijdrage geleverd aan dit proefschrift. Door jullie inleidende experimenten ben ik in staat geweest om snel goede resultaten te behalen.

Niet alleen de praktische hulp is van belang geweest tijdens dit promotie-project, maar ook technische assistentie was noodzakelijk om de benodigde apparatuur continu te kunnen laten functioneren. Ruth en Ben waren instaat om elk technisch probleem in een korte op te lossen. Siep Massalt heeft uitstekend geholpen bij het afdrukken van foto. Voor software problemen met Lotus of WordPerfect kon ik altijd terecht bij Steef de Bruin. De door jouw ontworpen "Lotus-macro" voor het omrekenen van piekoppervlaktes naar suikergehaltes heeft mij veel tijd bespaard, waardoor ik meer experimenten heb kunnen uitvoeren.

De totstandkoming van dit proefschrift is niet alleen te danken aan mensen die een directe bijdrage hebben geleverd, maar er is een grote groep collega's die ik moet bedanken vanwege hun indirecte bijdrage. Zij hebben er namelijk mede voor gezorgd dat er een zeer goede werksfeer op de vakgroep aanwezig was, waardoor inspanning op de juiste tijden werd afgewisseld met ontspanning. Ik herinner mij maar al te goed de vietnamese loempia's op woensdagmiddag, de kipkerrie-broodjes en worstenbroodjes van de warme bakker en de koffiediscussies in onze illegale kantine aan de rand van de

"Banaan". Mark en Jaap moet ik in deze speciaal bedanken omdat zij zich bereid hebben verklaard om mij te ondersteunen als paranympen.

Ik moet tevens mijn huidige werkgever Incotec International BV bedanken voor de financiële steun die zij mij hebben gegeven om mijn kosten voor dit proefschrift tot een minimum te beperken.

Als laatste wil ik Liesbeth bedanken voor de ruimte en ondersteuning die jij mij hebt gegeven gedurende deze promotie-periode. Ik hoop dat wij samen nog lang van deze investering kunnen genieten.

Frans

List of abbreviations

ABA	: abscisic acid
BHAM	: benzhydroxamate
CF	: 5(6)-carboxyfluorescein
2.4-D	: 2,4-dichlorophenoxy-acetic acid
DW	: dry weight
EF	: extraplasmatic face
FFA	: free fatty acids
FTIR	: fourier transform infrared spectroscopy
FW	: fresh weight
HPLC	: high performance liquid chromatography
IMP	: intramembraneous particles
LTSEM	: low temperature scanning electron microscopy
MC	: moisture content
PCV	: packed cell volume
PEG	: polyethylene-glycol
PEM	: pro-embryogenic mass
PF	: plasmatic face
PL	: phospholipid
RH	: relative humidity
SD	: standard deviation
SEM	: scanning electron microscopy
T_m	: gel-to-liquid crystalline phase transition temperature
TEM	: transmission electron microscope
v_{tot}	: total respiration
v_{cyt}	: respiration due to the cytochrome pathway
v_{alt}	: respiration due to the alternative pathway
v_{res}	: residual respiration

Chapter 1

General Introduction

Somatic embryogenesis

In 1838 Schwann and Schleiden have hypothesised that cells are autonomous and therefore have the unique property of being totipotent (historical review, Pierik, 1985). Totipotency is defined as the capacity of a cell to regenerate a complete new individual. However, it took more than a century before this theory could be experimentally verified for plant cells, due to the availability of the plant hormones auxin and cytokinin. Somatic embryogenesis can be considered as the ultimate form of totipotency. Somatic embryogenesis is an *in vitro* regeneration system, whereby an organized bipolar structure is formed out of one somatic cell. The bipolar structure contains a root apex as well as a shoot apex and thus morphologically resembles a zygotic embryo. In this thesis the bipolar structure will be referred to as (somatic) embryoid.

Somatic embryogenesis is potentially the most efficient method for large scale plant micropropagation, because root and shoot meristems are regenerated simultaneously on one structure (Redenbaugh, 1993). This is in contrast with conventional micropropagation techniques, whereby only roots or shoots are formed at a time. An extra advantage of somatic embryogenesis is the large amounts of individuals that can be produced simultaneously in liquid culture media.

Thirty seven years ago the first embryoids were regenerated *in vitro*, independently by Reinert (1958) and by Steward and coworkers (1958). They both subcultured carrot (*Daucus carota* L.) cell suspensions at low density in a hormone-free

culture medium. The original cell suspensions, induced and multiplied on auxin medium, contained small cytoplasm-rich cells and cell clusters, which were called proembryogenic masses (PEMs) by Halperin (1966). Within 14 days on hormone-free medium, these PEMs developed successively into globular, heart and torpedo-shape embryogenic stages. These embryogenic stages are typical for dicotyledon zygotic morphogenesis (Raghavan, 1986). Later on during the *in vitro* culture on hormone-free medium, the root and shoot meristems of these embryoids simultaneously grew out and developed into a complete plantlet, comparable to germinating seeds. Since this first discovery in carrot a multitude of reports on somatic embryogenesis of other plant species have been published (reviewed by Williams and Maheswaran 1986; Raghavan, 1986; Gray and Purohit, 1991; Redenbauch, 1993). At the moment more than 300 different plant species can be regenerated through somatic embryogenesis. Even from recalcitrant species of different plant groups such as conifers (Pinus, Abies), trees (banana, palm), cereals (rice, maize), vegetables (cucumber, soybean) and ornamentals (Euphorbia, Pelargonium) embryoids can be regenerated. Most of these recalcitrant species could be regenerated through somatic embryogenesis because of the prudent choice of explant source. A complete overview of all plant species that can be regenerated by this method is not in the scope of this thesis.

Carrot somatic embryogenesis still remains the primary model system. It is used to study the fundamental aspects of this phenomenon and to compare it to zygotic embryogenesis. Both processes are poorly understood up until now (see review Zimmerman, 1993).

Growth regulators play an important role during the induction and regeneration of somatic embryoids. Auxins, especially the strong synthetic 2,4-dichlorophenoxy-acetic acid (2.4-D), trigger the induction of somatic embryoid development. However, they also inhibit further development at the globular stage. Therefore, the auxin has to be removed from the culture medium to continue proper embryoid development. The role of exogenously supplied auxin as a trigger can be argued, because it has also been shown possible to induce somatic embryogenesis in carrot through a pH shift in auxin-free

medium (Smith and Krikorian, 1989, 1990). This example indicates how little we understand of the induction of embryogenesis.

Abscisic acid (ABA) plays a major role during zygotic embryogenesis, especially during the maturation phase of developing seeds (Kermode 1990; Bewley and Black, 1994). Ammirato (1974) was the first to recognize the importance of ABA in somatic embryogenesis, while studying cultures of caraway (*Carum carvi*). ABA synchronized the development of the embryoids, it induced a quiescent phase at the torpedo stage of development, it prevented precocious germination and it inhibited abnormal proliferations (Ammirato, 1974, 1983). Since these discoveries more attention has been given to the role of ABA in embryogenesis (reviewed by Skriver and Mundi, 1990). Koornneef et al. (1989) and Meurs et al. (1992) demonstrated unequivocally that endogenous ABA is also involved in the acquisition of desiccation tolerance of seeds. To establish this relationship they have used recombinants of ABA-deficient and ABA-insensitive mutants of *Arabidopsis thaliana* L..

In 1977 Murashige has for the first time publicly discussed the idea of synthetic seeds (further on referred to as artificial seed) (reviewed by Redenbauch, 1993). Several concepts for artificial seed were put forward in the literature, but most correctly, an artificial seed is defined as a somatic embryoid encapsulated with a coating which is directly analogous to a true zygotic seed (Redenbauch, 1993). Kitto and Janick (1982) were the first to put this idea into practice, however, with little success. Their desiccated artificial seeds of carrot germinated very poorly. The commercial interest on somatic embryogenesis rapidly increased, when Redenbauch et al. (1986) came with the concept of hydrated artificial seeds. They encapsulated alfalfa (*Medicago sativa* L.) embryoids with a hydrogel, e.g. sodium alginate, to get a propagule which resembled a true seed. The hydrogel served as synthetic endosperm containing carbon sources, nutrients, growth regulators, anti-microbial agents and minerals. These beads could be sown directly in the field or greenhouse, however the conversion rate (outgrowth into plantlets) was very low. These hydrated beads were also difficult to store, because the freely available water prevented the embryoids to reach a quiescent phase, in spite of the addition of ABA.

The latest achievement in somatic embryogenesis is the induction of desiccation tolerance. In 1989, McKersie and coworkers produced desiccation tolerant alfalfa embryoids, which had a moisture content less than 10 %. They also could confirm the involvement of ABA in the acquisition of desiccation tolerance of somatic embryoids (McKersie et al., 1989; Seneratna et al., 1989a and b). Not only alfalfa embryoids were able to survive almost complete dehydration, but also those of white spruce (*Picea glauca* [Moench] Voss.) (Attree et al., 1991), grape (*Vitis longii* L.) (Gray, 1990) and zonal geranium (*Pelargonium x hortorum*) (Marsolais et al., 1991). The role of exogenous ABA in the acquisition of desiccation tolerance can be discussed, because Anandarajah and McKersie (1990 and 1991) were able to induce tolerance to dehydration in alfalfa embryoids through heat or osmotic stress instead of ABA. Such stress treatments might have raised the endogenous ABA levels (Skriver and Mundy, 1990) and thus induced desiccation tolerance. In all the previous studies on induction of desiccation tolerance in embryoids the plant recovery rates were always significantly less than 100%. Poor embryoid quality, caused by sub-optimal protocols, or asynchronic embryoid development could have been the main reason for the low recoveries. More fundamental knowledge of seed physiology is required to advance in artificial seed technology (Gray and Purohit, 1991; Redenbauch, 1993).

Commercial breakthrough of artificial seeds may only be achieved through the combination of desiccation tolerance and dry encapsulation of high quality embryoids. With this combination one may unite the genetic uniformity of vegetative propagation with the storability and easy handling of seeds.

Desiccation tolerance

The Dutch pioneering microscopist Antonie van Leeuwenhoek was the first human to witness the phenomenon of desiccation tolerance. He had put some dry sediment out of his gutter into a glass with water and in a short time afterwards he saw with his microscope hundreds of small animals swimming around. This phenomenon was

originally called anabiosis or "return to life", because biologists at that moment believed that dried viable organisms actually were dead and returned to live after rehydration. Nowadays, the term anhydrobiosis or "life without water" (Keilin, 1959) is generally used, because dry organisms only cease their metabolism and seem without life, however they regain each vital metabolism under favourable environmental conditions.

Anhydrobiosis is exhibited in both the animal and the plant kingdom, for example nematodes, rotifers, tardigrades, cysts of brine shrimp, bacteria, fungi spores, algae spores, yeast cells, lichens, mosses, ferns, pollen, seeds of plants and even whole plants (for a review, see Crowe et al., 1996 and references therein). Many scientist have been fascinated by this phenomenon and have tried to elucidate the underlying fundamental mechanisms. Desiccation sensitivity always coincides with extensive leakage of intracellular solutes upon rehydration. The leakage often coincides with severe damage to the organism, and even to its death. Therefore, most research efforts have been focused on membrane behaviour, because it is believed that the phospholipid bilayer is the primary target of injury. Leakage of intracellular constituents indicates problems at the membranes (phospholipid bilayer). In the following paragraphs the three major concepts concerning the mechanisms of desiccation (in)tolerance with respect to membranes are described.

Imbibitional damage

During rehydration (imbibition), dry organisms leak solutes into the surrounding medium, which is associated with loss of viability. This kind of leakage can excellently be explained through the biophysical behaviour of the plasma membrane during rehydration, as incontrovertibly demonstrated by Crowe et al., (1989a,b). They extensively studied imbibitional damage in pollen and liposomes (phospholipid vesicles), that were used as simplified model systems for membranes. They developed the following hypothesis, which is depicted schematically in Figure 1. In order to explain this theory, it is necessary to describe the construction of membranes and their components. Membranes consist mainly of phospholipids (50%), proteins (40%) and steroids (10%). Their

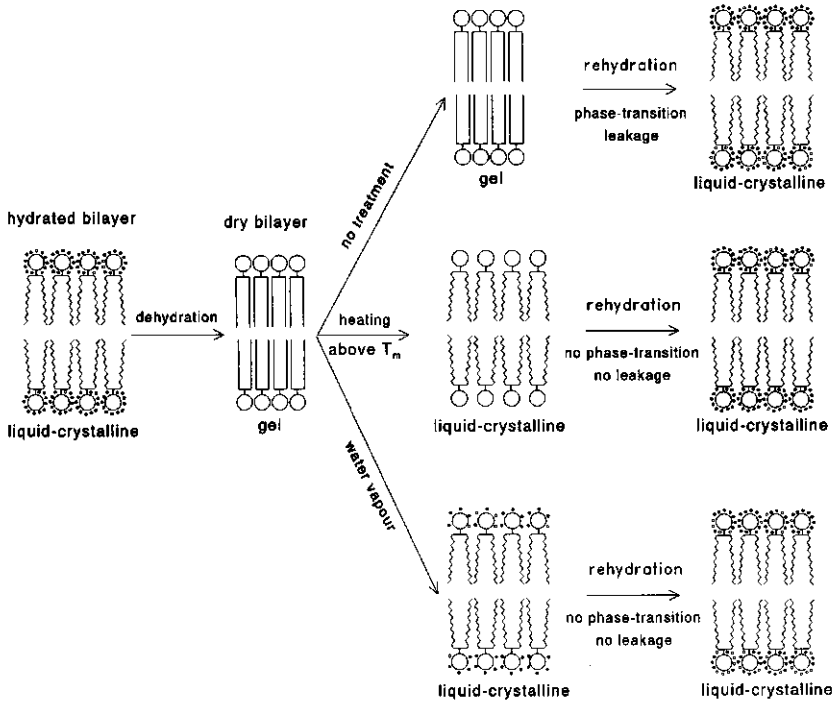

Figure 1: Diagrammatic representation of the proposed mechanism for imbibitional damage. As a hydrated bilayer (water molecules are depicted as small circles) in liquid crystalline phase is dehydrated, it may enter gel phase, depending on temperature. If this dry bilayer is not heated to above its transition temperature (T_m) before it is returned to water (upper pathway) it will undergo a phase transition during rehydration during which it would be expected to leak. On the other hand, if it is heated to above T_m or partially hydrated by exposure to water vapor (lower two pathways), the bilayer can pass through the phase transition in the absence of bulk water. Under these conditions it will not undergo a phase transition when it is placed in water and it will not leak (redrawn from Crowe et al., 1989b).

biophysical behaviour is principally specified by the phospholipids. In a aqueous solution the phospholipids tend to form a lamellar bilayer structure, because of their amphipathic character. The hydrophobic acyl chains turn inwards, whereas the hydrophilic phosphate head groups are located at the outside of the bilayer (Fig. 1). Because of the polarity of the phosphate groups the water molecules are surrounding them and form hydrogen bonds. Phospholipid bilayers exist mainly in two forms: liquid-crystalline-phase (fluid) or gel-phase (solid). At the transition temperature (T_m) the bilayer changes from the fluid to

the solid state or the reverse. The T_m depends on the composition of the acyl chains of the lipids. For instance, DPPC (di-palmitoyl-phosphatidyl-choline) vesicles in water undergo a phase change at 41°C (Chapman et al., 1967), whereas DLPC (di-linolenoyl-phosphatidyl-choline) vesicles in water have a T_m of -60 °C (Lynch and Steponkus, 1989). The latter vesicles have poly-unsaturated acyl chains and are, therefore, much more fluid.

A phase transition may also occur when water is removed from the bilayer, also depending on the composition of the acyl chains of the lipids. In other words, during the removal of water T_m of the membrane increases. Dry DPPC vesicles have a T_m of approximately 110°C (Crowe and Crowe, 1988). The increase due to dehydration can be explained as follows: the lateral spacing of the polar head groups decreases due to water removal, leading to increased opportunities for van der Waals' interactions between the hydrocarbons of the acyl chains (reviewed Crowe et al., 1996). Therefore, most dry phospholipid bilayers, constructed from isolated plant phospholipids, are in the gel-phase at physiological temperatures. As phospholipid bilayers are rehydrated they pass through their gel-to-liquid crystalline-phase transition, their permeability is transiently increased, and, in the case of unilamellar liposomes, all entrapped soluted are lost (Crowe et al., 1996; Fig. 1). During dehydration, this process occurs in reverse, also with increased permeability; however no leakage is noticed because of the limited availability of water as carrier of solutes.

During the thermotropic phase transition the lipid bilayer contains lipids in gel-phase as well as in liquid-crystalline-phase. The two types of lipids may become phase-separated and thus form gel-phase domains and liquid-crystalline-domains. The transient leakage during the phase transition is currently explained as the result of packing defects at the boundaries between the domains of these two phases. This effect might be exacerbated in plasma membranes, because they have a complex mixture of phospholipids (reviewed by Crowe et al., 1996).

Imbibitional leakage can be avoided through careful rehydration in water vapour (e.g. pollen, review Hoekstra et al., 1989) or through raising the imbibitional temperature above the T_m (e.g. yeast, Van Steveninck and Ledeboer 1974) (Fig. 1). Both methods

prevent phase transition of the plasma membranes in the presence of bulk water and, thus, the dry organisms are able to survive rehydration.

Water replacement theory

Anhydrobiotic organisms acquire the ability to withstand desiccation through several biochemical adaptations. Webb (1965) noticed an increase of inositol in desiccation tolerant bacteria. In cysts of *Artemia* an increase of trehalose to 20% of the DW was found during the adaptation to dehydration (Madin and Crowe, 1975). Trehalose, the only non-reducing disaccharide of glucose, was also found in large quantities in yeast and bacterial spores (Crowe et al., 1984). Trehalose does not seem to occur generally in higher plants, but instead, sucrose is the mayor disaccharide which accumulates during acquisition of desiccation tolerance (Amuti and Pollard, 1977). However, not only sucrose but also tri- and oligosaccharides are found in large amounts in seeds (Koster and Leopold, 1988).

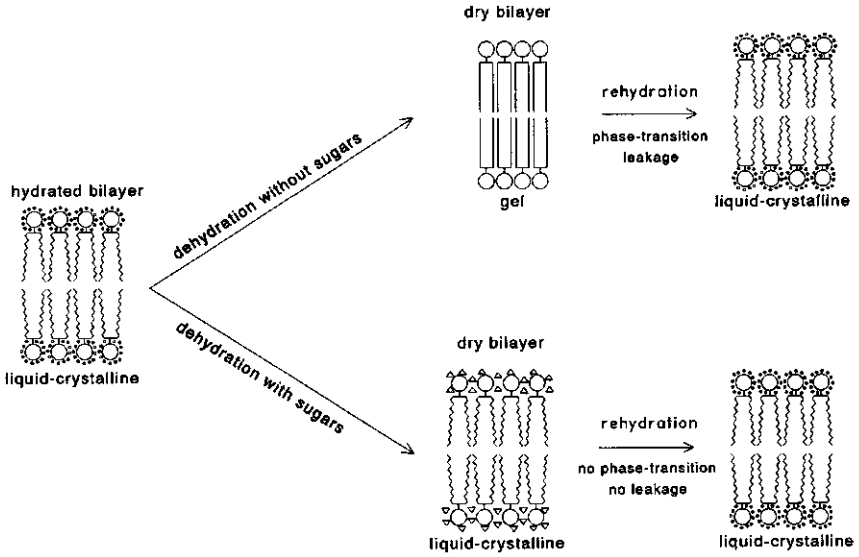

Figure 2: Diagram illustrating the mechanism by which carbohydrates (e.g. trehalose, ▵) is thought to stabilize dry phospholipid bilayers. Trehalose inhibits fusion during dehydration, but also reduces the gel to liquid crystalline transition temperature in the dry phospholipids. As a result of the latter effect, a phase transition during rehydration and the associated leakage can be avoided (redrawn from Crowe et al., 1992).

The apparent changes in carbohydrate metabolism of these anhydrobiotic organisms suggest that sugars must have an essential function in the survival of the dry state. Webb (1965) proposed that accumulating polyols could provide hydrophilic components which may replace the water molecules around labile macromolecules of biological importance during drying. This water replacement idea has been taken up by Crowe and coworkers in the late 1970's (reviewed by Crowe et al., 1996). Since, they have done some excellent research to elucidate the possible role of carbohydrates in anhydrobiosis. They found that sugars can maintain the structural integrity of plasma membranes even under dry conditions and that sugars prevent membranes from fusing, which maintains compartmentalization in the dry organisms (Fig. 2). During dehydration of the phospholipid bilayer the sugar molecules replace the water molecules around the polar head groups and also form hydrogen bonds with the phosphate group. Thus, the liquid-crystalline-phase in the dry state is retained (Fig. 2). No phase transition is taking place and thus, no leakage occurs, and the organism will survive the dehydration.

It has been incontrovertibly confirmed that proteins are also protected by carbohydrates through the water replacement mechanism (Carpenter et al., 1987; Crowe et al., 1987). The secondary structure of the dry protein (Prestrelski et al., 1993) and its functioning remain intact through hydrogen bonding of the sugar molecules.

Irreversible membrane damage

The fore-mentioned changes in membrane phase behaviour with drying are reversible in the sense that water addition restores the original situation. Permanent gel-phase lipid was found after lethal desiccation of germinating soybean seeds (Senaratna et al., 1984, McKersie et al., 1988), which was ascribed to a changed composition of the plasma membranes. An accumulation of unsaturated free fatty acids (FFA) was detected in these soybean seeds, which caused an irreversible increase of the transition temperature of the membranes far above physiological temperatures. FFAs are also able to induce fusion of membranes (Crowe et al., 1996). Both phenomena have been identified as major damaging events. The rise in T_m could also be induced in liposomes by the addition of unsaturated FFA (Senaratna et al., 1987). The question remains where do these FFA

come from? There are two lines of evidence that explain the origin of the FFA A: oxidative attack by free radicals which promotes phospholipid de-esterification and/or lipid peroxidation (Leprince et al., 1993) and B: enzymatic de-esterification of the phospholipids (Crowe et al., 1996). In germinating maize seeds, which have lost desiccation tolerance, respiration increases with incubation time, and this coincides with a dramatic increase of free radicals after redrying. This respiration in the hydrated state is held responsible for the elevated levels of free radicals after drying (Leprince et al., 1990, 1992). Free radical attack can be prevented through antioxidants (e.g. tocopherol, ascorbic acid) or free radical scavenger systems (e.g. SOD, catalase, glutathione reductase). Senaratna et al. (1987) demonstrated that membranes isolated from desiccation tolerant soybean axes were less susceptible to free radicals than the intolerant membranes, because the latter lacked lipid soluble antioxidants. FFA might also originate from enzymic de-esterification of the phospholipids through the activity of lipases such as phospholipase-A_2 (Crowe et al., 1996).

Other concepts

Not only the plasma membrane has to survive the dry state but also other cell parts, such as cytoplasm and DNA. Therefore, it has been suggested that vitrification (glassy state) of the cytoplasm is also an important parameter in the survival of almost complete dehydration (Burke, 1986; Bruni and Leopold, 1991; Koster, 1991). Glass formation, or vitrification, is the creation of liquid solution with the viscosity of a solid (Williams and Leopold, 1989). It is formed either by increasing the solution concentration or lowering the temperature. Carbohydrates not only protect membranes and proteins, but also enhance vitrification. A glassy state is beneficial for anhydrous organisms because degradative chemical reactions are inhibited, crystallization of cell constituents is prevented, cellular collapse is limited and quiescence and dormancy are ensured. The existence of a glassy state in anhydrous organisms does not exclude the interaction between phospholipids or proteins with carbohydrates (Crowe et al., 1996).

Another alternative explanation for the loss of viability in desiccation sensitive embryoids is that physical forces during drying and subsequent rehydration cause an

extensive disruption of certain tissues, with gross leakage of soluble cellular compounds as the result (Speath, 1987).

Desiccation tolerance is exceptionally complex and many parameters (imbibition, membrane behaviour, carbohydrate metabolism, vitrification etc.) are involved. Therefore it is difficult to classify these mechanisms as to which is the most important for survival of almost complete dehydration. Most likely, organisms have developed several pathways to attain this goal.

Scope of this thesis

Clonal propagation of a selected superior plant genotype through somatic embryogenesis has enormous advantages over generative seed propagation. Difficult long term breeding schemes can be avoided and the genetic information can easily be fixed. However, true seeds also have certain advantages, because they are in a dry quiescent phase. They are easy to handle, they can be stored for long periods of time without viability loss and they can survive extreme temperatures (Leopold, 1990). Combination of these advantageous properties is necessary to further commercialize artificial seeds. In order to develop and produce dry artificial seeds, it is essential to have more fundamental knowledge about the ongoing processes during embryoid development, maturation, dehydration and subsequent imbibition.

"Induction and characterization of desiccation tolerance in somatic embryoids" was the title of this project. This title comprises the two objectives of this study. In the first place induction of desiccation tolerance was investigated. We had to develop a method to produce desiccation tolerant carrot embryoids, which could be dehydrated to 5% moisture content (corresponding to carrot seeds) without the loss of viability. We have chosen to work mainly with carrot somatic embryogenesis, because it is utilized generally as a model system to study the induction of somatic embryogenesis. Furthermore, with this system it is easy to produce large amounts of embryoids (Redenbaugh, 1993). We used also embryoids of alfalfa (chapter 4), because the protocol for the induction and

development of tolerant embryoids had already been described (Senaratna et al., 1989a, 1989b). Some parameters concerning desiccation tolerance, such as ABA (Koornneef et al., 1989), drying rate (Senaratna et al., 1989a, b) and imbibition (Hoekstra et al., 1989), have been mentioned earlier in the literature for other plant species or experimental systems. In chapter 2 we have described the development of a method to induce and regenerate completely desiccation tolerant carrot embryoids.

Secondly, we focused on the characterization of desiccation tolerance. The elucidation of some of the mechanisms involved in the acquisition of desiccation tolerance would assist us to develop dry artificial seeds. Membranes are proposed to be the primary target for injury during drying. This was demonstrated with leakage experiments and freeze fracture analysis of the plasma membranes (Chapter 3).

In chapter 4 and 5 we have studied the carbohydrate metabolism of embryoids and the involvement of ABA hereupon. The function of the soluble carbohydrates as protectants of the phospholipid bilayer, proposed in the water replacement theory (Webb, 1965), was examined.

Chapter 6 deals with the effect of ABA on the respiration of carrot embryoids. Respiratory metabolism is a major source of free radicals, that may harm the membrane physical structure through peroxidation and deesterification of the acyl chains of the phospholipids.

Not only membranes have to be protected in the dry state, but also other cell components such as proteins or DNA. In chapter 7 we report on the relation between DNA replication and desiccation tolerance of carrot embryoids.

Another, alternative explanation for the loss of viability in desiccation sensitive embryoids is that physical forces during drying and subsequent rehydration cause an extensive disruption of certain tissues, with gross leakage of soluble cellular compounds as the result (Speath, 1987). Structural analysis of desiccation sensitive versus tolerant embryoids might reveal the possible physical background as is described in chapter 8.

The last chapter (9) of this thesis, the general discussion, combines all the results and gives an overview of the obtained knowledge of desiccation tolerance in somatic embryoids and puts it in perspective.

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Chapter 2

Induction of complete desiccation tolerance in carrot (*Daucus carota* L.) embryoids [1]

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Summary

Successful induction of complete desiccation tolerance in carrot embryoids (*Daucus carota* L.) depends on at least four different factors: developmental stage, abscisic acid (ABA), drying rate and rehydration mode. Embryoids may become desiccation tolerant when they have reached the torpedo stage of development. In contrast, at the earlier globular and heart stages, the embryoids never germinated after any drying treatment. Addition of at least 20 μM ABA after 7 days of culture of pro-embryogenic masses in 2,4-dichlorophenoxyacetic acid-free B_5 medium was necessary to induce complete desiccation tolerance. Less ABA resulted in desiccation tolerance of the roots only, while higher ABA concentrations retarded developmental growth. Slow drying is essential for the acquisition of complete desiccation tolerance. Slowly dried embryoids (moisture content 0.05 g $H_2O.g^{-1}$ dry weight) germinated for 100%, while rapidly dried ones germinated for only 0-30%. Initially viable dry embryoids may suffer injury when they are imbibed in water without prehydration in water vapour. Hundred percent germination was reached by prehydration of the embryoids in moist air for 4 to 8 hours at 24°C before imbibition in B_5 medium. With the optimized protocol we were able to produce desiccation tolerant embryoids of two genotypes having completely different genetic backgrounds.

[1] Also published in Journal of Plant Physiology **145**, 349-356 (1995)

Introduction

Since its discovery (Reinert, 1958; Steward et al., 1958), somatic embryogenesis in carrot (*Daucus carota*) has become a well established model system to study the fundamental aspects of embryo development. Meanwhile, regeneration of embryoids has been described for a large number of species. Redenbaugh et al. (1986) were the first to create artificial seeds by encapsulation of embryoids in alginate gel beads. However, the still hydrated artificial seeds were difficult to store, because they lacked quiescence, and the conversion rate (outgrowth into plantlets) was very low. The recent achievement of induction of tolerance to complete desiccation (Senaratna et al., 1989a) may give new opportunities to the artificial seed technology. Desiccated embryoids are better suited for this, because they possess the quiescent state comparable to dry seeds, and therefore have better storage properties. Gray (1989) even stated that dried grape embryoids germinated better than fresh ones.

Desiccation tolerance is defined by us as the ability of embryoids to regrow after storage at 25 °C for 2 weeks under low moisture conditions (e.g. 10% H_2O, comparable to dry seeds).

Production of desiccation tolerant embryoids has been published for the following species: alfalfa (McKersie et al., 1989; Senaratna et al., 1989a,b; Anandarajah and McKersie, 1990 and 1991), geranium (Marsolais et al., 1991), soybean (Parrott et al., 1988), spruce (Roberts et al., 1990; Attree et al., 1991), grape (Gray, 1989) and carrot (Iida et al., 1992; Lecouteux et al., 1992). In most studies the plant hormone, ABA, was used to induce desiccation tolerance. By adding the appropriate amounts of ABA to the culture medium at the appropriate stage of development, depending on species and genotype, embryoids could be dehydrated to MCs of less than 10% (on a DW basis) with retention of some viability. Through the addition of ABA, it was even possible to induce desiccation tolerance in callus cultures of carrot (Nitzsche, 1980) and *Craterostigma plantagineum* (Bartels et al., 1990). Endogenous ABA is involved in the acquisition of desiccation tolerance in seeds, as demonstrated unequivocally by Koornneef et al. (1989)

and Meurs et al. (1992). To establish this relationship, these authors used recombinants of ABA-deficient and ABA-insensitive mutants of *Arabidopsis thaliana*.

Not only exogenously supplied ABA, but also several stress treatments can induce the capacity to survive dehydration in embryoids (Anandarajah and McKersie, 1990, 1991). However, stress treatments such as heat or osmotic shock may raise the endogenous ABA levels (Skriver and Mundy, 1990) and thus induce desiccation tolerance.

The capacity of the embryoids to survive dehydration is also dependent on the drying method. Only Senaratna et al. (1989b) gave a well defined description of the drying method, including drying rates and final MC in alfalfa embryoids. They demonstrated that slow drying over 6 days enhanced germination and vigour as compared with rapid drying.

Hoekstra et al. (1989) showed that regrowth of initially viable, dry organisms is impaired because of imbibitional damage. This kind of injury is ascribed to leakage of soluble cell components due to a phase change in the membranes from gel phase to liquid crystalline phase during imbibition (Crowe et al., 1989). The leakage and poor regrowth do not occur when the dry organisms are prehydrated in moist air prior to imbibition. This treatment melts gel phase phospholipids, and thus, a phase change during imbibition is circumvented.

In all the previous studies on induction of desiccation tolerance in embryoids the plant recovery rates were always less than 100%. Poor embryoid quality, caused by suboptimal protocols or asynchronic embryoid development could be the reason for the low recoveries. More fundamental knowledge is required to advance in artificial seed technology.

In this paper we describe the development of a protocol to produce fully desiccation-tolerant embryoids of carrot. The specific roles of ABA, sucrose, the drying rate and prehydration treatment in the tolerance to desiccation are dealt with.

Material and Methods

Plant material

Two *Daucus carota* L. genotypes were used with entirely different genetic backgrounds. One is a commercial variety cv. "Trophy" and the other a breeding line "RS 1". Seeds and cell suspension cultures of cv. "Trophy" were kindly provided by Dr. S. de Vries of the Department of Molecular Biology, Wageningen Agricultural University. Seeds of "RS 1" were obtained from Royal Sluis, Enkhuizen, The Netherlands.

Media preparation and culture conditions

All culture media were based on the Gamborg's B_5 basal composition (Gamborg et al., 1968). Before autoclaving the pH was adjusted to 5.8. The media were sterilized for 20 min at 121 °C. However, ABA dissolved in 0.2 % $NaHCO_3$ as stock solution, was filter-sterilized (0.2 μm pore size disposable filter) before addition to the cooled medium. The cultures were grown in a climate chamber with a 16 $h.d^{-1}$ photoperiod and a continuous temperature of 25 °C.

Suspension culture

After surface sterilization with 2 % NaOCl, the seeds were germinated on solid B_5 medium (8 $g.l^{-1}$ agar). Sterile hypocotyl explants of 10-day-old seedlings were used to produce friable callus on solid B_5 medium supplemented with 2.3 μM 2,4-D and 20 $g.l^{-1}$ sucrose (later referred to as 2,4-D-B_5). Cell suspension cultures were started with 1 g callus per 50 ml 2,4-D-B_5 medium in 250 ml Erlenmeyer flasks on a rotary shaker at 100 rpm. The suspensions were maintained by subculturing 2 ml PCV (packed cell volume) in 50 ml fresh medium, every 14 days. Seven days after refreshing, the cell suspensions were used to regenerate embryoids.

Embryoid production

Regeneration of embryoids occurred after transfer of the PEMs to 2,4-D-free B_5 medium with 20 $g.l^{-1}$ sucrose ($0B_5$) at low density (approximately 30,000 $cells.ml^{-1}$) (De Vries et al., 1988). In order to synchronize the embryoid development only the PEM fraction of the cell suspension with the size between 50 μm - 125 μm diameter was used.

This fraction was collected with the aid of nylon sieves. When the PEMs had grown for 7 days on $0B_5$, the medium was refreshed to prevent exhaustion of the nutrients and to eliminate single cells that did not develop into embryoids. Also in this stage of development different amounts of ABA and sucrose were supplemented to the suspension. The refreshing of the ABA-containing medium was repeated after another 7 days. The embryoids (torpedo stage) were harvested on a 500 μm nylon sieve after a culture period of 18 to 20 days.

Desiccation and germination

Prior to dehydration the embryoids were thoroughly rinsed with $0B_5$ medium in a Buchner-funnel with applied vacuum. Approximately 1 g of the freshly harvested embryoids was transferred to a sterile plastic Petri dish (9 cm) by forceps. The embryoids were equally spread out over the surface of the Petri dish. The Petri dishes were closed and placed in hygrostats (Weges, 1987). Drying rates were varied by exposure to different RHs inside the hygrostat at 25°C, generated by different saturated salt solutions (RH between brackets): Na_2CO_3 (90 %), NaCl (73 %), $Ca(NO_3)_2$ (50 %), $CaCl_2$ (30 %) and LiCl (13 %). Embryoids remained in the hygrostat until their MC was in equilibrium with the RH as measured by their weight loss. Rapid drying was obtained by placing the Petri dishes without cover in an air flow cabinet. Dry weights of the embryoids were determined after freeze-drying for 24 h. The MC was calculated as g $H_2O.g^{-1}$ DW.

Desiccation tolerance of the dry embryoids was evaluated by counting the number of germinated specimens. Approximately 100 dry embryoids were placed on filter paper in a sterile plastic Petri dish (6 cm). Prior to imbibition, the embryoids inside the closed Petri dish were prehumidified in moisture-saturated air for 4 hours to prevent possible imbibitional damage (Hoekstra et al., 1989). Following this treatment, 1 ml $0B_5$ medium was provided to the embryoids. The Petri dish was sealed with Parafilm and placed in an incubator with a 16 $h.d^{-1}$ photoperiod at 25 °C. Embryoids were recorded as desiccation tolerant when they showed clear root growth within 10 days.

Results

Mode of dehydration

In an attempt to regulate the drying rate in a repeatable manner, mature embryoids were dried at different constant RHs (Table 1). Embryoids dried rapidly in an air flow of 20-30% RH were not able to germinate, whereas those dehydrated slowly over a saturated Na_2CO_3 solution turned brown and died before they reached their equilibrium MC. Maximal survival (49% germination) was achieved when the embryoids were dried above a saturated $CaCl_2$ solution. Drying over the different saturated salt solutions not only varied in rate, but also the final MCs differed.

Table 1: Effect of the drying method on desiccation tolerance of *Daucus carota* embryoids (genotype "Trophy"). Before desiccation the torpedo-shaped embryoids were cultured for 12 days on B_5 medium containing 3.8 μM ABA and 20 $g.l^{-1}$ sucrose. Prior to imbibition in $0B_5$ medium the embryoids were prehydrated in water-saturated atmosphere for 4 hours. The number of embryoids tested ranged from 91 to 210. Different letters represent a significant difference at $P \leq 0.05$ (Fishers Exact Probability Test).

Drying Method (RH)	Approx. Drying Time*	Moisture Content	Embryoid Germination
	h	g $H_2O.g^{-1}$ DW	%
sterile air flow (20-30%)	5	0.04	0 a
saturated LiCl (13%)	20	0.02	40 c
saturated $CaCl_2$ (30%)	30	0.05	49 c
saturated $Ca(NO_3)_2$ (50%)	96	0.11	18 b
saturated NaCl (73%)	144	0.21	0 a
saturated Na_2CO_3 (90%)	336	4.20	0 a
combination of 73, 50 and 30 % RH (3 days each)	168	0.05	76 d

*time to reach moisture equilibrium.

To optimize the results we exposed the embryoids to a descending range of RHs, each of which lasted for 3 days. This was based on the method of Senaratna et al. (1989a) with slight modifications. Due to a suboptimal ABA concentration (3.8 μM) during the

maturation phase (Table 1), germination could only be increased with this method to maximally 76%. To establish the rate of drying with this descending RH treatment, the MCs of embryoids grown under the optimal maturation conditions of 37.9 μM ABA and 60 $g.l^{-1}$ sucrose were measured. Most water was lost in the first 4 days, and after some additional days the equilibrium MC of 0.05 g $H_2O.g^{-1}$ DW was reached (Fig. 1). To determine as to how far desiccation tolerance had progressed during this slow drying, embryoids were quickly dehydrated to a MC of 0.05 g $H_2O.g^{-1}$ DW, at intervals. Germination of the rehydrated embryoids increased to 100% after a previous slow drying for 4 days (Fig. 1).

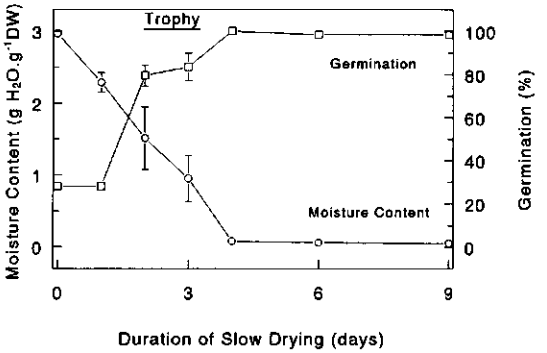

Figure 1: Effect of slow drying on germination of dry *Daucus carota* embryoids (genotype "Trophy"). The embryoids were cultured for 1 week on $0B_5$ medium followed by 4 days on maturation medium with 37.9 μM ABA and 60 $g.l^{-1}$ sucrose. At intervals during slow drying (3 days at 75%, 50% and 30% RH, given successively), the embryoids were rapidly dried in a sterile air flow for 4 hours to 0.05 g $H_2O.g^{-1}$ DW. Before imbibition in $0B_5$ medium, the embryoids were prehydrated in moist air for 4 hours. The MC data are the means $\pm$ SD of 4 replicates, the germination data are the means $\pm$ SD of duplicate measurements.

Imbibition and germination

To prevent imbibitional damage caused by too rapid a water uptake, embryoids were pretreated in a water vapour-saturated atmosphere for different lengths of time. Figure 2 shows that germination improved with increasing prehydration time up to 8 hours, after which germination decreased. As the embryoids lack endosperm, they may need additional nutrition for proper regrowth. Table 2 shows the germination data of

embryoids imbibed in B_5 medium or water. Without the nutrition the embryoids germinated very poorly (4-5%), while with B_5 medium the regrowth was optimal (98%). Potassium leakage measurements revealed that embryoids imbibed in water after a 4 h prehydration treatment leaked at a considerably higher rate than those imbibed in B_5 medium (data not shown).

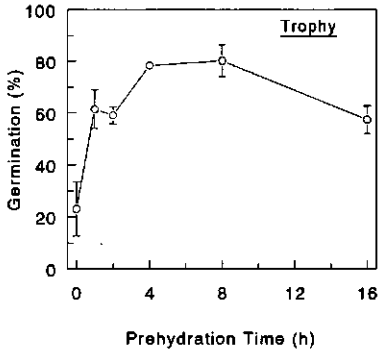

Figure 2: Influence of duration of moist air pretreatment on the germination of *Daucus carota* embryoids (genotype "Trophy"). The embryoids were cultured for 1 week on $0B_5$ medium followed by 4 days on maturation medium with 37.9 μM ABA and 60 $g.l^{-1}$ sucrose. The embryoids were slowly dried for 3 days at 75%, 50% and 30% RH each, to a MC of 0.05 g $H_2O.g^{-1}$ DW. The germination data are the means $\pm$ SD of duplicate measurements.

Table 2: Influence of nutrition and prehydration on desiccation tolerance of slowly dried *Daucus carota* embryoids (genotype "RS 1"). Following 1 week induction on $0B_5$ medium, the embryoids were cultured for 12 days on maturation medium, containing 3.8 or 37.9 μM ABA. The embryoids were dried for 3 days at 75%, 50% and 30% RH each, to a MC of 0.05 g $H_2O.g^{-1}$ DW. Final germination figures were determined 14 days after imbibition. The number of embryoids tested ranged from 82 to 121. Statistical analysis is within rows. Different letters represent a significant difference at $P \leq 0.05$ (Fishers Exact Probability Test).

	Embryoid Germination, %		
	in water	in B_5 medium	
ABA μM	with prehydration	without prehydration	with prehydration
3.8	5 a	4 a	34 b
37.9	4 a	52 b	98 c

Embryoid maturation

Osmotic stress and ABA are the main parameters that play a role in embryoid maturation. Therefore, concentrations of sucrose and ABA in the maturation medium were varied. ABA was supplemented to the medium 1 week after the start of the embryoid development. Earlier addition of ABA hindered embryoid development, whereas too late an addition did not prevent precocious germination (data not shown). The concentration of added ABA had a clear effect on desiccation tolerance. Germination reached its maximum between 19 and 37.9 μM of added ABA (Fig. 3). At higher ABA concentrations desiccation tolerance was still high, but the yield of torpedo-shaped embryoids decreased due to an impediment of development at earlier stages. At lower ABA concentrations desiccation tolerance was less. Only the roots elongated under such conditions, while the hypocotyls and cotyledons turned brown.

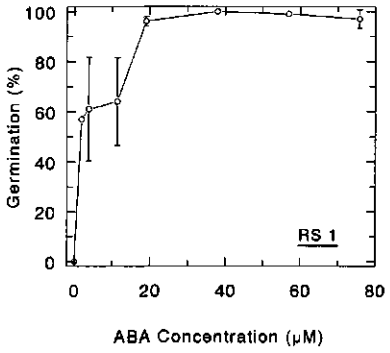

Figure 3: Effect of ABA concentration on the desiccation tolerance of *Daucus carota* embryoids (genotype "RS 1") after 20 days in culture. ABA was added on the 7th day of the culture. The embryoids were successively dried for 3 days at 75%, 50% and 30% RH, to a MC of 0.05 g $H_2O.g^{-1}$ DW. Before imbibition in $0B_5$ medium, the embryoids were prehydrated in moist air for 4 hours. The germination data are the means $\pm$ SD of 2 or 4 replicates.

Embryoids grown without ABA had a much lower percentage of DW than with ABA addition, because the latter embryoids were already germinating and had elongated roots and hypocotyls (results not shown). Elevated ABA concentrations hardly further increased the percentage of dry matter. The sucrose concentration had no effect on desiccation tolerance at the optimal ABA concentration of 37.9 μM, particularly not in

genotype "Trophy" (Table 3). But at 3.8 μM ABA high sucrose concentrations (osmotic stress) had a positive effect on the germination capacity. Elevated sucrose levels increased the percentage of dry matter of the embryoids (Fig. 4). However, the higher sucrose concentrations impeded embryoid development similarly as the elevated ABA concentrations, and the number of embryoids was reduced. The optimal sucrose concentration of the maturation medium for embryoid yield and regrowth performance was 60 $g.l^{-1}$ for "Trophy" and 20 $g.l^{-1}$ for "RS 1" (data not shown).

Table 3: Effect of ABA and sucrose concentration of B_5 maturation medium on desiccation tolerance of *Daucus carota* (genotypes "Trophy" and "RS 1") embryoids. Drying treatment was similar as for Table 2. The embryoids were prehydrated for 4 hours in moist air, before imbibition in $0B_5$ medium. The number of embryoids tested ranged from 41 to 93 for "RS1" and from 186 to 492 for "Trophy".

	Embryoid Germination, %			
Sucrose	3.8 μM ABA		37.9 μM ABA	
($g.l^{-1}$)	Trophy	RS 1	Trophy	RS 1
20	81	34	100	98
40	94	92	100	100
60	90	100	100	100
80	89	100	100	100
100	100	95	100	-
120	92	85	100	73

Embryoid development

Through subculturing at low density in 2,4-D-free B_5 medium, PEMs develop into the subsequent embryogenic stages: globular, heart and torpedo shape. The transition from undifferentiated to differentiated growth is characterized by a decrease of MC (Fig. 5). The "RS 1" embryoids reached the torpedo stage after 10 days. The torpedo-shaped embryoids have a MC of approximately 6 g $H_2O.g^{-1}$ DW. Without ABA the embryoids then started to germinate precociously, which caused an increase in MC. When ABA (37.9 μM) was added, the torpedo embryoids continued their development, while their

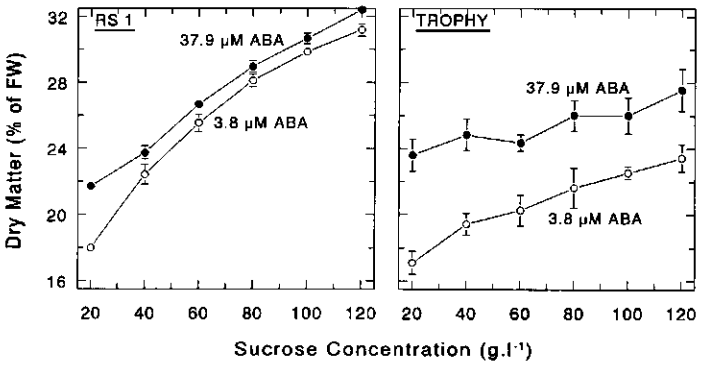

Figure 4: Effect of sucrose concentration on the percentage dry matter of *Daucus carota* embryoids after 20 days in culture. The embryoids were cultured on $0B_5$ medium. On day 7 the embryoids were transferred to media containing different sucrose concentration and either 3.8 (-○-) or 37.9 μM ABA (-●-). The data are the means $\pm$ SD of triplicate measurements.

MC dropped to 3 to 4 g $H_2O.g^{-1}$ DW. The decrease of the MC of the ABA-treated embryoids after 20 days is due to the drying treatment over the saturated salt solutions. As a comparison the MCs of carrot seeds during their development (data of Gray and Steckel, 1982) are also shown in Fig. 5.

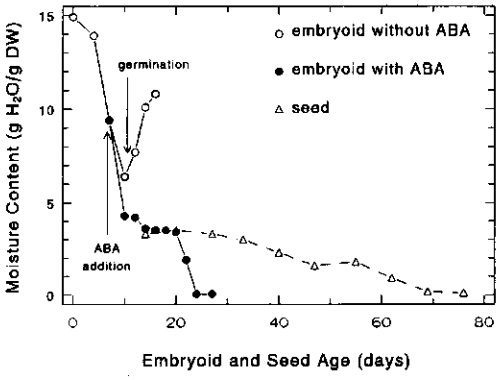

Figure 5: Changes in MC during development of *Daucus carota* embryoids (genotype "RS 1"), with or without ABA, and of seeds of the same species. ABA (37.9 μM) was added on the 7th day of the culture. After 20 days the embryoids, treated with ABA, were slowly dried above saturated salt solutions as described in Fig. 2. The seed MC data are redrawn from Gray and Steckel (1982).

Exposure of torpedo embryoids for 3 days to ABA was sufficient to induce desiccation tolerance for genotypes "RS 1" and "Trophy" (Fig. 6). The germination percentage increased within this period for both genotypes to 100%. Globular- and heart-shaped embryoids, never showed regrowth. Without ABA maximally 20 % of the "RS 1" embryoids and 45 % of the "Trophy" embryoids germinated after a slow dehydration to a MC of 0.05 g $H_2O.g^{-1}$ DW. Exogenous ABA seems not to be the only factor, but it enhances the desiccation tolerance significantly. When the "Trophy" embryoids were cultured for more then 11 days on ABA containing media the capacity to survive dehydration decreased, probably because they were producing secondary embryoids on their axes.

Discussion

During production of desiccation tolerant carrot embryoids we distinguish 4 subsequent phases; embryoid development, maturation, dehydration and germination. It is demonstrated in the present paper that 100% germination of rehydrated embryoids can only be reached when the importance of these 4 phases is recognized. Iida et al. (1992) reached 75% germination because they only optimized the maturation phase by varying the ABA treatment. Lecouteux et al. (1992) also claimed complete desiccation tolerance in carrot embryoids, with retention of viability for up to 8 months at 4°C. However, their embryoids still had a MC of 0.35 g $H_2O.g^{-1}$ DW (25% MC on a FW basis) during the quiescent phase, which is far more than the usual MC of seeds in storage (e.g. dry carrot seeds, 10 %). According to our definition, these embryoids can not be called desiccation tolerant. The embryoids might have been able to survive the storage period of 8 months, because of partial dehydration at low temperature, under conditions of which metabolism is much reduced.

Desiccation tolerance of embryoids has been reported also for other plant species, but the methods described so far fail to attain 100 % regrowth of the dried specimen (Parrott et al., 1988; McKersie et al., 1989; Senaratna et al., 1989a,b; Anandarajah and

McKersie, 1990 and 1991; Gray, 1990; Roberts et al., 1990; Attree et al., 1991; Marsolais et al., 1991). The authors did not pay full attention to the 4 subsequent phases in the embryoid production, which are discussed in more detail hereafter.

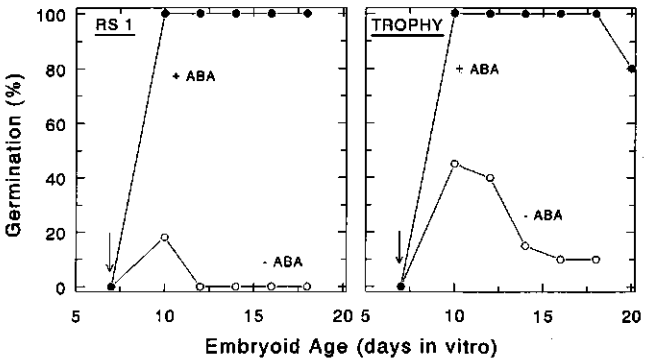

Figure 6: Influence of ABA on the development of desiccation tolerance of *Daucus carota* embryoids. Upper panel: embryoids of the genotype "RS 1"; Lower panel: embryoids of the genotype "Trophy". On day 7 embryoids were transferred to fresh B_5 media either without (-○-) or with 37.9 μM ABA (-●-). Embryoids were removed from the media after the indicated cultivation periods. Before germination embryoids were slowly dried. See Fig. 3 for description of slow drying and germination method. "Trophy" embryoids were cultured the first week on B_5 medium with 20 $g.l^{-1}$ sucrose and afterwards on B_5 medium with 60 $g.l^{-1}$ sucrose. "RS 1" embryoids were grown on B_5 medium with 20 $g.l^{-1}$ sucrose throughout the culture period. Arrows indicate the time of ABA addition.

Embryoid development

Only torpedo-shaped embryoids, formed 7-10 days after the start of the culture, were able to tolerate the drying treatment (Fig. 6). The preceding embryogenic stages, globular- and heart-shaped, were never desiccation tolerant. In a similar way barley zygotic embryos acquired desiccation tolerance after a developmental period of 16 days after pollination and *Arabidopsis* embryos 12 days after pollination (Bartels et al., 1988; Koornneef et al., 1989). Senaratna et al. (1989a, b) demonstrated with alfalfa that only torpedo and cotyledonary embryoids were able to germinate after desiccation. Iida et al. (1992) with carrot obtained similar results and suggested that only the torpedo embryoids were responsive to ABA. These data support the idea that somatic embryogenesis mimics zygotic embryogenesis.

Maturation

As soon as the histodifferentiation is completed the embryoids start to mature. The maturation phase is characterized by the deposition of lipids, proteins and carbohydrates (Kermode, 1990) and the acquisition of desiccation tolerance, while no apparent morphological changes occur. Abscisic acid and osmotic stress play an important role during embryoid maturation. Both parameters are involved in the expression of a specific set of genes, and they both can inhibit precocious germination (Kermode, 1990; Skriver and Mundy, 1990; Huet and Jullien, 1992). From our experiments it is apparent that exogenous ABA promotes desiccation tolerance. However, without the addition of ABA a much lower percentage of the embryoids still survived the drying treatment. This might indicate that induction of desiccation tolerance resides in the developmental program of the embryoid and that it is not only due to exogenous ABA. Tolerance is lost when embryoids are switched precociously from the embryogenic program (maturation) to the germination program, which will occur when ABA is left out from the medium. During a small time window just before embryoids may commence precocious germination, they are desiccation tolerant (Fig. 6), probably because they contain some endogenous ABA (Iida et al., 1992). Also in a double mutant of *Arabidobsis thaliana*, lacking both ABA synthesis and ABA sensitivity, some desiccation tolerance (15%) was observed during embryo development (16 days after pollination) (Koornneef et al., 1989). We only obtained 20% regrowth after dehydration with genotype "RS 1". This percentage probably is so low, because of asynchronous embryoid development: some were still too young (heart shaped), others already proceeded into the germination phase.

In accordance with the experiments by Huet and Jullien (1992), inhibition of precocious germination by osmotic stress (60 $g.l^{-1}$ sucrose in the maturation medium), increased the percentage of desiccation tolerant "Trophy" embryoids to 45% in the absence of ABA (Fig. 6, lower panel). In Table 3 we also show that in the case of cv. "RS1", high sucrose concentrations (80-120 $g.l^{-1}$, osmotic pressure -0.6 to -1.1 MPa) with low ABA concentration (3.8 μM) gave similar results as high ABA concentration (37.9 μM) without osmotic treatment, demonstrating that osmotic stress can replace ABA. Anandarajah and McKersie (1990, 1991) were also able to induce desiccation tolerance in

somatic embryoids of alfalfa through elevated sucrose concentrations. Also the vigour of the dry alfalfa embryoids was enhanced, an effect that we have not noticed with our carrot embryoids. These data might be explained by an osmotically induced increase of the endogenous ABA concentration (Skriver and Mundy, 1990). However, it has to be realized that sucrose not only acts as an osmoticum but also as the sole carbohydrate source. Sucrose had a significant effect on the dry matter content of the embryoids (Fig. 4). An optimal maturation protocol apparently requires both ABA and sucrose. This is further supported by the observation that embryoids after 1 week on ABA medium with 2% sucrose produced secondary embryoids on their axes, thereby losing their desiccation tolerance. Embryoids grown in ABA at high sucrose concentrations never showed secondary embryogenesis.

Dehydration

The rate of drying has been identified as a crucial factor during the acquisition of desiccation tolerance, not only in embryoids (McKersie et al., 1989; Senaratna et al., 1989a, b) but also in desiccation tolerant nematodes (Madin and Crowe, 1975), slow drying being essential for survival. Carrot embryoids behaved similarly, only the slowly dried embryoids were able to germinate (Table 1; Fig. 1). These observations implicate that during the slow drying changes occur within the organisms which protect them from the deleterious effects of dehydration. Tolerant nematode species produce large amounts of trehalose (a disaccharide), that protects membranes and proteins in the dry state (Crowe et al., 1987). Also in plants large quantities of di- and oligo-saccharides occur in desiccation tolerant seeds (Koster and Leopold, 1988) and pollen (Hoekstra and Van Roekel, 1988).

Not only the carbohydrate content might change during dehydration but also the protein content. Nordin et al. (1991) and Grossi et al. (1992) have demonstrated that during drought stress a specific set of genes is expressed. Most of these genes are also induced by ABA, but some are exclusively expressed during drought stress. The resulting proteins might be crucial to survive desiccation stress. This suggestion might also explain why Iida et al. (1992) found such low germination percentages with their rapidly dried

(3h) carrot embryoids. During this short drying period probably insufficient amounts of proteins and oligo-saccharides were synthesized for optimal regrowth.

Germination

The best way to measure desiccation tolerance is to determine germination. Non-germinating embryoids are not necessarily desiccation intolerant, because germination can be hindered by dormancy or by the wrong germination procedure. Embryoids are naked, not protected by a seed coat and endosperm, and therefore might be very sensitive to imbibitional damage and nutritional shortage. We have demonstrated that prehydration significantly enhances regrowth (Fig. 2). The positive effect of prehydration on the germination percentage is an indication that stability of membranes may play a role in the desiccation tolerance of embryoids. Membrane phospholipids of dry organisms may occur partially in the gel phase (Hoekstra et al., 1989), which may also hold for dry embryoids. During imbibition the membrane changes from the gel phase to the liquid-crystalline phase. Such transition can cause leakage of cell solutes when free water is available for solute transport, which may be catastrophic for the embryoid. Prehydration with moist air prevents leakage, because the transition then occurs in the absence of free water. The lower percentage germination on water compared to B_5 medium (Table 2), might be explained by a lack of nutrition.

Comparison of zygotic and somatic embryogenesis

In Fig. 5 we have shown the development of seeds and embryoids of *Daucus carota* on the basis of the MC. The curves look similar but the main difference between the two types lies in the time that the embryo(id)s need to decrease the MC from 3 to 0.5 g $H_2O.g^{-1}$ DW, which coincides with the maturation part of the development. The zygotic embryo has a prolonged maturation phase with an extended accumulation of storage reserves compared to the embryoid. We have to take into account that the data are from whole seeds, that is from embryos with endosperm. The condensed maturation of the embryoids might reduce the regrowth potential, because the embryoids may not have been able to synthesize all the necessary proteins, lipids and carbohydrates in the 10-12 days

maturation period. In contrast zygotic embryos have a 40-50 days maturation phase. In this respect somatic embryogenesis does not mimic zygotic embryogenesis.

In conclusion, our data clearly demonstrate that it is possible to induce complete desiccation tolerance in *Daucus carota* embryoids of two different genotypes. Therefore, the system may be an excellent model to study the working mechanisms of ABA and osmotic stress on embryoid maturation and on the induction of desiccation tolerance. Both parameters can easily be manipulated during the process.

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Chapter 3

Characterization of membrane properties in desiccation-tolerant and -intolerant carrot embryoids [1]

Frans Tetteroo, Anette de Bruin, Rob Henselmans, Wim Wolkers, Adriaan van Aelst, Folkert Hoekstra

Summary

In previous studies we have shown that carrot (*Daucus carota* L.) embryoids acquire complete desiccation tolerance when they are treated with abscisic acid during culture and subsequently dried slowly. With this manipulable system at hand, we have assessed damage with desiccation intolerance. Fast drying caused loss of viability, and all K^+ and carbohydrates leached from the embryoids within 5 min of imbibition. The phospholipid content decreased by about 20% and the free fatty acid content increased, which was not observed after slow drying. However, the extent of acyl chain unsaturation was unaltered, irrespective of the drying rate. These results indicate that during rapid drying irreversible changes occur in the membranes which are associated with extensive leakage and loss of germinability. The status of membranes after 2 h of imbibition was analyzed in a freeze-fracture study and by Fourier transform infrared spectroscopy. Rapidly dried embryoids had clusters of intramembraneous particles in their plasma membranes and the transition temperature (T_m) of isolated membranes was above room temperature. Membrane proteins were irreversibly aggregated in an extended ß-sheet conformation and had a reduced proportion of α-helical structures. In contrast, the slowly dried embryoids had irregularly distributed, but non-clustered, intramembraneous particles, T_m was below room temperature and the membrane proteins were not aggregated in a ß-sheet conformation. We suggest that desiccation sensitivity of rapidly dried carrot embryoids is indirectly caused by an irreversible phase separation in the membranes due to deesterification of phospholipids and accumulation of free fatty acids.

[1] Also accepted for publication in Plant Physiology (1996)

Introduction

Anhydrobiosis is a general phenomenon in nature. Animals, like nematodes or *Artemia* cysts, and also plant organs such as pollen and seeds, are able to survive almost complete dehydration (reviewed by Crowe and Crowe, 1992). Many studies have been undertaken to unravel the mechanisms involved in desiccation tolerance. It has been postulated that phase changes of membranes are involved in the desiccation sensitivity of dry organisms (Simon, 1974; Senaratna and McKersie, 1983a,b; Crowe and Crowe, 1992).

Carrot embryoids survive desiccation to low moisture contents (5% of DW) when they are treated with ABA (38 μM) at the proper stage of development (Tetteroo et al., 1995). Furthermore, the drying time has to be sufficiently slow (at least 4 d) to secure survival, and prehydration of the dry embryoids in moist air prior to imbibition is necessary to prevent imbibitional injury. The mechanism of imbibitional injury has been characterized as the transient loss of permeability of the plasma membrane during its transition from the gel to the liquid-crystalline phase (Crowe et al., 1989a; Hoekstra et al., 1989). Imbibitional damage can be avoided by prehydration over water vapor or by heating above T_m. The finding that dry carrot embryoids also must be prehydrated before imbibition (Tetteroo et al., 1995), points to the occurrence of a similar type of phase change in the membranes of these embryoids.

The conformational status of membranes is predominantly determined by the prevailing PLs and can be excellently analyzed by FTIR spectroscopy, both *in vitro* and *in situ* (Crowe et al., 1989b). In hydrated conditions the membranes of living organisms generally are in the liquid-crystalline phase. During water removal (drying) the distance between the PL headgroups decreases, leading to increased van der Waals' interactions between the acyl chains (reviewed by Crowe et al., 1992). The PL bilayer will then pass into the gel phase. Due to defects in the bilayer at the interphases of gel and liquid-crystalline domains during the phase transition, the membranes become permeable to solutes (Chapman et al., 1967; Hammoudah et al., 1981; Cameron and Dluhy, 1987). This often results in extensive damage and even cell death.

Avoidance of phase transitions in membranes is considered to be important for survival under dry conditions (Crowe et al., 1992). Phase transitions due to dehydration can be prevented by replacing the water molecules around the polar headgroup by di- and oligosaccharides (Crowe et al., 1984; Crowe et al., 1987). Another way of avoiding phase transitions is by formation of more unsaturated acyl chains, which decrease T_m of membrane lipids (Lynch and Steponkus, 1989). This occurs in pollen, as has been demonstated by *in vivo* T_m measurements of membranes (Hoekstra et al., 1992b). During dehydration T_m will rise but may not exceed ambient temperatures, due to the high content of 18:3 acyl chains. Thus, organisms can become desiccation tolerant by enhancing their unsaturation level. However, double bonds are very sensitive to free radical attack, and the resulting lipid peroxides may cause disruption of the membrane structure, leading to extensive leakage.

Extensive leakage of desiccation sensitive tissues may be caused not only by a phase change of the PLs, but also by problems with membrane proteins. Proteins may become irreversibly aggregated due to phase separation of the PLs (Hemminga et al., 1992). Protein secondary structures can be exquisitely studied *in planta* with FTIR spectroscopy (Wolkers and Hoekstra, 1995).

In the present paper, we use carrot embryoids as the experimental system to study desiccation tolerance mechanisms. Using slow and fast drying techniques, which provide viable and dead embryoids respectively, we investigated whether phase changes or phase separation in the membranes are the main cause of desiccation sensitivity. We measured leakage, PL content and acyl chain composition. TEM studies of freeze-fracture replicas of plasma membranes were compared with FTIR studies on membrane phase behavior and protein secondary structure. Several causes of membrane leakage in carrot embryoids are discussed.

Material and Methods

Plant material

Carrot embryoids, genotype "Trophy", were produced according to earlier described methods (Tetteroo et al., 1995). Survival of desiccation was varied by different drying regimes and prehydration treatments before imbibition. Desiccation tolerance was measured as germination percentage after 10 d of imbibition on B_5 medium (Gamborg et al., 1968). Embryoids were scored as having survived desiccation when they showed elongated roots and green cotyledons.

K^+-Leakage

Approximately 10 mg dry embryoids were homogenized with a mortar and pestle in 250 μL 0.1 N HCl to extract total K^+. After centrifugation the supernatant was diluted 50 times, and subsequently the K^+-content was measured with a flame photometer (PFP 7, Jenway Ltd, England).

To assess K^+-leakage, approximately 10 mg of dry embryoids were prehydrated in moist air for 4 h and then imbibed in 5 mL K^+-free B_5 medium in a 9 cm Petri dish. During imbibition the embryoids were continuously agitated on a rotary shaker. In the course of imbibition 40 μL samples of the medium were regularly taken, diluted to 4 mL with Milli-Q water, and immediately analyzed with the flame photometer. K^+-leakage was expressed as percentage of the total K^+-content.

Carbohydrate leakage

Total carbohydrates from approximately 20 mg dry embryoids were extracted for 15 min at 76°C in 1 mL 80% methanol. Raffinose was used as the internal standard. The methanol was evaporated with a Speed Vac (Savant Instruments Inc. Farmingdale, NY). The dry samples were suspended in water and centrifuged with an Eppendorf centrifuge. After dilution of the supernatant in Milli-Q water, the samples were analyzed on a Dionex HPLC (Dionex Corporation, Sunnyvale, CA), equipped with a Carbopac-1 column and a pulsed electrochemical detector (for details see Tetteroo et al., 1994). One hundred mM

NaOH was used as the eluent. The data were captured with a Spectra Physics integrator model SP4400 and Spectra Physics software (Labnet, Chromnet; San Jose, CA).

To assess carbohydrate leakage, approximately 20 mg of dry embryoids were prehydrated in moist air for 4 h and then imbibed in 4 mL sucrose-free B_5 medium in a 9 cm Petri dish, to which raffinose (1 $mg.mL^{-1}$) was added as the internal standard. During the imbibition the embryoids were continuously agitated on a rotary shaker. In the course of imbibition 20 μL samples of the medium were regularly taken. Carbohydrate extraction and analysis were performed as indicated above. Leakage was expressed as percentage of the total carbohydrate content.

Phospholipid and free fatty acid analyses

Lipid extraction and analysis were performed according to methods previously described by Hoekstra and van Roekel (1988). Briefly, embryoids were homogenized with a mortar and pestle in $CHCl_3$/methanol (2:1) with diheptadecanoyl-phosphatidylcholine and heptadecanoic acid as the internal standards. After 5 min of mild ultrasonic treatment, the homogenate was centrifuged and the supernatant was washed with 0.2 volumes of 0.9% NaCl solution. After centrifugation the $CHCl_3$ layer was recovered and passed over an anhydrous Na_2SO_4 column. The dried $CHCl_3$ was evaporated, and the residue was dissolved in 1 mL $CHCl_3$. Phospholipids were separated from neutral lipids and FFA with a SEP-PAK silica cartridge (Waters Associates Inc., Milford, MA, cat no. 51900) according to Juaneda and Rocquelin (1985). The PL fraction was transmethylated with 3 mL of 0.3 N KOH in methanol for 15 min at 70°C under vigorous shaking. The samples were cooled and, subsequently, 1 mL of a saturated NaCl solution and 1.5 mL hexane were added. After mixing and phase separation (centrifugation), the hexane fraction containing the methylated fatty acids was collected and passed over an anhydrous Na_2SO_4 column. The samples were then injected in a Perkin-Elmer 8320 Capillary Gas Chromatograph, equipped with a flame ionization detector and a CPsil 88 column, 50 m, 0.50 mm diameter. The oven temperature during an analysis was 2 min at 60°C, then further heating at 30°C.min^{-1} to 150°C, and then 3°C.min^{-1} to 240°C. The amounts of

the fatty acids were calculated on the basis of peak areas after identification on the basis of retention times of known standards.

The FFA were separated from di- and tri-acyl glycerides by TLC, using a mixture of hexane, ether and acetic acid (80:20:1) as the developing solvent. After scraping off, the FFA were directly methylated with freshly prepared diazomethane. GC analysis was performed as described for PL.

Electron microscopy

Embryoids, enveloped with B_5 medium, were mounted in golden specimen carriers (3 mm, Balzers AG, Liechtenstein). Directly afterwards the carriers were plunged into liquid propane (at -180°C) and stored in liquid N_2 (-196°C). Freeze-fracture replicas were prepared in a BAF 400 freeze-fracture apparatus (Balzers AG, Liechtenstein) at -120°C and 10^{-7} Torr. Platina was evaporated at an angle of 40°, and carbon was evaporated to support the replicas. To digest all embryoid material, the replicas were treated with 3% cellulase Y/C (Seishin Pharm. Co. Ltd, Japan) and 0.05% macerozym R-10 (Serva, Heidelberg, Germany) in 0.1% MES buffer (pH 5.5) containing 8% mannitol. Replicas were cleaned with 50% CrO_3 solution for 2 h and overnight soaking in 9% NaOCl solution. After mounting them on EM grids, electron micrographs were made with a transmission electron microscope (JEM-1200 EX II, Jeol, Japan) at 80 kV. Intramembraneous particle analyses were performed with an image analysis program (TIM version 3.3, Difa Measuring System, The Netherlands). Only plasma membranes were used for this quantative analysis. We consider IMP distribution as clustered when more than 5-10 IMPs are grouping together, with distinct spaces between the clusters.

Fresh torpedo-shaped embryoids were directly freeze-fixed in liquid propane after the culture period in vitro. Slowly and rapidly dried embryoids were first prehydrated in moist air for 4 h (100% RH) at 25°C and further imbibed in B_5 medium for 2 h before the freeze-fixation.

The membrane fracture face nomenclature of Branton et al. (1975) is used for the description of the freeze-fracture images.

Microsomal membrane isolation

Embryoids were prehydrated in moist air for 4 h at 25°C and subsequently incubated in B_5 medium for 2 h. The embryoids (200 mg DW) were then homogenized with a mortar and pestle, using 1 mM EDTA, 1 mM EGTA, 1 mM diethylenetriamine-pentaacetic acid, 5 mM ascorbic acid and 1 mM DTT in 10 mM Tes buffer, pH 7.4. The homogenate was centrifuged 2 times at 10,000 x g. The supernatant was then subjected to high speed centrifugation (35 min at 100,000 x g) and after resuspension of the pellet in either H_2O or D_2O, the high speed centrifugation was repeated. The isolated membrane pellet was directly used for FTIR analysis.

IR Spectroscopy

FTIR spectra were recorded on a Perkin-Elmer 1725 Fourier transform infrared spectrometer equipped with a liquid nitrogen-cooled mercury/cadmium/telluride detector and a Perkin-Elmer microscope interfaced to a personal computer. Membrane pellets were sandwiched between two CaF_2 windows and loaded in a temperature-controlled cell. For membrane fluidity studies, 50 to 60 FTIR spectra were recorded in the range between -60 and 80°C. For protein studies, spectra were recorded at room temperature. The optical bench was purged with dry CO_2-free air (Balston; Maidstone, Kent, England) at a flow rate of 25 $L.min^{-1}$. The acquisition parameters were: 4 cm^{-1} resolution, 512 co-added interferograms (32 in the membrane fluidity studies), 2 $cm.s^{-1}$ moving mirror speed, 3500-900 cm^{-1} wave number range, and triangle apodization function. The time needed for acquisition and processing of a spectrum (512) was 4.5 min.

Spectral analysis and display were carried out using the Infrared Data Manager Analytical Software, versions 2.5 and 3.5 (Perkin Elmer). The spectral region between 3000 and 2800 cm^{-1} was selected and second derivative spectra were calculated. The second derivative spectrum was normalized and the band position was calculated as the average of the spectral positions at 80% of the total peak height. Membrane fluidity was monitored by observing the band position of the CH_2 symmetric stretching band at approximately 2850 cm^{-1}. T_m was estimated from the discrete shifts in these band positions with temperature, *i.e.* the temperature at which half of the hydrocarbon

containing compounds have melted. The spectral region between 1800 and 1500 cm^{-1} was selected for protein studies. This region contains the amide-I and the amide-II absorption bands of the protein backbones. Procedures to generate second derivative spectra and deconvolved spectra were carried out according to Wolkers and Hoekstra (1995).

Results

Leakage

For maximum germination dry embryoids must be prehydrated for at least 4 h in moisture-saturated air before imbibition in B_5 medium (Tetteroo et al., 1995). Without prehydration, embryoids leaked more K^+ and at a faster rate over the first five minutes than with prehydration (Fig. 1). These results clearly indicate that dry embryoids are sensitive to imbibitional stress, and that leakage of endogenous solutes is one of the reasons for their non-viability. However, the prehydrated ones geminated for approximately 100% still leaked over 50% of the total K^+ present which makes the relationship of solute loss with viability less clear cut.

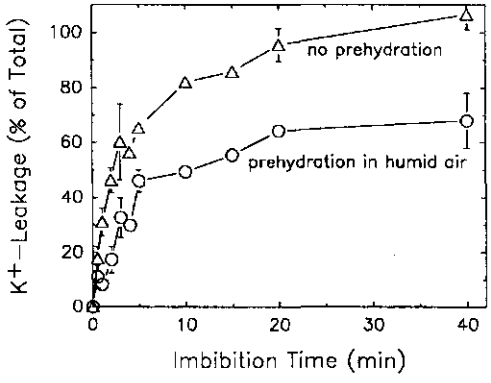

Figure 1: K^+-leakage during imbibition of slowly dried, viable carrot embryoids with and without prehydration in humid air for 4 h. Data are means of triplicate leakage experiments. Error bars (±SD) are indicated when they exceed symbol size.

The drying rate also determines whether embryoids retain their viability after dehydration to 5% moisture content (Tetteroo et al., 1995). Fast drying was detrimental, whereas slow-dried embryoids survived completely. Figure 2 demonstrates the difference in K^+-leakage between slowly and rapidly dried embryoids, both of which have been subjected to the 4 h prehydration treatment. In spite of prehydration which reduces imbibitional damage (see Fig. 1), the intolerant embryoids leaked more K^+ and at a higher rate. The drying rate had the same effect on leakage of umbelliferose and sucrose from the embryoids (Fig. 3).

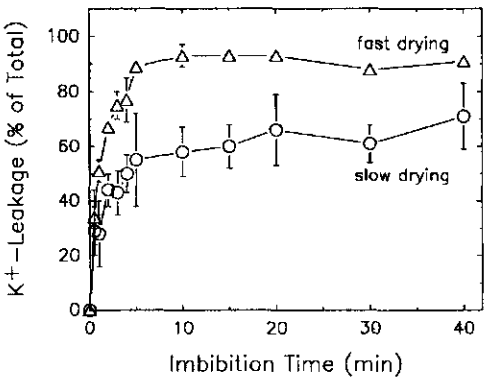

Figure 2. K^+-leakage during imbibition of desiccation tolerant (after slow drying) and intolerant (after fast drying) carrot embryoids. Average of triplicate imbibition experiments. All embryoids were prehydrated in saturated air for 4 h. Error bars (±SD) are indicated when they exceed symbol size.

Phospholipid analysis

The extensive leakage of K^+ and sugar from the intolerant embryoids (rapidly dried) cannot be simply explained by a phase change of membranes associated with rehydration, - the underlying chemical changes should also be considered. Therefore, we have analysed the PL content and acyl chain composition of fresh non-dried embryoids, of dried tolerant and intolerant embryoids and of the latter two after imbibition for 2 h (Table I). Because data on acyl chain composition are the averages of measurements of five independent embryoid cultures at different seasons of the year, fairly large variations

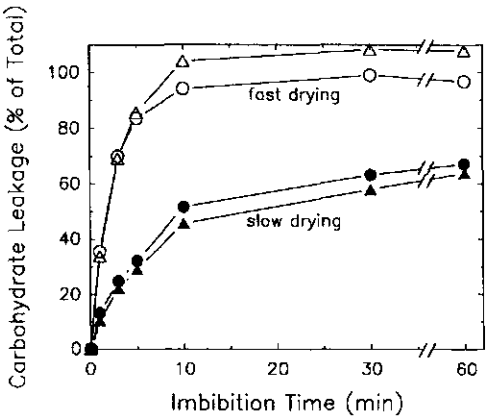

Figure 3. Leakage of umbelliferose (circles) and sucrose (triangles) during imbibition of desiccation tolerant (slow drying) and intolerant (fast drying) carrot embryoids. Prehydration conditions as in Fig. 2. Single extraction.

were observed. No substantial differences in acyl chain composition were found. This implies that lipid peroxidation could not have played an important role in the observed leakage of solutes from the imbibed, desiccation-sensitive embryoids.

Table I: Content and acyl chain composition of PLs in fresh, desiccation tolerant (after slow drying) and intolerant (after fast drying) carrot embryoids. The latter two specimens were also rehydrated in vapor-saturated air for 4 h followed by 2 h imbibition in B_5 medium. The FFA contents are also given. Data are averages of five independent experiments, except the FFA content data which are the average of 2 experiments.

Treatment	FFA content	PL content	Mol percent fatty acids in PL				
	(mg g^{-1})	(mg g^{-1})	16:0	18:0	18:1	18:2	18:3
Fresh, no drying	2.0	35.35	23.5	2.2	18.7	52.8	2.5
dry after slow drying	1.9	33.96	21.9	2.2	17.9	55.3	2.3
dry after rapid drying	4.7	27.43	24.9	2.5	20.4	49.8	2.3
2 h imbibed (slow dr.)	1.5	20.54	24.0	1.6	14.0	57.1	3.1
2 h imbibed (rapid dr.)	8.1	9.60	27.1	1.9	16.2	48.9	5.5
LSD (P=0.05)	2.6	7.05	11.3	3.0	12.3	8.6	2.9

However, the PL content of the dried intolerant embryoids decreased by about 20% during rapid drying, in all the five replicates (Table I). Such decrease was not observed when the embryoids were slowly dried. This means that lipid breakdown must have taken place during the 4 h of fast drying. Prehydration in humid air for 4 h followed by 2 h of incubation in B_5 medium further decreased the PL content, much more in the desiccation sensitive specimens than in the tolerant ones.

Analysis of the content of FFA in the dried desiccation sensitive and tolerant embryoids revealed elevated deesterification in the sensitive embryoids, which is in agreement with the loss of PLs (Table I). The 2 h imbibed sensitive specimens, particularly, had increased FFA contents.

Freeze-fracture

To visualize a possibly altered ultrastructure of membranes, freeze-fracture replicas were studied with TEM. An electron micrograph of the PF side of a plasma membrane in a fresh embryoid is shown in Fig. 4. The embryoids were treated with 38 μM ABA, which renders them potentially desiccation tolerant when they are dehydrated slowly. The membranes had a random IMP distribution, and also plasmodesmata were observed (see arrows in Fig. 4). These ultrastructural features correspond to the results of Emons et al. (1992) with carrot PEMs and are customary for a physiologically functional membrane (Leshem, 1992). Quantitative data of the IMP distribution and other features of these membranes are indicated in Table II. The extraplasmatic face and PF sides were initially scored separately, but since no differences in distribution were found between them, we have combined the data in Table II. Of all the 102 membranes from fresh, nondried embryoids scored, 40% showed microfibril imprints and 97% had a flat surface, which indicates that the fresh embryoid cells were fully turgid (Pearce, 1985). Only 10% of the membranes had exocytose configurations (not shown on micrographs), which have earlier been described in carrot PEMs by Emons et al. (1992).

Plasma membranes in 2 h-imbibed tolerant embryoids looked similar to those in fresh embryoids, except that they had less microfibril imprints (not shown on micrographs) and were more undulated (Fig. 5, Table II). This can be explained by the

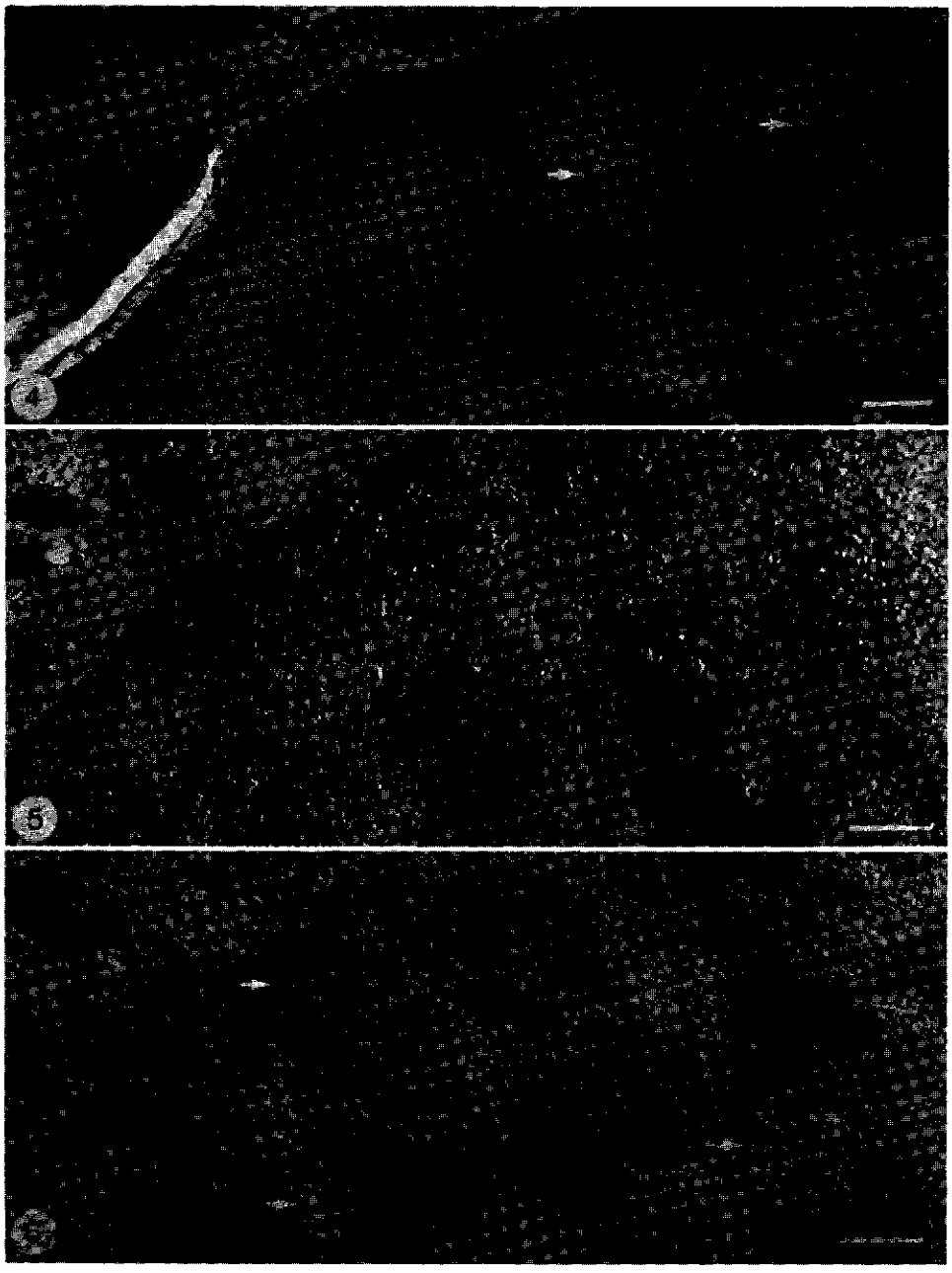

Figure 4: TEM micrograph of a freeze-fractured, fresh somatic embryo cultured in liquid medium containing 38 μm ABA. The IMPs in the PF face of the plasma membrane are randomly distributed and also plasmodesmata are visible (arrow). Bar = 200 nm.

Figure 5: TEM micrograph of a freeze-fractured, desiccation tolerant somatic embryo, cultured as in Fig. 4. After slow drying to 0.05g H_2O g DW^{-1} the embryoids were rehydrated in humid air for 4 h and imbibed in B_5 medium for 2 h. The IMPs in PF face are irregularly distributed in an undulated plasma membrane. Bar = 100 nm.

Figure 6: TEM micrograph of a freeze-fractured, desiccation intolerant somatic embryo, cultured as Fig. 4. After fast drying embryoids were rehydrated in humid air for 4 h and imbibed in B_5 medium for 2 h. An undulated PF face of the plasma membrane shows a distinct clustering of IMPs (arrows). Bar = 100 nm.

fact that the 2 h imbibition period is not sufficiently long to allow the cells to become fully turgid. These membranes also contained three times more exocytose configurations (Table II) compared to those in fresh embryoids. These exocytose configurations might function as storage organs for PLs during the water stress, because the decrease in cell volume during dehydration necessitates a reduction of membrane area. The IMPs in the plasma membranes of the desiccation tolerant embryoids were somewhat irregularly distributed (Fig. 5), but clustering was rare (Table II).

Membranes in imbibed intolerant embryoids (rapid drying) clearly showed different features (Fig. 6). Almost all replicas (98%, see Table II) displayed a distinct clustering of IMPs, which is indicative of domain formation of PLs and membrane proteins. Moreover, plasmodesmata, microfibril imprints and exocytose configurations were lacking, which is an indication that these membranes have lost their physiological functions.

We also analyzed freeze fracture images of plasma membranes in embryoids developed on a medium with the suboptimal ABA concentration of 3.8 μM (Table II). These embryoids only acquire partial desiccation tolerance (25%) when they are dried slowly (Tetteroo et al., 1995). This partial survival was also reflected in their membrane features. As much as 93% of the plasma membranes in the slowly dried embryoids had clustered IMPs, whereas no plasmodesmata, microfibril imprints and exocytose configurations were found.

Table II: Quantative data of the visual properties of freeze-fracture replicas of plasma membranes in fresh nondried carrot embryoids and in rehydrated tolerant (after slow drying) and intolerant (after fast drying) embryoids. Rehydration was for 4 h in water vapor-saturated air, followed by 2 h in B_5 medium. Embryoids were either treated with 38 μM ABA or the marginal concentration of 3.8 μM, one week before the drying procedures.

Treatment and status	Viability	Number of membranes	IMP distribution (%)		membrane surface (%)		other features (%)		
of embryoids	(percent germination)	observed	random	clustered	flat	undulated	plasmodesmata	microfibril imprints	exocytose configurations
38 μM ABA									
fresh	100	102	100	0	97	3	28	40	10
slowly dried	100	154	92	8	51	49	6	8	31
rapidly dried	0	54	2	98	22	78	0	2	0
3.8 μM ABA									
fresh	100	106	99	1	85	15	24	26	16
slowly dried	25	42	7	93	55	45	0	0	0
rapidly dried	0	20	0	100	60	40	0	0	0

Membrane fluidity

At a certain point during the rapid drying or the subsequent rehydration procedure membranes in the desiccation sensitive embryoids had undergone a phase separation (Fig. 6). The phase-separated intramembraneous proteins might have irreversibly aggregated, from which it could be erroneously concluded that the membrane lipids are still in gel phase after 2 h of imbibition. This question was resolved by FTIR analysis of the CH_2 vibrational freedom (wavenumber range from 3000 to 2800 cm^{-1}). A typical plot of the position of the CH_2 symmetric stretching band (at approximately 2850 cm^{-1}) against the temperature is shown in Fig. 7.

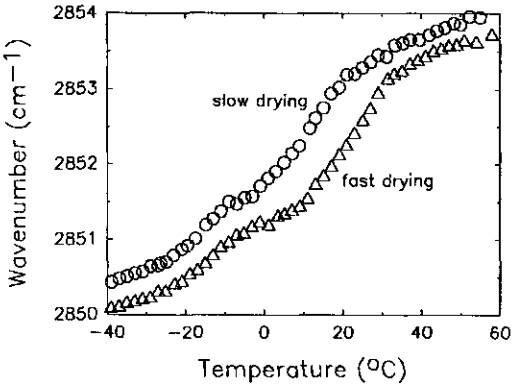

Figure 7: Wavenumber versus temperature plot (FTIR) of microsomal membranes isolated from slowly and rapidly dried carrot embryoids (representative plots). The data points represent the symmetric CH_2 stretching vibration of slowly dried embryoids (circles) and rapidly dried embryoids (triangles).

The shape of the curve suggests the existence of two lipid populations in each membrane pellet, one with a T_m between -20°C and -10°C, and one with a T_m above 0°C. Of the many explanations that could be generated, the most likely is that the lowest T_m is due to neutral lipid (oil) that was entrapped during the membrane isolation procedure and that the higher T_m is from the membrane phospholipids. Nonetheless, a considerable difference exists between the phase transition curves of the isolated membrane preparations. Membranes from the rapidly dried embryoids had a higher average T_m (22°C) than those from the slowly-dried ones (8°C). The measurements had to be conducted on isolated microsomal membranes, since *in situ* FTIR measurements of the CH_2 absorbance in the

intact embryoids are difficult to interpret due to oil interference (Hoekstra et al., 1993). At room temperature, membranes isolated from the rehydrated viable embryoids are mainly in the liquid crystalline phase, whereas at least part of the membrane fraction from the nonviable embryoids is in gel phase.

Membrane protein secondary structure

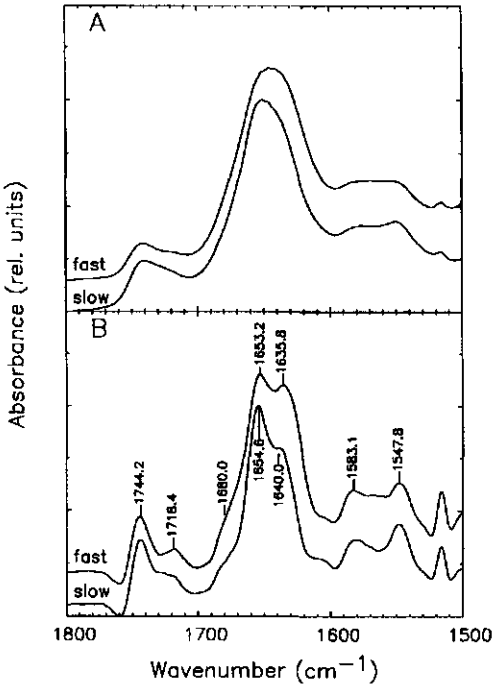

Figure 8. Absorbance (A) and deconvolved absorbance (B) FTIR spectra of isolated membranes from imbibed embryoids that were either slowly or rapidly dried (representative spectra). Membranes were prepared in D_2O. The prehydration was for 4 h in humid air, followed by 2 h incubation in B_5 medium.

To analyse whether the clustering of IMPs is associated with a possible irreversible aggregation of intramembraneous proteins in the desiccation sensitive embryoids, FTIR absorbance spectra were made of the isolated membranes in D_2O over the region between 1800 and 1500 cm^{-1} (Fig. 8A). Three major absorption bands could be observed. The absorption band at approximately 1744 cm^{-1} could be assigned to ester

bonds of the lipids. Those at approximately 1655 cm^{-1} and 1548 cm^{-1} represent the amide-I band and amide-II band of proteins, respectively (Susi et al., 1967). From the areas of the bands at approximately 1744 and 1655 cm^{-1}, it was calculated that the lipid to protein ratio decreases by a factor of 1.7 in the membranes from rapidly dried embryoids, which is in agreement with the considerable loss of PLs as shown in Table I.

To resolve the protein secondary structures in the isolated membranes, the spectra were deconvolved (Fig. 8B). Three bands could be distinguished in the amide-I region of the IR-spectra of the isolated membranes from slowly dried embryoids, namely at 1640, 1655 and 1680 cm^{-1}. In the isolated membranes from rapidly dried embryoids the amide-I bands shift partly to lower wavenumbers, namely 1636 and 1653 cm^{-1}. The band at approximately 1655 cm^{-1} is dominated by α-helical structure (Surewicz and Mantsch, 1988; Haris et al., 1989; Bandekar, 1992). The band at approximately 1640 contains contributions from random coil and turn structures (Surewicz and Mantsch, 1988). The band at 1680 most likely represents turn-like structures. Rapid drying decreased the amount of α-helical structure as compared to slow drying, which was deduced from the lineheight ratios of the amide-I bands. The band at 1636 cm^{-1} indicates the presence of β-sheet structures (Susi et al., 1967) in rapidly dried embryoid membranes. Our results indicate that rapid drying of the embryoids is correlated with a decoiling of the α-helical structure in favor of ß-sheet structures, characterized by peptide C=O groups having more colinearly oriented hydrogen bonds with the NH groups.

Discussion

Preservation of membrane integrity in the dry state has been proposed to be a key factor in the survival of anhydrobiotic organisms (Crowe and Crowe, 1992). Membrane integrity is generally determined by measuring leakage of cytoplasmic solutes like K^+ or sugars in the imbibition medium (McKersie and Stinson, 1980; Senaratna and McKersie, 1983a; Hoekstra et al., 1992a). Our leakage experiments also demonstrate that membranes play a crucial role in the acquisition of desiccation tolerance by carrot

embryoids. Within 5 min all cytoplasmic solutes had leached from intolerant embryoids, which indicates a complete loss of membrane integrity during dehydration and rehydration, similar to that in desiccation intolerant soybean embryos (Senaratna and McKersie, 1983a,b).

However, tolerant embryoids leached as much as 50% of their endogenous K^+ and sugars, but at a lower rate. This considerable loss from viable embryoids might be explained by the location of these solutes. K^+ will also be located in intracellular spaces because the embryoids are developed in B_5 medium which is high in K^+. However, the losses of umbelliferose and sucrose are not related with their original presence in B_5-medium. Umbelliferose was never added to the medium and the added sucrose is rapidly converted to glucose and fructose. These sugars might be actively transported to the outside of the plasma membrane during drying to enhance protection. The absolute requirement for such sugar transport was demonstrated in yeast mutants lacking the trehalose transporter in the membrane (Crowe et al., 1996). Thus, the K^+ and sugars in the imbibition medium may in part originate intercellularly and not from leakage through the plasma membrane. Another explanation for the considerable leakage from tolerant embryoids might be that not all embryoid cells are viable and that the solutes originate from dead cells in the nevertheless 100% viable embryoids. However, the lack of browning reactions in the rehydrated viable embryoids does not favor this explanation. Alternatively, a transient leakage independent of phase changes may occur upon imbibition regardless of tolerance state.

Our data indicate the occurrence of two types of membrane injuries (cf Figs. 1 and 2). Both types seem similar because they show similar leakage kinetics. However, the first type of injury (imbibitional damage) can be circumvented by prehydration (Fig. 1). Imbibitional damage has been previously described in pollen (Hoekstra and van der Wal, 1988). Zygotic embryos of different plant species also demonstrate this kind of damage but usually only when the testa is ruptured (Duke and Kakefuda, 1981). This again indicates that somatic embryoids resemble zygotic embryoids and that the seed coat has an important function in desiccation tolerance. The second type of damage, arising during the rapid drying, is irreversible (Figs. 2 and 3).

We analyzed freeze-fracture replicas of membranes in rapidly dried embryoids to explain the increased permeability. The electron micrographs clearly showed the occurrence of lateral phase separations of PLs and membrane proteins. Such phase separations were also detected in rehydrated microsomes of lobster muscles, which had lost their physiological function during lyophilization (Crowe and Crowe, 1992) and in membranes of drought-stressed wheat leaves, which showed increased leakage (Pearce, 1985).

Phase transitions can be caused by dehydration of membranes and may lead to domain formation. After rehydration the components may be fully mixed again. However, the membranes in rapidly dried, desiccation intolerant embryoids remain phase separated, which might be explained in two ways. On the one hand, the PLs in the membranes might remain in gel phase after rehydration, because of the accumulation of FFA and lipid peroxides, both originating from free radical activity (Senaratna et al., 1984, 1985a,b). On the other hand, the intramembraneous proteins might have irreversibly aggregated in extended ß-sheet structures (Sanders et al., 1993), which prevents them from redistributing in the liquid crystalline membrane. The aggregated proteins might thus form the disturbances in the membrane through which the leakage occurs.

Our PL and FFA analyses support the first hypothesis. A decrease of the PL content and an increase of the FFA content were found in the rapidly dried embryoids, typically the result of deesterification of acyl chains from the glycerol backbone (Table I). As reported by Senaratna et al. (1985b), we also did not detect changes in composition and saturation level of the acyl chains. Furthermore, the FTIR data on the fluidity of the membranes supports the occurrence of gel phase in desiccation intolerant embryoids but not in the tolerant ones (Fig. 7).

However, we also gathered evidence for the second hypothesis in relation to protein structure. FTIR spectroscopy is one of the most suitable techniques to study protein secondary structure *in situ* and also in isolated membranes (Surewicz et al., 1993). Both theoretical and experimental studies with model polypeptides and proteins have shown that there is a good correlation between the amide-I band frequency and the type of secondary structure (Surewicz and Mantsch, 1988; Haris et al., 1989; Bandekar, 1992).

The spectra of the amide-I region of membranes isolated from *Daucus* embryoids are typical of membrane proteins (Haris et al., 1989; Garcia-Quintana et al., 1993), where a major contribution from α-helical structures has been reported. We interpret the shift to lower wavenumbers of the amide-I band and the decrease in the amount of α-helical structure (Fig. 8) as a decoiling of α-helical proteins into intermolecular extended ß-sheet conformation in the isolated membranes from the rapidly dried embryoids.

We have demonstrated with TEM studies in combination with FTIR analysis that both gel phase lipid and membrane protein aggregation occur in membranes during rapid drying of carrot embryoids. However, it could also be that there are secondary effects, because membranes from 2 h imbibed intolerant embryoids were used, which were already leaky directly after imbibition. Because it is almost impossible to get good quality freeze fracture replicas of plasma membranes of dry materials, we chose to analyse rehydrated specimens. As is shown in Table I, breakdown reactions may have taken place during the 2 h imbibition period.

An important remaining question is which processes occurring during slow drying are involved in the prevention of phase transitions/separations and irreversible protein aggregation. Rapid drying almost certainly prevents gene expression and extensive protein synthesis, which may be required for desiccation tolerance.

We conclude that the integrity of the plasma membrane has to be preserved for carrot embryoids to survive dehydration to low moisture contents. The occurrence of lateral phase separations of PLs and membrane proteins during removal of the water could be the main cause of the loss of membrane function. The resulting extensive leakage of cytoplasmic solutes causes the embryoids to lose their viability.

Acknowledgements

We would like to thank Elena Golovina and Lekha Sreedhar for critically reading the manuscript and Tineke van Roekel for excellent technical assistance.

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Chapter 4

Effect of abscisic acid and slow drying on soluble carbohydrate content in developing embryoids of carrot (*Daucus carota*) and alfalfa (*Medicago sativa*) [1]

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Summary

In the presence of abscisic acid (ABA), contents of glucose and fructose decreased in carrot embryoids, whereas umbelliferose increased during dehydration. The acquisition of desiccation tolerance was tested by scoring germinated embryoids. It did not keep pace with the changes in soluble carbohydrate contents during development, which suggests that in these somatic embryoids di- and oligosaccharides are not the determining factors for anhydrobiosis. However, on slow dehydration, umbelliferose increased, while sucrose decreased and the monosaccharides declined completely. These changes were positively correlated with increased desiccation tolerance. A similar analysis of slowly dried, ABA-treated alfalfa embryoids showed that stachyose and sucrose increased and the monosaccharides declined with the acquisition of desiccation tolerance. These data support the contention that carbohydrates are involved in anhydrobiosis.

[1] Also published in Seed Science Research 4, 203-210 (1994)

Introduction

Higher plants have developed a wide variety of strategies to survive periods of harsh conditions. One of them is the formation of desiccation-tolerant embryos within seeds. Desiccation tolerance develops during the maturation phase, which is concluded by dehydration. In the dry state, the embryo is able to survive unfavourable conditions. Here, we define desiccation tolerance as the capacity of a tissue to regrow after dehydration to moisture contents of 5-15% on a dry weight basis. Abscisic acid (ABA) is the essential hormone involved in the acquisition of desiccation tolerance during seed development, as demonstrated by Koornneef et al. (1989) in studies with the recombinant of an ABA-deficient and an ABA-insensitive mutant of *Arabidopsis thaliana*. Embryoids of several plant species, which are regenerated from callus or suspension cultures, resemble these recombinant seeds in many aspects. Thus, embryoids do not experience a form of developmental arrest, but germinate directly after development. Also, like the double mutant, they become desiccation tolerant by the addition of ABA during culture (Senaratna et al., 1989a,b; Attree et al., 1991; Iida et al., 1992; Lecouteux et al., 1992; Tetteroo et al., 1995). Senaratna et al. (1989a) and Tetteroo et al. (1995) have shown that, besides ABA, slow drying for a few days and hydration prior to imbibition are also essential for proper regeneration of the dry embryoids. Embryoids treated with the optimal ABA concentration in the appropriate stage of development (torpedo) will not germinate when they are rapidly dried within a few hours. Furthermore, hydration before imbibition of dry embryoids is necessary to prevent imbibitional damage (Hoekstra et al., 1989).

The mechanisms involved in the acquisition of desiccation tolerance are still not fully understood. A better understanding of these mechanisms is essential for the perfection of dry artificial seed technology (McKersie et al., 1989). Accumulation of soluble carbohydrates (di-, tri- and tetrasaccharides) has been reported as a major factor in the acquisition of desiccation tolerance. These sugars may act *in situ* as protectants of membranes (Crowe et al., 1987, 1989) and proteins (Carpenter et al., 1986; Crowe et al.,

1987), elicit a stable glassy state (Bruni and Leopold, 1991; Koster, 1991), and form a carbon reserve for germination (Hopf and Kandler, 1976; Amuti and Pollard, 1977).

There is a discrepancy in the literature about the causal relationship between soluble carbohydrates and desiccation tolerance. The loss of desiccation tolerance during germination is correlated with decreasing oligosaccharides and increasing monosaccharides (Koster and Leopold, 1988). However, it is questionable whether the reverse process always occurs during seed development. On the one hand, Leprince et al. (1990) and Blackman et al. (1992) found that, in soyabean and *Brassica campestris,* oligosaccharides increased during acquisition of desiccation tolerance, but, on the other hand, the ability to withstand desiccation in pea seeds was preceded by a sharp decline in soluble sugars (Rogerson and Matthews, 1977). Furthermore, a negative correlation was found in seeds of various ABA-insensitive mutants of *Arabidopsis thaliana*, which differ in the extent of desiccation tolerance, (Ooms et al., 1993b): the most desiccation-sensitive mutants contained the most sucrose and *vice versa*, whereas the differences between the genotypes in raffinose and stachyose were minute. Moreover, Ooms et al. (1993a) were able to induce desiccation tolerance in *A. thaliana* double-mutant seeds by treatment with the ABA analog LAB 173 711, which did not change the sugar content. These authors suggested that carbohydrate accumulation is not a prerequisite for desiccation tolerance.

To investigate further the involvement of soluble carbohydrates in the extent of desiccation tolerance during organ development, we studied embryoids of carrot and alfalfa as the experimental systems. Embryoids have several advantages over developing seeds: it is easy to obtain large amounts of them and to manipulate the growth conditions, and embryoids are easy to handle. In the present paper, we describe the changes in soluble carbohydrate content of embryoids during development as affected by the addition of ABA and slow drying. The correlation between acquired desiccation tolerance and sugar content is discussed.

Materials and methods

Plant material

Seeds of the carrot (*Daucus carota* L.), genotypes RS 1 and Trophy were provided by Royal Sluis, Enkhuizen, Netherlands, and by Dr S. de Vries of the Department of Molecular Biology of the Agricultural University, Wageningen, Netherlands, respectively. The alfalfa (*Medicago sativa* L.) cultures were started from dry embryoids of the genotype Rangelander line RL-34, which were kindly provided by Drs T. Senaratna and B. D. McKersie of the University of Guelph, Canada.

Production of carrot embryoids

Callus regenerated from hypocotyl explants was used to initiate cell suspension cultures. Cell suspensions were subcultured on B_5 medium (Gamborg et al., 1968) containing 20 g sucrose l^{-1} and 2.3 μM 2,4-D, every 14 days. Only the 50-125 μm pro-embryogenic mass (PEM) fraction of 7-day-old cultures was used to produce embryoids (De Vries et al., 1988). The PEMs were grown at low density on hormone-free B_5 medium with 20 g sucrose l^{-1} (developmental phase). After 7 or 8 days, depending on the stage of development, the medium was renewed, and, optionally, ABA was added (maturation phase). This maturation medium also contained 20 g sucrose l^{-1} (Tetteroo et al., 1995).

Production of alfalfa embryoids

Desiccation-tolerant alfalfa embryoids were produced according to the method of Senaratna et al. (1989a,b). To enhance embryoid formation, 30 μM proline was added to the development medium (Stuart and Strickland, 1984a,b; Shetty and McKersie, 1993).

Desiccation

Embryoids were harvested regularly from the first day of the developmental phase until the end of their maturation. The collected embryoids, approximately 1 g FW, were then spread in a 9-cm disposable Petri dish and consecutively exposed to a range of

declining RHs. Alfalfa embryoids were dried at 25°C by subsequent exposure for 3 days each to several steps of a decreasing range of RHs, established over saturated salt solutions of Na_2CO_3 (90% RH), NaCl (73% RH), $Ca(NO_3)_2$ (50% RH) and $CaCl_2$ (30% RH). The carrot embryoids were dried in a similar way, except that the 90% RH step was omitted. Embryoids were kept aseptically during the entire drying procedure because they were to be germinated *in vitro* on B_5 medium (Tetteroo et al., 1995).

Germination test

Before imbibition, the dry embryoids were hydrated in air saturated with water vapour under aseptic conditions, to prevent imbibitional damage. Carrot embryoids needed only 4 h of prehydration (Tetteroo et al., 1995), whereas alfalfa embryoids were prehydrated for 24 h (Senaratna et al., 1989a,b). Subsequently, the carrot embryoids were imbibed in B_5 medium with 20 g sucrose l^{-1}, and the alfalfa embryoids in sterilized tap water (Senaratna et al., 1989a,b). Embryoids that developed green cotyledons and elongated radicles were scored as having survived the dehydration procedure.

Carbohydrate analysis

Approximately 10 mg lyophilized embryoids were mixed with 1 ml 80% methanol containing 1 mg internal standard (raffinose and melezitose for carrot and alfalfa embryoids, respectively). The samples were kept at 76°C in a water bath for 15 min to extract the soluble carbohydrates and to inactivate enzymes. Subsequently, the methanol was evaporated in a Speedvac (Savant Instruments Inc. Farmingdale, NY, USA). The samples were then suspended in 1 ml milli-Q water. After centrifugation in an Eppendorf centrifuge, the supernatants were diluted 50 times for HPLC analysis.

Carbohydrates were separated isocratically with a Dionex HPLC system (Dionex Corporation, Sunnyvale, CA, USA) equipped with a pulsed amperometric detector. For the separation of carbohydrates, a Carbopac PA-100 column with guard column was used for alfalfa and a Carbopac PA-1 column with guard column in carrot. Identification of carbohydrate peaks was by comparison with retention times of standard solutions at two different elution programmes. Umbelliferose was purified from embryoids (chapter 5) to

obtain the response factor for the quantitative analysis. A 100 mM solution of NaOH was used as the eluant, and 1.1 M sodium acetate in 100 mM NaOH was used to clean the column after each run. The data were analysed using a Spectra Physics integrator model SP 4400 and Spectra Physics software (Labnet, Chromdat; San Jose, CA, USA).

Results

Effect of ABA on fresh carrot embryoids

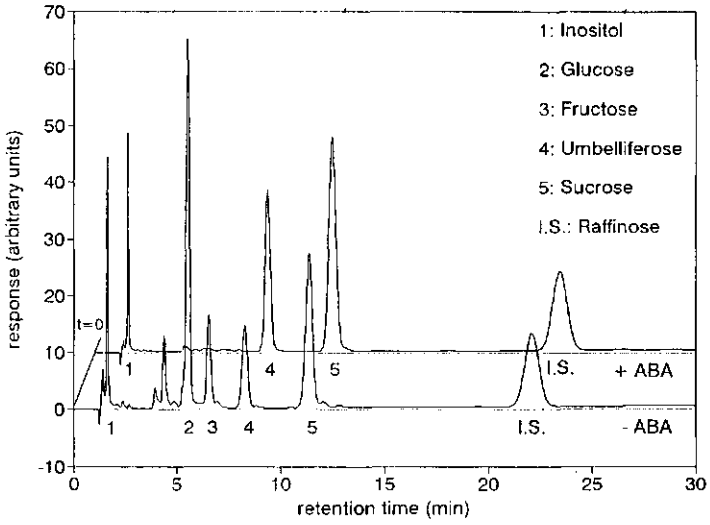

Figure 1: Carbohydrate Dionex HPLC chromatograms of 14-d-old fresh carrot embryoids regenerated with 38μM abscisic acid (ABA) added on the 7th d of culture and without ABA; (I.S. internal standard).

An example of a Dionex HPLC elution pattern of the soluble carbohydrates extracted from fresh RS 1 carrot embryoids with and without prior ABA treatment is shown in Fig. 1. Independently of ABA addition, carrot embryoids contained the same types of soluble carbohydrate as reported earlier for zygotic carrot embryos by Hopf and Kandler (1976), namely glucose, fructose, sucrose and the trisaccharide, umbelliferose. Umbelliferose (*O*-α-D-galactopyranosyl-(1-2)-*O*-α-D-glucopyranosyl-(1-2)-β-D-

fructofuranoside) was first identified by Wickstrom et al. (1956) as the typical trisaccharide in the family Apiaceae (formerly Umbelliferae).

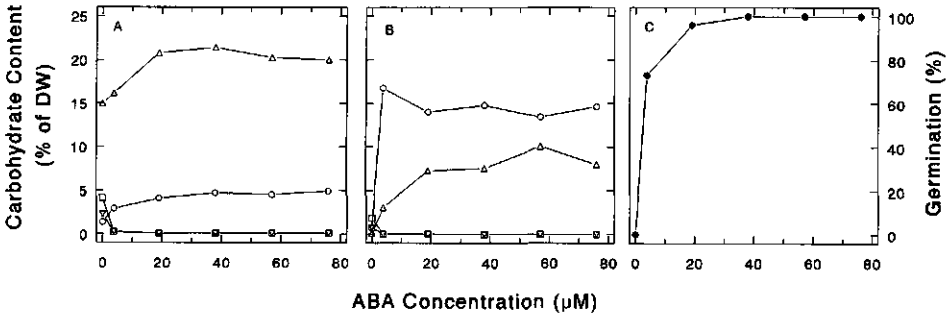

Figure 2: Effect of abscisic acid (ABA) concentration on the soluble carbohydrate contents (□ glucose, ▽ fructose, ○ umbelliferose, △ sucrose) of (A) fresh and (B) slowly dried embryoids of carrot genotype RS 1; embryoids were harvested after 20 d in culture. (C) Germination after slow dehydration, as affected by ABA concentration; before imbibition the embryoids were humidified for 4 h in a moisture-saturated atmosphere.

ABA was added as a supplement to the embryoids on the seventh day of 2,4-D-free culture to prevent precocious germination and to induce desiccation tolerance. In 20-day-old fresh embryoids, even low ABA concentrations influenced the carbohydrate content (Fig. 2), but application of high ABA concentrations hardly changed carbohydrate content compared with low concentrations. After 10 days embryoids without ABA were germinating in the Erlenmeyer flasks, whereas embryoids with ABA continued embryonic development, even at the lowest concentration tested (3.8μM).

A considerable decrease in glucose and fructose occurred in the fresh PEMs of RS 1 carrot during the first 7 days of subculture in 2,4-D-free B_5 medium (Fig. 3A and B). ABA was not added until the 7th day of the culture to permit the proper development of torpedo-shaped embryoids. Within 3 days of addition of ABA, changes in the soluble carbohydrates were observed (Fig. 3A, B, C and D). Without addition of ABA, the content of glucose and fructose remained at the moderate level of 2-5% of the DW; but,

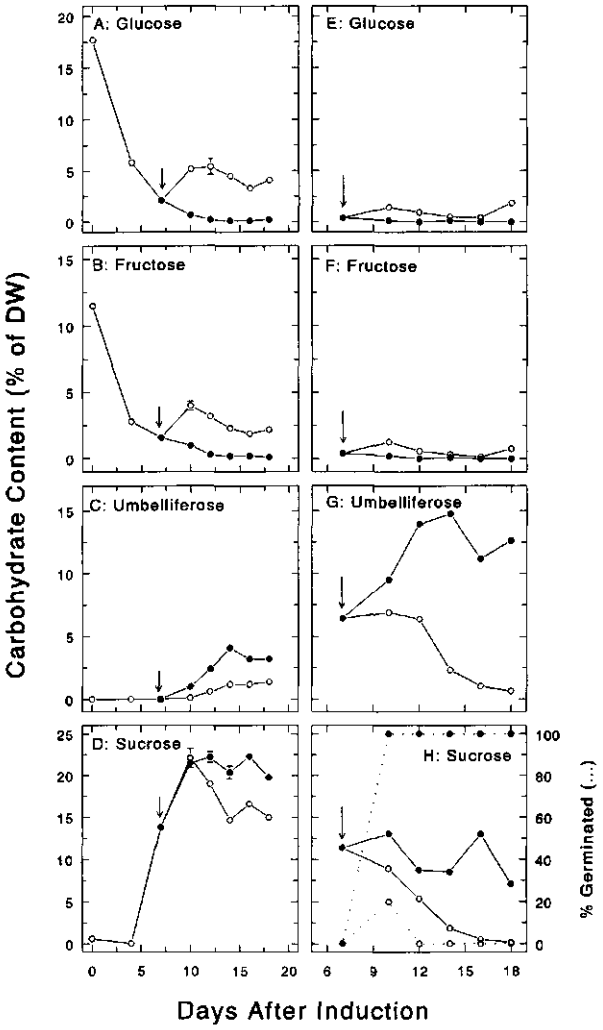

Figure 3: Changes in soluble carbohydrate contents of fresh (A-D) and slowly dried (E-H) embryoids of the carrot genotype RS 1, during development in 2,4-D- free B_5 medium. On the 7th d of culture (arrows), 38 μM abscisic acid (ABA) (●) was added to the embryoids, or the culture was continued without ABA (○). Data are means of duplicate measurements of a representative experiment (error bars are indicated when they exceed symbol size). Note different scales for different carbohydrates. Acquisition of desiccation tolerance of slowly dried embryoids is also indicated (H,┈┈).

in embryoids treated with ABA, there was a decline in monosaccharides to nearly undetectable levels. The sucrose content continued to increase to approximately 20% of the DW for both treatments, but decreased again when germination started in the absence

of ABA. Umbelliferose content rose to only 4% of the DW after 14 days in the presence of ABA. Without ABA the umbelliferose content rose slightly at a later stage of development, but did not exceed approximately 2% of the DW. Similar results (data not shown) were observed in the genotype Trophy, which has a different genetic background.

Effect of slow drying

We have found that slow dehydration is essential for the acquisition of desiccation tolerance in carrot embryoids. The effect of slow drying on carbohydrates was studied in embryoids that had been treated with 38 μM ABA on the 8th day of culture on 2,4-D free

Table 1: Effect of the critical first 4 d of slow drying in carrot RS 1 embryoids on moisture content, soluble carbohydrate content and desiccation tolerance. Eight days after the transfer to hormone-free medium the embryoids were grown on B_5 medium with 38 μM ABA for 10d. Embryoids were then subjected to slow drying. After the indicated number of days of slow drying, embryoids were rapidly dehydrated within a few hours to a moisture content of 0.05 g H_2O g^{-1} DW and stored at 25 °C and 30% RH for at least one week before germination was tested. Data are means of duplicate extractions.

Slow drying	Moisture content	Carbohydrate content (% of DW)				Germinated
(days)	g H_2O g^{-1} DW	Glucose	Fructose	Sucrose	Umbelliferose	%
0	2.6	0.27	0.26	15.41	6.11	0
1	2.2	0.08	0.05	9.32	7.98	4
2	1.2	0.05	0.11	6.47	12.64	55
3	1.1	0.04	0.03	6.32	13.21	68
4	1.0	0.04	0.08	4.09	13.95	89

B_5 medium. Rapidly dehydrating embryoid samples to a moisture content of 0.05 g H_2O g^{-1} DW did not alter the carbohydrate composition (data not shown). The entire slow-drying procedure above saturated salt solutions lasted for 9 days, but most of the water was lost in the first 4 days (Table 1). In the remaining 5 days the embryoids reached the equilibrium of 0.05 g H_2O g^{-1} DW moisture content. Within the first day of dehydration, glucose and fructose decreased to < 0.1% of DW (Table 1). Sucrose contents decreased from 15.4% to 4.1% of DW within 4 days, whereas the umbelliferose content increased

from 6.1% to 13.9% of DW. Germination percentage rose from 0%, when embryoids were immediately subjected to rapid drying, to 89% after 4 days of slow dehydration. From these data, it can be concluded that there is a strong positive correlation between umbelliferose content and desiccation tolerance, whereas contents of the other carbohydrates are negatively correlated with desiccation tolerance. The same experiment was also performed with genotype Trophy, with similar results (data not shown).

Effect of ABA on slowly dried carrot embryoids

Compared with the control without ABA, low ABA concentration (3.8 μM) raised the umbelliferose content significantly, to 17% of DW. Treatment with a higher concentration of ABA had no further effect. Sucrose content increased to a maximum of 10% of DW at 57 μM ABA. Monosaccharides were present in the embryoids only in the absence of ABA. Desiccation tolerance, measured as percentage germination, was greatest at 19-38 μM ABA (Fig. 2C). It increased sharply from 0 to 3.8 μM ABA, in close correlation with the increase in umbelliferose content. A further increase in germination to 100% was not correlated with an increase in umbelliferose content, but was with sucrose content.

The effect of slow drying was also tested in time-course measurements of carbohydrate contents (Fig. 3E-H). Embryoids were harvested at intervals after ABA addition, slowly dried to 5% moisture content, and soluble sugars were determined. After dehydration of ABA-treated carrot embryoids, the glucose and fructose was lost, whereas the control embryoids still contained low but detectable amounts of monosaccharides (Fig. 3E and F). Drying increased the umbelliferose content of 14-day-old embryoids from 4% to 14% of DW (compare Fig. 3C and 3G), while the sucrose content decreased from 22% to < 10% of DW (compare Fig. 3D and 3H). During the first 7 days of embryoid development, no desiccation tolerance was found. Embryoids acquired complete desiccation tolerance (100% germination) within 3 days of ABA addition, and this remained high throughout the experimental period (Fig. 3H). Without ABA, germination of embryoids was only 18% at the 10th day of development, to return to 0% at later stages. Comparison of the data on the carbohydrate content of the slowly dried embryoids

(Fig. 3E-H) with their desiccation tolerance does not indicate a strong positive correlation. The subsequent germination percentage increased sharply within 3 days of ABA addition (Fig. 3H), whereas the rise in umbelliferose content took 7 days. Without ABA, the umbelliferose content decreased after 10 days, associated with poor germination (18%) and loss of desiccation tolerance. Sucrose content of ABA-treated embryoids showed a variable course, whereas the untreated embryoids showed a clear decrease (Fig. 3H).

Effect of ABA on slowly dried alfalfa embryoids

Senaratna et al. (1989a,b) have developed a method for inducing desiccation tolerance in somatic embryoids of alfalfa. Using their technique, we obtained desiccation-tolerant alfalfa embryoids and analysed the soluble sugars for comparison with our carrot data. After slow drying the embryoids showed few monosaccharides, and raffinose remained low (Fig. 4A, B and C). The contents of stachyose and sucrose were significantly higher in ABA-treated embryoids than in untreated ones (Fig. 4D and E). The rise of the germination curve of ABA-treated embryoids (Fig. 4F) coincided with an increase of sucrose and stachyose. Embryoids did not survive drying after development on ABA-free medium.

Discussion

Our results clearly demonstrate that, in embryoids, ABA not only induces desiccation tolerance, but also influences soluble carbohydrate content. Carrot embryoids of two different genotypic backgrounds showed an increase in umbelliferose content during ABA treatment, whereas the monosaccharide content decreased to undetectable amounts, and the sucrose content remained more or less the same. Hopf and Kandler (1976) have described similar changes in soluble carbohydrate content during development in seeds of *Carum carvi* and *Foeniculum vulgare*, which also belong to the family Apiaceae. They found increasing amounts of umbelliferose and decreasing amounts of

glucose and fructose during seed development, but they did not determine the exact time of acquisition of desiccation tolerance. Consequently, no clear correlation with carbohydrates could be established. They also detected trehalose in *Carum carvi*, which is in contrast to our observations in carrot embryoids. Alfalfa embryoids showed similar trends to carrot embryoids in soluble sugar contents when treated with ABA, their sucrose and stachyose contents rose.

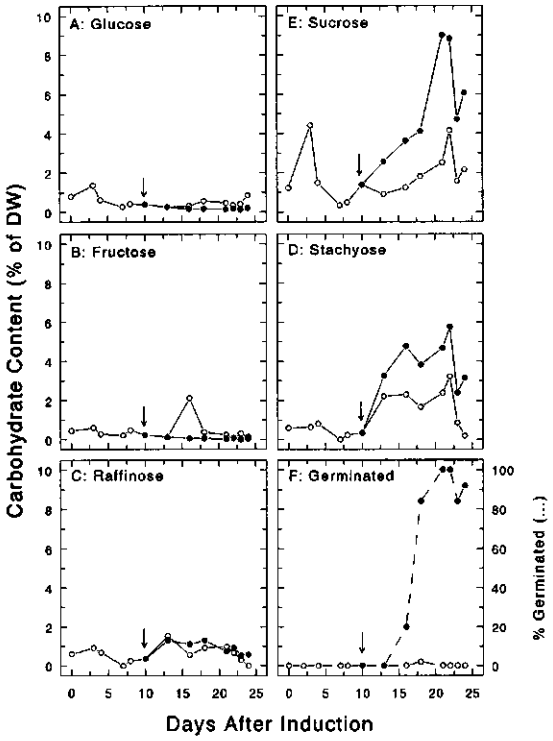

Figure 4: Changes in soluble carbohydrate contents of slowly dried (A-E) embryoids of the alfalfa genotype Rangelander, line RL34, during development in 2,4-D-free medium. On the 10th d of culture (arrows) 10μM abscisic acid (ABA) (●) was added to the embryoids, or the culture was continued without ABA (○). Acquisition of desiccation tolerance of the embryoids is also indicated (F,·····)

Besides ABA, embryoids also need slow drying to become desiccation tolerant. Excised immature embryos of *Ricinus communis* remain viable only after slow drying, whereas mature embryos survive fast drying treatments (Kermode and Bewley, 1985;

Kermode, 1990). This indicates that embryoids do not reach the same level of maturity as zygotic embryos. Immature soyabean embryos (Blackman et al., 1992) and immature *Papaver dubium* pollen (Hoekstra and van Roekel, 1988) remained viable only after slow drying.

During slow dehydration, the carbohydrate content of both carrot and alfalfa embryoids changed in a similar way. Oligosaccharides increased, and glucose and fructose contents decreased. The same trends in soluble sugar contents occur in slowly dried immature embryos of soyabean, whereas the embryos stored at high RH do not accumulate oligosaccharides and lose desiccation tolerance (Blackman et al., 1992).

If correlations between carbohydrate content and desiccation tolerance are to be meaningful, germination data have to be compared with the carbohydrate content of dried embryoids, because interaction of carbohydrates with phospholipids and proteins occurs at water contents of $< 30\%$ on a DW basis (reviewed by Crowe et al., 1996). The fact that both somatic (our data) and zygotic embryos (Hopf and Kandler, 1976) show the same pattern of changes, supports the hypothesis that carbohydrates play a general role in anhydrobiosis. Koster and Leopold (1988) demonstrated that loss of desiccation tolerance during germination of corn, pea and soyabean seeds is correlated with a change in soluble carbohydrate content. During imbibition, the content of monosaccharides increases, and that of sucrose and oligosaccharides decreases. We have obtained similar results with germinating carrot seeds (unpublished results). The correlation between loss of desiccation tolerance and changes in soluble carbohydrate content during germination suggests a similar correlation, but in the reverse way, during seed development. Indeed, Fisher et al. (1988) and Leprince et al. (1990) have shown that during seed development of *Sinapis alba* and *Brassica campestris* di- and oligosaccharides increased whereas the contents of monosaccharides decreased. However, Ooms et al. (1993a,b) reported that desiccation tolerance tends to coincide with low sugar content. Seeds of the desiccation-intolerant double mutant of *Arabidopsis thaliana* (lacking ABA and insensitive to ABA) contain twice as much sucrose as the wild type. Treatment of this double mutant with the ABA analog LAB 173 711 induces desiccation tolerance, but does not change the soluble sugar

content. A decline in soluble sugars during development has been found in peas (Rogerson and Matthews, 1977).

The above data conflict with each other, and often do not agree with our data. An important difference with the present approach is that none of the studies cited above applied slow drying methods, which were shown to be of the utmost importance in our studies. Another reason for the discrepancy might be that carbohydrates may be localized differently in seeds of different species. For the soluble carbohydrates to be effective at protecting membranes and proteins, their presence is also required in the cytoplasm. Seeds with high contents of oil bodies (e.g. *Arabidopsis*) or starch grains (e.g. peas), are comparatively limited in cytoplasm. Such seeds may have less of the carbohydrates and still be desiccation tolerant. We only measured bulk sugar composition and not location of sugars in the dry embryoids.

When we compare desiccation tolerance of carrot embryoids with their fresh sugar contents, the correlations are poor. Comparison of the soluble carbohydrate content of dried embryoids with germination percentages gave much better correlations, but a causal relationship could not be established. The germination percentage of dry carrot embryoids increased sharply from 0 to 100% soon after ABA addition on day 7, whereas the umbelliferose content rose only gradually. The same holds true for the stachyose and sucrose contents in alfalfa embryoids. These data suggest that di- and oligosaccharides are not limiting anhydrobiosis, but that a minimal threshold amount of these sugars is required. If this is true, the question remains why do ABA and slow drying have such a significant effect on the soluble carbohydrate content?. It seems very likely that di- and oligosaccharides have a function in dry embryoids.

Several hypotheses for the role of di- and oligosaccharides during anhydrobiosis have been put forward. Apart from the function as carbon reserve (Hopf and Kandler, 1976; Amuti and Pollard, 1977), soluble carbohydrates may protect membrane phospholipids and proteins in the dry state (Carpenter et al., 1987; Crowe et al., 1987, 1989; Hoekstra et al., 1989). Carbohydrates may also be important in the formation of a glassy state during dehydration (Bruni and Leopold, 1991; Koster, 1991). The glassy state is stabilized by oligosaccharides, whereas monosaccharides disrupt it. We found

monosaccharides to be present in very small amounts in desiccation-tolerant embryoids. Moreover, glucose and fructose are reducing sugars that might be involved in Maillard reactions in dry tissues (Koster and Leopold, 1988) with deleterious effects. We conclude that our data on carbohydrate content in dried embryoids corroborate the water replacement theory and the possible involvement of a stable glassy state in desiccation tolerance.

Acknowledgements

We thank Henk Schols of the Department of Food Science, Wageningen Agricultural University, for his support with the carbohydrate analysis on the Dionex HPLC, Steef de Bruin for his assistance with Lotus 123 and Marc Hagendoorn and Jaap Ooms for critically reading the manuscript.

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Chapter 5

Protective role of umbelliferose and sucrose in desiccation tolerant carrot (*Daucus carota* L.) embryoids.

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Summary

Previously, we have demonstrated that carrot (*Daucus carota* L.) embryoids can acquire desiccation tolerance. Then, we could not demonstrate a strict correlation between survival of the dry embryoids and the contents of sucrose and umbelliferose, being the major endogenous soluble carbohydrates. However, in the present paper we report on the apparent minimum requirement of sucrose plus umbelliferose for surviving severe dehydration, suggesting that these sugars play an important role in anhydrobiosis. Using FT-IR spectroscopy, we show that both sucrose and umbelliferose depress the transition temperature of dry liposomal membranes, which is indicative of the interaction with the phospholipid polar headgroups. Furthermore, both sugars prevent leakage from dry liposomes during drying and subsequent rehydration. We interpret this in the sense that both sugars are able to form a stable glass in the dry state at room temperature. Fructose and glucose were lacking in dry viable embryoids. In the light of the plasticizing effect of these monosaccharides on sugar glasses, a stable glassy state seems important during anhydrobiosis of carrot somatic embryoids. We show that umbelliferose can protect a protein that is desiccation sensitive.

Introduction

Desiccation tolerance is the capacity of organisms or tissues to regain vital metabolism after almost complete dehydration. This capacity can be encountered in seeds, pollens, nematodes, tardigrades, yeasts, fungi and bacteria (reviewed in Crowe et al., 1992). Considerable effort has been spent on elucidating the mechanisms of this desiccation tolerance (reviewed in Crowe et al., 1996a). Di- and oligosaccharides, such as trehalose, sucrose and raffinose play a key role in the protection of membranes and proteins, which is known as the "water replacement theory" (Carpenter et al., 1987; Crowe et al., 1987). During dehydration sugar molecules replace the water molecules around the polar headgroups of the phospholipids. The hydroxyl-groups of the sugars form hydrogen bonds with the phosphate of the headgroups, thereby preventing the membranes to pass from the liquid-crystalline phase into the rigid gel phase. Thus, the transiently increased permeability of the phospholipid bilayer and the associated leakage are prevented (Hammoudah et al. 1981; Crowe et al., 1989a, 1989b). Loss of cytoplasmic solutes often results in extensive cell damage or even in death of the cells (Simon 1974; Tetteroo et al., 1996).

Carrot (*Daucus carota* L.) zygotic embryos are desiccation tolerant, whereas somatic embryos only acquire this property when they are treated during their development according to earlier described methods (Tetteroo et al., 1995). Both embryo types contain sucrose and the trisaccharide, umbelliferose, which is characteristic for the Apiaceae (Hopf and Kandler, 1976; Tetteroo et al., 1994). Umbelliferose has been chemically identified as *O*-α-D-galactopyranosyl-(1-2)-*O*-α-D-glucopyranosyl-(1-2)-β-D-fructofuranoside by Wickstrom and Svendsen (1956). Until now, the function of this trisaccharide has not been investigated with respect to desiccation tolerance.

Umbelliferose might be involved in the protection in situ of dry membranes and proteins, similarly as suggested for sucrose and trehalose (Crowe et al., 1987). However, the causal relationship between desiccation tolerance and the presence of umbelliferose and sucrose in dry carrot embryoids still has to be demonstrated. During the indispensible slow drying of the embryoids umbelliferose seems to increase at the expense of sucrose,

which process continues after desiccation tolerance has been acquired (Tetteroo et al., 1994). This explains our conclusion that the correlation between desiccation tolerance and the contents of sucrose or umbelliferose was poor, but it does not exclude that a minimum threshold level of these sugars is crucial for desiccation tolerance. We have also demonstrated that leakage of solutes, such as K^+ and carbohydrates, through the plasma membrane, may be the main cause of the failing germination after imbibition of the dehydrated embryoids (Tetteroo et al., 1996a). This indicates that plasma membranes are a main target of injury during desiccation of carrot embryoids. Therefore, membranes will need protection in the dry state to allow the embryoids to survive.

In this study we have purified and identified umbelliferose as the sole soluble oligosaccharide in carrot somatic embryoids. We have elucidated possible functions of this trisaccharide during anhydrobiosis.

Material and Methods

Plant material

Seeds of carrot (*Daucus carota* L.), genotypes "RS 1" and "Trophy", were provided by Royal Sluis, Enkhuizen, The Netherlands, and by Dr S. de Vries of the Department of Molecular Biology of the Agricultural University, Wageningen, The Netherlands, respectively.

Embryoid production

Embryoids were produced, dried and germinated as described earlier (Tetteroo et al., 1995). The degree of desiccation tolerance of the different embryoid lots was varied by applying a large number of different treatments. Variables were: genotype, developmental stage of the embryoids, concentration or the added ABA, sucrose concentration in the maturation medium, addition of PEG during maturation, and dehydration rate. From each lot the carbohydrate content and composition were analysed and the germination capacity was determined.

Extraction, purification and identification of umbelliferose

Dry embryoids (10 g) were boiled in 200 mL of 80% methanol for one h. The embryoids were then filtered and washed with 50 mL of 80% methanol and the filtrates were combined. The methanol was removed from the filtrate by vacuum evaporation, and the remaining aqueous suspension was defatted by passing it through a C18 reversed phase column (Waters Assoc. Milford MA, USA). The suspension was then purified by passing it through a column of Polyclar T (insoluble polyvinylpyrrolidone). After reduction of the volume the suspension was layered on a Sephadex QAE-A-25-formate column and a Sephadex SP-C-25-H^+ column (Pharmacia, Sweden) and eluted with milli-Q water according to Redgwell (1980). The eluate containing the sugar fraction was freeze-dried to reduce volume. If required, the Sephadex column purification procedure was repeated. Umbelliferose was separated from the other sugars by preparative HPLC on a Shodex OH pak Q-2002 column (20x500 mm; Waters, Milford, MA, USA), using milli-Q water of 55°C as the eluent at 3 mL min^{-1} and a refractive index detector (Spectra Physics model SP 8430, San Jose, CA, USA). Using Dionex HPLC (analytical PA-1 column, 9x250 mm; see also under "carbohydrate analysis"; Dionex Corporation, Sunnyvale, CA, USA) we further purified the umbelliferose. The NaOH in the eluent recovered from the column was removed using a Dionex anion self-generating supressor (4mm) and 50 mN H_2SO_4 as the regenerant, flow rate 5 mL min^{-1}. The purified umbelliferose solution was then lyophilised and used for NMR and FT-IR measurements and also to determine the response factor for the quantitative analysis of umbelliferose by Dionex HPLC. The umbelliferose preparation purified according to the above procedure was characterized by one single peak in the Dionex HPLC chromatogram. Alternatively, extractions were made starting from 50 g of seed that was grinded in 80% methanol in a mortar with a little sand.

NMR measurements

For all experiments a Bruker AMX500 NMR spectrometer operating at 500 MHz was used. The samples (50 mg) were dissolved in aceton for ^{13}C NMR or in D_2O for ^1H

NMR and two dimensional NMR analysis. COSY-spectra were recorded to determine the ^{1}H-^{1}H correlations, whereas HETCOR-spectra were recorded to determine ^{13}C-^{1}H correlations. Raffinose was used as the control for comparison of peak positions and the correlations.

Carbohydrate analysis

Per lot of lyophilized embryoids approximately 10 mg were mixed with 1 mL 80 % methanol containing 1 mg raffinose as the internal standard. The samples were kept at 76 °C in a water bath for 15 min to extract the soluble carbohydrates and to inactivate enzymes. Subsequently, the methanol was evaporated in a Speedvac (Savant Instruments Inc. Farmingdale, NY, USA). The samples were then suspended in 1 mL milli-Q water. After centrifugation in an Eppendorf centrifuge the supernatants were diluted 50 times for HPLC analysis.

Carbohydrates were separated isocratically with a Dionex HPLC system (Dionex Corporation, Sunnyvale, CA, USA) equipped with a pulsed amperometric detector and a 4x250 mm Carbopac PA-1 column with guard column. Identification of carbohydrate peaks was by comparing with retention times of standard solutions at two different elution programs. Hundred mM NaOH was used as the eluent, and 1.1 M sodium acetate in 100 mM NaOH was used to clean the column after each run. The data were analysed using a Spectra Physics integrator model SP 4400 and Spectra Physics software (Labnet, Chromdat; San Jose, CA, USA).

FT-IR spectroscopy

Infrared absorption measurements of lipid and protein samples were carried out with a Perkin-Elmer series 1725 FT-IR spectrometer equipped with an external beam facility to which a Perkin-Elmer IR-microscope was attached. The microscope was equipped with a LN_2-cooled narrow band Mercury/Cadmium/Telluride IR-detector. Data were acquired with a MS-DOS computer using Perkin Elmer software (versions 2.5 and 3.5). Each spectrum was the average of 100 scans at each temperature in the IR region 3500-900 cm^{-1}. Temperature control of the sample in the instrument was with a liquid

nitrogen cooled brass cell with a resistance heater under computer control. The instrument was purged of water vapor with a Balston dry air generator (Balston, Maidstone, Kent, England).

Hydrated or air-dried lipid samples were precooled and slowly warmed up (40°C h^{-1}). Approximately 60 spectra were recorded over the temperature range from -60 to 90°C. Second derivative spectra were calculated, and after normalizing, the band position around 2852 cm^{-1}, attributed to the CH_2 symmetric stretch of the egg-PC acyl chains, was calculated as the average of the spectral positions at 80% of the total peak height. The T_m was estimated by observing the shift with temperature of this absorption band around 2852 cm^{-1} (temperature at mid-value of the shift).

For protein studies the spectral region between 1700 and 1500 cm^{-1} was selected, and each spectrum was the average of 256 scans. This region contains the amide-I and the amide-II absorption bands of the protein backbones.

Preparation of liposomes for FT-IR analysis and leakage studies

Egg-PC in $CHCl_3$ (Fluka, Buchs, Switzerland) was used without further purification. After removal of the $CHCl_3$ in vacuum overnight, the dry egg-PC was rehydrated in water at a concentration of 10 mg mL^{-1}. When required, carbohydrates were added externally to the egg-PC suspension to give a mass ratio of 5:1 (sugar:egg-PC). Subsequently, unilamellar vesicles were produced by passing the suspension 35 times through one 100-nm pore size polycarbonate filter (Nuclepore Corp., Pleasanton, CA, USA) as described previously (Van Bilsen et al., 1994).

For infrared spectroscopy, 5 μL samples were dried directly on CaF_2 windows for at least three h in a stream of dry air at 23°C (RH < 3%). Before the samples were removed from the dry air box another window was placed on top of the sample window, with a rubber ring in between, to prevent rehydration of the samples during transfer to the spectrometer and during FT-IR analysis. Hydrated liposome samples were concentrated by ultracentrifugation and the pellet was used for FT-IR analysis.

For leakage studies the vesicles were produced also at 10 mg mL^{-1}, in 1 mM Tes, pH 7.5, containing 0.25 M sucrose and 100 mM CF [Serva, Heidelberg, Germany,

purified according to Klausner et al. (1981)]. After the 35 times passage through the polycarbonate filter, the external sucrose and CF were removed from the liposomes by gel filtration (Sephadex G50). Typical concentration of egg-PC after filtration was 3 mg mL^{-1}. Samples of about 30 μg egg-PC containing different concentrations of the various sugars in a total volume of 30 μl, were dried in the caps of Eppendorf tubes for 3 h in dry air (RH < 3%). Leakage of CF was measured after rehydration of the sample in 1 mL Tes buffer, pH 7.5, in the closed Eppendorf tubes. The excitation wavelength was 490 nm and the emission wavelength was 515 nm. Percent CF retention was calculated according to Crowe and Crowe (1988).

Results

Purification and identification of umbelliferose

Table I: Peak positions in ^{13}C NMR spectra of raffinose and umbelliferose, in ppm, with acetone as the reference

Raffinose:	C_1	C_2	C_3	C_4	C_5	C_6
Galactose	99.3	69.3	70.2	70.0	71.8	61.9
Glucose	92.9	71.8	73.5	70.2	72.2	66.7
Fructose	62.2	104.6	77.1	74.8	82.2	63.3
Umbelliferose:	C_1	C_2	C_3	C_4	C_5	C_6
Galactose	97.2	68.9	70.2	70.0	71.8	61.8
Glucose	90.4	76.2	72.9	69.8	72.0	61.0
Fructose	62.6	105.0	77.0	74.5	82.0	62.9

Umbelliferose had to be purified for its identification and quantification (determination of the response factor) in the HPLC-chromatograms. Furthermore, purified umbelliferose was needed to study its interaction with phoshoplipids and to measure its protective properties on dried liposomes and proteins.

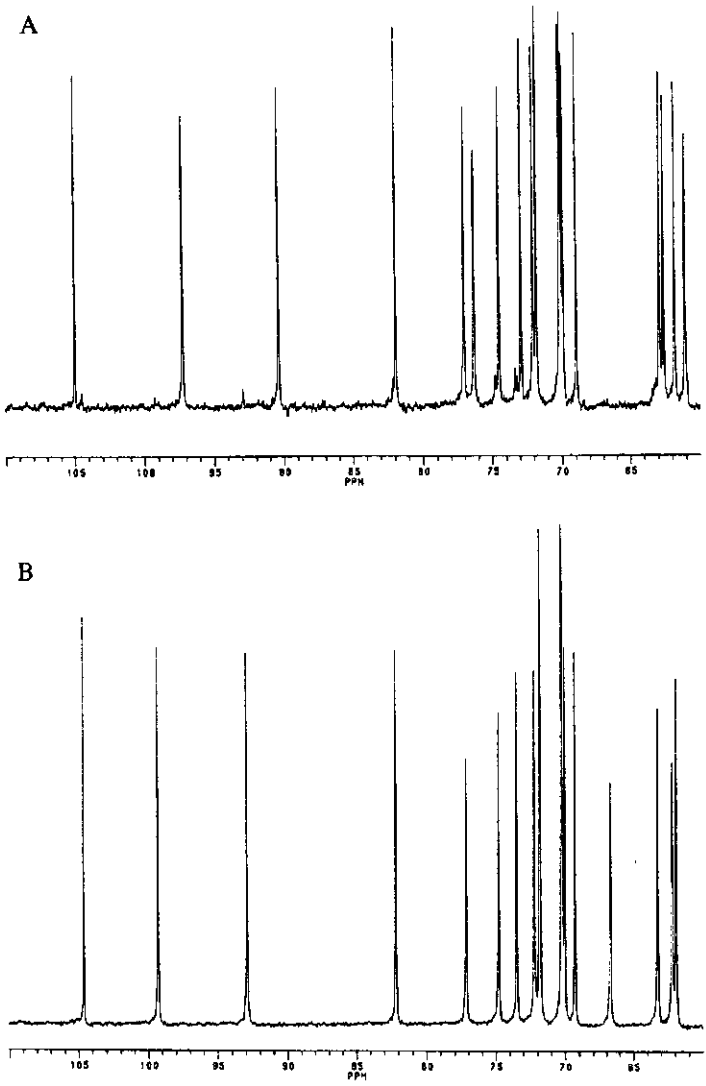

Figure 1: ^{13}C-NMR spectra of umbelliferose (A) and raffinose (B).

HPLC analysis of the trifluoroacetic acid hydrolysed trisaccharide from the embryoids showed that it consisted of glucose, fructose and galactose, similarly as raffinose. Therefore, raffinose was used as a control in the NMR analysis. The molecular

structure of umbelliferose was confirmed by using ^{13}C NMR (see Fig. 1 and Table I). The difference between umbelliferose and raffinose is at the linkage between the glucose and the galactose moiety. Galactose is linked to glucose at the C_6 position in raffinose, whereas in umbelliferose the galactose is linked to the glucose at the C_2 position. As expected, the ^{13}C NMR-spectrum of umbelliferose resembled that of raffinose, except for the Glc-C_2 and Glc-C_6 peak positions (Fig. 1 and Tab. I). The lack of large unidentified peaks in the NMR spectrum of umbelliferose is an indication of purity. Employing two dimensional COSY- and HETCOR-spectra we were able to identify the shifts of the Glc-C_2 (from 71.8 to 76.2 ppm) and of the Glc-C_6 (from 66.7 to 61.0 ppm) when comparing raffinose with umbelliferose (Table I). The ^{13}C-NMR spectra of raffinose were similar to earlier published spectra (Bock et al., 1984).

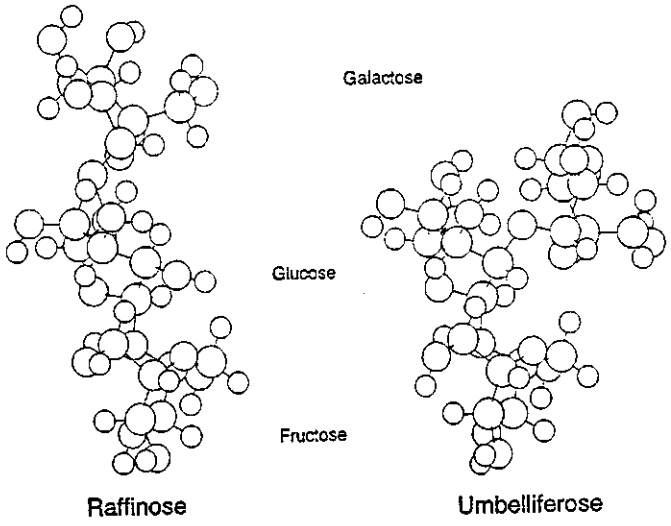

Figure 2: Diagram of molecular structure of umbelliferose and raffinose as calculated by Alchemy III v2 software.

The impact of the linkage of galactose at the Glc-C_2 position on the molecular structure of umbelliferose as calculated with Alchemy III v2 software (Tripos Assoc. Inc.

USA) is shown in Fig. 2. Compared to the relatively elongated molecular structure of the raffinose, the umbelliferose molecule is compact, in its size fairly similar to sucrose.

Carbohydrate content and composition in dry embryoids

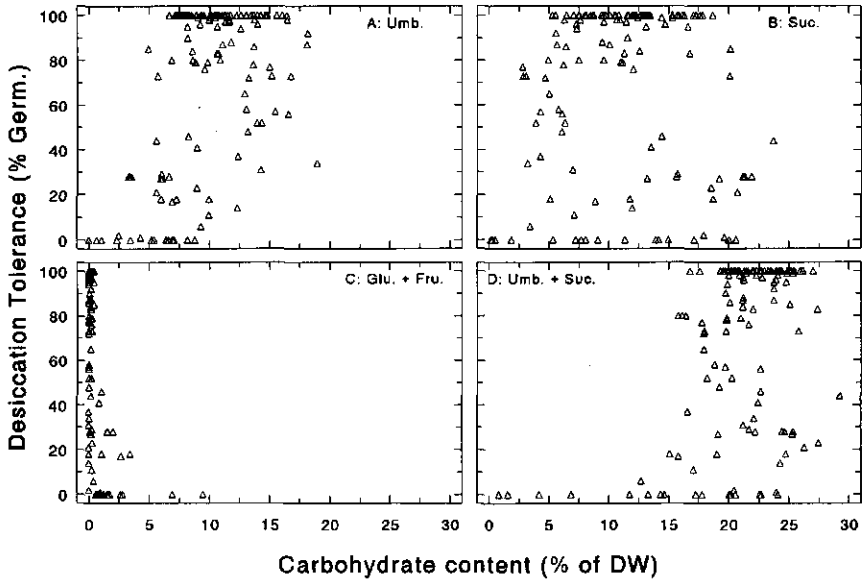

Fig. 3: Plots of the contents of different soluble carbohydrates in 137 dry carrot embryoid lots against the desiccation tolerance (germination percentage of the rehydrated lot). The variables in these 137 embryoid lots were genotype and developmental stage of the embryoids, concentration of added ABA and sucrose in the maturation medium, and rate of dehydration. A) umbelliferose content; B) sucrose content; C) glucose + fructose content; D) content of umbelliferose plus sucrose in each lot.

During protocol development for optimizing desiccation tolerance of the embryoids a large number of different treatments were given. The variables in these experiments were genotype and developmental stage of the embryoids, concentration of added ABA and sucrose in the maturation medium, and rate of dehydration. Figure 3 shows plots of the contents of the different carbohydrates in 137 dry embryoid lots from 15 independent experiments against the germination percentage as a measure of desiccation tolerance.

There was no clear correlation between desiccation tolerance and the contents of either sucrose or umbelliferose. Embryoids only survived dehydration, when they contained umbelliferose or sucrose for at least 3% of their DW and no monosaccharides. However, this does not mean that the soluble carbohydrates are of limited importance to desiccation tolerance. The minimum threshold level of sucrose and umbelliferose associated with desiccation tolerance became much more pronounced when the contents in each lot were added (Fig. 3D). Thus, an empty space could be observed (in plot 3D), indicating that a minimum of at least 15% of the DW had to be present before embryoids became desiccation tolerant. This threshold level is much higher than the sum of the individual minimum threshold levels of either umbelliferose (3%) or sucrose (3%). It is an indication that the occurrence of these two carbohydrates is not independent and that they may be both important in relation to desiccation tolerance. The interchangeability between sucrose and umbelliferose is further elaborated on in Fig. 4. Data of nongerminating embryoids were left out of this graph because we were only interested in the interchangeability in tolerant embryoids.

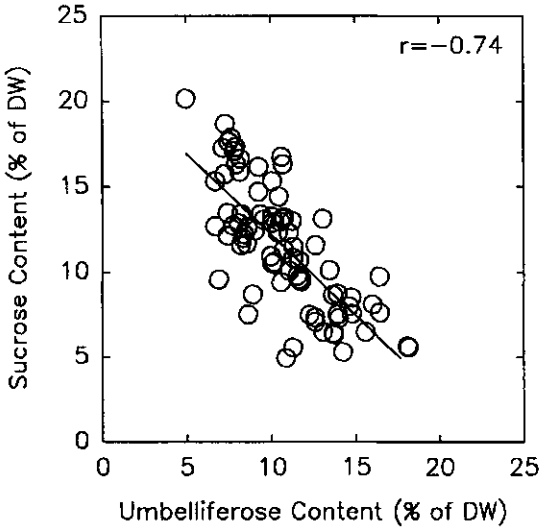

Figure 4: Correlation between sucrose and umbelliferose contents in each lot of viable embryoids also used in Fig. 3 (germination percentage ranging from 80-100%).

Similar results as in Fig. 3 were obtained with embryoids that were, in addition to ABA, treated with different amounts of PEG during the maturation phase, and always slowly dried (Fig. 5). Now the threshold level was merely 1.5% of the DW for umbelliferose and 2.5% for sucrose. The minimum threshold level for the added content of umbelliferose and sucrose in each lot was only 8% of the DW, which is almost 50% lower than the threshold level in Fig. 3 (without PEG). Still, the added threshold level (8%) was twice as high as the sum of two individual levels of umbelliferose (1.5%) and sucrose (2.5%), again indicating the interchangeability of the two sugars.

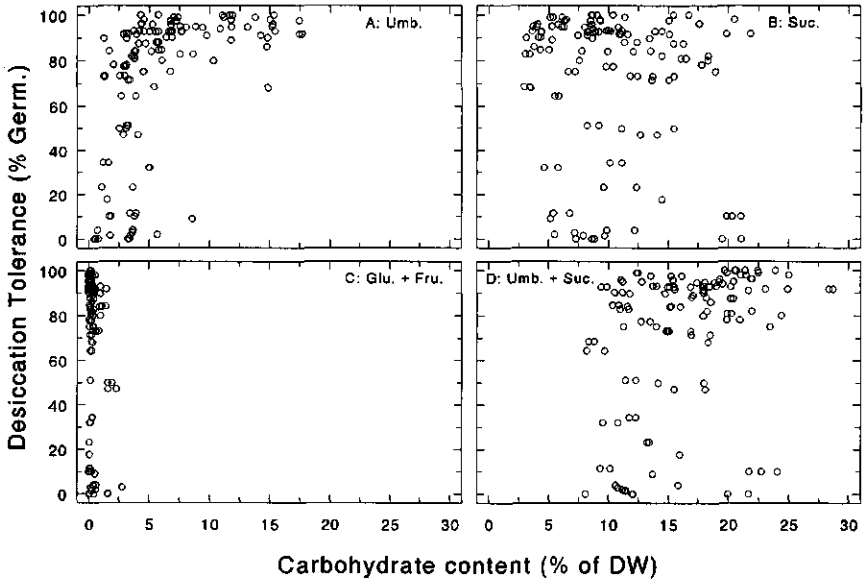

Figure 5: Plots of the contents of different soluble carbohydrates in PEG-treated carrot embryoid lots against the desiccation tolerance (germination percentage). A) umbelliferose content; B) sucrose content; C) glucose + fructose content; D) content of umbelliferose plus sucrose in each lot.

Interaction of umbelliferose and sucrose with dry liposomes

Using IR-spectroscopy we measured the position of the absorption band attributed to the CH_2 symmetric stretching vibration of the acyl chains in egg-PC liposomes. During the transition from the gel-phase to the liquid-crystalline-phase there is a wavenumber shift

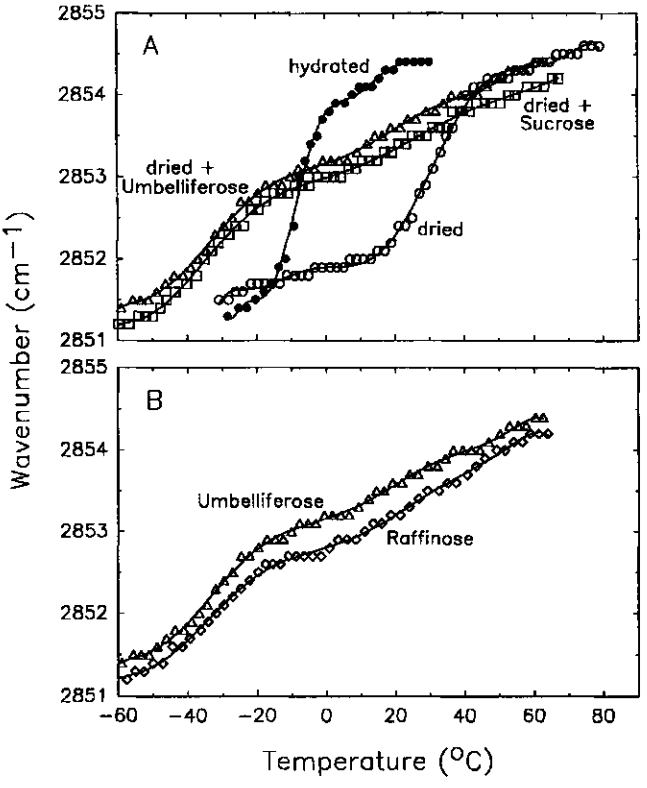

Figure 6: Wavenumber versus temperature plots (FT-IR) of egg-phosphatidylcholine (egg-PC) liposomes. The different conditions were: A: hydrated, air-dried (3% RH) and air-dried (3% RH) in the presence of sucrose or umbelliferose; B: air-dried (3% RH) in the presence of either umbelliferose or raffinose. Representitive plots.

from 2851 to 2854 cm^{-1} (Hoekstra et al. 1989). Hydrated liposomes showed a wavenumber shift at approximately -8°C (Fig. 6A). Dry liposomes had an increased T_m at approximately 32°C. Liposomes dehydrated in the presence of sucrose or umbelliferose had depressed T_ms at approximately -35°C. This low T_m is indicative of a strong spacing of the phospholipid headgroups by the sugars (Nakagaki et al., 1992; Crowe et al., 1996c). Also a slight shift between 10 and 50°C could be observed, which points to a slightly inhomogeneous interaction of these two sugars with the headgroups. In that

respect umbelliferose and sucrose were very similar. Figure 6B shows the interaction of raffinose with the dry egg-PC liposomes. The amount having strong interaction with the headgroups seems somewhat less than for umbelliferose. Through this interaction the lipid bilayer remains mainly in the liquid-crystalline phase during dehydration at ambient temperatures, which is one of the prerequisites for the protection of liposomes in the dry state (Crowe et al., 1994; Crowe et al., 1996b).

Liposome protection by umbelliferose and sucrose

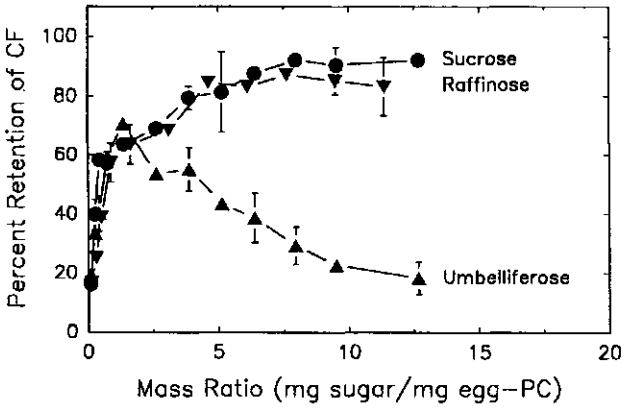

Figure 7: Retention of trapped CF in rehydrated egg-PC liposomes that were air-dried (3% RH) at room temperature for three h in the presence of varying amounts of sucrose, raffinose or umbelliferose.

Figure 7 shows the effect of air-drying on 30 μg egg-PC liposomes that were mixed with increasing amounts of either sucrose, umbelliferose or raffinose in a total volume of 30 μL. Sucrose and also raffinose provided excellent retention of entrapped CF at a sugar to lipid mass ratio of about 4 and higher. Umbelliferose provided similar protection as the two other sugars up to a mass ratio of approximately 2 (70% retention), but at higher mass ratio the protection declined gradually. It was observed that at these higher umbelliferose concentrations leakage occurred before the sample was dry. This

may be due to a persistent contaminant in our purified umbelliferose preparation, because earlier tests with less purified preparations gave no retention of CF, whatsoever. Generally, liposome tests for CF retention are sensitive to very small amounts of membrane soluble compounds and surfactants.

Protein protection by umbelliferose

Figure 8 shows the IR absorption spectra of polylysine (MW = 57.4 kD) dried on the laboratory bench (30% RH) in the presence or absence of umbelliferose. Polylysine is a synthetic polypeptide, that undergoes structural changes with drying (Prestrelski et al., 1993). In the hydrated state, at neutral pH, the protein attains a random coil conformation (Tiffany and Krimm, 1969). When polylysine was dried without umbelliferose, a large absorption band could be observed at 1625 cm^{-1} and at 1695 cm^{-1}, which is indicative of

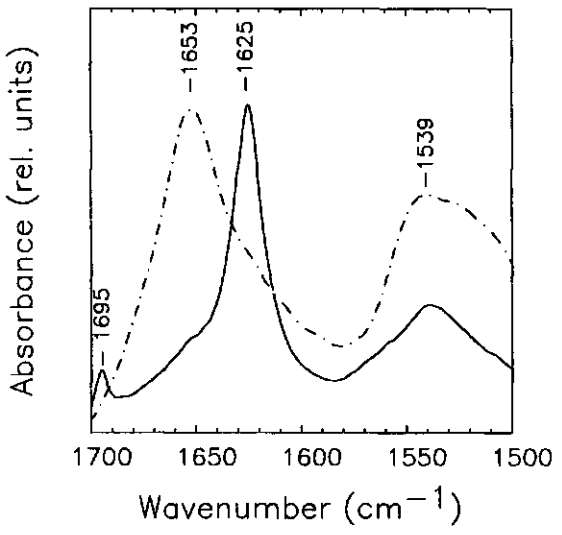

Fig. 8: FT-IR absorption spectra of polylysine (MW=57.4 kD), dried in the presence (dashed line) or absence (solid line) of umbelliferose.

extended ß-sheet structure. Furthermore, the sharp band-shape of the peak at 1625 cm^{-1} is typical of a highly ordered state of these protein aggregates. However, after drying in the

presence of umbelliferose a broad band at 1653 cm^{-1} dominated, representing random coil structure. From these spectra it can be concluded that umbelliferose can prevent a change of protein secondary structure with drying. In that respect, it is similarly effective as sucrose or trehalose (Prestrelski et al., 1993)

Discussion

Using NMR-spectroscopy we have conclusively demonstrated that the practically unknown, unique trisaccharide in carrot embryoids is umbelliferose. This trisaccharide was initially chemically recognized by Wickstrom and Svendsen (1956) in roots of *Angélica archangélica* L. subsp. *norvégica* (Rupr.) Nordh. Later on it was also found in carrot embryos and endosperm and in many other species of the Apiaceae (Hopf and Kandler, 1976). However, the chemical composition of umbelliferose had never been confirmed with NMR-spectroscopy. Dry, desiccation tolerant carrot embryoids not only contain umbelliferose, but also sucrose as soluble carbohydrate, monosaccharides being insignificant.

Our two independent data sets of carbohydrate contents in embryoids one with and one without PEG, undoubtedly show that a minimum threshold level of the combined contents of both sucrose and umbelliferose in each sample is required for survival of drying (Figs. 3D and 5D) and that both sugars are exchangeable to a certain extent (Fig. 4). There seems to be no preference for one of the two sugars in Fig. 3 and 4, but Fig. 5A may suggest a slight preference for umbelliferose in relation to desiccation tolerance. On account of the comparable molecular size of the two sugars, one would expect a similar type of interaction with the headgroups, which is indeed, suggested by the curves in Fig. 6A. Raffinose has a somewhat less effective interaction (Fig. 6B). From the above we cannot conclude that umbelliferose would be better at providing desiccation tolerance than sucrose. Moreover, oligosaccharides disappear during seed priming and are partly converted into sucrose, without loss of desiccation tolerance (Hoekstra et al., 1994).

The substantially lower threshold level in the PEG-treated embryoids might be explained as follows. Because of their longer maturation phase, the PEG-treated embryoids produced more dry matter than control embryoids without PEG (Tetteroo et al., 1996b, chapter 8), probably in the form of neutral lipids or proteins. Thus, the relative contribution of soluble carbohydrates to the dry matter will be less in the PEG-treated embryoids, while the amount of sugar available to the cytoplasm may be similar.

The disaccharides sucrose and trehalose can protect membranes and proteins during dehydration (Crowe et al., 1987; Carpenter et al., 1987). They prevent leakage from liposomes caused by fusion and phase transitions of the phospholipids during dehydration and subsequent rehydration (Crowe et al., 1994; Crowe et al., 1996b). Prevention of phase transitions by these disaccharides also has been demonstrated in intact organisms such as pollen and seed (Hoekstra et al., 1991; Hoekstra et al., 1996) and bacteria (Leslie et al., 1994). The function of umbelliferose in anhydrobiosis was still unknown up until now, but the present FT-IR data of Fig. 6A demonstrate the strong interaction of umbelliferose with the polar headgroups and the depression of T_m of the dry phospholipid.

Another prerequisite for an effective protection of liposome structure is prevention of fusion, which can be provided by any good glass forming compound (Crowe et al., 1994, 1996b). The retention of CF inside liposomes after drying in the presence of umbelliferose (Fig. 7) is evidence that the trisaccharide provides such a glass in the dry state at ambient temperatures. Likewise, raffinose (Fig. 7) and stachyose have this ability, but monosaccharides do not (Crowe et al., 1996b,c). We suggest that umbelliferose also exerts the above mentioned effects *in situ*. The conversion of sucrose into umbelliferose during slow drying (Tetteroo et al., 1994) suggests that a possible role for umbelliferose may be in extended longevity through a superior glass forming ability, rather than in desiccation tolerance *per se*. The low content of monosaccharides in dry desiccation tolerant carrot embryoids (Figs 3C and 5C) further corroborates the view that the presence of a stable glass is of vital importance. Monosaccharides, namely, have a plasticizing effect on glasses formed by dry di- and oligosaccharides (Levine and Slade, 1988).

It has been suggested that disaccharides also have a role in the protection of proteins during drying (Carpenter et al., 1987). The sugar molecules may replace the water molecules also around the dry protein, thereby preventing the loss of secondary structure (Prestrelski et al., 1993). The results presented in Fig. 8 clearly demonstrate that also umbelliferose is able to stabilize protein secondary structure in the dry state.

We conclude that umbelliferose and/or sucrose are necessary for the survival of carrot embryoids after (almost) complete dehydration. They definitely can function as protectants of membranes and proteins during anhydrobiosis. We suggest that they are able to form stable glasses at room temperature.

Acknowledgements

We would like to thank Mr B. van Veldhuizen of the Dept of Organic Chemistry, and G. de Ruiter and Dr. H. Schols of the Dept. of Food Science for their assistance with the different sugar analyses and Dr. E.A. Golovina for critically reading the manuscript.

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Chapter 6

Abscisic acid reduces respiration and sugar metabolism in developing carrot (*Daucus carota*) embryoids [1]

Frans Tetteroo, Adrie Peters, Folkert Hoekstra, Linus van der Plas, Marc Hagendoorn

Summary

Addition of abscisic acid (ABA) to developing carrot embryoids affects respiration and carbohydrate metabolism. Non-treated embryoids have a high level of respiration expressed per gram protein and consume almost all of their endogenous carbohydrates during the ten day culture period. In contrast, embryoids grown with either 1.9 or 38 μM ABA, have a lowered respiration rate and maintain their carbohydrate contents at 20% of the DW. Embryoids acquire complete desiccation tolerance, when they are treated with 38 μM ABA, whereas only 65% of the embryoids survive desiccation with 1.9 μM ABA. The reduced respiration of the developing embryoids might result in lower free radical levels after dehydration, in this way preventing a subsequent viability loss. We suggest that there is a relation between viability loss due to desiccation and respiration rate, although the latter is not the only factor involved.

[1] Also published in Journal of Plant Physiology **145**, 477-482 (1995)

Introduction

Carrot embryoids can acquire desiccation tolerance when during culturing a number of conditions are fulfilled. These conditions are the addition of abscisic acid (ABA) to torpedo-shaped embryoids at the proper time, slow dehydration and prehydration before imbibition (Tetteroo et al., 1995). In this manner, it is possible to dry embryoids to 5% moisture content without loss of viability.

Omitting one of these essential steps leads to inability to survive dehydration which is accompanied by extensive leakage of cytoplasmic solutes (chapter 3). Such leakage, caused by the loss of membrane function and integrity, is attributed to lateral phase separations of phospholipids and membrane proteins (Senaratna et al., 1984, 1985a,b; McKersie et al., 1988). These authors demonstrated that the phase separations are associated with gel phase domains, resulting from the accumulation of free fatty acids in the membranes. Using isolated membranes of desiccation tolerant soyabean embryos, Senaratna et al. (1984 and 1985a) could evoke both free fatty acids and gel phase domains through the addition of a xantine-xantine oxidase system, which produces free radicals. Based on these results Senaratna et al. (1985a) assume that the free fatty acids *in situ* similarly are the product of free radical-mediated deesterification of phospholipids. In desiccation sensitive carrot embryoids we have measured a tripled free fatty acid content and a 20% loss in phospholipid compared to tolerant embryoids (chapter 3), indicating the same mechanism.

Leprince et al. (1990, 1992, 1994) have demonstrated in germinating maize seeds that the loss of desiccation tolerance was associated with a rise in free radical content. They postulated that the free radicals originate from the respiration because the loss of desiccation tolerance during germination was tightly correlated with the increase of oxygen uptake and monosaccharide content. Mitochondria and chloroplasts contain the highest level of free radicals because of their oxygen-associated metabolic activity (reviewed by Elstner, 1982; Benson, 1990). In our ABA-treated carrot embryoids, chloroplasts had not yet developed, therefore the electron transport chain in mitochondria is a more likely source of free radicals. Also during seed development in pea, the

acquisition of desiccation tolerance coincides with a sharp decrease of respiration rate and in soluble carbohydrates (Rogerson and Matthews, 1977).

ABA is the essential endogenous hormone involved in desiccation tolerance during seed development (Koornneef et al., 1989). When applied to the culture medium, it also enhances the survival of somatic embryoids of carrot after dehydration to low moisture contents (Iida et al., 1992; Tetteroo et al., 1995). The mechanism of ABA regarding the acquisition of desiccation tolerance still has to be elucidated. ABA regulates specific gene expression (Skriver and Mundy, 1990), prevents precocious germination of embryoids (Tetteroo et al., 1995) and induces dormancy cq quiescence (Koornneef et al., 1989). Gude et al. (1988) have shown that ABA significantly decreases the respiration rate of potato tuber explants. ABA also decreases the contents of the respiratory substrates, glucose and fructose, during development of carrot embryoid genotype "RS 1" (Tetteroo et al., 1994).

Only a few studies deal with changes of respiration during seed development or germination and the correlation with desiccation tolerance (Rogerson and Matthews, 1977; Leprince et al., 1990; 1992), but the respiration of developing carrot embryoids and the effect of ABA on this process has yet to be determined. In this paper we have addressed two questions. 1) Does ABA affect the respiration rate and carbohydrate metabolism of developing carrot embryoids? 2) Is the effect on respiration correlated with desiccation tolerance? To answer these questions we have studied the effect of ABA in an optimal and suboptimal concentration, giving full survival of desiccation and partial recovery, respectively.

Material and methods

Plant material

Embryoids of the carrot genotypes "RS 1" and "Trophy" were regenerated, slowly dehydrated and germinated as described earlier (Tetteroo et al. 1995) with one minor modification: during the ABA treatment the sucrose concentration of the B_5 medium was

kept at 20 g/l instead of 60 g/l.

Respiration analysis

An oxygen electrode (Rank Bros., Bottisham, Cambridge, UK) was used to measure oxygen consumption. Approximately 50 mg fresh PEMs or embryoids were used for the measurement in a closed compartment, resuspended in 2.5 ml of spent B5 medium. The total oxygen uptake (v_{tot}) is the sum of the respiration due to the cytochrome pathway (v_{cyt}), the respiration due to the alternative pathway (v_{alt}) and the residual respiration (v_{res}). In order to calculate the mitochondrial oxygen consumption, the cytochrome pathway was specifically inhibited with 0.50 mM KCN, whereas the alternative pathway was blocked with 10 mM benzhydroxamate (BHAM). These inhibitor concentrations were experimentally determined with titration curves (data not shown) and were similar to the inhibitor concentrations used by Leprince et al. (1992). At this concentration, BHAM does not stimulate peroxidase activity (Møller et al., 1988).

Carbohydrate analysis

Approximately 50 mg fresh embryoids were lyophilized and subsequently mixed with 1 ml 80 % methanol containing 1 mg raffinose as the internal standard. The samples were placed in a water bath at 76 °C for 15 min to extract the soluble carbohydrates and to inactivate enzymes. Subsequently, the methanol was evaporated in a Speedvac (Savant Instruments Inc. Farmingdale, NY, USA). The samples were then suspended in 1 ml Milli-Q water. After centrifugation in an Eppendorf centrifuge the supernatants were diluted 50 times for HPLC analysis.

Carbohydrates were separated isocratically with a Dionex HPLC system (Dionex Corporation, Sunnyvale, CA, USA) equipped with a pulsed amperometric detector and a Carbopac PA-1 column with guard column. Identification of carbohydrate peaks and determination of response factors were done by comparing with standard solutions at two different elution programs. Umbelliferose was purified from dry embryoids (chapter 5) to obtain the response factor for its quantitative analysis. NaOH (100 mM) was used as the eluent, and 1.1 M sodium acetate in 100 mM NaOH was used to clean the column after

each run. The data were analyzed using a Spectra Physics integrator model SP 4400 and Spectra Physics software (Labnet, Chromdat; San Jose, CA, USA).

Carbohydrates from the culture media were diluted in Milli-Q water after lyophilization and directly analyzed without any extraction procedure.

Protein analysis

Fresh embryoids were homogenized in liquid nitrogen and dissolved in a 1 M NaOH solution (Bradford 1976). The sample was centrifuged and the supernatant was used for a protein analysis according to Bradford (1976). Supernatant (10 μl) plus 20 μl Milli-Q water were mixed in a cuvette with 1.5 ml dye reagent (100 mg Serva Blue G, 100 ml 85% phosphoric acid, 50 ml 96% ethanol and 850 ml Milli-Q water). After 5 min incubation at room temperature the absorption at 590 nm was measured with a Perkin Elmer 550 SE UV/VIS spectrophotometer. Quantification of protein contents was done by using bovine serum albumin as a standard.

Results

Carbohydrate metabolism

The effect of ABA concentration on carbohydrate metabolism was determined by measurement of the changes in soluble sugars in the culture medium and the embryoids during the first 10 days of ABA addition (Figs. 1 and 2). The composition of the sugars in the medium changed drastically during embryoid development; sucrose was converted (by extracellular invertase) into glucose and fructose within 5 days, both in the presence and absence of ABA (Fig. 1). Without ABA the glucose and fructose are so rapidly consumed by the precociously germinating embryoids, that no sugars are left in the medium after 10 days. The monosaccharide consumption of embryoids treated with 1.9 μM ABA was slower, whereas with 38 μM ABA the uptake of glucose and fructose ceased completely after day 5. The latter embryoids showed no further morphological changes and remained torpedo-shaped. In contrast, the embryoids on low (1.9 μM) ABA

medium developed green cotyledons after 5 days and showed some root elongation.

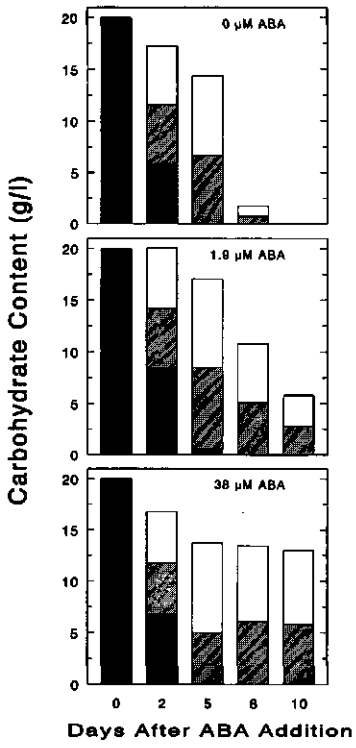

Figure 1: Changes in carbohydrate composition of B_5 medium of developing carrot embryoids (genotype "Trophy") as effected by the addition of ABA. Fresh medium and ABA were supplemented to the embryoids 7 days after the start of embryoid induction (black bar: sucrose, hatched bar: glucose, white bar: fructose). Data from a representative experiment are depicted.

The effect of ABA on the endogenous soluble carbohydrate content in fresh "Trophy" embryoids is shown in Fig. 2. In the absence of ABA the monosaccharides and sucrose decreased slowly from the second day on to very low levels at day ten. Only a small amount (3% of DW) of umbelliferose was formed. In the presence of ABA, the monosaccharides decreased to almost undetectable levels, whereas the umbelliferose content increased to 5% of the DW with 1.9 μM ABA and to approximately 10% of the DW with 38 μM ABA. Sucrose content increased and remained high with 1.9 μM ABA while it decreased to 8% with 38 μM ABA. Similar carbohydrate changes were reported

for embryoids of genotype "RS 1" (Tetteroo et al., 1994).

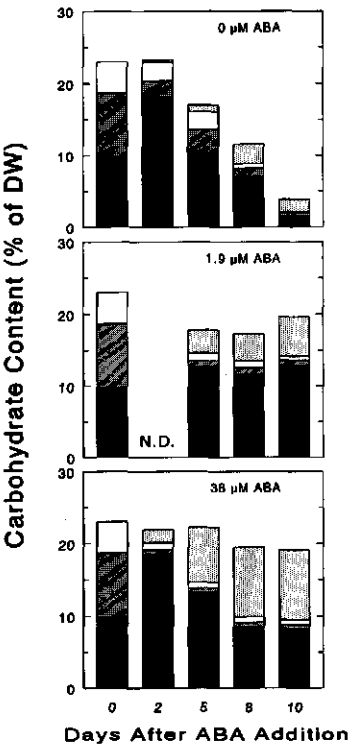

Figure 2: Effect of ABA on the changes of endogenous carbohydrates of carrot embryoids (genotype "Trophy"). Fresh medium and ABA were supplemented to the embryoids 7 days after the start of embryoid induction (dotted bar: umbelliferose, black bar: sucrose, hatched bar: glucose, white bar: fructose). N.D., not determined. Data from a representative experiment are depicted.

During the development of the embryoids we also measured changes in percent DW and protein content as affected by ABA (Fig. 3). In the presence of either concentration of ABA the DW percentage increased over the first two days and remained at approximately 20% with 38 μM ABA, whereas it decreased again to 15% with 1.9 μM ABA. In the absence of ABA the DW percentage decreased continuously to approximately 7%. The protein content, expressed per gram dry weight, decreased in all cases. In the presence of ABA, however, the decrease was less, resulting in a protein content of ca. 25% after 8 days. In embryoids that were not treated with ABA the protein content

decreased to less than 10% of the dry weight.

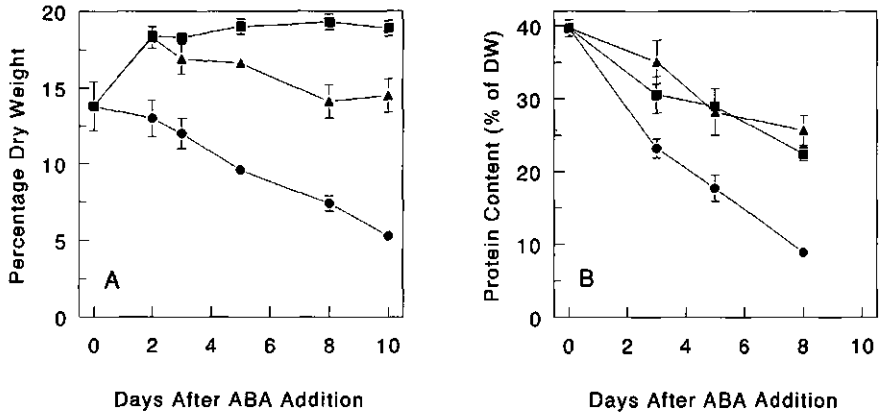

Figure 3: Changes in percent DW and protein content during the development of carrot embryoids at different ABA concentration (-●- 0 μM ABA, -▲- 1.9 μM ABA, -■- 38 μM ABA). Data are means of four replicates and the standard deviations are indicated with error bars, when they exceed symbol size.

Respiration

In carrot embryoids total mitochondrial respiration consisted largely of v_{cyt}. The oxygen uptake due to the alternative oxidase was very low during the entire embryoid development. The total respiration peaked directly after the addition of ABA with fresh medium, containing 20 g sucrose/l. After ca. 4 days a constant level was reached which continued for at least 6 days, independent of the ABA concentration (data not shown). Embryoids grown without ABA generally respired at a slightly higher level.

In order to further elucidate the differences in respiration, we measured the oxygen uptake after 8 days of ABA treatment of embryoids (Fig. 4A). The respiration of embryoids treated with 38 μM ABA was significantly less than that of embryoids without ABA (Student's t-test, $p<0.01$). The embryoids treated with 1.9 μM ABA showed an intermediate oxygen uptake, which was not significantly different from the two other treatments.

However, comparing respiration data per gram DW, can be argued. Embryoids cultured in the absence of ABA develop into seedling-like structures with many different cell types not present in the embryoids. Therefore, it may be better to express the

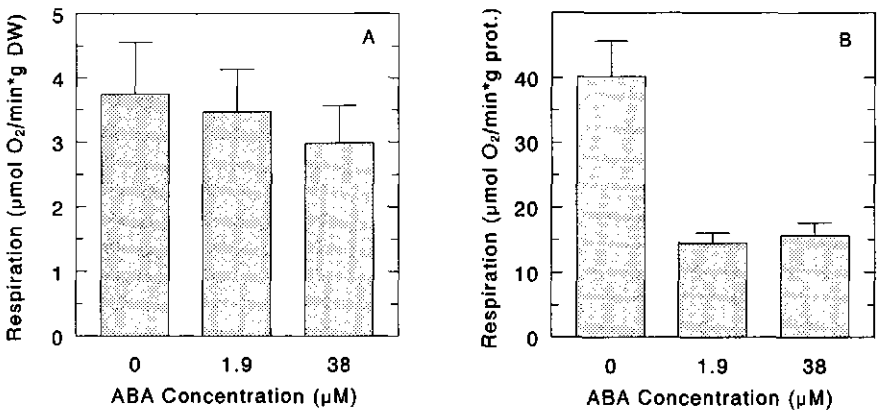

Figure 4: Effect of ABA concentration on the total respiration (corrected for v_{res}) of carrot embryoids. Respiration was measured after 8 days of ABA treatment. Error bars indicate standard deviation. Panel A shows the respiration per gram dry weight. Data are means of six separate experiments with at least 4 replicates each. Significant differences (Student's T-test $P<0.01$) were only found between 0 and 38 μM ABA treatment. Panel B shows the respiration per gram protein. The data are means of 8 replicates. Significant differences (Student's T-test $P<0.01$) were found between the treatment with and without ABA. Data of 1.9 and 38 μM ABA were not significantly different.

respiration per gram protein, the protein level being a better measure for the amount of cytoplasm, protein also being one of the most expensive compounds of the cells. Respiration in the presence of ABA (both 1.9 and 38 μM), expressed in this way, was only about one third of the control level (see Fig. 4B), and differed significantly from the respiration of the non-treated embryoids (Students t-test, $P<0.01$).

Desiccation tolerance

Embryoids (genotype "Trophy") not treated with ABA lost their viability completely during dehydration (Fig. 5), because they developed elongated roots and green cotyledons during the culture period. This precocious germination in culture could be prevented by the addition of low (1.9 μM) ABA as demonstrated earlier by Tetteroo et al. (1995) with genotype "RS 1". With "Trophy" the same results were obtained with regard to desiccation tolerance. From the embryoids treated with 1.9 μM ABA only 65% survived the dehydration (Fig. 5). Moreover, approximately 50% of these surviving embryoids showed only root growth, the shoot meristems being particularly sensitive to

dehydration (Fig.5). In the presence of 38 μM ABA almost all embryoids survived the desiccation. In this case 75% of the surviving embryoids showed complete germination.

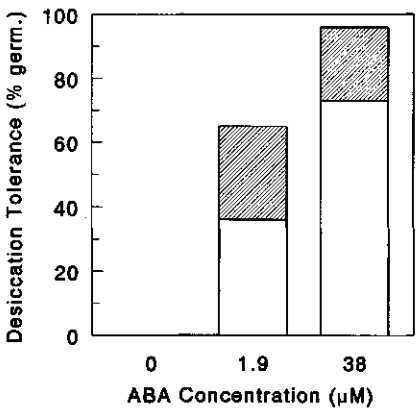

Figure 5: Effect of ABA on the desiccation tolerance of carrot embryoids, genotype "Trophy". After 8 days of ABA treatment the embryoids were slowly dehydrated to 5% moisture content and stored for 14 days at 30% RH. Before imbibition in B_5 medium, the dry embryoids (approximately 100 per treatment) were prehydrated in moist air for 4 hours. Embryoids were scored as desiccation tolerant when they showed at least root growth (hatched bar), or root and shoot growth (white bar).

Discussion

The rate of uptake of sugars from the medium by the developing embryoids is restrained by ABA which was particularly manifest after the fifth day of culture (Fig. 1). In the absence of ABA, this rapid sugar uptake and fast germination are indications of a high metabolic activity of these embryoids. Morphological differences e.g. green cotyledons, between embryoids developed on low and high ABA concentrations were only noticeable after 5 days, whereas physiological changes in protein level and DW percentage between the three treatments, occurred within two days (Fig. 3). Embryoids treated with high ABA (38 μM) became quiescent and remained white and torpedo-shaped. With low ABA the sugar consumption of the embryoids was relatively slow, whereas embryoids developed on high ABA medium completely ceased sugar uptake, in agreement with their quiescent stage. The endogenous soluble sugar content data after the

high ABA treatment also point to this quiescence (Fig. 2). In the absence of ABA total carbohydrate content rapidly decreased which is indicative for unrestricted metabolism.

Not only the total sugar contents of fresh embryoids are changed by ABA (Fig. 2) but also the composition, which was already shown in a previous report for another carrot genotype (Tetteroo et al. 1994). ABA stimulates the accumulation of the trisaccharide umbelliferose. This is in agreement with ABA-treated *Craterostigma plantagineum* callus, which accumulates sucrose and becomes desiccation tolerant (Bianchi et al., 1992). Di- and trisaccharides have been demonstrated to function as protectant of membranes and proteins in the dry state (Crowe et al., 1987) and to enhance the formation of a glassy state (Bruni and Leopold, 1991). The umbelliferose content (and the monosaccharide content to a lesser extent) of the embryoids treated with low ABA was intermediate between those of the 0 and 38 μM ABA treatments (Fig. 2). This intermediate umbelliferose content corresponds with the partially acquired desiccation tolerance (Fig. 5).

Several mechanisms can be envisaged for the inhibitory effect of ABA on respiration. In the first place, ABA might affect substrate competition between the glycolysis and the formation of sucrose and/or the trisaccharide umbelliferose. It may stimulate this formation and/or inhibit the glycolysis. Both processes need glucose and fructose as substrate/precursors. We have already demonstrated that the monosaccharide levels become very low with ABA, whereas the umbelliferose content is increased by ABA (Fig. 2). Another possibility is that ABA induces changes in the tissue which leads to decreased permeability of oxygen as shown for potato explants in which ABA stimulates suberization (Soliday et al., 1978; Cottle and Kolattukudy, 1982). The lowered oxygen diffusion then may lead to a decreased v_{tot}. However, the absence of clearly suberized or waxy layers, as well as the high O_2 affinity of cytochrome oxidase, the terminal oxidase that is chiefly involved, does not make this explanation very plausible. A third possibility is that ABA primarily affects the development of the embryoids; the low need for ATP in the embryoids with a restrained development might be responsible for the decreased respiratory rate.

Comparison between germination (Fig. 5) and respiration data (Figs. 4A and 4B)

of the different ABA treatments suggests that there might be a relation between desiccation tolerance and respiration. Although the difference in respiration per gram DW is relatively small (Fig. 4A), respiration on a protein basis is only one third of that of control embryoids, for both high and low ABA treatments. This is in agreement with similar correlations observed for germinating maize kernels by Leprince et al. (1990; 1992) and for developing pea seeds by Rogerson and Matthews (1977). In both cases, desiccation tolerance coincided with relatively low respiration rates before desiccation. The low respiration in 24 h imbibed maize seeds was accompanied by a low free radical level in the desiccated state; at this time the seeds were still desiccation tolerant. But after 72 h of imbibition respiration had increased, resulting in the loss of desiccation tolerance and an increased free radical level in the dried seeds. Carrot embryoids treated with high or low ABA have the same decreased respiration rate per gram protein (Fig. 4B) possibly leading to comparable levels of respiration-derived free radicals during dehydration. However, the low respiration of the 1.9 μM ABA treatment results in only partial germination (Fig. 5). This indicates that the initial respiration rate is not the only determining factor in desiccation tolerance of the carrot embryoid system.

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Chapter 7

Effect of ABA and slow drying on DNA replication in carrot (*Daucus carota*) embryoids [1]

Frans Tetteroo, Raoul Bino, Jan Bergervoet, Birgit Hasenack

Summary

Addition of abscisic acid (ABA) at the torpedo-shaped stage of development and slow dehydration are two parameters necessary to produce completely desiccation tolerant carrot (*Daucus carota* L.) embryoids. The mode of action of these parameters is still largely unidentified. Employing flow cytometry we investigated their effect on DNA replication and cell cycle activity of the developing embryoids. DNA replication was determined as percentage of 4C nuclei. Addition of ABA did not alter DNA replication and cell cycle during embryoid development in vitro, in spite of the putative quiescent state of the torpedo-shaped embryoids. In contrast, during slow drying the nuclei were preferentially arrested in the presynthetic G_0/G_1-phase and the amount of G_2 nuclei decreased. Dry zygotic carrot embryos do not contain any G_2 nuclei and are completely desiccation tolerant. The decline of G_2 nuclei in dry somatic embryoids seems to coincide with the increase in desiccation tolerance, which is incomplete compared to zygotic embryos. Our results suggest that in order to withstand anhydrobiosis, DNA replication may be controlled during the embryoid developmental program and slow dehydration, but not by the plant growth regulator ABA.

[1] Also published in Physiologia Plantarum **94**, 154-158 (1995)

Introduction

Previously, we developed a somatic embryogenesis system in carrot (*Daucus carota* L.), that provides dry (5% moisture content) embryoids which are completely viable (Tetteroo et al., 1995a). Requirements for the acquisition of full desiccation tolerance are a suitable developmental stage, the proper time of addition of ABA, slow drying, and prehydration prior to imbibition (Tetteroo et al., 1995a). If one of these parameters is not properly applied, the dry embryoids fail to germinate upon imbibition. The large number of embryoids that can be propagated and the simple procedure for manipulating viability render this system ideal for the study of the survival mechanisms of tissues after severe dehydration.

Recent studies on desiccation tolerance focused mainly on membrane behaviour and protection by carbohydrates of proteins and phospholipids in the dry state (reviewed by Crowe et al., 1996). However, not only membranes and proteins have to withstand dehydration to low moisture contents, but also all cell organelles including the DNA-containing nucleus.

Flow cytometry is a well established and valuable technique for measuring DNA content and studying stages of nuclear replication (Galbraith et al., 1983, Bino et al., 1993, Faure et al., 1993), particularly because of its simplicity and rapid sample preparation. Furthermore, flow cytometry is a reliable technique, since data are based on large numbers of nuclei that can be measured in a short time (de Laat et al., 1987, Bino et al., 1993).

Many studies have been performed on DNA content and cell cycle stages in seeds during dry storage using microdensitometry or flow cytometry (Brunori and D'Amato, 1967, Bino et al., 1993). In many species the nuclei of the quiescent zygotic embryos are completely arrested in the presynthetic G_0/G_1-phase (for a review, see Bewley and Black, 1994). This indicates a stringent control of the cell cycle during maturation and dehydration of seeds of these species. Deltour (1985) suggested that the general arrest in G_0/G_1 might render seeds more tolerant to stress conditions. However, in seeds of other species, such as *Vicia faba* (Brunori, 1967) and tomato (*Lycopersicon esculentum*) (Liu et

al., 1994), also nuclei in the postsynthetic G_2-phase were detected, mainly originating from root tip cells. Likewise, primed seeds contained considerable numbers of nuclei in G_2-phase (Bino et al., 1992, Lanteri et al., 1993), and although they are highly viable, they are more sensitive to dry storage (Lanteri et al., 1994).

It has been proposed that control of the nuclear division during maturation might be regulated through seed water activity (Brunori, 1967) or ABA (Liu et al., 1994). Brunori (1967) demonstrated in maturing *Vicia faba* seeds that DNA synthesis ceased below 75% water content on a fresh weight basis, whereas mitotic activity was inhibited below 65% moisture content. This notwithstanding, a certain percentage of G_2 nuclei remained. However, Bino et al. (1993) demonstrated that the recalcitrant seed of *Castanea sativa*, which lacks maturation drying, contained cells mostly in G_0/G_1-phase. This indicates that the arrest of the cell cycle activity is not directly linked to the seed water status. ABA plays an important role in the development, maturation and acquisition of desiccation tolerance of seeds (Koornneef et al., 1989, Bewley and Black, 1994). It is also an inhibitor of cell division (Evans, 1984, Bouvier-Durant et al., 1989). Levi et al. (1993) and Elder and Osborne (1993) have shown that added ABA also inhibits the reactivation of the cell cycle of imbibed seeds. However, seeds of tomato (Money Maker and *sit*^{w}) imbibed in ABA clearly activated nuclear replication but did not germinate (Liu et al., 1994).

Until now no study has been carried out on DNA behaviour and nuclear replication stages during carrot embryoid development after the addition of ABA and after dehydration. In this paper we present data for the effects of ABA and slow drying on the cell cycle status of developing carrot embryoids and the relation to desiccation tolerance.

Materials and methods

Plant material

Embryoids of two carrot (*Daucus carota* L.) genotypes (Trophy and RS 1) were regenerated and dried according to methods described by Tetteroo et al. (1995a). ABA

was added to the embryoids when they reached the torpedo stage of development (8 days after induction). After 10 days of ABA treatment the embryoids were either rapidly dehydrated within 4 h or slowly within 9 days. Before germination on filter paper soaked with B_5 medium, the dry embryoids were prehydrated in moist air for 4 h to prevent imbibitional damage. Young leaves of greenhouse grown plants of genotype RS 1 were used to isolate nuclei for the control.

Preparation of nuclear samples

Approximately 30 somatic embryoids (fresh or dry) and 10 zygotic embryos, that were dissected from seeds imbibed for 2 h and subsequently dried, were chopped with a razor blade in nucleus isolation buffer. This buffer contained 0.2 M mannitol, 10 mM 2(N-morpholino) ethanesulfonic acid, 10 mM NaCl, 10 mM KCl, 10 mM spermine tetrahydrochloride, 2.5 mM ethylenediamine-tetraacetic acid, 2.5 mM dithiothreitol, 0.05% (w/v) Triton X-100 and 0.05% (w/v) Na-azide at pH 5.8 (Bino et al., 1993). To detect DNA, 1 mg l^{-1} of DAPI (4',6-diamidino-2-phenylindole) was added to the isolation buffer. After chopping, the suspension was passed through a 25 μM nylon mesh and immediately analyzed.

Flow cytometry

A PAS II flow cytometer (Partec GmbH, Münster, Germany) was equipped with an HBO-100 mercury arc lamp. The optical configuration consisted of a KG1 and a BG38 filter for heat protection; a UG1 filter and a TK-420 dichroic mirror were used for UV excitation and a GG-435 long pass filter was used to measure fluorescent emission. All analyses were performed using peak height detection and logarithmic amplification according to Bino et al. (1993). The fluorescent signals are presented as frequency distribution histograms over 500 channels, starting from channel 20. The amount of DNA is proportional to the fluorescent signal and is expressed as arbitrary C values in which the 1C value comprises the DNA content of the unreplicated haploid chromosome component. DNA replication is determined as percentage of 4C nuclei resulting from the flow cytometric analysis.

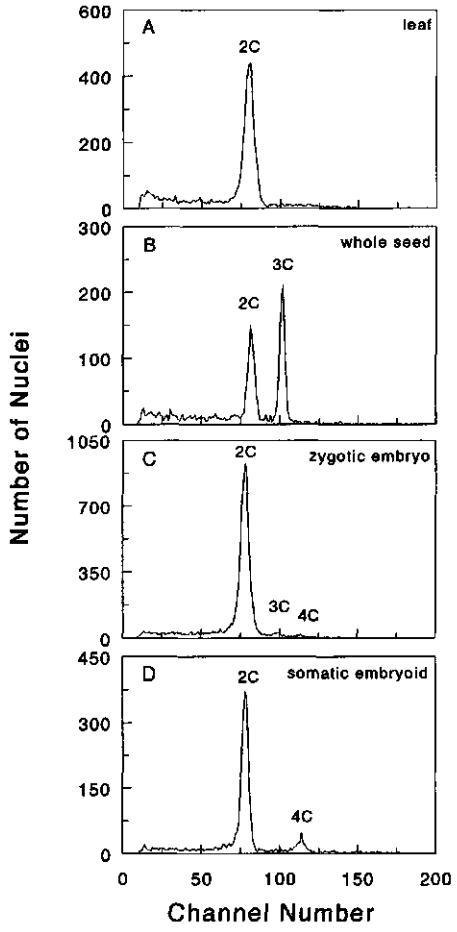

Figure 1: Typical DNA-histograms of DAPI (4',6-diamidino-2-phenylindole) stained nuclei of carrot RS 1 leaves (A), mature dry seeds (B), dissected dry mature zygotic embryos (C) and fresh torpedo-shaped somatic embryoids (D).

Results

To identify the DNA replication stages of carrot embryoid cells, we used nuclei isolated from carrot leaves as a control (Fig. 1A). Only one peak at channel 78 was

detected, which comprises DNA in the 2C configuration. Mature dry seeds of carrot RS 1 revealed a second peak at channel 101, corresponding to the 3C configuration of the endosperm DNA (Fig. 1B). The 3C peak was negligible for the dissected dry embryo (Fig. 1C). Fresh torpedo-shaped embryoids of RS 1 (Fig. 1D) showed a distinct signal at channel 114 of 4C nuclei, which was also slightly visible in the dry zygotic embryos (Fig. 1C). The 4C peak results from cells in the G_2 state of the cell cycle, most likely located around the root and shoot apex, i.e. the meristematic parts of the embryoid. Nuclei in the 4C stage were also detected in dried embryoids regardless of their ABA treatment and viability, but in smaller quantities than in the fresh embryoids (histogram not shown). Similar results were obtained with genotype Trophy.

Table 1: Effect of ABA and dehydration on cell cycle activity, expressed as percentage nuclei in the G_2-phase, of carrot embryoids after treatment for 10 days. The data are means $\pm$ SE of at least 6 replicates. Different letters indicate a significant difference between the treatments according to Student's t-test at P=0.01.

	Percentage G_2 nuclei		
ABA (μM)	fresh	rapidly dried	slowly dried
0.0	13.6 ± 0.9^{a}	13.2 ± 0.8^{a}	6.0 ± 0.7^{bc}
3.8	12.5 ± 0.9^{a}	12.2 ± 1.6^{a}	5.3 ± 0.3^{c}
38.0	12.3 ± 0.9^{a}	10.9 ± 0.6^{a}	7.4 ± 0.4^{b}

Table 1 shows the effect of different ABA concentrations on DNA content expressed as percentage nuclei in G_2-phase, of torpedo-stage carrot embryoids. These data indicate a nonsignificant decrease in 4C nuclei in fresh embryoids with increasing ABA concentration. Slow drying decreased significantly the percentage of nuclei in the G_2 phase, whereas fast drying barely changed the DNA content compared to that of the fresh embryoids (Tab. 1). The combined effect of ABA and slow drying was not significant. Rapidly dried embryoids treated with 38 μM ABA contained less G_2 nuclei compared to the untreated embryoids, whereas slowly dried embryoids treated with ABA contained more 4C nuclei than untreated controls.

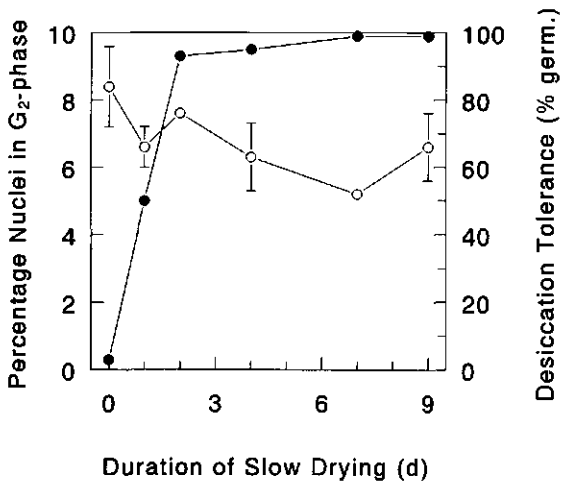

Figure 2: Effect of slow drying on DNA replication status (-○-) and desiccation tolerance (-●-) of developing carrot embryoids treated with 38 μM ABA. Immediately after the slow drying treatment the embryoids were rapidly dried to 5% moisture content and subsequently DNA analysis was performed. Data are means of 3 replicates. Error bars indicate SE.

In a sequel experiment we tried to specify the effect of slow drying and to correlate the DNA content data with the development of desiccation tolerance (Fig. 2). The torpedo-shaped embryoids treated with 38 μM ABA were slowly dried for the indicated periods, whereafter they were rapidly dried to 5% moisture content. Subsequently, DNA replication stages of these dry embryoids were determined. The drying effect on number of nuclei in G_2-phase was not as distinct as in Tab. 1, but still one can notice that during the dehydration period the amount of nuclei in the G_2-stage decreased, although it was not significant (Fig. 2). The sharp increase of desiccation tolerance during the first two days of slow drying did not coincide with a sharp decrease of G_2 nuclei. These data suggest that there was no strict correlation between DNA replication and desiccation tolerance.

However, by combining all DNA data and germination data of several experiments in one plot (Fig. 3), it is evident that desiccation tolerance coincides with a decreasing amount of cells in G_2-phase. Data for embryoids without ABA treatment are not included, because these embryoids were already germinating before dehydration and thus had lost

their ability to develop desiccation tolerance. Dry zygotic embryos contained only 2C nuclei (Fig. 3). During their maturation and drying all the embryo cells were arrested in the G_0/G_1-stage and were fully desiccation tolerant.

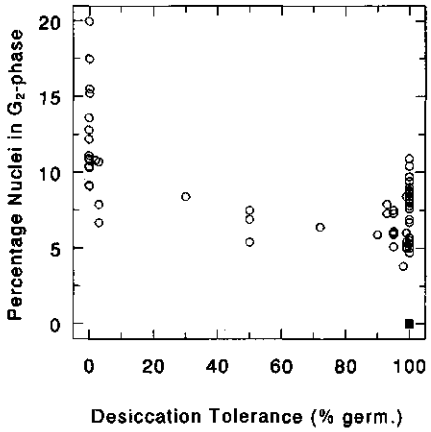

Figure 3: Correlation between DNA replication status, represented as percentage nuclei in G_2-phase, and desiccation tolerance of ABA-treated carrot embryoids (○). Data for zygotic carrot embryos indicated by ■.

Discussion

Using flow cytometry, we were able to measure DNA content and study the cell cycle of developing carrot embryoids. Our results from leaves, seeds and zygotic embryos correspond to and can be explained by earlier findings (de Laat et al., 1987, Bino et al., 1993). Carrot embryoids contained mainly 2C nuclei and 4C nuclei. This nuclear distribution has also been demonstrated for fresh *Vitis vinifera* embryoids (Faure and Nougarède, 1993).

Addition of ABA induced a putative quiescent state, since no morphological changes were noticeable and the embryoids remained in torpedo stage of development in the first week (Tetteroo et al., 1995b). Despite this apparent quiescence, the present data indicate the presence of 4C nuclei in the G_2-phase (Tab. 1). This could be ascribed to the maintenance of DNA replication or to a total inhibition of the cell cycle caused by ABA. Nevertheless, continuation of growth and cell division can be observed morphologically in

the second week of the ABA treatment (Tetteroo et al., 1995a). The embryoids then produce secondary embryoids on their radicles. The basal part of the radicle is covered with globular structures within 7 days. Thus, it can be concluded that ABA does not inhibit DNA replication and subsequent cell division. This conclusion was also drawn by Liu et al. (1994), using imbibed tomato seeds to measure the reactivation of DNA replication. However, these findings contrast with the results of Elder and Osborne (1993) and Levi et al. (1993), who used similar systems. This discrepancy might be explained by differences in the ABA concentrations used (Liu et al., 1994). Apparently, only high levels of ABA (100 μM) can completely inhibit DNA synthesis. In the case of carrot embryoids, ABA did not function as a regulator of DNA replication, but ABA is nevertheless essential for the induction of desiccation tolerance. Osborne and Boubriak (1994) suggested that LEA (late embryogenesis abundant) proteins and/or dehydrins, the synthesis of which is regulated by ABA, might function as structural protectors of DNA in the dry state, like the small acid-soluble proteins do in bacteria. Our data do not support this hypothesis.

The dehydration data conclusively demonstrate the inhibitory effect of drying on DNA replication. This was also demonstrated by Brunori (1967) during maturation drying of *Vicia faba* seeds. During seed dehydration DNA synthesis ceased at and below 75% moisture content, while G_2-phase nuclei could continue their cell cycle and were later arrested in the G_0/G_1-phase. The water activity seemed to be more important in the regulation of the cell cycle than ABA. In general, zygotic embryos of orthodox and recalcitrant seeds do primarily contain cells in the G_0/G_1-phase (Elder and Osborne, 1993, Bino et al., 1993). However, slow drying of carrot embryoids was not sufficient to decrease the amount of nuclei in G_2-stage to the same low level as for the dry zygotic carrot embryos (Fig. 3). This means that carrot somatic embryoids are not comparable to zygotic carrot embryos, because in the dry state the carrot embryoids still contained cells with 4C nuclei. This may indicate that the embryoids need a higher ABA concentration to sufficiently decrease the cell cycle activity. We added only 38 μM ABA, whereas Levi et al. (1993) and Elder and Osborne (1993) used 100 μM to inhibit reactivation of the DNA replication.

The combination of the DNA data with the germination data suggest that there is a correlation between desiccation tolerance and the phase of the DNA replication (Fig. 3). It seems that cells with DNA in S- and G_2-stage are more sensitive to low moisture contents. In general, compared to cells in G_0/G_1, cells in G_2 are more sensitive to factors that affect nuclear division, such as radiation and free radicals (Sybenga, 1972), because there is twice as much DNA in G_2 cells as there is in G_0/G_1 cells. However, slowly dried embryoids without ABA treatment also had a low number of nuclei in G_2-phase, but lost the ability to survive dehydration (Tetteroo et al., 1995a). This indicates that not only the DNA replication stage determines whether embryoids survive dehydration, but that also other parameters are involved. The fact that our desiccation tolerant embryoids still contain about 7% of 4C nuclei might explain why the quality of regrowth is poorer than for zygotic embryos (Tetteroo et al., 1995a).

We conclude that the putative quiescence of fresh torpedo-staged embryoids, brought about by 38 μM ABA, is not due to an inhibition of the cell cycle, although ABA is essential for their capacity to acquire desiccation tolerance. In contrast to fast drying which severely injures the embryoids, slow drying down-regulates the cell cycle. We suggest that this down-regulation contributes to the desiccation tolerance of the slowly dried embryoids.

Acknowledgments

We thank Eloy Boon of Iribov, Bovenkarspel, Holland for performing some of the measurements and Folkert A. Hoekstra for critically reading the manuscript.

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Chapter 8

Scanning electron microscopy studies on desiccation-tolerant and -intolerant carrot (*Daucus carota* L.) embryoids

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Summary

It has been shown previously that carrot (*Daucus carota*) somatic embryos treated with abscisic acid during their development, acquire complete desiccation tolerance when they are slowly dried, but fail to do so when they are rapidly dried. Cryofixation and analysis by low temperature scanning electron microscopy (LTSEM) are excellently suitable to compare the morphology of specimens with different moisture contents. Using LTSEM we have examined dry and hydrated carrot zygotic embryos and compared them with fresh, rapidly dried, and slowly dried carrot somatic embryos, including after rehydration. Somatic embryos always had reduced and abnormal cotyledons, mostly fused, and the surface was irregular. The surface of the dry somatic embryos was also more wrinkled than that of zygotic embryos. No morphological differences were detected between tolerant (slowly dried) and intolerant (rapidly dried) somatic embryos prior to and shortly after rehydration. However, clear morphological differences were detected after imbibition for three days. It is concluded that LTSEM is a powerful technique to study plant materials in their native state.

Introduction

Carrot somatic embryos (embryoids) are perfectly suited to study the mechanisms of desiccation tolerance (Tetteroo et al., 1995). It is easy to obtain large amounts of them and to manipulate the regrowth capacity of the dry embryoids by varying the ABA concentration, dehydration rate or prehydration prior to imbibition. However, the quality of the embryoids is less than that of zygotic embryos (Tetteroo et al., 1995). We have hypothesized that this difference in vigour is due to the difference in length of the maturation phase between embryoids (14 d) and zygotic embryos (50-60 d; Gray and Steckel 1982; Tetteroo et al., 1995). The maturation of white spruce somatic embryos could be prolonged by adding PEG to the culture medium (Attree et al., 1991, 1992). Using PEG in a bioreactor, Attree et al. (1994) were even able to produce desiccation tolerant white spruce somatic embryos with better vigour than excised zygotic embryos.

Our research activities are focussed on the elucidation of desiccation tolerance mechanisms. We have demonstrated that intolerant carrot embryoids, independent of treatment, have a higher leakage of potassium and soluble carbohydrates than desiccation tolerant embryoids (chapter 3). This indicates that reduced membrane integrity may be a major reason for the lack of regrowth of the intolerant embryoids. However, it is still not clear whether this membrane injury occurs during the dehydration or the rehydration and whether it is a primary type of injury or not. For a better understanding of the mechanisms involved in desiccation tolerance it is necessary to carry out a histological study of the different embryoid types. Recently, Fowke et al. (1994) have compared four different SEM techniques on desiccation tolerant white spruce somatic embryos. They concluded that plastic replicas and LTSEM are the best methods.

Since the early 1980's SEM has become a powerful tool for studying hydrated plant materials, because of the development of LTSEM and cryopreparation systems (Jeffree and Read 1991). The use of a field emission SEM in combination with a cold stage allows the use of low voltage of the electron beam, which produces images with maximum surface information, and minimum charging and beam damage of the specimen. The chemical fixation formerly necessary and critical point drying were a major source of

surface artifacts. For the LTSEM the specimens only have to be cryofixed before examination in the microscope. To enhance resolution and avoid charging, a thin layer of platinum is usually sputtered on the specimen. The artefacts that can occur during cryofixation are limited compared to conventional fixation systems (Jeffree and Read 1991). Therefore, LTSEM is the only technique that ideally allows for surface comparison of plant tissues with different moisture contents, such as white spruce somatic embryos (Fowke et al., 1994) or carrot somatic embryos during development of desiccation tolerance.

The objectives of the present LTSEM study were first to make a morphological comparison between carrot somatic and zygotic embryos in the hydrated as well as in the dry state, with particular emphasis on the low vigour of the somatic embryos; secondly, to study possible differences between desiccation tolerant and intolerant embryoids and to determine the type of injury; thirdly, to examine the effect of PEG given during culture on DW accumulation and vigour.

Material and methods

Plant material and treatments

Carrot embryoids were produced according to methods earlier described (Tetteroo et al., 1995). Briefly, hypocotyl explants of 10-day-old seedlings were used to produce friable callus on solid B_5 medium (Gamborg et al., 1968) supplemented with 2.3 μM 2,4-D and 20 $g.L^{-1}$ sucrose. Cell suspension cultures were started and maintained by subculturing, every fourteen days. Seven days after refreshing, the pro-embryogenic masses were transferred to 2,4-D-free B_5 medium with 20 $g.L^{-1}$ sucrose to regenerate embryoids. After 7 days, the medium was refreshed to prevent exhaustion of the nutrients and to eliminate single cells that did not develop into embryoids. Then, 38 μM ABA also was added to the medium. Refreshing of the ABA-containing medium was repeated after another 7 days. The embryoids (torpedo stage) were harvested on a 500 μm nylon sieve after a culture period of 18 to 20 days. Embryoids were also cultured for three weeks in B_5 medium

containing 38 μM ABA and 150 g.L^{-1} PEG 6000 to prolong the maturation period without the formation of secondary embryoids.

Approximately 1 g of embryoids, rinsed with B_5 medium, was transferred to a sterile plastic Petri dish (9 cm) by forceps and equally spread out over the surface of the Petri dish. The Petri dishes were closed and placed in hygrostats. Slow drying was carried out by exposure to different RHs inside the hygrostat at 25°C, generated by different saturated salt solutions [NaCl (73 % RH), $Ca(NO_3)_2$ (50 % RH) and $CaCl_2$ (30 % RH)]. Embryoids remained in each hygrostat for 3 days, in descending order of RH. Rapid drying within approximately four h was obtained by placing the Petri dishes without cover in an air flow cabinet. Dry weights of the embryoids were determined after freeze-drying for 24 h.

Approximately 100 dry embryoids were placed on filter paper in a sterile plastic Petri dish (6 cm). Prior to imbibition, the embryoids inside the closed Petri dish were prehumidified in moisture-saturated air for four hours Then, 1 mL B_5 medium was provided to the embryoids. The Petri dish was sealed with Parafilm and placed in an incubator with a 16 h.d^{-1} photoperiod at 25 °C. Embryoids were recorded as desiccation tolerant after 10 days, when they showed elongated roots and green cotyledons.
Zygotic embryos were isolated by excision from one-hour-imbibed seeds and subsequently redried.

Scanning electron microscopy

A field emission scanning electron microscope (FESEM JEOL 6300 F, Japan) was used to examine all embryos, at least 5-10 for each stage or treatment. The dry samples were mounted on stubs with double sticky tape, whereas the hydrated samples were mounted on stubs with wet filter paper (Whatman nr 3). The stubs were placed on the specimen holder and subsequently frozen in liquid nitrogen (-196°C). The frozen samples were brought into a cryo-transfer unit (CT 1500 HF Oxford Instruments, UK). This cryo-transfer unit consisted of a cryo-preparation chamber at high vacuum ($1*10^{-6}$ Pa) attached to the SEM and a cryo-stage inside the microscope. The specimen was placed inside the cryo-chamber at -85°C, kept there for 2 minutes to sublimate the contaminating water

vapour. Finally the sample was sputter-coated with 3 nm platinum. The coated specimen was placed inside the SEM and observed at 1-5 KV. The temperature of the specimen inside the SEM was kept at -180°C.

Results

Hydrated zygotic embryo

Figure 1a represents a hydrated isolated zygotic embryo. The two well developed cotyledons are close to half of the total length of the embryo. The surface of the epidermal cells is very smooth and the cells are fully turgid (Fig. 1b and 1c). Cell size at the radicle tip is smaller than that of the axis epidermal cells.

Fresh hydrated somatic embryos

Figure 2a shows a typical example of a fresh embryoid after 12 days of ABA (38 μM) treatment. The three cotyledons are fused and are relatively small, with an unusual irregular appearance. The surface of the embryoid is irregular and some cells seem to grow off the surface. All cells are fully turgid, but cell size and shape are variable. No morphological differences are visible between the radicle and the hypocotyl/cotyledon regions of the fresh embryoid (Fig. 2b and 2c).

Desiccation intolerant somatic embryos

Rapid drying, within four hours, causes embryoids to lose their ability to grow after rehydration, despite their culture in ABA-containing B_5 medium (Tetteroo et al. 1995). Figure 3 shows a rapidly dried embryoid with details of the radicle and hypocotyl regions. This embryoid also has abnormal, incompletely developed cotyledons, similar to those in Fig. 2, and the surface is entirely wrinkled. The surface of the radicle is more wrinkled than that of the hypocotyl and the cell size of the hypocotyl is more regular and elongated than that of the radicle (Fig. 3b and c). Cotyledon cells are intermediate in that

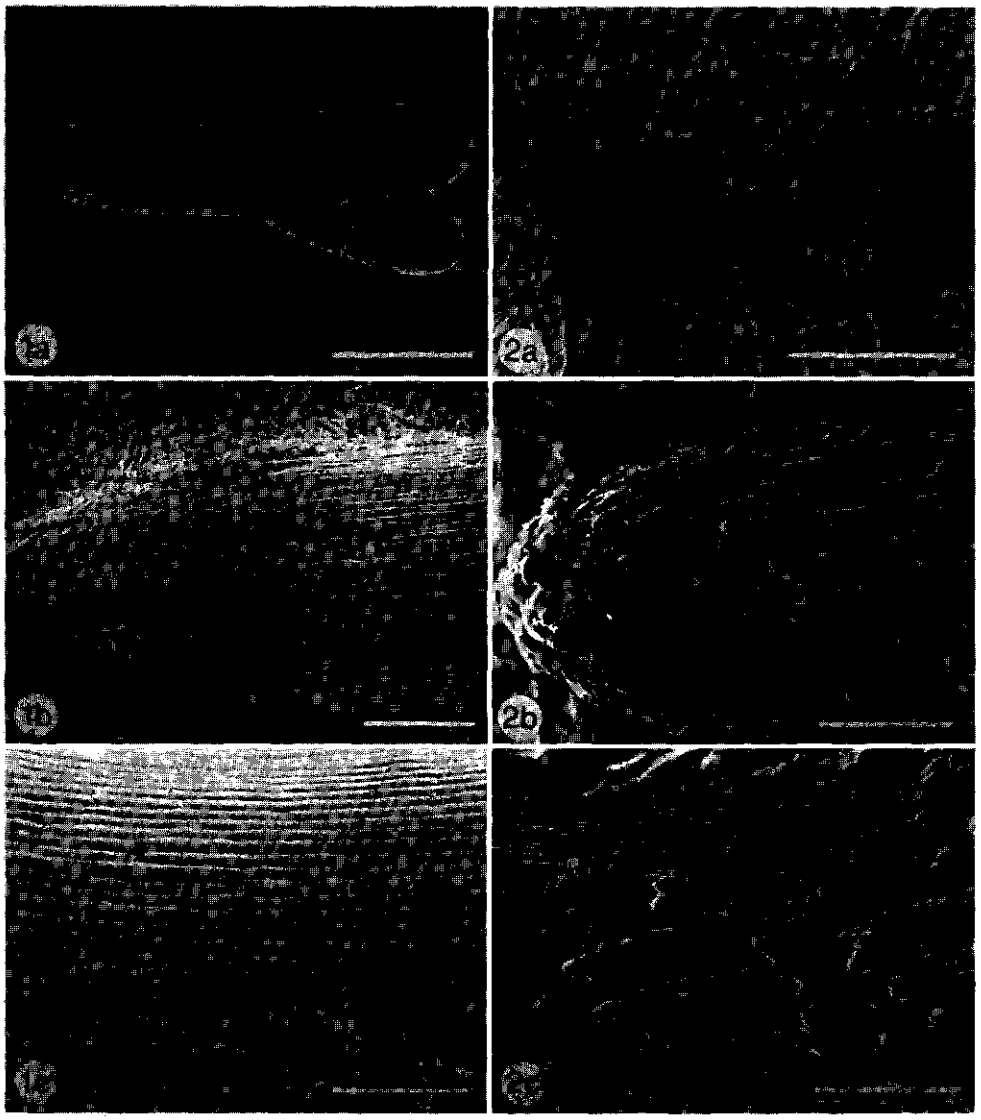

Figure 1: SEM micrograph of a hydrated zygotic embryo four hours after start of *imbibition*. a: whole embryo (bar = 500 μm); b: detail of the radicle (bar = 100 μm); c: detail of the hypocotyl (bar = 100 μm).

Figure 2: SEM micrograph of a fresh somatic embryo developed in B_5 medium containing 38 μM ABA. a: whole somatic embryo (bar = 500 μm); b: detail of the radicle (bar = 100 μm); c: detail of the hypocotyl (bar = 100 μm).

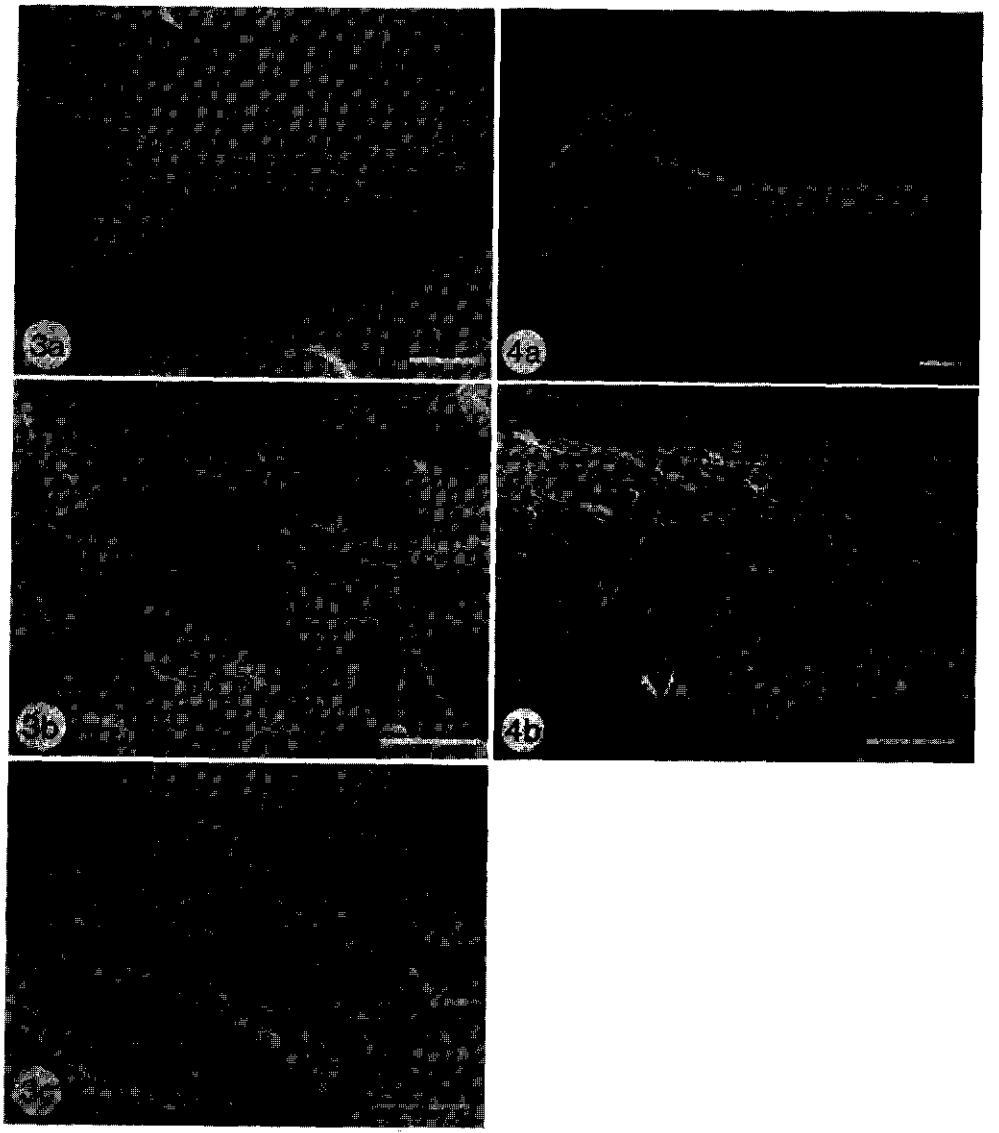

Figure 3: SEM micrograph of a dry, rapidly dried somatic embryo (desiccation intolerant). a: whole somatic embryo (bar = 100 μm); b: detail of the radicle (bar = 10 μm); c: detail of the hypocotyl (bar = 10 μm).

Figure 4: SEM micrograph of a rehydrated, rapidly dried somatic embryo (desiccation intolerant). a: whole somatic embryo two hours after start of imbibition (bar = 100μm); b: detail of the hypocotyl three days after start of imbibition (bar = 100 μm).

respect (not shown). During 2 h of imbibition (Fig. 4a) the embryoid swells to its original size and resembles a fresh hydrated embryoid (cf. Fig. 2a). No surface injuries were observed in all the intolerant embryoids that we have examined.

Desiccation tolerant somatic embryos

A desiccation tolerant embryoid is depicted in Figure 5a. The surface of the embryoid is similarly wrinkled as that of the intolerant embryoid (Fig. 3a). Details of the radicle and hypocotyl region again reveal that radicle cells are more wrinkled and irregular than hypocotyl cells (Fig. 5b and 5c), with cotyledon cells being intermediate (not shown). We have examined a great number of dry tolerant and intolerant embryoids, but no differences in surface morphology between them were detected. Also directly after rehydration the tolerant embryoids cannot be distinguished from hydrated intolerant embryoids (Fig. 4a and Fig. 6a). Only after three days of imbibition differences become noticeable. The cells of tolerant embryoids are fully turgid and elongated (Fig. 6b), whereas the cells of intolerant embryoids are only fully imbibed and not elongated (Fig. 4b).

Desiccation tolerant embryoids matured in PEG

Addition of PEG 6000 to the medium prolongs the maturation period. Similarly to untreated embryoids, the PEG-treated embryoids completely retain their viability after slow drying (100% germination). The average embryoid dry matter increases from 0.2 mg per embryoid without PEG to 0.3 mg per embryoid with PEG, while the average percentage DW on a fresh weight basis before drying increases from 23 to 32%. However, the vigour of the embryoids does not improve. The higher DW is also associated with changes in morphology. The cells of PEG-matured embryoids are less wrinkled compared to control cells (cf. Fig. 7 and 5). There is not much difference in extent of wrinkling between radicle cells and hypocotyl cells of dry PEG-treated embryoids (Fig. 7b and 7c). However, both cell types are irregular in size and shape. After imbibition the embryoids treated with PEG have the same morphology as desiccation tolerant embryoids that are not treated with PEG (Figure not shown).

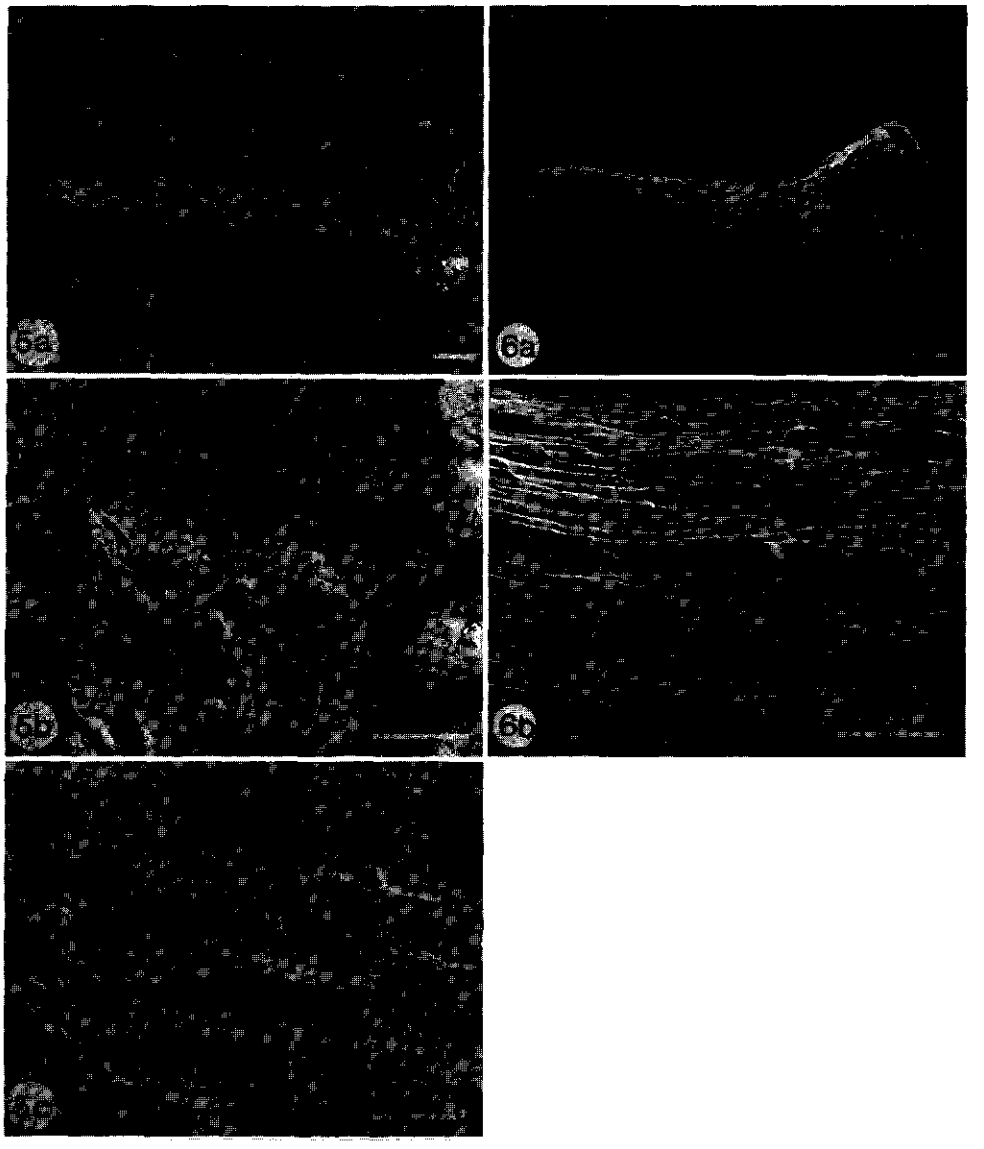

Figure 5: SEM micrograph of a slowly dried somatic embryo (completely desiccation tolerant). a: whole somatic embryo (bar = 100 μm); b: detail of the radicle (bar = 10 μm); c: detail of the hypocotyl (bar = 10 μm).

Figure 6: SEM micrograph of a rehydrated, slowly dried somatic embryo (completely desiccation tolerant). a: whole somatic embryo two hours after start of imbibition (bar = 100μm); b: detail of the hypocotyl three days after start of imbibition (bar = 100 μm).

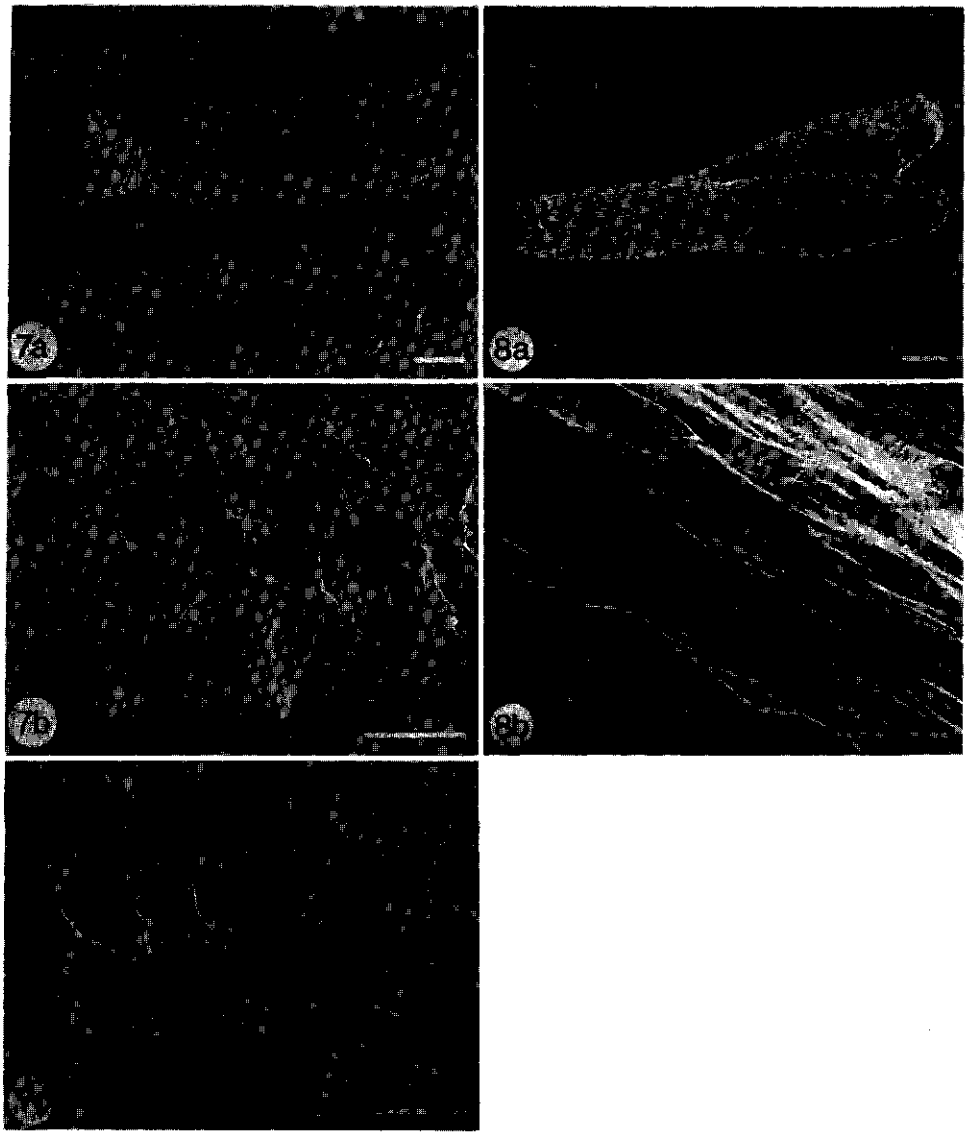

Figure 7: SEM micrograph of a slowly dried somatic embryo (desiccation tolerant) which was matured for three weeks in medium with PEG. a: whole somatic embryo (bar = 100 μm); b: detail of the radicle (bar = 10 μm); c: detail of the hypocotyl (bar = 10 μm).
Figure 8: SEM micrograph of a dry zygotic embryo. a: whole embryo (bar = 100 μm); b: detail of the radicle cells (bar = 10 μm).

Dry zygotic embryos

Figure 8a shows a typical dry zygotic embryo with two well developed, separated cotyledons. The epidermis of the dry embryo is much less wrinkled and also the cell size and shape are more regular than those of the somatic embryos (cf. Figs. 5 and 7). The surface is smooth without cells emerging from the epidermis (Fig. 8b). There are also no differences in morphology between the radicle and hypocotyl regions (details not shown).

Discussion

Zygotic versus somatic embryos

Our data clearly demonstrate differences in structural development between carrot zygotic and somatic embryos. In contrast, mature white spruce somatic embryos resemble morphologically their corresponding zygotic embryos (Fowke et al., 1994). These white spruce somatic embryos have even a higher vigour than excised zygotic embryos. The most prominent differences between the two carrot embryo types are the abnormally developed cotyledons which have the tendency to be fused and the wrinkled appearance of the dry somatic embryo cells. Zygotic embryo cells are very uniform in size and shape, whereas somatic embryo cells show great variation in size and shape. These features may be due to the much shorter maturation phase of the somatic embryos, which may precociously discontinue DW accumulation and cotyledon development. Prolonging of the maturation period by the PEG treatment does increase the DW of the embryoids and thereby diminishes the wrinkling of the dried epidermal cells. This was also demonstrated with white spruce somatic embryos (Attree et al., 1991, 1992). The PEG treatment also inhibits secondary embryogenesis on the axes of the carrot embryoids. However, the PEG maturation is incapable of improving cotyledon development of the carrot embryoids, whereas PEG treatment of white spruce somatic embryos results in well developed cotyledons similar to zygotic embryos (Fowke et al., 1994).

The poorly developed cotyledons and the rough surface of the carrot embryoids may also be caused by the different physical environments in which the embryoids

develop. A zygotic embryo is enveloped by endosperm and seed coat, whereas a somatic embryoid develops in a liquid medium without any restrictions, such as osmotic stress. It has been conclusively demonstrated that these stresses during seed maturation induce specific genes such as LEA-proteins, which may promote cotyledon development (Skriver and Mundi, 1990).

We conclude that carrot somatic embryoids do not completely resemble zygotic embryos. Embryoids are nevertheless able to survive dehydration even without the proper maturation. However, the vigour of carrot embryoids still needs further improvement before embryoids can become a commercial substitute for zygotic embryos in true seeds.

Desiccation-tolerant versus intolerant somatic embryos

In spite of the large number of embryoids that we have examined with LTSEM no large differences are found in morphology between dry tolerant and intolerant carrot embryoids. However, cell surface wrinkling of dry hypocotyl cells is less extensive than of dry radicle cells, which may indicate that fresh hypocotyl cells have accumulated more dry matter at the onset of drying. Thus, the hypocotyl cells will lose less volume than the radicle cells.

The lack of morphological differences between tolerant and intolerant embryoids may indicate that cell shrinkage does not induce lethal injuries during dehydration. Membrane injury, which is likely to prevent the regrowth of intolerant embryoids (Tetteroo et al., 1996), could not be detected, because only the cell wall is examined in detail with LTSEM. Also directly after imbibition no morphological differences are observed between tolerant and intolerant embryoids. This may indicate that there are only minor morphological injuries occurring during dehydration of the desiccation sensitive embryoids. We conclude that LTSEM is a very powerful technique to study native plant materials with different moisture content.

Acknowledgements

We would like to thank S. Massalt for making the prints, B. Hasenack and M. Lammertink for their practical assistance and Dr L. Fowke for critically reading the manuscript.

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Chapter 9

General discussion

With the last chapter of this thesis, the general discussion, we try to place the obtained results in a broader perspective of somatic embryogenesis and desiccation tolerance research. We have succeeded to realize both specified goals of this PhD project. Firstly, we have developed a protocol for the production of fully desiccation tolerant carrot embryoids. Secondly, with this innovative method at hand we were able to characterize aspects of the mechanism of desiccation tolerance. In the following paragraphs both items will be discussed.

Somatic embryogenesis

The production of viable dry carrot embryoids includes the following successive processes: induction, histo-differentiation, maturation, desiccation, rehydration and germination. These subsequent developmental phases with their respective moisture contents are schematically depicted in Figure 1.

The induction process of carrot cells to become embryogenic, is controlled by either an auxin (2.4-D) or an acidity shift. In the presence of 2.4-D, the PEMs proliferate, maintaining their high moisture content, and cease further embryogenic development, whereas in the abscence of 2.4-D embryogenic development proceeds with the formation of globular embryoids (I in Fig. 1). Proliferation of PEMs can also be maintained by means of a low pH of the culture medium (pH 4.0), whereas embryoids

develop when the pH of the culture medium is buffered at 6.0. The induction process has been studied in much detail. However, until now it is still not known how 2.4-D or acidity triggers somatic embryogenesis (Sterk and de Vries, 1993; Zimmerman, 1993). Some progress has been made by means of molecular biological techniques, but it is still poorly understood which genes are expressed during early embryogenic development (Zimmerman, 1993). The extensive list of species that nowadays can be regenerated through somatic embryogenesis has been established only by empirical research (reviewed by Redenbaugh, 1993).

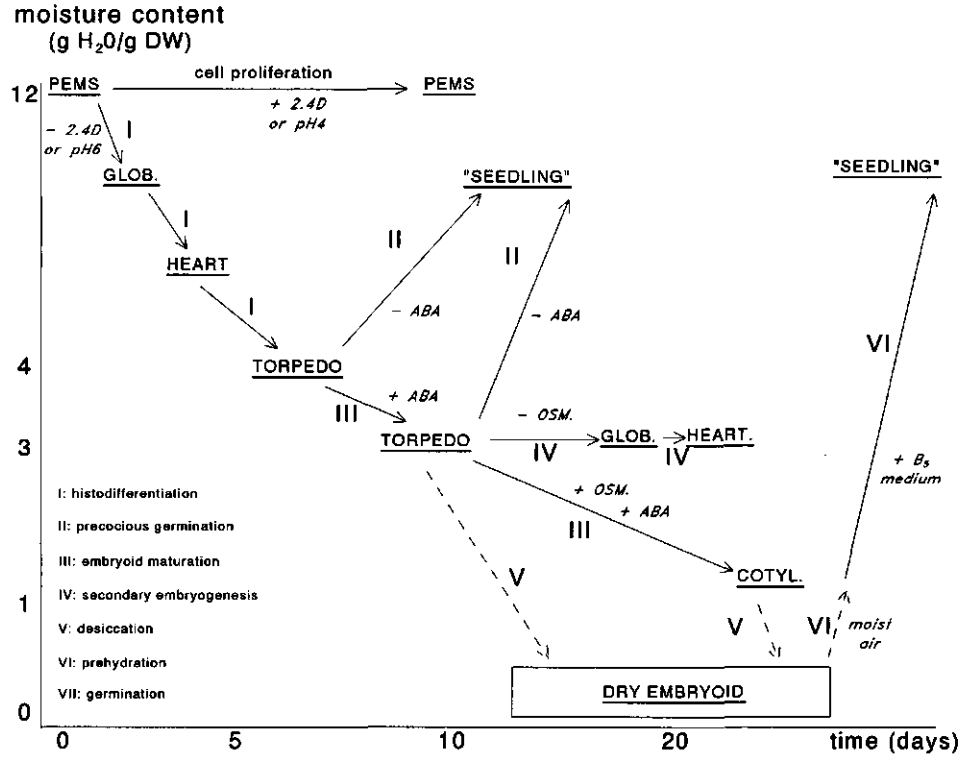

Figure 1: Schematic diagram of the developmental program of carrot embryoids (osm. stands for osmotic stress).

The lack of fundamental knowledge not only includes the induction of somatic and zygotic embryogenesis, but also the subsequent developmental processes. Especially maturation (III in Fig. 1) and slow dehydration (V in Fig. 1) are important phases in the development of carrot somatic embryos, because during these phases embryoids acquire desiccation tolerance (Tetteroo et al., 1995). It has been confirmed that during maturation the growth regulator abscisic acid (ABA) and osmotic stress are among the most important parameters involved in the acquisition of desiccation tolerance (Ooms et al., 1994). The role of ABA has been unequivocally demonstrated in seed of an *Arabidobsis thaliana* double mutant, which is both ABA-deficient and ABA-insensitive (Koornneef et al., 1989), and also in alfalfa somatic embryoids (Senaratna et al., 1989) and carrot somatic embryoids (this thesis).

In the absence of ABA and osmotic stress the torpedo-shaped carrot embryoids increased their moisture content and started precocious germination (II in Fig. 1). By adding ABA (38 μM) the maturation period could be prolonged for approximately seven days. Although the moisture content of the torpedo-shaped embryoids slightly decreased under these conditions, secondary embryoids were formed on the axes of the primary embryoids after these seven days (IV in Fig. 1). Thereby, the primary embryoids lost their ability to survive dehydration. We considered extension of the maturation phase important for the further accumulation of storage reserves, which could enhance vigour of the embryoids. However, this extension in vitro was difficult in water-based culture media. Lowering the osmotic potential of the media with PEG or sugars was possible to a certain extent and gave extension of the maturation phase (Tetteroo et al., 1995), but we did not succeed in obtaining similarly long maturation periods for somatic embryoids in vitro as for zygotic embryos.

Although we managed to increase the maturation period of the carrot embryoids to some extent, the vigour (regrowth capacity) was still inferior to that of zygotic embryos. This inferior vigour might not only be due to a shorter maturation phase, but also to the lack of interaction of the somatic embryoids with endosperm and seed coat. Recently, Senaratna et al. (1995) have demonstrated the importance of seed coats during the development of alfalfa somatic embryoids. By adding isolated seed coats to the culture

medium, they were able to induce quiescence and desiccation tolerance. This effect could be entirely explained through the production of ABA by the seed coat. However, *in vivo* seed coats may be also important for embryo viability, because they form a semipermeable barrier, which regulates water uptake and prevents imbibitional damage. Endogenous solutes that leak from the seed tissues during water uptake remain available to the embryo because of this semipermeability (reviewed by Mohamedyasseen et al., 1994). Somatic embryos lack such a seed coat and are, therefore, unable to retain their leaked solutes. Reduced vigour may be the result.

Not only vigour, but also genetic stability determines the quality of somatic embryoids. Somaclonal or epigenetic variation in somatic embryos has been often described and attributed to the use of the strong synthetic auxin, 2.4-D (Caligari and Shohet, 1993). Frequent genetic variation may limit commercial application, especially in genetically homogeneous crops like tomato and corn. However, cross pollinating crops such as carrot, still have a large genetic variability in commercial cultivars (hybrids), so that some somaclonal variation may be acceptable in commercial artificial seeds.

The induction of complete desiccation tolerance in carrot embryoids could contribute to the further development of artificial seed technology and its commercial application. However, before dry embryoids can be commercially applied, there is the need for large scale production in bioreactors in a synchronized manner. Large scale production of bacteria and yeasts in fermentors has been developed. Successful production of somatic embryoids in several small bioreactor systems has only been demonstrated for a few species (e.g. carrot, *Euphorbia*, *Clematis*, *Picea*) (Scragg and Komamine, 1994). Further research is absolutely necessary to optimise these large scale techniques. Moreover, it has to be taken into consideration that regeneration of embryoids is strongly dependent on genotype, which means that for each genotype or commercial cultivar, a new optimum protocol has to be developed.

In order to produce desiccated artificial seeds with similar properties as true seeds, a dry encapsulation technique has to be developed. The applied coating should provide nutrients, thereby substituting the endosperm, regulate water uptake and protect the fragile embryoids against adverse environmental conditions during sowing. Despite the

substantial current knowledge on pelleting seeds, up until now no satisfactory dry encapsulation method has been developed.

It is concluded that a commercial breakthrough in artificial seed technology can be expected only when we understand more about the induction and maturation of somatic embryos and combine this knowledge with an excellent dry encapsulation technique.

Desiccation tolerance

Several mechanisms (ultrastructural, biochemical as well as biophysical) have been proposed to be involved in desiccation tolerance. An overview of some of these mechanisms in relation to hydration level of the dehydrating tissue is given in Fig. 2 (reprinted from Vertucci and Farrant, 1995). This diagram is specifically based on data from orthodox and recalcitrant seeds, but it may also be used for somatic embryoids, because somatic embryogenesis in many respects resembles zygotic embryogenesis. The diagram shows that desiccation tolerance is a complex and dynamic phenomenon. During development the embryos can survive an increasing fall in moisture levels until quiescence is reached. After imbibition and subsequent germination, they gradually lose their desiccation tolerance. In order to survive complete dehydration to low moisture contents an embryoid has to possess all the features mentioned in Fig. 2. If one of these mechanisms fails, the embryoid is incapable of surviving dehydration.

During this research project we were able to elucidate parts of the mechanisms by which carrot embryoids become desiccation tolerant. It was demonstrated with leakage experiments and freeze fracture studies that membranes are a primary target of injury during dehydration and subsequent rehydration (chapter 3). Because of the suggested role of soluble carbohydrates in the protection of membranes, we further analyzed the carbohydrate metabolism of the developing carrot embryoids. It was shown that carrot embryoids accumulate di- and trisaccharides, whereas the monosaccharides disappear. This result confirms the so called "water replacement theory" for membranes and proteins (reviewed by Crowe et al., 1996). Tolerant embryoids indeed contained sufficient

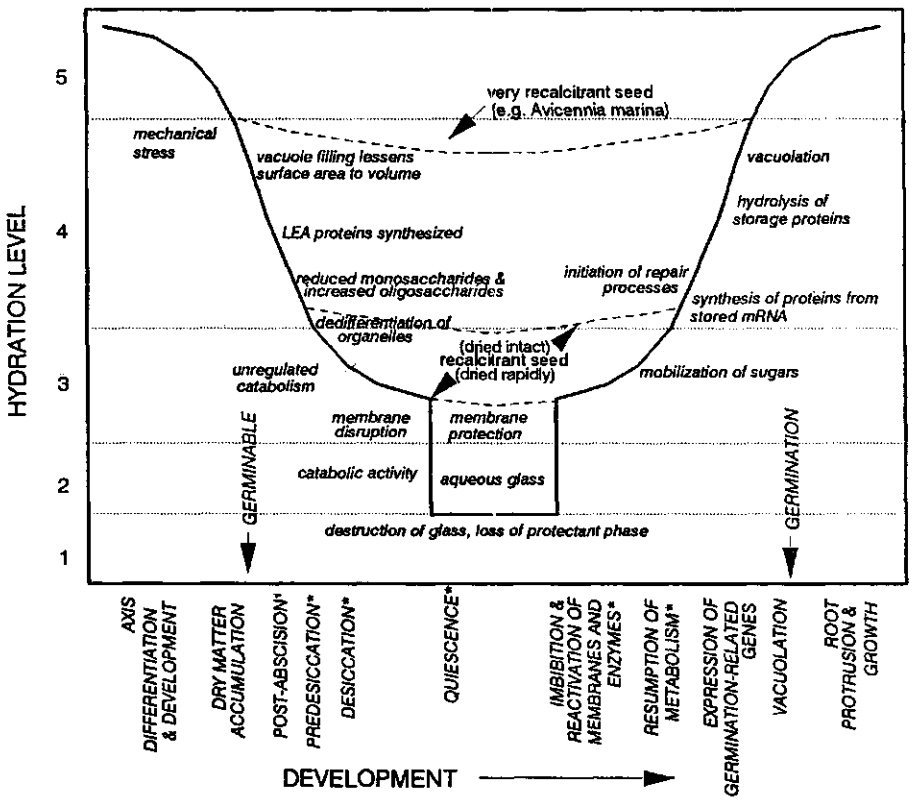

Figure 2: Diagram of the desiccation tolerance of developing seeds in relation to the changing requirements for different types of water. The solid line represents the moisture level below which drying is lethal for orthodox seeds. Dashed lines represent recalcitrant embryos' responses to drying. The text outside the solid lines represents different types of desiccation damage, and the text within the solid lines represents mechanisms to avoid the damage. Developmental processes are described along the abscissa; the timing of these processes is not represented in the figure and will depend on the species as well as on environmental factors. Processes that are starred (*) do not occur in recalcitrant seeds, and so it is expected that the corresponding protective mechanisms also do not occur (Vertucci and Farrant, 1995).

amounts of sucrose and umbelliferose to be able to protect membranes and proteins (chapter 5). In other words, embryoids possessed enough soluble sugars to maintain dry membranes in the liquid crystalline phase. Thus, phase transitions of the phospholipid bilayer should be prevented and the embryoids are not expected to leak during

rehydration. However, we found that tolerant embryoids have to be prehydrated before imbibition to avoid imbibitional damage. This suggests that the membrane PLs are partly in gel phase after drying. Also, intolerant embryoids contained similarly high amounts of soluble carbohydrates (chapter 4 and 5), but they showed extensive leakage of cellular solutes. Freeze fracture replicas of the plasmamembranes in intolerant embryoids indicate that the PLs and IMPs are phase-separated after two hours of imbibition. Both observations indicate that carbohydrates alone are not sufficient to protect the membranes and to provide complete desiccation tolerance. Thus, it must be concluded that more parameters are involved.

Desiccation tolerant organisms may need to form a glassy state (a condition of very high viscosity) upon dehydration. This glassy state prevents formation of crystals in the cytoplasm and may, by the resulting immobilization, prevent phase separation of PLs and IMPs in the membranes. It also could immobilize toxic components such as free radicals, so that they are not able to migrate to sensitive targets. Thus, deterioration of vital compounds of the embryoids is prevented. Oligosaccharides are known for their ability to form stable glasses and would therefore be benefical to survival.

It has been suggested that desiccation tolerant organisms need the synthesis of specific proteins (e.g. dehydrins and/or LEAs). This protein synthesis depends on either ABA treatment and/or water stress (slow dehydration). We have also determined such gene expression in carrot embryoids (data not shown). However, we were not able to detect a complete correlation with desiccation tolerance. These proteins might function also as protectant for membranes or other desiccation sensitive proteins, or they might be used for ion-sequestration or as channel proteins for water or solutes (reviewed by Bray, 1993).

Loss of desiccation tolerance has also been linked to free radical activity, with deesterification of the phospholipids as the result (Leprince et al., 1993). Free radicals emerge from the abrupt interruption of the metabolism during drying. The presence of sufficient amounts of antioxidants or free radical scavengers can prevent this damage. Lack of these systems may cause desiccation sensitivity. It is unlikely that this is the case

in the carrot embryoids, because of their high amounts of sugars that could act as hydroxyl radical scavengers (Smirnoff and Cumbes, 1989).

It has been suggested that membrane permeability increases during dehydration, because of the accumulation of endogenous amphiphilic compounds (e.g. flavonoids) in the phospholipid bilayer (Hoekstra et al., 1996). The partitioning of these compounds into the membrane is stimulated by the increased solute concentration of the dehydrating cytoplasm. During imbibition the reverse occurs and membranes regain their normal semipermeability. However, during repartitioning into the cytoplasm at rehydration, leakage may ensue, associated with reduced desiccation tolerance or reduced vigour. This mechanism is a possible explanation for the extensive sugar and K^+ leakage (60%) of the prehydrated tolerant carrot embryoids during imbibition (chapter 3). The partitioning may have also beneficial effects, because amphiphiles depress T_m and keep the dry membranes in the liquid crystalline phase. Thus, imbibitional leakage is prevented. An additional advantage is that some of the amphiphiles (e.g. flavonoids) have antioxidant properties, which would provide long term protection to the membranes (Terao et al., 1994).

Loss of structural integrity resulting from decompartmentalisation or membrane fusion, or the failure of repair processes during rehydration can cause desiccation sensitivity (Vertucci and Farrant, 1995). Elucidation of the individual contributions will be very difficult, because of the interaction of these mechanisms.

Another point of discussion is the role of ABA in the acquisition of desiccation tolerance. For carrot embryoids we demonstrated that ABA is one of the essential parameters involved in the production of completely desiccation tolerant embryoids (chapter 2). Without ABA the embryoids continue their development with precocious germination instead of further maturation and lose their desiccation tolerance. It seems that ABA only inhibits germination, allowing the genetic program of embryoid development and maturation to continue. However, ABA also influenced the carbohydrate metabolism and reduced the respiration of the embryoids (chapter 4 and 6). These ABA effects might be secondary effects, because alfalfa embryoids are able to acquire desiccation tolerance by means of osmotic or heat stress without exogenous ABA (Senaratna et al., 1989). Moreover, desiccation sensitive double mutant seeds of

Arabidopsis thaliana, which are both ABA-deficient and ABA-insensitive, survive dehydration after osmotic stress or slow dehydration (Ooms et al., 1994). This may indicate that ABA is not necessary for the acquisition of desiccation tolerance as long as the embryoids follow their developmental program.

Conclusion

A protocol was developed to regenerate completely desiccation tolerant carrot embryoids. Embryoids can be desiccated to 5% moisture content, comparable with true carrot seeds, without losing viability even after cool dry storage at 15°C for two years. We have identified four critical parameters involved in the acquisition of desiccation tolerance: developmental phase, ABA, dehydration rate and prehydration in humid air. Despite the optimization of these four parameters, the regrowth capacity of the dry carrot embryoids is still inferior to that of dry zygotic embryos. More fundamental physiological knowledge of signal transduction and gene expression, and the elucidation of the function of the gene products (proteins) during induction, maturation and dehydration are needed to improve embryoid quality.

Carbohydrate metabolism and respiration are altered by ABA and slow drying. The accumulated di- and tri-saccharides (sucrose and umbelliferose) probably play a role in desiccation tolerance as protectants of membranes and proteins and/or by forming a stable glassy state. Embryoids must contain a minimum level of these sugars to withstand dehydration to low moisture contents.

Although we have elucidated some aspects of the mechanisms that are involved in desiccation tolerance, we believe that the essence of these mechanisms remained unresolved.

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Summary

This thesis describes the research performed on the subject "Desiccation tolerance in somatic embryoids". Somatic embryoids are bipolar structures formed in tissue culture, with both a shoot and a root apex, which resemble very much zygotic embryos found in seeds. Through simultaneous development of root and shoot, these embryoids can grow out into complete plantlets.

In Chapter 2 we describe an optimized method to produce completely desiccation tolerant carrot (*Daucus carota*) embryoids. Using this method at least four different factors are important: developmental stage, abscisic acid (ABA) concentration, drying rate and rehydration mode. Embryoids may become desiccation tolerant when they have reached the torpedo stage of development. In contrast, at the earlier globular and heart stages, embryoids never germinated after any drying treatment. Addition of at least 20 μM ABA to the pro-embryogenic masses after 7 days of culture in 2,4-dichlorophenoxyacetic acid-free B_5 medium was necessary to induce complete desiccation tolerance. Less ABA resulted in desiccation tolerance of the roots only, whereas high ABA (>80 μM) concentrations retarded developmental growth. Slow drying is essential for the acquisition of complete desiccation tolerance. Slowly dried embryoids (moisture content 0.05 g $H_2O.g^{-1}$ dry weight) germinated for 100%. However, rapidly dried ones germinated for only 0-30%. Initially viable dry embryoids may suffer injury when they are imbibed in water without prehydration in water vapour. Hundred percent germination was reached by prehydration of the embryoids in moist air for 4 to 8 hours at 24°C before imbibition in B_5 medium. With the optimized protocol we were able to produce

desiccation tolerant embryoids in two genotypes having completely different genetic backgrounds.

With this manipulable protocol at hand, we have assessed damage associated with desiccation (Chapter 3). Fast drying caused loss of viability, and all K^+ and soluble carbohydrates leached from the embryoids within 5 min of imbibition. The phospholipid content decreased by about 20% and the free fatty acid content increased, which was not observed after slow drying. However, the extent of acyl chain unsaturation of the phospholipids was unaltered, irrespective of the drying rate. These results indicate that during rapid drying irreversible changes occur in the membranes which are associated with extensive leakage and loss of germinability. The status of membranes after 2 h of imbibition was analyzed in a freeze-fraction study and by Fourier transform infrared spectroscopy (FTIR). Rapidly dried embryoids had clusters of intramembraneous particles in their plasma membranes and the transition temperature (T_m) of isolated membranes was above room temperature. Membrane proteins were irreversibly aggregated in an extended ß-sheet conformation and had a reduced proportion of α-helical structures. In contrast, the slowly dried embryoids had irregularly distributed, but nonclustered, intramembraneous particles, T_m was below room temperature and the membrane proteins were not aggregated in a ß-sheet conformation. We suggest that desiccation sensitivity of rapidly dried carrot embryoids is indirectly caused by an irreversible phase separation in the membranes due to deesterification of phospholipids and accumulation of free fatty acids.

In Chapter 4 and 5 we have studied the role of the endogenous soluble carbohydrates during acquisition of desiccation tolerance. For carrot embryoids we demonstrated an apparent minimum requirement of umbelliferose plus sucrose for surviving severe dehydration, suggesting that these sugars play an important role in anhydrobiosis. We show with FT-IR spectroscopy, that both sucrose and umbelliferose depress the transition temperature (T_m) of dry liposomal membranes, which is evidence for their interaction with the phospholipid polar headgroups. Furthermore, both sugars prevent leakage from dry liposomes during drying and subsequent rehydration. We interpret this in the sense that both sugars are able to form a stable glass in the dry state. Fructose and glucose were lacking in dry viable embryoids. In the light of the plasticizing effect of these monosaccharides on sugar glasses, a stable glassy state seems important

during anhydrobiosis of carrot somatic embryoids. We show that umbelliferose can protect a protein that is desiccation sensitive.

To characterize desiccation tolerance we studied not only membranes and carbohydrate metabolism, but also the role of repiration (Chapter 6) and DNA replication (Chapter 7). Addition of ABA to developing carrot embryoids affected respiration and carbohydrate metabolism. Non-treated embryoids had a high level of respiration expressed per gram protein and consumed almost completely their endogenous carbohydrates during the ten day culture period. In contrast, embryoids grown with either 1.9 or 38 μM ABA, had a reduced respiration rate and maintained their carbohydrate contents at 20% of the DW. Embryoids acquired complete desiccation tolerance, when they were treated with 38 μM ABA, whereas only 65% of the embryoids survived desiccation with 1.9 μM ABA. The reduced respiration of the developing embryoids might result in reduced free radical levels after dehydration, in this way preventing a subsequent viability loss. We suggest that there is a relation between viability loss due to desiccation and respiration rate, although the latter is not the only limiting factor involved.

Employing flow cytometry we investigated the effect of ABA addition and slow drying on DNA replication and cell cycle activity of developing carrot embryoids. DNA replication was determined as percentage of 4C nuclei. Addition of ABA did not alter DNA replication and cell cycle during embryoid development in vitro, in spite of the putative quiescent state of the torpedo-shaped embryoids. In contrast, during slow drying the nuclei were preferentially arrested in the presynthetic G_0/G_1-phase, and the amount of G_2 nuclei decreased. Dry zygotic carrot embryos that are completely desiccation tolerant, did not contain any G_2 nuclei. The decline of G_2 nuclei in dry somatic embryoids seems to coincide with the increase in desiccation tolerance, which is incomplete compared to zygotic embryos. Our results suggest that in order to withstand anhydrobiosis, DNA replication may be controlled by the embryoid developmental program and by slow dehydration, but not by the plant growth regulator ABA.

Finally, we performed scanning electron microscopy studies to establish the possible changes during desiccation (Chapter 8). Cryofixation and analysis by low temperature scanning electron microscopy (LTSEM) are excellently suitable to compare the morphology of specimens having different moisture contents. Using LTSEM we

examined dry and hydrated carrot zygotic embryos and compared them with fresh, rapidly dried, and slowly dried carrot somatic embryoids, also after rehydration. Rapidly dried somatic embryoids were not able to germinate, whereas approximately 100% of slowly dried embryoids germinated. Somatic embryoids always had reduced and abnormal cotyledons, mostly fused, and the surface was irregular. The surface of the dry somatic embryoids was also more wrinkled than that of zygotic embryos. No morphological differences were detected between tolerant (slowly dried) and intolerant (rapidly dried) somatic embryoids before and shortly after rehydration. However, clear morphological differences were detected after imbibition for three days. Tolerant embryoids showed clear cell expansion, whereas intolerant ones did not. It is concluded that LTSEM is a very powerful technique to study plant materials in their native state.

Samenvatting

Dit proefschrift beschrijft het onderzoek naar de inductie en karakterisering van droogtetolerantie van somatische embryoiden. Somatische embryoiden zijn bipolaire structuren met zowel een scheut- als wortelgroeipunt, die alleen in weefselkweek onder steriele omstandigheden kunnen worden geregenereerd. Deze somatische embryoiden lijken in sterke mate op zygotische embryo's die zich ontwikkelen na de bevruchting van eicellen in zaden. Door de gelijktijdige uitgroei van scheutje en worteltje kunnen embryoiden zich ontwikkelen tot volledige planten.

In Hoofdstuk 2 wordt een geoptimaliseerde methode beschreven voor de productie van volledig uitdroogtolerante embryoiden van wortel (*Daucus carota*). Deze methode bevat minimaal vier essentiële parameters: ontwikkelingsstadium van de embryoiden, toediening van abscisinezuur (ABA), drogingssnelheid en methode van rehydratatie. Embryoiden kunnen droogtetolerant worden nadat zij het torpedo-vormig stadium van hun ontwikkeling bereikt hebben. Embryoiden, die gedroogd zijn in vroege ontwikkelingsstadia, zoals globulair en hartvormig, kiemden na imbibitie nooit. Toediening van tenminste 20 μM ABA aan de ontwikkelende embryoiden op de zevende dag van hun cultuur in 2.4-D-vrij B_5 medium, was noodzakelijk voor de inductie van uitdroogtolerantie. Toediening van lagere ABA-concentraties resulteerde in gedeeltelijke uitdroogtolerantie: alleen het worteltje overleefde. Te hoge ABA-concentraties (meer dan 80 μM) veroorzaakten een groeiremming van de embryoiden, zodat het torpedo-vormig stadium niet werd bereikt. Langzaam drogen van de embryoiden bleek eveneens essentieel te zijn voor het verkrijgen van uitdroogtolerantie. Langzaam gedroogde embryoiden (vochtgehalte van 5%) kiemden volledig na imbibitie in B_5 medium, terwijl snel

gedroogde embryoiden slecht of helemaal niet kiemden (0-30%). Droge embryoiden die optimaal behandeld zijn en daardoor aanvankelijk droogtetolerant zijn, verloren hun kiemkracht wanneer zij zonder opdampen in vochtige lucht (rehydratatie) gekiemd werden in B_5-medium. De opdampfase moest 4-8 uur duren bij 24°C om 100% kieming te verkrijgen. Met dit optimale protocol konden van twee genotypen met sterk verschillende genetische achtergrond, droge vitale embryoiden geproduceerd worden.

Met de hierboven beschreven methode hebben wij de schade vastgesteld die optreedt bij het verlies van droogtetolerantie d.m.v. snel uitdrogen. Na 5 minuten imbibitie waren alle K^+-ionen en suikermoleculen uit de snel gedroogde embryoiden weggelekt. Het fosfolipidengehalte verminderde met ongeveer 20% en het vrije-vetzuurgehalte steeg, hetgeen niet werd waargenomen in langzaam gedroogde embryoiden. Echter, de onverzadigingsgraad van de vetzuurketens van de fosfolipiden bleef onveranderd, ongeacht de methode van drogen. Deze resultaten geven aan dat gedurende snel drogen onomkeerbare veranderingen in het plasmamembraan zijn opgetreden, welke samengaan met grote lekkage en verlies van kiemkracht. De toestand van de membranen na twee uur imbibitie werd onderzocht met Fourier transform infrarood spectroscopie (FTIR) en met behulp van vriesbreken en transmissie-electronenmicroscopie. Snel gedroogde embryoiden bevatten samenklonteringen van intramembraanpartikels in hun plasmamembraan, en de transitie-temperatuur van geïsoleerde membranen was hoger dan de kamertemperatuur. De membraaneiwitten hadden gedeeltelijk een "extended ß-sheet" conformatie aangenomen en hadden minder α-helix structuur. De langzaam gedroogde embryoiden daarentegen hadden onregelmatig verdeelde, maar niet samengeklonterde intramembraanpartikels, de transitietemperatuur was beneden de kamertemperatuur, en de membraaneiwitten hadden geen "extended ß sheet" conformatie aangenomen. Verondersteld wordt dat de uitdrooggevoeligheid van snel gedroogde embryoiden indirect veroorzaakt is door een onomkeerbare fasenscheiding in het membraan als het gevolg van een de-esterificatie van de fosfolipiden en ophoping van vrije vetzuren.

In Hoofdstuk 4 en 5 is de functie van oplosbare suikers in de ontwikkeling van uitdroogtolerantie bestudeerd. Voor dit deel van het onderzoek zijn somatische embryoiden gebruikt van zowel wortel als lucerne (*Medicago sativa*). In beide gevallen

kon geen duidelijke correlatie worden aangetoond tussen suikers en kieming. Echter, voor wortel-embryoiden werd duidelijk bewezen dat er een minimum hoeveelheid aan saccharose en umbelliferose (een trisaccharide die alleen voorkomt bij de Apiaceae) aanwezig moet zijn om uitdrogen te overleven. Dit is een mogelijke aanwijzing dat deze suikers een belangrijke functie hebben tijdens overleven in droge toestand. Met FTIR-spectroscopie hebben we laten zien dat zowel saccharose als umbelliferose in staat zijn de transitietemperatuur van droge liposomale membranen te kunnen verlagen, hetgeen een bewijs is voor de interactie van de suikermoleculen met de polaire hoofdgroepen van de fosfolipiden. Daarnaast hebben we aangetoond dat beide suikers lekkage kunnen voorkomen van carboxy-fluoresceïne uit gedroogde liposomen na imbibitie. Dit geeft aan dat beide suikers liposomen kunnen beschermen door de vorming van een stabiel glas in droge toestand. Glucose en fructose daarentegen zijn niet in staat om bij kamertemperatuur een stabiel glas te vormen. Deze beide monosacchariden werden niet aangetroffen in droge embryoiden, hetgeen een indicatie kan zijn voor het belang van een stabiel glas voor overleven na uitdrogen. Met FTIR-spectroscopie hebben we tevens aangetoond dat umbelliferose in staat is om de secondaire structuur van een uitdrooggevoelig eiwit te behouden na drogen.

De karakterisering van droogtetolerantie had niet alleen betrekking op membranen en suikermetabolisme, maar wij hebben ook de ademhaling (Hoofdstuk 6) en de DNA-replicatie (Hoofdstuk 7) bestudeerd. Toediening van ABA aan ontwikkelende wortel-embyoiden had niet alleen een duidelijk effect op het suikermetabolisme maar ook op de ademhaling. Onbehandelde embryoiden hadden een hoge ademhalingssnelheid uitgedrukt per gram eiwit en zij consumeerden in tien dagen bijna alle endogene suikers. Daarentegen hadden embryoiden, die behandeld waren met 1,9 of 38 μM ABA, een veel lagere ademhalingssnelheid en waren in staat om hun suikergehalte op 20% van de totale droge stof te houden. Echter, alleen embryoiden die 38 μM ABA kregen toegediend ontwikkelden volledige uitdroogtolerantie. De verlaagde ademhaling van de met ABA behandelde embryoiden zou kunnen leiden tot een verminderde hoeveelheid vrije radicalen in de droge embryoiden, waardoor er geen vitaliteitsverlies optreedt. Wij veronderstellen dat er een verband bestaat tussen ademhaling en droogtetolerantie, maar het blijkt dat de ademhalingssnelheid niet de enige beperkende factor is.

Het effect van ABA-toediening en langzaam drogen van wortelembryoiden op de DNA-replicatie en de celcyclus hebben we bestudeerd met behulp van een flowcytometer. De status van de DNA-replicatie werd bepaald door het percentage 4C-kernen te meten in de populatie geïsoleerde kernen. De toediening van ABA aan de embryoiden veroorzaakte een schijnbare rustsituatie, de embryoiden bleven in een torpedo-vormig stadium van ontwikkeling, echter de DNA-replicatie bleek onveranderd: Er waren nog steeds cellen aan het delen. Bij langzaam drogen, daarentegen, ging de DNA-replicatie wel degelijk omlaag. De celcyclus werd bij voorkeur gestopt in de presynthese G_0/G_1-fase en het aantal kernen in de G_2-fase verminderde. Droge zygotische wortelembryo's die volledig uitdroogtolerant zijn, bevatten in het geheel geen kernen in de G_2-fase. Er lijkt dus een verband te bestaan tussen verminderde DNA-replicatie en mate van droogtetolerantie van wortelembryoiden.

Tot slot hebben we scanning-electronenmicroscoopopnamen gemaakt bij zeer lage temperatuur (-180°C) van zowel droge als natte wortelembryoiden om de morfologie te vergelijken (Hoofdstuk 8). De embryoiden werden voor deze techniek met vloeibare stikstof gefixeerd waarna zij direct in de microscoop bekeken konden worden. Met deze eenvoudige fixatietechniek was het uitstekend mogelijk om weefsels c.q. embryoiden met verschillende watergehalten onder gelijke omstandigheden te bestuderen. Ter vergelijking hebben we ook zygotische embryo's uitgeprepareerd, nat en droog gefixeerd, en bekeken. Somatische embryoiden hadden bijna altijd kleine abnormale cotylen, in de meeste gevallen vergroeid, en vaak meer dan twee, terwijl het oppervlak van de droge cotylen zeer onregelmatig was. Zygotische embryo's daarentegen hadden altijd maar twee cotylen met een zeer regelmatig egaal oppervlak. We hebben geen morfologische verschillen kunnen waarnemen tussen droge tolerante (langzaam gedroogd) en droge intolerante (snel gedroogd) embryoiden. Echter na drie dagen imbibitie was duidelijk waarneembaar dat de cellen van de tolerante embryoiden waren gestrekt, terwijl de cellen van intolerante embryoiden geen strekking vertoonden. Onze resultaten hebben laten zien dat scanning-electronenmicroscopie bij lage temperatuur een uitstekende techniek is om plantmateriaal te bestuderen in hun natuurlijke staat.

Curriculum Vitae

De schrijver van dit proefschrift, Franciscus Abraham Antonius Tetteroo werd op 14 november 1960 in Den Haag geboren. Na het behalen van het VWO-b diploma aan het Aloyssius College te Den Haag in mei 1979, werd een aanvang gemaakt met de studie Plantenveredeling aan de toenmalige Landbouwhogeschool in Wageningen. De doctoraal fase omvatte twee hoofdvakken, plantenveredeling en tuinbouwplantenteelt, en een bijvak agrarische bedrijfseconomie. In november 1986 werd de studie afgerond met het behalen van het doctoraalexamen. Van januari 1987 tot juni 1995 was de auteur werkzaam bij Royal Sluis. Gedurende deze periode werd hij in de gelegenheid gesteld om vanaf februari 1991 tot februari 1994 promotie-onderzoek te verrichten bij de vakgroep plantenfysiologie van de Landbouwuniversiteit in Wageningen. De resultaten van dit onderzoek naar droogtetolerantie van somatische embryoiden staan in dit proefschrift beschreven. Sinds 1 juni 1995 is de auteur werkzaam bij Incotec International B.V. te Enkhuizen, waar hij onderzoek verricht naar incapsuleringstechnieken van zaden.