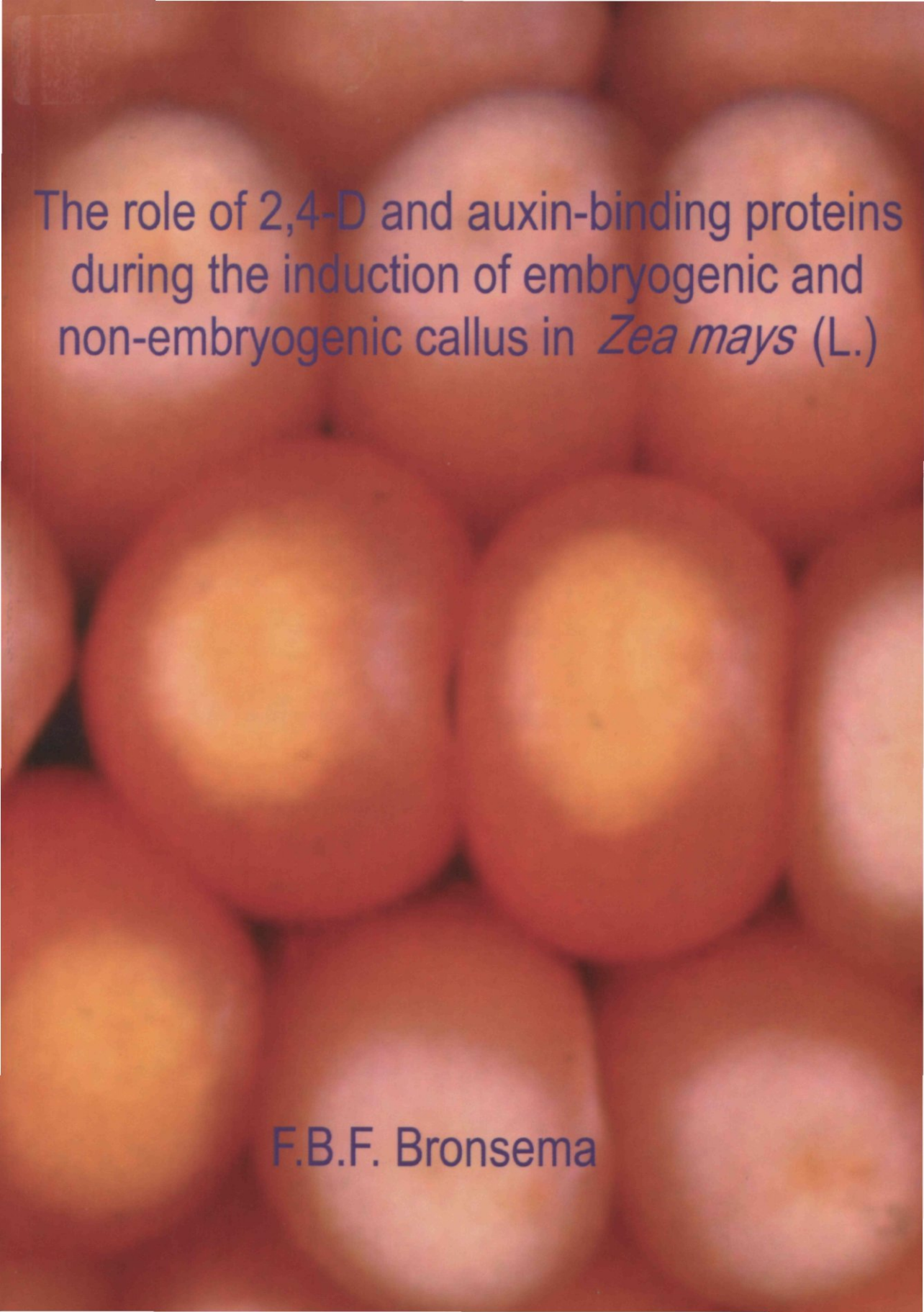

The role of 2,4-D and auxin-binding proteins during the induction of embryogenic and non-embryogenic callus in *Zea mays* (L.)

F.B.F. Bronsema

Stellingen

1. Opname van 2,4-D induceert somatische embryogenese in inteeltlijn A188, maar niet in inteeltlijn A632.
   (Hoofdstukken 2 en 3 van dit proefschrift)

2. Met behulp van autoradiografie kan ^{14}C-2,4-D zichtbaar gemaakt worden in weefsels en organen.
   (Hoofdstuk 4 van dit proefschrift)

3. ABP-1 komt niet voor in celwanden van maïscoleoptielen.
   (Jones and Herman, 1993, Plant Physiol 101:595-606)

4. Er bestaat geen verband tussen de distributie van ABP-1 en de plaats van inductie van embryogeen callus in inteeltlijn A188.
   (Hoofdstukken 4 en 5 van dit proefschrift)

5. Een wetenschappelijk experiment moet door minstens twee onafhankelijke personen uitgevoerd kunnen worden.
   (German technician's confession spurs check on suspect data, 1998, Nature 393:293)

6. Met *in vitro* vermeerdering is de natuurlijke zeldzaamheid van inheemse orchideeën niet op te lossen.

7. De extra hoge prijs voor melk in polycarbonaat-flessen is de prijs die ten onrechte moet worden betaald voor het milieuvriendelijk consumeren van melk.

8. Mensen zonder kinderen hebben arbeidspieken in de schoolvakanties.

9. Het nemen van een rotonde is in Nederland een door gemeenten aangelegd kansspel.

10. Het bezit van een bijnaam leidt tot meer verbale communicatie.

11. Bij de Nederlandse Spoorwegen is drie minuten te laat op tijd.

Stellingen behorend bij het proefschrift:
"The role of 2,4-D and auxin-binding proteins during the induction of embryogenic and non-embryogenic callus in Zea mays (L.)"
F.B.F. Bronsema
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The role of 2,4-D and auxin-binding proteins during the induction of embryogenic and non-embryogenic callus in *Zea mays* (L.)

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The role of 2,4-D and auxin-binding proteins during the induction of embryogenic and non-embryogenic callus in *Zea mays* (L.)

Folbert Bronsema

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op gezag van de rector magnificus
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in het openbaar te verdedigen
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Cover: Detail of maize cross A632*A188 after selfing.

Aan Fokke Bronsema

Aan Wim Schuurmans

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Chapter 1

General introduction

At the Laboratory of Plant Cytology and Morphology of the Wageningen Agricultural University, research is performed on the embryogenesis of maize (*Zea mays* L.), in which tissue culture techniques are used to study zygotic and somatic embryogenesis under controlled conditions (Van Lammeren 1987, 1988, Fransz 1988). Tissue culture techniques are used to induce embryogenic callus and to obtain plants by somatic embryogenesis on cultured immature zygotic embryos. In general new plants can be obtained from explants either without the use of hormones or growth regulators as for instance with microspore cultures of *Brassica napus* after heat shock treatment (Hause and Hause 1996), or with the application of hormones or growth regulators in the culture medium as for instance with *Daucus carota* (Timmers 1993). The composition of the culture medium determines whether the normal embryogenesis of the excised and immature embryos is continued, or whether there is renewed cell division and formation of callus, followed by the formation of adventitious organs or somatic embryos on the zygotic embryo. With respect to the adventitious organ formation and somatic embryogenesis, members of the *Poaceae* are rather recalcitrant. Only strong stable growth regulators, like 2,4-dichlorophenoxyacetic acid (2,4-D) or 3,6-dichloro-o-anisic acid (Dicamba), are capable to induce embryogenic callus and somatic embryogenesis in maize. Although 2,4-D is a synthetic growth regulator and differs from natural auxins, like indoleacetic acid (IAA), is it necessary in the experiments, because only with 2,4-D induction of cell division and subsequent differentiation is established.

In recent years considerable evidence for the existence of hormone receptors in plants cells came up. In the case of auxins, the maize auxin-binding protein (ABP-1) is a receptor which has been studied intensively by several groups (Löbler and Klämbt 1985a,b, Napier and Venis 1988). Because the growth response in cultured maize embryos is caused by 2,4-D, the presence, localisation and subcellular distribution of both 2,4-D and ABP-1 was studied in relation to the induction of embryogenic callus.

Thus, the aim of the study has been to provide more insight into the role of the growth regulator 2,4-D and the maize auxin-binding protein (ABP-1) during the induction of callus from immature embryos of maize in relation to somatic embryogenesis by the use of cytological, morphological, immunocytochemical, physiological, biochemical and radiological techniques.

The herbicide 2,4-dichlorophenoxyacetic acid (2,4-D)

The phenoxycarboxylic acids, like 2,4-D and 2,4,5-trichlorophenoxyacetic acid (2,4,5-T), are among the oldest synthetic herbicides. They are used primarily for postemergence management of dicotelydonous weeds in grass crops, pastures and lawns. The butyric acid derivative of 2,4-D, 4-(2,4-dichlorophenoxy)-butyric acid (2,4-DB) is a proherbicide, that is metabolised to 2,4-D in sensitive species. Some legumes that are sensitive to 2,4-D, are tolerant to 2,4-DB, because they lack the β-oxidation activity required to activate it. Although introduced in the 1940s, it is still in use in many countries. Phenoxycarboxylic acids have been off patent for many years and are very effective and inexpensive.

Tolerance of grasses to herbicides appears to be related to rapid irreversible metabolic conversion to non-toxic products, whereas in dicotyledonous species the herbicide is often found in the form of reversible conjugates (Devine *et al.* 1993). Several weed species have evolved resistance to 2,4-D (LeBaron 1991). The resistance appears to be due to accelerated metabolic detoxification.

The phenoxy herbicides, such as 2,4-D, are active as plant growth regulators or hormones with auxin activity. They are absorbed through the leaves and are translocated initially for short distances by intracellular movement to the phloem and then over long distances in the phloem to the shoot meristems. At the meristem 2,4-D interferes with cell development, presumably through saturation effects arising from mimicry of auxin action on cell elongation (Dodge 1989). Characteristic damage to plants by low levels of 2,4-D is severe twisting and spindly growth of vegetative and floral tips and organs, followed by the death of meristems and eventually the plant.

Detoxification of the phenoxy herbicides may play an important part in the selectivity of these chemicals for different plant species. 2,4-D can undergo a variety of chemical modifications in

insensitive plants, including side chain degradation, side chain elongation, ring hydroxylation, and conjugation with proteins, amino acids, and glucose (Ashton and Crafts 1981). Monocotyledonous plants are considerable less sensitive to phenoxyacetic herbicides and hence these herbicides are of particular use for broadleaf weed control in monocot crops such as cereals and sugarcane.

2,4-D is an important auxin in the tissue culture of cereals and is an active growth regulator for these species. It is not surprising, therefore, that cereals are sensitive to 2,4-D at particular developmental stages and can be seriously affected if this herbicide is used inappropriately (Evans 1968).

The growth regulator 2,4-dichlorophenoxyacetic acid (2,4-D) and induction of callus in immature embryos of *Zea mays* (L.)

In experiments with the highly embryogenic white dent inbred line A188 (Green and Phillips 1975, Green and Rhodes 1982, Tomes 1985) it was shown that cultured zygotic embryos showed polarity for the formation of callus and somatic embryos. Callus formation and formation of somatic embryos are first observed at the basal side of the cotyledon, *i.e.* the scutellum, if the zygotic embryos are cultured with their meristem side on the culture medium (Fransz and Schel 1987, van Lammeren, 1988). During the first 24h of culture, the cells of the scutellum become active, the number of organelles increases, and vacuolation and nuclear morphology change. This happens during the shock response phase and is independent of the presence of 2,4-D in the medium (Fransz and Schel 1987). The growth response depends on the presence of 2,4-D and is characterised by mitotic activity of cells of the scutellum. In embryos of inbred line A188 a broad scutellar meristematic zone is observed and a second meristematic region around the coleorhiza (Fransz and Schel 1994). A second maize inbred line A632 which was studied, does not form somatic embryos, independent of the presence or absence of 2,4-D in the culture medium (Fransz 1988). Culture conditions for the induction and proliferation of embryogenic callus have improved (Emons *et al.* 1993) ever since, e.g. maturation of the somatic embryos on MS medium with 6% sucrose and regeneration on MS with 2% sucrose, led to the establishment of fertile maize plants. Chapter 2 presents the growth responses of the inbred lines A188 and A632 under the influence of various improved media

selected for micropropagation. The carbon source sucrose was replaced by the sugar alcohol sorbitol to test whether embryogenic capacity could be improved in inbred A188 or induced in immature embryos of inbred A632. Furthermore, the inheritance of embryogenic capacity was investigated in reciprocal crosses between the two inbred lines.

In summary, the growth response of the two inbred lines was studied after exposure to 2 mg/l 2,4-D in the induction medium of various compositions. The differences in the two inbred lines were compared with respect to the induction of embryogenic and non-embryogenic callus, and the formation, maturation, and regeneration of somatic embryos.

Uptake and biochemical analysis of 2,4-D in cultured embryos

Having analysed the differences in culture response the question arose whether the differences in growth response of the two inbred lines were caused by differences in uptake of 2,4-D, or by differences in sensitivity to 2,4-D in the scutellum cells. To answer these questions, the uptake of 2,4-D from the induction medium by the two inbred lines was examined in Chapter 3. After uptake the nature of the 2,4-D had to be established to determine whether the 2,4-D was present as free, conjugated, or metabolised inactive 2,4-D. Free 2,4-D is considered to be active as an auxin, and conjugated 2,4-D can be set free after hydrolysis.

Thus we questioned whether differences in the uptake and nature of 2,4-D would explain the difference in growth response observed between the two inbred lines. To this end the uptake was quantified and the biochemical nature of the 2,4-D was determined.

Distribution of 2,4-D in cultured embryos

Differences in growth response by the cells of the scutellum of both inbred lines could well be explained by the presence or absence of 2,4-D in certain regions of the embryo. The uptake and biochemical analysis of 2,4-D both do not tell where the 2,4-D is present in the embryo. Therefore uptake and distribution were analysed after 16 h of culture on induction medium labelled with ^{14}C-2,4-D. The distribution of 2,4-D was studied in median sections of embryos of the two inbred lines by autoradiography of ^{14}C-2,4-D. The redistribution of 2,4-D was

determined after 24 h and 72 h of subculture on medium without ^{14}C-2,4-D. The results of these experiments are described in Chapter 4.

Thus the distribution of 2,4-D over the tissues of the cultured embryos was studied to answer the question, whether differences in the distribution of 2,4-D are the reason why the same zones of the cultured A188 and A632 zygotic embryos show different growth responses.

Influence of concentration and duration of exposure to 2,4-D on the culture response of maize embryos

Under standard culture conditions 2 mg/l 2,4-D is used in the induction medium and embryos are cultured on such medium for 14 days. Because it was found that such culture can be considered as a culture under exhaustive conditions (Chapter 3), the influence of short pulses (0.5 h - 24 h) and long pulses (1-13 days) with 2 mg/l 2,4-D on the culture reaction of embryos of A188 was studied. After exposure, the influence of 2,4-D on morphogenesis was determined to elucidate the minimal culture time needed for the induction of embryogenic callus and somatic embryos in A188. Additionally, the concentration of 2,4-D in the induction medium was varied from 1.10^{-3} to 1.10^{+3} mg/l to determine the influence of the concentration of 2,4-D on the morphogenetic response in both inbred lines. These experiments are described in Chapter 7.

Thus the morphogenetic consequences of short pulses with 2,4-D are investigated, to answer the question of the duration of exposure to 2,4-D needed to obtain embryogenic callus. Embryos were cultured under a range of 2,4-D concentrations to answer the question which concentration is optimal for embryogenic callus induction and what the consequences are when culture is performed at another 2,4-D concentration.

Influence of TIBA and 3,5-D on the culture of maize embryos

Polar auxin transport inhibitors influence the transport of auxins from cell to cell by interference with the efflux carrier in the plasma membrane of cells. The influence of tri-iodobenzoic acid (TIBA) was investigated in both inbred lines under conditions where

embryos were cultured without 2,4-D and with 2,4-D. Morphogenetic responses were studied. Additionally, the auxin analogon 3,5-dichlorophenoxyacetic acid (3,5-D) was studied. This to answer questions on its influence on embryogenesis and induction of callus in cultured embryos of maize, alone or in combination with various concentrations of 2,4-D in both inbred lines. These experiments are described in Chapter 7.

Thus the morphogenetic responses on TIBA, an auxin transport inhibitor, and on the auxin analogon 3,5-D are investigated to answer the question of their influence during differentiation in cultured embryos of maize.

Auxin binding proteins (ABP-1) and auxin signal transduction

Addition of 2,4-D to the medium leads to a growth response in the scutellum cells of cultured embryos. The 2,4-D, which mimics the action of the natural auxin indoleacetic acid (IAA), causes the competent cells in the scutellum of the immature embryo to redifferentiate to dividing cells, resulting in the formation of callus. Although not the whole signal transduction pathway of auxins is known today, there is evidence for the existence of several auxin binding proteins in plant cells, which might function as hormone receptors (Barbier-Brygoo *et al.* 1989, Venis *et al.* 1990). Several theories for auxin and ABP action have been proposed over the years (Klämbt 1990, Cross 1991, Jones and Prasad 1992). Applying immunocytochemical methods we approached the question on the (sub)cellular distribution of maize auxin-binding protein (ABP-1) in coleoptiles and embryos, as well as the possible role of ABP-1 during the induction of embryogenic callus in cultured embryos of A188. These experiments are described in Chapter 5.

Thus the presence and distribution of ABP-1 in coleoptiles and cultured embryos was studied with polyclonal antibodies to answer the question to what extent ABP-1 might play a role in the induction of callus in the embryogenic inbred line A188.

mRNA synthesis during induction of embryogenic and non-embryogenic callus

The induction of callus in the two inbred lines is initiated after the uptake of 2,4-D. By this uptake the cells of the scutellum, competent for renewed cell division, change fate, expressed in amongst others renewed mRNA synthesis. The reprogramming of mRNA synthesis and the subsequent induction of callus were studied in the two inbred lines by the use of oligo-dT probes to answer the question of the difference in growth response of the lines with respect to the distribution of unprocessed mRNA. The experiments are described in Chapter 6.

Thus, the unprocessed mRNA distribution during early stages of embryogenic and non-embryogenic callus development in maize was studied in relation to the difference in culture response of the two inbred lines.

Outline of the thesis

The various chapters of the thesis describe the influence of the growth regulator 2,4-D on the induction of callus in two maize inbred lines: the embryogenic line A188 and the non-embryogenic line A632. In Chapter 2 the induction of callus and regeneration of somatic embryos is compared in the two inbred lines under various culture conditions and in reciprocal crosses. In Chapter 3 the uptake of the growth regulator 2,4-D is studied during the first 14 days of culture by the two inbred lines on induction medium. In Chapter 4 the distribution of ^{14}C-2,4-D is investigated by autoradiography during the first 3 days of culture, in which embryogenic and non-embryogenic callus formation is induced. In Chapter 5 the maize auxin-binding protein (ABP-1) is studied in coleoptiles and in cultured zygotic embryos in order to investigate the distribution and the possible role during induction of callus on cultured maize embryos. In Chapter 6 the distribution of poly(A)+ containing mRNA is studied during induction of callus in the embryogenic and non embryogenic inbred line. In Chapter 7 the morphogenetic influence of the duration of exposure to 2,4-D, as well as the concentration of 2,4-D in the induction medium are investigated. The morphogenetic influences of 3,5-D and TIBA are studied, first alone, then in combination with 2,4-D in the induction medium.

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Chapter 2

Comparative analysis of callus formation and regeneration on cultured immature maize embryos of the inbred lines A188 and A632

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Abstract

Induction, maintenance, differentiation and embryogenic capacity of callus obtained from immature embryos by culture on induction medium, proliferation medium, maturation medium and regeneration medium respectively, were compared for two inbred lines of maize, *i.e.* A188 and A632. The callus of inbred line A188 was embryogenic and maintained embryogenic capacity for at least 1 year. Immature embryos of inbred line A632 formed callus that was not embryogenic. It only produced roots. When sucrose was replaced by sorbitol to induce or improve embryogenesis, again only A188 formed embryogenic callus. Subculture of this callus, however, allowed 4 week intervals in stead of 2 week intervals without loss of embryogenic capacity.

When A188 was pollinated with A632 pollen, embryogenic callus was obtained from cultured immature "F_1" embryos, showing that embryogenic capacity was inherited through the mother. The callus did not differ from the embryogenic callus generated on selfed A188 embryos. When A632 was pollinated with A188 pollen, embryogenic callus was obtained too, showing that embryogenic capacity was also inherited through the father, though the embryogenic capacity diminished quickly, and the stability of the callus was lower than in the reciprocal cross.

Key Words

somatic embryogenesis, tissue culture, *Zea mays* (L.)

Abbreviations

DAP = days after pollination, DIC = days in culture, 2,4-D = 2,4-dichlorophenoxy acetic acid, Dicamba = 3,6-dichloro-o-anisic acid, IM = induction medium, PM = proliferation medium, MM = maturation medium, RM = regeneration medium

Introduction

The first report of regeneration from tissue culture of *Zea mays* was by Green and Phillips in 1975. Since then culture conditions have been improved, and in general MS (Murashige & Skoog 1962) and N6 (Chu et al. 1975) media, in combination with growth regulators like 2,4-D and Dicamba, have been used for the induction of embryogenic callus of *Zea mays* (Duncan et al. 1985; Kamo et al. 1985). The uptake of 2,4-D in cultured maize embryos and the successive biochemical changes were recently analyzed by Bronsema et al. (1996).

When immature embryos of the embryogenic white dent inbred line A188 (pedigree: 4-29 (Silver King)* 46 (N.W.Dent)4^4, Minnesota Agric. Exp. Stn., USA), Green & Phillips 1975; Green & Rhodes 1982; Tomes 1985a) are excised and placed onto culture medium, the scutellum cells become active, the numbers of organelles increase, and vacuolation and nuclear morphology change (Fransz & Schel 1987). This reaction is called the shock response, it lasts for 24h, and is independent of the presence of 2,4-D in the medium. After the shock response a second response is the induction of callus. This is called the growth response and depends on the presence of 2,4-D. It is characterized by mitotic activity in the cells that are sensitive to 2,4-D. In the scutellum of A188 embryos, proliferation was activated strongly, resulting in the appearance of a broad scutellar meristematic zone (Fransz & Schel 1994). A second meristematic region was observed around the coleorhiza.

Two types of embryogenic callus can be distinguished, i.e., Type I and Type II callus (Armstrong & Green 1985). Type I callus, white and compact in appearance, is found in

cultures on MS medium with a high sucrose concentration (6%). Type II callus, soft, white or pale yellow and friable, is obtained when MS or N6 culture medium is used with a low sucrose concentration (2%). The friable callus is valuable for the production of maize suspension cultures or protoplasts with regeneration capacity (Kamo et al. 1987; Rhodes et al. 1988; Prioli & Söndahl 1989; Shillito et al. 1989). A third type of callus formed in maize cultures is rhizogenic callus. This type is also found in non-embryogenic inbred lines as the yellow dent A632 (pedigree (Mt42*B14)B14^3, Minnesota Agric. Exp. Stn., USA; Green & Phillips 1975; Green & Rhodes 1982; Tomes 1985a).

The present study aims at comparing growth responses of an embryogenic line with those of a non-embryogenic line. To this end the induction and growth responses of embryogenic callus of inbred line A188 are compared from the morphological point of view, with the induction and growth of callus in the non-embryogenic line A632. We monitored callus formation under the influence of various media, called induction medium, proliferation medium, maturation medium and regeneration medium, applied in a regeneration protocol adapted from Emons & Kieft (1995), and of carbon sources as sucrose and sorbitol. Additionally the inheritance of embryogenic capacity was investigated in reciprocal crosses between the two inbred lines.

Materials and Methods

Plant material

Plants of the maize inbred lines A188 and A632 (kindly provided by Dr. C.E. Green, University of Minnesota) were grown under greenhouse conditions (22°C/18°C day/night). Additional light was provided by Philips HPI-T 400W metal-halide lamps located 1 m above the plants. Phytotron conditions were 24°C during the 16h light period and 20°C during the 8h dark period, at 70% relative humidity. Light was provided by Philips 50W/84 HF fluorescent tubes at an intensity of 50 W/m^2. Additional red light was provided by Philinea tubes. Cobs were covered with paper bags before silks appeared. Fresh pollen from other plants was collected in the morning and directly used for controlled pollination, 2-3 days after the appearance of the silks. Although donor plants have been raised all year round there was a seasonal response with greenhouse cultured plants to such a degree that they were only used from May till September.

Culture media

The induction medium (IM) consisted of the macronutrients, micronutrients and vitamins of the N6 medium (Chu et al. 1975), supplemented with 20 mM L-proline, 200 mg l^{-1} casein hydrolysate, 2% or 6% sucrose and 8.3 mM 2,4-dichlorophenoxyacetic acid (2,4-D). The medium was solidified by 0.7% Difco Noble agar, autoclaved at 121°C for 20 min and dispersed into 9 cm plastic Petri dishes (25 ml per plate).

Three additional solidified media were prepared comparably: proliferation medium (PM) consisting of induction medium supplemented with 3% mannitol; maturation medium (MM) consisting of MS medium (Murashige & Skoog 1962) supplemented with 6% sucrose without growth regulators; and regeneration medium (RM) consisting of MS medium with 2% sucrose without growth regulators.

Tissue culture

Cobs were taken from the greenhouse or phytotron 11-12 days after pollination (DAP). After removal of the husks, the whole cob was surface sterilized in 1% sodium hypochlorite solution for 15 min. After 2 rinses in sterile water for 10 min each, the upper parts of the caryopses were cut with a surgical blade and up to 10 undamaged opaque zygotic embryos with a length between 1 and 2 mm were placed onto the solidified induction medium in a Petri dish with the root-shoot axis down. Embryos were cultured at 25 ± 2°C in a climate chamber in the dark for 2 weeks. They formed callus which was subcultured on proliferation medium. To keep the callus of A188 embryogenic on media containing 2% sucrose, regular subculture was required every 2 wks. Globular somatic embryos, which were formed on induction and proliferation media, developed further on maturation medium. They were gradually exposed to light by covering the Petri dishes with two layers of paper tissue during the first 3 days of culture on the maturation medium in an illuminated climate chamber. Once transferred onto regeneration medium, plantlets arose from somatic embryos after 10 days. They were subcultured in test tubes or Petri dishes in the phytotron for 1 week and then transplanted to soil and covered by a plastic cup to maintain high relative humidity for the first few days. Plants were grown to maturity to control flowering and seed set.

Sorbitol as carbon source

In induction and proliferation medium, 2% sucrose was replaced by equimolar (1.6%) sorbitol.

Analysis

The influence of the various nutrient media on induction, growth and regeneration of callus was monitored at the onset of culture and then weekly with a Wild binocular dissecting microscope using a WV-E550 Panasonic CCD camera.

The influence of the carbon source (2% sucrose, 6% sucrose or 1.6% sorbitol) in the induction medium on growth was measured after 7 and 14 days of culture. To this end at least 5 Petri dishes with 10 embryos each were set up for each treatment. All embryos were weighed individually. Data of the various treatments were compared using the analysis of variance with two blocks, one for influence of the cob and one for influence of the carbon source (F-test). Significance of the means of the treatments was determined using an t-test (g=0.05).

Genetic component

To determine the inheritance of ability to form embryogenic callus, reciprocal crosses were made between A188 and A632. Immature zygotic F_1 embryos of A188*A632 and A632*A188 were excised 11-12 DAP, placed on induction medium with 2% sucrose and analyzed as described above.

Results

Callus formation on induction medium

Zygotic embryos responded to culture on induction medium with 2% sucrose by increasing in size during the first day of culture. Their weight almost doubled during this time. In line A188 the first signs of callus formation were seen after 4 days of culture, as the swelling of the middle and basal sides of the scutellum (Figure 1d). Despite the high concentration of 2,4-D which normally inhibits cell growth, the coleoptile of the zygotic embryo elongated (see Figure 1a), often in a rotated or corkscrew manner. From 1 week of culture onwards friable embryogenic callus appeared on the basal side of the scutellum. Small globular somatic

embryos were now seen at the surface of the soft friable callus (see Figure 1e). After 14 days of culture some compact embryogenic callus (Type I), but more soft friable embryogenic callus (Type II) was formed on the scutellum of the A188 embryos. Compact embryogenic callus was formed at the middle part of the scutellum and friable embryogenic callus still at the basal side of the scutellum. Maintenance required two weekly subculture on proliferation medium. Growth on induction medium was monitored after 1 and 2 wks (Figure 5). Embryos with callus appeared to have increased in weight almost 100 times after 2 wks.

When immature embryos were cultured on induction medium with 6% sucrose significant more callus was formed (over 180 fold weight increase in 2 wks, Figure 5) but maintenance required weekly subculture on proliferation medium to prevent browning and senescence of the callus. The callus consisted predominantly of the compact Type I callus, which exhibited less but larger somatic embryos than found on the friable Type II callus predominantly obtained on induction medium with 2% sucrose. Embryos were dissected from several cobs, but this appeared to have only a small, though statistical significant, influence on the increase of fresh mass compared to the larger influence of the carbon source. Culture on induction medium in the light was compared with culture in the dark. The growth of the callus did not differ under those two conditions.

During the first 4 days of culture on 2% sucrose induction medium the zygotic embryos of line A632 responded similarly to those of line A188 (see Figure 1g). Then the middle part of the scutellum formed callus. The coleoptile of the zygotic embryos elongated too, but the shape of the coleoptile was straight in comparison with the corkscrew shape of A188. The surface of the scutellum was sometimes hairy as shown in Figure 1h. Contrary to A188, no callus formation was observed at the basal part of the scutellum. The callus was soft and non-embryogenic. When cultured on induction medium with 6% sucrose A632 exhibited increased growth too. Weekly subculture was required to prevent browning and senescence.

Subculture on proliferation medium

A188 embryos with embryogenic friable callus were subcultured on proliferation medium (Figure 1b). Subculture of the embryogenic callus was done every 2 weeks after selection based on the presence of globular somatic embryos. Such callus remained embryogenic for at

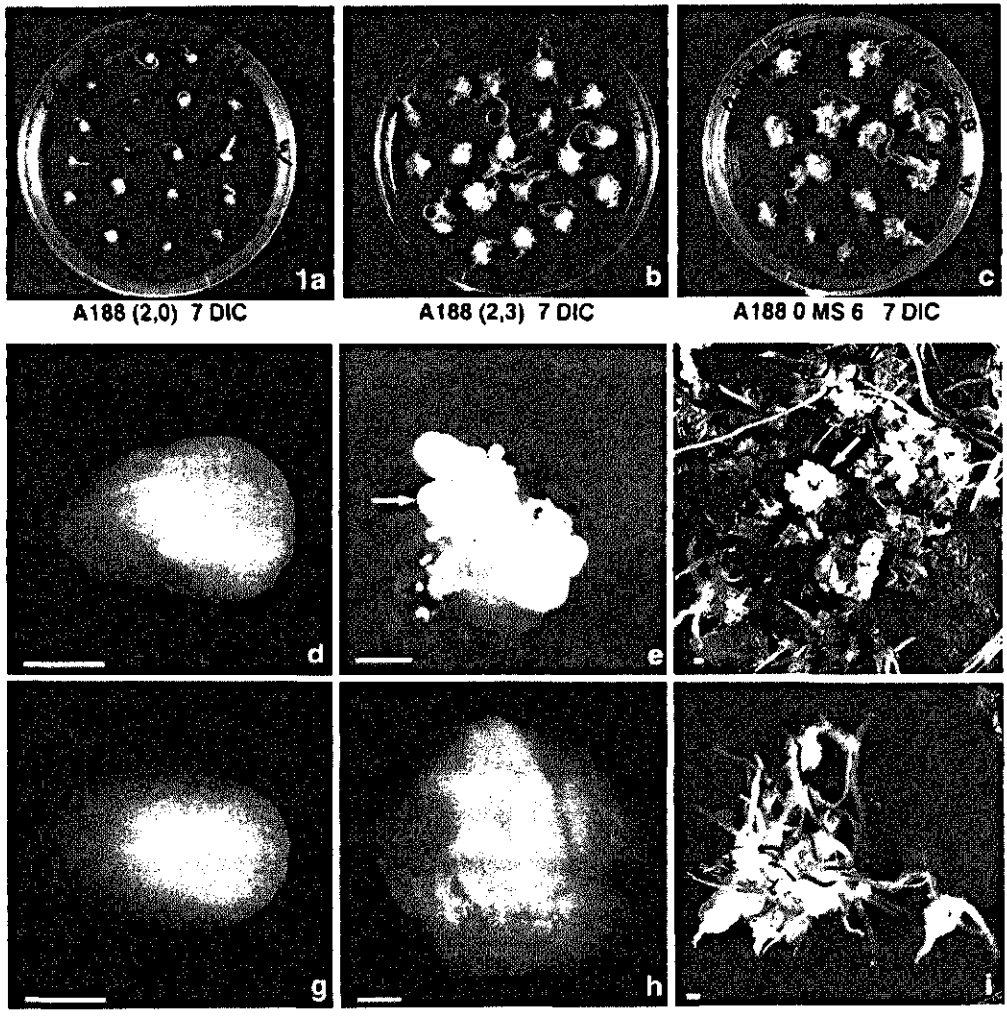

Figure 1: *In vitro* culture of immature zygotic embryos of maize inbreds A188 (a-f) and A632 (g-i) in 9 cm Petri dishes. **a**: Zygotic embryos 7 days in culture (DIC) on induction medium (IM). **b**: Zygotic embryos 7 DIC on proliferation medium (PM) after 14 DIC on IM. Note the increase in size of the proliferating embryos. **c**: Zygotic embryos 7 DIC on maturation medium (MM) after 14 DIC on IM and 14 DIC on PM. **d**: Zygotic embryo 4 DIC on IM. **e**: Globular somatic embryos (arrow) on a piece of friable callus after 14 DIC on IM.
f: Zygotic embryos 7 DIC on regeneration medium (RM) after 14 DIC on IM, 14 DIC on PM and 10 DIC on MM. Note the formation of regions with white scutella (arrow), leaves and roots. **g**: Zygotic embryo 4 DIC on IM. **h**: Zygotic embryo 14 DIC on IM showing a hairy surface. Note the absence of somatic embryos. **i**: Zygotic embryos 7 DIC on MM after 14 DIC on IM and 14 DIC on PM. Root formation is abundant. Bars = 1 mm.

least 1 year. Without mannitol we observed loss of embryogenic capacity after some cycles of subculture. A632 embryos with callus were also subcultured on proliferation medium for several months, but this culture resulted in very poor growth of the callus; roots were formed but often the callus turned brown and died.

Maturation of somatic embryos

The embryogenic callus of A188 was placed on maturation medium, gradually exposed to light, and cultured for at least 10 days (Figure 1c). During this period the globular somatic embryos matured, i.e., they formed a scutellum, a coleoptile-like structure and a rootlet. The scutellum turned white and the coleoptile turned green. Callus from A632 was also placed on maturation medium. The callus responded by the outgrowth of roots which often exhibited red pigmentation (Figure 1i). Some callus turned brown and died.

Regeneration of somatic embryos

Single mature somatic embryos of A188 were excised from callus aggregates and placed on regeneration medium. After 1 week of culture plantlets were formed (Figure 1f) which then were transplanted to soil. Twenty plants were grown to maturity in the greenhouse or phytotron. They showed normal cob formation and appeared fertile. The overall process of somatic embryogenesis from the induction of embryogenic callus to the regeneration of plants is represented schematically in Figure 2.

Callus of A632 was also put on regeneration medium. No part of the callus turned green or formed embryos; only roots were observed.

Sorbitol as carbon source

When sorbitol was used in the induction medium instead of sucrose, A188 formed callus, too (Figure 3a), but there was only a 60 fold weight increase after 14 days (Figure 5). The callus was not as friable as Type II callus, but it was embryogenic and consisted of white- to cream-coloured tissue similar to the Type I embryogenic callus. Compared to inbred A188, A632 also formed less callus on sorbitol containing induction medium. It appeared non-embryogenic.

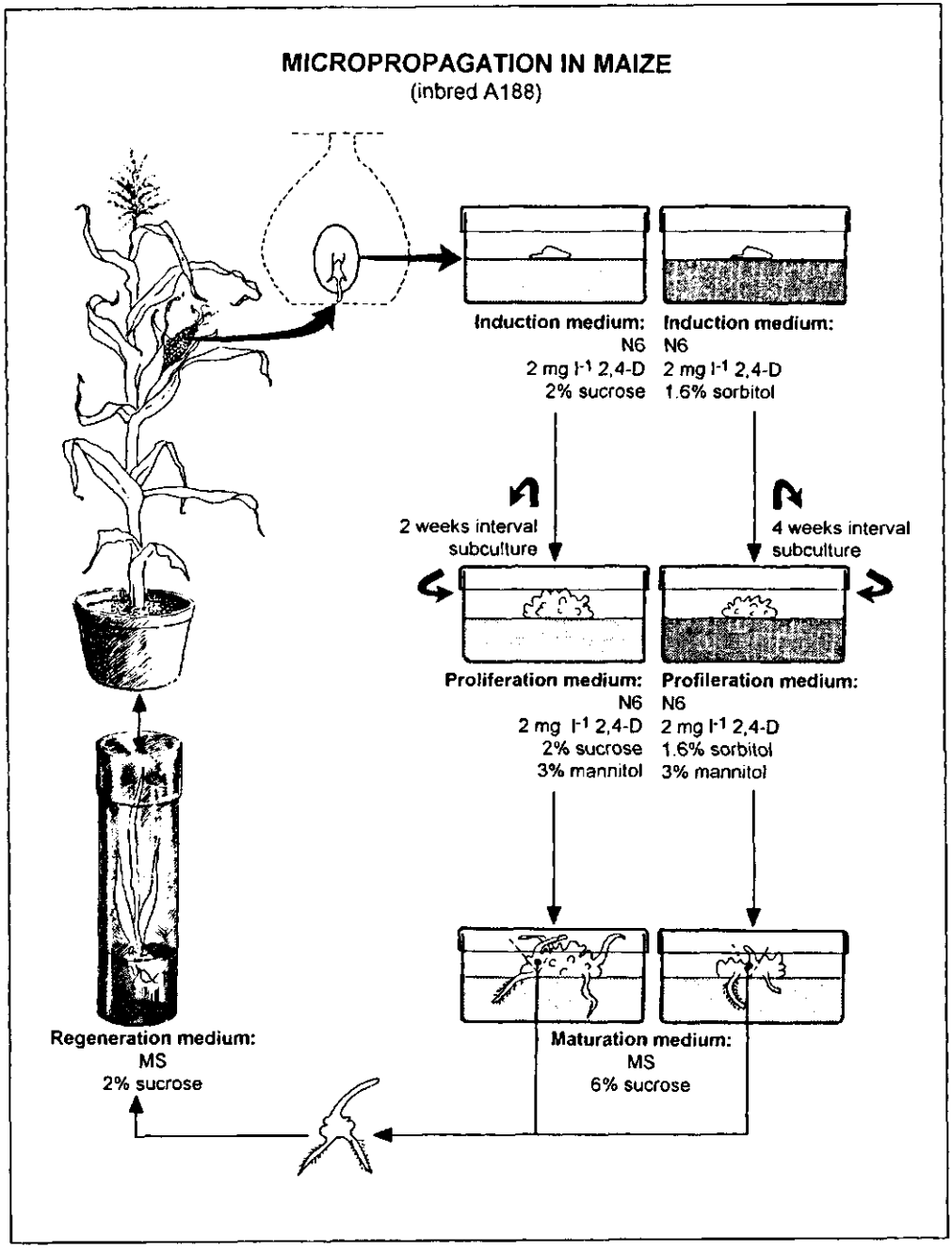

Figure 2: Schematic representation of micropropagation by somatic embryogenesis in maize inbred line A188 on two different sets of culture media.

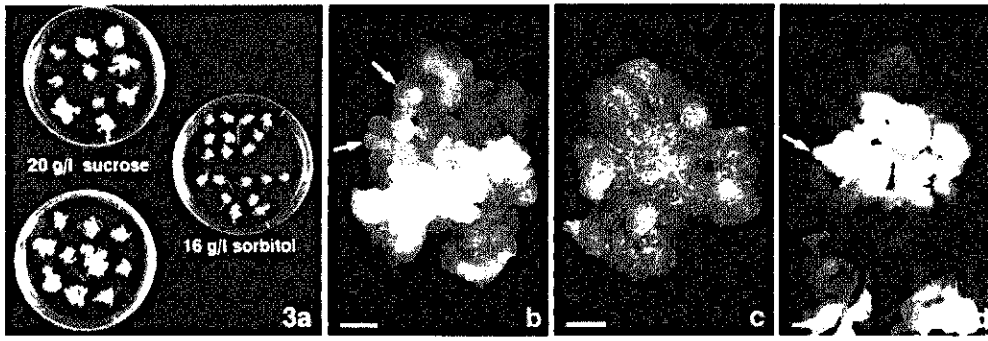

Figure 3: Callus of the maize inbreds A188 and A632 grown on media in which sucrose or sorbitol was the carbon source. **a**: Callus formation of A188 after 4 weeks of culture on induction medium with sucrose as carbon source (left hand side Petri dishes) and induction medium with sorbitol as carbon source (right hand side Petri dish). Note the reduced callus growth on the sorbitol containing medium. **b**: Embryogenic callus of A188 after 6 months of subculture on proliferation medium with sorbitol. Somatic embryos are indicated by arrows. **c**: Callus of A632 formed on proliferation medium with sorbitol. Note the absence of somatic embryos. **d**: Maturation of somatic embryos of A188 after 14 days in culture (DIC) on maturation medium after 14 DIC on induction medium and 14 DIC on proliferation medium. Arrows point to somatic embryos with scutellum-like structure. Bars = 1 mm.

When callus of line A188 was subcultured on proliferation medium with sorbitol instead of sucrose, subculture could be done every 4 weeks instead of every 2 weeks without loss of embryogenic potential (Figure 3b). Somatic embryos further developed on maturation medium with 6% sucrose (Figure 3d) and plants were obtained on regeneration medium (Figure 2). Frequencies of embryo and plantlet formation were similar to those obtained with sucrose containing media. Callus from line A632 was also subcultured on proliferation medium with sorbitol but it hardly grew and did not result in the formation of embryogenic callus (Figure 3c).

Genetic component

F_1 embryos formed after the crosses A188*A632 and A632*A188 all generated embryogenic callus on induction medium. When line A188 was the female parent, friable embryogenic callus was obtained (Figure 4a). It was grown for 1 year and remained equally embryogenic to A188*A188 embryogenic callus.

When line A632 was the female parent the morphology of the embryogenic callus tended more to Type I than to Type II callus, since no soft and friable callus was observed. Somatic embryos were, however, formed (Figure 4b). Subculture of this embryogenic callus on proliferation and maturation medium resulted in maturation of somatic embryos (see Figure 4c) and culture on regeneration medium (Figure 4d) to the regeneration of plants. Despite regular subculture, embryogenic potential diminished quickly and disappeared within 3 months.

Discussion

The inbred lines A188 and A632 were studied in relation to their response to tissue culture (Fransz & Schel 1987, 1991a, 1991b; Van Lammeren 1988). Medium factors such as L-proline (Armstrong & Green 1985), sucrose (Fransz et al. 1987), mannitol (Emons & Kieft 1995) and sorbitol (Swedlund & Locy 1993) affect the formation and maintenance of embryogenic callus. Originally, high sucrose concentrations (6%) were applied in the induction medium of maize cultures (Fransz et al. 1987). This culture condition resulted in the compact Type I callus in inbred A188. Low sucrose concentrations (2%), however, stimulate Type II formation (Lu et al. 1983; Vasil et al. 1985; Tomes 1985a). In our experiments with inbred line A188 we confirmed the change from Type I to Type II by lowering the sucrose concentration from 6 to 2%. Moreover the subculture with 6% sucrose was more laborious since weekly subculture was required. Thus, Type I and type II callus in A188 are governed by the sucrose concentration in the medium. The results with A632, however, show that this influence is genotype dependent.

Subculture

Embryogenic callus was subcultured on proliferation medium which included mannitol. Emons and Kieft (1995) used mannitol in the proliferation medium of genotype 4c1 cultures to maintain the competence of the callus to form embryos rather than to become rhizogenic. The embryogenic callus of A188 was subcultured with mannitol for at least a year and the callus remained embryogenic throughout the culture. Without mannitol we indeed observed loss of embryogenic capacity after some cycles of subculture. Subcultured rhizogenic callus of A632

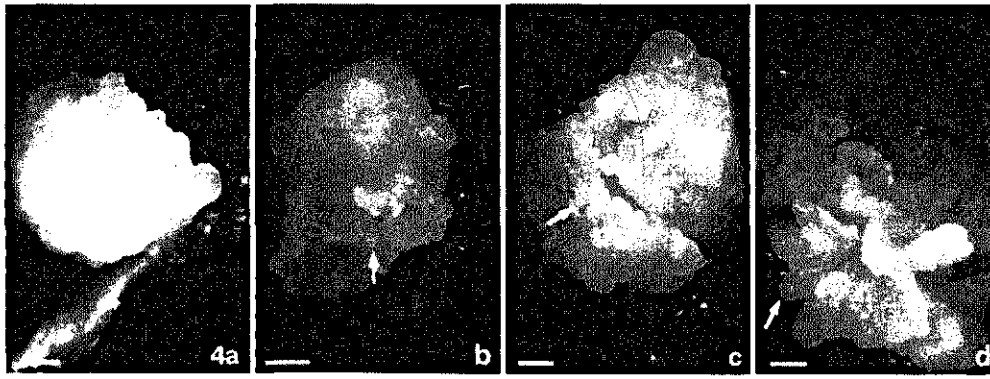

Figure 4: Embryogenic maize callus of A188*A632 (a) and A632*A188 (b-d). **a**: Zygotic embryo of A188*A632 12 days in culture (DIC) on induction medium (IM). Note small globular somatic embryo (arrow). **b**: Zygotic embryo of A632*A188 13 DIC on IM. Arrow points to somatic embryo. **c**: Zygotic embryo of A632*A188 1 DIC on maturation medium (MM) after 14 DIC on IM and 14 DIC on proliferation medium (PM). Arrow points to somatic embryos. **d**: Zygotic embryo of A632*A188 1 DIC on regeneration medium after 14 DIC on IM, 14 DIC on PM, and 10 DIC on MM. Bars = 1 mm.

hardly grew on mannitol containing proliferation medium and often died. This characterizes again the difference with A188.

The maturation phase of the somatic embryos of 4c1 on maturation medium (MS medium with 6% sugar) was characterized by the formation of meristems in the globular embryos, by the formation of a starchy scutellum, and by the repression of precocious germination (Emons et al. 1993). By reducing the sucrose concentration in the medium from 6 to 2%, that is transfer from maturation to regeneration medium, calli formed roots and shoots via organogenesis (Emons & Kieft 1991), and thus somatic embryos developed to fertile plants. With our culture system, embryogenic callus of inbred line A188 was obtained in a way comparable to 4c1, a hybrid containing A188 as one of the 4 parents (Mórocz et al. 1986). Subculture of embryogenic callus on maturation medium and regeneration of somatic embryos was carried out routinely and showed that the method provides a suitable tool for vegetative propagation of maize (Figure 2). Since the inbred line A632 only produced rhizogenic callus and could not be regenerated under our culture conditions it appears that vegetative propagation still depends on the genetic properties of the varieties or inbred lines used. It does, however, not exclude the possibility that deficiencies in the medium cause the

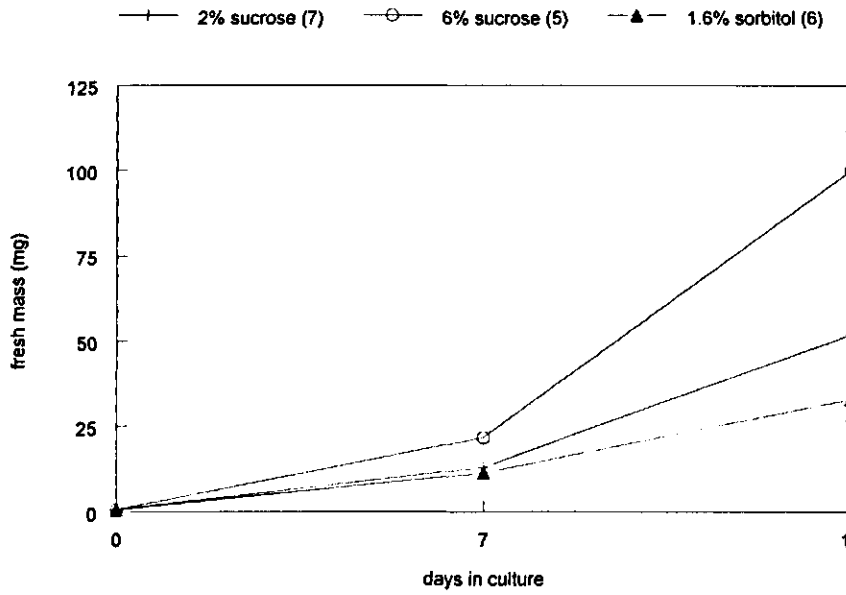

Figure 5: Influence of the carbon source on callus initiation and growth on immature maize embryos of inbred line A188 cultured on induction medium during 14 days. Data points are the means of at least 5 replicates per treatment with their standard deviation. Each replicate consisted of the average fresh weight of 10 embryos from 1 Petri dish, individually measured at day 7 and 14. Embryos were excised from 3 different cobs. Initial average embryo weight was 0.53 mg.

different responses because Duncan et al. (1985) reported successful regeneration with A632 when cultures were treated with Dicamba.

Sorbitol

Growth of embryogenic callus on media containing sucrose is typified by both embryogenic (regenerable) and non-embryogenic (non-regenerable) callus. Because non-embryogenic callus is formed, selection is required with each subculture. Swedlund and Locy (1993) reported that the use of sorbitol only supports the growth of embryogenic callus. Culture on sorbitol would therefore simplify the culture procedure since the time-consuming selection of

embryogenic callus would be abolished. They also observed a greater regenerative capacity (more plants per gram fresh weight of callus) for callus grown on sorbitol than callus grown on sucrose. To determine the influence of the type of carbohydrate on embryogenesis of A188 and A632 cultures, the source of carbon was changed from sucrose to the six-carbon sugar alcohol sorbitol. We found that there was induction of embryogenic callus on A188 but also induction of non-embryogenic callus in the case of A632. Since non-embryogenic callus was also formed with A188 at the onset of culture we cannot confirm the findings of Swedlund and Locy (1993) and still needed selection of embryogenic callus in the first round of subculture to keep the culture embryogenic. Zygotic embryos taken from the same cob produced less callus on sorbitol than on sucrose containing medium. So the overall lower growth rate of cultures grown on sorbitol compared to cultures grown on sucrose was confirmed in our experiments (cf Swedlund & Locy 1993). It might explain the longer subculture intervals possible on sorbitol containing media (4 vs. 2 weeks).

For maintenance purposes the 4 weekly subculture on sorbitol containing media is advantageous because it is less laborious whereas the embryogenic capacity is not lost (Figure 2).

Genetic component

Tomes (1985b) found that the callus type response varied considerably among genotypes capable of plant regeneration. It was suggested that Type I and II culture responses are conditioned by separate nuclear determinants. Hodges et al. (1985) found that the inheritance of somatic embryogenesis and plant regeneration in maize primarily involved two genes. These genes come to expression in the abaxial region of the middle and basal side of the scutellum of immature zygotic embryos when cultured under the proper conditions. In A188 these regions proliferate and form embryogenic callus. These regions do not proliferate in the case of A632; only non-embryogenic callus is produced in the adaxial region of the scutellum close to the coleorhiza. It is known that embryogenic callus meristems are formed exogenously while in rhizogenic callus they are formed endogenously (Emons et al. 1993). Likely the formation of exogenous meristems is blocked in A632 under the conditions applied but it

remains to be established whether A632 is really non-embryogenic in the presence of 2,4-D or only recalcitrant.

In our experiments with the crosses between two inbred lines we determined to what extent the capacity to form embryogenic callus under the given culture conditions was influenced maternally and paternally. The genetic information needed for embryogenic callus formation in the case of A632 being the mother, was transferred through A188 pollen. This clearly demonstrates the genotype effect. The callus obtained from 12 day old immature F_1 embryos, however, lost its embryogenic capacity rather quickly. As it is unlikely that twelve day old embryos still have paternal influences other than through the genome, it is likely that the genes needed for the formation of embryogenic callus were switched on only shortly, resulting in the early loss of embryogenic capacity by ongoing culture.

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Chapter 3

Uptake and biochemical analysis of 2,4-D in cultured zygotic embryos of *Zea mays* L.

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Summary:

In order to determine the influence of 2,4-D on the regeneration of zygotic embryos of maize, the uptake and metabolism of 2,4-D by immature embryos were compared in an embryogenic inbred line (A188) and a non-embryogenic inbred line (A632), cultured on induction medium with 2 mg/L 2,4-D which was partly ^{14}C-labelled.

Uptake of 2,4-D under exhaustive conditions, i.e. without subculture, was analyzed from the onset of culture until 14 days of culture. During the so called shock response period, i.e. the first 24h of culture, uptake of 2,4-D was observed in both lines, with a higher uptake in the embryos of A632. The availability of 2,4-D in the medium became a limiting factor for uptake from 3 days of culture onwards. Then the concentration of 2,4-D per gram fresh weight was up to 125 times higher than the concentration in the induction medium for both inbred lines. Differences in uptake between the two lines were observed until 5 days of culture. In this period a lower concentration of 2,4-D per gram fresh mass was found in A188. After 5 days of culture, however, the concentrations of 2,4-D per gram fresh mass were comparable in both lines.

Therefore the difference in embryogenicity is likely not caused by differences in uptake of 2,4-D. Supplementing TIBA to the induction medium under exhaustive conditions caused a drop in the uptake of 2,4-D in both inbred lines, but TIBA neither inhibited the uptake completely nor prevented the induction of callus. As killed embryos only showed a residual uptake, not influenced by TIBA, it is concluded that immature embryos of the two inbred lines do accumulate the greater part of the 2,4 D in an active manner.

The ^{14}C labelled 2,4-D which had accumulated in cultured embryos was analyzed biochemically. Up to 70% of the radioactive 2,4-D accumulated in A188 embryos was present as free 2,4-D after 24h of culture, and 37% in A632 embryos. Conjugation of 2,4-D to sugars and amino acids started after 16h of culture. Metabolization of 2,4-D was observed from 2h onwards and increased with ongoing culture. As compared to A188, A632 embryos showed a higher metabolization rate of 2,4-D. It might well be that the higher levels of free 2,4-D in A188, together with the higher metabolization of 2,4-D in A632 do cause differences in the influence of 2,4-D on the 2 inbred lines, i.e. the different response on tissue culture.

Thus, it is concluded that the developmental differences found in cultured immature embryos of embryogenic and non-embryogenic inbred lines might be caused by differences in levels of free 2,4-D and metabolization of 2,4-D. This could be regulated by genetical differences between the two inbred lines. However, other genetical factors might determine the sensitivity of the embryos to 2,4-D as well.

Keywords

Zea mays L., A188, A632, 2,4-D, 2,4-dichlorophenoxyacetic acid, hormone analysis, somatic embryogenesis, TIBA, triiodobenzoic acid.

Abbreviations

DAP = days after pollination, DIC = days in culture, 2,4-D = 2,4-dichlorophenoxyacetic acid, GC-MS = gas chromatography-mass spectrometry, IAA = indoleacetic acid, IS-HPLC = ion suppression-high pressure liquid chromatography, IAA = indoleacetic acid, LC-MS = liquid chromatography-mass spectrometry, TIBA = triiodobenzoic acid

Introduction

When immature zygotic embryos of inbred lines A188 and A632 of maize are cultured on complex nutrient medium containing 2,4-D, A188 forms embryogenic callus, whereas A632 forms non-embryogenic callus. The embryogenic callus is maintained in the presence of 2,4-D, but after lowering the concentration of 2,4-D or its removal, somatic embryos will mature and develop to fertile plants when cultured appropriately (Green, 1983). Somatic embryos not only developed from callus, but also directly from zygotic embryos (Fransz *et al.*, 1987). Thus 2,4-D appears necessary for the induction of callus formation and for the onset of somatic embryogenesis. Recently, maize lines were compared with respect to sexual (Van Lammeren, 1986) and vegetative propagation (Fransz and Schel, 1987; Fransz and Schel, 1991; Emons and De Does, 1993b). The sites of callus formation and somatic embryogenesis were not randomly distributed over the embryo but restricted to certain zones (Fransz and Schel, 1987). Embryogenesis generally occurred in areas away from the zone where the embryo had contact

with the nutrient medium and only after a period of at least one week of growth on the nutrient medium. Because 2,4-D enters the immature embryo from the nutrient medium, and is necessary for the induction of callus formation in both lines, it is of interest to know when and how the 2,4-D is taken up by the embryo, how the uptake proceeds during the culture period, and if there are differences in these aspects between embryogenic and non-embryogenic inbred lines.

In this study we therefore aim at investigating the uptake of 2,4-D in cultured zygotic embryos of A188 and A632. The 2,4-D, taken up by the embryo, is analyzed biochemically to determine whether the growth regulator was free, conjugated or metabolized (Cohen and Bandurski, 1982). The effects of the application of triiodobenzoic acid (TIBA) which blocks polar transport of auxins, on the uptake of 2,4-D, and the effect of 2,4-D on the levels of the endogenous auxin IAA were analyzed as well.

Materials and methods

Culture conditions and uptake of ^{14}C-2,4-D

Maize plants (*Zea mays* L.) were grown in a growth chamber with a 16h/8h day/night regime at 24/20°C. Immature embryos of both embryogenic (A188) and non-embryogenic (A632) inbred lines (kindly provided by dr. C.E. Green, Minnesota) with sizes ranging from 1-2 mm, were excised 11-12 days after pollination (DAP). The embryos were cultured in the dark on a modified N6 medium (Chu *et al.*, 1975) containing 2 mg/L 2,4-D, 2.3 g/L L-proline, 200 mg/L casein hydrolysate, and 2% sucrose, pH 5.8 (Emons *et al.*, 1993a). Twenty five embryos were cultured in a petridish (ϕ 9-cm) containing 25 mL medium. To determine the uptake of the growth regulator by scintillation counting, 0.5 µCi/L of 2-^{14}C labelled 2,4-D (specific activity: 12.7 mCi/mmol) was added to the culture medium, now containing 2.009 mg/L 2,4-D.

The uptake of labelled 2,4-D during the first 14 days was studied under three different conditions. 1) Embryos were kept on the same medium for the whole period, called exhaustive condition. 2) Embryos were cultured on exhausting medium for varying periods of time ranging from zero to 13 days, and thereafter they were subcultured on ^{14}C-labelled 2,4-D medium during another 24h. 3) Embryos were subcultured daily on fresh, labelled medium with 2 mg/L 2,4-D to prevent exhaustion. After various hours or days of culture samples of 10-

25 embryos were removed from the culture medium, dried on filter paper, weighed, frozen in liquid N_2, and stored at -20°C until further processing. For measurements of the concentration of 2,4-D in the medium, pieces of agar were taken, before and after culture, frozen in liquid nitrogen and used for scintillation counting. Each sample was collected in 0.5 mL millipore water and boiled for 30 min. Finally 4 mL of scintillation fluid were added and the samples were measured twice in a Beckman LS 6000TA scintillation counter for 30 min with a 2.5% error count and background subtraction. Data of the uptake in the embryos are depicted per embryo and per gram fresh mass.

In order to examine the influence of TIBA on the uptake of 2,4-D, some embryos were preincubated for 8h on a culture medium with TIBA (5 mg/L) but without 2,4-D and then they were cultured on media with TIBA and 2,4-D. Other embryos were not preincubated with TIBA but grown on culture medium with various combinations of 2,4-D (2 mg/L) and TIBA (2 or 10 mg/L). Embryos which were killed by freezing in liquid N_2, were placed on the same media and analyzed as controls.

Identification of ^{14}C-labelled compounds by IS-HPLC.

In order to determine the nature of the accumulated ^{14}C compounds, embryos were cultured on the modified N6 medium with 2 mg/L ^{14}C-2,4-D (specific activity 12.7 mCi/mmol). After various hours of culture, samples of 25 embryos each were dried on filter paper, frozen in liquid nitrogen, and extracted overnight in 100% MeOH. Each extract was then divided into 2 parts. One part was used for the separation of 2,4-D and 2,4-D conjugates by Ion-Suppression-HPLC (Rosil C18-RP Column, MeOH/H_2O/HAc : 50/49.5/0.5, flow 0.5 L/min). Fractions of 0.5 mL were collected, 3 mL scintillation fluid was added, and the ^{14}C contents was counted. Concentrations of 2,4-D were calculated by means of the specific radioactivity of the ^{14}C-2,4-D. The second part of the extract was submitted to a strong alkaline hydrolysis (7N NaOH, 100°C for 3h under N_2) and analyzed for total (free, esterified and amide bound) 2,4-D (Bialek and Cohen, 1989). After hydrolysis the sample was diluted tenfold, brought to pH 2.5 with 2N HCl at 0°C and purified on a bond-elut C18-cartridge. The sample was further submitted to IS-HPLC as described above.

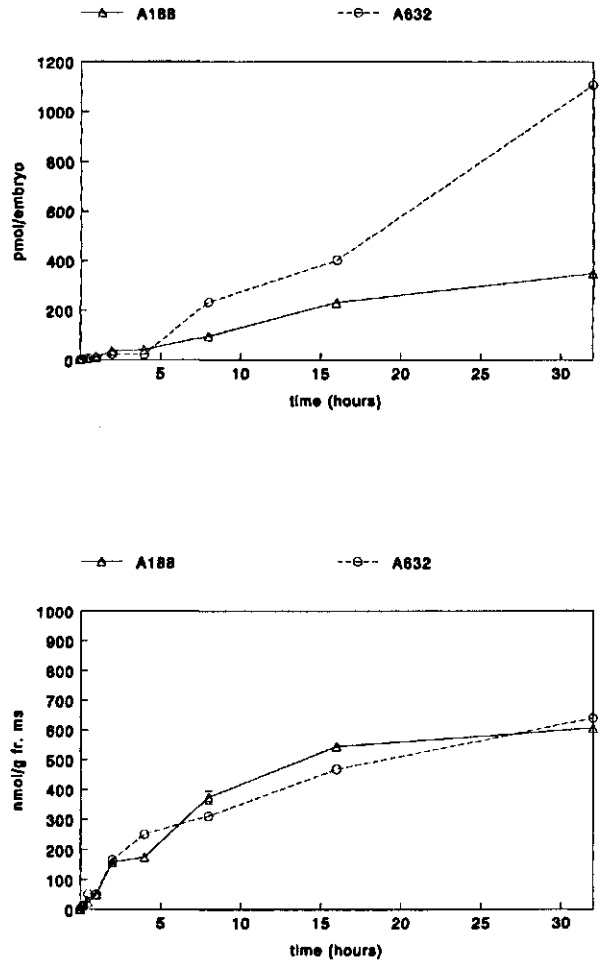


Figure 1: a Uptake of 2,4-D in excised immature maize embryos during the first 32h of culture as measured by scintillation counting of ^{14}C-2,4-D. Data depicted as pmol 2,4D per embryo. **b** Data depicted as nmol 2,4D per gram fresh mass. Each data point is determined by counting a sample of 25 embryos. All embryos originated from one cob for each inbred line. The data for A188 are given as means of two independent experiments with their standard deviation.

Analysis of IAA and IAA conjugates by GC-MS.

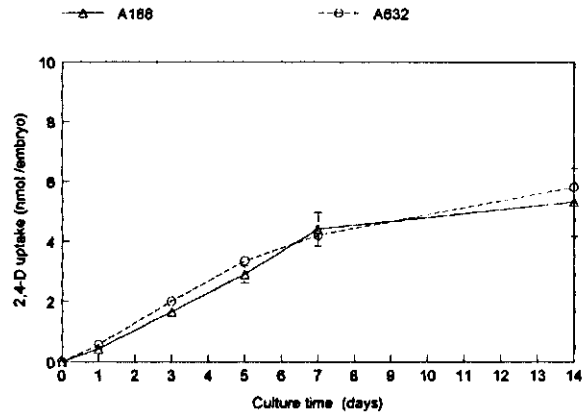
To determine the effect of 2,4-D on the endogenous indoleacetic acid (IAA) content, we measured free and conjugated IAA in embryos of the two inbred lines after cultivation on

medium with or without 2,4-D. Embryos were extracted overnight in 100% MeOH and 200 ng $^{13}C_6$-IAA (Cambridge Isotope Lab) was added. Part of the sample was submitted to alkaline hydrolysis (see above). The free IAA was purified on a DEAE-sephadex column (formiate conditions), eluted with 6% formic acid and bound to a bond-elut C18 column with 5 mL diethylether and dried *in vacuo* (Prinsen *et al.*, 1991). After the alkaline hydrolysis and purification on a C18-cartridge, the fraction which contained the total amount of IAA was further purified and analyzed as the free IAA mentioned above. Prior to analysis on GC-MS, the samples were methylated with diazomethane (Schlenk and Gellerman, 1960) and derivatized (Pilet and Saugy, 1985). After drying the methylated fraction under N_2, it was derivatized with heptafluorobuturylimidazole (HFBI) (Pilet and Saugy, 1985). The reaction took place in sealed reaction vials at 85°C for 2h. After cooling, the samples were diluted with milliQ H_2O and the Me-IAA-HFBI was extracted with hexane and dried under N_2. GC-MS analysis was performed on a VG TRIO 2000 Mass Spectrometer (EI^+) on line with a Hewlett Packard 5890 series II gas chromatograph equipped with 14 m DB 1, 0.25mm I.D., 0.25 df column, and a temperature gradient from 50 to 300°C. The diagnostic ions were at m/z 326 and 332 corresponding to Me-IAA-HFB and $^{13}C_6$-Me-IAA-HFB, monitored in SIM.

Results

Uptake of ^{14}C-2,4-D.

The uptake of 2,4-D in embryos was first studied under exhaustive conditions. During the first 32h of culture, embryos of both inbred lines took up 2,4-D from the medium. **Figures 1a, b** show the dynamics of uptake measured from 0.5h of culture onwards. Embryos of A632 took up more 2,4-D compared to embryos of A188. Measured per gram fresh mass only a slightly higher concentration for A632 was observed, due to the higher average weight of A632 embryos at the time of excision. Prolonged culture showed that embryos accumulated ^{14}C-2,4-D until day 7 (**Fig. 2**). The two inbred lines did not differ in the uptake of 2,4-D per embryo. In the same experiment the concentration of 2,4-D was determined in the culture medium used throughout the culture period. It was found that the concentration of 2,4-D decreased from the onset of the experiment falling down to 20% of the initial concentration



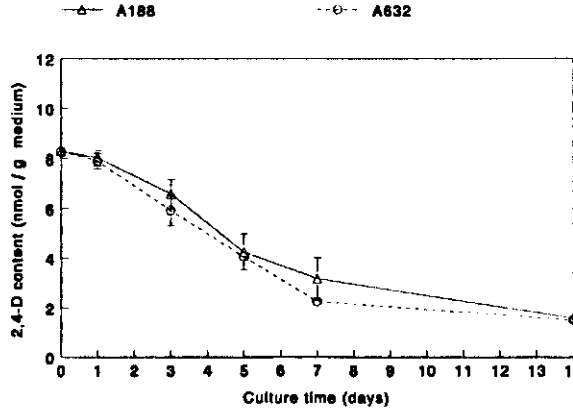
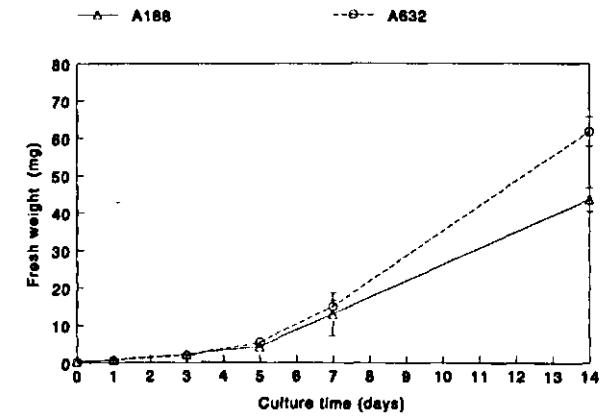


Figure 2: Uptake of 2,4-D per embryo during the first 14 days of culture as measured by scintillation counting of ^{14}C-2,4-D. Data given as means of two independent experiments with their standard deviation.

Figure 3: Concentration of 2,4-D in the modified N6 culture medium during the first 14 days of culture as measured by scintillation counting of ^{14}C-2,4-D. Data given as means of two independent experiments with their standard deviation.



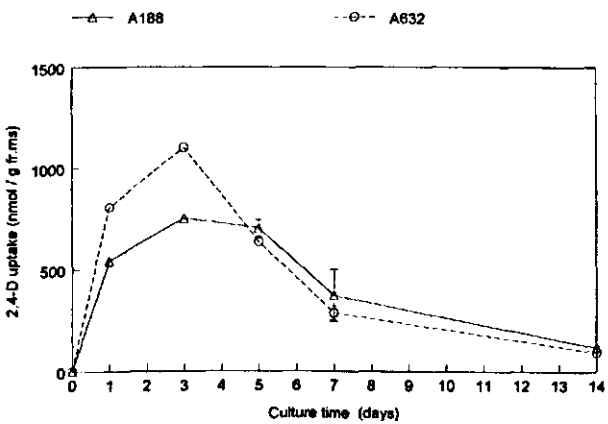
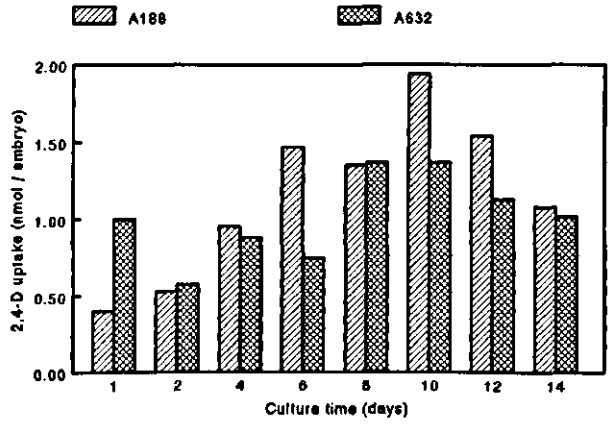


Figure 4: Increase in fresh weight of the zygotic embryos during the first 14 days of culture. Data given as means of two independent experiments with their standard deviation.

Figure 5: Uptake of 2,4-D per gram fresh mass during the first 14 days of culture as measured by scintillation counting of ^{14}C-2,4-D. Data given as means of two independent experiments with their standard deviation.

at day 14 (**Fig. 3**). Measurements showed that the increase in 2,4-D in the embryos coincided with the decrease in concentration in the agar medium. The ratio of 2,4-D in the embryo tissue versus the nutrient medium rose up to 125 (cf. **Figs. 3** and **5**). Comparing the accumulation of 2,4-D in embryos and taken the increase of weight into account (**Fig. 4**), it was found that inbred line A632 exhibited a faster uptake per gram fresh mass than the inbred line A188 during the first 3 days of culture (**Fig. 5**). After 5 days of culture the concentration per gram fresh mass was comparable in the two lines. The decrease per gram fresh mass during further culture coincided with the formation of callus. In embryos of A188 embryogenic callus was formed whereas in A632 only non-embryogenic callus was obtained.
In a second series of experiments embryos were also cultured under exhaustive conditions for increasing periods of time, and then pulse labelled with ^{14}C-2,4-D during the last 24h of culture in order to analyze the uptake capacity of the embryos at the end of the culture period. The uptake of 2,4-D is given in the **Figs. 6** and **7**. Embryos of both A188 and A632 accumulated ^{14}C-2,4-D. There was an increase in uptake per embryo in the culture period until day 10, especially for A188. The uptake of ^{14}C-2,4-D per gram fresh mass, however, decreased gradually until day 14 due to the increase of the average weight of the embryos in both lines (**Fig. 7**). The uptake per gram fresh mass was highest at the onset of culture (day 1 and 2) and highest in A632.
In a third experiment the exogenous 2,4-D concentration of 2 mg/L was maintained by daily subculture on fresh medium. A trace of ^{14}C-2,4-D was added to the medium to quantify the uptake. The accumulation of 2,4-D by embryos grown under such conditions is given in **Figs. 8** and **9**. During the first 10 days of culture the accumulation of 2,4-D increased at higher rates than observed in **Fig. 2** in which embryos were cultured under exhaustive conditions, and the accumulation of 2,4-D in A632 was higher than in A188 embryos. From 12 days onwards the accumulation in A188 was highest. The accumulation of 2,4-D, expressed as nmol per gram fresh mass (**Fig. 9**), was comparable to that found in **Fig. 5** during the first 4 days under exhaustive conditions. Thereafter uptake under non-exhausting conditions decreased, however, much slower than under exhausting conditions (**Fig. 5**). Embryos, cultured under non-exhaustive conditions, accumulated up to 2.5 times the concentration of



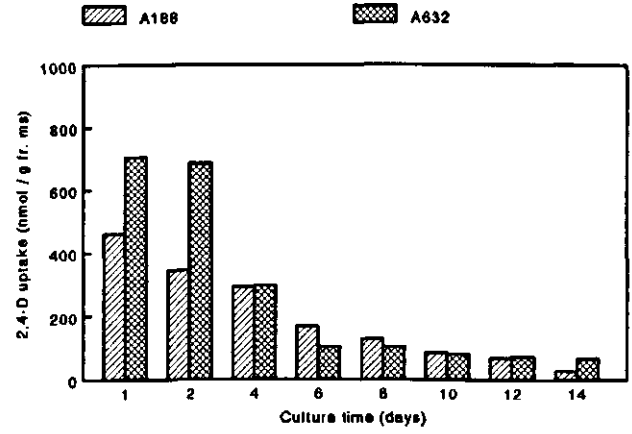


Figure 6: Uptake of 2,4-D per embryo during the last 24h of culture on modified N6 medium after preincubation on exhausting medium for zero up to 13 days, measured by scintillation counting of ^{14}C-2,4-D. Each data point is determined by counting a sample of 25 embryos. All embryos originated from one cob for each inbred line.

Figure 7: Uptake of 2,4-D per gram fresh mass during the last 24h of culture on modified N6 medium after preincubation on exhausting medium for zero up to 13 days, measured by scintillation counting of ^{14}C-2,4-D. Each data point is determined by counting a sample of 25 embryos. All embryos originated from one cob for each inbred line.

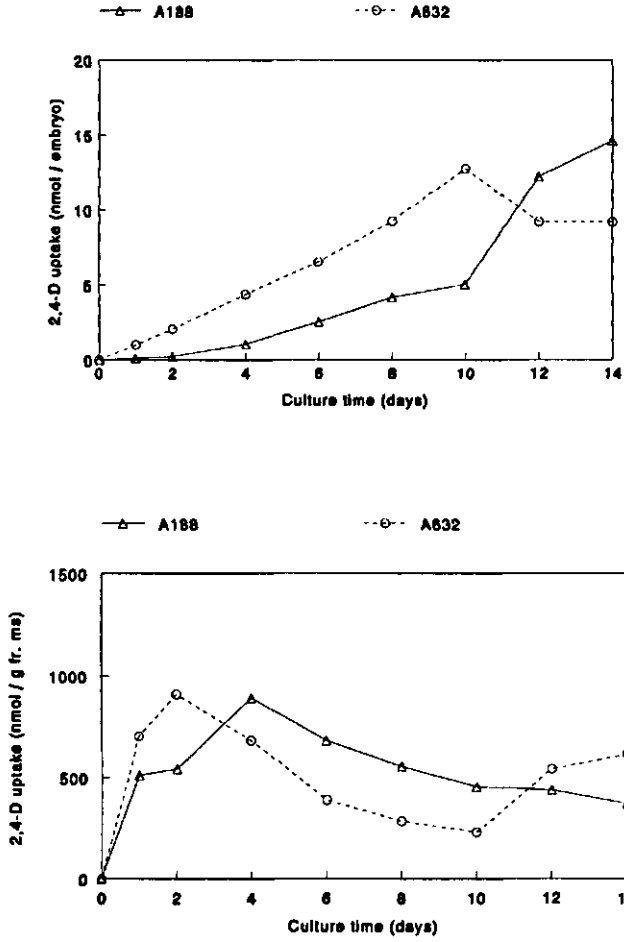


Figure 8: Uptake of 2,4-D per embryo during the first 14 days of culture on medium with a constant 2,4-D concentration, measured by scintillation counting of ^{14}C-2,4-D. Each data point is determined by counting a sample of 25 embryos. All embryos originated from one cob for each inbred line.

Figure 9: Uptake of 2,4-D per gram fresh mass during the first 14 days of culture on medium with a constant 2,4-D concentration measured by scintillation counting of ^{14}C-2,4-D. Each data point is determined by counting a sample of 25 embryos. All embryos originated from one cob for each inbred line.

2,4-D found in embryos cultured under exhaustive conditions (cf. **Figs. 2** and **8**). Under these conditions both inbred lines still formed callus although less than observed under exhaustive conditions.

Influence of TIBA on the uptake of 2,4-D.

TIBA blocks the polar transport of auxins. It was added to the culture medium to investigate its influence on 2,4-D uptake (**Table 1**). Pretreatment with TIBA (5 mg/L) reduced the uptake of 2,4-D by 30% for A188 and by 21% for A632. Competition of 2 mg/L TIBA with 2 mg/L 2,4-D during the uptake, reduced the uptake of 2,4-D by 47% for A188 and by 60% for A632 (**Table 1**). Pretreatment with TIBA, before competition, showed that this reduction was only slightly enlarged.

Culture with 10 mg/L TIBA as competitor reduced the uptake to 31% for the embryogenic and to 24% for the non-embryogenic line. When control embryos were first killed by submersion in liquid nitrogen and then cultured, they accumulated only 15% (A188) to 10% (A632) of the ^{14}C-2,4-D normally found. Pretreatment of these dead embryos with TIBA did not influence the residual uptake.

Although a lower uptake of 2,4-D was observed during competition experiments with TIBA, embryogenic callus was still formed in A188, whereas A632 again only formed non-embryogenic callus.

Identification of the ^{14}C-labelled compounds by IS-HPLC.

When embryos were cultured for 2h on medium containing ^{14}C-2,4-D, IS-HPLC analysis showed that most of the radioactive signal was still free 2,4-D (**Table 2**). After 24h of culture up to 70% (A188) and 37% (A632) of the total uptake of 2,4-D was free 2,4-D. Conjugation of 2,4-D started at 16h after the onset of the culture. Hydrolysis showed that 2,4-D was conjugated to sugars and amino acids. In both inbred lines first an accumulation of free 2,4-D was observed, then conjugation started (**Table 2**). These conjugation data were confirmed by LC-MS analysis (unpublished). The quantity of metabolized 2,4-D rose from 0.5% at 2h to 10.5% after one day of culture for A188 and from 8.3% to 40.8% for A632.

Table 1: Influence of 8h pretreatment with 5 mg/L TIBA, and competition with 2 or 10 mg/L TIBA on the uptake of 2,4-D by immature excised maize embryos cultured with 2 mg/L ^{14}C-2,4-D for 16h. The uptake of 2,4-D after 16h without pretreatment is given as 100%. Reduction in uptake of 2,4-D given in brackets. Each measurement is the value obtained from a sample of 25 embryos. All embryos originated from one cob.

pretreatment	treatment	uptake A188 %	uptake A632 %
0 mg/L TIBA	0 mg/L TIBA	100	100
0 mg/L TIBA	2 mg/L TIBA	53 (47%)	40 (60%)
0 mg/L TIBA	10 mg/L TIBA	31 (69%)	24 (76%)
0 mg/L TIBA	killed embryo	15 (85%)	10 (90%)
5 mg/L TIBA	0 mg/L TIBA	70 (30%)	79 (21%)
5 mg/L TIBA	2 mg/L TIBA	48 (52%)	37 (63%)
5 mg/L TIBA	10 mg/L TIBA	24 (76%)	22 (78%)
5 mg/L TIBA	killed embryo	16 (84%)	10 (90%)

Analysis of IAA and IAA conjugates by GC-MS.

To determine the influence of 2,4-D on the endogenous IAA levels, embryos were cultured in the presence or absence of 2,4-D and analyzed for free and conjugated IAA (**Table 3**). A rise in free IAA was observed after 2h of culture in the absence of 2,4-D for the inbred line A188. This augmention in free IAA was absent when A188 embryos were cultured in the presence of 2,4-D. The inbred line A632 contained less total IAA initially, and a rise of free IAA at the onset of culture, as observed in line A188 in the absence of 2,4-D, was not observed. Culture in the presence of 2,4-D resulted in an initial drop (during the first 8h of culture) in the levels of conjugated IAA in both inbred lines.

Discussion

Uptake of ^{14}C-2,4-D on exhausting medium

Immature maize embryos put in culture show a shock response during the first 24h of culture. In this period the numbers of organelles increase, vacuolation and nucleolar morphology change but cells do not divide yet (Fransz and Schel, 1987). It was reported that the shock response occurred in both inbred lines and was independent of the presence of 2,4-D in the

Table 2: IS-HPLC analysis of ^{14}C-2,4-D after uptake by the immature embryos of A188 (A,B) and A632 (C,D) during the first 24h of culture. A and B, and C and D are data of independent experiments. Data in brackets are the standard errors of two replicate injections. The 2,4-D is distinguished as free, conjugated (conj), metabolized (met) or total (tot) amount and expressed in nmol per gram fresh mass. Each measurement is the value obtained from a sample of 25 embryos. All embryos of each experiment originated from one cob.

A A188

Time (h)	2,4-D free	2,4-D conj	2,4-D met	2,4-D tot
2	40 (± 4)	0	0.4(± 0.1)	40.4
4	60 (± 7.5)	0	5 (± 1.2)	65
8	90 (± 10)	0	10 (± 2.5)	100
16	160 (± 8)	0	40 (± 5)	200
24	220 (± 11)	50 (± 6)	20 (± 3)	290

B

Time (h)	2,4-D free	2,4-D conj	2,4-D met	2,4-D tot
2	50 (± 3.7)	0	0	50
4	140 (± 8)	0	8 (± 1.6)	148
8	180 (± 10)	0	20 (± 3.4)	200
16	240 (± 23)	60 (± 4.3)	50 (± 4.2)	350
24	270 (± 18)	50 (± 6)	60 (± 3)	420

C A632

Time (h)	2,4-D free	2,4-D conj	2,4-D met	2,4-D tot
2	50 (± 5)	0	6 (± 1)	56
4	100 (± 12)	0	10 (± 2)	110
8	140 (± 21)	0	12 (± 3)	152
16	140 (± 11)	100 (± 12)	14 (± 3)	254
24	120 (± 9)	80 (± 8)	100 (± 10)	300

D

Time (h)	2,4-D free	2,4-D conj	2,4-D met	2,4-D tot
2	80 (± 8)	0	5 (± 1)	85
4	140 (± 7)	0	10 (± 2)	150
8	260 (± 13)	0	40 (± 3)	300
16	370 (± 43)	0	180 (± 13)	550
24	200 (± 10)	100 (± 10)	280 (± 22)	580

medium (Fransz and Schel, 1987). In the present study we analyzed whether uptake of 2,4-D already took place during the 24h shock response and if uptake differed in the embryogenic and non-embryogenic lines. It is now shown that both inbred lines accumulated 2,4-D against

the concentration gradient during the shock response period. Only minor differences in uptake between the two inbred lines were observed. Since excised embryos only form callus in the presence of 2,4-D, the regulator appears necessary for the growth response following the shock response. The early uptake of 2,4-D is therefore a prerequisite for further development but does not influence the first cytological changes during the shock response period.
During the first two weeks of culture the uptake of 2,4-D per embryo was comparable for the two inbred lines. Values of up to 125 times the concentration of 2,4-D in the medium were found in embryos of both inbred lines after 3 days of culture. Embryos of A632 were the smallest at the first three days of culture but they grew faster than A188 embryos and hence showed less 2,4-D per gram fresh mass after two weeks. The reduction of the 2,4-D concentration per gram fresh mass appears to be mainly caused by the intensive growth in the root-shoot axis. This is the region where rhizogenic callus is formed (Fransz *et al.*, 1990).

Uptake of 2,4-D at the end of the culture period

The uptake of 2,4-D from fresh medium during the last 24h of culture was determined to reveal the uptake capacity and dynamics of the embryos at the end of culture, when first cultured under exhaustive conditions for various periods of time. Up to 10 days of culture, uptake increased per embryo but it decreased per gram fresh mass already from the first day of culture onwards. Because the medium contained less and less 2,4-D by ongoing culture we expected to find increased uptake of 2,4-D in time. This appeared not to be the case. The limited uptake might be caused by the restricted contact area between embryo and agar medium and physiological limitations in velocity of uptake which is an active process as demonstrated above with the experiments with TIBA.

Culture on non-exhausting medium

Compared to culture on exhausting medium, culture in the constant presence of 2 mg/L 2,4-D resulted in higher 2,4-D uptake per gram fresh mass from 4 days of culture onwards. In both inbred lines 2,4-D now accumulated during the whole culture period although the increase per gram fresh mass was maximal at day 3 again and decreased thereafter, but less than

Table 3: GC-MS analysis of IAA and IAA-conjugates in cultured maize embryos during the first 24h of culture. Embryos were cultured without (A and C) or with (B and D) 2 mg/L 2,4-D in the medium. Data in brackets are the standard errors of two replicate injections. Concentrations of IAA are given in nmol per gram fresh mass. Each measurement is the value obtained from a sample of 25 embryos. All embryos originated from one cob.

A A188 - 2,4-D

Time (h)	IAA free	IAA conj
0	1.4 (± 0.1)	140 (± 7)
2	100 (± 5)	90 (± 5)
4	66 (± 3)	990 (± 50)
8	2.0 (± 0.1)	160 (± 8)
16	2.0 (± 0.1)	4.0 (± 0.2)
24	1.2 (± 0.6)	50 (± 3)

B A188 + 2,4-D

Time (h)	IAA free	IAA conj
0	1.4 (± 0.1)	600 (± 30)
2	1.7 (± 0.1)	14 (± 1)
4	4.00 (± 0.02)	17 (± 1)
8	0.50 (± 0.03)	150 (± 8)
16	7.40 (± 0.04)	3.2 (± 0.2)
24	5.70 (± 0.03)	1.0 (± 0.1)

C A632 - 2,4-D

Time (h)	IAA free	IAA conj
0	4.0 (± 0.2)	23 (± 1)
2	1.8 (± 0.1)	780 (± 39)
4	2.3 (± 0.1)	190 (± 10)
8	1.9 (± 0.1)	100 (± 5)
16	4.4 (± 0.2)	22 (± 1)
24	3.8 (± 0.2)	330 (± 17)

D A632 + 2,4-D

Time (h)	IAA free	IAA conj
0	11.0 (± 0.6)	19 (± 1)
2	2.0 (± 0.1)	5.7 (± 0.3)
4	2.7 (± 0.1)	5.4 (± 0.3)
8	0.9 (± 0.1)	4.2 (± 0.2)
16	2.2 (± 0.1)	140 (± 7)
24	3.4 (± 0.2)	100 (± 5)

observed under exhaustive conditions. Thus the uptake by the embryos under exhaustive conditions is limited by the culture medium rather than by saturation of the embryos with 2,4-D. The lower rate of callus formation, as compared to culture on exhausting medium, is likely due to the higher concentrations of 2,4-D in the embryos. This is in agreement with the finding that the culture of embryos on exhausting medium with starting concentrations of 20 and 200 mg/L 2,4-D, gave less callus formation, too (Bronsema, 1996). Embryogenic callus was still formed in A188 but the formation of somatic embryos was restrained. A632 only formed rhizogenic callus. The suppression of embryogenesis in A188 is likely caused by the increased accumulation of 2,4-D, too. A comparable relation was also reported for in the *in vitro* induction and outgrowth of somatic embryos on maize callus explants (Emons, 1994). The 2,4-D kept somatic embryos at the globular stage, but prolonged culture on the same medium resulted in germination of the somatic embryos probably due to exhaustion of 2,4-D in the medium. Although embryos are usually cultured on exhausting conditions to form somatic embryos, the data shown here indicate that embryos can accumulate more 2,4-D when cultured under non-exhausting conditions (cf. the differences in uptake under non-exhausting conditions in **Fig 8** and **9** and under exhausting conditions in **Fig 2** and **5**). So under normal, exhaustive culture conditions, the availability of 2,4-D becomes limited after 3 days of culture. Since it is known that induction of embryogenesis and the outgrowth of embryos are triggered by high and low levels of 2,4-D, respectively, our findings indicate that the induction of embryogenesis is triggered within 3 days, whereas outgrowth of embryogenic cells comes thereafter (Fransz and Schel, 1987). This observation is confirmed by experiments in which 2,4-D was given for defined periods of time at the onset of the culture followed by subculture on medium without 2,4-D (Bronsema, 1996).

Influence of TIBA on the uptake of 2,4-D

It should be emphasized that endogenous and applied auxins move through the plant by polar transport. Auxin inhibitors have the ability to specifically inhibit polar transport of auxins and they act at the site of efflux (Sussman and Goldsmith, 1981). TIBA is a synthetic auxin transport inhibitor which does not bind to the phytotropin binding protein of the efflux carrier system and which itself is polarly transported. It acts at a site distinct from the auxin binding

site on the efflux carrier and is thus active in a non-competitive manner to auxins (Lomax *et al.*, 1995). To distinguish whether TIBA prevents the uptake of 2,4-D or interferes with 2,4-D during uptake in cultured maize embryos, we successively treated the immature embryos with TIBA and 2,4-D, or applied both compounds simultaneously.

Pretreatment of excised embryos with TIBA resulted in some reduction of 2,4-D uptake. Culture with 2 or 10 mg/L TIBA in combination with 2 mg/L 2,4-D further reduced the uptake of 2,4-D, and most reduction was found after a combination of pretreatment and culture with 2,4-D together with 10 mg/L TIBA. As it was found that the simultaneous incubation of 2,4-D with 2 mg/l TIBA was more effective than pretreatment with 5 mg/l TIBA, we suggest that the blocking of polar transport by TIBA might well be based upon a reversible interaction between TIBA and the transport mechanism.

The remaining uptake of about 24%, was partly due to aspecific binding since control experiments with killed embryos showed that 2,4-D uptake was not neglectable but reduced to 15%. The residual concentration of 2,4-D accumulated above the background, i.e. 9%, appeared still high enough to induce embryogenic callus. It was recently confirmed that 2,4-D applied in concentrations ranging from 0.2 to 2 mg/L, all induced callus formation (Bronsema, 1996). Only high concentrations of TIBA i.e. up to 100 mg/L prevented callus formation. This points to a further reduction of 2,4-D uptake when higher concentrations of TIBA are used (Bronsema, 1996).

The residual uptake of 2,4-D in freeze-killed embryos was not influenced by TIBA, as expected. The uptake might be due to diffusion into the embryo, which cellular structure was damaged by the formation of ice crystals. Additionally it might be caused by residual 2,4-D, present in the water film surrounding the embryo.

Identification of the ^{14}C compounds

As only free 2,4-D induces callus formation and embryogenesis, it is of great interest to determine whether the radioactive signals detected are free, conjugated or metabolized forms of 2,4-D. Hydrolysis of inactive conjugates forms a source for active free 2,4-D (Cohen and Bandurski, 1982). During the first 24h of culture the bulk of the supplied 2,4-D is available as free 2,4-D for the induction of callus formation. From 16h onwards, 2,4-D is partly conjugated

to amino acids and sugars, through amide and ester bonds, respectively. The time of initiation of this conjugation varies, depending on the inbred line, and the developmental stage of the embryos. In all cases there remained a level of free 2,4-D sufficiently high to initiate callus formation during the early stages of culture. It is remarkable that A188 showed 70% of the radioactive 2,4-D was free 2,4-D, whereas A632 only showed 37% in free form. This difference might well influence or explain the difference in reaction of the two inbred lines upon culture.

Initially only a small percentage of the 2,4-D was metabolised, but after 24h this percentage increased significantly. The metabolization of 2,4-D was higher in A632 than in A188 after 24h of culture. Again this might point to a different influence of 2,4-D in the two inbred lines. The metabolism of 2,4-D as well as the exhaustion of the nutrient medium contributed to the decrease of 2,4-D, needed for the outgrowth of callus and somatic embryos in the later period of culture. This also explains why the maintenance of embryogenic callus and suppression of embryo formation requires regular subculture.

IAA and IAA-conjugates

We questioned whether the developmental differences in the two inbred lines, A188 and A632, were due to a difference in IAA contents caused by the application of 2,4-D. Upon 2,4-D treatment a reduction of total IAA in both inbred lines was observed. The kinetics of free IAA, after the application of 2,4-D, are similar in both lines and thus not responsible for the differences in development. The general effect on total IAA, however, indicates that 2,4-D might prevent germination of embryos, when cultured without 2,4-D, by lowering the total IAA contents in both lines.

Our findings that the use of 2,4-D results in a drop of IAA levels, is in contradiction with results found by Michalczuk *et al.*, 1992. They found increased IAA levels after the application of 2,4-D in the medium in their experiments with callus and developing embryos of *Daucus carota* cultured in suspension. The differences in IAA contents, observed between embryogenic and non-embryogenic *Daucus* lines, were not found in our maize cultures.

In summary, the 2,4-D uptake does not differ markedly between the two inbred lines, but conjugation and metabolism do. The influence of 2,4-D on the endogenous auxin IAA contents

is quite comparable in both inbred lines in culture with 2,4-D. Therefore we conclude that the developmental differences during tissue culture might be caused by different biochemical changes of the 2,4-D, i.e. levels of free 2,4-D and levels of metabolism of 2,4-D. However, genetic components could also determine the different sensitivity of the embryos for 2,4-D. Such a postulated genetic component is, however, not an absolute block for embryogenesis in the recalcitrant A632 since Duncan reported successful induction of somatic embryogenesis in a later experiment using Dicamba as growth regulator (Duncan *et al.*, 1985). Our results might point to the possibility that metabolic conditions can be created under which sufficiently high levels of free 2,4-D are created in A632, needed for embryogenic pathways.

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Chapter 4

Distribution of [^{14}C] dichlorophenoxyacetic acid in cultured zygotic embryos of *Zea mays* L.

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Abstract

The uptake of 2,4-dichlorophenoxyacetic acid (2,4-D), necessary for the *in vitro* induction of callus formation and somatic embryogenesis in cultured immature maize embryos, was quantified after culture on nutrient medium with [^{14}C]2,4-D. The identity of the ^{14}C label in the embryos was determined by high performance liquid chromatography (HPLC), and its distribution within embryos was visualized on sections of plastic embedded material. Quantification of the ^{14}C label after a pulse label of 16 h showed a hundredfold accumulation of 2,4-D in the embryos with respect to the initial medium concentration. During tissue processing for *in situ* detection of ^{14}C, however, up to 70% of the label disappeared because of the embedding process. The best structural preservation was obtained after ethanol-mediated infiltration of Technovit 7100. Water-mediated infiltration of Technovit 7100 gave the highest retention of ^{14}C. HPLC analysis showed that more than 95% of the residual ^{14}C label found in embryos was still 2,4-D. Autoradiography showed that the embryogenic inbred line A188 contained ^{14}C label in distinct regions of the scutellum, coleoptile, and suspensor. The nonembryogenic inbred line A632 contained more label after 16 h of culture in a different distribution compared with A188. Subculture of the embryos for 24 and 72 h and histologic analysis showed that cell proliferation and callus formation were restricted to specific regions of the embryo in both inbred lines. The pattern of 2,4-D distribution did not codistribute with regions of proliferation, indicating that 2,4-D is not the only trigger for proliferation.

Abbreviations: 2,4-D, 2,4-dichlorophenoxyacetic acid; TIBA, triiodobenzoic acid; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; HPLC, high performance liquid chromatography; IS, ion suppresssion.

Key Words: 2,4-D, *Zea mays* L., callus induction, autoradiography, TIBA

Introduction

Immature maize embryos excised 11-12 days after pollination form callus when cultured on modified N6 medium (Chu et al. 1975) with 9 μM 2,4-dichlorophenoxyacetic acid (2,4-D) or DICAMBA (Green and Phillips 1975, Duncan et al. 1985, Van Lammeren 1988, Emons and de Does 1993, Emons et al. 1993). Depending on the genotype 2,4-D also induces the formation of somatic embryos, e.g. in the inbred line A188. In contrast, the inbred line A632 is unable to regenerate somatic embryos (Van Lammeren 1988, Bronsema et al. 1996, 1997). In both inbreds, the 1st day of culture is characterized by cytologic changes, such as an increase in the number of organelles, and changes in vacuolation and nuclear morphology. Determination of the incorporation of [^{3}H]thymidine in the scutellum showed that the lines had similar mitotic indexes (Fransz et al. 1990). This early phase is called the shock response phase, and aforementioned characteristics also occur in the absence of 2,4-D (Fransz and Schel 1987, 1991). A second phase, the growth response phase, starts after day 1, and, depending on the genotype and culture conditions, variations in developmental patterns occur (Fransz and Schel 1987). After 3 days, A188 embryos form a broad meristematic zone in the scutellum and a meristematic zone around the coleorhiza. These meristematic zones give rise to somatic embryos. In A632, the recalcitrant line, a meristematic zone develops only in the coleorhizal region (Fransz and Schel 1987, Fransz et al. 1990). Our aim was to determine whether the uptake and distribution of 2,4-D differed in cultured embryos of A188 and A632 and whether the distribution pattern was related to the zones of cell proliferation. To this end we quantified the uptake of [^{14}C]2,4-D after the first 16 h of culture, and we visualized the distribution of ^{14}C within the embryos immediately after labeling and after two periods of chase by autoradiography on sections of Technovit-embedded embryos.

Materials and Methods

Plants and culture conditions

Plants of the embryogenic maize inbred line A188 and the nonembryogenic maize inbred line A632 (both kindly provided by Dr. C.E. Green, St. Paul, MN) were grown in a growth chamber with a 16 h/8 h day/night regime at 24/20°C and a light intensity 50 W/m^2. Immature embryos with sizes ranging from 1.5-2.0 mm, were excised 11-12 days after pollination and cultured in Petri dishes (diameter, 6 cm) with 5 mL of modified N6 medium (Chu et al. 1975) for 16 h in the dark at 25°C (Emons et al. 1993). They had their shoot meristem side in contact with the medium, which contained 8 g/liter agar, 2.3 g/liter L-proline, 200 mg/liter casein hydrolysate, 2% sucrose, pH 5.8, and 9 μM [2-^{14}C]2,4-D (Sigma, St. Louis, MO; specific activity, 12.7 mCi/mmol). Some of the embryos were analyzed for the uptake and distribution of [^{14}C]2,4-D immediately after culture. Other embryos were subcultured for 24 h or 72 h on medium in which the ^{14}C-labeled 2,4-D was replaced by unlabeled 2,4-D. Embryos that were killed after excision by submersion in liquid nitrogen were cultured on [^{14}C]2,4-D-containing medium as a control.

The influence of triiodobenzoic acid (TIBA) on the uptake and distribution of [^{14}C]2,4-D was examined by preculturing embryos on N6 culture medium with 10 μM TIBA for 8 h and then culturing them on [^{14}C]2,4-D-containing medium as described above.

Quantification of ^{14}C in cultured embryos

Uptake of [^{14}C]2,4-D and the loss of label during fixation procedures and embedding in Technovit 7100 were determined. After 16 h of culture on labeled medium, samples of five embryos of line A188 were collected, dried on filter paper, weighed, and either (A) processed immedately for scintillation counting (see below); or (B) fixed in a mixture of 2% paraformaldehyde and 3% glutaraldehyde in 0.1 M phosphate buffer for 2 h at room temperature, rinsed in phosphate buffer for 1 h and in Milli-Q H_2O for 30 min, and processed for scintillation counting; or (C) fixed in aldehydes as described above and then processed further in aqueous solutions until the 100% Technovit 7100 embedding step. Procedures B and C were also performed after prefixation in an aqueous solution of 2% 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) for 30 min to bind 2,4-D to structural proteins in

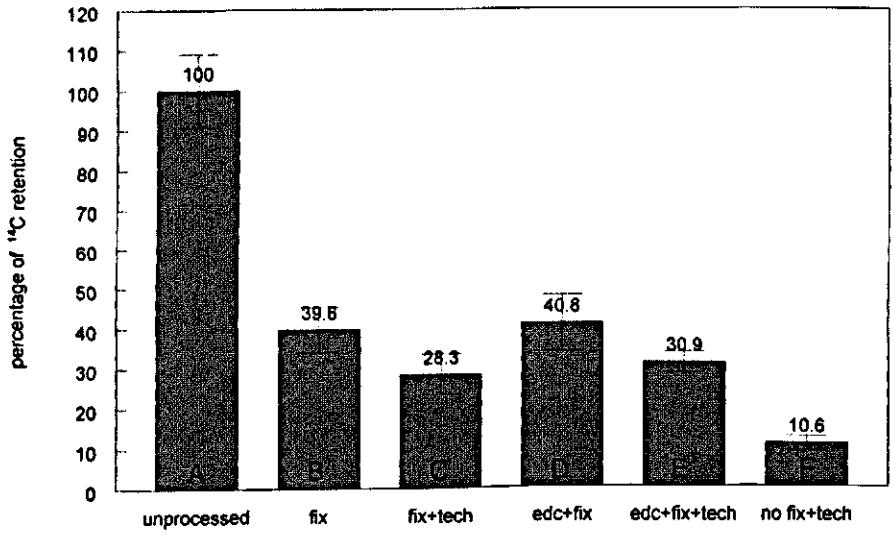

Figure 1. Influence of prefixation, fixation, and embedding in Technovit 7100 on the retention of 2,4-D in cultured maize embryos of A188 after 16 h of culture with 9 μM [^{14}C]2,4-D. Uptake before fixation is 100%. Data are the means of independent experiments (n) with the standard error of the mean. **A**, embryos analyzed immediately after culture without processing (n = 4). **B**, after fixation with glutaraldehyde and paraformaldehyde and rinses (n = 2). **C**, after fixation with glutaraldehyde and paraformaldehyde and water-mediated embedding in Technovit 7100 (n = 4). **D**, after treatment with EDC and fixation with glutaraldehyde and paraformaldehyde and rinses (n = 2). **E**, after treatment with EDC and fixation with glutaraldehyde and paraformaldehyde and water-mediated embedding in Technovit 7100 (n = 4). **F**, no fixation and water-mediated embedding in Technovit 7100 (n = 2).

the cells (Shi et al. 1993) (procedures D and E). As a control, unfixed embryos were kept in water for 3.5 h and then processed through a graded series until the 100% Technovit step (procedure F). Each sample was collected in 0.5 ml Milli-Q H_2O, and boiled for 30 min. Finally, 4 mL scintillation fluid (Ready Safe, Beckman, Fullerton, CA) was added, and the samples were measured in a Beckman LS 6000TA scintillation counter for 30 min with a 2.5% error count and background subtraction.

Fixation and embedding of cultured embryos in Technovit 7100

After culture, embryos were removed from the Petri dish and put on filter paper to reduce the film of attached liquid medium. They were prefixed in an aqueous solution of 2% EDC for 30 min, rinsed in 0.1 M phosphate buffer, pH 7.2, for 30 min, and fixed in a mixture of 2% freshly prepared paraformaldehyde and 3% glutaraldehyde in 0.1 M phosphate buffer for 2 h at room temperature. After rinsing in phosphate buffer for 1 h and Milli-Q H_2O for 30 min, embryos were either infiltrated directly with monomer Technovit 7100 by incubation in aqueous mixtures with increasing concentrations of Technovit up to 100%, or embryos were first dehydrated through an ethanol series and then infiltrated with mixtures of ethanol and increasing concentrations of Technovit. Once in 100% Technovit, the samples were put in plastic molds and polymerized for 1 h at room temperature and for 2 h at 40°C.

In situ localization of ^{14}C in sectioned embryos

Sections of 2 and 4 µm thickness were cut on a Leitz rotation microtome. The median sections of the embryos were mounted on slides by stretching them on drops of Milli-Q H_2O at 40°C and drying them overnight. Slides were dipped in Amersham LM-1 photoemulsion and left for exposure in the dark for 3 weeks at 4°C. The photoemulsion was developed in Kodak D19 (Eastman Kodak, Rochester, NY) for 5 min, rinsed in water, and fixed in Kodak fix for 7 min. Sections were stained with 1% toluidine blue in water, rinsed with water, dried, mounted in DePeX (BDH Laboratory Supplies, Poole, UK), and dried overnight at room temperature. Black and white micrographs were made using bright-field and epipolarization microscopy with a Labophot (Nikon).

Identification of [^{14}C]2,4-D

To identify the nature of the ^{14}C label that caused the autoradiography in the Technovit sections, embryos were first cultured on [^{14}C]2,4-D, prefixed with EDC, fixed with aldehydes, and processed through a graded aqueous series of Technovit. Then four samples of five embryos each were homogenized and extracted overnight in 2 mL 80% methanol instead of polymerizing the 100% Technovit. The tissue was separated by centrifugation (13,000 xg for 15 min at room temperature using a MSE microcentaur, UK). The extracts (supernatants),

containing the nonbound fraction of 2,4-D in the tissue, were counted for ^{14}C by liquid scintillation counting (Tri-Carb 1500, Packard Instruments Inc. Meridan, USA) using Ultima gold (Packard) as scintillator. Data were expressed in dpm. The pellets (remaining tissue) were resuspended in 8N NaOH. Direct tissue hydrolysis was performed for 3 h at 100°C under water-saturated nitrogen conditions (Bialek and Cohen 1989) to hydrolyze all 2,4-D that was apparently bound by the fixation. After hydrolysis, the extracts were titrated to pH 2 and desalted using a C18 cartridge (Varian). These extracts were submitted to HPLC-IS (Shimadzu LC9A pump with on line UV detection at 260 nm; Applied Biosystems 757 absorbance detector, 60/39.5/0.5; methanol/H_2O/acetic acid; v/v/v, flow 0.8 mL/min, Alltech Alltima C18 5 µm, 150 mm x 4.6 mm). The retention time for 2,4-D under these conditions was 15 min. Fractions of 0.8 mL were collected, 4 mL scintillation fluid (Ultima gold) was added, and the ^{14}C content was counted. Concentrations of 2,4-D were calculated by means of the radioactivity at the 2,4-D-specific retention time and the specific radioactivity of [^{14}C]2,4-D (12.7 mCi/mmol). The metabolized 2,4-D was calculated by means of the fraction of radioactivity in the total extract after hydrolysis and desalting before HPLC subtraction by the amount of radioactivity at the 2,4-D-specific retention time where eventual recovery losses during HPLC were taken into account using a [^{14}C]2,4-D reference solution.

Results

Retention and biochemical analysis of ^{14}C in embryos cultured with [^{14}C]2,4-D

The uptake and retention of ^{14}C in unfixed and fixed embryos were quantified by scintillation counting. The filter paper, used to dry the five embryos of each sample, contained 110 dpm of ^{14}C/embryo. Embryos had accumulated an average of 17,668 dpm immediately after culture, which is equivalent to 24,203 dpm/mg or 858 µM 2,4-D. This concentration is 95 times the initial medium concentration (9 µM). About 60% of the label taken up by embryos, was rinsed away during the fixation and rinsing (**Fig. 1**). Further loss of label during the water mediated infiltration of Technovit was about 10%, but unfixed embryos lost up to 90% of the label during the whole embedding procedure (**Fig. 1**). EDC did not improve the retention of ^{14}C during fixation and Technovit embedding.

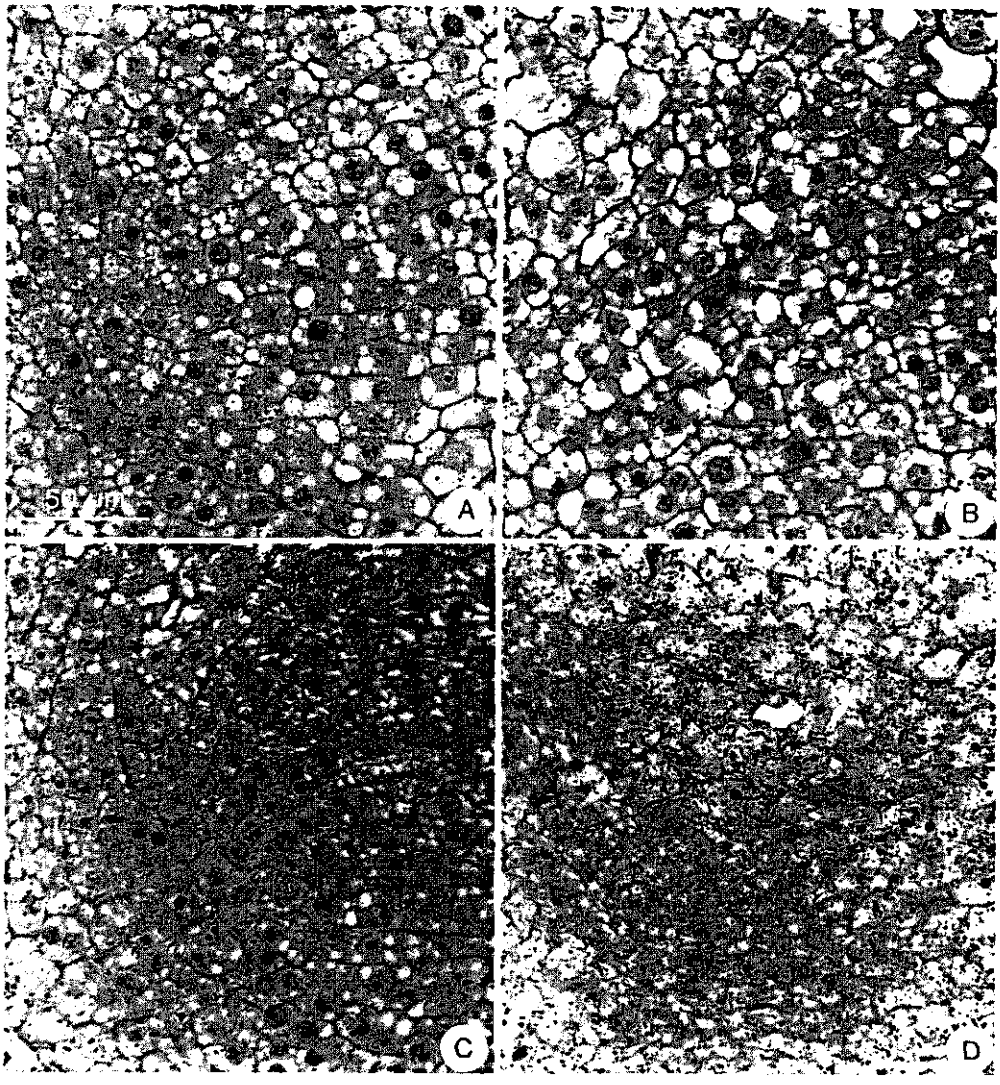

Figure 2. Autoradiographs of 2-μm-thick median sections of cultured maize embryos showing the structural preservation and ^{14}C labeling of the root zone of A188 embryos, fixed and embedded in various ways after pulse label with 9 μM [^{14}C]2,4-D for 16h. Embryos were either obtained after ethanol-mediated Technovit infiltration (**A** and **B**), or via a water-mediated Technovit infiltration (**C** and **D**). Embryos in **A** and **C** were not treated with EDC before aldehyde fixation; embryos in **B** and **D** were treated. The best structure preservation was obtained in **A**, but the most label was observed in **D**. The *bar* represents 50 μm for all figures.

Some embryos were processed until the 100% Technovit step via procedure E, but they were not treated with high temperature for polymerization. Instead they were used to identify the nature of the residual ^{14}C label by methanol extraction and HPLC. A minor part of 3.6 ± 0.9% of the total radioactivity was extracted by methanol. After direct tissue hydrolysis and HPLC, 95.6 ± 0.9% of the radioactivity was detected at the 2,4-D-specific retention time. A minor portion of 0.8 ± 0.1% was not detected at the 2,4-D-specific retention time. Once embedded in Technovit and sectioned, embryos did not loose ^{14}C label as was determined by analysis of the water in which sections were rinsed for 0.5 h.

In situ localization of ^{14}C in embryos cultured with [^{14}C]2,4-D

The influence of tissue processing on the retention of ^{14}C and the preservation of the structure was analyzed on median sections of Technovit-embedded embryos. **Fig. 2** presents four autoradiographs of A188 embryos obtained after 16 h of culture with 9 µM [^{14}C]2,4-D, fixed without or with EDC, and embedded in Technovit after ethanol- or water-mediated infiltration. Ethanol-mediated Technovit infiltration resulted in good preservation of the structure, but labeling was most abundant after water-mediated Technovit embedding (compare **Fig. 2, A** and **B** with **C** and **D**). Prefixation with EDC resulted in decreased cell preservation; but in the case of water-mediated Technovit infiltration, a slightly increased density of silver grains was observed (compare **Fig. 2, C** and **D**).

The distribution of ^{14}C was studied in median sections of embryos after various periods of culture. The topography of a median section is given in **Fig. 3A**. After 16 h of culture with 9 µM [^{14}C]2,4-D, A188 embryos showed labeling in the basal part of the scutellum, in the top part of the scutellum opposite the shoot meristem, in the coleoptile near the scutellum, and in the lower part of the suspensor (**Fig. 3B**; see **Fig. 5**). Most label was found in the scutellum opposite the shoot meristem. In the nonembryogenic line A632 label was observed in the top, middle, and basal part of the scutellum except for the procambium strand, in the coleoptile near the scutellum, and in the lower part of the embryo axis away from the scutellum (**Fig. 4A** and **5**). Compared with A188, line A632 exhibited more silver grains on sections. In both lines low amounts of label were found in the root and shoot meristems of embryos and in the central

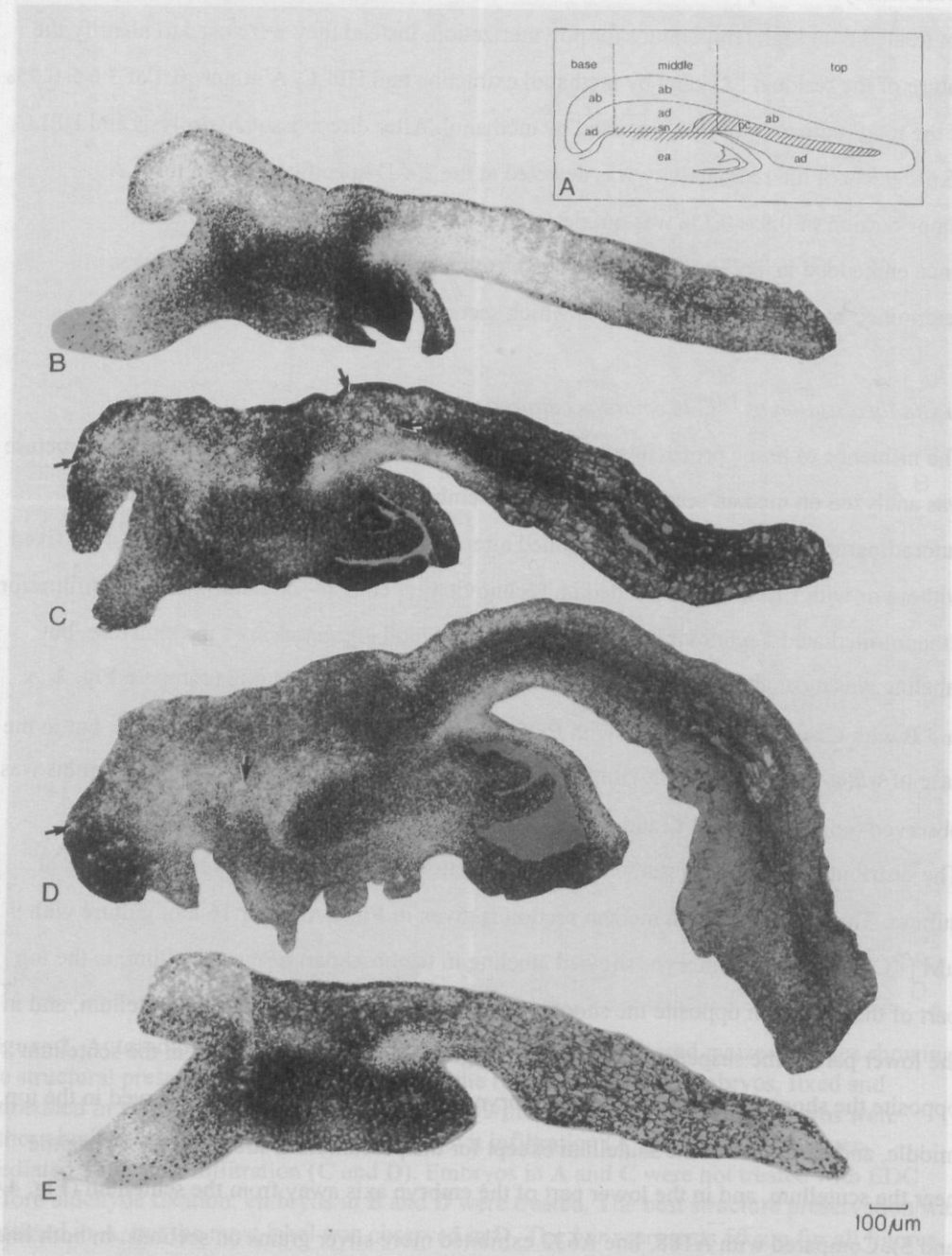
base
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Figure 3. Median sections of immature maize embryos showing histology (**A**) or morphogenesis and distribution of [^{14}C]2,4-D (**B-E**). **A**, schematic representation showing the various subregions. *ab*, abaxial side; *ad*, adaxial side; *ea*, embryo axis; *pc*, procambium strand; *sn*, scutellar node. (Adapted from Fransz et al. 1990). **B-E**, light micrographs showing distribution of [^{14}C]2,4-D by autoradiography and epipolarization illumination in 4-µm sections of A188 embryos. The *white* regions and *spots* in the micrographs are caused by illumination of the silver grains by epipolarization and represent the label in the various tissues of the embryo. The *dark* regions represent the tissues of the embryos after staining with toluidine blue. **B**, distribution after 16 h of pulse with 9 µM [^{14}C]2,4-D. Note the accumulation of label in the basal part of the top region of the scutellum and in its basal zone. **C**, distribution after 16 h of pulse with 9 µM [^{14}C]2,4-D and 24 h of chase with 9 µM 2,4-D. *Arrows* indicate spots of silver grains. **D**, distribution after 16 h of pulse with 9 µM [^{14}C]2,4-D and 72 h of chase with 9 µM 2,4-D. *Arrows* indicate spots of silver grains. **E**, distribution after 8-h pretreatment with 10 µM TIBA and a 16-h pulse with 9 µM [^{14}C]2,4-D. Note the reduced autoradiography signal compared with **B**.

region of the embryo axis. Control embryos, frozen in liquid nitrogen before culture, showed no label above the background after 16 h of culture with 9 µM [^{14}C]2,4-D (**Fig. 4D**).

Other embryos were first cultured on [^{14}C]2,4-D medium for 16 h and then subcultured on chase medium with 9 µM unlabeled 2,4-D for another 24 or 72 h to examine changes in the distribution of ^{14}C label during subculture. After a chase of 24 h, fixation, and embedding, A188 embryos showed most label in the scutellum, predominantly in those parts where it was also found without subculture (see **Fig. 3, B** and **C**). The intensity, however, diminished, especially the high amount of label in the top part of the scutellum opposite the shoot meristem. Distinct spots of label appeared in this region. After a chase of 72 h the distribution of label had changed in A188 embryos. More label was observed in the basal part of the scutellum and in the area around the coleorhiza. Tissues with low concentrations of label had increased in size, especially in the top part of the scutellum (**Figs. 3D** and **5**).

When A632 embryos were subcultured on unlabelled medium for 24 h, the general distribution of label had not changed, but the label intensity had decreased, and distinct spots of label appeared in all scutellar parts (**Figs. 4B** and **5**). After 72 h of subculture, label was still observed in the basal and top parts of the scutellum. The middle part of the scutellum opposite the coleorhiza had enlarged and showed less silver grains. The embryo axis and coleoptile, which had increased in size, exhibited low concentrations of label (**Figs. 4C** and **5**).

In situ localization of ^{14}C in embryos cultured with [^{14}C]2,4-D after pretreatment with TIBA

The influence of an 8-h pretreatment with 10 μM TIBA on the uptake of [^{14}C]2,4-D and the distribution of the label in embryos is shown in **Fig. 3E**. Less label is seen but with a similar distribution, indicating a lower uptake of [^{14}C]2,4-D (compare **Fig. 3, B and E**). Most label accumulated in a region between the scutellum and coleoptile and less at the base of the scutellum and base of the suspensor. After 24 and 72 h of chase the densities of label were lower than shown in **Fig. 3E** (data not shown). A spot-like accumulation was observed at the adaxial side of the scutellum. The regions with most label exhibited fewest enlargement.

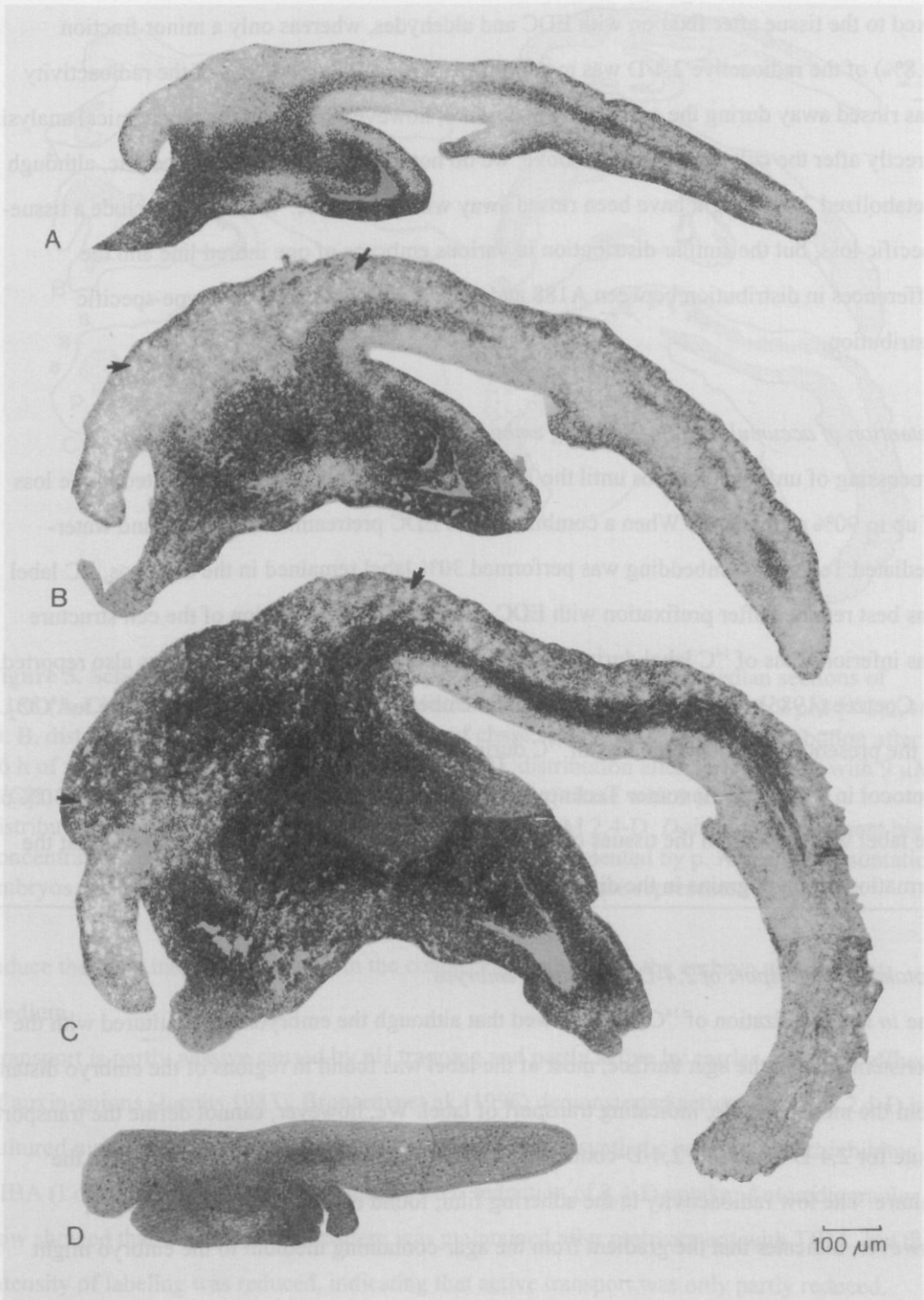
Discussion

Fate of 2,4-D after uptake by cultured embryos

Autoradiography of ^{14}C label on sectioned material cannot distinguish among free, conjugated, or metabolized forms of 2,4-D, because all ^{14}C label will cause the formation of silver grains in the dipping film. Quantitative and qualitative analysis of ^{14}C after uptake in cultured embryos of A188 has shown, however, that after a pulse of 16 h with [^{14}C]2,4-D, 73% of the ^{14}C was free 2,4-D, 11% was conjugated 2,4-D, and 16% was metabolized 2,4-D; embryos took up 275 μM 2,4-D (Bronsema et al. 1996). For A632, 63.5% of the 2,4-D was free after 16 h of culture, 12.5% conjugated, and 24% metabolized, and embryos took up 402 μM 2,4-D. The present results show an accumulation of 858 μM 2,4-D which is about a hundredfold with respect to the initial medium concentration and about three times as much as found previously (Bronsema et al. 1996). The difference could have been caused by a variation in metabolic activity due to seasonal influences.

Determination of the nature of the ^{14}C present in the plastic-embedded embryos is crucial for the interpretation of the autoradiographs. From the biochemical analysis with HPLC it is

Figure 4. Distribution of [^{14}C]2,4-D shown by autoradiography and epipolarization illumination in 4-μm median sections of A632 embryos. **A**, distribution after 16 h of pulse with 9 μM [^{14}C]2,4-D. **B**, distribution after 16 h of pulse with 9 μM [^{14}C]2,4-D and 24 h of chase with 9 μM 2,4-D. **C**, distribution after 16 h of pulse with 9 μM [^{14}C]2,4-D and 72 h of chase with 9 μM 2,4-D. *Arrows* indicate spots of label. **D**, distribution of [^{14}C]2,4-D by autoradiography and epipolarization illumination in 4-μm median sections of an A188 embryo submerged in liquid nitrogen after 16-h pulse with 9 μM [^{14}C]2,4-D.


A
B
C
D
100 µm

concluded that 95% of the ^{14}C analyzed corresponded to free and conjugated 2,4-D which was fixed to the tissue after fixation with EDC and aldehydes, whereas only a minor fraction (0.8%) of the radioactive 2,4-D was metabolized. With A188 up to 70% of the radioactivity was rinsed away during the embedding procedure; however, based on the biochemical analysis directly after the culture mentioned above, we do not expect that the loss is specific, although metabolized 2,4-D might have been rinsed away with preference. We cannot exclude a tissue-specific loss, but the similar distribution in various embryos of one inbred line and the differences in distribution between A188 and A632 rather indicate a genotype-specific distribution.

Retention of accumulated 2,4-D during embedding procedures

Processing of unfixed embryos until the 100% Technovit embedding step resulted in the loss of up to 90% of the label. When a combination of EDC pretreatment, fixation, and water-mediated Technovit embedding was performed 30% label remained in the embryos. ^{14}C label was best retained after prefixation with EDC, although the preservation of the cell structure was inferior. Loss of ^{14}C label during fixation, rinsing, and embedding steps was also reported by Coetzee (1985) who fixed, dehydrated, and embedded bean leaves after exposure to $^{14}CO_2$. In the present experiments the loss of ^{14}C during ethanol dehydration was reduced by a protocol in which the monomer Technovit 7100 was dissolved in water. Although only 30% of the label was retained in the tissues of the embryo, the biochemical analysis showed that the formation of silver grains in the dipping film represents the presence of [^{14}C]2,4-D.

Uptake and transport of 2,4-D in cultured embryos

The *in situ* localization of ^{14}C label showed that although the embryos were cultured with the meristem side on the agar surface, most of the label was found in regions of the embryo distant from the meristem side, indicating transport of label. We, however, cannot define the transport route for 2,4-D because a 2,4-D-containing water film always surrounded embryos in the culture. The low radioactivity in the adhering film, found by counting the filter paper, however, indicates that the gradient from the agar-containing medium to the embryo might

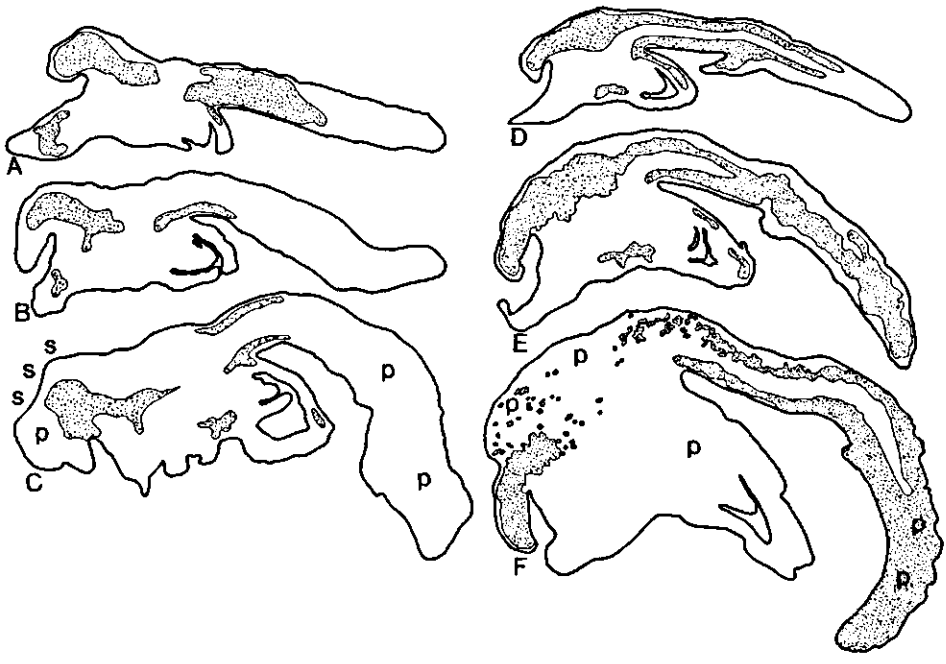


Figure 5. Schematic representation of the distribution of [^{14}C]2,4-D in median sections of A188 (**A-C**) and A632 (**D-F**) embryos. **A**, distribution after 16 h of pulse with 9 µM [^{14}C]2,4-D. **B**, distribution after 16 h of pulse and 24 h of chase with 9 µM 2,4-D. **C**, distribution after 16 h of pulse and 72 h of chase with 9 µM 2,4-D. **D**, distribution after 16 h of pulse with 9 µM [^{14}C]2,4-D. **E**, distribution after 16 h of pulse and 24 h of chase with 9 µM 2,4-D. **F**, distribution after 16 h of pulse and 72 h of chase with 9 µM 2,4-D. *Dotted* areas represent high concentrations of [^{14}C]2,4-D; areas of proliferation are represented by p. Areas where somatic embryos and friable embryogenic callus are formed in A188 are represented by s.

induce the most intensive transport in the contact region between the embryo and nutrient medium.

Transport is partly passive caused by pH trapping and partly active by carrier-mediated efflux of auxin-anions (Jacobs 1983). Bronsema et al. (1996) demonstrated active uptake of 2,4-D in cultured maize embryos by applying pretreatment with the synthetic polar transport inhibitor TIBA (Lomax et al. 1995), which caused a 30% reduction of 2,4-D uptake. Autoradiography now showed that the distribution pattern was maintained after pretreatment with TIBA, but the intensity of labeling was reduced, indicating that active transport was only partly reduced.

Since freeze-killed embryos did not show any labeling because of the destruction of cell membranes, it is concluded that pH trapping also causes a significant contribution to the uptake of 2,4-D in living embryos.

Distribution of ^{14}C label in embedded and sectioned embryos

Random labeling of 2,4-D in embryonal tissues was not observed in any of the embedding protocols used. Procambium strands were only weakly labeled, indicating that the transport of 2,4-D might either be cell to cell transport, not mediated by the cells of the procambial strands, or that the procambial strands do not accumulate 2,4-D in a way seen in the parenchyma cells of the embryo.

Comparing the two lines for their distribution of label, the more intensive labeling found in A632 is in agreement with data of the quantitative analysis (Bronsema et al. 1996). Differences in distribution are presented schematically in **Fig. 5** together with the sites of proliferation and callus formation. Spots of intensive label appeared in both lines. These spots were found in cells that, however, did not differ in morphology from cells surrounding the spots. Cells that formed callus were never found in regions with dense labeling and regions with a high label intensity did not proliferate. In A632, however, the top part of the scutellum enlarged despite the presence of label. Thus, the pattern of 2,4-D distribution did not codistribute with proliferation.

Cell proliferation in cultured gramineous embryos is generally initiated first in the nodal and basal region of the scutellum. Both regions are in close proximity to the root meristem and the procambium of the embryo axis (Vasil et al. 1985). It has been suggested that this may be related to the high levels of plant growth regulators presumed to be present in root and procambium (Vasil and Vasil 1982). With our autoradiographic localization we indeed observed high levels of label in the nodal and basal regions of the scutellum but not in the root meristem and the procambium.

The suggestion that the uptake of 2,4-D by embryos cultured *in vitro* is probably channeled more effectively through the embryo suspensor, and the coleorhiza (Vasil et al. 1985) could not be confirmed by our results, because the highest amount of label observed in A188 was found in the scutellum near the coleoptile, distant from the suspensor.

In all, the detection of 2,4-D by autoradiography visualizes the distribution of the growth regulator over the various tissues of the zygotic embryo. The regulator is taken up rapidly from the medium by passive and active processes and distributed throughout the embryo. The patterns of 2,4-D distribution did not codistribute with regions of proliferation. On the contrary, the regions of high accumulation of label always showed reduced enlargement and proliferation. These observations indicate that the presence of 2,4-D in a certain tissue itself is not the only prerequisite for triggering cell division and callus formation.

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Chapter 5

Immunocytochemical localisation of auxin-binding proteins in coleoptiles and embryos of *Zea mays* L.

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Protoplasma 1998 (202): 65-75

Napier et al. 1988). Three epitopes on ABP-1 to a number of antisera were mapped. The epitopes are clustered around the glycosylation site. Two of the three epitopes are conserved in monocotyledonous and dicotyledonous plants (Napier and Venis 1992). An essential portion of the auxin-binding site has been identified by raising antibodies to a synthetic peptide. These antibodies display auxin agonist activity (Venis et al. 1992).

Löbler and Klämbt (1985b) localised 90% of the ABP-1 in the outer epidermal cells of maize coleoptiles by immuno-fluorescence microscopy applying polyclonal anti ABP-1. At the electron microscopic level, Jones and Herman (1993) found a KDEL-containing ABP-1 secreted via the Golgi system to the plasma membrane and cell wall. Henderson et al. (1997) confirmed the presence of ABP-1 in the ER but did not detect it in Golgi bodies and cell walls. Thus the results are equivocal on this aspect.

Much of the signal transduction pathway of auxin is still unclear, but despite this fact several theories for auxin and ABP action have been proposed (Klämbt 1990, Cross 1991, Jones and Prasad 1992). Goldsmith (1993) reviewed the action of auxins with respect to cellular signalling. Thiel et al. (1993) observed a modulation of K^+ channels in stomatal guard cells caused by peptides, homologous to the auxin-binding protein C-terminus. Physiological data on tobacco protoplasts give mounting evidence that ABP-1 functions as an auxin receptor on the outside of the plasma membrane (Barbier-Brygoo et al. 1989, 1991, Venis et al. 1990), and patch-clamp analysis of maize protoplasts established a role for auxin-binding proteins in the auxin stimulation of plasma membrane current (Rück et al. 1993).

In a study on growth responses of immature embryos of the embryogenic inbred line A188 on the supply of 2,4-D we questioned how 2,4-D was distributed in cultured embryos (Bronsema et al. 1997, 1998). Here we aim at localising ABP-1 with polyclonal antibodies. The present study gives an overview of the presence and distribution of ABP-1 in etiolated coleoptiles of seedlings and in immature embryos of A188 observed by light and electron microscopy.

Material and methods

Plant material

The maize inbred line A188 (kindly provided by Dr. C.E. Green, University of Minnesota, MN, U.S.A.) was grown in the greenhouse. Cobs were hand-pollinated and immature embryos were excised at 11-12 days after pollination. They were either directly processed for immunocytochemical labelling, or first cultured *in vitro* on modified N6 medium containing 2 mg 2,4-D per liter (for details, see Bronsema et al. 1996) for 5-7 days and then sampled. Mature caryopses were imbibed in tap water for 6 h, and germinated at 25 °C on moist filter paper in the dark. After 5 days the etiolated coleoptiles of the seedlings were sampled for further processing.

Preparation of coleoptiles and embryos for light and electron microscopy

For each coleoptile a 5-mm long segment was cut 3 mm below its tip and fixed in a mixture of 3% formaldehyde (freshly prepared from paraformaldehyde) and 0.1 % glutaraldehyde for light microscopy (LM), and in 1% glutaraldehyde for electron microscopy (EM), all in 0.1 M phosphate buffer, pH 7.2, by vacuum infiltration for 2 h at room temperature. After fixation embryos and coleoptiles were rinsed in phosphate buffer for 2 h and dehydrated through a graded series of ethanol: 10%, 30%, 50%, 70%, 90%, and absolute ethanol (twice) for 30 min for each step and embedded in polyethylene glycol (PEG) for light microscopy according to van Lammeren et al. (1985). Semi-thin transversal sections (2 μm) were prepared with a Kulzer Histoknife (Kulzer, Friedrichsdorf, Federal Republic of Germany) mounted on a rotation microtome (Microm HM 340). Sections were picked up with hanging drops of 40% PEG 6000 in water to permit stretching, transferred onto poly-L-lysine coated slides and rinsed twice in phosphate buffered saline (PBS; 135 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 8 mM Na_2HPO_4), pH 7.2, to remove the PEG.

For EM the dehydrated samples were infiltrated with increasing concentrations of London Resin White (LR White) dissolved in absolute EtOH (1:3, 1:1, 3:1) for 2 h for each step, and in pure resin overnight. The resin was replaced with fresh LR White and polymerised in gelatine capsules for 24 h at 55°C. Ultrathin sections, cut with a diamond knife on an LKB Bromma

2088 ultratome, were collected on formvar-coated nickel grids. Embryos were processed similarly but longitudinal sections were prepared.

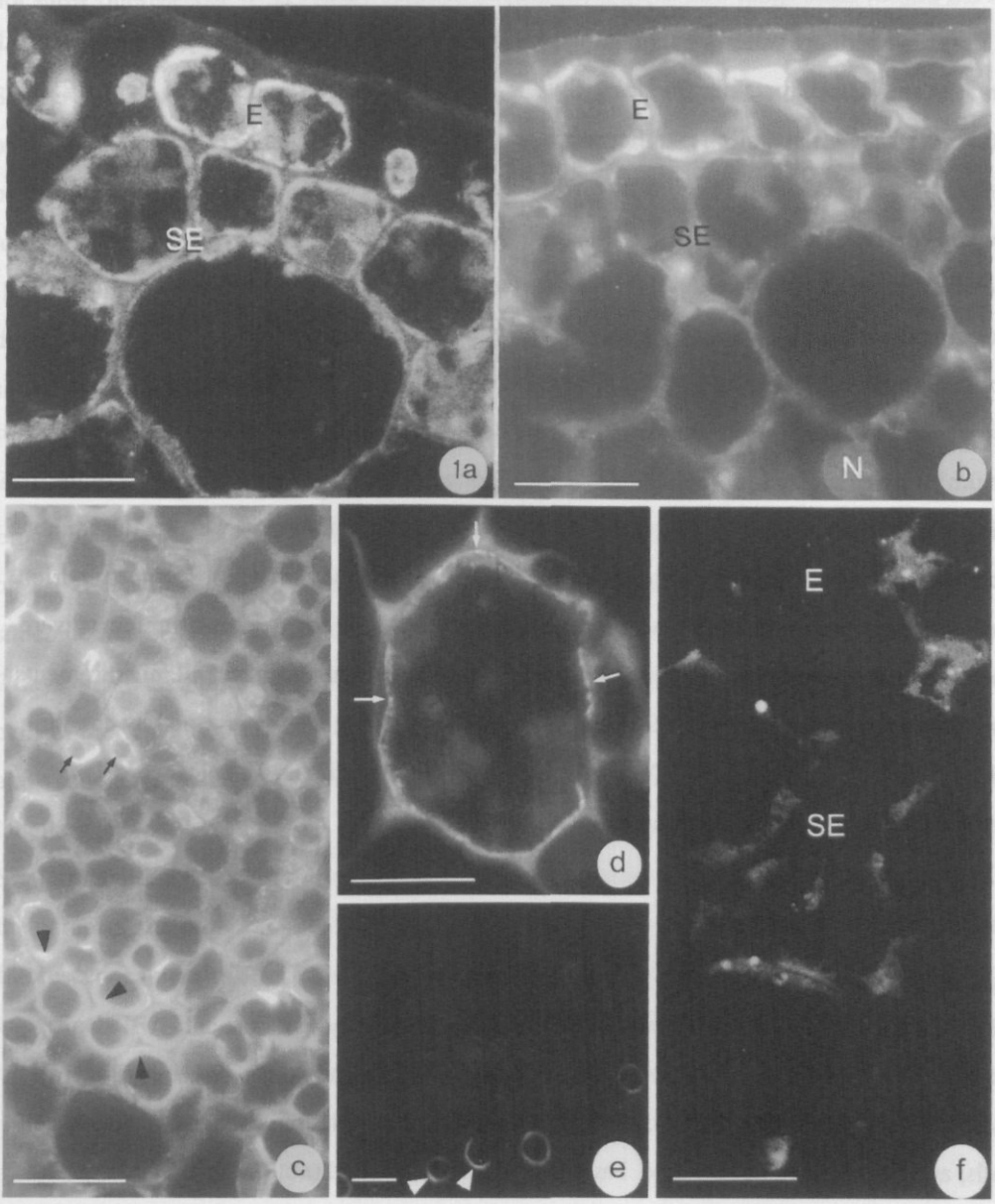
Immunocytochemistry

For the immunolocalisation of ABP-1 two polyclonal antisera directed against ABP-1 were used. They were kind gifts of Dr. D. Klämbt (Löbler and Klämbt 1985b) and of Drs. M.A. Venis and R.M. Napier (Napier et al. 1988).

For light microscopy, sections were rinsed in freshly prepared 15 mM $NaBH_4$ in PBS and in 0.1 M NH_4Cl in PBS (both for 5 min) to quench aldehydes and then blocked with 1% bovine serum albumin (BSA) in PBS for 30 min to reduce aspecific labelling. They were incubated with anti-ABP-1 diluted 1:10 to 1:100 in 0.1% acetylated BSA (BSA-C; Aurion, Wageningen, the Netherlands) in PBS. Incubation with PBS or with rabbit preimmune serum served as controls, all for 45 min at 37°C. Sections were rinsed in PBS for 45 min and exposed to a secondary fluorochrome labelled antibody (goat anti-rabbit IgG-FITC or GaR-Bodipy; Sigma, St. Louis, MO, U.S.A., diluted 1:40 in 0.1% BSA-C in PBS) for 45 min at 37°C, rinsed in PBS, embedded in Citifluor in glycerol, and analysed with a Microphot-FXA epifluorescence microscope (Nikon) with a xenon lamp with the filter set: excitation filter 470-490, dichroic mirror 510, barrier filter 515IF. Black and white images were recorded on Kodak TMY 135-film, colour images on Kodak EES 135-film. Alternatively the second antibody was labelled with 10-nm gold particles. After labelling at a dilution of 1:30 and silver enhancement sections were analysed by epipolarisation microscopy and double exposure with UV light.

Fig. 1 a-f. Light-microscopic distribution of ABP-1 in PEG crosssections of etiolated coleoptiles of maize inbred line A188. The ABP-1 was visualised immunocytochemically with GaR-FITC or GaR-Bodipy. **a** and **b** Labelling in the epidermal cells (*E*) and subepidermal cells (*SE*) with the polyclonal anti-ABP of Klämbt (**a**) and with the polyclonal anti-ABP of Venis (**b**). Note labelling in cytoplasm and absence in cell walls and cuticle. *N* Nucleus. **c** Labelling within a vascular bundle with the anti-ABP of Venis. The cytoplasm of the companion cells was highly fluorescent, but the nuclei were negative (arrows). Xylem elements exhibited autofluorescence in the cell wall (arrowheads). **d** ABP-1 labelling with the anti-ABP of Klämbt in a slightly plasmolysed mesophyll cell especially in the region of the cell membrane (arrows). **e** Control with PBS instead of the first antibody in the vascular bundle. Fluorescence in the lower part is autofluorescence of xylem elements (arrowheads). **f** Control with preimmune serum of the Venis antibody in the epidermal (*E*) and subepidermal cells (*SE*). Bars: 25 μm

Nickel grids with ultrathin sections were incubated in 0.05 M glycine in PBS (10 mM phosphate buffer, 150 mM NaCl, pH 7.6) for 15 min, and in BSA-C buffer (0.1% BSA-C in PBS) with 5% normal goat serum for 15 min to reduce aspecific labelling. Grids were then incubated in diluted primary antibody for 1 h (dilution 1:100). Control grids were incubated



either in preimmune serum of the anti ABP-1 of Venis or in PBS for the anti-ABP-1 of Klämbt. After six washes in BSA-C buffer, grids were incubated in goat anti-rabbit IgG conjugated with 10-nm gold particles (Aurion, Wageningen, the Netherlands), at a dilution of 1:30 in BSA-C buffer. After incubation for 45 min at 37 °C the grids were washed six times in BSA-C buffer, fixed again with 2% glutaraldehyde in PBS, washed twice in PBS and rinsed six times in Millipore water. Finally the sections were post-stained with uranyl acetate and lead citrate in an LKB Ultrostainer Carlsberg System. Sections were observed with a JEOL JEM-1200 EXII transmission electron microscope.

Quantitative analyses

The distribution of gold particles over the various tissues of the coleoptiles was determined on images in the transmission electron microscope with a video camera by the TIM computer programme (Difa Measuring Systems, Breda, the Netherlands). Surfaces of cell organelles were measured, numbers of gold particles were determined, and the numbers of gold particles per μm^2 were calculated for the various regions. For each antibody treatment 3 cells of the epidermis, of the mesophyll, and of the vascular tissue (companion cells) were analysed. For each tissue various subcellular regions were selected, i.e., nucleus, cytoplasm, mitochondrion, plastid, endoplasmic reticulum, vacuole, and cell wall. For each region 6 measurements were made. The data of one labelling procedure were all from the same grid, and all measurements were done at the same EM magnification. Background labelling was determined by counting gold particles on a part of the LR White section without maize tissue. The data were statistically analysed using the Student's t-test.

Results

Light microscopy

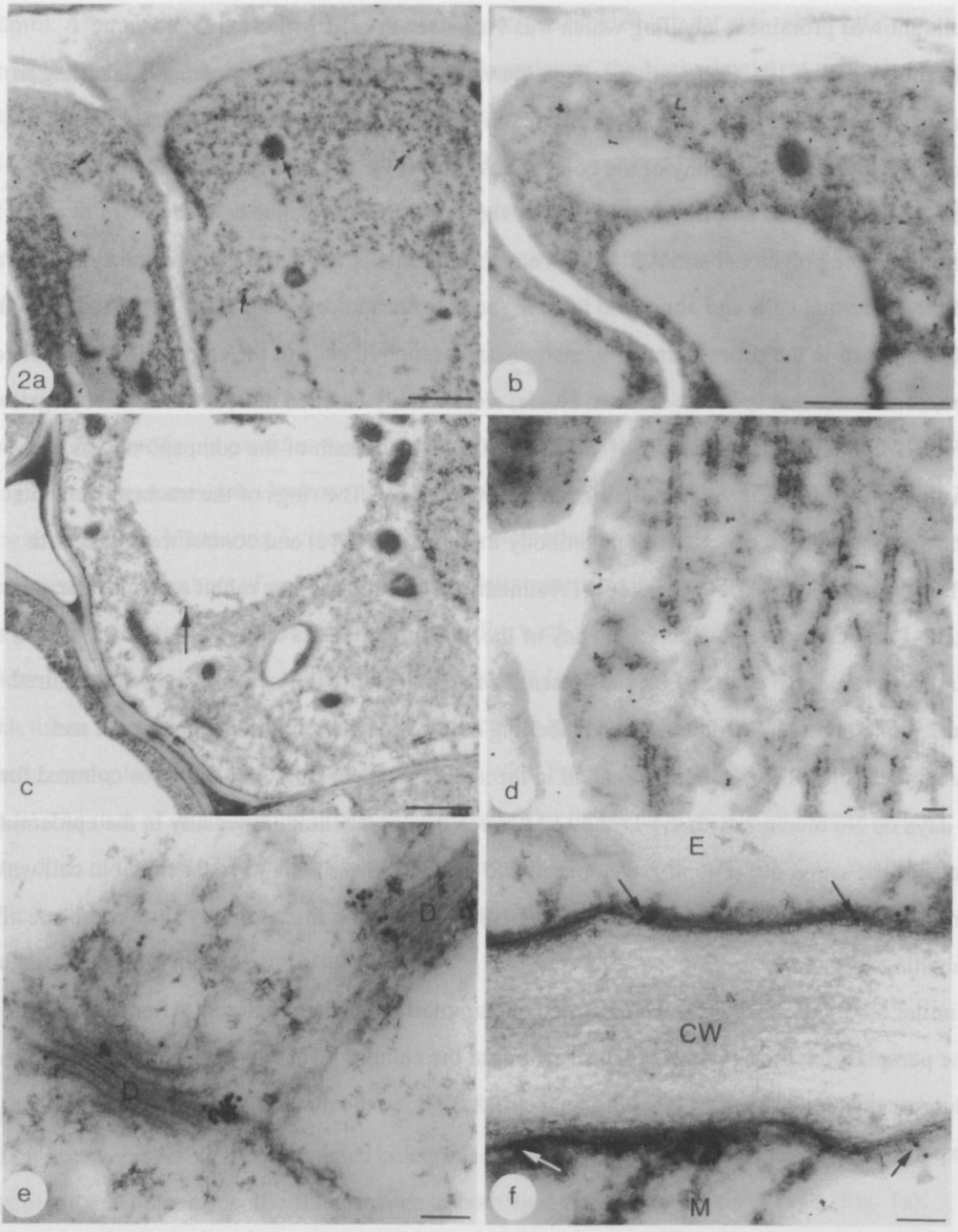
When transversal sections of etiolated coleoptiles were incubated with the anti-ABP-1 of Klämbt, they showed specific fluorescence in both epidermal and mesophyll cells (Fig. 1a). The fluorescence was distributed over the cytoplasm of the epidermal cells but vacuoles, cell walls, and cuticle were not labelled. Nuclei were faintly labelled (not shown). The large parenchymatic mesophyll cells showed fluorescence in the thin layer of cytoplasm aligning the

cell membrane, but again the vacuoles were not labelled. The cell membrane regions of these cells showed prominent labelling which was visualised in cells which exhibited some plasmolysis (Fig. 1d). Companion cells in the vascular bundles showed intensive labelling in the cytoplasm (data not shown). Control treatments with PBS instead of the first antibody did not show fluorescence in any of the coleoptile tissues (Fig. 1e). The fluorescence within the vascular bundle was autofluorescence of the rings of tracheary elements.

Similarly, the polyclonal anti ABP-1 of Venis caused intensive fluorescence in the cytoplasm of the epidermal cells and almost no labelling in cuticles and cell walls (Fig. 1b). The thin layer of cytoplasm in the subepidermal parenchymatic mesophyll cells of the coleoptile was labelled too, sometimes in a spot-like fashion. Nuclei were weakly labelled but cell walls were negative (Fig. 1b). In the vascular bundle of the coleoptile the cytoplasm of the companion cells was distinctly labelled (Fig. 1c) but their nuclei were negative. The rings of the tracheal elements showed autofluorescence after both antibody treatment (Fig. 1c) and control treatment with PBS instead of anti-ABP-1 (Fig. 1e). Treatment with the preimmune rabbit serum of the anti-ABP-1 of Venis gave no labelling in any of the coleoptilar tissues either (Fig. 1f).

Embryos were also analysed for the presence of ABP. Freshly excised embryos, not cultured on 2,4-D containing medium showed labelling at low intensity both in the epidermal and subepidermal cells of the scutellum and in the shoot meristem (Fig. 4a). Embryos cultured for 7 days on N6 medium with 2,4-D showed a much denser labelling, especially in the epidermal cells of the scutellum (Fig. 4b). Cytoplasm and nuclei of these cells were labelled but cell walls and vacuoles were negative. Controls with PBS instead of the first antibody showed no specific labelling (Fig. 4c).

Similar labelling procedures were applied on paraplast embedded material. After removal of the paraplast the preservation of the structures at the cellular level was very poor as was the fluorescence intensity after labelling with anti-ABP-1, so the method was not used in further experiments.


2a
b
c
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e
E
CW
f
M

Fig. 2. Electron-microscopic distribution of 10-nm gold particles in ultra thin sections of etiolated maize coleoptiles indicating the location of ABP-1 in coleoptile cells of maize after labelling with the anti ABP-1 of Klämbt (**a**) or Venis (**b** and **d-f**). **a** Distribution of gold particles in epidermal cells (arrows). Note the absence of labelling in the cell wall. **b** Same view after labelling with the Venis anti ABP-1. **c** Control with first antibody replaced by PBS; the arrow points to a single silver grain. **d** A typical area with labelled ER within the epidermal cell. **e** Epidermal cell of the coleoptile in which dictyosomes (*D*) are densely labelled with gold particles. **f** Detail of the cell wall between epidermis (*E*) and mesophyll (*M*) cells with gold particles on the plasma membranes (arrows). The cell wall (*CW*) was without labelling. Bars: in a-c, 1 μm; in d-f, 100 nm

Electron microscopy

The subcellular distribution of the ABP-1 in coleoptiles was determined with the electron microscope on ultra thin sections which were labelled with either of the two polyclonal antibodies and with a second antibody conjugated to 10-nm gold particles.

Cross sections of coleoptiles exhibited gold labelling within the cells of the epidermis with both antibodies (Fig. 2a, b). Most intensive labelling was observed within the ER (Fig. 2d) and the dictyosomes (Fig. 2e), but vacuoles, cell walls and cuticles were almost negative (Fig. 2a, b, d). The cytoplasm of the mesophyll cells and companion cells of the phloem was labelled in a similar way as found in the epidermis. Labelling at the plasma membrane was regularly observed in all tissues (Fig. 2f), though never at high densities. Controls with PBS instead of the first antibody were only slightly labelled (Fig. 2c).

In freshly excised embryos we observed label at low densities comparable to the labelling in cells in coleoptile tissues. After 5 days of culture, label intensity had increased. High label intensities were observed in the epidermal cells of the scutellum opposite the coleoptile. Especially the ER and the nucleus were densely labelled, the cytoplasm at intermediate density, and vacuoles at background level (Fig. 4d).

Quantitative analysis

The distribution of gold particles was quantified by electron microscopy for the coleoptile tissues. The results after labelling with the anti-ABP-1 of Klämbt and the anti-ABP-1 of Venis are given for the epidermis, the mesophyll and companion cells of the phloem (Fig. 3).

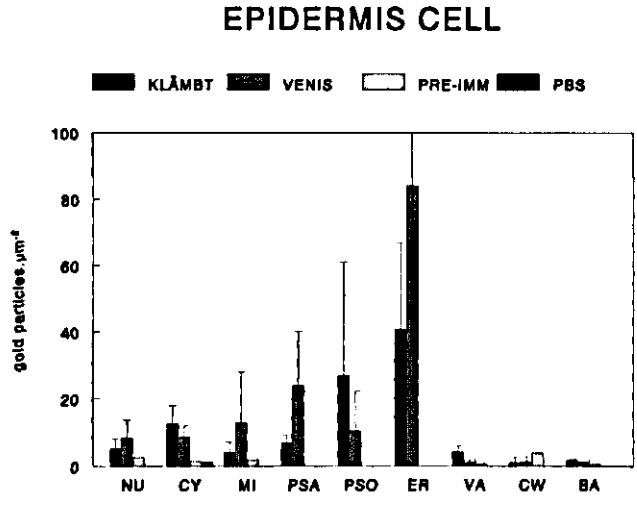
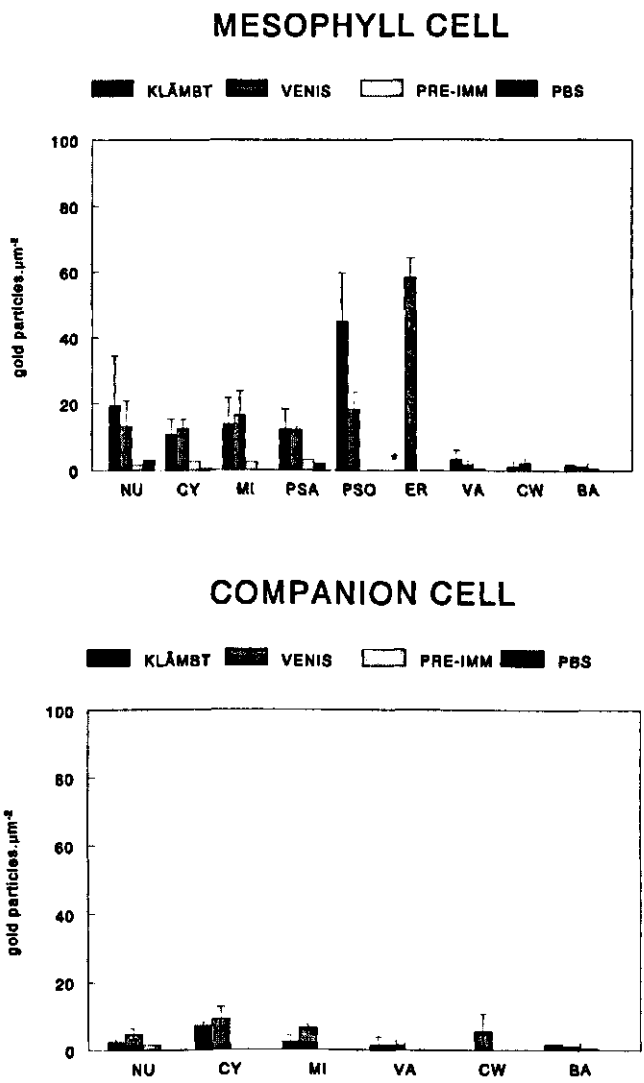


Fig. 3. Distribution of the number of gold particles per μm^2 in **a** epidermal, **b** mesophyll, and **c** companion cells of maize coleoptiles after labelling with the Klämbt polyclonal anti-ABP-1 and the Venis polyclonal anti-ABP-1 as observed by electron microscopy. Results are the averages of 6 counts per organelle and 3 cells per treatment. In control treatments the first antibody was replaced by preimmune serum or PBS. Totals are based on 6 counts per cell and one cell per control treatment. *NU* Nucleus, *CY* cytoplasm, *MI* mitochondrion, *PSA* starch of plastid, *PSO* stroma of plastid, *ER* endoplasmic reticulum, *VA* vacuole, *CW* cell wall, *BA* background outside the section, * not enough data.

With the Klämbt anti-ABP-1, epidermal cells had highest densities of gold particles within ER, plastid stroma and cytoplasm. Nuclei, mitochondria, and vacuoles were moderately labelled. The lowest labelling, equal to the background, was found for the cell wall (Fig. 3a). Mesophyll cells exhibited higher densities of gold particles per square micrometre as compared to epidermal cells (Fig. 3b). They, however, had large vacuoles and only a thin layer of cytoplasm aligning the plasma membrane. Nucleus and plastid stroma were densely labelled. Less particles were found on cytoplasm, mitochondria, and starch grains, and fewest in vacuoles and on cell walls at a level comparable to the background. As compared to epidermis and mesophyll cells, companion cells in the vascular tissue showed a similar density of label in the cytoplasm (Fig. 3c). Plastids, mitochondria, and ER were not observed in companion cells, probably due to the state of differentiation or to the weak fixation which was applied to



preserve the antigenicity of the tissue. Vacuoles and cell walls had low densities comparable to the background.

With the Venis anti-ABP-1, epidermal cells showed the highest numbers of gold particles per square micrometre in the ER (Fig. 3a). The nucleus, cytoplasm, mitochondria, and plastids exhibited intermediate numbers of gold particles, and the vacuole and cell wall the lowest. Compared to the other cell types, mesophyll cells had the highest amount of label per square micrometre for the parameters measured (Fig. 3b). The ER had the highest density of label, the

nucleus, cytoplasm, mitochondria, and plastids an intermediate density, and the vacuole and cell wall the lowest. Companion cells exhibited similar labelling intensities in the cytoplasm as found in the epidermal and mesophyll cells (Fig. 3c). The other regions measured were labelled at background level. Preimmune serum and PBS treatments of the sections resulted in labelling intensities at background level for all regions. Table 1 presents the statistical analysis of the data by the Student's t-test. There was no significant difference between the overall labelling obtained with the two antibodies. Amongst the cell types, the labelling of the mesophyll cells was significantly higher than that of the epidermal cells and the companion cells. Amongst the subcellular regions, vacuoles and cell walls were significantly less labelled than nuclei, cytoplasm, and mitochondria. The data for ER and plastids were not used in the statistical analysis since they were not available for all cell types.

Discussion

Localisation of auxin-binding proteins in coleoptiles of maize

The results of the light-microscopic labelling of PEG-sections with polyclonal antibodies and preimmune sera confirm the epidermal localisation by Löbler and Klämbt (1985b). Additionally we also localised ABP-1 in the cytoplasm of mesophyll and companion cells. Löbler and Klämbt (1985b) thought the concentration of ABP-1 in these cells too low for detection by immunofluorescence. In their experiments paraplast was chosen as embedding medium. It might well be that the higher label intensity in the present experiments is caused by an improved preservation of the antigenicity of the epitopes on ABP-1, since PEG was applied as embedding medium. Another advantage with respect to paraplast embedding is the improved preservation of the structure of the tissues and cellular constituents such as cytoplasm, vacuoles, and nuclei.

The immunocytochemical procedures applied, do not aim at quantifying ABP-1 levels as we cannot determine how many epitopes are preserved during fixation and are accessible in the sections. Despite this limitation, controls and comparison of LM and EM data, show that the localisation of ABP-1 is in agreement with biochemical data.

Subcellular distribution of ABP-1

In general light microscopy revealed that ABP-1 was present in the cytoplasm of epidermal cells, mesophyll cells, and companion cells of the phloem. Since we aimed at determining not only the distribution of ABP-1 in the various tissues but at the subcellular level as well, the labelled tissues were investigated in detail with transmission electron microscopy. Applying immuno-electron microscopy and comparing the distribution of gold particles over plastids, mitochondria, ER, dictyosomes, and regions of cytoplasm without these organelles, the two polyclonal antibodies of Klämbt and Venis gave similar results for these regions, as shown by the quantitative analysis of the areas measured (Fig. 3). If, like in case of the mesophyll cells, the cytoplasm is densely labelled, but only contributes to the total surface for a minor part, its relative contribution to the labelling of the whole cell surface is small. This might explain the 10% distribution outside the epidermis observed by Klämbt.

Cytoplasm and organelles

For the cytoplasm without organelles similar numbers of gold particles were observed with TEM in epidermis, mesophyll and companion cells. Mitochondria were labelled at similar densities as found in the cytoplasm. Plastids, especially the stroma, had a relatively high density. Starch was often labelled too, but controls with the preimmune serum or in which the first antibody was substituted by PBS still had gold particles on the starch grains, so at least part of localisation on plastids is due to nonspecific binding to starch. It is known that ABP is preferentially found in ER (Ray 1977), being the site of synthesis. Its presence in the cytoplasm, mitochondria, and plastid stroma as found in the present study, points to an escape from the ER. According to Henderson et al. (1997), this escape is caused by leakage from the ER, rather than by an auxin-induced conformational change in ABP-1 by which the KDEL retention sequence is masked and thus no longer functional in retaining the ABP-1 in the ER (Napier and Venis 1990). Additionally it suggests that these subcellular parts attained the capacity to bind auxins and thus to respond to the presence of these signal molecules.

Endoplasmic reticulum

The highest density of gold particles was observed at the ER. This presence at the ER was expected since the ABP-1 has an N-terminal signal peptide for entry into the ER, and both polyclonals were directed against proteins purified from isolated ER (Löbler and Klämbt 1985b, Napier et al. 1988). When present, dictyosomes showed a high number of gold particles too (Fig. 2a). However, the mild fixation conditions of the tissue, needed for immunocytochemistry, made the detection of dictyosomes hard, but postfixation of coleoptiles with OsO_4 resulted in good preservation of ER and dictyosomes within the coleoptilar tissues (results not shown). When ABP-1 was detected in dictyosomes, the number of gold particles was relatively high and they were detected at the cis and trans sides indicating that epitopes did not change during processing in the dictyosomes.

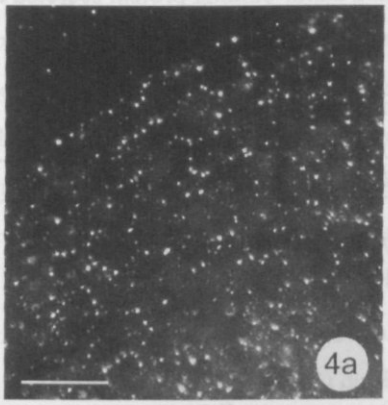
The presence of ABP-1 in dictyosomes might show the route of the ABP being transported to the plasma membrane, confirming the model of Cross (1991), where ABP cycles through the cell from ER to the plasma membrane, secreted via dictyosome vesicles. Although dictyosomes were labelled in our experiments, the vesicle-mediated secretion to the plasma membrane was not observed, and thus not confirmed by this cytological method.

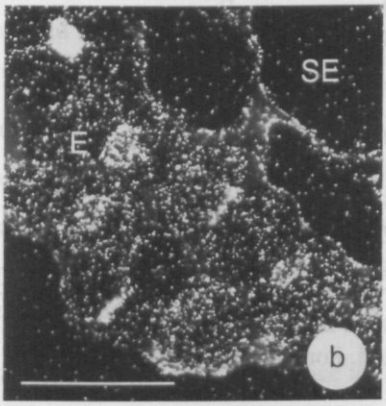
Plasma membrane

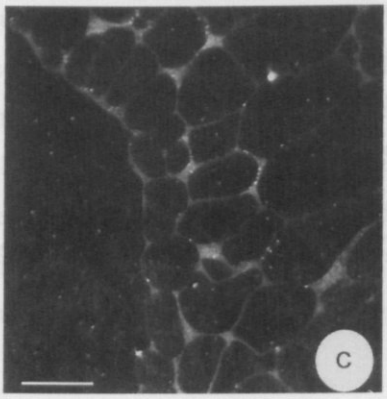
Localisation of ABP-1 at the plasma membrane was observed in all the tissues examined. The number of gold particles as observed by TEM, however, was never high. Although the plasma membrane is considered to be the site where ABP functions in signalling auxins (Barbier-

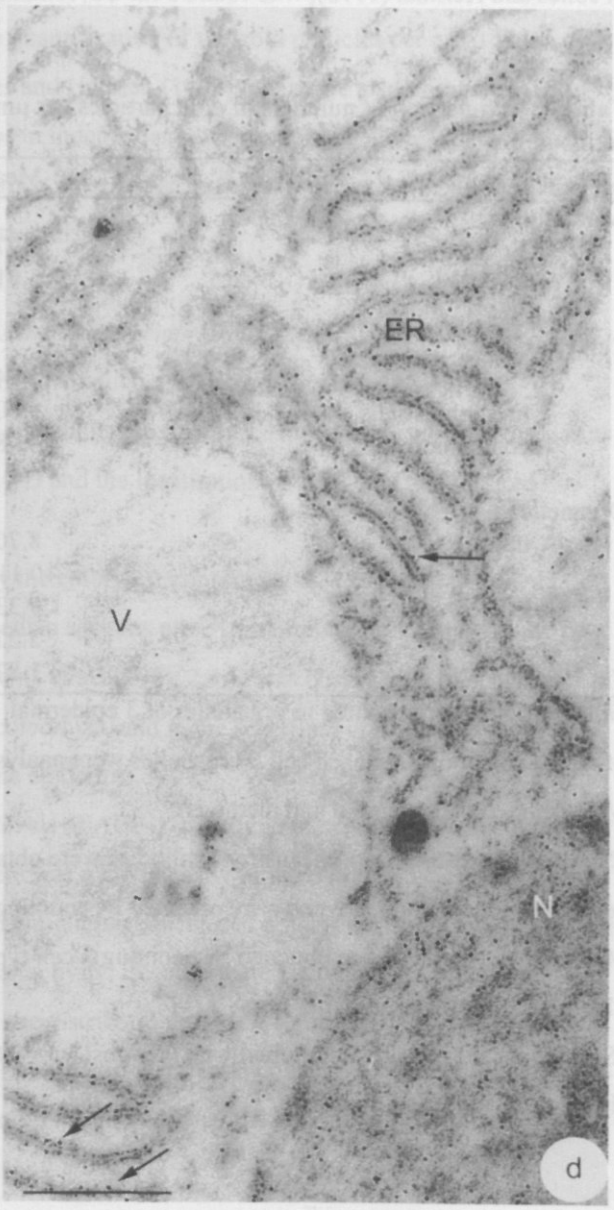
Fig. 4. Light-microscopic and electron-microscopic distribution of ABP-1 in cross sections of fresh and cultured immature zygotic embryos of maize inbred line A188, after labelling with anti-ABP of Klämbt and visualised with silver enhanced gold labelling at LM level (**a-c**) and 10-nm gold labelling at EM level (**d**). **a** Distribution of silver grains in the shoot meristem region of a freshly excised embryo. **b** Labelling in epidermal (*E*) and subepidermal (*SE*) cells of the scutellum after culture for 7 days on 2,4-D containing medium. **c** Control labelling of the same material after omission of the anti-ABP. The faint fluorescence is caused by autofluorescence of the cell walls due to double exposure with UV light. Note the low number of silver-enhanced gold particles. **d** Labelling after 5 days of culture in the epidermal cells of the scutellum; *ER* Endoplasmic reticulum, *N* nucleus, *V* vacuole. Note the high density of label in the ER and in the nucleus. Arrows point to gold particles on the ER. The vacuoles are negative. Bars: in a-c, 25 μm; in d, 1 μm

Brygoo et al. 1989, 1991; Rück et al. 1993), indeed low signal levels were to be expected. However, the low number, also mentioned by Diekmann et al. (1995), might be due to surface labelling applied with immuno-electron microscopy. With this respect it is noteworthy that labelling on embedment-free semithin sections with anti-ABP-1 and FITC revealed a thin but prominent fluorescent line marking the cell membrane in slightly plasmolysed cells. Hence this









signal is suggested to be the sign of membrane-bound ABP-1.

Cell wall and vacuoles

In our electron microscopic examinations we did not observe specific labelling in cell walls and vacuoles of maize coleoptile cells. Although this result is in contradiction to the findings of Jones and Herman (1993), who found a KDEL-containing auxin-binding protein secreted to

Table 1: Comparison of numbers of gold particles per μm^2 found after labelling with antibodies of Venis or Klämbt.

	N[a]	Means[b]	Significance[c]
Antibody			
Venis	15	6.97	A
Klämbt	15	5.91	A
Cell type			
epidermis cells	10	5.94	A
mesophyll cells	10	9.30	B
companion cells	10	4.08	A
Organelle			
nucleus	6	8.70	B
cytoplasm	6	10.11	B
mitochondrion	6	9.33	B
vacuole	6	2.22	A
cell wall	6	1.83	A

Gold particles were counted in organelles of 3 epidermal, 3 mesophyll, and 3 companion cells of maize coleoptiles. In each cell 5 organelles were analysed. For each organelle 6 regions were counted (see Material and methods).

[a] Number of means tested. Means for antibodies were obtained by counting 5 organelles in 3 cell types. Means for cell types were obtained by counting 5 organelles with 2 antibodies. Means for organelles were obtained by counting 3 cell types with 2 antibodies.

[b] Number of gold particles per μm^2.

[c] Significance levels are determined with the t-test ($\alpha = 0.05$). Different capital letters indicate significantly different means.

the cell wall and even to the culture medium of BMS suspension cells, the observation is in agreement with the data obtained by Henderson et al. (1997).

Nuclei

At the EM level nuclei were labelled in all tissues. At the LM level we observed only faint labelling of the nuclei in epidermis and mesophyll, in companion cells hardly any. These observations were confirmed with the quantification of the gold particles per area. This localisation was not observed by Jones and Herman (1993), but as we also observed ABP labelling in nuclei of cultured embryos in independent experiments, we conclude that ABPs must be present in nuclei of the epidermis, possibly as a receptor mediating auxins and nuclear activity at the transcription level (Dietz et al. 1990).

Localisation of auxin-binding proteins in cultured embryos of A188

In our study on the role of 2,4-D during the induction of embryogenic callus in A188, we cultured immature embryos on 2,4-D containing N6 medium. Now we ask whether there is a relation between the localisation of 2,4-D and the localisation of ABP-1 in the cultured embryo.

During culture, 2,4-D was taken up by the embryo (Bronsema et al. 1996) and its distribution was studied by autoradiography on median sections after 16 h of pulse and 72 h of chase on ^{14}C-2,4-D-containing medium (Bronsema et al. 1998). 2,4-D was observed in the basal part of the scutellum, in the area around the coleorhiza, and in the scutellum opposite the shoot meristem but not with preference in the epidermis.

Cultured embryos showed increased levels of ABP-1, mainly in the epidermal cells of the scutellum. At the subcellular level there was a high density of label in the nuclei both at LM and EM level, indicating that the 2,4-D might have increased sensing for auxins in the nucleus which might lead to increase in nuclear activity. Because there is no direct relation found between the distribution of 2,4-D and the distribution of ABP-1, the 2,4-D found in cultured embryos might be accumulated by pH trapping rather than by binding to ABP-1. In such case an induction relation between epidermis and subepidermal tissues can still not be excluded.

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Chapter 6

Analysis of poly(A)+RNA distribution during maize somatic embryogenesis using digoxigenated oligo-dT probes.

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Abstract

The pattern of total transcription activity in terms of steady state levels of poly(A)+ containing mRNA during callus initiation and somatic embryogenesis in a high (A188) and a low (A632) embryogenic line of maize was analyzed using digoxigenin labelled oligo-dT probes. A gradual increase and a preferential accumulation of label was observed in both lines, differing temporally up to 4 days in culture. In the A188 line of maize the callus gave rise to somatic embryos. The globular embryos showed less label than the callus; this labelling mostly was present in the basal part of the embryos. At a later stage upper embryogenic and lower non-embryogenic layers were observed in the A188 callus, showing conspicuous differences in the amount of label. In the late globular stage the poly(A)+ RNA signals were seen all over the embryo but at the junction of the suspensor and the callus tissue no label was observed.

Keywords

Zea mays L. - Histochemistry - Embryogenesis - RNA synthesis

Abbreviations

BCIP - 5-bromo-4-chloro-3-indolylphosphate; 2,4D - 2,4 dichlorophenoxy acetic acid; DIG - digoxigenin; NBT - nitroblue tetrazolium

Introduction

Somatic embryogenesis in plants, including maize, besides offering an excellent system to study early development, also has considerable commercial value. Therefore, many aspects already have been investigated, last years focussing on differential gene expression and molecular markers for embryogenesis (see e.g. De Jong et al., 1993). Although these approaches are of great value, not only the expression of specific genes during callus initiation and induction of embryogenesis is important, but also insight in the overall reprogramming of mRNA synthesis. This approach is emphasized e.g. by Raghavan (1981) who studied mRNA synthesis during pollen embryogenesis in *Hyoscyamus* using tritiated poly-U probes for *in situ* hybridization. More recently, Chandra Sekhar and Williams (1992) have studied the *in situ* localization of poly(A)+RNA during pollen development in *Nicotiana* using non-radioactive digoxigenated oligo-dT probes. We have adapted this sensitive and fast method to compare unprocessed mRNA distribution during early stages of embryogenic and non-embryogenic callus development in maize.

Materials and methods

Plant material and culture conditions.

Plants of both inbred lines A188 (high embryogenic response) and A632 (low embryogenic response) were grown in a climate chamber with 16 h light at 24°C and 8 h dark at 20°C at a relative humidity of 70%. Light was provided by Philips 50W/84 HF fluorescent tubes with an intensity of 50 W/m^2. Additional red light was provided by Philinea lights. Immature zygotic embryos with a length between 1 and 2 mm were excised 11 days after pollination of the cobs.

The embryos were transferred into Petri dishes with solidified N6 medium (Chu et al., 1975), containing 0.7% agar, 2 mg/L 2,4-D, 20 mM L-proline, 200 mg/L casein hydrolysate and 2% sucrose. They were placed with the embryo axis in contact with the medium. Callus initiation took place in the dark at 25° ± 1°C. For more details, see Fransz and Schel 1991a, 1991b.

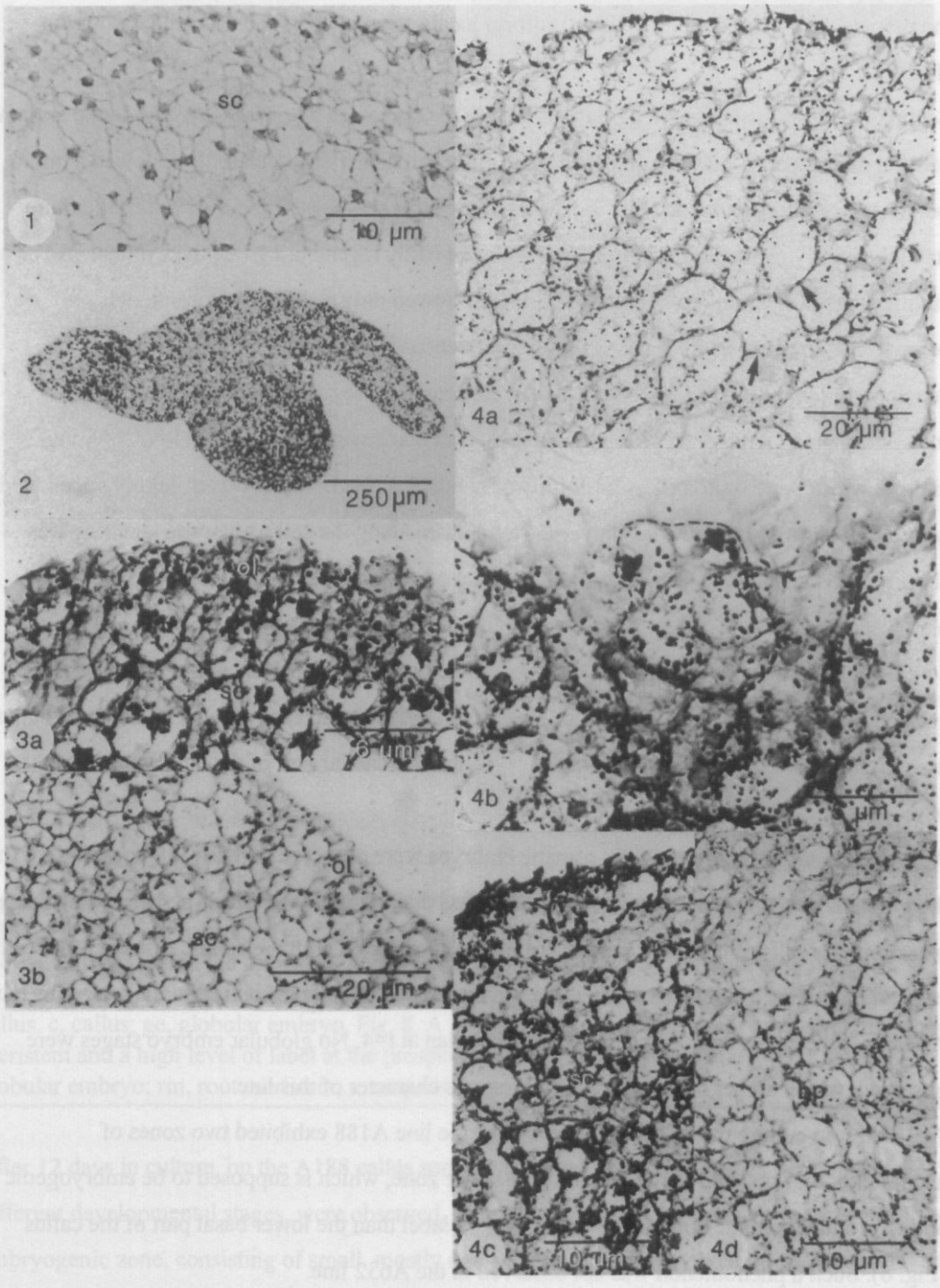
Histochemistry.

With 2 day intervals, samples were fixed in 1% glutardialdehyde in 0.1 M cacodylate buffer (pH 7.2) for 3 hours, dehydrated in a graded series of ethanol, infiltrated with xylol and finally embedded in paraffin. Semithin (5 mm) sections were made and mounted on organosilane-coated slides. The slides were dried overnight at 40°C. After that, they were incubated with digoxigenin-labelled oligo-dT probes, which were prepared from a terminal transferase kit (Boehringer Mannheim, Germany). The hybridization regions were detected using an anti-digoxigenin antibody conjugated with alkaline phosphatase (Boehringer Mannheim, Germany). Alkaline phosphatase activity was indicated by a reaction with NBT (nitroblue tetrazolium) and BCIP (5-bromo-4-chloro-3-indolyl phosphate), giving rise to blue precipitates. After these histochemical treatments, the sections were washed twice in distilled water, air dried and mounted in Depex. For a more detailed description of the procedures, see Bimal et al. (1995).

Results

Sections of cultured zygotic maize embryos, hybridized with DIG-labelled poly(A) as a control, did not show any label (Fig. 1). After hybridization with the oligo-dT probe at the onset of culture (t=0) the embryogenic line A188 differed from the badly regenerating line A632

Figs. 1-4. *In situ* hybridization of DIG-labelled poly(A) control probe or oligo-dT probes with poly(A)+RNA in sections of maize. Fig. 1. Section of a maize zygotic embryo (t=2) hybridized with the control probe. Fig. 2. Zygotic embryo of A188 (t=0) showing more label in the meristematic zone. Fig. 3a. Accumulation of poly(A)+RNA signals in the scutellum in A188 (t=2). Fig. 3b. Zygotic embryo of A632 (t=2) showing no preferential localization of signals in the scutellum. Fig. 4a. A188 culture (t=4) showing a conspicuous increase in signal. Highly vacuolated cells have very few or no label (arrows). Fig. 4b. Embryogenic unit of A188 callus (t=4) surrounded by a cell layer having few or no label. Fig. 4c. Increase in label in the scutellar part of an A632 embryo (t=4). Fig. 4d. Less label is present in the basal part of the A632 embryo (t=4). bp, basal part; m, meristem; ol, outer layer; sc, scutellum.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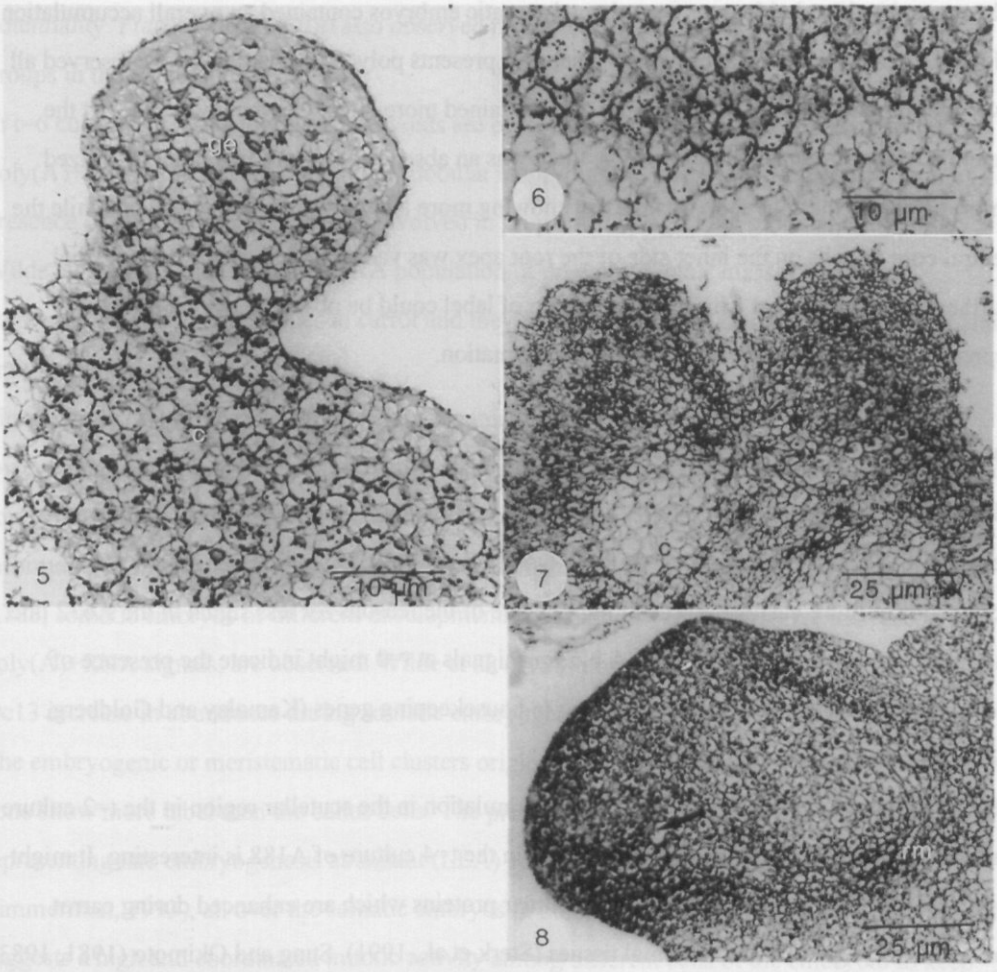
in three aspects: i. presence of more label all over the embryo, ii. concentration of more label in the meristematic zone, and iii. a rather uniform localization of signals in the scutellar region (Fig. 2).

Sections of A188 embryos after 2 days in culture (t=2) showed an overall increase in the amount of label as compared with t=0. Especially the outer cell layers of the scutellum showed a high poly(A)+RNA accumulation (Fig. 3a). Most of the label was distributed over onset of culture (t=0) the embryogenic line A188 differed from the badly regenerating line the cytoplasm. The t=2 culture stage of the non-embryogenic line A632 showed only a slight increase in label as compared with t=0 (Fig. 3b). There was no preferential localization of label at this stage of development in the A632 line as was seen in the scutellum region of A188.

In a 4 days old culture of A188 there was a conspicuous increase in poly(A)+RNA binding signals, which were more or less uniformly distributed throughout. However, highly vacuolated cells either showed very few label over the cytoplasm along the cell wall or the labelling was absent in such cells (Fig. 4a). Incidentally a group of cells, probably an embryogenic unit, was observed at the surface of the scutellar region (Fig. 4b). The cells surrounding this cell group contained either very few or no label. There was an increase in label as compared with the t=2 culture. In the non-embryogenic line A632 there was also an increase of label at 4 days of culture as compared with t=2. More label was observed in the scutellar part of the embryo than in the basal part (Figs. 4c, d).

At 6 days in culture small globular somatic embryos were observed in the A188 line (Fig. 5). In the globular structures the amount of label was less than in the callus cells.The label was mostly present in the cells of the lower part of the embryo connected with the callus. However, the callus cells adjoining the globular structures showed more accumulation of poly(A)+RNA signals. In the A632 line, the callus cells at t=6 had more label than at t=4. No globular embryo stages were observed, which agrees with the non-embryogenic character of this line.

At 10 days in culture the callus of the embryogenic line A188 exhibited two zones of accumulation of poly(A)+RNA signals. The upper zone, which is supposed to be embryogenic and to produce somatic embryos, contained more label than the lower basal part of the callus (Fig. 6). Such a phenomenon was not observed in the A632 line.



Figs. 5-8. *In situ* hybridization of DIG-labelled oligo-dT probes during somatic embryogenesis in A188. Fig. 5. A globular embryo (t=6) showing less label than the callus. Fig. 6. Callus (t=10) showing more label in the upper cell layers (embryogenic zone). Fig. 7. Large globular cell clusters at the periphery of the embryogenic zone in A188 (t=12) contain more label than the callus. c, callus; ge, globular embryo. Fig. 8. A more organized somatic embryo showing the root meristem and a high level of label at the prospective site of the shoot apex (arrow). c, callus; ge, globular embryo; rm, root meristem; s, suspensor.

After 12 days in culture, on the A188 callus somatic embryos of various shapes, representing different developmental stages, were observed. Large globular cell clusters at the periphery of the embryogenic zone, consisting of small, mostly non-vacuolated cells, showed more label and were separated from the rest of the callus by vacuolated cells having comparatively less label (Fig. 7).

The more developed oblong or pear-shaped somatic embryos contained an overall accumulation of label. In late globular stages, with a suspensor presents poly(A)+RNA label was observed all over the somatic embryo, but the upper part contained more label than the lower part. At the junction of the suspensor and the callus, there was an absence of signal. In a more organized embryo (Fig. 8) the root apex was present showing more label towards the outer side, while the central core of cells on the inner side of the root apex was vacuolated and showed less label. Further, in the upper part a high concentration of label could be observed, which probably represents the prospective site of shoot apex formation.

Discussion

The presence of more poly(A)+RNA label in the embryogenic line (A188) and the difference between A188 and A632 in poly(A)+RNA distribution at t=0 seems to be genotypic. The occurrence of more vacuolated cells might be one of the reasons for less signal in the A632 line. In both maize lines, the poly(A)+RNA binding signals at t=0 might indicate the presence of messengers for polypeptides corresponding to housekeeping genes (Kamalay and Goldberg, 1980; Bertrand-Garcia et al., 1992).

The gradual increase in poly(A)+RNA, its accumulation in the scutellar region in the t=2 culture and the further increase in the number of label in the t=4 culture of A188 is interesting. It might represent transcripts responsible for extracellular proteins which are enhanced during carrot somatic embryogenesis in the dermal tissues (Sterk et al., 1991). Sung and Okimoto (1981, 1983), Chen and Luthe (1987) and Stirn and Jacobsen (1987) have also observed polypeptides specific for embryogenic growth in carrot, rice and pea respectively. The increase in poly(A)+ containing mRNA during t=2 and t=4 might also be related to the biphasic model of early callus formation as proposed by Fransz et al. (1990) for cultured maize embryos.

A comparatively less but gradual increase in poly(A)+RNA in t=2 and t=4 cultures of the non-embryogenic line (A632) also points to active mRNA synthesis. The preferential accumulation of label in the scutellum in the t=4 cultures in A632 seems to represent messages for callus initiation, although in A188 this was observed in t=2 cultures. In the t=4 culture of A188 the embryogenic groups with labels were separated by a cell layer having very few or no labels indicating a kind of isolation or imposition of stress which may be a pre-requisite for the acquisition of embryogenic

potentiality. Fransz et al. (1991a) also observed physiological isolation of such embryogenic groups in the A188 line.

In t=6 cultures of A188 globular embryoids are observed, showing a differential distribution of poly(A)+RNA. The lower basal part of globular embryoids has more label. This suggests the presence of informational molecules involved in proper nurturing of the globular embryoids. Wilde et al. (1988) studied the mRNA population of proembryogenic masses (PEM), embryogenic and mature tissues in carrot and they found 21 polypeptides specific for PEMs and embryogenic tissues.

Significantly, in t=10 cultures of the embryogenic line A188 the upper embryogenic and the lower somatic zone can be distinguished based on the presence of more label in the embryogenic zone than in the somatic zone. It seems that all the cells of the upper embryogenic zone have informational molecules conductive to the development of somatic embryos. In t=12 cultures of A188, somatic embryos of different developmental stages, showing progressive increase in the poly(A)+RNA signals, are observed. Wilde et al. (1988) observed that cDNAs Dc3, Dc5 and Dc13 increase in abundance during somatic embryogenesis in carrot.

The embryogenic or meristematic cell clusters originating at the periphery of the embryogenic zone show more label than the callus cells. The presence of more poly(A)+RNA signals, possibly representing late embryogenesis abundant (LEA) gene transcripts (Wurtele et al., 1991; Zimmerman, 1993), all over the somatic embryos in oblong or late globular stages probably suggests a high and coordinated mitotic activity among different cells of the embryoid to keep pace with the growth. It also could indicate a state of readiness for organogenesis.

In the late globular stage the presence of less label in the lower basal part and decrease or loss of informational molecules at the place of attachment of the suspensor and the callus might suggest a kind of strained source-sink relationship or maybe a stress signal, triggering the somatic embryos to develop their own root meristem. It also might indicate the prospective site of detachment of the embryoid from the mother callus.

In more developed and organized embryos the upper part has more poly(A)+RNA label, representing the prospective shoot apex while the cells outside the newly formed root meristem have more labels than the cells on the innerside of the root meristem. A gradual accumulation of EMB-1 LEA gene mRNA has been shown as somatic embryogenesis proceeds further from

globular to late developmental stages predominantly over peripheral regions of the embryo, indicating initiation of polarity (Wurtele et al., 1991).

In the past, many, mostly biochemical, studies have been made to register metabolic changes during somatic embryogenesis (e.g. Montague et al., 1978; Fujimura et al., 1980; Sengupta and Raghavan, 1980; Ashihara et al., 1981; Sung et al., 1981). We conclude that the use of oligo-dT probes to localize poly(A)+RNA is an additional way to follow stage and genotype specific differences in gene expression.

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Chapter 7

Influence of 2,4-D, TIBA and 3,5-D on the growth response of cultured maize embryos

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Abstract

Callus induction and regeneration of somatic embryos on immature zygotic embryos of maize require the presence of 2,4-D in the culture medium. Pulse-chase experiments with 2,4-D, and the application of various concentrations of 2,4-D in the induction medium were tested in relation to the induction of callus in the embryogenic inbred line A188 and the non-embryogenic inbred A632. Additionally, interactions of 2,4-D, 3,5-D and the auxin transport inhibitor TIBA were studied.

Pulse-chase experiments showed that exposure to 2,4-D influenced the culture response from 0.5 h onwards. After a pulse of 0.5 h shoot and root elongation was stimulated. A pulse of 16 h or longer induced outgrowths and callus formation at the basal side of the scutellum. Pulses of 7 days and longer resulted in the induction of friable embryogenic Type II callus in A188.

Embryos were cultured at various 2,4-D concentrations ranging from 2.10^{-3} till 2.10^{+3} mg/l. The optimal concentration for the induction of embryogenic callus in A188 was 2 mg/l 2,4-D. At lower concentrations there was a transition between callus formation and germination; at increasing concentrations callus induction was reduced and finally growth responses became blocked.

When embryos of both lines were cultured with their scutellum side on the medium instead of on their meristem side, uptake of 2,4-D was 2-7 times higher, but only induced changes in the growth response in A632 embryos.

TIBA did influence morphogenesis. When added to medium without 2,4-D, root elongation decreased in a dose dependent way suggesting the need of polar transport of endogenous auxins for root elongation. When added to medium with 2,4-D at concentrations ranging from 0.2-20 mg/l, TIBA caused suppression of callus formation. When added at concentrations higher than 200 mg/l, callus formation was blocked, pointing to the necessity of polar transport of 2,4-D for callus formation.

Analysing the influence of 3,5-D on morphogenesis, it was found that addition of 3,5-D to medium without 2,4-D, did not influence germination. In combination with 2,4-D, culture with 3,5-D resembled culture at lower 2,4-D concentrations, pointing to a competitive interaction between 3,5-D and 2,4-D.

Abbreviations

DAP = days after pollination, DIC = days in culture, 2,4-D = 2,4-dichlorophenoxyacetic acid, 3,5-D = 3,5-dichlorophenoxyacetic acid, Dicamba = 3,6-dichloro-o-anisic acid, TIBA = triiodobenzoic acid, IM = induction medium, PM = proliferation medium, MM = maturation medium, RM = regeneration medium, GM = growth medium

Introduction

The presence of 2 mg/l 2,4-D in culture media is a prerequisite for callus induction on immature embryos of maize (Green and Phillips, 1975). Using an embryogenic inbred line (A188), two types of callus were formed, *i.e.* compact Type I callus and friable Type II callus, on which somatic embryos were induced. When subcultured on 2,4-D-free maturation and regeneration media, the somatic embryos matured and developed to plants (Bronsema et al., 1997). Quantitative analysis showed that the 2,4-D was rapidly taken up during the first 5 days of culture (Bronsema et al., 1996). A comparative study was made with respect to callus induction and regeneration with the non-embryogenic inbred A632 (Bronsema et al., 1997). Qualitative analysis showed that the greater part of the accumulated 2,4-D was free 2,4-D. A minor part was conjugated or metabolised. Analysing the 2,4-D which accumulated in A188 and A632, it appeared that A632 embryos exhibited the highest 2,4-D metabolism (Bronsema et al., 1996). There was also a difference in the distribution of 2,4-D, as observed by autoradiography on sections of embryos after culture on ^{14}C-2,4-D containing medium (Bronsema et al., 1998).

Next to the presence of 2,4-D, the composition of the nutrient medium influences the growth response of cultured maize embryos. Bronsema et al. (1997) compared nutrient media for the culture of A188 and A632 embryos. They found that culture on N6 based media with 2% sucrose and supplemented with L-proline and casein hydrolysate enhanced the formation of friable, Type II callus. Mannitol was added to the proliferation medium to maintain the embryogenic capacity of the callus at ongoing culture (Emons et al., 1993). Selection of the callus for subculture remained, however, necessary since non-embryogenic and Type I callus was also induced and only Type II callus was desired for establishing suspension cultures.

Culture on MS based media with 6% sucrose resulted in the formation of Type I callus and was not selected for further use.

Although 2,4-D is required initially for the induction of callus formation and somatic embryogenesis, a reduction of its concentration is needed for further embryo development and germination. Such a change in concentration happens during embryo culture under exhaustive conditions. After 14 days of culture most of the 2,4-D present in the culture medium is taken up by the embryos (Bronsema et al., 1996). Subculture on proliferation medium supplies the embryogenic callus with new 2,4-D for maintenance in an undifferentiated state (Emons and Kieft, 1995).

In this study we aim at understanding the influence of 2,4-D on the growth response of cultured maize embryos. Exhaustive conditions were simulated either by applying pulse-chase experiments with 2,4-D, or by culturing under conditions in which the concentration of 2,4-D was varied, to observe the morphological influence of 2,4-D. It was further tested whether reduced polar transport of auxins, caused by triiodobenzoic acid (TIBA), had an impact on callus induction and regeneration. Bronsema et al. (1996, 1998) analysed the uptake and distribution of ^{14}C-2,4-D under the influence of TIBA. They found that pre-treatment with 5 mg/l TIBA in medium without 2,4-D, resulted in a lower uptake of 2,4-D after 16 h of culture on induction medium with 2,4-D. Here it is questioned what influence TIBA has on morphogenesis when added alone to the induction medium or in combination with 2,4-D. As a third aspect, the specificity of the morphogenetic response on 2,4-D was analysed by monitoring the effects of competition between 2,4-D and the non-inducing analogon 3,5-D which competes with auxins for binding sites in the cell (Felle, 1991).

Thus, this article presents data on the influence of the duration and concentration of 2,4-D on embryos, the interactions of 2,4-D with the auxin transport inhibitor TIBA and the inactive auxin 3,5-D, all in relation to the induction of callus and further differentiation of immature embryos of the embryogenic inbred line A188 and the non-embryogenic A632.

Material and methods

Plant material and culture

Maize plants of the embryogenic inbred line A188 and the non-embryogenic inbred line A632 (kindly provided by Dr. C.E. Green, Minnesota) were grown in the greenhouse during the summer. Immature embryos with sizes ranging from 1.5 to 2.0 mm were excised 11-12 days after controlled hand pollination (DAP) and placed in 9-cm Petri dishes containing 25 ml induction medium. The *induction medium* consisted of a modified N6 medium (Chu et al., 1975) supplemented with 2.3 g/l L-proline, 200 mg/l casein hydrolysate, 2% sucrose, 8 g/l agar and 2 mg/l 2,4-D, pH 5.8 (Emons et al., 1993). Embryos were cultured in the dark at 25°C. Embryogenic callus was subcultured on *proliferation medium, i.e.* induction medium supplemented with 3% mannitol. With biweekly subculture on the 2,4-D-containing proliferation medium, the callus remained embryogenic for at least one year (Bronsema et al., 1997). Somatic embryos matured on growth regulator-free MS medium (Murashige and Skoog, 1962) with 6% sucrose (*maturation medium*) and regenerated to plants on MS medium with 2 % sucrose (*regeneration medium*). For each series of experiments embryos were excised from one cob to exclude cob dependent influences.

Influence of duration of 2,4-D treatment

Embryos of inbred A188 were cultured on induction medium with 2 mg/l 2,4-D for various periods of time ranging from 0 to 24 h and from 1 to 13 days to determine the influence of the length of the pulse with 2,4-D on further development. Hereafter they were subcultured for 7 days on *growth medium, i.e.* induction medium without 2,4-D, and investigated morphologically.

Culture under exhaustive and non-exhaustive culture conditions

The effect of daily subculture on the increase in weight and morphology of cultures of A188 was compared with the normal, exhaustive culture, where the embryos were maintained in the same Petri dish with induction medium for 14 days. Under non-exhaustive conditions the embryos were daily subcultured on fresh induction medium. Weight increase was determined after 1, 2, 4, 6, 8, 10, 12 and 14 days in culture (DIC). Each sample consisted of 25 embryos. Under exhaustive conditions embryos were sampled at 0, 7 and 14 days in 3 independent experiments. For each data point again 25 embryos were sampled.

Effect of changes in 2,4-D concentration

The influence of the concentration of 2,4-D in the induction medium on germination, callus induction and morphogenesis was investigated for the two inbred lines. Embryos were cultured under exhaustive culture conditions on induction medium with 2,4-D concentrations ranging from 0, 2.10^{-3}, 2.10^{-2}, 2.10^{-1}, 2, 20, 200 to 2000 mg/l. The influence of the 2,4-D concentration was determined after 2-4 weeks of culture.

Effect of embryo orientation

The influence of the positioning of excised embryos, with their meristem side or with their scutellar side on the induction medium, was investigated in relation to the uptake of 2,4-D and morphogenesis. The medium was supplemented with ^{14}C-2,4-D. Differences in uptake of 2,4-D were quantified by scintillation counting after 16 h of culture (Bronsema et al., 1996). The morphology of embryos was compared after 14 and 28 days of culture.

TIBA treatment

The influence of the auxin transport inhibitor triiodobenzoic acid (TIBA) on morphogenesis was tested for both A188 and A632 embryos cultured on induction medium. The influence of TIBA was first tested in the absence of 2,4-D by applying TIBA in concentrations ranging from 0.2 to 20 mg/l. The influence of TIBA in combination with 2,4-D was then studied: combinations of 2, 20 and 200 mg/l TIBA with 0.2 mg/l 2,4-D were tested, as well as concentrations of 20, 200 or 2000 mg/l TIBA with 2 and with 20 mg/l 2,4-D. The effects on callus induction and germination were determined after 3 weeks of culture.

3,5-D treatment

The influence of the non-auxin 3,5-D on morphogenesis was tested for both A188 and A632 embryos. First the influence of 3,5-D was investigated in medium without 2,4-D by varying 3,5-D concentrations from 0.2 to 20 mg/l. Next combinations of 3,5-D with 2,4-D were studied: a concentration of 0.2 mg/l 2,4-D was combined with 2, 20 and 200 mg/l 3,5-D, and 2 mg/l 2,4-D was combined with 20, 200 and 2000 mg/l 3,5-D. The effects on callus induction and germination were determined after 2-3 weeks of culture.

Results

Influence of the duration of 2,4-D treatment on the culture of A188 embryos

Embryos of A188 were cultured on 2,4-D containing medium (*induction medium*) for 0-24 h, subcultured on medium without 2,4-D (*growth medium*) for 7 days, and then monitored for shoot and root development, callus formation and embryogenesis (Fig. 1). When embryos were cultured without 2,4-D (pulse period 0 h), embryos germinated *i.e.* roots and shoots elongated. After a 2,4-D pulse of 0.5 h the length of the shoot increased. After a pulse of 1 and 2 h the elongation reduced. A pulse of 4 h or longer highly reduced the outgrowth of the shoot. Root outgrowth was influenced from 0.5 h treatment onwards as well. Culture for 0.5 and 1 h resulted in an increase in length of the roots. After a 2 h pulse reduction of root length was observed, and after a 4 h pulse or longer only the coleorhiza of the embryos grew out, without emergence of the root. The results on outgrowth of shoots and roots are summarised in Table 1 and Graph 1a.

The relation between the period of culture on 2,4-D containing medium and the rate of germination after 7 days of culture on 2,4-D free medium (growth medium) is depicted in Graph 1b. Shoots and roots showed a reduced outgrowth after 4 h of culture on medium with 2,4-D, resulting in a reduction of germination to 48 %. When embryos were subcultured on growth medium for 2 weeks, up to 64 % of the embryos germinated, and after 4 weeks they all germinated (Graph 1b), this in contrast to embryos which were continuously cultured on induction medium as a control, these embryos never germinated (Fig. 1i). Induction of callus was incidentally observed when embryos had first been on induction medium for 16 h and then on growth medium for 7 days. It was regularly seen after a pulse of 24 h on induction medium and subculture for 7 days (Fig. 1h). Such embryos exhibited crown-like outgrowths at the basal side of the scutellum. The outgrowths were in contact with the agar and consisted of compact non-embryogenic callus. Friable embryogenic callus was not formed when the induction period was limited to 24 h (see also Table 1).

When embryos of A188 were first cultured on induction medium for 1-14 days and then cultured on growth medium for 7 days, the enlargement of the shoot was generally restricted to outgrowth of the coleoptile in a corkscrew manner. Root outgrowth, expressed as the length of the coleorhiza, was inhibited when culture on induction medium lasted at least 3 days.

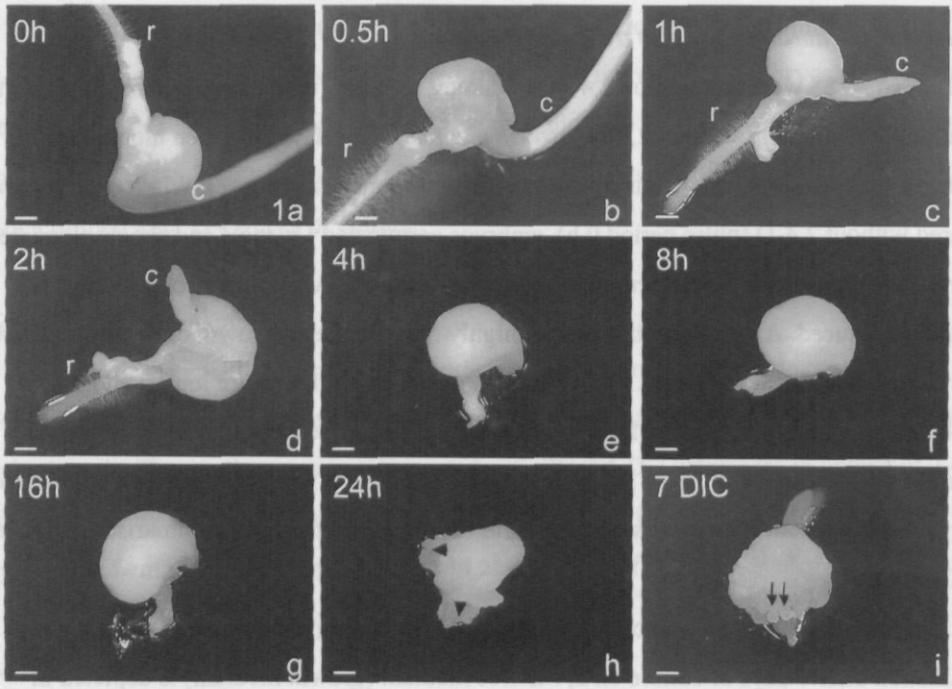


Figure 1: Influence of the duration of 2 mg/l 2,4-D in the induction medium (IM) and subsequent culture on growth medium (GM) for 7 days, on the growth of embryos of inbred line A188. ***a***: Culture on GM for 7 days without pre-treatment on IM with 2,4-D. ***b***: Culture for 0.5 h on IM and GM for 7 days. ***c***: Culture for 1 h on IM and GM for 7 days. ***d***: Culture for 2 h on IM and GM for 7 days. ***e***: Culture for 4 h on IM and GM for 7 days. ***f***: Culture for 8 h on IM and GM for 7 days. ***g***: Culture for 16 h on IM and GM for 7 days. ***h***: Culture for 24 h on IM and GM for 7 days, note the formation of crown-like outgrowths (arrowheads). ***i***: Culture for 7 days on IM without subculture on GM, arrows point to somatic embryos. r = root, c = coleoptile, arrowheads = crown-like outgrowths, arrows = somatic embryos, bars in the figures represent 1 mm.

Formation of compact non-embryogenic callus was observed when pulses were 1 day or longer (Fig. 2). When embryos were on induction medium for 1-5 days, and then subcultured on growth medium for 7 days, the non-embryogenic callus formed shoots and leafy structures. Embryogenic callus was incidentally observed after 5 days of culture on induction medium (Fig. 2d) and frequently from 7 days of inductive culture onwards (Figs. 2e-i). The callus was friable Type II callus (Green et al., 1983), which is the type routinely obtained when embryos are first cultured on induction medium for 14 days and then put onto proliferation

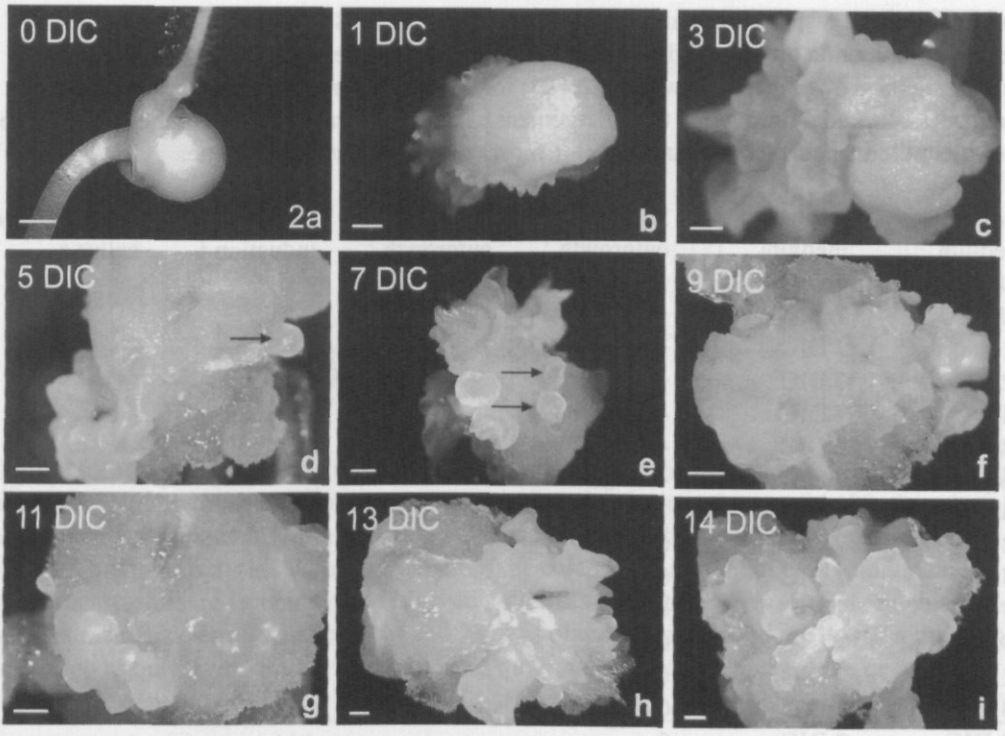


Figure 2: Influence of the duration of 2 mg/l 2,4-D in the induction medium (IM) and subsequent culture on growth medium (GM) for 7 days, on the growth of embryos of inbred line A188. ***a***: Culture for 7 DIC on GM, without pre-treatment on IM with 2,4-D . ***b***: Culture for 1 DIC on IM and 7 DIC on GM. ***c***: Culture for 3 DIC on IM and 7 DIC on GM. ***d***: Culture for 5 DIC on IM and 7 DIC on GM. ***e***: Culture for 7 DIC on IM and 7 DIC on GM. ***f***: Culture for 9 DIC on IM and 7 DIC on GM. ***g***: Culture for 11 DIC on IM and 7 DIC on GM. ***h***: Culture for 13 DIC on IM and 7 DIC on GM. ***i***: Culture for 14 DIC on IM and 7 DIC on GM, arrows point to somatic embryos. Bars in the figure represent 1 mm.

medium (Bronsema et al., 1997). The culture response of A188 embryos is summarised in Table 1.

Influence of exhaustive and non-exhaustive culture conditions.

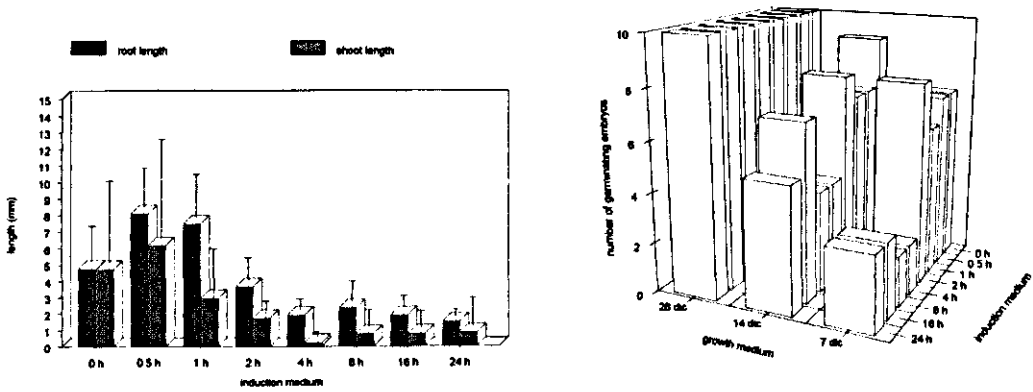
The effect of culture under exhaustive and non-exhaustive conditions on the morphology of A188 embryos is given in Fig. 3. Embryos of the two cultures showed morphological differences after 7 days. Under exhaustive conditions more growth was observed at the basal

Table 1: Culture response of maize embryos of inbred line A188. Influence of the duration of culture on induction medium (with 2 mg/l 2,4-D) for various periods of time (0-24h) and subculture on growth medium (without 2,4-D) for 7 days. [1] ++ normal or slightly affected, + reduced, - blocked outgrowth, 0 the enlargement of the shoot was restricted to the outgrowth of the coleoptile in a corkscrew manner. [2] +++ most, ++ mediate, + little, - none.

Induction medium	Growth medium	Shoot outgrowth [1]	Root outgrowth [1]	Callus induction [2]	Embryogenic callus [2]
0 h	7 DIC	++	++	-	-
0.5 h	7 DIC	++	++	-	-
1 h	7 DIC	+	+	-	-
2 h	7 DIC	+	+	-	-
4 h	7 DIC	-	-	-	-
8 h	7 DIC	-	-	-	-
16 h	7 DIC	-	-	+	-
24 h	7 DIC	-	-	+	-
3 DIC	7 DIC	0	-	+	-
5 DIC	7 DIC	0	-	+	-
7 DIC	7 DIC	0	-	++	+
9 DIC	7 DIC	0	-	++	+
11 DIC	7 DIC	0	-	++	++
13 DIC	7 DIC	0	-	+++	++
14 DIC	7 DIC	0	-	+++	++

side of the scutellum compared to culture under non-exhaustive conditions. After 14 days of culture more friable callus with somatic embryos was obtained on embryos grown under exhaustive conditions. Under non-exhaustive culture conditions more leaf-like structure were observed, although friable, embryogenic callus was formed as well. When embryogenic callus of both cultures was subcultured on proliferation medium, the callus, obtained under exhaustive conditions, grew faster and had a higher embryogenic capacity than the callus formed under non-exhaustive conditions. The increase of weight of A188 embryos was determined with samples of 25 embryos each and appeared higher during the whole culture period when cultured under exhaustive culture conditions than under non-exhaustive culture conditions (Fig. 4).

Influence of the concentration of 2,4-D in the induction medium.



Graph 1: Influence of 0-24 h of exposure to 2 mg/l 2,4-D in the induction medium and 7 days of culture on growth medium on the growth of cultured zygotic embryos of A188. Samples of ten embryos per treatment from the same cob were compared for their response to 2,4-D. **a.** The outgrowth of root (including the length of the coleorhiza) and shoot of 10 individual embryos are measured and the mean is given with SD values. **b.** Germination of ten embryos per treatment is determined after 7, 14 and 28 days of culture on growth medium. Germination is defined as outgrowth of both coleoptile and root (including coleorhiza) of the excised embryo.

The influence of the concentration of 2,4-D on the development of cultured embryos is shown in Figs. 5a, c and Graph 2a for the embryogenic inbred line A188 and in Figs. 5b, d and Graph 2b for the non-embryogenic inbred A632. In the standard induction medium 2 mg/l 2,4-D is used (Bronsema et al., 1997). When the concentration was raised from 2 mg/l to 20 and 200 mg/l, less embryogenic callus was formed in both inbreds. In the case of 2000 mg/l 2,4-D embryos did not form callus at all, and became static (Figs. 5a, b). They remained in this condition for several months, before they started to degrade.

When A188 embryos were cultured at 2.10^{-1} mg/l 2,4-D there was still formation of embryogenic callus and germination did not occur (Fig. 5c). At 2.10^{-2} mg/l 2,4-D there was a shift from formation of embryogenic callus to germination (Fig. 5c), and at 2.10^{-3} and 0 mg/l 2,4-D the embryos germinated and did not form callus at all (Graph 2a).

In the case of A632, lowering the concentration to 2.10^{-1} mg/l 2,4-D instead of 2 mg/l resulted in less callus formation (Fig. 5d). By ongoing culture for 4 weeks some callus resembled Type I callus. At a concentration of 2.10^{-2} mg/l 2,4-D we observed transition from induction of non-

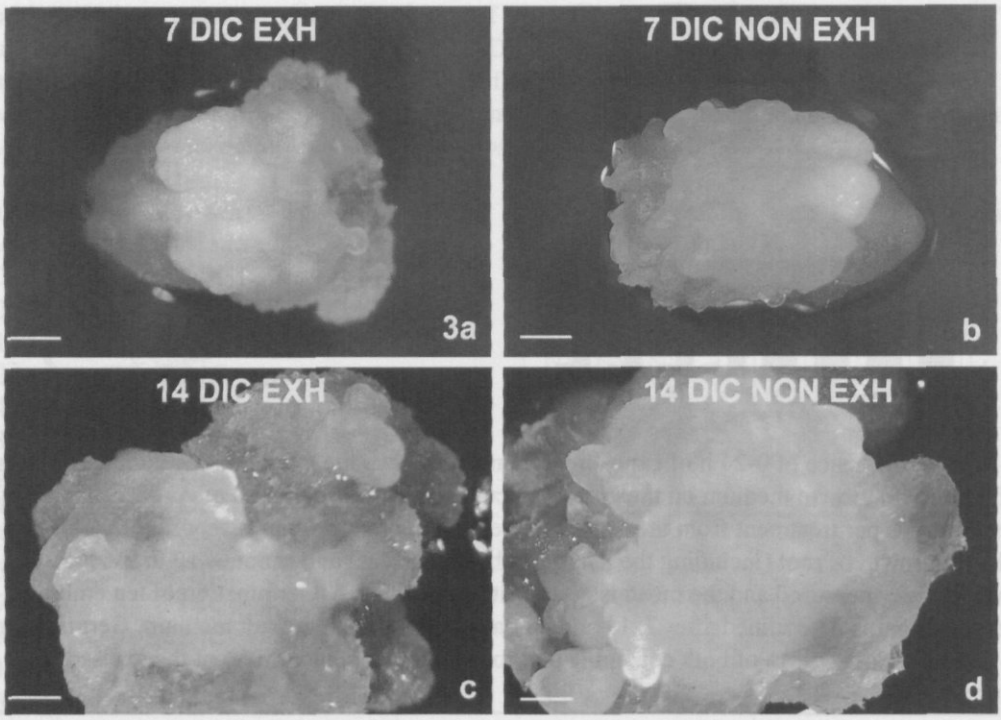


Figure 3: Influence of culture under exhaustive and non-exhaustive culture conditions on the growth of embryos of A188. ***a***: After 7 DIC on exhaustive induction medium. ***b***: After 7 DIC on non-exhaustive induction medium. ***c***: After 14 DIC on exhaustive induction medium. ***d***: After 14 DIC on non-exhaustive induction medium. Note the slight increase in size compared to **c**. Bars in the figure represent 1 mm.

embryogenic callus to germination, *i.e.* some embryos formed callus and some germinated. When the concentration of 2,4-D was further lowered to 2.10^{-3} or 0 mg/l all embryos germinated and callus formation was absent (Graph 2b). When embryos were first cultured on 0.2 mg/l 2,4-D for 14 days and then subcultured on proliferation medium with 0.2 or 2 mg/l 2,4-D (Figs. 6a,b), only non-embryogenic callus was obtained. When further subcultured on maturation and regeneration medium all calli only formed roots.

Influence of the positioning of the embryos on the induction medium

Under standard culture conditions A188 embryos are cultured with their meristem side in contact with the medium. They regularly formed a coleoptile in a corkscrew manner and

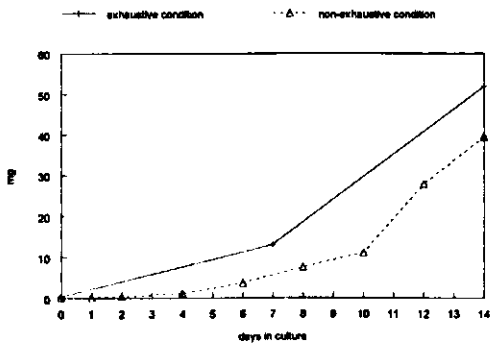


Figure 4: Influence of culture for 14 days under exhaustive and non-exhaustive conditions on the weight of embryos of A188. Each data point represents a sample of 25 embryos, originating from the same cob for each treatment.

always embryogenic callus (Fig. 7a, c). Culture with the scutellum side in contact with the medium resulted in similar development (Fig. 7a, c). In case of the A632 we observed formation of non-embryogenic callus and outgrowth of coleoptiles after 14 DIC in both ways of culture (Fig. 7b), but when the embryos had been cultured on the meristem side for another 14 days more callus was formed (Fig. 7d) than when they had been cultured on the meristem side, and more roots when cultured on the scutellum side (Fig. 7d). Incidentally callus formation was dominant.

Uptake of ^{14}C-2,4-D was measured for both culture procedures and for both inbreds. Samples of 5 embryos of A188 took up 5619 DPM after culture on the meristem side, and culture on the scutellum resulted in an uptake of 12881 DPM, which is twice as much. For A632 the uptake was 2674 DPM for culture on the meristem and 18979 DPM for culture on the scutellum, which is 7 times as much.

Influence of TIBA and combinations of 2,4-D and TIBA on morphogenesis.

The influence of the addition of TIBA to induction medium *without* 2,4-D is shown in Fig. 8a. Embryos of both lines were cultured in the same Petri dish. Except for a slight reduction in root formation there was no remarkable difference between culture without TIBA (Fig. 5c, d) and with 0.2 mg TIBA (Fig. 8a). Addition of 2 mg/l TIBA caused a decreased elongation of shoots and a blocked root formation, especially in A632 embryos. At a concentration of 20 mg/l TIBA the coleoptiles were smaller and again root formation was highly reduced. When

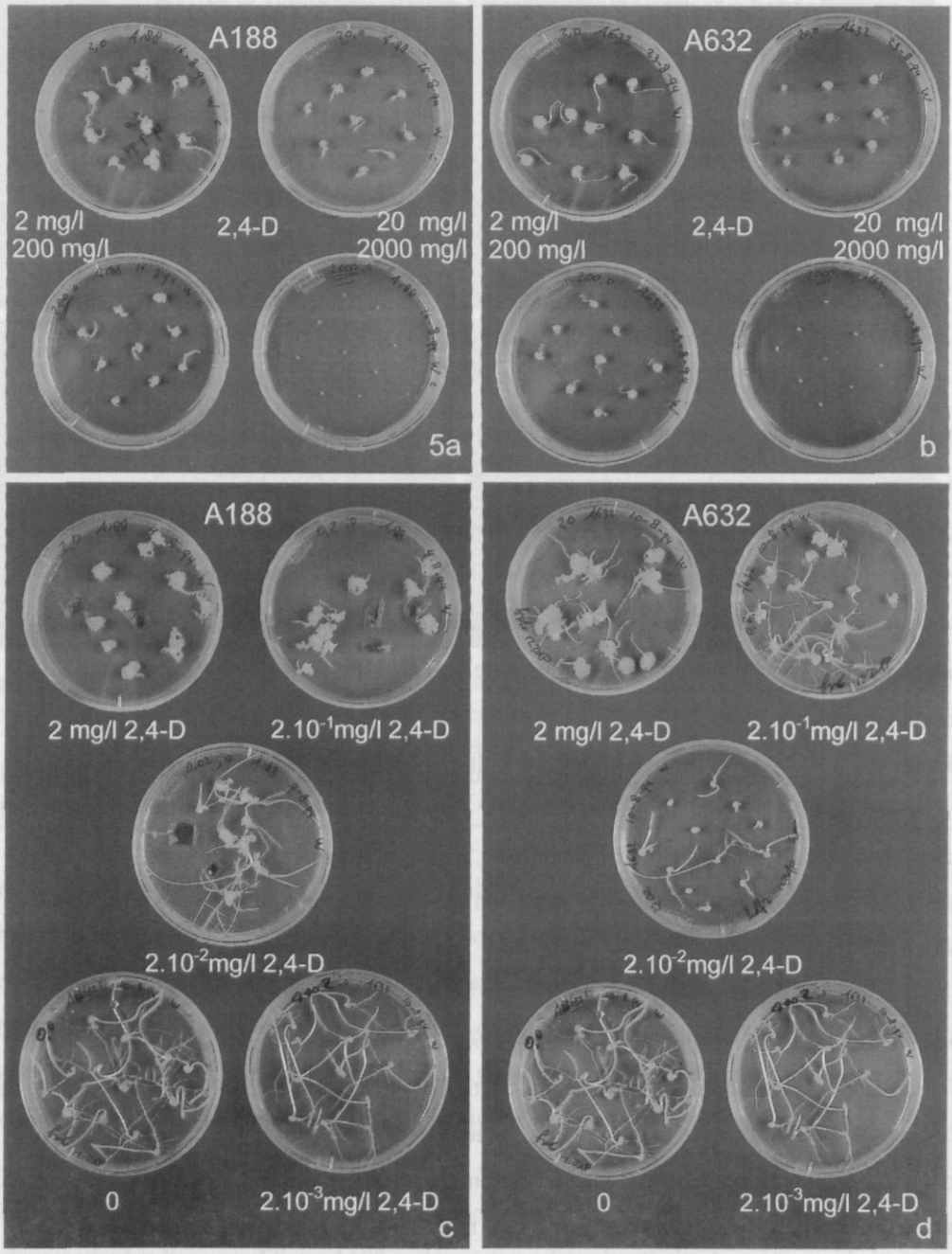

A188
2 mg/l
200 mg/l
2,4-D
20 mg/l
2000 mg/l
5a
A632
2 mg/l
200 mg/l
2,4-D
20 mg/l
2000 mg/l
b
A188
2 mg/l 2,4-D
2.10-1mg/l 2,4-D
2.10-2mg/l 2,4-D
0
2.10-3mg/l 2,4-D
c
A632
2 mg/l 2,4-D
2.10-1mg/l 2,4-D
2.10-2mg/l 2,4-D
0
2.10-3mg/l 2,4-D
d

Figure 5: Influence of the concentration of 2,4-D in the induction medium on the growth of embryos of inbred lines A188 and A632 as observed after 2-4 weeks of culture. ***a***: Influence of 2, 20, 200 and 2000 mg/l 2,4-D after 21 days in culture (DIC) for A188. ***b***: Influence of 2, 20, 200 and 2000 mg/l 2,4-D after 14 DIC for A632. ***c***: Influence of 2, 2.10^{-1}, 2.10^{-2}, 2.10^{-3} and 0 mg/l 2,4-D after 28 DIC for A188. ***d***: Influence of 2, 2.10^{-1}, 2.10^{-2}, 2.10^{-3} and 0 mg/l 2,4-D after 28 DIC for A632.

cultured for 3 more weeks all embryos, however, germinated. So the application of TIBA in the induction medium without 2,4-D did slow down shoot and root elongation, but did not prevent germination after prolonged culture.

The effect of combinations of TIBA *with* 2,4-D on the growth of cultured embryos is shown in Fig. 9. The development of the embryos was determined after three weeks of culture with respect to germination and callus induction and is summarised in Graphs 2c,d,e,f. When 2 mg/l TIBA was added to induction medium with 0.2 mg/l 2,4-D, (Figs. 9a,d,g, 10a), A188 and A632 embryos formed less callus but more roots than cultures at the same 2,4-D concentration without TIBA (cf. Fig. 9d with Fig 12a,b). Addition of 20 mg/l TIBA to media with 0.2 mg/l 2,4-D resulted in more outgrowth of coleoptiles in inbred line A188 and less root formation in A632 (Graphs 2c,e). Callus formation was greatly reduced in both inbred lines (cf. Fig. 9d with 12a,b, Graphs 2d,f). Addition of 200 mg/l TIBA resulted in a static state of the embryos for both inbred lines. The embryos remained in the initial state for several months without necrosis. The addition of 20 mg/l TIBA to the normal culture condition of 2 mg/l 2,4-D resulted in less callus formation in both inbred lines (cf. Figs. 9e,h with 5a,b). Addition of higher concentrations of TIBA, *i.c.* 200 and 2000 mg/l, resulted in static embryos (Figs. 9b,e,h, 10b, Graphs 2c,d,e,f). At the highest concentration of 2,4-D tested, *i.e.* 20 mg/l, addition of 20 mg/l TIBA resulted in comparable or slightly better outgrowth of the coleoptile and less callus formation for A188 (cf. Fig. 9i and 5a). In case of A632 coleoptile growth was improved and less callus was formed on the scutellum (cf. Fig. 9f and 5b). Addition of more TIBA in the induction medium resulted in a static state of the embryos of both inbred lines (Figs. 9c,f,i, Graphs 2c,d,e,f).

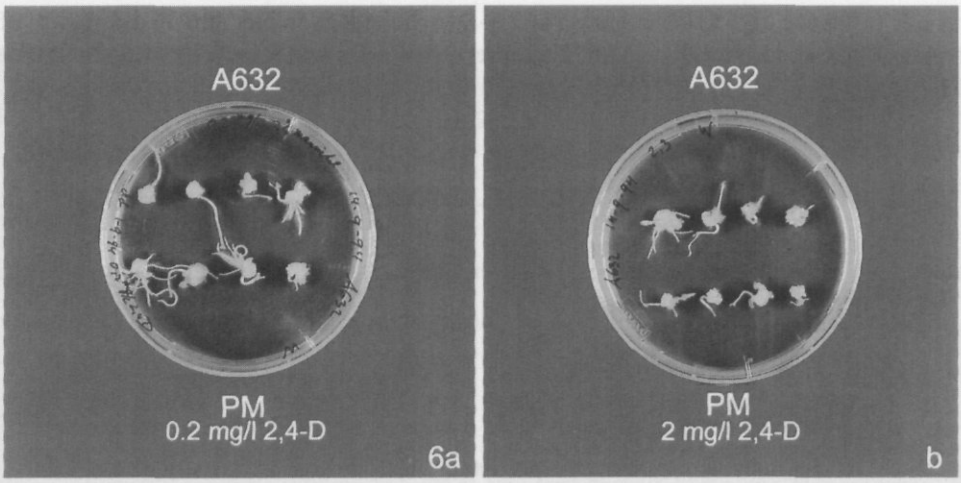


Figure 6: Cultured embryos of inbred line A632 on proliferation medium with 3% mannitol after 7 days in culture (DIC), obtained after 14 DIC on induction medium with 0.2 mg/l 2,4-D. ***a***: Embryos cultured with 0.2 mg/l 2,4-D in the proliferation medium. ***b***: Embryos cultured with 2 mg/l 2,4-D in the proliferation medium.

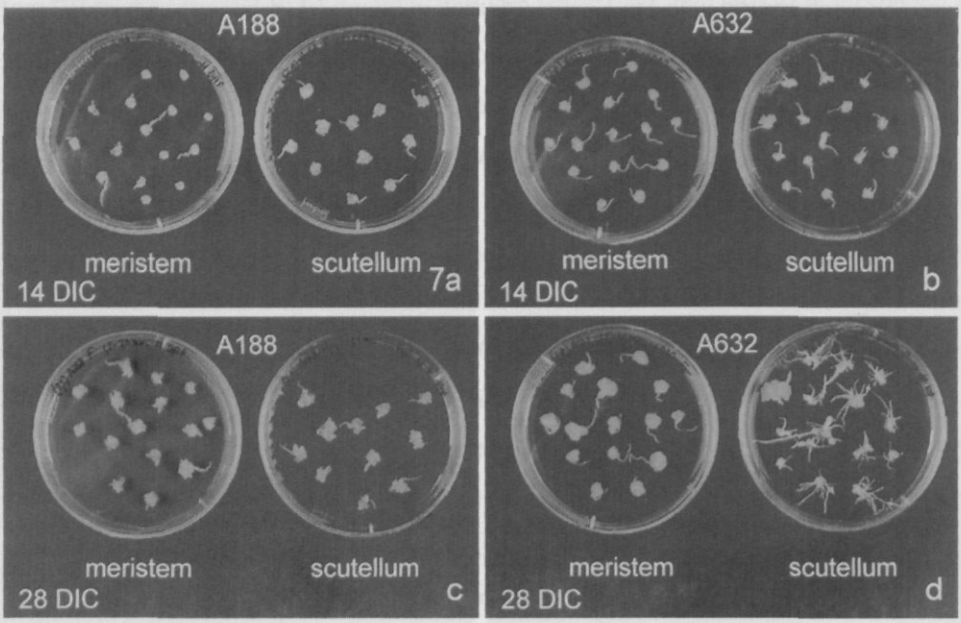


Figure 7: Influence of the positioning of the excised embryo on the induction medium. The meristem side or the scutellum side is in contact with the induction medium. ***a***: After 14 days in culture (DIC) on induction medium (IM) for inbred A188. ***b***: After 14 DIC on IM for inbred A632. ***c***: After 28 DIC on IM for inbred A188. ***d***: After 28 DIC on IM for inbred A632.

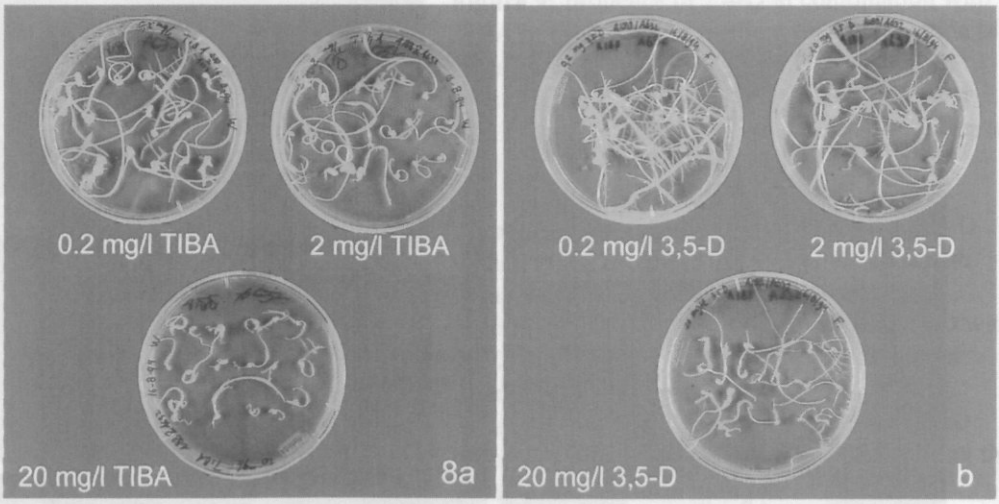
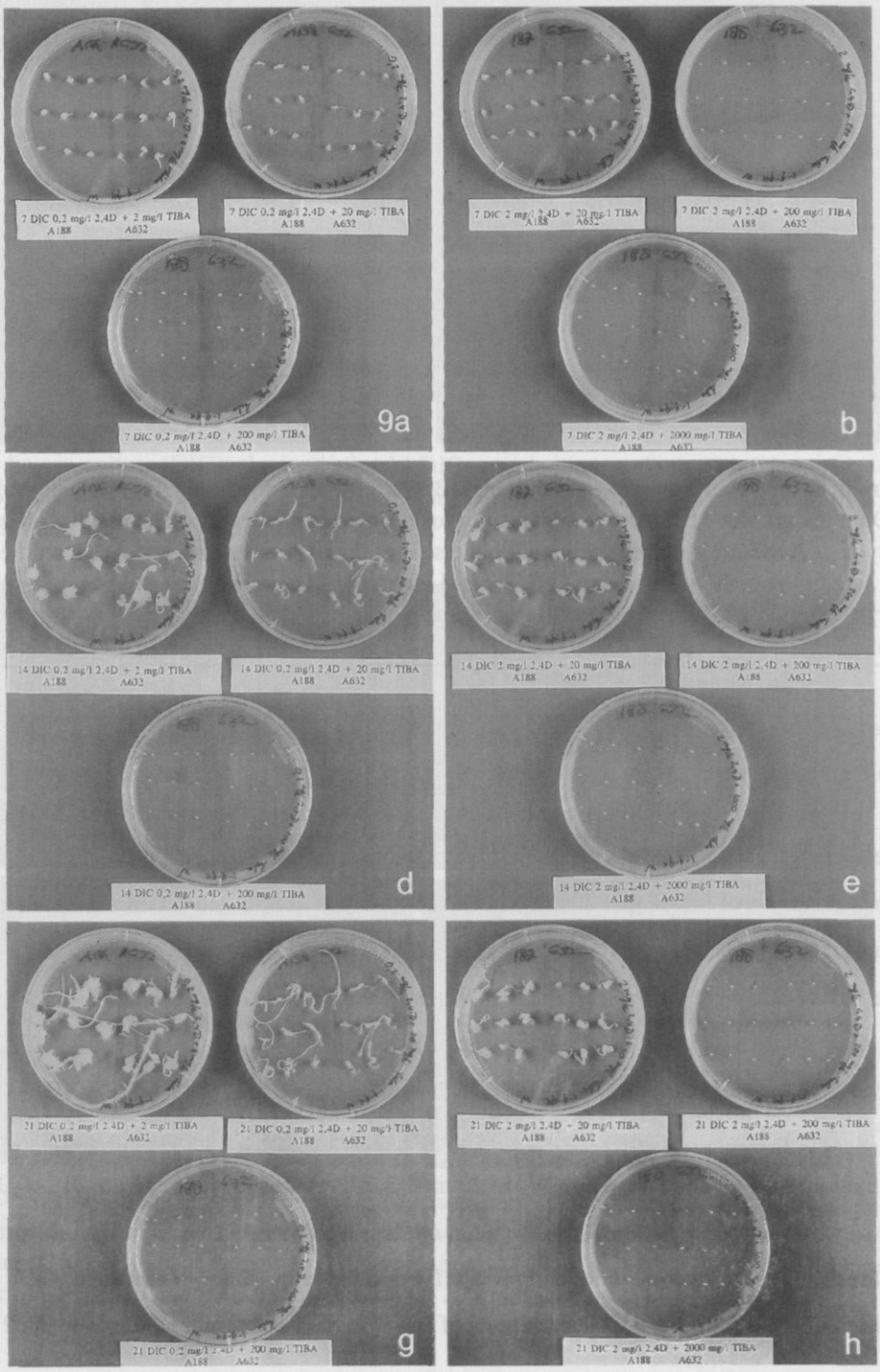


Figure 8: Influence of the concentration of TIBA and 3,5-D in the induction medium without 2,4-D on the growth of immature maize embryos of inbreds A188 and A632. In all dishes 9 embryos of A188 are cultured on the left hand side of the dish, 9 embryos of A632 on the right hand side of the dish. ***a***: Influence of 0.2, 2 and 20 mg/l TIBA on the growth of immature embryos after 21 days in culture (DIC). ***b***: Influence of 0.2, 2 and 20 mg/l 3,5-D on the growth of immature embryos after 21 DIC.

Influence of the concentration of 3,5-D and combinations of 2,4-D and 3,5-D on morphogenesis.

The effect of addition of 3,5-D to the induction medium *without* 2,4-D is presented in Fig. 8b. At concentrations of 0.2, 2, and 20 mg/l 3,5-D the embryos of both inbred lines germinated, although germination retarded with increasing 3,5-D concentrations.

The effect of combinations of 3,5-D *with* 2,4-D on the induction of callus in the inbred lines is shown in Figs. 11a and b. The low concentration of 0.2 mg/l 2,4-D was combined with 2, 20 and 200 mg/l 3,5-D (Fig. 11a). At 2 mg/l 3,5-D callus formation was not influenced in A188 (cf. Fig. 11a with 12a), whereas in A632 we observed less callus formation and better germination (cf. Fig. 11a with 12b). When 20 mg/l 3,5-D was added to the 0.2 mg/l 2,4-D there was a large reduction of callus formation on the scutella of A188 and A632, and an increased outgrowth of coleoptiles and roots (cf. Figs. 11a with 12a,b), resembling the condition of growth with low concentrations of 2,4-D in the induction medium (Graphs 2


7 DIC 0,2 mg/l 2,4D + 2 mg/l TIBA
A188 A632
7 DIC 0,2 mg/l 2,4D + 20 mg/l TIBA
A188 A632
7 DIC 0,2 mg/l 2,4D + 200 mg/l TIBA
A188 A632
9a
7 DIC 2 mg/l 2,4D + 20 mg/l TIBA
A188 A632
7 DIC 2 mg/l 2,4D + 200 mg/l TIBA
A188 A632
7 DIC 2 mg/l 2,4D + 2000 mg/l TIBA
A188 A632
b
14 DIC 0,2 mg/l 2,4D + 2 mg/l TIBA
A188 A632
14 DIC 0,2 mg/l 2,4D + 20 mg/l TIBA
A188 A632
14 DIC 0,2 mg/l 2,4D + 200 mg/l TIBA
A188 A632
d
14 DIC 2 mg/l 2,4D + 20 mg/l TIBA
A188 A632
14 DIC 2 mg/l 2,4D + 200 mg/l TIBA
A188 A632
14 DIC 2 mg/l 2,4D + 2000 mg/l TIBA
A188 A632
e
21 DIC 0,2 mg/l 2,4D + 2 mg/l TIBA
A188 A632
21 DIC 0,2 mg/l 2,4D + 20 mg/l TIBA
A188 A632
21 DIC 0,2 mg/l 2,4D + 200 mg/l TIBA
A188 A632
g
21 DIC 2 mg/l 2,4D + 20 mg/l TIBA
A188 A632
21 DIC 2 mg/l 2,4D + 200 mg/l TIBA
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21 DIC 2 mg/l 2,4D + 2000 mg/l TIBA
A188 A632
h

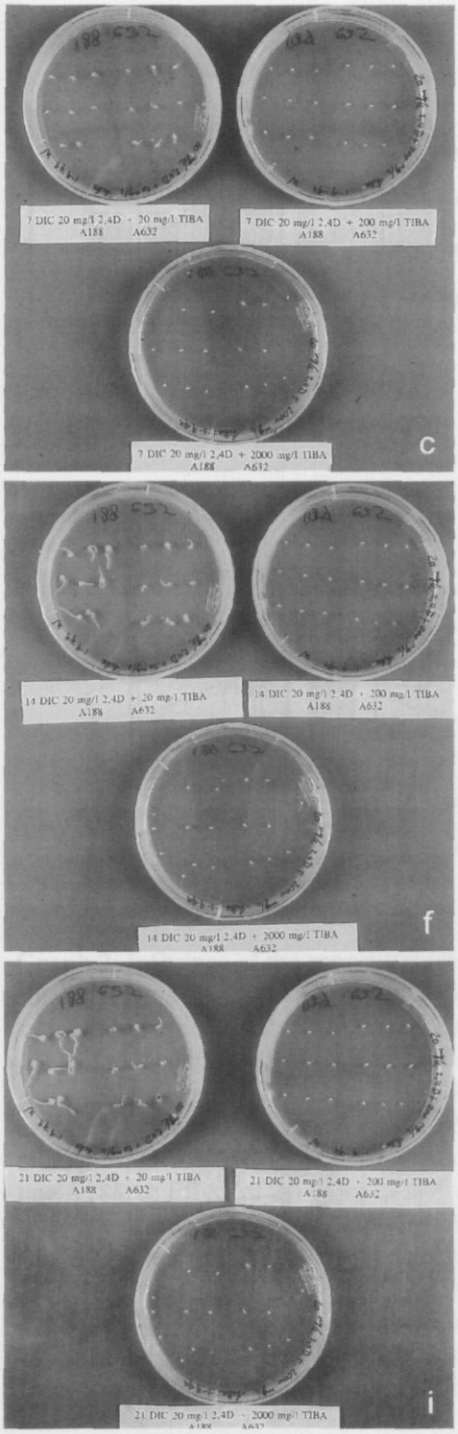
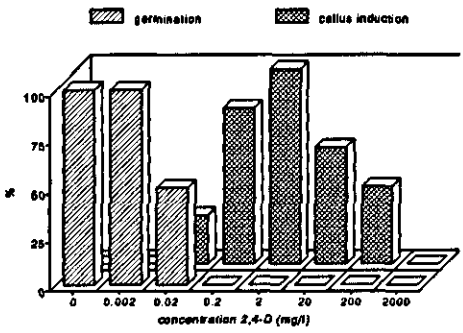
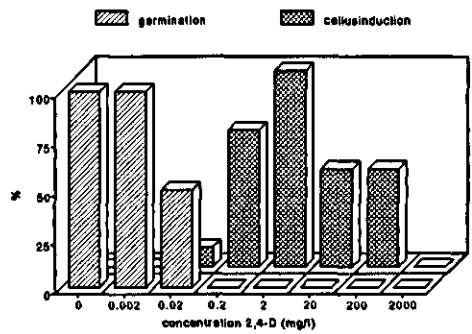


Figure 9: Influence of 2,4-D in combination with TIBA in the induction medium on the induction of callus on immature embryos after 7, 14 and 21 days of culture (DIC) in the dark for A188 and A632. In all dishes 9 embryos of A188 are cultured on the left hand side of the dish, 9 embryos of A632 at the right hand side of the dish.
a: Influence of the combination of 0.2 mg 2,4-D with 2, 20 or 200 mg/l TIBA after 7 DIC on the induction of callus in both inbred lines.
b: Influence of the combination of 2 mg 2,4-D with 20, 200 or 2000 mg/l TIBA after 7 DIC on the induction of callus in both inbred lines.
c: Influence of the combination of 20 mg 2,4-D with 20, 200 or 2000 mg/l TIBA after 7 DIC on the induction of callus in both inbred lines.
d: Influence of the combination of 0.2 mg 2,4-D with 2, 20 or 200 mg/l TIBA after 14 DIC on the induction of callus in both inbred lines.
e: Influence of the combination of 2 mg 2,4-D with 20, 200 or 2000 mg/l TIBA after 14 DIC on the induction of callus in both inbred lines.
f: Influence of the combination of 20 mg 2,4-D with 20, 200 or 2000 mg/l TIBA after 14 DIC on the induction of callus in both inbred lines.
g: Influence of the combination of 0.2 mg 2,4-D with 2, 20 or 200 mg/l TIBA after 21 DIC on the induction of callus in both inbred lines.
h: Influence of the combination of 2 mg 2,4-D with 20, 200 or 2000 mg/l TIBA after 21 DIC on the induction of callus in both inbred lines.
i: Influence of the combination of 20 mg 2,4-D with 20, 200 or 2000 mg/l TIBA after 21 DIC on the induction of callus in both inbred lines.

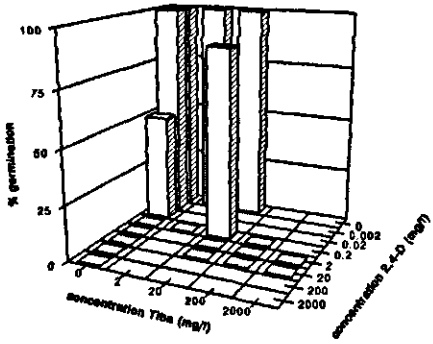
a: A188



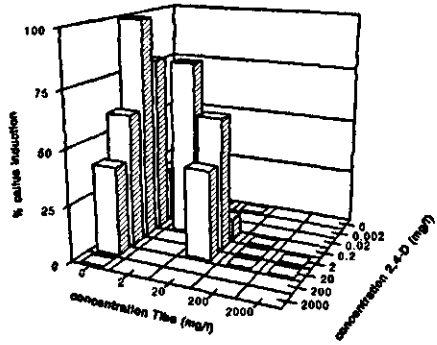
b: A632



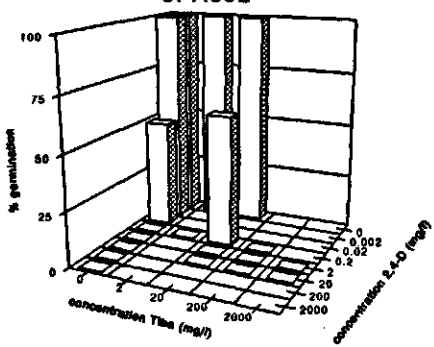
c: A188



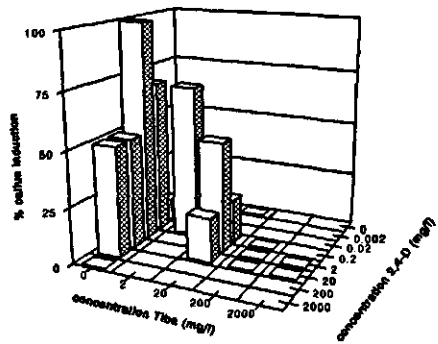
d: A188

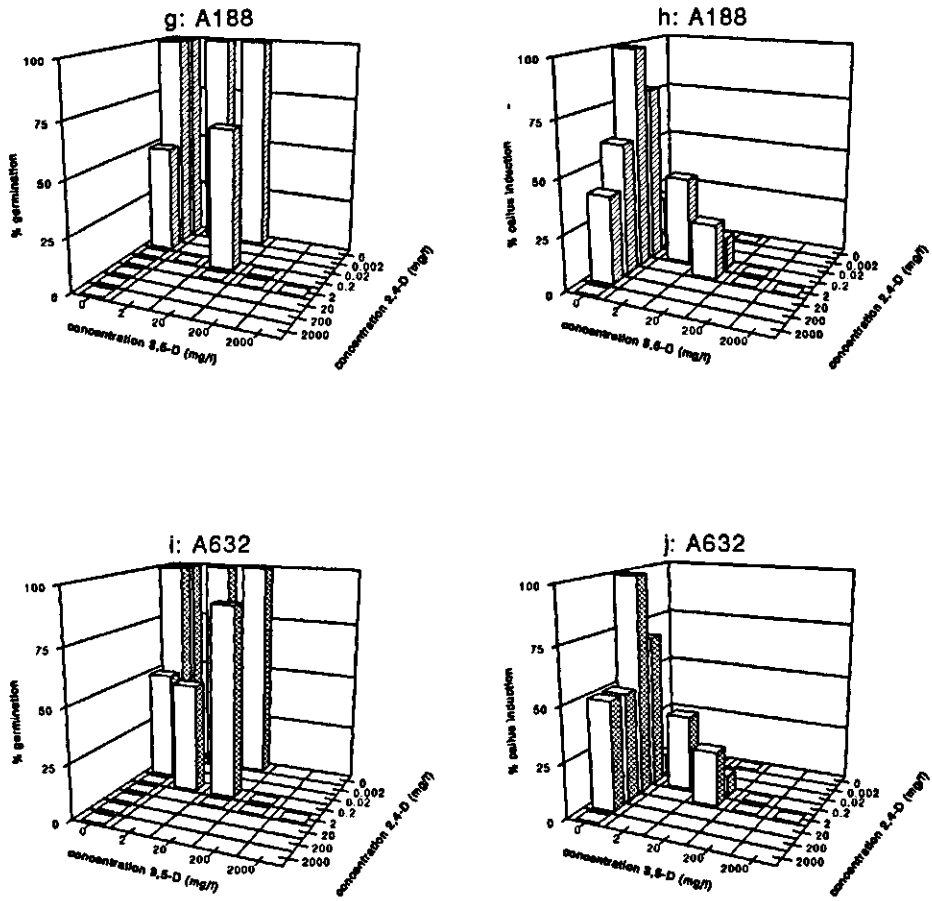


e: A632



f: A632





Graph 2: Influence of the concentration of 2,4-D in the induction medium on the growth of cultured zygotic embryos of A188 and A632. In both inbred lines the germination and callus formation are measured. Germination is defined as outgrowth of both coleoptile and root (including the coleorhiza) of the excised embryo. Callus induction was compared to callus induction at the standard culture condition of 2 mg/l 2,4-D, which is given as 100%. ***a***: Effect of 2,4-D on the embryogenic inbred line A188. ***b***: Effect of 2,4-D on the non-embryogenic inbred line A632. ***c***: Effect of 2,4-D and TIBA on the germination of the embryogenic inbred line A188. ***d***: Effect of 2,4-D and TIBA on the callus induction of the embryogenic inbred line A188. ***e***: Effect of 2,4-D and TIBA on the germination of the non-embryogenic inbred line A632. ***f***: Effect of 2,4-D and TIBA on the callus induction of the non-embryogenic inbred line A632. ***g***: Effect of 2,4-D and 3,5-D on the germination of the embryogenic inbred line A188. ***h***: Effect of 2,4-D and 3,5-D on the callus induction of the embryogenic inbred line A188. ***i***: Effect of 2,4-D and 3,5-D on the germination of the non-embryogenic inbred line A632. ***j***: Effect of 2,4-D and 3,5-D on the callus induction of the non-embryogenic inbred line A632.

g,h,i,j). If 200 mg/l 3,5-D was added to the 0.2 mg/l 2,4-D almost all callus formation and growth of coleoptiles and roots was prevented (cf. Figs. 11a with 12a,b).
Culture with 2 mg/l 2,4-D to which 20 mg/l 3,5-D was added, resulted in less callus formation especially with A632 (cf. Figs. 11b with 12a,b). Addition of 200 mg/l 3,5-D resulted in reduction of almost all callus formation and outgrowths of the coleoptiles (cf. Figs. 11b with 12a,b). Culture in combination with 2000 mg/l 3,5-D resulted in absence of a growth response (cf. Figs. 11b with 12a,b).

Discussion

Immature embryos of maize form embryogenic callus when they are excised 11-12 days after pollination and cultured with strong growth regulators like 2,4-D (Green and Phillips, 1975) or Dicamba (Duncan et al., 1985). The uptake of 2,4-D and the biochemical fate after uptake were studied previously for both the embryogenic inbred A188 and the non-embryogenic A632 (Bronsema et al., 1996). Under exhaustive conditions embryos took up 2,4-D rapidly during the first 3 days of culture but the embryogenic line accumulated less 2,4-D than the non-embryogenic line and metabolisation rates differed, too, as did culture responses such as germination, callus formation and somatic embryogenesis. Because of these differences, we now studied various aspects of treatment with 2,4-D in the two inbred lines in more detail, by analysing the influence of culture for defined periods on 2,4-D containing media, by culture under exhaustive and non-exhaustive conditions, by culture with a range of 2,4-D concentrations, by analysing the effects of TIBA and 3,5-D, and by changing the positioning of the embryo during culture.

Influence of the duration of 2,4-D treatment

When embryos were cultured on their meristem side and were exposed to 2,4-D for a short period of time (0-16h), and subcultured on growth medium, they showed hardly any callus formation and germinated after subculture. When exposed to 2,4-D for 16-24 h, the embryos exhibited crown-like outgrowths at the basal side of the scutellum, but after subculture still all

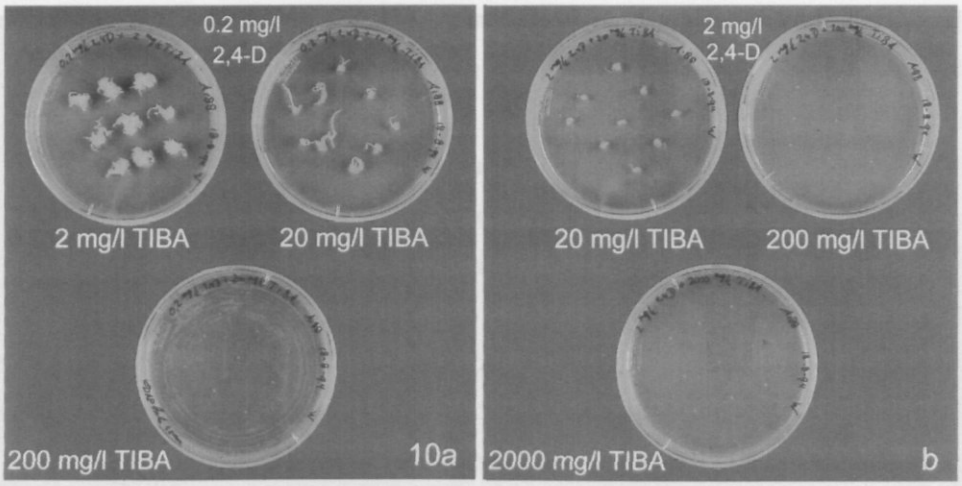


Figure 10: Influence of the concentration 2,4-D in combination with TIBA in the induction medium on the growth of immature embryos of A188. ***a***: Influence of 0.2 mg/l 2,4-D in combination with 2, 20 and 200 mg/l TIBA on the induction of callus in A188 after 21 DIC. ***b***: Influence of 2 mg/l 2,4-D in combination with 20, 200 and 2000 mg/l TIBA on the induction of callus in A188 after 21 DIC.

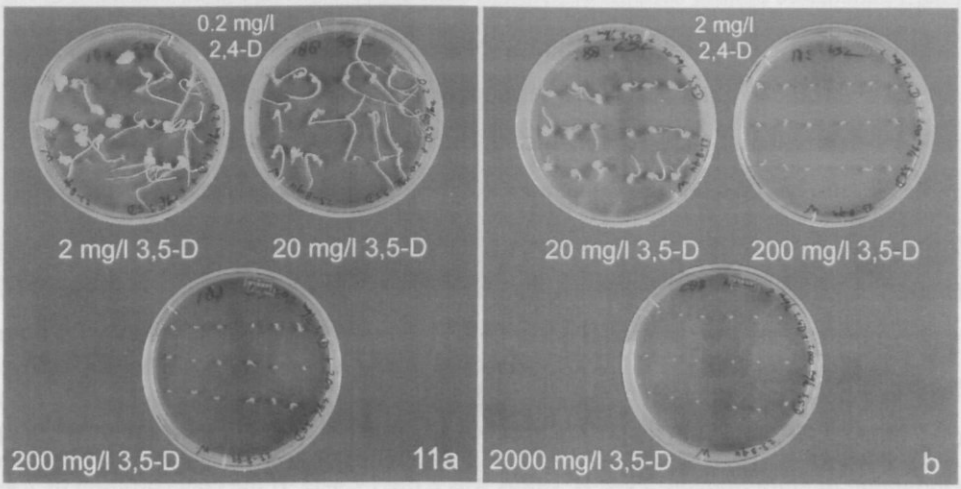


Figure 11: Influence of the concentration 2,4-D in combination with 3,5-D in the induction medium on the growth of immature embryos of A188 and A632. In all dishes 9 embryos of A188 are cultured on the left hand side of the dish, 9 embryos of A632 on the right hand side of the dish. ***a***: Influence of 0.2 mg/l 2,4-D in combination with 2, 20 and 200 mg/l 3,5-D on the induction of callus in both inbred lines after 14 DIC. ***b***: Influence of 2 mg/l 2,4-D in combination with 20, 200 and 2000 mg/l 3,5-D on the induction of callus in both inbred lines after 14 DIC.

embryos germinated after 4 weeks. Thus the amount of 2,4-D in the cells of the scutellum was neither high enough to induce callus formation nor to prevent embryo germination.
When embryos were exposed to 2,4-D for 1-14 days, the effect of 2,4-D became pronounced with increasing incubation periods. With exposure up to 7 days, leaf-like structure were formed. With exposure longer than 7 days, embryogenic callus and somatic embryos were induced in A188. As it was found that by ongoing culture more 2,4-D accumulated in the embryos (Bronsema et al. 1996) it is suggested that immature embryos need a certain amount of 2,4-D to form embryogenic callus and somatic embryos. To cross this threshold level the embryos of A188 needed to accumulate at least 4 nmol 2,4-D per embryo, *i.e.* 350 nmol per gram fresh mass after 7 days of culture (Bronsema et al., 1996).

Exhaustive and non-exhaustive culture conditions
Uptake of 2,4-D was studied previously under both exhaustive and non-exhaustive culture conditions and was 2.5 times higher under non-exhaustive culture conditions than under standard exhaustive culture conditions (Bronsema et al., 1996). The higher uptake of 2,4-D coincided with a lower increase of weight of the embryos which is caused by both the inhibition of germination as well as suppression of callus induction and growth. Besides, subculture on proliferation medium resulted in better embryogenic capacity for embryos that were first cultured under exhaustive conditions. This points to the existence of a restricted period of induction of embryogenic callus occurring during the initial culture under exhaustive condition.

Influence of the 2,4-D concentration in the induction medium
During culture on media with a range of 2,4-D concentrations, the optimum concentration for the induction of embryogenic, Type II, callus was 2 mg/l 2,4-D. If the concentration of 2,4-D was lowered to 2.10^{-2} mg/l, there was a transition from callus formation to germination. In embryos of A632 a similar phenomenon was observed. Most callus was formed at 2 mg/l 2,4-D, and callus induction changed to germination at 2.10^{-2} mg/l 2,4-D. The threshold level for the induction of embryogenic callus in A188 was 0.2 mg/l 2,4-D. This concentration is high enough, by ongoing, exhaustive culture, to induce the formation of embryogenic callus in the

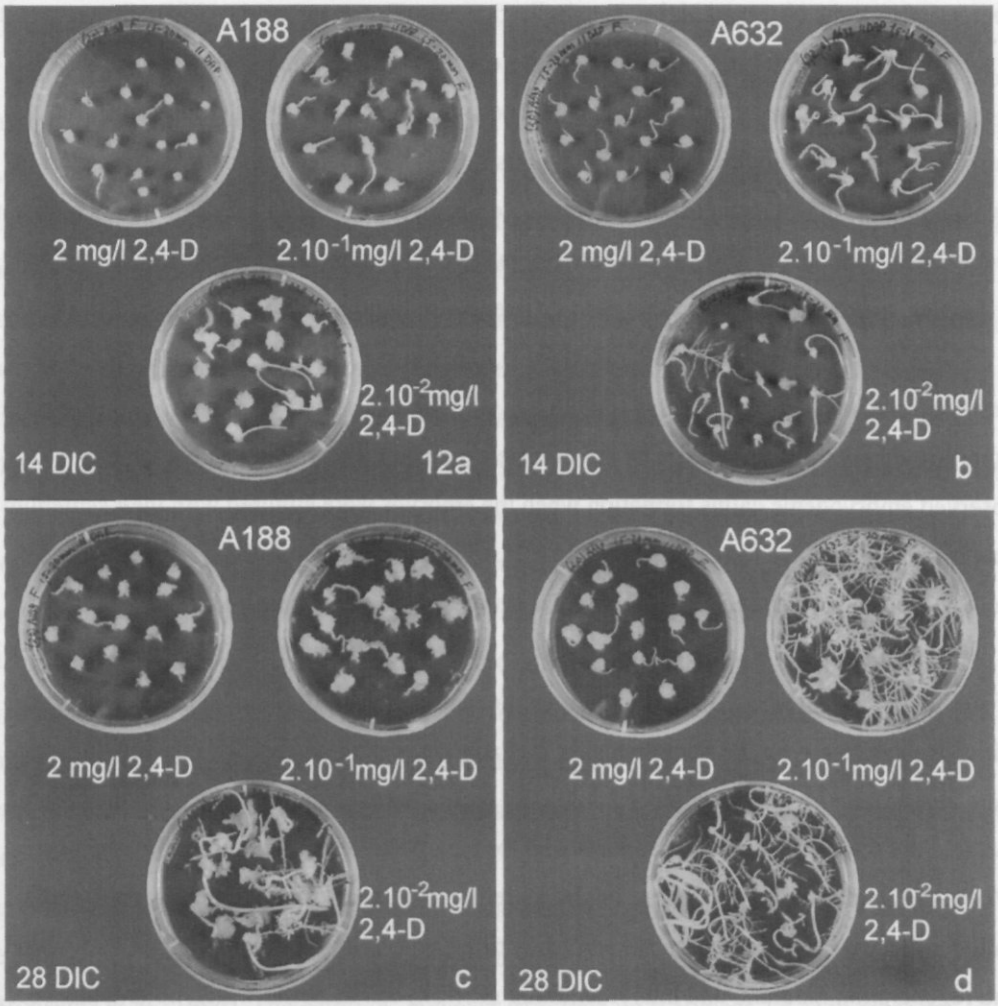


Figure 12: Influence of the concentration of 2,4-D in the induction medium on the growth of embryos of inbred lines A188 and A632. a: Influence of 2, 2.10^{-1}, 2.10^{-2} mg/l 2,4-D after 14 days in culture (DIC) for A188. b: Influence of 2, 2.10^{-1}, 2.10^{-2} mg/l 2,4-D after 14 DIC for A632. c: Influence of 2, 2.10^{-1}, 2.10^{-2} mg/l 2,4-D after 28 DIC for A188. d: Influence of 2, 2.10^{-1}, 2.10^{-2} mg/l 2,4-D after 28 DIC for A632.

scutellum. In A632 the same phenomenon was observed. A concentration of 0.2 mg/l 2,4-D enabled the formation of non-embryogenic callus by ongoing culture.

The non-embryogenic character of the callus of A632 did not depend on the concentration of 2,4-D in the medium. A higher concentration of 2,4-D in the induction medium, applied to

counterbalance the observed higher metabolisation rate (Bronsema et al., 1996), did not result in the formation of embryogenic callus. On the contrary, a lower concentration of 2,4-D seemed to produce Type I callus which might become embryogenic, but ongoing culture showed that this was not the case. Although this finding is in contradiction with the report of Duncan et al., 1985, who induced regeneration on embryos of A632 cultured with Dicamba, it seems likely that genetic differences between the two inbred lines are responsible for the difference in culture response rather than differences in uptake and metabolism of 2,4-D.

Positioning of the embryos

Embryos are normally cultured with the meristem side on the agar surface, but we also observed embryogenic callus formation when the embryos were cultured with their scutellum side in contact with the agar. The transport of 2,4-D does not necessarily need the suspensor and the procambium strands to reach the scutellum cells as was proposed by Vasil et al. (1985). Culture on the scutellum side led to a 7.1 times higher uptake of 2,4-D with embryos of A632, and for A188 a 2.3 times higher uptake was observed. It might be that the embryos which were cultured on the scutellum side, had a larger area of contact with the medium and were able to take up relatively more 2,4-D. The higher uptake might also be explained by the fact that the scutellum is an organ specialised for the uptake of nutrients from the endosperm. When A632 embryos were cultured on the scutellum side, roots were formed by rhizogenic callus a phenomenon normally observed when rhizogenic callus is subcultured on maturation and regeneration medium, and thus resembling growing conditions in which no or a low concentration of 2,4-D is present.

The amount of 2,4-D in the medium was investigated previously (Bronsema et al., 1996). After 14 days of culture under exhaustive conditions up to 90 % of the 2,4-D in the induction medium was taken up by the embryos. The lack of 2,4-D became a limiting factor by ongoing culture because embryos accumulated more 2,4-D when cultured at a constant 2,4-D level. When embryos did accumulate most of the 2,4-D, the ongoing culture mimics culture on medium without 2,4-D and thus permits regeneration.

Effect of TIBA

Culture at low 2,4-D concentrations resulted in transition from callus formation to germination in both inbred lines. We investigated whether it was possible to mimic this transition at higher 2,4-D concentrations, at which no transition is observed, by adding TIBA or 3,5-D to the culture medium. TIBA influences the polar transport of auxins from cell to cell, by modulating the efflux carrier for auxins (Lomax et al., 1995), and 3,5-D influences the effect of 2,4-D by competition at the binding sites for auxins (Felle et al., 1991). TIBA acts in a non-competitive manner to auxins on the efflux carrier (Lomax et al., 1995) and moves through the same channels as auxin. TIBA itself can be polarly transported (Thomson et al., 1973).

TIBA reduced callus induction at concentrations of 2 and 20 mg/l when added to medium with 2 mg/l 2,4-D, but the callus of A188 that was formed, was still embryogenic. In previous experiments embryogenic callus of A188 was cultured on proliferation medium containing 2 mg/l 2,4-D, with 2 and 10 mg/l TIBA. That callus remained embryogenic, too, and normal plants were formed when somatic embryos were cultured on maturation and regeneration medium (Bronsema et al., 1996). When added to globular zygotic wheat embryos, TIBA caused embryos with abnormal symmetry, but all phenotypes germinated when TIBA was removed from the medium (Fisher and Neuhaus, 1996). Such aberrant morphological features were not observed in maize.

Suppression of callus induction is probably caused by the hampered uptake of 2,4-D as was determined by pretreating embryos with TIBA before culture on medium with ^{14}C-2,4-D (Bronsema et al., 1996). The embryos, however, still took up sufficient 2,4-D to cross the threshold level for callus induction, but callus formation was lower compared to the standard induction on 2 mg/l 2,4-D without pre-treatment with TIBA. The distribution of the ^{14}C-2,4-D, as observed on median sections of embryos of A188 pre-treated with 5 mg/l TIBA, was similar, but less label was found than on embryos cultured with 2 mg/l 2,4-D without pre-treatment (Bronsema et al., 1998).

The block on callus induction and germination by high concentrations of TIBA (> 200 mg/l) in the induction medium can be explained by blocked polar transport of all auxins. It might also be a toxic effect on the growth of cells because Fisher and Neuhaus, (1996), observed that addition of 10 mg/l TIBA had a toxic influence on cultured globular wheat embryos leading to

a high rate of undifferentiated structures. In our cultures older and more differentiated embryos were used, which are probably less affected by TIBA, because the meristem and scutellum of the embryo were already differentiated.

Culture with TIBA in induction medium without 2,4-D did not inhibit germination at concentrations from 2 - 20 mg/l, but slowed down the speed of root elongation pointing towards disturbance of endogenous auxins. When embryos are cultured on medium with 0.2 mg/l 2,4-D, they failed to germinate and formed callus, but adding 20 mg/l TIBA did restore germination in embryos of both lines. Addition of less TIBA made the embryos cross the threshold level for callus induction, because sufficient 2,4-D was taken up. Germination could not be restored at higher concentrations of 2,4-D by addition of TIBA for both inbred lines, probably because high concentrations of TIBA have the tendency to bring the embryos in a static state.

Effect of 3,5-D

The ability of 2,4-D to induce and generate embryogenic callus was investigated by applying 2,4-D in combination with 3,5-D. The growth-inactive auxin 3,5-D should reduce the effect of the 2,4-D, because it will compete with 2,4-D at the binding sites for auxins with respect to cell elongation (Felle et al., 1991). At low concentrations of 2 mg/l, 3,5-D restored germination in A632 and repressed callus induction in both lines. Addition of 20 mg/l 3,5-D restored germination at low 2,4-D concentrations, too, and suppressed callus induction. Thus, the addition of 3,5-D to the 2,4-D does indeed resemble the morphogenetic response of culture at lower 2,4-D concentrations for both inbred lines. Addition of 200 or 2000 mg/l 3,5-D, however, resulted in suppression of germination and callus induction at the concentrations 2,4-D tested, probably caused by disturbance of the endogenous hormonal balance.

Conclusions

2,4-D is necessary for the induction of callus in immature embryos of maize. At least 0.2 mg/l is needed to cross the threshold level for the transition from germination to callus induction. In A188 friable embryogenic callus is formed from 7 days of induction with 2 mg/l 2,4-D onwards. In A632 only rhizogenic callus was induced under the conditions tested. The polar

transport inhibitor TIBA and the growth inactive auxin analogon 3,5-D do modulate the effects of 2,4-D on the culture of immature maize embryos, TIBA by influencing the transport, 3,5-D by competition.

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Chapter 8

General discussion

Tissue culture of immature embryos of Zea mays (L.)

Plant regeneration from immature embryos of maize follows two different pathways. It occurs either directly or indirectly. In the first case *de novo* development of meristems is needed from which new explants are formed. In the second case a callus phase mediates the development of new plantlets. A second distinction can be made between organogenesis such as shoot formation, and somatic embryogenesis. Shoots are directly formed upon the explant or indirectly on callus tissue. Somatic embryos develop in the same way, with or without an intervening callus phase (Fransz and Schel 1994).

Shock and growth response of the cultured embryos

Ultrastructural changes that occur during the first three days of embryogenic and non-embryogenic callus development have been studied by Fransz and Schel, 1987, in an embryogenic (A188) and a non-embryogenic inbred line (A632). On the first day of culture the two lines react similarly with changes in vacuolation and nuclear morphology, and by an increase in the number of organelles. After one day of culture they, however, behaved differently. A188 formed two meristematic regions, one in the scutellum and one in the coleorhiza. In A632 only a meristematic zone developed in the coleorhiza. Embryogenic callus only developed in line A188, originating from the scutellar meristematic area.

Callus types

In maize two types of embryogenic callus are distinguished, Type I and Type II callus (Armstrong and Green 1985). The Type I callus is white and compact in appearance, and is normally found in tissue culture with MS medium. Friable Type II callus is soft and white or pale yellow, and is the less differentiated type of regenerable callus. It is obtained when N6 or MS culture media are used with low sucrose concentrations (2%). It consists of aggregates of undifferentiated cells and groups of small isodiametric meristematic cells on the outside of the

aggregate (Emons and Kieft 1991). The cells of Type II callus are tightly packed, thin walled, cytoplasm rich, highly basiophilic, and contain many small vacuoles, lipid droplets, and starch grains (Fransz and Schel 1991, 1994). The friable Type II callus can only be subcultured on medium containing sufficient 2,4-D. If the concentration of 2,4-D decreases, the globular somatic embryos start differentiation and begin to mature, forming a scutellum. Type II friable callus is an important source for the production of maize suspension cultures and protoplasts with regeneration capacity (Kamo *et al.* 1987, Rhodes *et al.* 1988, Prioli and Söndahl 1989, Shilito *et al.* 1989). The Type I embryogenic callus is no more than an advanced differentiation step. Its white appearance is caused by the starch containing scutellar-like structures. Type I callus can not be subcultured itself, it may produce friable callus, although it can also be regenerated into plants. A third type of callus in maize is non-embryogenic callus. Because it will only form roots when the 2,4-D concentration decreases, the term rhizogenic is often used. The most important difference between embryogenic and rhizogenic callus is the origin of the meristems. In embryogenic callus they are formed exogenously: from a protodermal cell layer at the outside of the tissue. In rhizogenic callus they are formed endogenously: from a parenchymatic cell layer within the tissue (Emons *et al.* 1993).

Influence of medium composition on culture response

The immature embryos of both inbred lines were cultured on a modified N6 medium to which 2 mg/l 2,4-D, 20 mM L-proline, and 200 mg/l casein hydrolysate were added (Emons *et al.* 1993). Their culture response was compared in Chapter 2. The immature embryos of A188 formed friable Type II callus which could be subcultured, after selection, on proliferation medium to which 3% mannitol was added. Embryos of A632 formed no embryogenic callus, only rhizogenic callus on the same induction medium as used for the A188 embryos. Some callus was obtained which had the appearance of Type I callus but it could not be regenerated into plants. This might be in agreement with the observation by Duncan *et al.*, 1985, who scored embryos of A632 as producing embryogenic callus, but who could not regenerate plants from them as well.

The carbon source in the induction medium plays an important role. High percentages of sucrose (6%) induce Type I callus, low percentages (2%) enhance Type II callus. With the

modified induction medium we indeed obtained Type I callus when the sucrose concentration was raised to 6%. For embryos of A632 this did not lead to induction of embryogenic Type I callus. Swedlund and Locy, 1993, obtained highly regenerable maize callus when the carbon source sucrose was replaced by the sugar alcohol sorbitol. They observed that embryogenic callus had the capability to use sorbitol as a carbon source because the embryogenic callus contained sorbitol dehydrogenase. They stated that only embryogenic callus could grow on media containing sorbitol. With our embryogenic inbred line we indeed obtained embryogenic callus, with an appearance more resembling Type I callus. It was not needed to subculture this callus type each two weeks. It could be subcultured every four weeks. In the case of A632 we were also able to grow callus on the medium with sorbitol, indicating that this callus type also contained sorbitol dehydrogenase, but despite its growth the callus never formed somatic embryos. Thus, the A632 inbred line could not be forced to induce embryogenic callus under these culture conditions.

Auxin binding proteins

The auxin-binding protein 1 (ABP-1), purified first from maize microsomal membranes, has been of particular interest as a candidate receptor because it has been shown to be necessary on the plasma membrane for the auxin induced hyperpolarisation of tobacco and maize protoplasts (Barbier-Brygoo *et al.* 1989, Rück *et al.* 1993). For this reason ABP-1 has been extensively studied. The gene structure and protein sequence have been analysed in detail. Despite this, the characteristics of the protein *in vivo* are poorly understood. There is considerable uncertainty for example how the protein reaches the plasma membrane. The "ER paradox" (Jones 1990) makes it unclear how ABP-1 reaches the plasma membrane because in all the sequences reported the ER retrieval motif KDEL is conserved.

A lot of speculation on the function of ABPs has been the fate of these proteins. At this moment we know that the maize ABP-1 is involved in the hyperpolarization of the plasma membrane in tobacco (Barbier-Brygoo *et al.* 1989). Furthermore it is reported that ABP-1 is present on the surface of freshly prepared protoplasts of maize (Rück *et al.* 1993). Until now these membranes were also the sites in the cell where modulation of the auxin signal was observed.

Löbler and Klämbt, 1985, were the first to localise ABP-1 immunocytochemically in sectioned tissues of maize coleoptiles. The overall view is that ABP-1 is present at the plasma membrane of the cells, while it is formed in the ER of the same cells. Jones and Herman, 1993, also observed ABP-1 in the cell walls and in the culture medium by immunological methods, but these observations were not confirmed by Henderson *et al.*, 1997, who did not observe ABP-1 in the cell walls of the cells after immunocytochemical detection.
Although we detected ABP-1 in both the ER regions as well as in the plasma membrane, it is not clear which mechanism translocates ABP-1 (see o.a. Henderson *et al.* 1997). On the other hand the presence of ABP-1 in the dictyosomes points to a Golgi-mediated transport.
With respect to the function of ABP-1 in the induction of embryogenic callus in immature embryos of the inbred line A188, we did not observe any particular distribution of ABP-1 in the cultured zygotic embryo. Only the epidermal cells of the scutellum showed a higher label intensity. Between the remaining cells of the scutellum we did not observe any particular difference of distribution of ABP-1. The labelling of ABP-1 is most remarkable in cytoplasm-rich cells of the maize coleoptile and of the cultured embryo. Our localisation at the light microscopic level also revealed that ABP-1 is present in the subepidermal cells of the coleoptile and in the companion cells of the phloem. The last being a place not reported before in literature.
Our initial assumption that there might be a relation between the presence and distribution of ABP-1, and the potency of certain regions in immature embryos to form embryogenic or non-embryogenic callus in maize inbred lines, has to be rejected, because such a co-distribution could not be demonstrated.

Differences in culture response of A188 and A632 embryos

Uptake of 2,4-D

The uptake of 2,4-D by the immature embryos of the inbred lines revealed that both lines accumulate 2,4-D from the medium during the first 14 days of culture. So the difference in growth response between the lines can not be explained by the fact that the non-embryogenic line does not take up 2,4-D from the induction medium. The non-embryogenic line even takes

up more 2,4-D after 3 days of culture, if the uptake is quantified per gram fresh mass. This higher uptake is observed during the so called growth response phase of the immature embryos, in which the callus is initiated in the scutellum. The high concentration might force the embryos of A632 to form rhizogenic callus. When, however, lower concentrations of 2,4-D were applied in the culture medium the callus still remained non-embryogenic. The uptake of 2,4-D per gram fresh mass is similar for the inbreds from 5 days of culture onwards.

Metabolism of 2,4-D

The qualitative analysis of the 2,4-D after uptake by the embryos, revealed that after 24 h of pulse with ^{14}C-2,4-D, the embryos of A632 had a higher metabolic capacity for 2,4-D than embryos of A188. This might explain why A632 embryos are not able to form embryogenic callus: the higher uptake per gram fresh mass is counteracted by an higher metabolic capacity, so as a result less free 2,4-D, considered to be the active form of the growth regulator, is available for redifferentiation of the scutellum cells. However, applying higher 2,4-D concentrations in the culture medium did not result in induction of embryogenic development.

Distribution of 2,4-D

The distribution of 2,4-D in cultured embryos was visualised by autoradiography after a pulse of 16 h with ^{14}C-2,4-D. Although a large part of the 2,4-D is rinsed away during the embedment process, the distribution observed in the two inbred lines points to differences in distribution of the label between the two inbred lines. The redistribution of the label after chases for 24 h and 72 h, so during the growth response phase of the embryos, revealed that there was not a relation between the sites with abundant labelling by ^{14}C-2,4-D and the regions that showed induction of callus. In the embryogenic line A188 the area around the coleorhiza, where embryogenic callus is formed, shows label, while on the outside of the basal side of the scutellum, where embryogenic Type II callus is formed, no label was observed. In A632 the whole scutellum is labelled while only on a part of the scutellum rhizogenic callus is induced. So the difference in growth response cannot be explained by the presence or absence of 2,4-D in a certain region of the cultured embryo, at least not with the cytological methods used in our localisation experiment.

mRNA syntyhesis during callus induction

The induction of callus in the scutellum cells of the immature embryos is caused by redifferentiation of competent cells into dividing cells, which are capable to form embryogenic or non-embryogenic callus. With the help of digoxigenin labelled oligo-dT probes the unprocessed mRNA distribution during early stages of callus development was studied in both lines. Globular somatic embryos of A188 showed less label than the callus. At later stages of development highly labelled embryogenic layers were observed, compared to the lower labelled non-embryogenic layers. The method proved successful for the visualisation of genotype and stage specific differences with respect to poly(A)+mRNA.

Modulation of the action of 2,4-D

Concentration of 2,4-D in the induction medium

The morphogenetic effect of 2,4-D was examined by culturing zygotic embryos on induction media with a range of 2,4-D concentrations. We observed an optimum concentration for the induction of embryogenic callus at 2 mg/l 2,4-D for A188. At lower 2,4-D concentrations we noticed a shift between the induction of callus and germination, and embryos germinated when they were cultured on media with less than 0.02 mg/l 2,4-D. For A632 we observed that at a concentration of 0.2 mg/l 2,4-D callus was formed resembling Type I callus. Subculture did, however, not result in maturation of somatic embryos and regeneration into plants. This observation at a lower 2,4-D concentration in the induction medium is not in accordance with the higher capacity of A632 embryos to metabolise 2,4-D, but at concentrations higher than 2 mg/l 2,4-D the zygotic embryos of A188 and A632 formed less embryogenic and non-embryogenic callus, and the outgrowth of the formed callus was reduced.

Influence of TIBA

The addition of the polar auxin transport inhibitor TIBA to culture medium without 2,4-D reduced elongation of the shoot and especially of the root of the zygotic embryo, it could, however, not prevent germination. Culture in combination with 2,4-D caused suppression of callus formation, caused by lower uptake of 2,4-D and disturbance of the polar transport of

auxins from cell to cell. Autoradiography showed that there was a lower uptake after a pulse of 16 h, but the distribution of the label did not differ from embryos cultured without TIBA. At high concentrations of TIBA embryos did not respond to 2,4-D at all, so TIBA disturbed the polar transport of auxins completely.

Influence of 3,5-D

The shift from induction of callus to germination of the embryos of both inbred lines was observed between 0.2 and 0.02 mg/l 2,4-D. We tried to mimic these culture conditions by applying 3,5-D in combination with 2,4-D, and observed that cultures with 3,5-D were morphologically comparable to cultures at lower 2,4-D concentrations, because the 3,5-D competed with 2,4-D for the same binding sites in the cell. At high concentrations the 3,5-D resulted in the absence of a growth response.

Genetic inheritance

The ability to form embryogenic callus of either Type I or Type II is present in the inbred line A188 (Green and Phillips 1975). In inbred line A632 this ability is not present, because during many cycles of induction of callus and cultures at several 2,4-D concentrations no callus, apart from rhizogenic callus could be induced.

The embryogenic capacity of the inbred line A188 could be transferred to the non-embryogenic inbred A632 by crossing. Fairly stable callus was obtained when A188 was the mother. When A632 was pollinated with A188 pollen, embryogenic callus was formed as well, but the stability of the embryogenic callus was poor; after a few cycles of subculture the regeneration capacity was lost. Selfing of the F1 plants was performed as well, but it was difficult to obtain the right size of explant, embryos with a size between 1-2 mm, because of the heterosis effect after the selfing of the F1.

Today no genes for somatic embryo formation in maize are known. The trait of somatic embryo formation is likely not controlled by one pair of genes. Hodges *et al.*, 1985, showed the participation of two genes in the regulation of morphogenesis in the analysis of hybrids between A188 and Mo17. In breeding practice promising lines are hybridised with lines with a

high regenerative capacity because of their dominant or codominant inheritance (Yu and Dolgykh 1994).

Conclusions

a. 2,4-D is required for the initiation of callus formation and somatic embryogenesis in immature zygotic embryos of maize.
b. The medium composition influences the regeneration capacity of both the embryogenic and the non-embryogenic inbred line.
c. The uptake of 2,4-D does not differ that much between the two inbred lines that it explains the difference in culture response with respect to induction of embryogenic callus.
d. The higher metabolic capacity of A632 embryos for 2,4-D might point to differences between the two inbred lines.
e. The ability of 2,4-D to induce embryogenic callus in A188 can be modulated by the application of 3,5-D and TIBA, but this does not induce embryogenic callus in A632.
f. ABP-1 is detected in coleoptiles of the embryogenic and non-embryogenic inbred lines and in cultured embryos of the embryogenic inbred line at histological and subcellular levels, but appears not to be a scale turning factor for the property of somatic embryogenesis.
g. The pattern of distribution of 2,4-D is inbred line specific and differs from regions with high mitotic activity and regeneration.
h. Because the distribution of 2,4-D and the sites of callus formation and regeneration do not colocalize, a direct site-related action of 2,4-D could not be demonstrated.
i. Crossing experiments showed that the genetic inheritance of somatic embryogenesis is probably a determining factor, but the presence of 2,4-D remains a prerequisite for induction of somatic embryogenesis in cultured immature embryos of maize.

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Summary

This thesis deals with the influence of the growth regulator 2,4-dichlorophenoxyacetic acid (2,4-D) on the induction of callus in cultured immature embryos of *Zea mays* (L). In maize, two types of embryogenic callus can be induced in immature zygotic embryos. Type I callus, which is compact in appearance and hard to subculture, and type II callus, which is friable, and is well suited for subculture without loosing its capability to produce somatic embryos. The somatic embryos mature on growth regulator free medium with 6% sucrose and grow out into normal fertile plants on regeneration medium. This process of somatic embryogenesis is important for large scale manipulation in biotechnological procedures. Friable embryogenic callus can be used for the production of suspension cultures or protoplasts with regeneration capacity. It is also well suited for particle gun experiments in which foreign DNA is transferred in the cells of the callus. The influence of the 2,4-D was studied in two inbred lines.

In Chapter 1 the aim of the thesis is described. The chapter presents an overview of the questions we raised and the way in which we approached the questions.

In Chapter 2 the callus formation and regeneration on cultured immature embryos is described for two inbred lines, the embryogenic A188 and the non-embryogenic A632. The induction, maintenance, differentiation and embryogenic capacity of callus was compared. Callus of inbred line A188 was embryogenic, Type II callus, and maintained its embryogenic capacity for at least one year. Callus formed on inbred line A632 was not embryogenic, it only produced roots. Replacement of the carbon source sucrose by the sugar alcohol sorbitol in the induction medium increased embryogenic callus induction in inbred line A188, but A632 remained non-embryogenic. The embryogenic callus resembled Type I callus. The enhanced nutrient medium permitted subculture intervals of 4 weeks instead of 2 weeks. Reciprocal crosses between the two inbred lines resulted in induction of embryogenic callus in both crosses. Only the cross with pollen from A188 lost its embryogenic capacity rather quickly.

In Chapter 3 the uptake and metabolism of 2,4-D by immature embryos was compared in the embryogenic (A188) and the non-embryogenic inbred line (A632). During the shock response period uptake of ^{14}C-2,4-D was observed in both lines, with a higher uptake in A632. The

availability of 2,4-D in the induction medium became a limiting factor from 3 days of culture onwards. The concentration of 2,4-D per gram fresh mass was up to 125 times the initial concentration in the induction medium after 3 days of culture for A632. Differences in uptake between the two inbred lines were observed during the first 5 days of culture. In A188 a lower concentration per gram fresh mass was observed. After 5 days of culture 2,4-D concentrations were comparable for both lines. Therefore the difference in embryogenicity is not caused by differences in uptake of 2,4-D by the inbred lines. Addition of the auxin transport inhibitor triiodobenzoic acid (TIBA) to the induction medium caused a drop in the uptake of 2,4-D in both inbred lines, but TIBA did not block the uptake completely, nor did it prevent the induction of callus. The ^{14}C-2,4-D, accumulated in the embryos, was analysed biochemically. After 24 h of culture 70% of the 2,4-D in A188 embryos was present as free 2,4-D. In A632 37% was free 2,4-D. Conjugation of 2,4-D to sugars and amino acids started after 16 h of culture. Metabolization of 2,4-D was observed from 2 h of culture onwards. A632 embryos showed a higher metabolization rate of 2,4-D compared to embryos of A188. The higher level of free 2,4-D and the lower metabolization rate of 2,4-D in A188 embryos might cause the developmental differences observed in cultured immature embryos of the embryogenic and the non-embryogenic inbred line. This could be regulated by genetical differences between the two inbred lines, however other genetical factors might determine the embryogenic response of the embryos to 2,4-D as well.

In Chapter 4 the uptake and distribution of ^{14}C labelled 2,4-D in cultured embryos was studied. After a pulse label of 16 h, embryos showed a hundredfold accumulation of 2,4-D in the embryos compared to the concentration in the induction medium. During the tissue processing for *in situ* detection of the ^{14}C label in the embryos, up to 70 % of the label disappeared from the embryo tissue. HPLC analysis after direct tissue hydrolysis showed that 95% of the residual ^{14}C label found in the embryos was non-metabolised 2,4-D. Plastic embedded embryos were sectioned and analysed for ^{14}C label by autoradiography. Water mediated infiltration of Technovit 7100 gave the highest retention of ^{14}C label, but a better structural preservation was obtained after ethanol mediated infiltration of Technovit 7100. After 16 h on pulse medium, embryos of A188 had label in the basal part of the scutellum, in the top part of the scutellum opposite the shoot meristem, in the coleoptile, and in the suspensor. In A632 more label was

observed compared to A188. Most label was observed in all parts of the scutellum except for the procambium strands, in parts of the shoot and in the coleoptile. Subculture of the embryos for 24 and 72 h on medium without ^{14}C-2,4-D and histological analysis showed that cell proliferation and callus formation were restricted to specific regions of the embryo in both inbred lines. The distribution of 2,4-D in the sectioned embryos did not codistribute with the regions of proliferation, indicating that 2,4-D is not the only trigger for proliferation.

In Chapter 5 the auxin binding protein 1 (ABP-1) was localised immunocytochemically in coleoptiles and immature embryos of *Zea mays* (L), by the use of two polyclonal antibodies. At the light microscopical level ABP-1 was localised in the epidermal cells of etiolated coleoptiles, in the subepidermal mesophyll cells and in the companion cells of the vascular bundle. Immature embryos showed low labelling throughout their tissues just after excision, but after 7 days of culture on 2,4-D containing induction medium, intensive labelling was observed in the epidermal cells of the scutellum. Quantitative electron microscopy confirmed the localisation in the cytoplasm of epidermal, mesophyll and companion cells of the coleoptile. No labelling was observed in cell walls or cuticle. Areas with endoplasmic reticulum and dictyosomes had a denser labelling with gold particles than elsewhere. Labelling at the plasma membrane was observed at low levels in all cells tested. Epidermal cells of embryos cultured for 5 days on induction medium, exhibited high levels of gold particles in endoplasmic reticulum and nuclei, lower levels in the cytoplasm. This distribution of ABP-1 is only partly in accordance with the model in which ABP cycles through the cell from the ER via the Golgi system towards the plasma membrane.

In Chapter 6 the pattern of total transcription activity in terms of steady state levels of poly(A)+ containing mRNA during callus initiation and somatic embryogenesis in the highly embryogenic (A188) and the non-embryogenic (A632) inbred lines of maize was analysed using digoxigenin labelled oligo-dT probes. A gradual increase and a preferential accumulation of label were observed in both lines, differing temporally up to 4 days in culture. In the A188 line of maize the callus gave rise to somatic embryos. The globular embryos showed less label than the callus; this label was mostly present in the basal part of the embryos. At a later stage upper embryogenic and lower non-embryogenic layers were observed in the A188 callus, showing conspicuous differences in the amount of label. In the late globular stage the poly(A)+

RNA signals were seen all over the embryo but at the junction of the suspensor and the callus tissue no label was observed.

In Chapter 7 the influence of the growth regulator 2,4-D, the inactive analogon 3,5-D and the polar transport inhibitor triiodobenzoic acid (TIBA) on the induction of embryogenic and rhizogenic callus in zygotic embryos of *Zea mays* (L) inbred lines is analysed. Pulse chase experiments, to mimic culture under exhaustive conditions, showed that 2 mg/l 2,4-D in the induction medium influenced the culture of A188 embryos from 0.5 h of culture onwards. A pulse of 16 h resulted in outgrowths and callus formation at the basal side of the scutellum. Pulses for 7 days or longer induced friable embryogenic Type II callus. Culture with a constant concentration of 2 mg/l 2,4-D in the induction medium resulted in less callus growth at the basal side of the embryo and lower weight of the embryos of A188 after 14 days of culture compared to embryos grown under exhaustive culture conditions. An optimum concentration of 2 mg/l 2,4-D was found for the induction of friable embryogenic callus in A188, when culture with a range of concentrations from 0 to 2000 mg/l 2,4-D was performed. Lower concentrations of 2,4-D in the induction medium resulted in a transition from callus induction to germination, higher concentrations led to a block of development of the embryos of both lines. Embryos, cultured with their scutellum in contact with the medium, took up more 2,4-D, than when cultured on their meristem, 2.3 times for A188 and 7.1 times for A632. Addition of TIBA without 2,4-D retarded the germination of the embryos; the outgrowth of the root was effected. Addition of TIBA in combination with 2,4-D led to suppression of callus formation and to a total block of development if TIBA concentrations were higher than 200 mg/l. Addition of 3,5-D, without 2,4-D, did not influence germination of the embryos. Culture in combination with 2,4-D resembled culture at lower 2,4-D concentrations. Addition of 2 mg/l 3,5-D restored germination, repressed callus induction, 20 mg/l 3,5-D resulted in germination of the embryo and suppression of callus formation. Concentrations of more than 200 mg/l 3,5-D suppressed germination and callus induction at the 2,4-D concentrations tested.

In Chapter 8 the results of the chapters 1 till 7 are discussed with respect to their influence on the process of induction of callus and somatic embryogenesis in cultured immature embryos of *Zea mays* (L).

Samenvatting

Dit proefschrift behandelt de invloed van de groeiregulator 2,4-dichlorofenoxy-azijnzuur (2,4-D) op de inductie van callus in gekweekte embryo's van *Zea mays* (L). In maïs kunnen twee types embryogeen callus geïnduceerd worden in onvolwassen, zygotische embryo's. Type I callus is compact in uiterlijk en is moeilijk door te kweken, type II callus is bros en geschikt voor doorkweek zonder het vermogen voor de vorming van somatische embryo's te verliezen. De somatische embryo's rijpen op groeiregulator vrij medium met 6% sucrose en groeien uit tot normale, fertiele planten op regeneratie medium. Dit proces van somatische embryogenese is belangrijk voor toepassingen op grote schaal in de biotechnologie. Bros embryogeen callus kan gebruikt worden voor de productie van suspensie cultures of voor de productie van protoplasten met regeneratievermogen. Het is ook geschikt voor particle gun-experimenten waarbij soortsvreemd DNA wordt overgebracht naar cellen van het callus. De invloed van 2,4-D is bestudeerd in twee inteeltlijnen.

In Hoofdstuk 1 is het doel van het proefschrift beschreven. Het hoofdstuk presenteert een overzicht van de vragen die wij gesteld hebben en de manier waarop wij deze vragen hebben aangepakt.

In Hoofdstuk 2 is de vorming van callus en de regeneratie in gekweekte onvolwassen maïsembryos beschreven voor twee inteeltlijnen, de embryogene lijn A188 en niet embryogene lijn A632. Callus van inteeltlijn A188 was embryogeen, type II callus en behield zijn embryogene karakter minstens 1 jaar. Callus gevormd op inteeltlijn A632 was niet embryogeen, het produceerde alleen wortels. Vervanging van de suikerbron sucrose door de suikeralcohol sorbitol in het inductiemedium verhoogde de embryogene callus-inductie in inteeltlijn A188, maar A632 bleef niet embryogeen. Het embryogene callus leek meer op type I callus. Het verbeterde medium liet intervallen van 4 weken toe voor doorkweek in plaats van intervallen van 2 weken. Reciproke kruisingen tussen de twee inteeltlijnen resulteerden in inductie van embryogeen callus in beide kruisingen. Alleen de kruising met pollen van A188 verloor zijn embryogene karakter vrij snel.

In Hoofdstuk 3 zijn de opname en de metabolisatie van 2,4-D door onvolwassen embryo's vergeleken in de embryogene (A188) en de niet-embryogene inteeltlijn (A632). Gedurende de shock response periode namen beide lijnen ^{14}C-2,4-D op, er was een hogere opname in A632.

De beschikbaarheid van 2,4-D in het inductie-medium werd een limiterende factor na 3 dagen van cultuur. De concentratie 2,4-D per gram versgewicht was tot 125 maal de aanvangsconcentratie in het inductie medium na 3 dagen van kweek in A632 embryo's. Gedurende de eerste 5 dagen van kweek werden er verschillen in opname waargenomen tussen de twee inteeltlijnen. In A188 werd een lagere concentratie per gram versgewicht waargenomen. Na 5 dagen kweek waren de 2,4-D concentraties vergelijkbaar voor beide lijnen. Daarom is het verschil in embryogeniciteit niet veroorzaakt door verschil in opname van 2,4-D door de inteeltlijnen. Toevoeging van de auxine transport inhibitor triiodobenzoëzuur (TIBA) aan het inductie medium veroorzaakte een dip in de opname van 2,4-D in beide lijnen, maar blokkeerde de opname niet helemaal, noch voorkwam de inductie van callus. De ^{14}C-2,4-D, opgehoopt in de embryo's, is biochemisch geanalyseerd. Na 24 uur kweek was 70% van de 2,4-D in A188 embryo's aanwezig als vrij 2,4-D. In A632 was dit 37% vrij 2,4-D. Conjugatie van 2,4-D aan suikers en aminozuren begon na 16 uur kweek. Metabolisatie van 2,4-D is waargenomen vanaf 2 uur kweek. De mate van metabolisatie was hoger in A632 embryo's dan in A188 embryo's. Het hogere aandeel vrij 2,4-D en de lagere metabolisatiegraad van 2,4-D in A188 kunnen de oorzaak zijn van de verschillen in ontwikkeling tussen de onvolwassen embryo's van de embryogene en de niet embryogene inteeltlijnen. Dit kan gereguleerd worden door genetische verschillen tussen de twee lijnen, hoewel ook ander genetische factoren de embryogene response op 2,4-D kunnen verklaren.
In Hoofdstuk 4 zijn de opname en de distributie van ^{14}C gelabeld 2,4-D in gekweekte embryo's bestudeerd. Na een puls van 16 uur, vertoonden embryo's een honderd maal hogere concentratie 2,4-D dan het inductie medium. Gedurende de weefselpreparatie voor de *in situ* detectie van ^{14}C label in de embryo's, verdween tot 70% van het label uit het embryoweefsel. HPLC analyse na "direct tissue hydrolysis" toonde aan dat 95% van het overgebleven ^{14}C label ongemetaboliseerd 2,4-D was. Embryo's zijn ingebed in plastic (Technovit 7100) en op de coupes is de, aanwezigheid van ^{14}C label geanalyseerd door middel van autoradiografie. Infiltratie van de Technovit 7100 met behulp van water gaf de hoogste retentie van ^{14}C label, maar het beste structuurbehoud werd verkregen na alcoholinfiltratie van de Technovit 7100. Na 16 uur incubatie op puls medium, vertoonden embryo's van A188 label in het basale deel van het scutellum, in het top deel van het scutellum tegenover het scheutmeristeem, in het coleoptiel en in de suspensor. In A632 werd meer label waargenomen. Het meeste label werd

waargenomen in alle delen van het scutellum met uitzondering van de procambium strengen, in delen van de scheut en in het coleoptiel. Doorkweek gedurende een periode van 24 en 72 uur op medium zonder ^{14}C-2,4-D en histologische analyse toonden aan dat celproliferatie en callusvorming beperkt waren tot specifieke regio's in de embryo's van beide lijnen. De distributie van 2,4-D in de geanalyseerde embryo's had niet dezelfde verdeling als de gebieden met proliferatie, hetgeen er op wijst dat 2,4-D niet de enige aanleiding is voor proliferatie.
In Hoofdstuk 5 is het auxine bindende eiwit 1 (ABP-1) immunocytochemisch gelocaliseerd in coleoptielen en onvolwassen embryo's van *Zea mays* (L) met behulp van twee polyclonale antilichamen. Op lichtmicroscopisch niveau is ABP-1 gelocaliseerd in epidermiscellen van geëtioleerde coleoptielen, in de subepidermale mesofylcellen en in de begeleidende cellen in de vaatbundel. Onvolwassen embryo's vertoonden weinig labeling in alle weefsels direct na het uitprepareren, maar na 7 dagen kweek op 2,4-D bevattend medium werd er een intensieve labeling in epidermis cellen van het scutellum waargenomen. Kwantitatieve electronenmicroscopie bevestigde de localisatie in het cytoplasma van de epidermis, mesofiel en begeleidende cellen van het coleoptiel. In de celwanden en de cuticula is geen labeling waargenomen. Gebieden met endoplasmatisch reticulum en dictyosomen hadden een hogere labeling met gouddeeltjes. Labeling op de plasmamembraan is waargenomen in alle onderzochte cellen maar met een lage dichtheid. Epidermis cellen van embryo's die 5 dagen gekweekt zijn op inductiemedium, vertoonden hoge dichtheden van gouddeeltjes in het endoplasmatisch reticulum en de kern, en lagere dichtheden in het cytoplasma. De distributie van ABP-1 is slechts ten dele in overeenstemming met het model waarin ABP door de cel wordt getransporteerd van het ER, via het Golgi systeem, naar de plasmamembraan.
In Hoofdstuk 6 wordt het patroon van de totale transcriptie-activiteit, in termen van steady state levels van poly(A)+ bevattend mRNA, geanalyseerd met behulp van digoxigenine gelabelde oligo-dT probes gedurende de callus inductie en somatische embryogenese in de embryogene (A188) en de niet embryogene (A632) inteeltlijn van maïs. Een graduele verhoging en een ophoping van label is waargenomen in beide lijnen, in tijd verschillend tot 4 dagen in kweek. In lijn A188 ontstonden somatische embryo's. De globulaire embryo's vertoonden minder label dan het callus, dit label was voornamelijk aanwezig in het basale deel van de embryo's. In latere stadia werden meer naar buiten gelegen embryogene en meer naar binnen gelegen, niet-embryogene lagen waargenomen in het A188 callus, met duidelijk

waarneembare verschillen in de hoeveelheid label. In het laat globulaire stadium werden poly(A)+ RNA signalen over heel het embryo waargenomen, maar op de aanhechtingsplaats van de suspensor aan het callus weefsel is geen label waargenomen.

In Hoofdstuk 7 is de invloed onderzocht van de groeiregulator 2,4-D, het inactieve analogon 3,5-D en de polaire transport-inhibitor triiodobenzoëzuur (TIBA) op de inductie van embryogeen en rhizogeen callus in zygotische embryo's van *Zea mays* (L) inteeltlijnen. Pulse/chase experimenten, die cultuur onder uitputtende omstandigheden simuleren, toonden aan dat 2 mg/l 2,4-D in het inductie-medium de kweek van A188 embryo's vanaf 30 minuten beïnvloedde. Een puls van 16 uur resulteerde in uitgroeiingen en callusvorming aan de basale zijde van het scutellum. Pulsen van 7 dagen of langer induceerden embryogeen, type II callus. Kweek met een constante concentratie van 2 mg/l 2,4-D in het inductie-medium resulteerde in minder callusgroei aan de basale zijde van het embryo en een lager gewicht van de embryo's na 14 dagen kweek vergeleken met embryo's gekweekt onder uitputtende omstandigheden. In een experiment met een concentratiereeks van 0 tot 2000 mg/l 2,4-D is gevonden dat 2 mg/l 2,4-D de optimale concentratie is voor de inductie van bros, embryogeen callus in A188. Lagere concentraties 2,4-D in het inductie medium resulteerden in een transitie van callusinductie naar kieming; hogere concentraties in een blokkade van ontwikkeling van de embryo's van beide lijnen. Embryo's gekweekt met hun scutellum, in plaats van hun meristeem op het medium, namen meer 2,4-D op; embryo's van A188 2,3 maal meer, embryo's van A632 7,1 maal meer. Toevoeging van TIBA aan het inductie medium zonder 2,4-D, vertraagde de kieming van de embryo's; de uitgroei van de wortel werd beïnvloed. Toevoeging van TIBA met 2,4-D leidde tot onderdrukking van callusvorming, en een totale blokkade van ontwikkeling wanneer de TIBA-concentratie hoger was dan 200 mg/l. Toevoeging van 3,5-D aan het inductie-medium zonder 2,4-D, beïnvloedde de kieming van de embryo's niet. Kweek in combinatie met 2,4-D kan vergeleken worden met de cultuur onder lagere 2,4-D concentraties. Toevoeging van 2 mg/l 3,5-D zorgde weer voor kieming, en onderdrukte callusvorming. Concentraties hoger dan 200 mg/l 3,5-D onderdrukten kieming en callusinductie bij alle 2,4-D concentraties die getest zijn.

In Hoofdstuk 8 worden de resultaten van de hoofdstukken 1-7 besproken met betrekking tot hun invloed op het proces van inductie van callus en somatische embryogenese in gekweekte, onvolwassen embryo's van *Zea mays* (L).

Nawoord

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Folbert Bronsema

Curriculum vitae

Folbert Bronsema werd geboren op 14 oktober 1964 te Groningen, waar hij in 1983 het diploma Gymnasium β aan het Willem Lodewijk Gymnasium behaalde. Hierna ging hij in 1983 plantenveredeling aan de Landbouwuniversiteit in Wageningen studeren. In 1990 voltooide hij deze studie. Hij volgde het afstudeervak Anatomie en -morfologie op de Vakgroep Plantencytologie en -morfologie in samenwerking met het ITAL, onder begeleiding van dr. J.H.N Schel en dr. L.J.W. Gilissen. Hierbij is het ontstaan van hairy roots in door *Agrobacterium tumefaciens* getransformeerde protoplasten van *Nicotiana plumbaginfolia* bestudeerd. Bij het afstudeervak *In vitro* cultuur van hogere planten op de Vakgroep Tuinbouwplantenteelt, onder begeleiding van prof. dr. ir. R.L.M. Pierik, werden de opkweekomstandigheden van *Phalaenopsis* zaailingen onderzocht. De praktijktijd plantenveredeling werd op het USDA Vegetable Laboratory in Charleston, SC, USA, doorgebracht. Er is gewerkt aan de resistentieveredeling van meloen, sweet potato, broccoli en paprika. In 1991 is hij begonnen met het promotieonderzoek op de vakgroep Plantencytologie en -morfologie te Wageningen, onder leiding van prof. dr. M.T.M. Willemse en dr. A.A.M. van Lammeren. Dit onderzoek werd gefinancierd door de Nederlandse Organisatie voor Wetenschappelijk Onderzoek (NWO) te Den Haag. De resultaten van dit onderzoek zijn in dit proefschrift beschreven.