Androgenesis in *Hordeum vulgare* L.:

Physiological and molecular aspects

Sietske Hoekstra

Androgenesis in *Hordeum vulgare* L.:

Physiological and molecular aspects

Proefschrift

ter verkrijging van de graad van Doctor
aan de Rijksuniversiteit te Leiden,
op gezag van de Rector Magnificus Dr. L. Leertouwer,
hoogleraar in de faculteit der Godgeleerdheid,
volgens besluit van het College van Dekanen
te verdedigen op donderdag 5 september 1996
te klokke 15.15 uur

door

Sietske Hoekstra
geboren te Amsterdam in 1963

Promotiecommissie

Promotor	Prof.dr. R.A. Schilperoort
Co-promotor	Dr. F. Heidekamp (TNO)
Referent	Prof.dr. E. Heberle-Bors (University of Vienna, Austria)
Overige leden	Prof.dr. K.R. Libbenga
	Prof.dr. J.W. Kijne
	Dr. S.C. de Vries (Landbouw Universiteit Wageningen)

The research described in this thesis, has been performed within the ABIN-project and from 1992 in the strategic collaboration between the TNO department Plant Biotechnology and the Institute of Molecular Plant Sciences section I of Leiden University, in the Center for Phytotechnology.
Financial support for the publication of this thesis by the TNO Nutrition and Food Research Institute is greatfully acknowledged.

Aan mijn ouders,

Voor Uli

Abbreviations

ABA = (±)2-*cis*-4-*trans*-abscisic acid
ABA_i = intracellular ABA
ABA_e = extracellular ABA
BAP = 6-benzylaminopurine
d.a.i. = days after isolation of microspores
ELS = embryo-like structures
IAA = indolacetic acid
LOS = lipo-oligosaccharides
2,4-D = 2,4-dichlorophenoxyacetic acid

Contents

Outline of the thesis

The development of pollen is an intriguing process, resulting in unicellular structures. These cells have a rigid cell wall, which enables a resistance to withstand various kinds of stress. Upon appropriate conditions, these structures are able to hydrate, germinate and when a pollen tube is formed, to participate in the fertilization process.

This gametophytic development can be arrested and changed into the sporophytic route. Immature pollen grains, also called microspores, can be induced at the verge of mitosis to become what are called embryogenic microspores. Under specific conditions, the microspore will develop into an embryo-like structure and from this a haploid plant can be formed. The mechanism of this switch from the gametophytic into the sporophytic pathway has attracted the attention of many researchers. Moreover, the product of the switch, a homozygous plant, has surplus value for plant breeding. By the use of haploids from immature pollen grains, the production of new varieties can be largely improved. The rapid and complete homozygosity of the offspring simplifies phenotype selection for quantitative inherited characters and, therefore, breeding is more efficient. Today, androgenic haploids have been produced in more than 50 genera, and the greatest effort has been given to economically important plants such as the cereals and vegetable crops. Although the anther culture method is used successfully in breeding companies, very little is known about the mechanism(s) responsible for changing microspore development into the sporophytic pathway. What is known is that an appropriate microspore developmental stage together with a specific stress treatment is required for the production of microspore-derived plants.

A few model species have been used for microspore-derived plant formation, namely tobacco, rapeseed and barley. For studies presented in this thesis we used the crop barley, as in the TNO department of Plant Biotechnology, studies are being performed aiming at adaptation of barley for industrial needs (see Bengtsson, 1992; McElroy and Jacobsen, 1995). The aim of the studies for this thesis was twofold: trying to understand the mechanism leading to formation of embryogenic microspores, and optimization of plant production efficiency from androgenesis. The importance of several parameters in androgenesis has been described in literatu-

re. We were specifically interested in parameters which are responsible for the initiation and early development of the sporophytic pathway. Three preconditions were identified for androgenesis in barley: a uninucleate developmental stage, an anther pretreatment and a growth regulator application.

A general introduction of androgenesis for the model species barley is given in chapter 1 of this thesis. In chapter 2, a comparison of the regeneration efficiency between anther and microspore culture is made. The basics of androgenesis is described, with an emphasis on the importance of the late uninucleate microspore developmental stage. Microspore culture proved to be more efficient in plant production than anther culture. Studies described in chapter 3 were focussed on the development of an efficient microspore culture protocol. After an optimized anther pretreatment, an embryogenic subpopulation was identified resulting in the production of 50 green plants per anther.
In chapter 4 a study on the effects of the growth regulator 2,4-dichlorophenoxyacetic acid (2,4-D) is presented. Pulse application of the synthetic auxin 2,4-D can induce plant formation at similar frequencies as described for the cytokinine benzyl aminopurine, which is generally used. In chapter 5 and 6, the importance of anther pretreatment is demonstrated. An appropriate osmotic level during pretreatment is necessary for plant formation. We also showed evidence for the need of specific abscisic acid levels, endogenously as well as exogenously, during the pretreatment. In chapter 6 is shown, that these hormonal and osmotic levels might be necessary for induction of apoptosis in anther tissue. Also, a possible role of lipo-oligosaccharides in the formation of embryogenic microspores was demonstrated.
In the last chapter of this thesis a hypothesis is presented, based on the results obtained from our studies, for a mechanism by which androgenesis is initiated.

References

Bengtsson B.O. Barley genetics - not only here for the beer. Trends I. Genet. 8: 3-5 (1992)
McElroy D. and J. Jacobsen. What is brewing in barley biotechnology? Biotechnology 13: 245-249 (1995)

Chapter 1

General Introduction

Haploidization

For variety development in most crop plants, a critical step is the establishment of true breeding lines. A stable, homozygous (or as much as possible) plant is defined as a true breeding line. Such plants are used as finished varieties or as parents for hybrid seed production. Traditionally, plant breeders have achieved homozygosity by using the time consuming processes of self-fertilization or backcrossing (Morrison and Evans, 1988). Homozygosity *in vivo* arises upon abnormal fertilization by reduction of the genetic information to the amount of DNA present in gametes; these plants, in which each gene is represented once, are called **haploids**. There are several origins of *in vivo* haploids, i.e. parthenogenesis (autonomous growth of the ovary without fertilization), gynogenesis (growth of the unfertilized egg in the presence of normal endosperm) and genome elimination (natural elimination of either gametic genomes after fertilization). Natural haploids of higher plants are, however, of rare occurence and restricted to only a few species (Maheshwari et al., 1980).

With the publication of the paper by Guha and Maheshwari in 1966, a new haploidization approach came into existence: development of haploid plants derived from immature pollen or **microspores** in cultured anthers, what is called (*in vitro*) **androgenesis**. Haploids derived from microspores opened a new dimension because of the abundance of microspores produced by the plants and their potentially general occurrence in the plant kingdom (Heberle-Bors, 1985). Subsequently, in many laboratories a great deal of research effort was immediately directed towards perfecting the technique so that it could routinely be used.

The plant which led to the initial success was a common weed, *Datura innoxia* Mill., belonging to the family *Solanaceae*. Today, androgenic haploids have been produced in more than 50 genera, but the greatest effort has been given to economically important plants such as the vegetable crops and cereals (Cao et al., 1995; Veilleux, 1994). Via androgenesis new varieties have been developed in a number of agricultural

crops such as *Brassica sp.*, tobacco, potato, asparagus, wheat, rice, maize, barley, etc. (Bajaj, 1990).

In this thesis, the development of a method for androgenesis in barley and the analysis of parts thereof, are described. For a good comprehension of the other chapters, the reproductive development, the process of microsporogenesis and various aspects of androgenesis are introduced.

The reproductive system and embryogenesis

Angiosperm plants produce flowers, very beautiful and intricate structures, in which their reproductive development takes place. In flowering plants a spore-producing generation or **sporophyte** alternates with a gamete-producing generation or **gametophyte**. Unlike some of the evolutionarily more primitive plants, the male and female gametophytes of angiosperms are reduced to microscopic structures that are dependent on the tissues of the sporophyte for their development. The flower contains specialized structures (see Figure 1), the **anthers** and the pistil in which the male and female gametophytes, respectively are formed. The role of the male gametophyte (i.e. microspores) is to produce two generative cells which are transported via a pollen tube, through the tissues of the style into the ovary. In the double fertilization that follows, fusion of one generative nucleus with the egg and of the second generative nucleus with the central cell results in formation of respectively the zygote and the endosperm, a nutritive tissue. The zygote continues to develop to the structure of an embryo within the ovule, and this process is called **zygotic embryogenesis**. In some species, the embryogenic pathway is well documented. In dicotyledonous species the stages of octant, globule, heart and torpedo are typical, whereas in monocotyledonous species the pro-embryo, the transition, and the coleoptilar stage with a scutellum are formed (Meinke, 1991).

A characteristic of plant cells is their totipotency, which means that they have the ability to regenerate into a plant under the appropriate *in vitro* conditions. If somatic cells replay *in vitro* a developmental program leading to the production of embryos, this process is called **somatic embryogenesis**. The embryos arise directly from the explant, or indirectly after a phase of dedifferentiated growth. Alternatively, somatic cells develop *in vitro* via organogenesis into a plant, which is mostly an adventitious shoot (Reynolds, 1994). Regenerants of somatic cells keep the genetic constitution of the mother plant under appropriate conditions, so no haploid or homozygous plants are to be expected.

Microsporogenesis

In flowering plants, the pollen is the male gametophyte. The male gametophyte completes its development within the anther, which is illustrated in Figure 2 and 3 and described in the following.

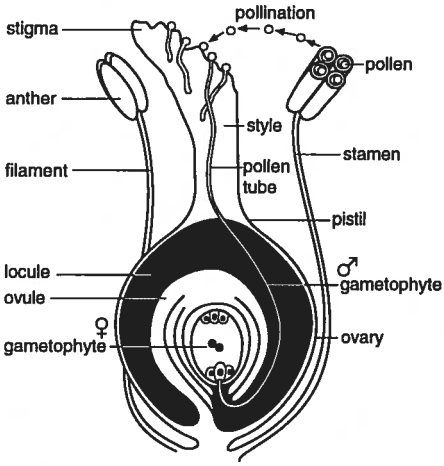


Figure 1: Reproductive development in a diagrammatic median longisection of a generalized flower. The component structures are labeled.

Data described herein are based on the reviews from Bedinger (1992), Goldberg et al. (1993 and 1995), Mascarenhas (1989 and 1990), McCormick (1993), Scott et al. (1991) and Vicente et al. (1991). During microsporogenesis a series of mitotic divisions of the archesporial cells give rise to both the primary parietal cells and the sporogenous cells; latter develop into microspore mother cells that are surrounded by a thick callose wall. Meiosis is initiated in the microspore mother cell and a tetrad containing four haploid cells is formed. The callose wall is digested by the action of ß(1,3)-glucanase produced by the tapetum and individual cells of the tetrad are released as free microspores. Upon release from the tetrad, the microspores change in shape. The germ pore (pore where the pollen tube can grow out) is formed. The intine, or inner pollen wall is first laid down over the site of the pore and then spreads to encase the entire microspore. The intine is thought to be derived largely from gametophytic gene expression and is composed mainly of polysaccharides. Deposition of the **exine**, or outer pollen wall, is reduced or absent over the germ pore. The microspores grow in circumference soon after their release from the tetrad, and the outer pollen cell wall synthesis increases during this stage. The exine is composed of sporopollenin, a highly chemically resistant substance formed by the oxidative polymerisation of carotenoids and carotenoid esters. The **tapetum** is largely responsible for the synthesis and deposition of wall materials to the developing exine. In most species, the tapetum begins to degenerate shortly after the first pollen mitosis and is usually absent by anthesis. The supply of nutrients, such as reducing sugars, amino-acids, and lipids to the developing microspores is an important role of the tapetum.

Cereal pollen, in contrast to other species, contain only one germ pore, which enables localization of nuclei at a specific developmental stage. In Figure 3 and below, the development into mature pollen is presented for the species barley. During pollen cell wall development, a vacuole develops opposite the germ pore. When the vacuole has reached maturity, the nucleus is located close to the germ pore. Thereupon, the nucleus migrates

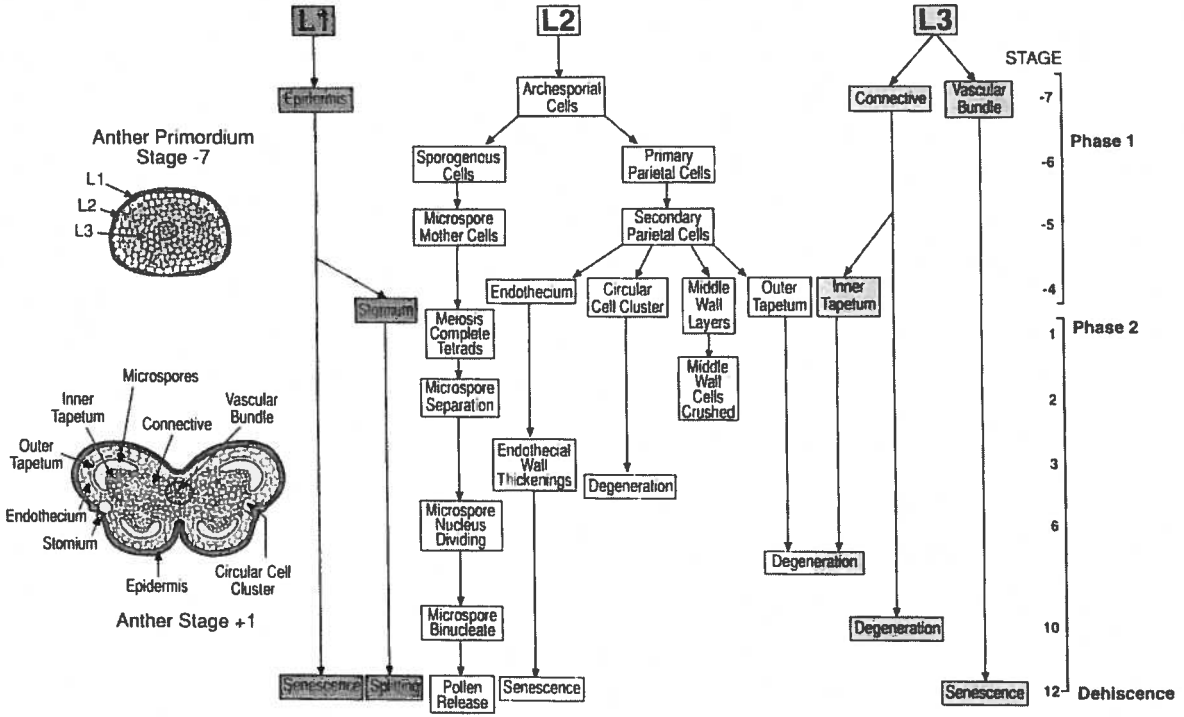


Figure 2: Cell lineages and major events that occur during anther development. Stages, timing of events and anther schematic representations are for tobacco (with premission, from Goldberg et al., 1995).

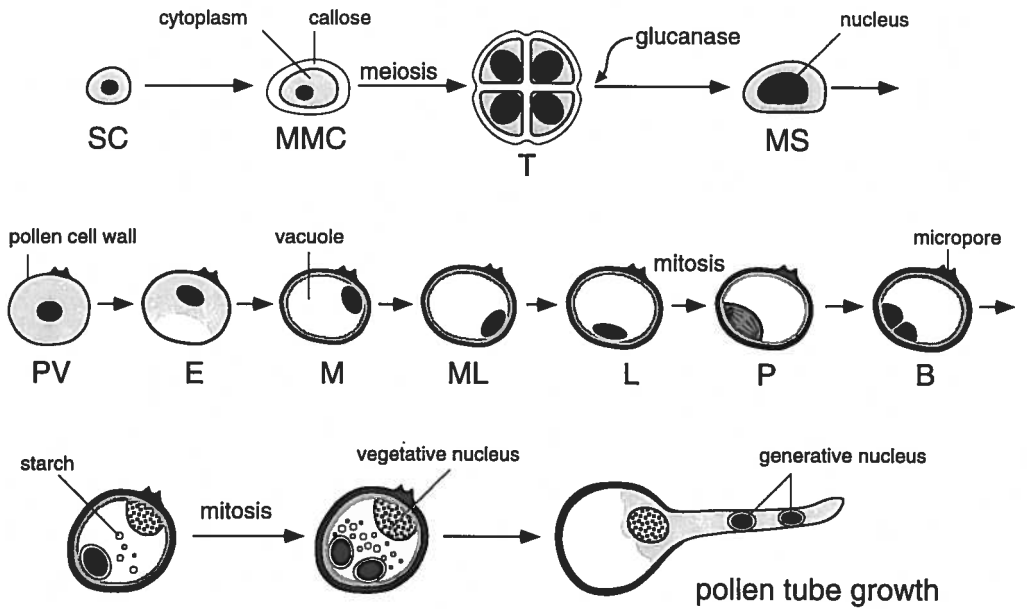


Figure 3: Stages of microsporogenesis in barley.
B = binucleate, E = early uninucleate, L = late uninucleate, M = mid uninucleate, ML = mid-late uninucleate, MMC = microspore mother cell, MS = microspore separation, P = first pollen grain mitosis, PV = pre-vacuolate, SC = sporogenous cell , T = tetrad

along the cell wall till it reaches a location opposite of the germ pore, what is called the **late uninucleate stage**, to undergo an asymmetric mitotic division or **first pollen mitosis**. This results in a pollen containing a large **vegetative** and a small **generative cell**, that is enclosed entirely within the vegetative cell. The vegetative nucleus does not undergo another mitosis, and its role is to serve as a "powerhouse" to drive the further development of the pollen and the growth of the pollen tube so that the two generative nuclei are delivered to the ovary. The generative cell has a condensed nucleus and a reduced amount of cytoplasm compared to the vegetative cell. The generative cell must undergo another mitotic division. Development of the immature pollen continues, including the further elaboration of the outer pollen cell wall and the deposition of reserves like starch granules. The release of the mature pollen from the anther is called **dehiscence** and takes place during **anthesis**. In approximately 70% of plant families (e.g. *Solananeae*, and *Liliaceae*), the pollen is released from the anther when it consists of just these two cells; the second mitotic division of the generative cell occurs while the pollen tube grows through the female pistil. In other plant families (e.g. *Cruciferae* and *Gramineae*), this second mitotic division occurs before the pollen is shed from the plant. There, the mature gametophyte is finally formed when a mitotic division of the generative cell produces a pair of gametes. Mature pollen contain a store of presynthesized mRNAs. It is known that a surprisingly large number of genes is expressed in pollen. In *Tradescantia*, about 60% as many genes are expressed in the male gametophyte as are expressed in shoots, that consits of several different cell types. Most of these genes are expressed in both pollen and sporophytic tissues while about 5% are pollen-specific. The transcripts stored in mature pollen will be translated into proteins that are needed for processes during and after pollen germination. For a while following dehiscence from the anther, the pollen exists as a free organism, protected under extreme conditions by the exine, until it is carried by wind, insects, or an other agent to the stigma of an appropriate or compatible flower, where the right conditions are present for germination of the pollen.

The development of the gametophyte can be arrested and changed into the sporophytic pathway. A plant can be produced from immature pollen, if they are cultured at the verge of mitosis in a specified medium and condition. Such a developmental switch results in the production of a plant, containing a haploid genome, from androgenesis presumably via embryogenesis.

Androgenesis in model systems

In this paragraph, the current status of the scientific knowledge concerning androgenesis of the three model species *Brassica napus*, *Nicotiana tabacum* and *Hordeum vulgare* is described.

In *Brassica napus* or rapeseed, the gametophytic development is maintained at a temperature of 18°C and can be changed into the sporophytic pathway by elevating the temperature to 32°C.

Microspores in the late uninucleate or early binucleate stage are competent for plant formation (Custers et al., 1994). The majority of vegetative nuclei re-enter the cell cycle after 12 hours of culture at 32°C (Binarova et al., 1993). About 8 hours of culture at 32°C is sufficient to induce this embryogenic development irreversibly (Pechan et al., 1991). Such a heat shock induces a program of gene expression in which the synthesis of a family of proteins, that is called heat-shock proteins, is highly induced. These proteins result from *de novo* synthesis and belong to the 70-kDa class (Cordewener et al., 1995). Upon heat treatment, the morphology of the cells is changed. The central vacuole becomes fragmented allowing the nucleus to assume a central position within the cell. Starch grains are not present, the cell develops a thick fibrillar wall, situated immediately adjacent to the intine, and large aggregates of globular material (Zaki and Dickinson, 1990). In embryogenic microspores, i.e. microspores destined to become embryos, new microtubular arrangements are observed. The first symmetrical division starts in the vegetative nucleus provided the generative nucleus is arrested near the pollen cell wall (Hause et al., 1993; Zaki and Dickinson, 1990). The cells divide randomly within the exine for 4 to 7 days following heat treatment. Then multicellular structures are released from the exine and undergo periclinal divisions resulting in protoderm differentiation of a globular embryo (Telmer et al., 1995).

In *Nicotiana tabacum* or tobacco, immature pollen develop into embryogenic microspores, if they are isolated at the unicellular or the mid-binucleate stage and cultured in a nutritionally poor medium lacking sucrose and nitrogen, what is called starvation. After an initial phase of gametophytic gene expression, the normally quiescent vegetative cell enters a phase of dedifferentiation during which the bulk of its cytoplasm is degraded. Organelles are virtually cleared from the cell and little more than the nucleus and a few structurally simplified plastids clustered around it remain (Dunwell and Sunderland, 1974 a and b; Garrido et al., 1995). The embryogenic microspores have similar characteristics as above described for rapeseed (Heberle-Bors, 1989). One of the crucial events during embryogenic induction is the derepression of the G_1 arrest in the cell cycle of the vegetative cell (Zarsky et al., 1992). Starvation induces *de novo* transcription of specific genes, a.o. a transcript for a low molecular weight heat shock protein (Garrido et al., 1993; Zarsky et al., 1993). These proteins, however, are not detected. Only recently Touraev et al. (1996 and in press) show that heat shock is a potent inductive treatment for tobacco. Apparently the mRNAs are not translated *in vivo* but accumulate in the embryogenic microspores in a translationally inactive form (Garrido et al., 1993). Further, changes in the activity of protein kinase have been observed during starvation (Garrido et al., 1993), suggesting that protein phosphorylation cascades a re involved in the transduction of the signal. Indeed, Kyo and Harada (1990a and b) detected phorphorylation of proteins

specific for embryogenic microspores.

Finally in *Hordeum vulgare* or barley, a cold pretreatment or a starvation (i.e. nutritionally poor medium as in tobacco) induces embryogenesis in mid-late to late uninucleate microspores (chapter 2). A cold pretreatment for 3 to 4 weeks is performed with spikes (Huang and Sunderland, 1982), whereas for a 3 to 4 day starvation pretreatment anthers are first dissected from the spikes (Roberts Oehlschlager and Dunwell, 1990). Upon cold pretreatment of spikes, degeneration of the tapetum is observed. In fact, changes in the morphology of the anther resemble those observed for a maturing anther, whereas the pollen development is blocked before the first pollen mitosis. Apparently, the normal sequence of events is disrupted in the cold (Sunderland et al., 1984).

In rapeseed and tobacco, haploid plants are produced via androgenesis. The majority of microspore-derived plants in barley, however, have a doubled haploid genome, resulting from spontaneous diploidization. Specifically in barley, a multiplicity of initial division patterns is revealed *in vitro* upon pretreatment of the microspores. Independent division of the generative and vegetative nucleus, results in the formation of chimerical units partitioned into distinct embryo- and endosperm- (or suspensor-) like components. Partitioning occurs only with anther excision during the mid uninucleate microspore developmental stage when the nuclei are probably still in the pre-DNA replication phase (G1 phase), but anther excision at the early uninucleate stage is ineffective and leads to rapid degeneration of the microspores. With excision at the late uninucleate stage, DNA replication is (nearly) completed and many microspores are in the G2 interphase. Independent contribution by the generative cell is blocked and the microspores develop into the A, B, or C pathways. The vegetative cell behaves either as a haploid embryo mother cell without any contribution from the generative cell (A pathway), or a non-haploid embryo mother cell derived from incorporation of the generative cell by nuclear fusion (C pathway). The microspore itself also sometimes functions as an embryo mother-cell, as two chromosome complements seemingly similar, and these might result from fusion either in interphase or in prophase (B pathway) (Sunderland et al., 1979; Sunderland and Evans, 1980). This multiple origin, from either the vegetative or the generative cell or both, can account for the different ploidies found in regenerated plantlets (Sunderland et al., 1979; Sunderland and Evans, 1980). Nuclear fusion has been emphasized as the major source of ploidy increase in barley, but endoreduplication (omission of the G2 and M-phase; Nagl, 1995) and nuclear restitution (mitosis stops because the spindle breaks down; Nagl, 1995) are not ruled out. Fusion of the generative and vegetative nuclei has been observed in cultured microspores of barley by Lee and Chen (1987), suggesting that ploidy increases are generated very early in culture.

In summary, in all three model species there is very superficial knowledge available about the mechanism of

androgenesis induction. In the model species barley, sofar only microscopical observations are reported. In contrast to the limited number of reports on the fundamental analysis of androgenesis, there is very much empirical information about factors that influence the number of plants produced through androgenesis. Information about such factors is oftensaid to be derived from "trial and error" experiments. An overview of the methodology and such factors is described below for the model species barley.

Barley androgenesis in practice

Impact of method

There are three different androgenesis methods to produce microspore-derived (doubled) haploids in barley. Haploidization can be achieved by culture of whole anthers, what is called **anther culture**. The male gametes can be mechanically isolated from the anthers, what is called (isolated) **microspore culture**. Alternatively, the microspores can be isolated naturally from dehiscing anthers, what is called shed culture (Sunderland and Xu, 1982; Ziauddin et al., 1990). Comparison between microspore and shed culture shows that shed culture is difficult to reproduce, as the period required for shedding and the shed microspore density varies, due to the physiological state of the donor plant at the time of harvest and the microspore developmental stage. In microspore culture on the contrary, such variations can be adjusted directly after isolation (at the end of this paragraph, this statement will be explained). Comparison between anther and microspore culture reveals that former is easier to perform. The simplicity of performing anther cultures has resulted in a strategy of culturing high numbers of anthers rather than to increase the efficiency of anther culture. In many species the anther wall provides a physical and chemical environment for microspore embryogenesis to a sufficient degree in many species. However, the anther wall provides also inhibitory substances (Heberle-Bors, 1984) and badly-defined nutrients (Sunderland and Xu, 1982) for sporophytic microspore development. The technique of anther culture suffers from a large disadvantage, i.e. plants may originate not only from the microspores, but also from various parts of the anther resulting in a mixed population of plants deriving both from gametic and somatic origin. Application of microspore or shed culture prevents production of anther-derived somatic regenerants. Besides, microspore culture is five times more efficient for plant production than anther culture (chapter 2). In the case of anther culture, it is extremely difficult to follow the development of the microspores within the anther and in general the culture efficiency can not be assessed reliably until 4 weeks after culture initiation. The efficiency of the development into plants is largely influenced by the density throughout culture. A significant advantage using microspore culture, is the possibility to follow the growth of the microspores under a light microscope and determine growth indices throughout culture. Further, in microspore culture the

culture density can be optimized at various time pointsduring culture. There are two indices that can be used to control the density during culture, starting from the day of microspore isolation. Directly after pretreatment the percentage of **embryogenic microspores** (i.e. a sub-population that is assumed to develop into plant; Bolik and Koop, 1991; Olsen, 1991; chapter 4 and 7) can be recognized. Further, the density of **embryo-like structures** (ELS, i.e. multicellular structures where the exine has been disrupted; these can have the morphology of a zygotic embryo) can be determined. Adjustment of both embryogenic microspore and ELS density significantly improves the reproducibility of the culture system for green plant development (chapter 4; Hoekstra et al., 1994).

The aim of androgenesis is stimulation of microspores to form embryos, maybe after an intermediate phase of dedifferentiated growth, and subsequently plants. Conditions of culture, like culture density, together with genetic determinants, affect the microspores in their new developmental pathway. Identification of such factors is of utmost importance for the establishment of a reproducible and efficient androgenesis protocol.

Impact of plant growth and culture conditions

The following contains an overview of factors of different natures which are reported to affect embryo formation in anther or microspore cultures in barley, and their possible modes of action. A more detailed overview of cereal microspore culture is written by Jähne and Lörz (1995).

The quality of the donor material such as its vigour, has a decisive influence on the success of androgenesis. Pest control procedures have a detrimental effect on microspore development (Jähne et al., 1991). Important environmental factors which influence the vigour of donor plants include light intensity and spectrum, photoperiod, temperature, and nutrition. For barley high light intensity (20,000 lux) and low temperature (12°C) are favourable for androgenesis (Foroughi-Wehr and Mix, 1979). Low temperature increases the time of development, therefore increasing the chance to harvest material in the right stage of development (Figure 4). A negative correlation between plant age and anther response with regard to androgenesis has been shown by various workers (Olsen, pers. comm.).

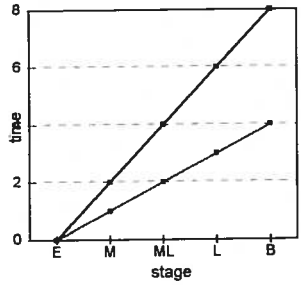


Figure 4: Schematic influence of high and low temperature on growth of the donor and the chance of harvesting material in the right stage of development.

The frequency of androgenesis is higher for anthers harvested at the beginning of the flowering period and declines with plant age. The reduced response has been ascribed to the deterioration in the gene-

ral condition of the plants, particularly during seed set. Spikes harvested at the verge of mitosis are most responsive (Gaul et al., 1976; Wheatley et al., 1986; chapter 2). Variation in stage per spike and spike number is found (Dunwell et al., 1987). The microspore developmental stage is a precondition for induction of androgenesis, as outlined earlier.
Another precondition for gametophytic embryogenesis is application of a pretreatment. Two pretreatment methods are generally used for haploidization in barley. Either a cold pretreatment of the spike for 3-5 weeks (Huang and Sunderland, 1982) or an anther pretreatment on a mannitol containing solution for 4 days (Roberts-Oehlschlager and Dunwell, 1990). Preference of mannitol pretreatment over cold period has been mentioned (Hoekstra et al., 1994). During pretreatment embryogenic microspores (chapter 4) develop, and a mechanism of action is proposed in chapter 7.

The number and percentage of embryogenic microspores obtained, is largely influenced by the method of microspore isolation (Hoekstra et al., 1994). Methods have been described in which pestle maceration (chapter 3 and 4), microblending (Olsen, 1991) or vortexing (Hu et al., 1995) of the anthers is used. However, observations in our laboratory indicated that gentle treatment of the material is necessary for obtaining a good population of embryogenic microspores and that was, in our hands, only possible with pestle maceration.

The plating efficiency is influenced by the culture medium applied. Major improvements of medium composition are cited. It has been shown that nitrogen composition of the culture medium plays a significant role in androgenesis. A reduced ammonium nitrate concentration together with additional glutamine as a nontoxic nitrogen source, is generally used (Olsen, 1987; Mordhorst and Lörz, 1993). The importance of the carbohydrate and its concentration resp. osmolarity is shown by Finnie et al. (1989), Cistué et al. (1995) and in chapter 4. Hunter (1987) demonstrates the benefit of glucose-based saccharides; in general, the traditional sucrose is replaced by maltose which is degraded at a slower rate. Scott and Lyne (1994) propose that metabolization of sucrose and glucose leads to accumulation of a toxic product resulting in cell death, whereas maltose can sustain development of embryos.

A third and last precondition for induction of androgenesis is the presence of a growth regulator in the culture medium. In general 4 μM benzyl-aminopurine (BAP), sometimes combined with a low amount of indolacetic acid (IAA), is used (Olsen, 1987; Jähne et al., 1991; Kuhlmann and Foroughi-Wehr, 1989). In chapter 5 the application of a synthetic auxin as growth regulator is presented, which is generally used to induce somatic embryogenesis.

A striking phenomenon in optimizing the physical conditions of anther culture is, that anthers orientated in the "up" position (i.e. with one lobe in contact with the medium) produce more green plants (Powell et al., 1988; Hunter, 1985; Shannon et al., 1985). An explanation for

inferior plant efficiency of anthers in the "flat" position (with two lobes in contact with the medium) is that they generally shed the microspores on the solid medium, which prevents further development of the sporophytic route. The shedding of microspores from anthers in the "flat" position during pretreatment on a mannitol containing solution, on the contrary, is positive (Roberts-Oehlschlager and Dunwell, 1990). These authors further find a positive effect on plant production if the anther density is reduced to 20 anthers; if 60 anthers are plated per dish, the number of embryos per responding anther doubled. Köhler and Wenzel (1985), however, prefer an increased plating density of the anthers (40 in stead of 10 per ml). The density of as well anthers or microspores as ELS is very important enabling development without an intervening dedifferentiation phase (Datta and Potrykus, 1989; chapter 4). The former density should be relatively high, whereas optimal embryo development requires strong dilution. These data explain contradictory results in the literature.

Specifically for barley, no chromosome doubling technique is required to obtain diploid plants after androgenesis, as about 80% of the plants obtained have spontaneously doubled chromosome numbers (see the paragraph androgenesis in model systems). The importance of culture conditions is demonstrated in the ratio green to albino plants, and optimization resulted in a decrease from 1:1 to 34:1 (chapter 3).

Applications of androgenesis

Plant breeding of barley

Methods for haploid plant production are summarized above. In barley, haploids can be relatively easy obtained *in vivo* via genome elimination in crossings with *Hordeum bulbosum*. Comparisons between the *Hordeum bulbosum* method and androgenesis have been performed. Björnstad et al. (1993) find no consistently negative impact of anther culture in barley, and although not identical, the methods may be considered equivalent. Devaux (1991) describes that androgenesis enables doubled haploids to be obtained from genotypes which respond poorly to the *H. bulbosum* method. Thus, the two techniques can be used parallel to each other to improve doubled haploid production efficiency. Foroughi-Wehr and Wenzel (1989) observe that even in species where parthenogenetic techniques had some importance, as barley, microspore approaches are overtaking.

For maximal incorporation of androgenesis-derived doubled haploids into breeding programs, the following criteria should be fulfilled:

> *Production of **large numbers** of doubled haploids of **all genotypes** should be **easy and consistent**, the plants should be **genetically normal and stable** and the population should contain a **random sample of the meiotically recombined gametes**.*

On basis of these criteria for ultimate application of doubled haploids in breeding, a description of advantages and

disadvantages of androgenesis is made. The major problem is that the method is very genotype dependent, implicating that only from certain genotypes large numbers of doubled haploids can be generated (e.g. Maheshwari et al., 1980; Foroughi- Wehr et al., 1982; Knudsen et al., 1989; Larsen et al., 1991). Another problem is the unpredictability of plant production efficiency per genotype, attributing to low and fluctuating frequencies (e.g. Sunderland et al., 1981). For the crop barley in general, about 80% of the regenerants are spontaneous doubled haploids (e.g. Islam et al., 1992). Further, in barley there is very little evidence of induced genetic changes. The level of instability is insignifcant when compared with that which is observed following meiotic recombination (Finnie et al., 1991). In other crops e.g. wheat the instable genetic background can be recognized in the regenerants. Especially in cereals formation of chlorophyll deficient plants is observed due to deletions of the plastid genome (Harada et al., 1991). Significant deviations from the expected Mendelian ratios are observed e.g. by Thompson et al. (1991) for four of the ten markers studied. On the other hand, in responsive genotypes, thousands of homozygous plants can be obtained within about 3 months, and save years of backcrossing or selfing. Resistances against nematode, viral and fungal infection have been found among regenerants from anther culture (e.g. Wenzel and Uhrig, 1981; Foroughi-Wehr and Friedt, 1984; Kintzios et al., 1994). Moreover, homogeneous parent material can be generated for F1 hybrid production. Reduction of the time needed for production of a new variety upon application of androgenesis is limited, as regenerants should be screened under field conditions for several years. The genuine advantages of application of microspore-derived plants are quality improvement by generation of new variability, fixation of traits (Boppenmeier et al., 1989), superior material for gene mapping, and determination of the genetics of multiple disease resistance (Steffenson et al., 1995). Selection of improved varieties can be enhanced if androgenesis is combined with *in vitro* selection or genetic engineering.

In vitro selection and genetic modification

Androgenesis can be combined with *in vitro* techniques, in order to enhance the chance on selection of the desired genotype. Especially when microspore culture is combined with such techniques, the advantage of treating haploid single cells is favourable. Below, the most important methods for combination with androgenesis are described.

In vitro selection is a low cost, and attractive technique for traits expressed at the cell biological level. This method is being combined with microspore culture for selection of various traits, like low pH and heavy metal tolerance (Karsai et al., 1994). Via *in vitro* selection of androgenesis-derived tissue, salt tolerance has been selected in rice (KrishnaRaj and SreeRangasamy, 1993), freezing tolerance in *Brassica napus* (Orr et al., 1990), and disease tolerance in wheat (Fadel and Wenzel, 1993). In addition, Touraev et al. (1995) show that *in vitro* selection can

be applied on mid-binucleate pollen during *in vitro* maturation, and therefore determine that gametophytic selection is feasible for enhanced transmission of genes to the next sporophytic generation.

Moreover, induction of genetic changes has been performed in rapeseed in an indirect way using gamma-ray mutation (Polsoni et al., 1988). Nowadays a tremendous interest is shown in more directed, targeted methods for changes in the (haploid) genetic background, using genetic transformation. For the generation of stably transformed plants, in general five transformation methods are used. In dicotyledonous species the method of DNA transfer is generally mediated by *Agrobacterium tumefaciens*. In the monocotyledonous species rice, cocultivation of seed derived callus with *Agrobacterium tumefaciens* resulted only recently in stable integration of foreign DNA (Hiei et al., 1994). The other methods for DNA transfer are electroporation, microinjection, PEG-mediated uptake and particle bombardment. An overview of the methods used for transformation of uni- or binucleate microspores is presented in Table 1.

Table 1: Overview of transformation methods used on uni- or binucleate microspores as target tissue, the result and their reference. S = stably transformed, T = transient expression.

References	Species	Method	Result
Fennel and Hauptmann, 1992	*Zea mays*	electroporation	T
Jardinaud et al., 1995	*Zea mays*	electroporation	T
Joersbo et al., 1990	*Hordeum vulgare*	electroporation	T
Olsen, 1991	*Hordeum vulgare*	microinjection	T
Bolik and Koop, 1991	*Hordeum vulgare*	microinjection	T
Gaillard et al., 1992	*Zea mays*	microinjection	T
Jones-Villeneuve et al., 1995	*Brassica napus*	microinjection	T
Kuhlmann et al., 1991	*Hordeum vulgare*	PEG	T
Fennel and Hauptmann, 1992	*Zea mays*	PEG	T
Stöger et al., 1992	*Nicotiana tabacum*	bombardment	T
Jähne et al., 1994	*Hordeum vulgare*	bombardment	S
Kasha et al., in press	*Hordeum vulgare*	bombardment	S
Stöger et al., 1995	*Nicotiana tabacum*	bombardment	S
Nishihara et al., 1995	*Nicotiana rustica*	bombardment	S
Toureav et al., pers. comm.	*Nicotiana tabacum*	bombardment	S

There is only one report from 1989 available on *Agrobacterium*-mediated DNA transfer to microspores of rapeseed (Pechan); this has proven to be non-reproducible.

Further, sofar only transient expression has been obtained in microspores after electro-

poration, microinjection and PEG-mediated DNA uptake. For transformation of microspores, particle bombardment has recently proven to be a suitable method. A system for the biolistic transformation of barley using freshly isolated microspores as the target tissue has been developed. Independent transformation events led, on average, to the reovery of 1 plant per 10^7 microspores. The transferred genes were inherited in all progeny plants indicating the homozygous nature of primary regenerants (Jähne, et al., 1994). This method is repeated and similar results with the same species are obtained (Kasha et al., in press). Recently, by using this approach, trangenic plants are produced from *Nicotiana sp.* (Stöger et al., 1995; Nishihara et al., 1995).

In addition, if microspores are used as target tissue in genetic transformation, the gametophytic developmental route can be completed in stead of the sporophytic pathway. DNA transfer to microspores by particle bombardment and subsequently *in vitro* maturation of these microspores generates mature transgenic pollen (Alwen et al., 1990). Via this elegant approach, laborious and time consuming steps of tissue culture can be omitted for the production of transgenic plants. Recently stable inheritance of the transgene has been achieved through bombardment of tobacco microspores, *in vitro* selection during pollen germination, and *in vivo* fertilization; the time required from microspore isolation until stable expression of the transgene is less than 3 weeks (Touraev et al., pers. comm.).

Aim of the thesis

Plant production through androgenesis has many attractive characteristics, which are outlined in detail above. Generation of (doubled) haploid plants is of utmost importance for breeding of improved plant varieties. Selection of the desired traits can be further accelerated by *in vitro* selection or transformation of the haploid cells. In many species (doubled) haploid plants have been produced using anther or microspore culture. However, large differences in regeneration frequencies between species and genotypes, prevent general applicability of the method. Even in model species, only one or a few genotypes, e.g. cv. Igri in barley and cv. Topas in rapeseed, are used in fundamental studies because of their excellent culture efficiency. As mentioned earlier, there is some empirical knowledge available about factors that influence the response and regeneration efficiencies of microspores. One of them is the physiological state of the donor material. Accumulation of fundamental knowledge about the switch from the gametophytic pathway to the sporophytic pathway is still very limited. However, it has not been possible sofar, to modify culture efficiency in such a way that all cultivars produce microspore-derived green plants.

In this thesis, preconditions for the production of microspore-derived plants are described using donor plants grown in a well conditioned environment. Determination of such factors will enable improvement of the reproducibility of the method. The aim of the research is to

identify both "direct-breeding-applicable" possibilities and more fundamental factors, in order to unravel part of the complicated mechanism necessary for the switch from the gametophytic to the sporophytic pathway in barley microspores.

References

Alwen A., N. Eller, M. Kastler, R.M.B. Moreno and E. Heberle-Bors. Potential of in vitro pollen maturation for gene transfer. Physiol. Plant. 79: 194-196 (1990)

Bajaj Y.P.S. *In vitro* production of haploids and their use in cell genetics and plant breeding. In: Biotechnology in agriculture and forestry vol. 12 Haploids in crop improvement I, ed. Bajaj Y.P.S., Springer Verlag Brlin Heidelberg pp. 1-44 (1990)

Bedinger P. The remarkable biology of pollen. Plant Cell 4: 879-887 (1992)

Binarova P., K. Straatman, B. Hause, G. Hause and A.A.M. VanLammeren. Nuclear DNA synthesis during the induction of embryogenesis in cultured microspores and pollen of *Brassica napus* L. Theor. Appl. Genet. 87: 9-16 (1993)

Björnstad A., H. Skinnes and K Thoresen. Comparisons between doubled haploid lines produced by anther culture, the *Hordeum bulbosum*-method and lines produced by single seed descent in barley crosses. Euphytica 66: 135-144 (1993)

Bold C.B., C. Alexopoulos, and T. Delevoras (eds.). Morphology of plants and fungi. Harper and Row Publishers, New York pp. 570 (1980)

Bolik M. and H.U. Koop, Identification of embryogenic microspores of barley (*Hordeum vulgare* L.) by individual selection and culture and their potential for transformation by microinjection. Protoplasma 162, 61-68 (1991)

Boppenmeier J., S. Zuechner and B. Foroughi-Wehr. Haploid production from barley yellow dwarf virus resistant clones of *Lolium*. Plant Breed. 103: 216-220 (1989)

Cao M.Q., Y. Li, F. Liu, T. Jiang and G.S. Liu. Application of anther culture and isolated microspore culture to vegetable crop improvement. Acta Hort. 392: 27-38 (1995)

Cistué L., A. Ziauddin, E. Simion and K.J. Kasha. Effects of culture conditions on isolated microspore response of barley cultivar Igri. Plant Cell Tiss. Org. Cult. 42: 163-169 (1995)

Cordewener J.H.G., G. Hause, E. Goergen, R. Busink, B. Hause, J.J.M. Dons, A.A.M. VanLammeren, M.M. VanLookeren Campagne and P. Pechan. Changes in synthesis and localization of members of the 70-kDa class of heat shock proteins accompany the induction of embryogenesis in *Brassica napus* L. microspores. Planta 196: 747-755 (1995)

Custers J.B.M., J.H.G. Cordewener, Y. Noellen, J.J.M. Dons and M.M. VanLookeren Campagne. Temperature controls both gametophytic and sporophytic development in microspore cultures of *Brassica napus*. Plant Cell Rep. 13: 267-271 (1994)

Datta S.K. and I. Potrykus. Artificial seeds in barley: encapsulation of microspore-derived embryos. Theor. Appl. Genet. 77: 820-824 (1989)

Devaux P. Investigations to improve doubled haploid production efficiency in a winter barley breeding programme. Cereal Research Comm 19: 51-58 (1991)

Dunwell J.M. and N. Sunderland. Pollen ultrastructure in anther cultures of *Nicotiana tabacum* II. changes associated with embryogenesis. J. Exp. Bot. 25: 363-373 (1974a)

Dunwell J.M. and N. Sunderland. Pollen ultrastructure in anther cultures of *Nicotiana tabacum* III. The first sporophytic division. J. Exp. Bot. 26: 240-252 (1974b)

Dunwell J.M., R.J. Francis and W. Powell. Anther culture of *Hordeum vulgare* L. Theor. Appl. Genet. 74: 60-64 (1987)

Fadel F. and G. Wenzel. *In vitro* selection for tolerance to *Fusarium* in F1 microspore populations of wheat. Plant Breed. 110: 89-95 (1993)

Fennel A. and R. Hauptmann. Electroporation and PEG delivery of DNA into maize microspores. Plant Cell Rep. 11: 567-570 (1992)

Finnie S.J., W. Powell and A.F. Dyer. The effect of carbohydrate composition and concentration on anther culture response in barley (*Hordeum vulgare* L.). Plant Breed. 103: 110-118 (1989)

Finnie S.J., B.P. Foster and K.J. Chalmers. Genetic stability of microspore-derived doubled haploids of barley: a cytological, biochemical, and molecular study. Genome 34: 923-928 (1991)

Foroughi-Wehr B. and W. Friedt. Rapid production of recombinant barley yellow mosaic virus resistant *Hordeum vulgare* lines by anther culture. Theor. Appl. Genet. 67: 377-382 (1984)

Foroughi-Wehr B., W. Friedt and G. Wenzel. On the genetic improvement of androgenetic haploid formation in *Hordeum vulgare* L. Theor. Appl. Genet. 62: 233-239 (1982)

Foroughi-Wehr B. and G. Mix. *In vitro* response of Hordeum vulgare L. anthers cultured from plants grown under different environments. Env. Exp. Bot. 19: 303-309 (1979)

Foroughi-Wehr B. and G. Wenzel. Androgenetic haploid production. IAPTC Newsletter May: 11-18 (1989)

Gaillard A., E. Matthys-Rochon and C. Dumas. Selection of microspore derived embryogenic structures in maize related to transformation potential by microinjection. Bot. Acta 105: 313-318 (1992)

Garrido D., N. Eller, E. Heberle-Bors and O. Vicente. *De novo* transcription of specific mRNAs during the induction of tobacco pollen embryogenesis. Sex. Plant Reprod. 6: 40-45 (1993)

Garrido D., O. Vicente, E. Heberle-Bors and M.I.Rodriguez-Garcia. Cellular changes during the acquisition of embryogenic potential in isolated pollen grains of *Nicotiana tabacum*. Protoplasma 186: 220-230 (1995)

Gaul H., G. Mix, B. Foroughi-Wehr and M. Okamoto. Pollen grain development of *Hordeum vulgare*. Zeitschrift für Pflanzenzüchtung 76: 77-80 (1976)

Goldberg B., T.P. Beals and P.M. Sanders. Anther development: basic principles and practical applications. Plant Cell 5: 1217-1229 (1993)

Goldberg B., P.M. Sanders and T.P. Beals. A novell cell-ablation strategy for studying plant development. Phil. Trans. R. Soc. Lond. B 350: 5-17 (1995)

Harada T., T. Sato, D. Asaka and I. Matsukawa. Large-scale deletions of rice plastid DNA in anther culture. Theor. Appl. Genet. 81: 157-161 (1991)

Hause B., G. Hause, P. Pechan and A.A.M. VanLammeren. Cytoskeletal changes and induction of embryogenesis in microspore and pollen cultures of *Brassica napus* L. Cell Biol. Int. 17: 153-168 (1993)

Heberle-Bors E. Genotypic control of pollen plant formation in *Nicotiana tabacum* L. Theor. Appl Genet. 68: 475-479 (1984)

Heberle-Bors E. *In vitro* haploid formation from pollen: a critical review. Theor. Appl. Genet. 71: 361-374 (1985)

Heberle-Bors E. Isolated pollen culture in tobacco: plant reproductive development in a nutshell. Sex. Plant Reprod. 2: 1-10 (1989)

Hiei Y., S. Ohta, T. Komari and T. Kumashiro. Efficient transformation of rice (*Oryza sativa* L.) mediated by *Agrobacterium* and sequence analysis of the boundaries of the T-DNA. Plant J. 6: 271-282 (1994)

Hoekstra S. M.H. VanZijderveld, S. VanBergen, F. VanderMark and F. Heidekamp. Genetic modification of barley for end use quality. In: Improvement of cereal quality by genetic engineering, Henry R.J., and J.A. Ronalds (eds.), Plenum Press, New York, pp. 139-144 (1994)

Hu T.C., A. Ziauddin, E. Simion and K.J. Kasha. Isolated microspore culture of wheat (*Triticum aestivum* L.) in a defined media I. Effects of pretreatment, isolation methods, and hormones. In Vitro Cell Dev. Biol. 31: 79-83 (1995)

Huang B. and N. Sunderland. Temperature-stress in barley anther culture. Ann Bot 49: 77-88 (1982)

Hunter C.P. The effect of anther orientation on the production of microspore-derived embryoids and plants of *Hordeum vulgare* cv. Sabarlis. Plant Cell Rep. 4: 267-268 (1985)

Hunter C.F. Plant generation method. European patent application number 87200773.7, publication number 0 245 898 (1987)

Islam M.R., S. Kintzios and G. Fischbeck. Anther culture responsiveness of *Hordeum spontaneum* derived spring barley lines and a genetic analysis of plant regeneration. Plant Cell Tiss. Org. Cult. 29: 235-239 (1992)

Jähne, A., D. Becker, R. Brettschneider and H. Lörz. Regeneration of transgenic, microspore-derived, fertile barley. Theor. Appl. Genet. 89: 525-533 (1994)

Jähne, A., P.A. Lazzeri, M. Jäger-Gussen and H. Lörz. Plant regeneration from embryogenic cell suspension derived from anther cultures of barley (*Hordeum vulgare* L.). Theor. Appl. Genet. 82: 74-80 (1991)

Jähne A. and H. Lörz. Cereal microspore culture. Plant Sci. 109: 1-12 (1995)

Jardinaud M.F., A. Souvre, M. Beckert and G. Alibert. Optimisation of DNA transfer and transient β-glucuronidase expression in electroporated maize (*Zea mays* L.) microspores. Plant Cell Rep. 15: 55-58 (1995)

Joersbo M., R.B. Jørgensen and P. Olesen. Transient eletropermeabilization of barley (*Hordeum vulgare* L.) microspores to propidium iodide. Plant Cell Tiss. Org. Cult. 23: 125-129 (1990)

Jones-Villeneuve E., B. Huang, I. Prudhomme, S. Bird, R. Kemble, J. Hattori and B. Miki. Assessment of microinjection for introducing DNA into uninuclear microspores of rapeseed. Plant Cell Tiss. Org. Cult. 40: 97-100 (1995)

Karsai I., Z. Bedoe, G. Kovács and B. Barnabás. The effect of *in vivo* and *in vitro* aluminium treatment on anther culture response of triticale x wheat hybrids. J. Gen. Breed. 48: 353-358 (1994)

Kasha et al., in press

Kintzios S., R.M. Islam and G. Fisckbeck. Distorted segregation for mildew resistance in doubled haploid lines of spring barley. Plant Breed. 112: 248-251 (1994)

Knudsen S., I.K. Due and S.B. Andersen. Components of response in barley anther culture. Plant Breed. 103: 241-246 (1989)

Köhler F. And G. Wenzel. Regeneration of isolated barley microspores in conditioned media and trials to characterize the responsible factor. J. Plant Physiol. 121: 181-191 (1985)

KrishnaRaj S. and S.R. SreeRangasamy. *In vitro* salt tolerance screening in long-term anther cultures of rice (*Oryza sativa* L.) variety IR50. J. Plant Physiol. 142: 754-758 (1993)

Kuhlmann U. and B. Foroughi-Wehr. Production of doubled haploid lines in frequencies sufficient for barley breeding programs. Plant Cell Rep. 8: 78-81 (1989)

Kuhlmann U., B. Foroughi-Wehr, A. Graner and G. Wenzel. Improved culture system for microspores of barley to become a target for DNA uptake. Plant Breeding 107: 165-168 (1991)

Kyo M. and H. Harada. Phosphorylation of proteins associated with embryogenic dedifferentiation of immature pollen grains of *Nicotiana rustica*. J. Plant Phys. 136: 716-722 (1990a)

Kyo M. and H. Harada. Specific phosphoproteins in the initial period of tobacco pollen embryogenesis. Planta 182: 58-63 (1990b)

Larsen E.T., I.K.D. Tuvesson and S.B. Andersen. Nuclear genes affecting percentage of green plants in barley (*Hordeum vulgare* L.) anther culture. Theor. Appl. Genet. 82: 417-420 (1991)

Lee F.M. and C.C. Chen. Nuclear fusion in cultured microspores of barley. Plant Cell Rep. 6:191-193 (1987)

Maheshwari S.C., A.K. Tyagi and K. Malhotra. Induction of haploidy from pollen grains in angiosperms - the current status. Theor. Appl. Genet. 58: 193-206 (1980)

Mascarenhas J.P. The male gametophyte of flowering plants. Plant Cell 1: 657-664 (1989)

Mascarenhas J.P. Gene activity during pollen development. Annu. Rev. Plant Physiol. Plant Mol. Biol. 41: 317-338 (1990)

McCormick S. Male gametophyte develop-ment. Plant Cell 5: 1265-1275 (1993)

Meinke D.W. Perspectives on genetic analysis of plant embryogenesis. Plant Cell 3: 857-866 (1991)

Mordhorst A.P. and H. Lörz. Embryogenesis and development of isolated barley (*Hordeum vulgare* L.) microspores are influenced by the amount and composition of nitrogen sources in culture media. J. Plant Physiol. 142: 485-492 (1993)

Morrison R.A. and D.A. Evans. Haploid plants from tissue culture: new plant varieties in a shortened time frame. Biotechnology 6: 684-690 (1988)

Nagl W. Cdc2-kinases, cyclins, and the switch from proliferation to polyploidization. Protoplasma 188: 143-150 (1995)

Nishihara M., M. Seki, M. Kyo, K. Irifune and H. Morikawa. Transgenic haploid plants of *Nicotiana rustica* produced by bombardment-mediated transformation of pollen. Transg. Res. 4: 341-348 (1995)

Olsen F.L. Induction of micropsore embryo-genesis in cultured anthers of *Hordeum vulgare*. The effects of ammonium nitrate, glutamine and asparagine as nitorgen sources. Carlsberg Res. Commun. 52: 393-404 (1987)

Olsen F.L. Isolation and cultivation of embryogenic microspores from barley (*Hordeum vulgare* L.). Hereditas 115: 255-266 (1991)

Orr. W., A.M. Johnson-Flanagan, W.A. Keller

and J. Singh. Induction of freezing tolerance in microspore-derived embryos of winter *Brassica napus*. Plant Cell Rep. 8: 579-581 (1990)

Pechan P.M. Successful cocultivation of *Brassica napus* microspores and proembryos with *Agrobacterium*. Plant Cell Rep. 8: 387-390 (1989)

Pechan P.M., D. Bartels, D.C.W. Brown and J. Schell. Messenger-RNA and protein changes associated with induction of *Brassica* microspore embryogenesis. Planta 184: 161-165 (1991)

Polsoni L., L.S. Kott and W.D. Beversdorf. Large-scale microspore culture technique for mutation-selection studies in *Brassica napus*. Can. J. Bot. 66: 1681-1685 (1988)

Powell W., F.M. Borrino and V. Goodall: The effect of anther orientation on microspore-derived plant production in barley (*Hordeum vulgare* L.). Euphytica 38: 159-163 (1988)

Reynolds J.F. *In vitro* culture of vegetable crops. In: Plant cell and tissue culture, Vasil I.K., Thorpe T.A. eds., Kluwer Academic Publishers, Dordrecht pp. 331-362 (1994)

Roberts-Oehlschlager S.L. and J.M Dunwell. Barley anther culture: pretreatment on mannitol stimulates production of microspore-derived embryos. Plant Cell, Tiss. Org. Cult. 20: 235-240 (1990)

Scott R., R. Hodge, W. Paul and J. Draper. The molecular biology of anther differentiation. Plant Sci. 80: 167-191 (1991)

Scott P. and R.L. Lyne. Initiation of embryogenesis from cultured barley microspores : a furter investigation into the toxic effects of sucrose and glucose. Plant Cell Tiss. Org. Cult. 37: 61-65 (1994)

Shannon P.R.M., A.E. Nicholson, J.M. Dunwell and D.R. Davies. Effect of anther orientation on microspore-callus production in barley (*Hordeum vulgare* L.). Plant Cell Tiss. Org. Cult. 4: 271-280 (1985)

Sheridan W.F. Genes and embryo morphogenesis in angiosperms. Developm. Genet. 16: 291-297 (1995)

Steffenson B.J., Y. Jin, B.G. Rossnagel, J.B. Rasmussen and K. Kao. Genetics of multiple disease resistance in a doubled-haploid population of barley. Plant Breed. 114: 50-54 (1995)

Stöger E., R.M. Benito Moreno, B. Ylstra, O. Vicente and E. Heberle-Bors. Comparison of different techniques for gene transfer into mature and immature tobacco pollen. Transg. Res. 1: 71-78 (1992)

Stöger E., C. Fink, M. Pfosser and E. Heberle-Bors. Plant transformation by particle bombardment of embryogenic pollen. Plant Cell Rep. 14: 273-278 (1995)

Sunderland N. and L.J. Evans. Multicellular pollen formation in cultured barley anthers II. The A, B, and C pathways. J. Exp. Bot. 31: 501-514 (1980)

Sunderland N., B. Huang and G.J. Hills. Disposition of pollen in situ and its relevance to anther/pollen culture. J. Exp. Bot. 35: 521-530 (1984)

Sunderland N., M. Roberts, L.J. Evans and D.C. Wildon. Multicellular pollen formation in cultured barley anthers I. Independent division of the generative and vegetative cells. J. Exp. Bot. 30: 1133-1144 (1979)

Sunderland N. and Z.H. Xu. Shed pollen culture in *Hordeum vulgare*. J. Exp. Bot. 33: 1086-1095 (1982)

Sunderland N., Z.H. Xu and B. Huang. Recent advances in barley anther culture. In: Barley genetics IV, pp. 699-703 (1981)

Telmer C.A., W. Newcomb and D.H. Simmonds. Cellular changes during heat shock induction and embryo development of cultured microspores of *Brassica napus* cv. Topas. Protoplasma 185: 106-112 (1995)

Telmer C.A., D.H. Simmonds and W. Newcomb. Determination of developomental stage to obtain high frequencies of embryogenic microspores in *Brassica napus*. Phys. Plant. 84: 417-424 (1992)

Thompson D.M., K. Chalmers, R. Waugh, B.P. Foster, W.T.B Thomas, P.D.S Caligari and W. Powell. The inheritance of genetic markers in microspore-derived plants of barley *Hordeum vulgare* L. Theor. Appl. Genet. 81: 487-492 (1991)

Touraev A., C.S. Fink, E. Stöger and E. Heberle-Bors. Pollen selection: a transgenic reconstruction approach. Proc. Natl. Acad. Sci. USA 92: 12165-12169 (1995)

Toureav A., A. Ilham, O. Vicente and E. Heberle-Bors. Stress-induced microspore embryogenesis in tobacco: an optimized system for molecular studies. Plant Cell Rep. 15: 561-565

(1996)
Touraev. A., M. Pfosser, O. Vicente and E. Heberle-Bors. Stress as the major signal controlling the developmental fate of tobacco microspores: towards a unified model of induction of microspore/pollen embryogenesis. Planta, in press.
Veilleux R.E. Development of new cultivars via anther culture. Hort. Sci. 29: 1238-1240 (1994)
Vicente O., R.M. Benito Moreno and E. Heberle-Bors. Pollen cultures as a tool to study plant development. Cell Biol. Rev. 24: 295-305 (1991)
Wenzel G. and H. Uhrig. Breeding for nematode and virus resistance in potato via anther culture. Theor. Appl. Genet. 59: 333-340 (1981)
Wheatley W.G., A.A. Marsolais and K.J. Kasha. Microspore growth and anther staging in barley anther culture. Plant Cell Reports 5: 47-49 (1986)
Zaki M.A.M. and H.G. Dickinson. Structural changes during the first division of embryos resulting from anther and free microspore culture in *Brassica napus*. Protoplasma 156: 149-162 (1990)
Zarsky V., D. Garrido, L. Rihova, J. Tupy, O. Vicente and E. Heberle-Bors. Derepression of the cell cycle by starvation is involved in the induction of tobacco pollen embryogenesis. Sex. Plant Reprod. 5: 189-194 (1992)
Zarsky V., D. Garrido, N. Eller, J. Tupy, O. Vicente, F. Schöffl and E. Heberle-Bors. The expression of a small heat shock gene is activated during induction of tobacco pollen embryogenesis by starvation. Plant Cell Env. 18: 139-147 (1995)
Ziauddin A., E. Simion, and K.J. Kasha. Improved plant regeneration from shed microspore culture in barley (*Hordeum vulgare* L.) cv. Igri. Plant Cell Rep. 9: 69-72 (1990)

Chapter 2

Anther and microspore culture of *Hordeum vulgare* L. cv. Igri

S. Hoekstra, M.H. van Zijderveld, J.D. Louwerse, F. Heidekamp & F. van der Mark

Summary

The influence of environmental and cultural changes was investigated on both anther culture and microspore culture of barley cv. Igri. The highest regeneration frequency for both culture systems was obtained when at least 50% of the microspore population was in the mid-late to late uninucleate stage, when the anthers had been pretreated for 4 days on mannitol and when culture was performed with oxygen supply at regular intervals. Furthermore, the supplement of vitamins and casein hydrolysate in the culture medium improved the isolated microspore culture, whereas these compounds had a negative effect, if any effect at all, on the culture of anthers. Anther culture is not as laborious as microspore culture, but turned out to be at least 5 times less efficient. When mechanically isolated microspores were cultured, under the conditions found to be optimal in the present study, a mean of 12.4 green plants per anther was obtained.

Introduction

The application of microspore-derived plants in breeding programs is an established technique today. Advantage is provided by accelerating and simplifying procedures (Morrison and Evans, 1988). Moreover, isolated microspores are unicellular and haploid and are therefore promising targets for *in vitro* manipulation with subsequent selection for dominant as well as recessive traits (Ahmad et al., 1991; Swanson et al., 1989; Ye et al., 1987). Microspores, microspore-derived embryoids or pollen are also very useful as target cells in experiments aiming at stable transformation, for example by cocultivation with *Agrobacterium* or particle bombardment (Creissen et al., 1990; Pechan, 1989; Twell et al., 1989).

A successful use of microspores for such applications requires optimized culture procedures to achieve a high regeneration frequency. In barley the techniques for anther culture are rather well established.

Olsen (1987) and Jähne et al. (1991) have reported a yield of 7 green plants per anther, but in all other studies much lower numbers were obtained.

Few studies have been directed towards the cultivation of isolated barley microspores. Furthermore, among these studies the experimental approach differs considerably. Kuhlmann et al. (1991) used the classic method of isolation, i.e. pestle maceration, and reported yields of up to 10 green plants per anther. The positive effect of microblending compared to pestle maceration described by Olsen (1991) resulted in 9.4 green plants per anther. A third approach for culturing isolated microspores was chosen by Ziauddin et al. (1990) who purified microspores, which were naturally shed from floating anthers, for culture and obtained up to 3 green plants per anther.

The aim of the present study was to compare the influence of several parameters on the efficiency of the anther and microspore culture systems. The investigations include identification of the stages in microsporogenesis that respond optimally with respect to microspore embryogenesis, optimization of the mannitol pretreatment, evaluation of the effect of two different media and, assessment of the effect of oxygen supply during culture.

Materials and Methods

Growth of the donor plants

Donor plants of *Hordeum vulgare* cv. Igri were grown in growth cabinets at 12°C with a 16-h light regime (24000 lx) as described in detail by Olsen (1987).

Table 1: Composition of media (mg/l); I modified according to Jähne et al. (1991), II and III modified MS according to Olsen (1987). [a] for anther culture, [b] for isolated-microspore culture, [c] glutamine (25%) is the active component (Bister-Miel et al., 1985), [d] expressed as g/l.

	I	II	III
NH_4NO_3	165	165	165
KNO_3	1750	1900	1900
$MgSO_4.7H_2O$	350	370	370
KH_2PO_4	200	170	170
$CaCl_2.2H_2O$	450	440	440
$FeNa_2EDTA$	40	40	40
$MnSO_4.H_2O$	15	22.3	22.3
H_3BO_3	5	6.2	6.2
KI	0.75	0.83	0.83
$ZnSO_4.7H_2O$	13.4	11.7	11.7
$Na_2MoO_4.2H_2O$	0.25	0.25	0.25
$CuSO_4.5H_2O$	0.025	0.025	0.025
$CoCl_2.6H_2O$	0.025	0.025	0.025
Nicotinamide	1	1	1
Thiamin HCl	1	10	10
Pyridoxine HCl	1	1	1
D-Calcium pantothenate	1	-	-
Choline chloride	1	-	-
L-ascorbic acid	2	-	-
Vit. B_{12}	0.02	-	-
Riboflavin	0.2	-	-
Folic acid	0.4	-	-
Biotin	0.01	-	-
Cholecalciferol	0.01	-	-
Vit. A	0.01	-	-
p-Aminobenzoic acid	0.02	-	-
Myo-inositol	100	100	100
Glutamine	750	750	750
Casein hydrolysate[3]	1000	-	-
Maltose	30[1]/50[2]	30[1]/60[2]	-
Sucrose	-	-	10/30
Ficoll 400	100,000[1]	100,000[1]	-
Agarose HMT (Sea Kem)	-	-	6,000
Benzyl aminopurine	1	1	-

Spike selection
Tillers were harvested when the interligule length was about 50 mm. Before the spikes were removed from the ensheathing leaves, the surface was sterilized with an aerosol of 70% ethanol. Microspores derived from anthers of the central floret were stained with acetocarmine. The developmental stage of at least 100 microspores per spike was determined. The influence of the microspore developmental stage on the efficiency of culture was assessed for spikes with distinct microspore populations numbered I to V.

Anther culture
Sixty anthers were placed on 1 ml 0.3 M mannitol and maintained for 4 days in the dark at 25°C. At the end of pretreatment the shed microspores were counted and the anthers were transferred to culture medium I (day 0) (Table 1). The cultures were incubated in a container sealed with Parafilm in the dark at 25°C. Pure oxygen was flushed through the container for 15 min. at days 0, 3 and 7. At days 3, 7 and 10 fresh medium was added and from day 10 onwards the cultures were incubated under continuous orbital shaking at 100 rpm. Proper dilution of the developing embryo-like structures (ELS) is important.
In addition to the culture regime outlined above, the influence of 3 days of mannitol starvation, culture on medium II (Table 1) and culture without oxygen supply was studied.

Microspore isolation and culture
The anthers to be used for microspore isolation were pretreated as described above. After pretreatment, microspores were isolated by very gentle maceration with a teflon rod and sieving through a nylon mesh (pore size 100 μm). The filtrate was rinsed twice in 0.3 M mannitol followed by centrifugation at 100 x *g* for 5 min. The microspores were then resuspended in 1 ml culture medium I or II (Table 1). After determining the number of isolated microspores with a haemocytometer (modified Fuchs-Rosenthal), the volume was adjusted with culture medium to give a final density of $1.5x10^4$ microspores per ml. From correctly staged spikes 2-$10x10^4$ microspores were isolated per spike. The microspores were cultured as described under anther culture.

Regeneration of plants
After 8 weeks of culture all ELS with a diameter larger than 1 mm were transferred with a forceps to medium III (Table 1; 1% sucrose, except B* on 3% sucrose) and incubated at 25°C for 7 days under 600 lx. Then the ELS and developing plants were grown under 3200 lx. After a total of 4 weeks of culture the plates were scored for number of green and albino plants. Plants were defined as distinct structures possessing one or more leaves at least 5 mm in length (in previous studies was shown, that 90% of these are able to develop *in vivo* into a plant). The ploidy level of representative plants was determined by flow cytometric analysis of the relative nuclear DNA content.

In order to be able to compare results obtained from anther and microspore culture, experiments were performed according to the split-spike approach described by Kao (1981). All results presented are the mean of two separate experiments. The general conclusions from these experiments are supported by at least 5 additional experiments.

Results

Types of microspores
Two different types of microspores could be distinguished during pre-treatment, after isolation and in culture. One type consisted of small, often plasmolysed, microspores with a diameter of 35-40 μm. The other type is characterized as enlarged and swollen, 40-60 μm in diameter and rich in cytoplasm which is mainly of a granular appearance; moreover, a red and/or blue interference around the exine was seen with the light microscope. The latter type of microspores can be compared with type A microspores described by Bolik and Koop (1991) and with the large microspores reported by Olsen (1991). It was apparent that only the large type of microspores divided and grew.

Influence of the microspore stage on embryogenesis

In barley microsporogenesis the microspore stage, from dissolution of the tetrad until the first mitosis, is usually divided into three major stages (early, mid and late) on the basis of the presence of the vacuole and the position of the nucleus relative to the micropore (see Figure 1 and Wheatley et al., 1986). We have investigated the cultural characteristics of five different anther and microspore populations spanning the interval from the early microspore stage until the binucleate pollen stage. As can be seen in Figure 1, there was, as expected, a considerable overlap of stages in the five populations. The data presented in Table 2 illustrate that only a minor fraction of the pollen, 5-16% of the populations, was shed from the anthers during mannitol pretreat-ment. The shed pollen were therefore not purified and cultured. Almost all shed microspores in population III were of the large type, while in population IV and V this type was less abundant. The data also illustrate that the yields of microspores isolated by maceration correlate positively with the progressive development of the cells. In populations III, IV and V, on average, 55% of the isolated microspores was enlarged.

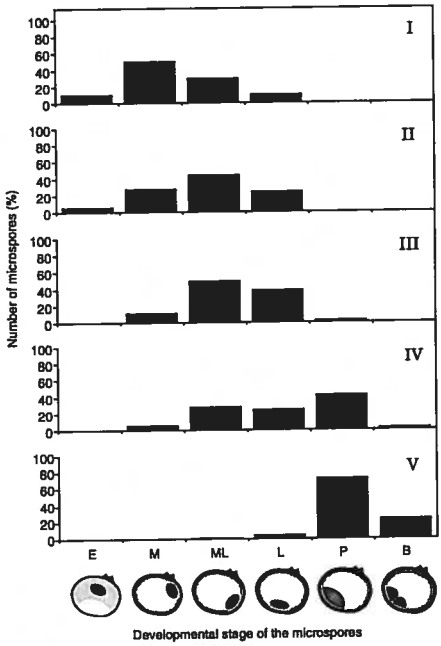


Figure 1: Populations of microspores (I-V) arranged according to their developmental stage. E = early uninucleate, M = mid uninucleate, L = late uni nucleate, P = first pollen mitosis, B = bicellular. S.D. of all populations ≤6.6%, except population V: P and B = 23%.

The embryogenic response of the five different populations of isolated microspores and anthers, as measured by the numbers of ELS at days 10 and 17, clearly differed among the that the microspores of populations III and IV, i.e.at the mid-late to late microspore stage, are embryogenic. Apparently, the presence of enlarged microspores is indicative of the plating efficiency when advanced in development than population II (Figure 1) which suggests that the period in which a microspore can be triggered to develop into an embryo occurs within a short period in the G1phase. Departing from the assumption that only one ELS is formed per large microspore, it can be deduced from the data in Table 2 that 15% of the enlarged microspores in population III developed

Table 2: Influence of the microspore developmental stage. (a) The yield of microspores at the end of pretreatment after natural dehiscence of the anthers without any mechanical pressure (yield shed in mannitol solution) and after gentle maceration with a teflon rod (yield after mechanical isoaltion). (b) The culture efficiency after 10 and 25 resp. 17 days in culture of the anther and the microspore system. % total into ELS = number of ELS present at day 17 divided by the total number of microspores isolated at day 0 (in percentage). % enlarged into ELS = number of ELS present at day 17 divided by the number of enlarged microspores isolated at day 0 (in percentage). (see Figure 1 for stage of populations I-V)

	day	I	II	III	IV	V
Yield shed in mannitol solution						
Total no. of microspores		630	<550	8000	9000	7500
% of enlarged microspores	0	0	0	94	68	25
Yield after mechanical isolation						
Total no. of microspores		17000	27000	51000	83000	140000
% of enlarged microspores	0	0	22	55	66	48
Anther culture						
Anther response (%)	10	0	2	90	97	100
Number of ELS	25	0	480	3200	2080	320
Microspore culture						
Number of ELS	10	0	496	2825	3226	224
	17	0	784	4330	5403	336
% total into ELS	17	0	2.9	8.5	6.5	0.2
% enlarged into ELS	17	0	13.2	15.5	9.9	0.5

into an ELS, while the frequency for population IV was about 10%. However, since the large microspores were isolated at a higher frequency in population IV, the total number of ELS formed in this population exceeded that found in population III. Regular examination of the cultures showed that for anther culture at day 25 the ELS had developed in a stage similar to that in microspore culture at day 17. The highest number of ELS was formed in population III indicating that the mid-late uninucleate stage is optimal for anther cultures. The number of ELS formed in this population was however only 59% of that found for the best performing microspore population IV (see Figure 2).

Influence of the duration of pretreatment, vitamin mixture supplementation in the culture medium, and oxygen supply on the efficiency of culture

The effects of the investigated parameters on the number of ELS produced during the first 17 days of microspore culture are presented in Table 3. Pretreatment had no influence on the percentage of enlarged microspores. At day 7 the 4-day pretreatment in combination with medium I was the best condition. Upon further culture the positive effect of oxygen supply became obvious. At day 17 the difference in number of ELS was pronounced, as the microcalli of the 3-day pretreatment culture and, even more so, the culture with the basal vitamin mixture

showed a strong delay in development. Especially medium II inhibited growth and proliferation of the microcalli. At the end of culture the difference in total number of ELS between the treatments with and without oxygen supply was no longer evident (Figure 3a).

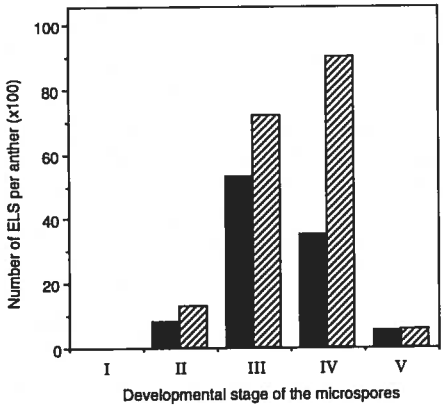


Figure 2: Influence of the microspore developmental stage on the efficiency of culture. Comparison between anther culture (black bars) and microspore culture (shaded bars) for absolute numbers of ELS obtained after resp. 25 and 17 days. (See Figure 1 for the developmental stage of populations I-V).

In anther culture there was no difference visible in response of anthers at day 10 for the parameters tested. In all cases about 95% of the anthers were responding. As for isolated microspore culture, a 4-day pretreatment was superior to 3 days of mannitol starvation. Extra oxygen supply at regular intervals during culture proved to have a positive effect, not only on the rate of growth as found for the microspore culture, but also on the number of ELS formed. In contrast to the results for microspore embryogenesis, the additional vitamin mixture present in medium I had no effect or even a negative effect.

In Figure 3 the number of green plants produced per anther are shown for the different parameters. The data presented have been corrected for the average number of $5x10^4$ microspores isolated per spike; this calculation method proved to allow for proper comparison of data. For both microspore and anther cultures there is a good correlation between number of ELS and number of green plants produced. In microspore culture, faster development of the ELS by means of additional oxygen supply combined with culture in medium I resulted in the highest number of green plants. Under this cultural regime a mean of 6.1 green and 1.2 albino plants were generated per anther, whereas the highest regeneration frequency in anther

Table 3:Development of ELS derived from isolated microspores, during the first 17 days in culture. A = 3 days pretreatment, medium I, with oxygen; B = 4 days pretreatment, medium I, with oxygen; C = 4 days pretreatment, medium I, no oxygen; D = 4 days pretreatment, medium II, with oxygen.

parameters	day	A	B	C	D
% of enlarged microspores	0	67	63	63	68
number of ELS	7	135	450	505	35
	10	620	1740	1195	1470
	17	565	1950	1355	1280

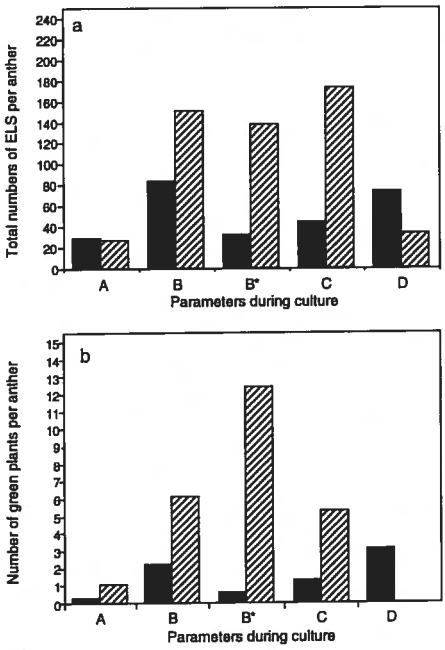


Figure 3: The influence of different parameters on the efficiency of anther cultures (black bars) and microspore cultures (shaded bars). (a) Total number of ELS produced; (b) Number of green plants produced per anther (See the legend to Table 3 for an explanation of A - D). * growth on medium III with 3% sucrose.

culture (a mean of 3.1 green and 0.4 albino plants) was found in medium II with extra oxygen. The green/albino ratio was around 8 for anther cultures, but only approx. 4 for microspore culture.

In a pilot study the effect of 3% rather than 1% sucrose in medium III was assessed for anther as well as microspore cultures. For anther cultures there was no effect or a negative effect, whereas 3% sucrose in medium III for a microspore culture resulted in a drastic increase in regeneration frequency with a mean of 12.4 green plants regenerating per anther. The green/albino ratio for the latter regime was 1.2, but about 11 in other experiments under the same conditions. A population of 60 representative plants comprised 88% doubled haploids, 10% of haploids, and one plant was tetraploid.

Discussion

In the present study the influence of several parameters on the regene-ration frequency of the model cv. Igri was evaluated. The effect of the developmental stage of microspores has been the subject of various investigations. Wheatley et al. (1986) specificied that barley microspores should preferably be in G1-phase. Slightly younger or older microspores had a drastically reduced embryogenic performance in *Brassica* (Kott et al., 1988; Telmer et al., 1992). Scrutinizing the stage and treatment on the basis of that knowledge, improved the response in culture considerably, even of cv. Igri.

Elongation of mannitol pretreatment to 4 days showed a 5-fold higher number of green plants produced per anther for both microspore and anther culture. Longer or shorter pretreatment, i.e. 2 or 5 days, had a negative effect on the cultures (results not shown). These results are in agreement with Roberts-Oehlschlager and Dunwell (1990) who reported improved microspore embryogenesis after 4-day mannitol pretreatment.

Our results revealed differences in time

between microspore and anther culture response. The anther wall apparently creates a closed environment until the internal pool of nutrients is depleted. Isolated pollen however, can respond immediately to the medium (Heberle-Bors, 1989) which results in earlier development in culture. Continuation of pollen development within the anther could also explain that in anther culture an earlier microspore stage (III) is optimal compared to microspore culture.

Furthermore, both anthers and isolated microspores responded differently when cultured in medium I and II. In barley anther culture extra vitamins (Kao et al., 1991) and amino acids (0.1 mM) (Zhu et al, 1990) have been supplied. Xu and Sunderland (1981) found an inhibitory effect of glutamine at 5.5 mM in anther culture which is consistent with our results (6.8 mM glutamine). Upon culture in medium with high concentrations of glutamine, this compound probably is redundant in anther culture, as the anther wall and connective tissue mediate nutrients, in particular glutamine, to the pollen grain (Maheshwari et al., 1980). In the case of isolated microspores, the additional medium components possibly replaced (in part) the anther wall and tapetal tissue.

The results obtained in this study indicate that the extra supply of oxygen had a positive effect on the development of both microspore and anther cultures. The benefit of proper aeration is documented by Lichter (1989) who used agitation of *Brassica* microspores, and by Luckett et al. (1991) and Olsen (1991) who applied membrane rafts.

In this study about 6.5 times more microspores were obtained when they were isolated mechanically instead of shed naturally. The dehiscence of anthers is dependent on free pollen present in the lumen of the anther loculi (Sunderland et al., 1984); mechanical isolation can therefore be necessary to obtain sufficient material. It is however crucial to apply very gentle forces for the isolation of the microspores, otherwise their viability will be very low. From the results presented it is evident that the isolated microspore system is easier to control during culture and provides at least 5 times more green plants than anther culture under the optimum conditions. For *Brassica napus* even a ten-fold higher efficiency of isolated microspores as compared to cultured anthers has been reported (Siebel and Pauls, 1989). The anther culture system, however, will be a good routine method for the production of doubled-haploid barley plants.

From the results obtained in this study it is clear that optimization of (1) the microspore stage, (2) the duration of pretreatment, (3) the medium composition and (4) the additional oxygen supply strongly improved the number of plants generated in microspore culture of barley cv. Igri.

Preliminary results show that the spring barley cultivars Dissa and Gimpel also develop optimally, when the optimized cultural regime is applied. Genotype specificity is reflected in the frequency of regeneration, which are below that observed for cv. Igri.

In summary, microspore culture does meet the requirement of high regene-

ration frequency enabling their application. Experiments aiming at stable transformation through particle bombardment of microspores are in progress.

Acknowledgement
We thank Dr. F.L. Olsen, Carlsberg Research Laboratory, for the pleasant introduction in this field and helpful discussions, Dr. P.B. Holm, Carlsberg Research Laboratory, for critically reviewing the manuscript, C. ten Bosch and S. An for pilot studies, J.M. Vink for growing the plants and P. Hock for the layout of figures and tables.
This work was supported by EUREKA grant EU270, and is ABIN publication 86.

References

Ahmad I., J.P. Day, M.V. MacDonald and D.S. Ingram. Haploid culture and UV mutagenesis in rapid-cycling *Brassica napus* for the generation of resistance to chlorsulfuron and *Alternaria brassicicola*. Ann. Bot. 67: 521525 (1991)

Bister-Miel F., J.L. Guignard, M. Bury and C. Agier. Glutamine as an active component of casein hydrolysate: Its balancing effect on plant cell cultured in phosphorus deficient medium. Plant Cell Rep. 4: 161-163 (1985)

Bolik M. and H.U. Koop. Identification of embryogenic microspores of barley (*Hordeum vulgare* L.) by individual selection and culture and their potential for transformation by microinjection. Protoplasma 162: 61-68 (1991)

Creissen G., C. Smith, R. Francis, H. Reynolds and P. Mullineaux. *Agrobacterium* - and microprojectile - mediated viral DNA delivery into barley microspore-derived cultures. Plant Cell Rep. 8: 680-683 (1990)

Heberle-Bors E. Isolated pollen culture in tobacco: plant reproductive development in a nutshell. Sex. Plant Reprod. 2: 1-10 (1989)

Jähne A., P.A. Lazzeri, M. Jäger-Gussen and H. Lörz. Plant regeneration from embryogenic cell suspensions derived from anther cultures of barley (*Hordeum vulgare* L.). Theor. Appl. Genet. 82: 74-80 (1991)

Kao K.N. Plant formation from barley anther cultures with Ficoll media. Z. Pflanzenphys. 103: 437-443 (1981)

Kao K.N., M. Saleem, S. Abrams, M. Pedras, D. Horn and C. Mallard. Culture conditions for induction of green plants from barley microspores by anther culture methods. Plant Cell Rep. 9: 595-601 (1991)

Kott L.S., L. Polsoni and W.D. Beversdorf. Cytological aspects of isolated microspore culture of *Brassica napus*. Can. J. Bot. 66: 1658-1664 (1988)

Kuhlmann U., B. Foroughi-Wehr, A. Graner and G. Wenzel. Improved culture system for microspores of barley to become a target for DNA uptake. Plant Breed. 107: 165-16 (1991)

Lichter R. Efficient yield of embryoids by culture of isolated microspores of different *Brassicaceae* species. Plant Breed. 103: 119-123 (1989)

Luckett D.J., S. Venkatanagappa, N.L. Darvey and R.A. Smithard. Anther culture of Australian wheat germplasm using modified C17 medium and membrane rafts. Aust. J. Plant Phys. 18: 357-367 (1991)

Maheshwari S.C., A.K. Tyagi and K. Malhotra. Induction of haploidy from pollen grains in angiosperms - the current status. Theor. Appl. Genet. 58: 193-206 (1980)

Morrison R.A. and D.A. Evans. Haploid plants from tissue culture: new plant varieties in a shortened time frame. Biotechnology 6: 684-690 (1988)

Olsen F.L. Induction of microspore embryogenesis in cultured anthers of *Hordeum vulgare*: the effects of ammonium nitrate, glutamine and asparagine as nitrogen sources. Carlsberg Res. Comm. 52: 393--404 (1987)

Olsen F.J. Isolation and cultivation of embryogenic microspores from barley (*Hordeum vulgare* L.). Hereditas 115: 255-266 (1991)

Pechan P.M. Succesful cocultivation of *Brassica napus* microspores and proembryos with *Agrobacterium*. Plant Cell Rep. 8: 387-390 (1989)

Roberts-Oehlschlager S.L. and J.M Dunwell. Barley anther culture: pretreatment on mannitol stimulates production of microspore-derived embryos. Plant Cell Tiss. Org. Cult. 20: 235-240 (1990)

Siebel J. and K.P. Pauls. A comparison of anther and microspore culture as a breeding tool in *Brassica napus*. Theor. Appl. Genet. 78: 473-479 (1989)

Sunderland N., B. Huang and G.J. Hills. Disposition of pollen *in situ* and its relevance to anther/pollen culture. J. Exp. Bot. 35: 521-530 (1984)

Swanson E.B., M.J. Herrgesell, M. Arnoldo, D.W. Sippell and R.S.C. Wong. Microspore mutagenesis and selection: Canola plants with field tolerance to the imidazolinones. Theor. Appl. Genet. 78: 525-530 (1989)

Telmer C.A., D.H. Simmonds and W. Newcomb. Determination of developmental stage to obtain high frequencies of embryogenic microspores in *Brassica napus*. Physiol. Plant. 84: 417-424 (1992)

Twell D., T.M. Klein, M.E. Fromm and S. McCormick. Transient expression of chimeric genes delivered into pollen by microprojectile bombardment. Plant Phys. 91: 1270-1274 (1989)

Wheatley W.G., A.A. Marsolais and K.J. Kasha. Microspore growth and anther staging in barley anther culture. Plant Cell Rep. 5: 47-49 (1986)

Xu Z.H. and N. Sunderland. Glutamine, inositol and conditioning factor in the production of barley pollen callus *in vitro*. Plant Sci. Lett. 23: 161-168 (1981)

Ye J.M., K.N. Kao, B.L. Harvey and B.G. Rossnagel Screening salt-tolerant barley genotypes via F1 anther culture in salt stress media. Theor. Appl. Genet. 74: 426-429 (1987)

Zhu M., X. Abing, Y. Miaobao, H. Chunnong, Y. Zhilong, W. Linji and Y. Jianjun. Effects of amino acids on callus differentiation in barley anther culture. Plant Cell Tiss. Org. Cult. 22: 201--204 (1990)

Ziauddin A., E. Simion and K.J. Kasha. Improved plant regeneration from shed microspore culture in barley (*Hordeum vulgare* L.) cv. Igri. Plant Cell Rep. 9: 69-72 (1990)

Chapter 3

Microspore culture of *Hordeum vulgare* L.: the influence of density and osmolality

S. Hoekstra, M.H. van Zijderveld, F. Heidekamp & F. van der Mark

Summary

Donor plants of *Hordeum vulgare* L. cv. Igri were grown in a conditioned environment to minimise fluctuations in the composition of the microspore population. After isolation different types of microspores were identified within each population, amongst others an embryogenic subpopulation. It was shown that the optimum plating density is achieved by adjusting the density to $2x10^4$ embryogenic microspores per ml, with a lower threshold at $5x10^3$ per ml. By increasing the osmolality of the pretreatment solution to 440 $mOs.kg^{-1}$ and that of the culture medium to 350 $mOs.kg^{-1}$, up to 15% of the population developed into embryo-like structures. When microspores of cv. Igri were cultured under the optimized conditions, the ratio of green/albino plants increased from 1:1 to 34:1, and 50 green plants per anther were formed.

Introduction

Microspore culture represents a potential tool for a range of biotechnological applications. Moreover, the microspore system is an attractive model to study the molecular mechanism that controls the switch from the gametophytic to the sporophytic pathway. However, the induction frequency and the efficiency of outgrowth into green plants remains low and unpredictable in the microspore system. In order to be able to enhance the efficiency of microspore culture, it is necessary to identify the type of microspore that is capable of undergoing embryogenesis and subsequent development into a green plant. The number of what are called embryogenic microspores, may vary within one variety due to the environmental conditions in which the donor plants are grown (Heberle-Bors 1989). Well-controlled growth conditions of barley donor plants and the use of an efficient isolation

procedure allowed us to perform reproducible experiments throughout the year (Hoekstra et al. 1992).

The effect of plating density of the microspores on the number of developing embryo-like structures (ELS) has been reported earlier. For rapeseed Huang et al. (1990) describe that a density of 3-4x104 microspores per ml is crucial for embryogenesis. For maize, a higher density viz. 6-8x10^4 microspores per ml, produces an optimum in culture efficiency (Gaillard et al. 1991). The effect of different types of microspores present in a population on the plating density and culture efficiency has not been investigated before.

Besides the density, the importance of the medium osmolality is known for anther culture (Kao 1981; Chu et al. 1990; Zhou et al. 1991). To date, only Pescitelli et al. (1990) applied different osmotical conditions for isolated microspore culture, reporting an optimum concentration of 7.9% sucrose for the development of embryo-like structures.

In this paper, for *Hordeum vulgare* L. cv. Igri a description is given of the different types of microspores which are present in a population directly after isolation, in order to recognize the type of microspore that has the capacity to develop into a plant. The influence of the density of this microspore-subpopulation on the culture efficiency is investigated. Furthermore, the effect of the osmolality of both the pretreatment and the culture medium on ELS and plant formation is studied.

Materials and Methods

The growth of the donor plants *Hordeum vulgare* L. cv. Igri, the selection of the material, the pretreatment and culture conditions were essentially as described previously (Olsen 1991; Hoekstra et al. 1992). In short, donor plants were grown at 12°C with a 16 h light regime (24,000 lux) and the anthers were harvested when at least 50% of the microspores were in the Mid Late to Late Uninucleate stage. Upon pretreatment of the anthers on mannitol solution for 4 days, microspores were isolated after pestle maceration (about 500 embryogenic microspores per anther) and plated as mass culture in medium I. Under optimal conditions, at least 30% of the microspore population was embryogenic (see results). Different types of microspores were recognized in a population by light microscopy using an Olympus inverted microscope IMT-2, on the basis of their diameter, colour interference of the exine, and appearance and colour of the cytoplasm. Their behaviour in culture was followed. Viability was assessed using the viable stain fluorescein diacetate (stock solution 5 mg/ml in acetone).

The influence of the density of embryogenic microspores on the ability to develop into ELS was investigated under different pretreatment conditions (viz. anthers for 2, 3 or 4 days on mannitol starvation (Hoekstra et al. 1992) or spikes incubated at 4°C for 28 days (Huang and Sunderland 1982)) in medium II (Hoekstra et al. 1992).

The effect of increased osmolality of the pretreatment solution (with mannitol) and of medium I (with maltose) on the formation of microspore-derived ELS has been tested.

The optimum density of the embryogenic microspores as well as the best osmotic conditions during pretreatment and culture have been applied to study their effect on the ability of the microspores to develop into ELS and on the number of plants subsequently formed. Plant formation was assessed on the first 1000 developing ELS after careful transfer with forceps to medium III (Hoekstra et al. 1992). The number of plants per anther was calculated as the number of plants on the first 1000 ELS, divided by the

number of anthers used for the microspores plated in 1 ml medium.
In the present study plants were defined as distinct structures possessing one or more leaves at least 5 mm in length; previous experiments have shown that 90% of these plants were able to develop *in vivo* into a fertile plant.
The ploidy level of representative plants was determined by flow cytometric analysis of the relative nuclear DNA content.

Results

Types of microspores

Different types of microspores could be recognized during the first stages of isolation and in culture. Using fluorescein diacetate as a viable stain, a varying percentage of each population was non-viable. This type was characterized by a small diameter (35-40 μm), no colour interference of the exine, and a collapsed cytoplasm (Figure 1a). The remaining microspores looked viable: enlarged diameter up to 65 μm, a red and/or blue interference of the exine and viable cytoplasm (Figure 1b and c). Only a fraction of these microspores, however, divided at day 3. The rest of the supposed viable microspores did not change in morphology or were plasmolysed. After thorough investigation it was evident that microspores which had a diameter of 40-50 μm and showed a blue interference of the exine (Figure 1b), plasmolysed upon culture (Figure 1d). They represented on average 15% of the isolated microspores. Visually recognizable microspores, for which later on many showed the capacity to divide, had a diameter of 50-60 μm, showed red interference of the exine and had a granular cytoplasm. This group of microspores was defined as the embryogenic subpopulation (Figure 1c), typically 60% of the microspores at the day of isolation. It should be noted that of this population about 25% (15% of total) divided at day 3 (Figure 1e).

Influence of embryogenic microspore density on the number of ELS

Data concerning plating density of embryogenic microspores are presented in Figure 2. These data are derived from 28 experiments under different pretreatment conditions, which resulted in fluctuations in the percentage of embryogenic microspores isolated. A minimum density of $5x10^3$ embryogenic microspores per ml was necessary to ensure further development of the microspores. At this threshold density, even in populations

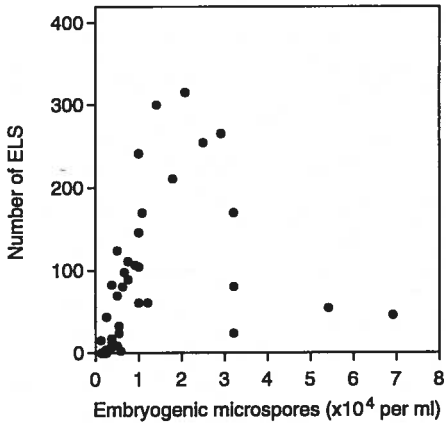


Figure 2: Correlation between the density of embryogenic microspores and the number of ELS formed per 10^4 microspores 10 days after isolation. Different pretreatments were applied in 28 experiments (see materials and methosds).

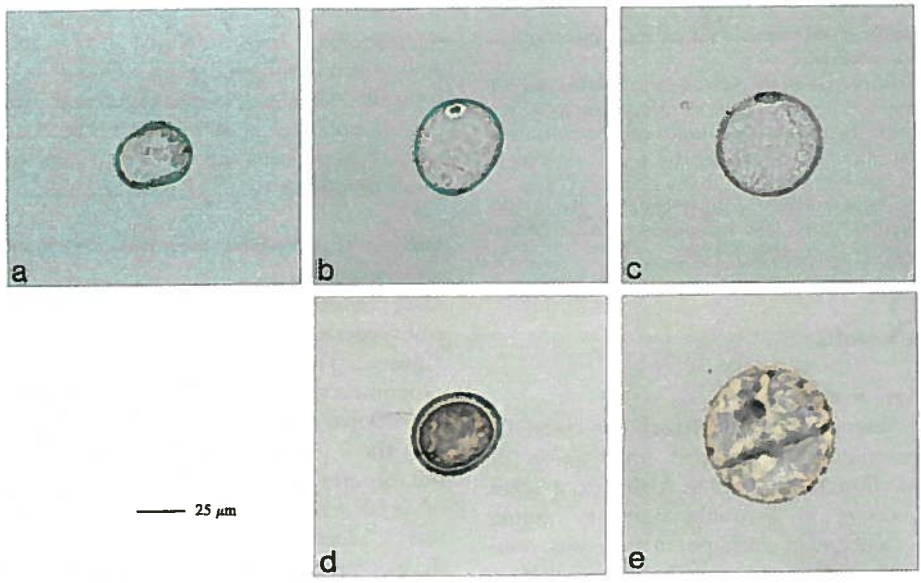


Figure 1: Differrent types of microspores present in a population. a. non-viable microspore b. viable microspore at day of isolation c. embryogenic microspore at day of isolation d. viable micropsore 3 days after isolation e. dividing microspore days after isolation.

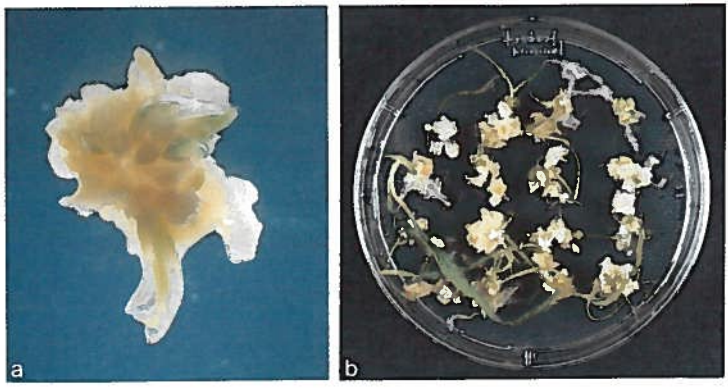
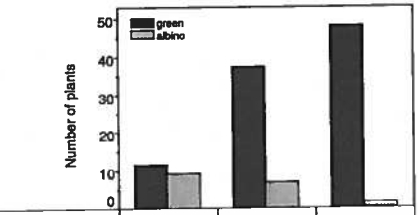


Figure 4: Microspore-derived regeneration. a. green structure b. Petri plate of 9 cm with 16 ELS after 30 days of incubation ($2x10^4$ embryogenic microspores, 440 $mOs.kg^{-1}$ mannitol starvation and 350 $mOs.kg^{-1}$ medium I).

Pretreatment in mOs. kg^{-1}	350	350	440	440
Culture in mOs. kg^{-1}	230	350	350	440

Figure 3: Influence of the osmotic conditions during pretreatment and in culture on the number of ELS formed 17 days after isolation. The density of embryogenic microspores was $9x10^3$. Data are based on results of 3 experiments.



Density of embryogenetic microspores per ml	9×10^3	2×10^4	2×10^4
Pretreatment in mOs. kg^{-1}	350	350	440
Culture in mOs. kg^{-1}	230	230	350

Figure 5: Effect of the density of embryogenic microspores and osmotic conditions on the number of green (black bars) and albino plants (dotted bars) produced per anther. Data are based on results of 4 experiments.

which consist of 95% non-viable microspores (after cold pretreatment), ELS were formed. The optimum plating density was around $2x10^4$ embryogenic microspores per ml, independent of the pretreatment applied. The number of ELS formed per density (i.e. the angle and height of the curve), is determined by the various microspore types present in the microspore population and the culture method applied.

Influence of the osmolality on the number of ELS

In Figure 3 the influence of the osmolality of the pretreatment solution and of the culture medium on the number of ELS formed is shown. The highest plating efficiency was observed when the medium had an osmolality of 350 $mOs.kg^{-1}$.
Further it was found that during pretreatment an increased osmotic pressure of 90 $mOs.kg^{-1}$ (compared to culture) greatly improved the response of the microspores.

Influence of density of embryogenic microspores and osmolality on the number of developing plants

The number of plants produced on the first 1000 developing ELS was determined, for different densities of embryogenic microspores and several osmotic conditions (Figures 4 and 5). There was less time required for ELS formation at $2x10^4$ embryogenic microspores per ml, thus in a shorter time more plants can be produced. At this density, moreover, there was a three-fold enhancement in the number of green plants formed. Under optimized conditions of both density and osmolality, a further increase in the number of green plants was observed, which resulted in an average of 50 green plants per anther.

A population of 41 plants consisted of

87% doubled haploids, 11% tetraploids and 1 plant was triploid. The change in ratio of green/albino plants from 1:1 to 34:1 under optimized conditions was remarkable.

Discussion

In general, embryogenic microspores are characterized by a large vacuole e.g. in tobacco (Kyo and Harada 1985), rapeseed (Deslauriers et al. 1991), maize (Coumans et al. 1989) and wheat (Datta and Wenzel 1987). Enlarged diameter accompanied by a red interference colour of the exine is characteristic for embryogenic microspores of rice (Cho and Zapata 1990). From previous experiments, it was evident that the distinction between viable and non-viable microspores on the basis of enlarged diameter was not reliable enough to obtain reproducible results, i.e. a fixed fraction of the viable population developing into plants. In barley, Olsen (1991) describes that microspores with a blue or red corona divide, and that only the blue type is able to break through the exine. The "Olsen blue type" is different from the blue type presented here, as the latter was already plasmolyzed after 3 days in culture. It should be emphasized that both diameter and colour of the exine are affected by the osmotic pressure applied (results not shown). Besides, the colour of the exine observed is a result of the specific refraction of light in a given microscope. The optical conditions and the osmotic environment described by Olsen (1992) are different from our experimental design.
Bolik and Koop (1991), furthermore, distinguished 2 types of cytoplasm in barley microspores: embryogenic type A being granular in appearance and non-embryogenic type B containing less cytoplasm, which in addition is smooth and star-like arranged. These data are based on a limited number of 5 regenerants. In our experiments star-like microspores were observed under suboptimal conditions e.g. low density of embryogenic microspores. We observed that the presence of small vacuole(s) or "dark-dotted" cytoplasm also were indicative under these circumstances (results not shown). Only 25% of the embryogenic population divided, which is in agreement with the results which Gaillard et al. (1992) show for maize. For a further identification of embryogenic microspores more investigations are required.

The appropriate culture density is dependent on the quality of the material i.e. the percentage of embryogenic microspores present, which is influenced by the pretreatment and the method of microspore isolation. Therefore, differences in optimum densities may vary between species and even laboratories. Our results showed a relatively low optimum density which can be explained by the use of good donor quality. Gaillard et al. (1991) reports 6-8x10^4 microspores per ml for maize, whereas for rapeseed Huang et al. (1990) finds 3-4x10^4 microspores per ml to be optimal. Cho and Zapata (1990) describe for rice that non-embryogenic microspores die earlier and that toxic substances released from these dead microspores probably are deleterious to the development of embryogenic microspores. Osmotic pressure of the media has been shown to be an important parameter in

anther culture (Chu et al. 1990; Kao 1981). The optimal osmotic environment from this barley study is comparable with the results described for maize (Pescitelli et al. 1990). At high osmolality, an increase in the number of green plants together with a decrease in the number of albino plants, is in agreement with the results obtained for anther culture of wheat (Zhou et al. 1991). It can be assumed, that this effect is due to the osmotic conditions as there probably was an abundance of carbon source. It can be concluded that the number of albino plants produced is not merely genotype dependent, but is also largely determined by yet unidentified physio-logical factors in the culture conditions used. Preliminary results showed a comparable positive effect at the level of ELS formation for cvs. Gimpel and Dissa when the optimizations described in this paper were applied.

In the literature reported so far, barley microspore cultures of cv. Igri produce up to 12 green plants per anther (Olsen 1991; Kuhlmann et al. 1991; Hoekstra et al. 1992). Our results show that for cv. Igri the optimizations resulted in a four-fold increase in the number of green plants formed; it should be emphasized that this calculation is an underestimation of plant formation based on only the first 1000 ELS. The formation of 50 green plants per anther on a routine basis affirms that applications for microspore culture are more feasible in the near future.

Acknowledgements

We thank Prof. R.A. Schilperoort for critically reviewing the manuscript, J.M. Vink and J.D.L. Louwerse for growing the plants and P. Hock for the layout of the figures. This work was supported by EUREKA grant EU270 and is ABIN publication 120.

References

Bolik M. and H.U. Koop. Identification of embryogenic microspores of barley (*Hordeum vulgare* L.) by individual selection and culture and their potential for transformation by microinjection. Protoplasma 162: 61-68 (1991)

Cho M.S. and F.J. Zapata. Plant regeneration from isolated microspores of Indica rice. Plant Cell Physiol. 31: 881-885 (1990)

Chu C.C., Hill RD and A.L. Brule-Babel. High frequency of pollen embryoid formation and plant regeneration in *Triticum aestivum* L. on monosaccharide containing media. Plant Sci. 66: 255-262 (1990)

Coumans M.P., S. Sohota and E.B. Swanson. Plant development from isolated microspores of *Zea mays* L. Plant Cell Rep. 7:.618-621 (1989)

Datta S.K. and G. Wenzel. Isolated microspore derived plant formation via embryogenesis in *Triticum aestivum* L. Plant Sci. 48: 49-54 (1987)

Deslauriers C., A.D. Powell, K. Fuchs and K.P. Pauls. Flow cytometric characterization and sorting of cultured *Brassica napus* microspores. Bioch. Biophys. Acta 1091:.165-172 (1991)

Gaillard A., E. Mattys-Rochon and C. Dumas. Selection of microspore derived embryogenic structures in maize related to transformation potential by microinjection. Bot. Acta 105: 313-318 (1992)

Gaillard A., P. Vergne and M. Beckert. Optimization of maize micropsore isolation and culture conditions for reliable plant regeneration. Plant Cell Rep. 10: 55-58 (1991)

Heberle-Bors E. Isolated pollen culture in tobacco: plant reproductive development in a nutshell. Sex. Plant Reprod. 2: 1-10 (1989)

Hoekstra S, M.H. VanZijderveld, J.D. Louwerse, F. Heidekamp and F VanderMark. Anther and microspore culture of *Hordeum vulgare* L. cv. Igri. Plant Sci. 86: 89-96 (1992)

Huang B., S. Bird, R. Kemble, D. Simmonds, W. Keller and B. Miki. Effects of culture density conditioned medium and feeder cultures on microspore embryogenesis in *Brassica napus* L. cv. Topas. Plant Cell Rep. 8: 594-597 (1990)

Huang B. and N. Sunderland. Temperature-stress in barley anther culture. Ann. Bot. 49: 77-88 (1982)

Kao K.N. Plant formation from barley anther cultures with Ficoll media. Z. Pflanzenphys. 103: 437-443 (1981)

Kuhlmann U., B. Foroughi-Wehr, A. Graner and G. Wenzel. Improved culture system for microspores of barley to become a target for DNA uptake. Plant Breed. 107: 165-168 (1991)

Kyo M. And H. Harada. Studies on conditions for cell division and embryogenesis in isolated pollen culture of *Nicotiana rustica*. Plant Physiol. 79: 90-94 (1985)

Olsen F.L. Isolation and cultivation of embryogenic microspores from barley (*Hordeum vulgare* L.). Hereditas 115: 255-266 (1991)

Pescitelli S.M., C.D. Johnson and J.P. Petolino. Isolated microspore culture of maize: effects of isolation technique reduced temperature and sucrose level. Plant Cell Rep. 8: 628-631 (1990)

Zhou H., Y. Zheng and C.F. Konzak. Osmotic potential of media affecting green plant percentage in wheat anther culture. Plant Cell Rep. 10: 63-66 (1991)

Chapter 4

The interaction of 2,4-D application and mannitol pretreatment in anther and microspore culture of *Hordeum vulgare* L. cv. Igri.

S. Hoekstra, S. van Bergen, I.R. van Brouwershaven, R.A. Schilperoort & F. Heidekamp

Summary

The influence of 2,4-dichlorophenoxyacetic acid (2,4-D) on embryo-like structures (ELS) and plant development from barley microspores was determined. Microspores cultured on filters enabled simple modification of growth regulator concentrations. Regeneration frequencies obtained with 2,4-D as growth regulator were similar to the results achieved with the generally applied cytokinin 6-benzylaminopurine. If 2,4-D was applied after a regular mannitol pretreatment, maximal plant regeneration was achieved if 10^{-6} mol/L 2,4-D was present continuously or for 7 days. Alternatively, maximal plant formation was induced, if 10^{-5} or 10^{-4} mol/L 2,4-D was present for 1 hour or if present resp. 3 days or 1 day. Induction of plant regeneration by a 1 h treatment with 10^{-4} mol /L 2,4-D is a more general observed phenomenon for single cells or small cell clusters of both dicotyledonous and monocotyledonous species.

Without mannitol pretreatment, in anther culture it was possible to induce plant production after 2,4-D treatment only in anther cultures. Without mannitol pretreatment no embryogenic type of microspores can be recognized at the moment of microspore isolation, and plating efficiency never reached 1%. In anther culture without mannitol pretreatment, a higher molarity and/or longer presence of 2,4-D was required and resulted only in about 1 green plant per anther. After application of a mannitol pretreatment, plant production increased at least 10 times.

To our knowledge this is the first report on microspore-derived barley plants via androgenesis without any pretreatment. The combination of 2,4-D and anther pretreatment with mannitol as trigger for microspore differentiation is discussed.

Introduction

Synthetic auxins such as 2,4-dichlorophe noxyacetic acid (2,4-D), are essential constituents of culture media that are used for production of embryogenic cultures of both dicotyledonous and monocotyledonous plants such as the *Gramineae* rice (Abdullah et al., 1986), wheat (Vasil et al., 1990), maize (Morocz et al., 1990) and barley (Lührs and Lörz, 1987). 2,4-D has been used to induce dedifferentiation or, if applied at a higher concentration, for induction of embryogenesis. Dudits et al. (1992) observed that a 10 times higher concentration than used for the initiation of dedifferentiation, induces embryogenesis in dedifferentiated microcallus suspensions of carrot and alfalfa. Comparable results are obtained for cucumber (Tabei et al., 1991) and the monocotyledonous species *Pennisetum* in florescense-derived callus and *Asparagus* in stem-derived callus (Talwar and Rashid, 1990; Levi and Sink, 1991).

From carrot cell cultures, it is known that there are at least two phases in the differentiation from single cells into embryos. The development of the first phase requires exogenous auxin, whereas the second phase is inhibited by the same growth regulator (Komamine et al., 1990). The response to exogenous auxin in somatic embryo induction appears to depend on the nature of the explant used in the experiment (Zimmerman, 1993).

Generally in microspore culture, if (synthetic) auxins are used they are present during the entire induction period. However in cereal microspore culture, auxins have rarely been used sofar as growth regulators (Tiwari and Rahimbaev, 1991; Hassawi et al., 1990; Ball et al., 1993). Generally 6-benzylaminopurine (BAP) is used (a.o. Olsen, 1987; Hoekstra et al., 1993). Datta and Potrykus (1989) stated that low auxin was important for the development of embryos, because it prevents the development of unorganized cultures. A significant negative effect of 2,4-D, if applied for a long period, on plant regeneration and green plant percentage is shown in wheat anther culture (Ball et al., 1993).

In the present paper we studied, whether 2,4-D can induce plant development in barley microspores and under which conditions maximal plant formation was achieved. Moreover, the importance of anther tissue in microspore-derived plant formation was investigated upon 2,4-D treatments with and without mannitol pretreatment.

Materials and Methods

Growth of the donor plants *Hordeum vulgare* L. cv. Igri, selection of material, mannitol pretreatment of anthers and culture conditions were as described previously (Hoekstra et al. 1993) with the following modifications. Anthers (without Ficoll) or microspores were cultured in medium I at 350 $mOs.kg^{-1}$ (Hoekstra et al., 1992) with 2.5% coconut water. When the majority of ELS were at least 1 mm in diameter, ELS were plated on medium III (Hoekstra et al., 1992) with 0.4 mol /L Kinetin (Sigma).

Microspore culture

After isolation, $5x10^3$ or $7x10^3$ embryogenic microspores were plated on Transwell

polycarbonate filters with pore size 12 μm (Costar). At 10 d.a.i., the filters were cut out of their frame, to enable culture of embryo-like structures (ELS) in 1 mL liquid medium. The ploidy level of representative plants was determined by flow cytometric analysis of the relative nuclear DNA content.

Anther culture

Thirty anthers (equivalent to 1/2 spike) of several spikes were randomnized used per parameter. There was no need for the use of filters.

The effect of the concentration and the time that 2,4-D was present in the medium, was studied in relation to both the ability of the microspores to develop into ELS and the number of plants subsequently formed (result section A). Taking the best variables found for plant regeneration, the capacity to develop plants of $5x10^3$ embryogenic microspores was assessed on the first 500 developing ELS after careful transfer with forceps to medium III (Hoekstra et al. 1992) containing 0.4 mol /L Kinetin. The regene-ration efficiency of microspore culture with the continuous presence of $4x10^{-6}$ mol/L 2,4-D in the above mentioned modified medium I (without the use of filters), and application of $4x10^{-6}$ Mol /L 6-BAP according to Hoekstra et al. (1993), was compared (result section B). The effect of 2,4-D with omittance of mannitol pretreatment was tested at 10^{-6} to 10^{-4} mol/L 2,4-D and different time periods of its presence in both anther and microspore culture (result section C).

No standard deviation is given in the figures, since due to differences in microspore developmental stage and plating density, the absolute numbers varied. However the patterns of the figures were reproducible, throughout 4 independent experiments.

Results

A. Effect of 2,4-D conditions after mannitol pretreatment on plant production

Microspore culture

In order to remove 2,4-D from the culture medium, microspores were cultured on a filter. The optimal density for culture efficiency of microspores on filter was assessed (Table 1). At a higher microspore density, a higher percentage of microspores developed into ELS. The regeneration into plants however, was maximal when $3x10^3$ microspores were present on the filter. The latter density was at the threshold level enabling development of ELS (indicated by the high standard deviations). In subsequent experiments the microspores were cultured at a density of $5x10^3$ and $7x10^3$ per filter.

The influence of continuous presence of 2,4-D on the development of microspores into plants was tested for the concentration range as indicated in Figure 1. The size of the ELS was reduced at 10^{-5} mol/L or higher. At these concentrations

Table 1: The influence of various microspore densities, grown on a filter, on the culture efficiency. Culture conditions were 10^{-6} mol/L 2,4-D continuously present, in microspore culture with pretreatment.

	microspore density on filter			
	$3x10^3$	$5x10^3$	$7x10^3$	10^4
(ELS/density) x100	0.67±0.94	3.74±1.33	4.96±1.85	5.74±1.14
(plants/ELS) x100	7.50±14.9	5.50±1.06	0.15±0.54	0.09±0.61

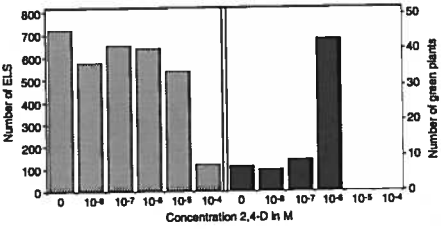


Figure 1: 2,4-D concentration range, continuously present, in microspore culture with pretreatment, and its effect on the number of ELS produced on day 14 and the number of green plants developed.

an accumulation of proembryos was observed; however, no development into green plants took place. The optimal concentration for green plant development was 10^{-6} mol/L. At lower concentrations of 2,4-D, the cell structure of the ELS loosened and the ELS developed into friable callus.

For each of 3 different molarities of 2,4-D the effect of the period of time that the auxin was present on the development of microspores into plants was investigated (Figure 2). In Figure 2a the positive effect of a continuous presence of 10^{-6} mol/L 2,4-D on plant regeneration was confirmed. This concentration had to be present at least for 7 days in order to get a strong induction of differentiation. A shorter application time resulted in structures with a friable appearance. In Figure 2b and 2c the concentration of 2,4-D was increased. No significant effect on the number of developing ELS was observed for 10^{-5} mol/L (Figure 2b), whereas reduced number and size of the ELS was visible if a concentration of 10^{-4} mol/L was tested for a prolonged period of time (Figure 2c). A high number of

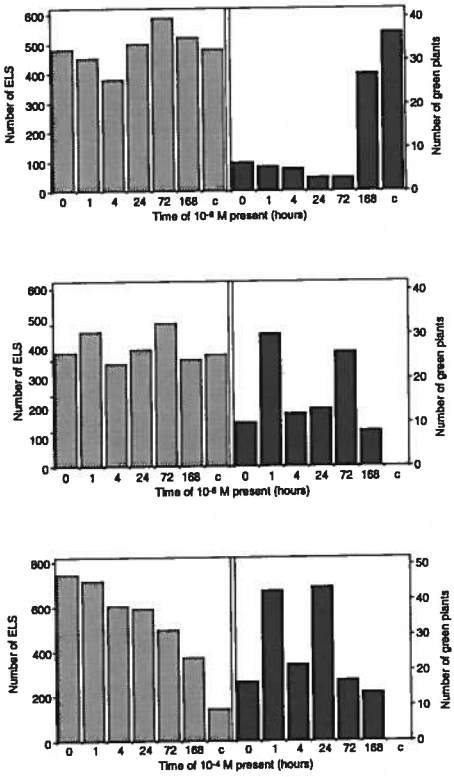


Figure 2: A time course of a. 10^{-6} mol/L, b. 10^{-5} mol/L and c. 10^{-4} mol/L 2,4-D after mannitol pretreatment in microspore culture and its effect on the number of ELS produced on day 14 and on the number of green plants developed.
c = continuously present

plants was produced if 10^{-5} mol/L was present either 1 h or 3 days and if 10^{-4} mol/L was present for 1 h or 1 day.

Anther culture

Anther culture also showed maximal plant formation after treatment with 10^{-5} or 10^{-4} mol/L 2,4-D for 1 h, or 10^{-6} mol/L

Table 2: Green plant production in microspore culture with pretreatment, for 5 different 2,4-D regimes. c = continuously present

	Concentration and time present of 2,4-D				
	10^{-6}M	10^{-5}M		10^{-4}M	
	c.	1h	3d	1h	1d
n plants per anther	20.3	12.9	14.9	14.7	15.8

continuously. However, the time of 2,4-D presence required for plant formation, is less restricted than in microspore culture (data not shown).

B. Plant production with 2,4-D under optimal conditions, after mannitol pretreatment

In A. several conditions were clearly suboptimal for regeneration. For the best 5 parameters as obtained in A., the number of regenerated plants upon 2,4-D treatment was determined in microspore culture under optimal regeneration conditions. No significant difference in plant production was found between these various culture conditions tested (see Table 2). Further, of 59 regenerants about 23% was haploid, 71% had the diploid level and their fertility (85%) was similar to the donor material. No changes in gross morphology of plants were visible, and no event of somaclonal variation was found by barley breeders in field-grown seed progeny from pretreated and subsequently 2,4-D treated microspores.

A comparison of microspore culture in the presence of $4x10^{-6}$ mol/L 2,4-D with the regularly applied $4x10^{-6}$ mol/L BAP resulted in similar frequencies of both green and white plants; depending on the developmental stage and culture conditions used, between 25 and 50 green plants per anther were obtained.

C. Effect of 2,4-D conditions without mannitol pretreatment on plant production

Microspore culture

Without mannitol pretreatment, no embryogenic type of microspores (Hoekstra et al., 1993) can be recognised at the moment of isolation. Even when the plating density was adjusted to 2.5-$3x10^4$ microspores per filter, the plating efficiency did not reach 1%. This result was also not improved either, when the optimal parameters for anther culture without pretreatment were applied in microspore culture.

Anther culture

For each of 3 different molarities of 2,4-D, the effect of the period of time that the auxin was present on the development of microspores into plants was investigated with omittance of the mannitol pretreatment (Figure 3). Determination of average ELS production was unreliable 14 days after anther culture. Therefore, the numbers of ELS were counted after approximately 25 days, and were thereafter plated on plant regeneration medium. Growth of microspores was

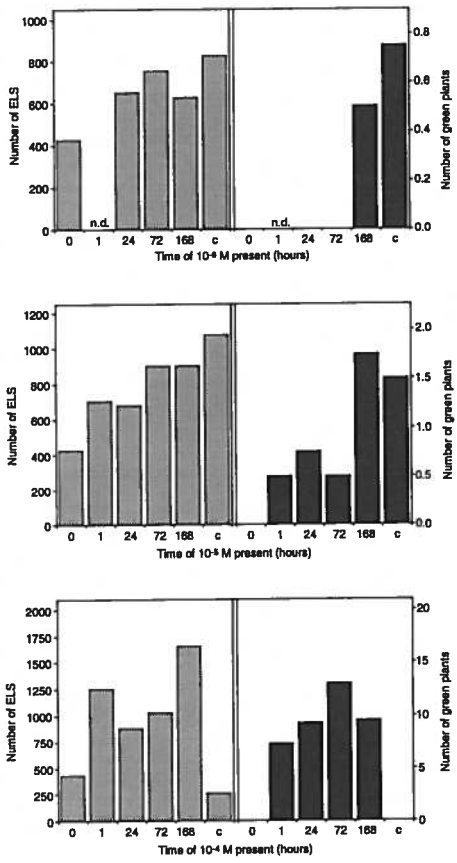


Figure 3: A time course of a. 10^{-6} mol/L, b. 10^{-5} mol/L and c. 10^{-4} mol/L 2,4-D without mannitol pretreatment in anther culture and its effect on the number of ELS before plating and on the number of green plants developed. c = continuously present.

reduced in all cases compared with their development after mannitol pretreatment. If no 2,4-D was applied or if 10^{-4} mol/L 2,4-D was present continuously, growth was even more negatively affected. Plant regeneration at 10^{-6} mol/L 2,4-D was very low and observed only if the growth factor was present for 7 days or longer (Figure 3a). Regeneration was visible when 10^{-5} mol/L 2,4-D was present for all periods of time tested, but a slightly better frequency was obtained when 2,4-D was applied for at least 7 days (Figure 3b). Using 10^{-4} mol/L 2,4-D (Figure 3c), maximal regeneration efficiencies were found when the growth regulator was present for 3 days. However, when 10^{-4} mol/L 2,4-D was continuously present in the culture medium, no regeneration was observed.

Discussion

In this report we showed that 2,4-D can be applied as successfully as 6-BAP for production of plants in anther and microspore culture. We observed a relationship between the concentration and the period of time that 2,4-D should be present in order to induce maximal regeneration. At low concentrations, 10^{-6} mol/L, optimal results are obtained if the growth factor is present continuously. At high concentrations e.g. 10^{-4} mol/L, the period that it is present, however, should be reduced drastically. These data are in agreement with the prediction of Ball et al. (1993), who argued that a putative positive effect will be found for a shortened application time at high 2,4-D concentrations in wheat anther culture. Furthermore, the necessity for a short 2,4-D application at higher concentrations might explain the negative effect of 2,4-D in cereal anther and microspore

culture, reported by Kasha et al. (1990), Tiwari and Rahimbaev (1991) and Hassawi et al. (1990). An alternative approach to prevent inhibition of embryo maturation by prolonged auxin presence, is the use of IAA, the easily degradable natural auxin (Datta and Potrykus, 1989).

The method of 2,4-D application for plant production in barley anther and microspore culture, is comparable to its use for somatic embryogenesis. At low concentrations, or if 2,4-D was not long enough present, unorganised tissue was generated. When app. 10^{-5} mol/L 2,4-D present for several days, embryogenesis is induced in various seed, seedling and plant tissues for carrot, cucumber, *Pennisetum* and *Asparagus* (resp. Dudits et al., 1991; Tabei et al., 1991; Talwar and Rashid, 1990; Levi and Sink, 1991). The 1 h pulse treatment with 10^{-4} mol/L 2,4-D in dicotyledonous microcallus of alfalfa resulting in morphogenesis (Dudits et al., 1991), gave, in our hands similar results in the monocotyledonous barley microspores that were pretreated on mannitol. When the synthetic auxin 2,4-D was applied at high concentration during a prolonged period, hampered growth of ELS was observed, together with complete inhibition of embryo maturation and germination (this could be due to high endogenous 2,4-D levels). Dudits et al. (1991) states that embryogenesis occurs in the presence of 2,4-D, at concentrations that already inhibit the growth of callus tissues, and this hypothesis is confirmed by our results.

A comparison between anther and microspore culture has been performed earlier in barley, and some differences are described (Hoekstra et al., 1992). Upon 2,4-D application in anther and microspore culture, a high similarity was observed. Furthermore in anther culture, the presence of 2,4-D in mannitol was investigated. No significant difference in efficiency of plant production was found, whether 2,4-D was applied during, after, or both during and after pretreatment (data not shown).

Comparison of the pattern of regeneration with and without mannitol pretreatment in anther culture, reveals high similarity. There are two remarkable differences however. The number of plants produced was strongly reduced without pretreatment. In addition the pattern in the figures of 2,4-D concentration and time period of its presence required for regeneration was shifted. Without pretreatment, microspores only in the presence of anther tissue, can to a limited extent produce plants upon application of a higher and longer presence of 2,4-D. Non-pretreated isolated microspores showed an extremely declined level of plating efficiency upon 2,4-D treatment, not enabling plant formation. After application of a certain shock treatment (e.g. sugar starvation, cold, high 2,4-D concentration), the anther tissue apparently acquires the capacity to mediate a switch from the gametophytic to the sporophytic pathway. We suppose that in anther culture with and without, and in microspore culture only with anther pretreatment, another inductive trigger for differen-

tiation is active besides exogenous applied 2,4-D. To our knowledge, all papers on microspore-derived (cereal) plant production describe a certain pretreatment. The pretreatment of barley microspores can be a cold period of spikes (Huang and Sunderland, 1982), or mannitol starvation of anthers (Roberts-Oehlschlager et al., 1990). During both treatments the microspores are surrounded by the anther tissue. We hypothesize that in anther culture and in culture of pretreated microspores, the trigger for induction of differentiation is mediated by more than one signal. The highest efficiency of plant production is achieved by application of a signal strength that is optimal for a specific cell competence. Fundamental research in this field will contribute to unraveling induction of plant development.

Acknowledgements
The authors wish to thank dr. F. VanderMark for discussions, M.H. VanZijderveld for the performance of pilot studies, J.M. Vink for growing the plants and P. Hock for the layout of the figures.

References

Abdullah R., E.C. Cocking and J.A. Thompson. Efficient plant regeneration from rice protoplasts through somatic embryogenesis. Biotechnol. 4: 1087-1090 (1986)

Ball S.T., H.P. Zhou, and C.F. Konzak. Influence of 2,4-D, IAA, and duration of callus induction in anther cultures of spring wheat. Plant Sci. 90: 195-200 (1993)

Datta S.K. and I. Potrykus. Artificial seeds in barley: encapsulation of microspore-derived embryos. Theor. Appl. Genet. 77: 820-824 (1989)

Dudits D., L. Bogre and J. Gyorgyey. Molecular and cellular approaches to the analysis of plant embryo development from somatic cells *in vitro*. J. Cell Sci. 99: 475-484 (1991)

Hassawi D.S., J. Qi and G.H. Liang. Effects of growth regulator and genotype on production of wheat and triticale polyhaploids from anther culture. Plant Breed. 104: 40-45 (1990)

Hoekstra S., M.H. VanZijderveld, J.D. Louwerse, F. Heidekamp and F. VanderMark. Anther and microspore culture of *Hordeum vulgare* L. cv. Igri. Plant Sci. 86: 89-96 (1992)

Hoekstra S., M.H. VanZijderveld, F. Heidekamp and F. VanderMark. Microspore culture of *Hordeum vulgare* L.: the influence of density and osmolality. Plant Cell Rep. 12: 661-665 (1993)

Huang B. and N. Sunderland. Temperature-stress in barley anther culture. Ann. Bot. 49: 77-88 (1982)

Kasha K.J., A. Ziauddin, E. Simion, A.A. Marsolais and C. Yu Rong. Barley and wheat microspore culture. International Association of Plant Tissue Culture 1990: hand-out.

Komamine A., M. Matsumoto, M. Tsukahara, A. Fujiwara, R. Kawahara, M. Ito, J. Smith, K. Nomura and T. Fujimura. Mechanisms of somatic embryogenesis in cell cultures - physiology, biochemistry and molecular biology pp. 307. In: Progress in plant cellular and molecular biology, eds. Nijkamp, van der Plas and van Aartrijk, Kluwer Academic Publishers (1990)

Levi A. and K.C. Sink. Somatic embryogenesis in asparagus: the role of explants and growth regulators. Plant Cell Rep. 10: 71-75 (1991)

Lührs R. and H. Lörz. Plant regeneration *in vitro* from embryogenic cultures of spring- and winter-type barley (*Hordeum vulgare* L.) varieties. Theor. Appl. Genet. 75: 16-25 (1987)

Morocz S., G. Donn, J. Nemeth and D. Dudits. An improved system to obtain fertile regenerants via maize protoplasts isolated from a highly embryogenic suspension culture. Theor. Appl. Genet. 80: 721-726 (1990)

Olsen F.L. Induction of microspore embryogenesis in cultured anthers of *Hordeum vulgare*: the effects of ammonium nitrate, glutamine and asparagine as nitrogen sources. Carlsberg Research Comm. 52: 393-404 (1987)

Roberts-Oehlschlager S.L. and J.M Dunwell. Barley anther culture: pretreatment on mannitol

stimulates production of microspore-derived embryos. Plant Cell Tiss. Org. Cult. 20: 235-240 (1990)

Tabei Y., T. Kanno and T. Nishio. Regulation of organogenesis and somatic embrogenesis by auxin in melon, *Cucumis melo* L. Plant Cell Rep. 10: 225-229 (1991)

Talwar. M. and A. Rashid. Factors affecting formation of somatic embryos and embryogenic callus from unemerged inflorescences of a graminaceous crop Pennisetum. Ann. Bot. 66: 17-21 (1990)

Tiwari S. and I. Rahimbaev. Effect of 2,4-dichloro-phenoxyacetic acid on barley anther culture in liquid and agar media. Indian J. Genet. 51: 112-117 (1991)

Vasil V., F. Redway and I.K. Vasil. Regeneration of plants from embryogenic suspension culture protoplasts of wheat (*Triticum aestivum* L.). Biotechnol. 8: 429-434 (1990)

Zimmerman L. Somatic embryogenesis: A model for early development in higher plants. Plant Cell 5: 1411-1423 (1993)

Chapter 5

Androgenesis in *Hordeum vulgare* L.: The role of ABA during anther pretreatment, on a solution containing mannitol and calcium

S. Hoekstra, I.R. van Brouwershaven, S. van Bergen, R.A. Schilperoort & M. Wang

Summary

Pretreatment induced androgenesis of *Hordeum vulgare* L. cv. Igri was investigated in order to find factors which control optimal plant production. The influence of what is called pre-medium, and of the concentration of both mannitol and calcium was studied. Increasing concentrations of each of the compounds, also resulting in a higher osmolality, improved plant production. Optimal plant production was obtained upon anther pretreatment on 30 mM $CaCl_2$ adjusted with mannitol to 440 $mOs.kg^{-1}$. The rather high calcium concentration and the observation that calcium could be replaced by potassium ions, suggested a role of these ions as osmotic agents. Since ABA is involved in several developmental processes and is known to be induced by osmotic stress we studied ABA_i and ABA_c levels during pretreatment. Optimal plant production was observed at a lower ABA_i concentration than the initial value. This suggested a negative correlation between androgenetic potential and ABA level, which became even more evident when the ABA_c values were considered. Our overall data indicated that both a defined ratio ABA_c/ABA_i level was required throughout pretreatment, but in particular in a period around 24 hours, for optimal microspore-derived plant production. The mechanism of pretreatment for induction of androgenesis is discussed.

Introduction

Integration of microspore-derived plants in plant breeding is hampered by large genotypic differences in anther response. Of many barley genotypes, doubled haploids can be produced via androgenesis if three requirements are met. First, the developmental stage of the microspores should be at the verge of mitosis (Hoekstra et al., 1992). Second, the right hormone treatment is necessary for induction of plant production. For

barley this is achieved either by 6-benzyl aminopurine (BAP) in culture medium (Olsen, 1987; Hoekstra et al., 1993) or by a specific 2,4-dichlorophenoxyacetic acid (2,4-D) application (Hoekstra et al., 1996). And third, the right pretreatment of the anthers is needed in order to switch the gametophytic pathway into a sporophytic development of the microspores. For barley, a pretreatment can be a cold treatment of spikes (Huang and Sunderland, 1982) or anthers can be pretreated on a mannitol solution (Roberts-Oehlschlager and Dunwell, 1990). The latter pretreatment enhances doubled-haploid production in barley; the microspore viability is increased and a higher efficiency of plant production is achieved (Hoekstra et al., 1994). Optimization of culture conditions, in particular taking into account the microspore developmental stage and the use of growth regulator, reduced the genotype dependency of androgenesis (unpublished data, Hoekstra et al.). Knowledge about biological processes involved in effects of pretreatment, might result in the development of a genotype independent protocol for androgenesis.

Little is known about the processes that are affected by the pretreatment. Heberle-Bors (1989) suggests that during pretreatment, starvation due to the absence of useful saccharides, is the basis for the occurence of certain degradation processes in pollen grains, resulting in the induction of pollen embryogenesis. Especially in tobacco the starvation effect is pronounced, if pretreatment is performed in nitrogen-free medium with mannitol. Pretreatment of anthers from barley, resulting in plant production, can be performed using similar conditions as for pollen preculture of tobacco, i.e. on mannitol dissolved in what is called a pre-medium. Mannitol is thought to create osmotic stress. The presence of the sugars mannitol during pretreatment and maltose during culture, are important to have proper osmotic pressure for microspore-derived barley plant production (Hoekstra et al., 1993). From literature, e.g. Davies and Jones (1991), it is known that, in response to certain stress treatments like osmotic pressure and cold, ABA is produced. ABA-induced gene expression by mannitol as osmotic agent is observed in e.g. cell suspension cultures (Leonardi et al., 1995). In addition, relatively high ABA_i levels are correlated with high embryogenic potential of *Pennisetum* and carrot cells (Rajasekaran et al., 1987a, b; Kiyosue et al., 1992). In the case of tobacco androgenesis, anthers are pretreated for 2 days on 0.5 M mannitol or by treatment with 10^{-5} M ABA for 1 to 3 days. If tobacco anthers, for the induction of androgenesis, are pretreated on 0.5 M mannitol for 1 day, a 2-fold increase in ABA_i concentration is measured (Imamura and Harada, 1980).

The other part of barley pretreatment is the pre-medium, which predominantly consists of $CaCl_2$, $MgSO_4$ and KNO_3. From literature it is known that calcium ions play a role in embryogenesis (Timmers et al., 1989; Overvoorde and Grimes, 1994; Jansen et al., 1990). No significant influence of magnesium ions has been reported for gametophytic embryogenesis (Reynolds, 1990).

Potassium ions, however, stimulate somatic embryogenesis in alfalfa (Shetty and McKersie, 1993) and have been used for pretreatment induced androgenesis of several species. For the production of doubled haploid plants of barley, rice and wheat, 10 to 20 mM KNO_3 have been used during mannitol pretreatment (resp. Wei et al., 1986; Ogawa et al., 1992; Hu et al., 1995), whereas tobacco microspores are pretreated in mannitol with 20 mM KCl (Kyo and Harada, 1986).

In this paper, the role of ABA in barley androgenesis as well as the anther pretreatment elements mannitol and pre-medium, was investigated.

Materials and Methods

Materials

Donor plants of *Hordeum vulgare* L. cv. Igri were grown in a phytotron under conditions described previously (Hoekstra et al., 1992). Monoclonal antibody to free (+)ABA was purchased from Idetek, Inc. (San Bruno, Calif., USA). Rabbit anti-mouse alkaline-phosphatase conjugate, (+)ABA and bovine serum albumin (grade suitable for enzyme-linked immunosorbent assay; ELISA) were obtained from Sigma (St. Louis, Mo., USA). Fluridone was kindly provided by the Bulb Research Laboratory, Lisse, Netherlands.

Androgenesis

Selection of material, mannitol pretreatment and culture conditions were as described previously (Hoekstra et al., 1992) with the following modifications. Anthers of several spikes were randomly distributed over the parameters to be tested and per parameter 30 anthers were used. Anthers were pretreated on 0.37 M mannitol dissolved in pre-medium, containing 10^{-2} M $CaCl_2$, 10^{-3} M $MgSO_4.7H_2O$, 10^{-3} M KNO_3, $2x10^{-4}$ M KH_2PO_4, 10^{-6} M KI and 10^{-7} M $CuSO_4.2\ H_2O$ (440 $mOs.kg^{-1}$, by Osmomat), what is called pretreatment solution. After 4 days the anthers were transferred to medium I without Ficoll at 350 $mOs.kg^{-1}$. The first 100 developing ELS were carefully transferred with forceps to medium III and the rest was plated directly, at the moment that the majority of ELS were at least 1 mm in diameter. After 3 weeks plant production was assessed per group of 30 anthers.

The osmolality of the pretreatment solution was adjusted with mannitol to values of 350, 440 and 540 $mOs.kg^{-1}$. $MgSO_4$ at 0, 0.01, 0.1, 1 mM and a concentration range of $CaCl_2$ were tested in pre-medium. Omittance of $CaCl_2$ was used in stead of EGTA to overcome the weak calcium buffering capacity of the latter at the pH of pre-medium. KCl, KNO_3 and $Ca(NO_3)_2$ were tested at 10 and 20 mM in modified pre-medium (with resp. 1 mM $Ca(NO_3)_2$, $CaCl_2$ and KCl in stead of KNO_3). Pre-medium with 10, 30 and 40 mM $CaCl_2$ was adjusted to an osmolality value of 440 $mOs.kg^{-1}$ with mannitol.

At defined times the pretreatment solution was removed and new pretreatment solution was added to the anthers. (±)2-*cis*-4-*trans*-ABA (Sigma) was added from a stock solution (4 mM in 5% ethanol) to obtain a range of concentrations (in a volume of max. 25 µl) at the start and removed at 24 hours of pretreatment. Fluridone was added at a concentration of 40 mg/l from the beginning and removed at 24 hours of pretreatment.

Note: Data in the Tables and Figures, are presented in percentages of regeneration on pre-medium since the absolute numbers varied, due to differences in microspore developmental stage and ELS density during culture. The patterns of the figures were reproducible, however, throughout at least 3 experiments. In table 1 numbers of regenerated plants are presented, to give an indication of the absolute numbers of plants obtained per 30 anthers.

ABA extraction

After treatment, anthers and pretreatment solution were seperately frozen in liquid N_2. Pestle maceration of the frozen anthers was not necessary for ABA extraction (unpublished results, M. Wang et al.). The contents of the Eppendorf vials were freeze dried. The samples were extracted at 150 rpm, 4°C with cold methanol (containing 100 mg/l butylated hydroxytoluene and 0.5 g/l citric acid

monohydrate). The anther samples were extracted twice each time in 0.5 ml for 24 hours. The pretreatment solution samples were treated similarly, except that the first extraction was performed in 1.0 ml. The vials were centrifuged at 8,000*g* for 15 min at 4°C and the supernatants of the samples were pooled. The samples were concentrated in a Speed-vac system (Savant) and the volume adjusted to 25 µl with methanol. The samples were stored at -80°C until assay.

ABA ELISA assay

The assay of the amount of ABA in anthers and in pretreatment solution was carried out by an enzyme-linked immunosorbent assay (ELISA) as described by Wang et al. (1995). No interference in the (+)ABA ELISA was observed by commercial mannitol, in contrast to what is noted by Belefant and Fong (1989).

Results

Pretreatment solution elements

For optimal doubled haploid plant production of barley, a pretreatment solution consisting of mannitol and premedium, was used. The influence of these elements on doubled haploid production was tested. Without pretreatment, no doubled haploid plants can be produced (Hoekstra et al., submitted). Anther pretreatment on water induced very limited plant production. The efficiency of plant production was significantly improved, if mannitol was present during pretreatment. A higher mannitol concentration accompanied by a higher osmolality of the pretreatment solution, resulted in an increase of green plant production upto a concentration of 0.37 M mannitol. At higher mannitol concentration the number of plant produced remained constant (Table 1).

Table 1: The influence on plant production of different mannitol concentrations without premedium, during pretreatment. The results of one experiment are shown and expressed in number of plants per 30 anthers. From other experiments similar data were obtained. Numbers in parentheses indicate the osmolality of the pretreatment solution (in $mOs.kg^{-1}$).

mannitol	n plants	
0	10	(0)
0.3	65	(310)
0.37	86	(400)
0.44	89	(500)

Table 2: The influence of Ca^{2+}, K^+, Cl^- and NO_3^--ion concentrations during pretreatment in premedium, on plant production. The number of plants obtained per 30 anthers, are expressed in percentages.

ions in mM				n plants
Ca^{2+}	K^+	Cl^-	NO_3^-	
10	1	20	1	100
20	1	40	1	114±33
10	1	1	20	123±49
20	1	1	40	106±37
1	10	10	2	80±17
1	20	20	2	81±51
1	10	1	10	84±37
1	20	1	20	74±30

Table 3: The influence of different $CaCl_2$ concentrations (in mM) in pre-medium without and with mannitol at 440 $mOs.kg^{-1}$, on plant production. The number of plants obtained per 30 anthers, are expressed in percentages. Numbers in parentheses indicate the osmolality of the pretreatment solution (in $mOs.kg^{-1}$).

$CaCl_2$	n plants			
	pre-medium		pre-medium with mannitol	
0	35±13	(0)	202±61	(400)
10	100	(31)	323±74	(450)
20	133±53	(58)	-	-
30	193±55	(82)	450±40	(450)
40	248±80	(93)	242±86	(440)

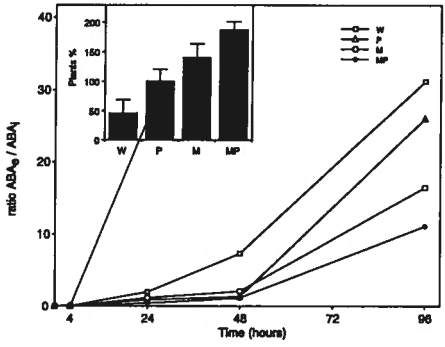


Figure 1: Changes in the exogenous ABA concentration for 4 different pretreatments each of 30 anthers, expressed as the ratio ABA_e/ABA_i (endo- and exogenously in pgr per ml).
Insert: Plant production efficiency after 4 different pretreatments for 96 hours; the number of plants obtained per 30 anthers, are expressed in percentages.
W = water, P = pre-medium, M = mannitol, MP = mannitol with pre-medium

Changes in the magnesium ion concentration did not significantly alter the number of plants produced (data not shown). Apparently there was no special Mg^{2+} concentration required for optimal induction of gametophytic embryogenesis. Calcium could be replaced by potassium for induction of androgenesis at 10 and 20 mM, independent of the anion used (Table 2).
Calcium appeared to be slightly more effective than potassium. Therefore, the effect of a wider range of $CaCl_2$ concentrations was tested on plant production (Table 3). The stimulatorory effect of calcium was confirmed, since omittance of $CaCl_2$ resulted in the same small number of plants, as found for pretreatment on water (Figure 1). Moreover, an increase of the $CaCl_2$ concentration, leading also to slightly higher osmolalities of the pretreatment solution, enhanced the number of plants produced. Higher concentrations, 50 mM and 60 mM, of $CaCl_2$ did not result in further improvement of plant production (data not shown). If the various $CaCl_2$ concentrations were combined with mannitol (pretreatment solution at 440 $mOs.kg^{-1}$), a clear stimulation of plant production was observed and an optimum was found at 30 mM $CaCl_2$ (Table 3).

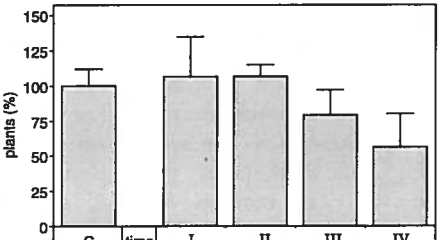
ABA_i and ABA_e concentrations during pretreatment. The observed stimulation of plant production by mannitol indicates, that osmotic pressure plays an important role in pretreatment induced androgenesis. In such cases ABA is reported to be involved (Davies and Jones, 1991). Therefore it could also in our case be speculated that ABA might be

Table 4: ABA concentration a. intracellular (ABA_i) in pgr per 10^{-5} gr DW and b. extracellular (ABA_e) in pgr per ml, measured between 4 and 96 hours of pretreatment of 30 anthers. Pre-medium contained 10 mM $CaCl_2$ and 0.37 M mannitol was applied. One experiment consisted of all samples, with anthers randomly distributed. DW = dry weight

	time	pretreatment solution			
		water	pre-medium	mannitol	mannitol with pre-medium
ABA_i	0	1.06±0.78	1.06±0.78	1.06±0.78	1.06±0.78
	4	2.50±1.09	2.56±1.10	4.29±1.99	3.10±0.95
	24	3.71±1.14	4.03±1.43	3.98±0.84	4.48±1.04
	48	2.27±1.10	3.33±0.13	2.82±1.99	2.98±0.80
	96	1.07±0.82	0.99±0.73	0.80±0.12	0.70±0.45
ABA_e	0	<45	<45	<45	<45
	4	<45	<45	<45	100±15
	24	1589±213	742±247	1317±171	477±209
	48	3672±96	673±128	1767±658	794±335
	96	5996±523	4344±308	3746±117	2355±278

involved in pretreatment induced barley androgenesis by mannitol, and perhaps supported by the $CaCl_2$ containing pre-medium. Therefore ABA_i and ABA_e levels were determined during anther pretreatment using four different conditons, namely water, pre-medium, mannitol, and mannitol with pre-medium (Table 4). The ABA_i concentration increased in all four conditions upto 24 hours and thereafter decreased again. The ABA_e concentration continuously increased dramatically in the course of 96 hours, except for pre-medium, resulting in $2.5x10^{-8}$ M (+)ABA for the water treatment after 96 hours (Table 4). The decrease of ABA_i concentration in the presence of mannitol with pre-medium resulted in a value that was lower than the initial ABA_i level. The ABA_i levels of the other three conditions after 96 hours were higher, but it can not be excluded that this is due to contamination of ABA_e, since ABA_e represents 96% of the total ABA concentration (Figure 1 and Table 4). A negative correlation was observed between the ratio ABA_e/ABA_i and the embryogenic capacity induced (Figure 1). For both cases with mannitol in the pretreatment, the ratio ABA_e/ABA_i gradually increased during pretreatment, whereas for the other conditions the ratio ABA_e/ABA_i reached not only a much higher level but also within a shorter

period. Summarizing, we see that for optimal plant production the ABA_e concentration in a period of about 24 hours to 48 hours should be relatively low, that mannitol has some additional positive effect, and that at the end of pretreatment the ABA_e concentration should not be too high. Furthermore, the ABA_e level in mannitol with pre-medium at 4 hours is significantly higher than in the other cases.



C	time	I	II	III	IV
400±87	12	400±87			400±87
	16	613±275			613±275
	20	622±356			622±356
477±209	24	776±141	477±209	477±209	776±141
794±335	48			1938±177	1822±101
	72			1366±191	1322±357
2355±278	96	2086±420	2098±264	1005±262	786±159

Figure 2: The influence of refreshment of the pretreatment solution (mannitol with pre-medium) at various times, on a. plant production upon 96 hours of pretreatment and b. exogenous ABA concentration in the removed pretreatment solution in pgr per ml, compared to the exogenous ABA level accumulating in time in the control. The number of plants obtained per 30 anthers, are expressed in percentages. C = control, no refreshment, ABA_e values are from Table 4.
I = refreshment at 12, 16, 20 and 24 h
II = refreshment at 24 h
III = refreshment at 24, 48 and 72 h
IV = refreshment at 12, 16, 20, 24, 48 and 72 h

Manipulation of the ABA_e concentration. The above results suggest that the ABA_e concentration somehow has an effect on the induction of androgenesis. Specifically the period around 24 hours was of interest in this respect. Therefore the influence of a reduction of ABA_e level was investigated at different time intervals before and beyond 24 hours of pretreatment. A reduction of the ABA_e concentration was achieved by refreshing the pretreatment solution at several time intervals (Figure 2). The ABA_e concentration was determined in the removed solution. The data indicated that there was not much difference in ABA_e concentration and plant production between one (after 24 hours) or more refreshments of the pretreatment solution in the period upto 24 hours, and no refreshment. If the pretreatment solution was refreshed repeatedly up to 24 hours, a slightly increased ABA_e level after 24 hours was observed. Refreshments of the solution up to and including 24 hours did not affect the ABA_e concentration of the pretreatment solution after 96 hours, nor the plant production. However, if the pretreatment solution was refreshed from 24 hours onwards, both a lower ABA_e concentration at the end of pretreatment and a reduced plant production was observed. The efficiency of plant production was even more reduced if the pretreatment solution was removed at several time intervals during the complete period from 12 to 96 hours. So, the minor changes that occurred in the ABA_e concentration up to and including 24 hours of pretreatment on pre-medium had a limited effect on plant production.

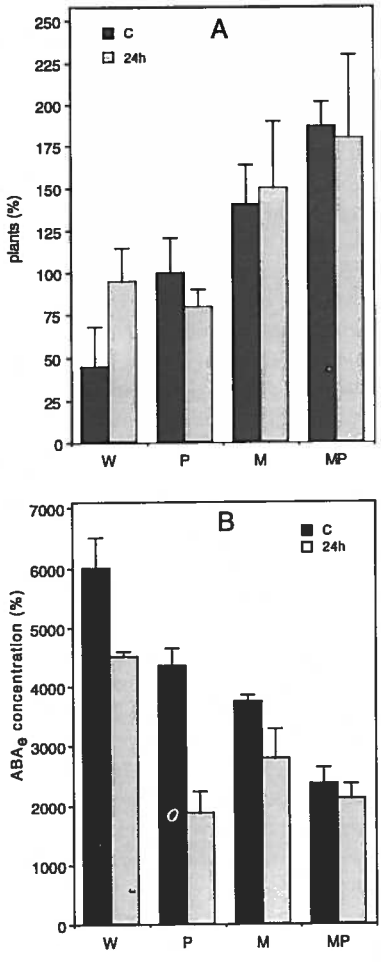


Figure 3: The influence of a. fresh pretreatment solution after 24 hours (24h) on green plant production after 4 different pretreatments of 96 hours (C) and b. the exogenous ABA level in pgr per ml after 96 hours of pretreatment. The number of plants obtained per 30 anthers are expressed in percentages.
W = water, P = pre-medium, M = mannitol, MP = mannitol with pre-medium

Changes thereafter, however, did have an effect. With respect to this it is of interest to note that the ABA_e values observed for 24 hours and 48 hours in the refreshment samples are clearly higher than found in the samples without refreshment (Table 4, mannitol with pre-medium). To see whether the composition of the pretreatment solution had an effect if a change in ABA_e level was made, by refreshment of the solution after 24 hours in the course of a 96 hours pretreatment, 4 different solutions were tested (Figure 3). The ABA_e concentration was determined after 96 hours of pretreatment and plant production was assessed. For pretreatment on mannitol with or without pre-medium, no significant effect of refreshment of the solution after 24 hours on total plant production was observed. The removal of ABA_e at 24 hours also did not much affect the ABA_e concentration determined after 96 hours in mannitol with pre-medium, whereas in the case of mannitol pretreatment the value was reduced. If anthers were pretreated on pre-medium, a slight negative effect on green plant production was observed if the solution was refreshed after 24 hours, while the ABA_e concentration at the end of pretreatment was found to be strongly reduced. A marked increase in green plant production, accompanied by a decrease in ABA_e level at 96 hours, was observed, if the pretreatment was performed on water with refreshment of the solution after 24 hours. These data show that the ABA_e level at 96 hours is not a reliable indication for plant production efficiency.

Moreover, the effect of ABA_e on induction of androgenesis was further

studied by application of exogenous (±)ABA. An ABA concentration range from 10^{-9} upto 10^{-4} M was tested in pre-medium. Only for 10^{-7} and 10^{-6} M a slightly increased plant production was found (data not shown). Therefore, the four different pretreatment solutions were used to study the effect during 24 hours, of the addition of 10^{-7} M (±)ABA at the start of the 96 hours pretreatment (Figure 4). The concentration of 6000 pgr (+)ABA_e per ml at 96 hours in water pretreatment (Table 4) equals $2.5x10^{-8}$ M (+)ABA, and $5x10^{-8}$ M (±)ABA. A clear positive effect of the ABA addition on plant production was observed for pretreatment on pre-medium. Exogenously applied ABA did not influence the regeneration efficiency for mannitol containing solutions, while a strong reduction was found for water. Without addition of ABA to mannitol with pre-medium (Table 4), ABA_e was already detected after 4 hours of pretreatment, whereas the level of ABA_e in the other pretreatment solutions was below level of detection. Apparently, the presence of a relatively high level of ABA_e during the first 24 hours of pretreatment only has an effect in the absence of mannitol. The effect is negative for water that already seems to produce a lot of ABA_e in the period between 24 hours and 96 hours, and positive for pre-medium that normally has much lower levels in the same period (Table 4). In preliminary experiments the presence of added 10^{-7} M ABA during the entire pretreatment was tested, and resulted in reduced plant production when anthers were pretreated on mannitol with pre-medium, whereas on water only a slight negative effect on plant production was found (data not shown).

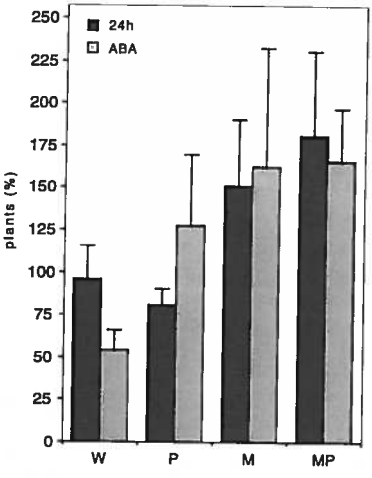


Figure 4: Plant production after 4 different pretreatment solutions with refreshement of the pretreatment solution after 24 hours (24h); and the influence of the added 10^{-7} M ABA during the first 24 hours of pretreatment (ABA). The number of plants obtained per 30 anthers, are expressed in percentages.
W = water, P = pre-medium, M = mannitol, MP = mannitol with pre-medium

Manipulation of the ABA_i concentration. It has been shown that ABA, synthesized in response to water stress (see also Table 4), is an apo-carotenoid; carotenoids are precursors of ABA (Parry and Horgan, 1991). Fluridone is an inhibitor of the carotenoid biosynthesis (Bartels and Watson, 1978). Fluridone-treated barley plants therefore do not accumulate ABA because of carotenoid deficiency rather than plastid dysfunction (Gamble and Mullet, 1986). For manipulation of the ABA_i concentration, the presence of

fluridone during the first 24 hours of pretreatment was investigated. The concentration of ABA_i was determined after 24 hours of pretreatment on the 4 different pretreatment solutions. The presence of fluridone, resulted in an ABA_i level of 3.19±0.65 pgr per 10^{-5} gr DW after 24 hours in all treatments (data not shown), which was only a small reduction compared to the control (Table 4). Therefore, the ABA_e concentration was determined after 24 hours and at the end of the pretreatment of 96 hours. Application of fluridone resulted in an ABA_e level of 325±74 pgr/ml after 24 hours in all treatments (data not shown), which for all cases except mannitol with pre-medium is much lower than found normally (Table 4). The ABA_e data after 96 hours and corresponding plant production efficiencies are shown in Figure 5. Fluridone inhibited *de novo* ABA synthesis, which resulted in a reduced ABA_e level of about 1400 pgr per ml after 96 hours of pretreatment in all conditions. The ABA_e level after 96 hours again does not reflect plant production efficiency. The presence of fluridone during the first 24 hours, resulted in all cases in an increase in bleached plant production and in a large decrease in plant production for mannitol. For mannitol with pre-medium the presence of fluridone resulted in a less pronounced decrease in the total number of plants. For the two pretreatments without mannitol, however, a strong stimulation of plant production was oberved. It can be concluded that a specific level of ABA_e is required in a period between 24 hours and 48 hours, and that only in the presence of mannitol *de novo* ABA synthesis was required in the first 24 hours for optimal plant production.

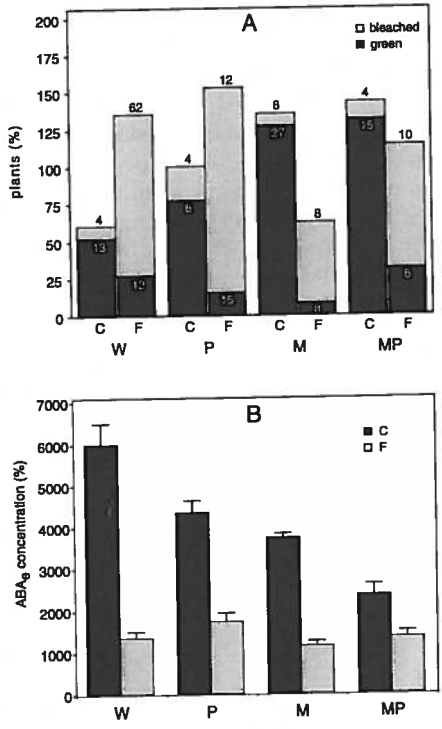


Figure 5: The influence of a. the presence of 40 mg/l fluridon during the first 24 hours (F) of pretreatment on green and white plant production for 4 different pretreatment solutions (C) and b. the exogenous ABA level in pgr per ml after 96 hours of pretreatment. The number of plants obtained on the first 100 ELS per 30 anthers are expressed in percentages of green (G) and bleached (B) plants upon pretreatment on pre-medium.
The numbers in the bars of Figure 5a are the standard deviations.
W = water, P = pre-medium, M = mannitol, MP = mannitol with pre-medium

Discussion

A proper pretreatment is one of the requirements for doubled haploid production in barley. We identified two factors in the pretreatment solution, mannitol and calcium, which were of crucial importance for efficient plant production. In this study, the optimal concentration of calcium for plant production is 30 mM (in the presence of mannitol), which is relatively high. For androgenesis in *Solanum sp.* the optimum calcium concentration is 3 mM (in the presence of 19 mM K^+; Reynolds, 1990). This author further reports that $CaCl_2$ can be substituted by $Ca(NO_3)_2$, and not by other bivalent ions such as e.g. magnesium. This is in agreement with our results; our data showed moreover, that Ca^{2+} could be replaced by K^+. This result and the relatively high concentration used, give an indication for a possible role of these ions as osmotic stress agents like mannitol in the pretreatment. However calcium might also have a specific function in the induction of androgenesis. Other data from our laboratory indicated that programmed cell death, also known as apoptosis, takes place in the anther tissue during pretreatment (preliminary results). The key characteristic of apoptosis is the systematic fragmentation and degradation of nuclear DNA (Raff, 1992). This DNA ladder is very often caused by the action of calcium-dependent nucleases (Bowen, 1993). From our present data, we conclude that for plant production an osmotic pretreatment of microspores (i.e. 0.3-0.4 M mannitol in the presence of minimal 10 mM Ca^{2+} or K^+), apparently is a general pre-condition for the species barley (Wei et al., 1986), wheat (Hu et al., 1995), rice (Ogawa et al., 1992), *Solanum carolinense L.* (Reynolds, 1990) and tobacco (Imamura and Harada, 1980; Kyo and Harada, 1986). Furthermore we assume, that differences in optimal mannitol (and calcium) concentration per species can be explained by differences in sensitivity towards osmotic pressure. Our data suggest that, the appropriate osmotic stress controls the optimal ratio ABA_e/ABA_i needed for embryogenic induction in the microspores. The importance of ABA_i levels has been determined for morphogenic competence in *Pennisetum sp.* by Rajasekaran et al. (1987a). Furthermore, these authors show that application of ABA enhances somatic embryogenesis, and that inhibition of plant production is observed by addition of fluridone in the induction medium. The use of fluridone on donor plants even causes the loss of capacity for embryogenesis (Rajasekaran et al., 1987b).

With regard to a correlation between androgenetic capacity and ABA level, the literature appears to be rather controversial. Johansson et al. (1982) report that appropriate pretreatment of *Anemona canadensis* to induce androgenesis, reduced the ABA_i concentration at the end of pretreatment nearly 4 times, whereas Imamura and Harada (1980) demonstrate the presence of a peak in ABA_i concentration after 24 hours of mannitol pretreatment in tobacco anthers. Both data, nevertheless, are in agreement with our study, showing a peak in ABA_i level after 24 hours and a

reduction in ABA_i concentration at the end of the mannitol pretreatment after 96 hours with or without pre-medium. These conditions were the best for plant production. In our study, a negative correlation between plant production efficiency and ABA concentration was most pronounced if we looked at the ABA_e level and the ratio ABA_e/ABA_i at the end of the pretreatment. The importance for plant production of the ABA_e level and the ratio ABA_e/ABA_i has not been reported earlier. Imamura and Harada already proposed a specific level for ABA_i in 1980. These authors have shown in a time range experiment using 10^{-5} M ABA, that a 1 to 3 days period of pretreatment stimulate plant production in tobacco androgenesis. For wheat androgenesis, Hu et al. (1995) observe a stimulation by application of ABA (about 10^{-5} M) throughout the pretreatment. Kyo and Harada (1985) show that application of $5x10^{-6}$ M ABA in the second half of the pollen pretreatment stimulated embryo production in tobacco. From our data we conclude that, during the period between 24 hours and 48 hours, specific ABA levels both inside and outside the cell must be present.

In our investigation, the presence of fluridone during the first 24 hours of pretreatment resulted in a large decrease of the number of plants for mannitol and and a lower decrease for mannitol with pre-medium, indicating that normally there is a need for *de novo* ABA synthesis during the first 24 hours in the presence of mannitol. So, a certain level of ABA_i is required for plant production. However, *de novo* ABA synthesis during the first 24 hours of pretreatment was apparently not required for optimal plant production if pretreatment occurred in pre-medium or water. Both data together suggest that in the latter cases, after removal of fluridone, sufficient ABA is synthesized to reach the right level for optimal plant production. Probably this level must be reached in the period between 24 hours and 48 hours. Fluridone is also known as a bleaching herbicide, inhibiting phytoene desaturation in the presence of $7x10^{-8}$ M fluridone (Mayer et al. 1989). In our study the number of bleached plants indeed was much higher for fluridone (about 10^{-4} M) during pretreatment than for the controls.

The increase of the ratio ABA_e/ABA_i during anther pretreatment was important for induction of androgenesis. The ratio ABA_e/ABA_i can be changed either by modification in (the rate of) ABA synthesis or by changes in the metabolism which result in reduction of ABA level. The presented data show that *de novo* ABA synthesis is important during the first 24 hours of pretreatment, but it remains to be tested whether this is also the case at a more progressed stage of pretreatment. In addition, changes of ABA catabolism could also be involved in the regulation of the ratio ABA_e/ABA_i and were not investigated in this study. Differences in ABA_e concentrations between treatments, also can be explained by transport or diffusion of ABA_i into the solution. Alternatively, the increase of ABA_e between 24 and 96 hours of pretreatment, could be due to leakage of ABA_i from dying cells. Preliminary data from our laboratory support the last possibility. We found that during pretreatment in mannitol with pre-

medium, anther tissue showed a high degree of systematic DNA fragmentation and degradation after 72 hours, while the DNA had disappeared completely by 96 hours. The microspores within this tissue, however, showed normal viability. Therefore we assume, that depending on the conditons of pretreatment, ABA synthesis is induced to a certain level that it corresponds to the level of (osmotic) stress experienced by the cell. The concentration of mannitol used in our study correlates with the appropriate stress level needed for induction of apoptosis (Cotter and Al-Rubeai, 1995) and probably protects the surviving microspores against a shock due to a sudden too high level of ABA_e, such as in the case of water pretreatment. The role of calcium is to enable action of calcium-dependent nucleases required for the process of apoptosis, thereby preventing the cell's contents (i.e. ABA_i in our study) spilled into the extracellular space (Raff, 1992).

Our presented data indicated that ABA is also acting from outside the cell, *in casu* on the microspores. There has been much debate as to the initial site of action of ABA eliciting cellular responses, in particular as to whether the ABA receptor is located in the cytosol (Assmann, 1994) or faces outward into the extracellular matrix (Allan and Trewavas, 1994). Recently, in 2 systems evidence for extracullular action of ABA has been reported. Wang et al. (1995) show that diffusion of ABA_i into the medium plays a role in germination of barley embryos. In other morphogenetic responses as turion production in *Spirodela polyrrhiza* L., it is demonstrated that ABA triggers a morphogenic switch. In the latter system, ABA most likely interacts with a plasmalemma-located receptor system to induce turion production (Smart et al., 1995). It is interesting to note that the morphogenetic switch for turion production, is induced by environmental effectors like nitrate deficiency and cold, which both also induce gametophytic embryogenesis. Apparently, the involvement of an ABA trigger is a more general phenomenon and not restricted to the morphogenetic switch from the gametophytic to the sporophytic pathway in pollen.

Acknowledgement

G. van Duijn (TNO, Zeist) is thanked for teaching the Elisa, R. van der Meulen for technical assitance, J. Vink for growth of the donor plants, P. Hock for the lay-out of the figures.

References

Allan C.A. and A.J. Trewavas. Abscisic acid and gibberellin perception: inside or out? Plant Physiol. 104: 1107-1108 (1994)

Assmann S.M. Ins and outs of guard cell ABA receptors. Plant Cell 6: 1187-1190 (1994)

Bartels P.G. and C.W. Watson. Inhibition of carotenoid synthesis by fluridone and norflurazon. Weed Sci. 26: 198-203 (1978)

Belefant H. and F. Fong. Abscisic acid ELISA: organic acid interference. Plant Physiol. 91: 1467-1470 (1989)

Bowen I.D. Apoptosis or programmed cell death? Cell Biol. Int. 17: 365-380 (1993)

Cotter T.G. and M. Al-Rubeai. Cell death (apoptosis) in cell culture systems. Tibtech. 13: 150-155 (1995)

Davies W.J. and H.G. Jones (eds). In: Abscisic Acid physiology and biochemistry. Bio's scientific publishers limited (1991)

Gamble P.E. and J.E. Mullet. Inhibition of carotenoid accumulation and abscisic acid biosynthesis in fluridone-treated dark-grown

barley, Eur. J. Biochem. 160: 117-121 (1986)
Heberle-Bors E. Isolated pollen culture in tobacco: plant reproductive development in a nutshell. Sex. Plant Reprod. 2: 1-10 (1989)
Hoekstra S., M.H. VanZijderveld, J.D. Louwerse, F. Heidekamp and F. VanderMark. Anther and microspore culture of *Hordeum vulgare* L. cv. Igri. Plant Sci. 86: 89-96 (1992)
Hoekstra S., M.H. VanZijderveld, F. Heidekamp and F. VanderMark. Microspore culture of *Hordeum vulgare* L.: the influence of density and osmolarity. Plant Cell Rep. 12: 661-665 (1993)
Hoekstra S., S. VanBergen, I.R. VanBrouwershaven, R.A. Schilperoort and F. Heidekamp. The interaction of 2,4-D application and mannitol pretreatment in anther and microspore culture of *Hordeum vulgare* L. cv. Igri. J. Plant Physiol. 148: 696-700 (1996)
Hoekstra S., M.H. VanZijderveld, S. VanBergen, F. VanderMark and F. Heidekamp. Genetic modification of barley for end use quality. In: Henry R.J. and J.A. Ronalds (eds). Improvement of cereal quality by genetic engineering. Plenum Press pp 139-144 (1994)
Hu T.C., A. Ziauddin, E. Simion and K.J. Kasha. Isolated microspore culture of wheat (*Triticum aestivum* L.) in a defined media I. Effects of pretreatment, isolation methods and hormones. In Vitro Cell. Dev. Biol. 31: 79-83 (1995)
Huang B. and N. Sunderland. Temperature-stress pretreatment in barley anther culture. Ann. Bot. 49: 77-88 (1982)
Imamura J. and H. Harada. Effects of abscisic acid and water stress on the embryo and plantlet production in anther culture of *Nicotiana tabacum* cv. Samsun. Z. Pflanzen- physiol. 100: 285-289 (1980)
Ishitani M., T. Nakamura, S.Y. Han and T. Takabe. Expression of the betaine aldehyde dehydrogenase gene in barley in response to osmotic stress and abscisic acid. Plant Mol. Biol. 27: 307-315 (1995)
Jansen M.A.K., H. Booij, J.H.N. Schel and S.C. DeVries. Calcium increases the yield of somatic embryos in carrot embryogenic suspension cultures. Plant Cell Rep. 9: 221-223 (1990)
Johansson L., B. Andersson and T. Eriksson. Improvement of anther culture technique: activated charcoal bound in agar medium in combination with liquid medium and elevated CO_2 concentration. Physiol. Plant. 54: 24-30 (1982)
Kiyosue T., M. Nakajima, I. Yamaguchi, S. Satoh, H. Kamada and H. Harada. Endogenous levels of abscisic acid in embryogenic cells, non-embryogenic cells and somatic embryos of carrot (*Daucus carota* L.). Biochem. Physiol. Pflanzen 188: 343-347 (1992)
Kyo M. and H. Harada. Studies on conditions for cell division and embryogenesis in isolated pollen culture of *Nicotiana rustica* Plant Physiol. 79: 90-94 (1985)
Kyo M. and H. Harada. Control of the developmental pathway of tobacco pollen *in vitro*. Planta 168: 427-432 (1986)
Leonardi A., S. Heimovaara-Dijkstra and M. Wang. Differential involvement of abscisic acid in dehydration and osmotic stress in rice cell suspension. Physiol. Plant. 93: 31-37 (1995)
Mayer P.M., D.L. Bartlett, P. Beyer and H. Kleinig. The *in vitro* mode of action of bleaching herbicides on the desturation of 15-cis-phytoene and cis-ζ-carotene in isolated daffodil chromoplasts. Pesticide Biochem. Phys. 34: 111-117 (1989)
Monroy A.F. and R.S. Dhindsa. Low-temperature signal transduction: induction of cold acclimation-specific genes of alfalfa by calcium at 25°C. Plant Cell 7: 321-331 (1995)
Ogawa T., T. Hagio and Y. Ohkawa. Plant regeneration from isolated pollen grains in indica type rice (*Oryza sativa* L.). Japan J. Breed. 42: 675-679 (1992)
Overvoorde P.J. and H.D. Grimes. The role of calcium and calmodulin in carrot somatic embryogenesis. Plant Cell. Physiol. 35: 135-144 (1994)
Raff M.C. Social controls on cell survival and cell death. Nature 356: 397-400 (1992)
Rajasekaran K., M.B. Hein and I.K. Vasil. Endogenous abscisic acid and indole-3-acetic acid and somatic embryogenesis in cultured leaf explants of *Pennisetum purpureum* Schum. Plant Physiol. 84: 47-51 (1987a)
Rajasekaran K., M.B. Hein, G.C. Davis, M.G. Carnes and I.K. Vasil. Endogenous growth regulators in leaves and tissue cultures of *Pennisetum purpureum* Schum. J. Plant Physiol. 130: 13-25 (1987b)
Reynolds T.L. Interactions between calcium and

auxin during pollen androgenesis in anther cultures of *Solanum carolinense* L. Plant Sci. 72: 109-114 (1990)

Roberts-Oehlschlager S.L. and J.M. Dunwell. Barley anther culture: Pretreatment on mannitol stimulates production of microspore-derived embryos. Plant Cell Tiss. Org. Cult. 20: 235-240 (1990)

Shetty K. and B.D. McKersie. Proline, thioproline and potassium mediated stimulation of somatic embryogenesis in alfalfa (*Medicago sativa* L.). Plant Sci. 88: 185-193 (1993)

Smart C.C., A.J. Fleming, K. Chaloupkova and D.E. Hanke. The physiological role of abscisic acid in eliciting turion morphogenesis. Plant Physiol. 108: 623-632 (1995)

Timmers A.C.J., S.C. DeVries and J.H.N. Schel. Distribution of membrane-bound calcium and activated calmodulin during somatic embryogenesis of carrot (*Daucus carota* L.). Protoplasma 153: 24-29 (1989)

Wang M., S. Heimovaara-Dijkstra and B. VanDuijn. Modulation of germination of embryos isolated from dormant and nondormant barley grains by manipulation of endogenous abscisic acid. Planta 195: 586- 592 (1995)

Wei Z.M., M. Kyo and H. Harada. Callus production and plant regeneration through direct culture of isolated pollen of *Hordeum vulgare* cv. Sabarlis. Theor. Appl. Genet. 72: 252-255 (1986)

Chapter 6

Androgenesis in *Hordeum vulgare* L.: Apoptosis and the effect of lipo-oligosaccharides during anther pretreatment

S. Hoekstra, M.W. van der Heijden, B.J. Oppedijk, I.R. van Brouwershaven, S. van Bergen, F. Heidekamp & R.A. Schilperoort

Summary

Characteristics of senescence were investigated during the pretreatment of anthers needed to induce gametophytic embryogenesis in cultured barley microspores. Degradation of chlorophyll in the anthers and a specific pattern of changes in the intracellular ABA were found both during and without pretreatment. During pretreatment, different RNA transcripts which may result from the corresponding ABA-induced genes were detected in the anther tissue as well as in the microspores. Pretreatment resulted in pronounced degradation of protein and RNA. However, breakdown of DNA was peculiar since a well-defined fragmentation pattern of the nuclear DNA was observed, suggesting programmed cell death, also known as apoptosis. Furthermore, expression of the hydrolytic enzyme glucanase throughout pretreatment was shown, whereas expression of the hydrolytic enzyme chitinase was induced during pretreatment. A stimulatory effect of lipo-oligosaccharides (LOS) application on microspore-derived plant production was observed only during pretreatment of anthers. In the discussion, a mechanism for induction of androgenesis is proposed.

Introduction

For the production of (doubled) haploid plants from microspores i.e. via gametophytic embryogenesis, pretreatment of anthers and microspores is generally essential. During this pretreatment, microspores change into cells that are called embryogenic microspores. These embryogenic microspores can be recognized microscopically by their increased size (50-60 μm in diameter) and red interference of the exine (Hoekstra et al., 1993). Little is known about the biological processes occurring during pretreatment. In the model

system barley, a few studies have been performed to investigate the effects of anther pretreatment on gametophytic embryogenesis. It was found that pretreatment on mannitol with pre-medium is the best condition for plant production (Hoekstra et al., submitted). Recently, for plant production in barley the importance of a certain concentration of endogenous as well as exogenous abscisic acid (ABA) during pretreatment in the period between 24 to 48 hours was demonstrated (Hoekstra et al., submitted). It is known that the hormone ABA plays a role in the process of senescence (Davies and Jones, 1991). Cho and Kasha (1992) suggest that the mannitol pretreatment in barley is responsible for the occurrence of senescence. These authors show that lipid peroxidation takes place in membranes of anthers during such a pretreatment. Inclusion of free radical scavengers not only delayed and suppressed lipid peroxidation and ethylene production during the early culture period, but also reduced embryoid production. During pretreatment, also changes in cell wall properties are observed (Sunderland et al., 1984). Senescence involves synthesis of hydrolytic enzymes, most importantly glucanases (Salisbury and Ross, 1991), which are responsible for cell wall degradation. Moreover, glucanase together with chitinase are detected in embryogenic cell suspension cultures of barley (Kragh et al., 1991). By the action of chitinases, lipo-oligosaccharides (LOS) are assumed to be released from plant-derived precursor molecules (probably cell wall fragments). These molecules are supposed to play a role in early embryogenesis (de Jong et al., 1993). In the model system carrot de Jong et al. (1993, 1995) demonstrate a reduced level of 32kD chitinase in a *ts*11 mutant cell line. In this mutant the embryogenic capacity is rescued by adding chitinase or by application of LOS, which are known as Nod-factors. Nod-factors are secreted by *Rhizobium* in the process of nodule induction and symbiotic nitrogen fixation (Spaink et al., 1991).

In the present study, characteristic features of senescence (Salisbury and Ros, 1991; Smart, 1994), such as degradation of chlorophyll, proteins and RNA, a constant DNA level, and a specific pattern of intracellular ABA (ABA_i) concentration were investigated during pretreatment induced androgenesis of barley. We also studied the expression of genes during pretreatment by differential display using family specific domain primers of genes that are known to be "ABA responsive" (a.o. Chandler and Robertson, 1994; Skriver and Mundy, 1990) and of chitinase and glucanase. Also, the effect of LOS on androgenesis was studied.

Materials and Methods

Materials

Donor plants of *Hordeum vulgare* L. cv. Igri were grown in a phytotron under conditions described previously (Hoekstra et al., 1992). Monoclonal antibody to free (+)ABA was purchased from Idetek, Inc. (San Bruno, Calif., USA). Rabbit anti-mouse alkaline-phosphatase conjugate, (+)ABA and bovine serum albumin (grade suitable for enzyme-linked immunosorbent assay; ELISA) were obtained from Sigma (St. Louis, Mo., USA). Lipo-

oligo saccharides, i.e. the *nodABCL*-dependent metabolites of *Rhizobium leguminosarum* bv. *viciae* (Spaink et al., 1991) were kindly provided by dr. H. Spaink, Institute of Molecular Plant Sciences, Leiden University, Netherlands.

Androgenesis

Selection of material and culture conditions of anthers were as described previously (Hoekstra et al., 1996) with a few modifications. In short, anthers containing late uninucleate microspores, were pretreated on mannitol with pre-medium for a period of 4 days (these days are indicated as day -4, -3, -2, -1 and 0); at day 0 anthers were transferred to medium I (Hoekstra et al., 1992) without hormones. Regeneration was assessed per 30 anthers on the first 100 developing embryo-like structures (ELS) and the remaining structures were plated at once on medium III (Hoekstra et al., 1992) with 1 mg/l Kinetine.

As a control for possible effect(s) of anther harvest conditions or *in vitro* culture conditions, 30 anthers were cultured without any pretreatment directly on medium I.

The number of viable microspores during anther pretreatment was determined by squashing 1 ml of the anther tissue and staining the squashed material with about 20 µl Fluoresceine Di-Acetate (stock 5 mg/ml in acetone).

A concentration range of lipo-oligosaccharides was added during or after pretreatment; in addition the anthers were cultured directly without pretreatment on medium I with or without lipo-oligosaccharides.

Weight and protein determination

Anther fresh weight as well as the dry weight was measured after 16 hours of freeze drying of the samples. Protein in frozen ground anthers was determined using the bicinchoninic acid protein assay. Bovine serum albumin (BSA) was used as a standard (Smith et al., 1985).

Chlorophyll measurement

Anthers were ground in 80% acetone with a pestle. The debris was removed by centrifugation. Chlorophyll specific absorption was measured on supernatan aliquots at 662.5 nm.

ABA extraction and ELISA assay

Anthers were put into Eppendorf vials and frozen with liquid N_2. Pestle maceration of the frozen anthers was not necessary for ABA extraction (unpublished results, M. Wang et al.). The contents of the Eppendorf vials were freeze dried. The samples were extracted at 150 rpm, 4°C with cold methanol (containing 100 mg/l butylated hydroxytoluene and 0.5 g/l citric acid monohydrate) twice each time in 0.5 ml for 24 hours. The vials were centrifuged at 8,000*g* for 15 min at 4°C and the supernatants of the samples were pooled. The samples were concentrated in a Speed-vac system (Savant) and the volume adjusted to 25 µl with methanol. The samples were stored at -80°C until assay.

The assay of the amount of ABA in anthers and in pretreatment solution was carried out by an enzyme-linked immunosorbent assay (ELISA) as described by Wang et al. (1995).

RNA isolation

Samples containing either anthers and/or microspores were used for total RNA isolation. Microspores were isolated according to Hoekstra et al. (1993) with the modification that the isolation was performed at 4°C. The material was harvested directly from the plant or after 1, 2, 3 or 4 days of pretreatment. The material and a steel pestle, used for grinding the material, were frozen in liquid nitrogen. Glass from the tips of pasteur pipets was ground and added in a ratio pollen:glass equivalent to 1:2 in an Eppendorf vial. Glass and plant material were gently mixed with a stainless steel spatula. Subsequently the mixture was ground for 10 seconds with the cold steel pestle (manufactured to fit in the vial) mounted in an electric screwdriver. The process of chilling in liquid nitrogen, mixing and squashing was repeated three times. The degree of pollen disruption was established by microscopy. In general, an average of 70% of the pollen was damaged. Total RNA was isolated from the crushed cells using a micro RNA isolation method according to Chomczynski and Sacchi (1987) using 4 M guanidinium-isothiocyanate, 0.02 M sodium citrate, 0.5% sarcosyl and a phenol/chloroform extraction step. A DNase treatment was applied to remove residual DNA from the RNA sample.

Total RNA was isolated from aleuron and from

embryos that were 2 days imbibed on water according to Wang et al. (1992), from leaf and shoot according to Chomczynski and Sacchi (1987).

DNA isolation

Samples of anthers containing microspores were used for genomic DNA isolation. Anthers were frozen in liquid nitrogen and homogenized as described in the section RNA isolation. DNA was isolated from the samples according to Koes et al. (1995).

Domain directed differential display

One μg total RNA was converted to first strand cDNA with the "First-strand cDNA synthesis kit" (Pharmacia) in a volume of 33 μl and 0.4 μM T_{17}Adapterprimer. 1.5 μl cDNA pool was amplified by PCR, using 0.5 μM Adapter primer, 0.5 μM family specific domain primer, 1.25 U *Taq* DNA polymerase (Pharmacia) and 0.2 mM dNTPs in 1x PCR buffer (Pharmacia). The family specific domain primers were chosen based on sequence comparison using the Wisconsin GCG software package, and directed against a conserved sequence located at 200-700 bp from the poly-A tail. For maximum specificity, the degree of degeneracy was kept as low as possible. Primers used in this work were as follows: T17-Adapter (5'TTTTTTTTT-TTTTTTTT-AGCTACAGCTGAGCTCAG3'), Adapter (5'GCTACAGCTGAGCTCAG3'), "ABA responsive" (5'CGAA-GAGGAACTA(C/G)AA-(C/G/A)AGG3'), Chitinase (5'C(G/C)GCAG-TAGGT(C/T)TTGGT) and Glucanase (5'CAA-GAG(C/T)AACTTGTACC(G/T)CT3').

Reaction mix-tures were heated to 95°C for 3 min. Thirty cyles of linear amplification were performed in a Perkin-Elmer machine, using denaturation for 45 sec at 95°C, annealing for 60 sec at 52°C for the Glucanase and "ABA responsive" primers and at 59°C for the Chitinase primer, the extension step was done for 60 sec at 72°C, followed by a final 10 min elongation at 72°C. A 3 µl of each PCR reaction was separated on a 2% (w/v) agarose gel in 1x TBE buffer and stained with ethidium bromide.

The protocol applied in this study was similar to the RACE method described by Frohman et al. (1988), who used a single 17-mer gene-specific oligonucleotide primer in combination with a (dT)17-adapter to amplify copies of the region at the 3'end. According to these authors, optimization of annealing temperature, number of cycles, buffer condition, and extension time of the initial (+) strand synthesis reaction reduced the production of aspecific bands. The method was further modified by using a primer for a conserved family domain (Fischer et al., 1995).

Results

Pretreatment induced changes

During anther pretreatment, the switch from the gametophytic to the sporophytic pathway takes place. When anthers were cultured directly on medium I without pretreatment, no plant production was observed (Hoekstra et al., 1996). Known characteristics of senescence were investigated during anther culture both on pretreatment solution and on medium. A clear colour change of the anthers from

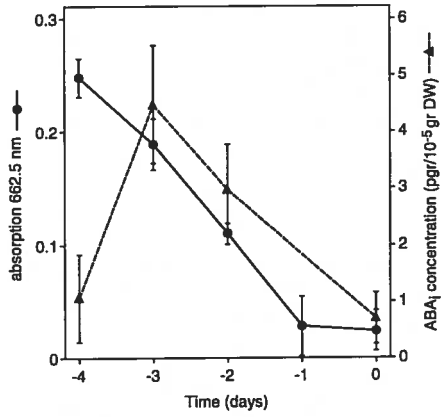


Figure 1: The chlorophyll content (absorption at 662.5 nm) and ABA_i concentration (in pgr/10^{-5} gr of DW) of 30 anthers during anther pretreatment.

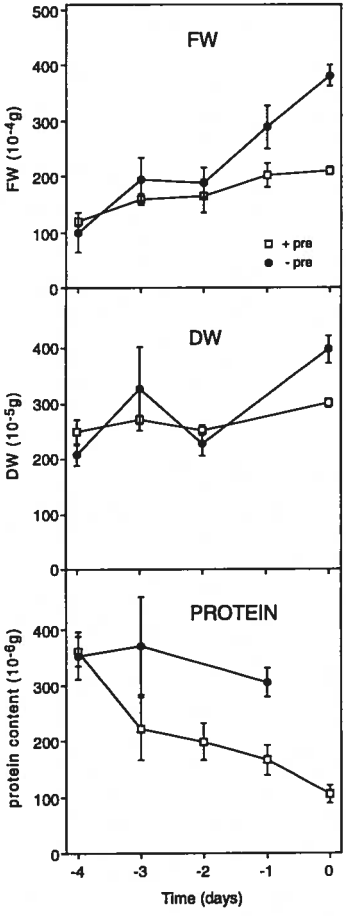


Figure 2: Fresh weight (FW, in 10^{-4} g), dry weight (DW, in 10^{-5} g) and protein content (in 10^{-6} g) per 30 anthers during pretreatment (+pre) and without pretreatment (-pre).

green to yellow was observed during pretreatment and could be quantified by spectrofotometric measurement of chlorophyll (Figure 1a). A similar degradation of chlorophyll was also observed if anthers were cultured without pretreatment (data not shown).

Furthermore, a peak in ABA_i concentration was measured at day -3, during pretreatment (Figure 1b) as well as without pretreatment.

Moreover, during pretreatment large changes in the rigidity of the anther wall were observed. Furthermore, the increase of anther fresh weight was quadrupled if anthers were cultured without pretreatment, whereas the value was only doubled at the end of pretreatment (Figure 2a). Much less difference was found in dry weight with or without pretreatment (Figure 2b). If anthers were cultured without pretreatment the protein level was relatively constant, whereas a considerable loss of protein was observed during pretreatment (Figure 2c).

Subsequently, the level of RNA and DNA in anthers was determined during pretreatment.

RNA isolation from microspores

Difficulties in isolating large amounts of RNA from (immature) pollen were experienced. In pilot experiments we obtained very low yields of RNA from microspores using a standard method for total RNA isolation from leaves. As a consequence, it took about 3 months to collect sufficient material to obtain one RNA sample of 10 μg. Traditional ways to disrupt cells, like sonification or squashing in the presence of sand did not result in a yield improvement of RNA isolated from the microspores. Most likely this was due to the presence of the exine, which is composed of sporopollenin, a

Table 1: Genomic DNA and total RNA (in μg per 30 anthers) isolated from anthers, total RNA (in μg per 30 anthers) isolated from microspores at day 0, and microspore vitality (in %) during pretreatment.
a = anther, m = microspore

day	DNA a	RNA a	RNA m	% vitality
-4	28.3±1.2	24.3±5.1	n.d.	27.5±4.9
-3	13.5±1.3	15.4±9.2	n.d.	21.0±0.0
-2	10.4±1.8	7.6±4.0	n.d.	24.5±2.1
-1	2.4±0.5	2.6±0.8	n.d.	24.5±4.9
0	2.1±0.3	1.7±0.7	1.5±0.6	26.0±4.2

complex substance that is very resistant to degradation (McCormick, 1993). Besides this, pollen contain large amounts of RNAses (Matousch and Tupy, 1985). So, the main problem was the disruption of the exine without degrading the RNA inside the cell. Application of glass splinters and a specially designed pestle, together with an operating temperature below 0°C, were essential factors for a succesfull RNA isolation. Typically, about 100 pgr total RNA per microspore was isolated at day -4. Using the above described protocol, for the most difficult stage, namely microspores at day 0, also about 100 pgr total RNA per embryogenic microspore was routinely isolated. The quality of the RNA was tested on agarose gels. No degraded RNA was detected in the samples (data not shown). The size of the mRNA molecules was assessed using RT-PCR amplification. This method resulted in a banding pattern containing cDNA fragments up to 2 Kb (data not shown).

Degradation processes in anthers during pretreatment

A considerable decrease of total RNA was found during pretreatment (Table 1). At day 0, the amount of total RNA obtained from anthers was almost similar to that of microspores. A decrease in the amount of RNA in the anthers might be explained by a strong reduction in the number of viable cells. This is supported by the observation that during pretreatment the decrease in the amount of DNA and RNA was of the same magnitude (Table 1). Gelelectrophoresis of the DNA samples showed a clear banding pattern, indicative for extensive intranucleosomal cleavage (Figure 3). Apparently the anther tissue was dying due to the pretreatment, as no decrease in RNA levels was found if anthers were cultured without pretreatment directly on medium I (data not shown). Therefore the viability of the microspores in the anther tissue during pretreatment was investigated. No difference was found in the percentage of microspores that fluoresced upon staining with FDA between anther culture with (Table 1) or without (data not shown) pretreatment.

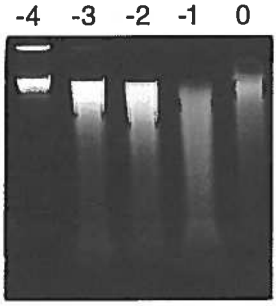


Figure 3: Genomic DNA (1 μg per lane), isolated from anthers during pretreatment, was loaded on a 1% (w/v) agarose in TBE buffer, stained with ethidium bromide and separated electrophoretically. The number above each lane corresponds with the day of pretreatment.

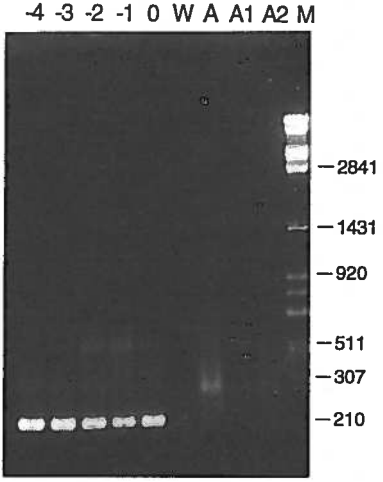


Figure 5: Domain directed differential display for glucanase of total RNA, derived from anthers during pretreatment on mannitol with premedium. The numbers above each lane correspond with the day of pretreatment. M = molecular weight marker (lambda DNA digested with SspI); A = aleurone; W = water; A_1 = aleurone with adaptor primer; A_2 = aleurone with family specific domain primer.

Domain directed differential display

Expression of three gene families was investigated during pretreatment in anthers isolated and cultured under sterile conditions. Using differential display with a family specific domain primer, several transcripts of "ABA responsive" genes were detected. One transcript of about 400 bp was pronouncedly present in the anther samples of day -4 and -3, and was also present in leaf and shoot samples.

In lane E1 and all microspore samples, an aspecific fragment of slightly higher molecular weight than the previous band, was obtained when only the adapter primer was present. Another RNA transcript of about 650 bp was clearly present at day -3 in the whole anther. Latter transcript was observed also in the samples derived from isolated microspores starting from day -3 and was visible till the end of pretreatment; expression was also found in the control samples of leaf, shoot and embryo (Figure 4). Fragments ranging from 300 upto 700 bp were expected deduced from the size of known "ABA responsive" genes in databanks.

If the primer, composed of base sequences of a conserved region in the glucanase family was used, one bright band was detected in all pretreatment samples of the anther tissue (Figure 5). Especially small fragments, like the strong but broad 200 bp glucanase band, were diffuse due to random annealing of the oligo-dT primer to the poly-A tail (a fragment of

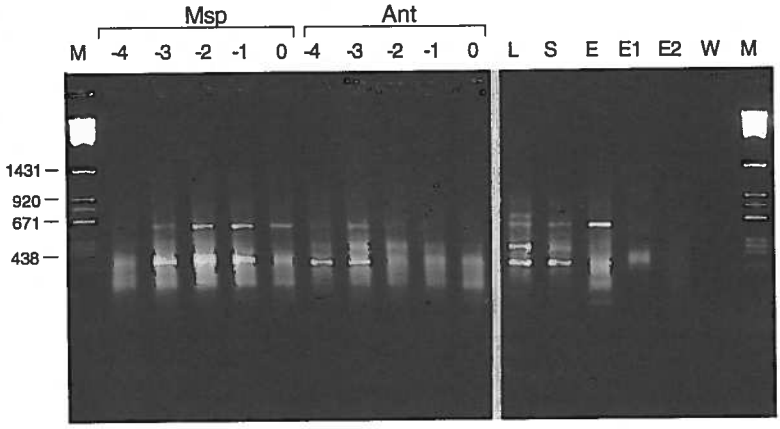


Figure 4: Domain directed differential display for "ABA responsive" genes of total RNA, derived from both anthers and microspores, during pretreatment on mannitol with pre-medium. The numbers above each lane correspond with the day of pretreatment. M = molecular weight marker (lambda DNA digested with SspI); Ant =anther; Msp = microspore; L = leaf; S = shoot; E = embryo; W = water; E_1 = embryo with adaptor primer; E_2 = embryo with family specific domain primer.

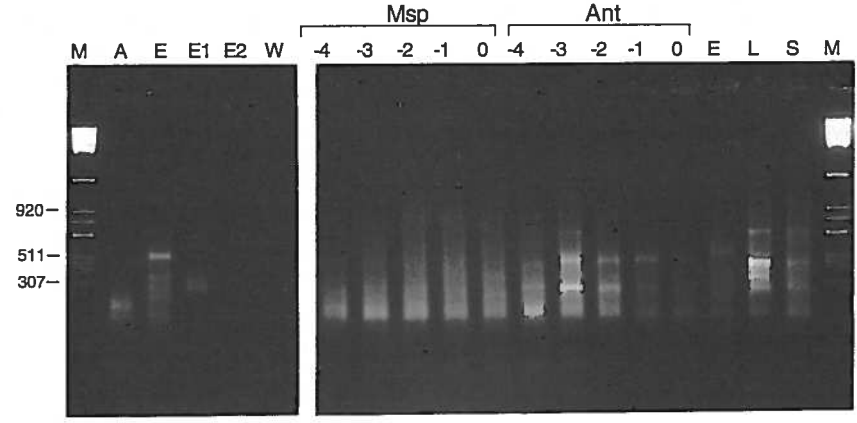


Figure 6: Domain directed differential display for chitinase, derived from both anthers and microspores, during pretreatment on mannitol with pre-medium. The numbers above each lane correspond with the day of pretreatment. M = molecular weight marker (lambda DNA digested with SspI); Ant = anther; Msp = microspore; L = leaf; S = shoot; E = embryo; W = water; E_1 = embryo with adaptor primer; E_2 = embryo with family specific domain primer.

200 to 300 bp was expected from databank information). Besides the strong band, also a faint band was visible at about 500 bp, which was due to aspecific adapter priming (lane A1). With the same approach, two chitinase RNAs of about 300 and 500 bp, showed highest expression in decreasing levels from day -3 to day 0 of pretreatment in the anther tissue, which correponded with the expected sizes ranging between 300 and 500 bp (Figure 6). The chitinases, however, appeared to be different from the observed chitinases expressed in leaf, shoot and embryo (Figure 6). The aspecific adapter band in lane E1 appeared to be slightly larger than the 300 bp band.

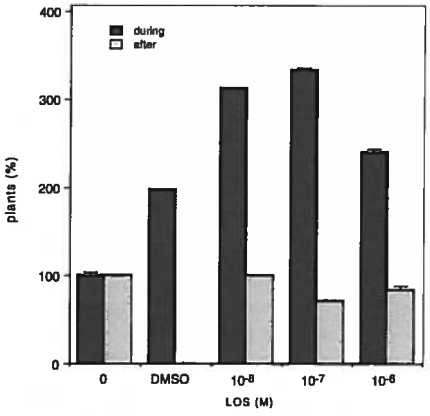


Figure 7: Plant formation in anther culture (in %) upon treatment with lipo-oligo saccharides (LOS, in M) applied during or after pretreatment.
Note: Data are presented in percentages of regeneration, since due to differences in microspore developmental stage and ELS density during culture, the absolute numbers varied. The patterns of the figures were however, reproducible throughout several experiments.

The influence of lipo-oligosaccharides on androgenesis

Application of LOS was performed for culture conditions, specified as during, after and without pretreatment, with no growth regulators present during pretreatment or in medium I.
If LOS were applied without pretreatment, nearly no plants developed (data not shown), as is usual under this condition (Hoekstra et al., 1996). Applying LOS after pretreatment, did not significantly improve plant production (Figure 7). However, if LOS were applied during pretreatment, a significant positive effect on plant production was found with an optimum at 10^{-7} M of LOS (Figure 7).

Discussion

The process of senescence can be described by features like a decline of chlorophyll, a specific ABA_i pattern, a decrease in proteins and RNA levels, while the DNA level remains constant, and an increase of hydrolytic enzyme concentration (Salisbury and Ross, 1991; Smart, 1994). The colour change of the anthers from green to yellow during pretreatment observed in this study, was accompanied by chlorophyll degradation and perhaps due to starvation of carbohydrates (Elamrani et al., 1994). The decline of chlorophyll as well as the specific pattern of ABA_i (an increase followed by a rapid decline) are typically described for senescence (Smart, 1994) and in this study observed upon culture *in vitro*. These phenomena, apparently, are not specific for the induction of

gametophytic embryogenesis. In the present work, under the conditions where androgenesis was induced, in addition to chlorophyll degradation, also protein and RNA break down (resulting in mainly microspore-derived RNA at day 0) was observed. In contrast to what happens during senescence however, we observed DNA cleavage into well defined fragments before total degradation occurred.

Degradation of DNA into oligomers of oligonucleosome-sized fragments as observed in this study, is a hallmark for programmed cell death, which also is called apoptosis (Raff, 1992). This author describes that another characteristic of apoptosis is shrinkage or fragmentation of the nucleus and cytoplasm, avoiding the cell's contents being spilled in the extracellular space and thereby preventing accidental cell death. For further proof for the occurence of programmed cell death during pretreatment, is in progress.

In many of the cases studied until now, an increase in the cytoplasmic calcium concentration appears to serve as a common, early signal for initiation of apoptosis (Ojcius et al., 1991). Calcium is also thought to activate preexisting endogenous endonucleases (Bowen, 1993; Mittler and Lam, in press). Some developmental pathways as well as some environmental insults, as disruption of cell-matrix (Frisch and Francis, 1994) or cell-cell (Bates et al., 1994) interactions, or exposure to stress (Bowen, 1993), result in the activation of apoptosis. According to Greenberg et al. (1994), active oxygen and peroxidation plays a prominent role in apoptosis. In animals, apoptosis is a ubiquitous phenomenon and plays an important role in the development of organs and whole organisms (Vaux et al., 1994) and in the control of tissue homeostasis (Raff, 1992).

In plants, little is known about the occurrence of apoptosis beyond the descriptive level, but apoptosis is thought to be involved in vascular tissue development (Chasan, 1994) and in hypersensitive response to pathogens (Greenberg et al., 1994; Mittler and Lam, in press). A specific level of stress or certain cellular conditions are needed for induction of apoptosis (Cotter and Al-Rubeai, 1995; Mittler et al., 1995). This could be in agreement with the specific pretreatment conditions required for induction of gametophytic embryogenesis.

According to Sunderland et al. (1984) for induction of androgenesis, degradation of the tapetum as well as properties of the anther wall are critical, and are more akin to those of maturing anthers near to anthesis (Sunderland et al., 1984). Comparison with literature data from microspore development *in vivo*, reveal that in most species the tapetum begins to degenerate shortly after the first pollen mitosis and is usually not present anymore at anthesis. So, anther dehiscence *in vivo* involves programmed destruction of specific cell types (Goldberg et al., 1993). The development of microspores, starting from the stage around the first pollen grain mitosis, into mature anthers normally takes 10 to 11 days (Hoekstra et al., unpublished). During anther pretreatment, degradation of the tapetum probably occurred earlier and did not have taken more than 3 days (see DNA level in Figure 3; Cho and Kasha, 1992).

From the data presented in this study, we propose the following mechanism of androgenesis induction. The anther is exposed to several kinds of stress. Upon harvest the cell-cell communication with

the vascular tissue is interrupted and the anther is placed on a solution containing mannitol and calcium as major components. In the anther tissue chlorophyll breaks down, and ABA is synthesized. At day -3 in the anther tissue a peak in gene expression is observed of stress-induced genes like "ABA responsive" genes and chitinase. The observed ABA_i peak at day -3 of pretreatment coincided with the occurence of anther-derived "ABA responsive" transcripts. Under the appropriate stress conditions, i.e. in the presence of mannitol and 30 mM calcium ions (Hoekstra et al., submitted), nucleases that initiate programmed cell death are activated. Accidental cell death is prevented and thereby leakage of endogenous ABA is limited. May be LOS are freed from the anther tissue during the process of apoptosis. Since the anther tissue is degrading, the microspores become gradually exposed to the pretreatment solution and a specific level of exogenous ABA. The microspores enlarge under these osmotic circumstances and microspore-derived "ABA responsive" transcripts, correspondig to the specific exogenous ABA level, are expressed: the production of what are called embryogenic microspores, is established at the end of pretreatment.

Degradation of the anther tissue for induction of androgenesis probably requires the activation of many genes that are also initiated during the process of *in vivo* anther dehiscence. An example are genes that encode hydrolytic enzymes required for cell wall degradation, such as glucanases (Goldberg et al., 1993). Four different glucanases have been isolated during *in vivo* anther development in *Lathyrus sp.* and the temporal pattern of expression of each glucanase gene is unique (Neelam and Sexton, 1995). In this study, one major glucanase transcript, being expressed throughout pretreatment, was detected. The expression of both glucanase and chitinase can be induced by pathogens (Ignatius et al., 1994), and a wide range of chemical compounds (van Loon, 1985) like ethylene (Siefert et al., 1994). Our data suggest that these enzymes can also be induced under aseptic conditions by apoptosis. Moreover, these enzymes have been detected in the medium of embryogenic cell suspension cultures of barley (Kragh et al., 1991) and are shown to play a role in early embryo development *in vitro* (DeJong et al., 1993). We detected different chitinase transcripts early during pretreatment in the anther tissue, which is slightly after the highest ethylene production at 12 hours of culture (Cho and Kasha, 1992). Indeed, Schmidt et al. (1993) mentioned that chitinase genes are also expressed in the absence of pathogens and that not all plant-produced chitinases do have an antifungal activity *in vitro*. The observation that expression of plant chitinases is correlated with plant development indicates that chitin-like molecules occur in uninfected plants and could play a role in plant development (Spaink and Lugtenberg, 1994). There is growing evidence that plants may be able to synthesize molecules analogous to Nod factors, which stimulate division in protoplasts derived from legumes and non-legumes (Schmidt et al., 1993b;

Spaink et al., 1993). Thus, rhizobial Nod factors may be mimicking endogenous plant factors and consequently receptors for Nod factor-like molecules could be present in both leguminous and non-leguminous species, having a more general role than symbiosis alone (Bono et al., 1995). In our study, a positive effect of application of Nod-like molecules was achieved, only when applied during pretreatment. We speculate that under optimal pretreatment conditions of androgenesis, apoptosis induces anther-derived glucanases and chitinases. Chitinase subsequently releases LOS from the anther wall, which contribute to the induction of embryogenesis in microspores.

Acknowledgement
Dr. A.M. Meijer (IMP, Leiden) is thanked for molecular assistance, J. Vink for growing the donor plants and P. Hock for the lay-out of the figures.

References

Bates R.C., A. Buret, D.F. VanHelden, M.A. Horton and G.F. Burns. Apoptosis induced by inhibition of intercellular contact. J. Cell Biol. 125: 403-415 (1994)

Bono J.J., J. Riond, K.C. Nicolaou, N.J. Bockovich, V.A. Estevez, J.V. Cullimore and R. Ranjeva. Characterization of a binding site for chemically synthesized lipo-oligosaccharidic NodRm factors in particulate fractions prepared from roots. Plant J. 7:253-260 (1995)

Bowen I.D. Apoptosis or programmed cell death? Cell Biol. Intern. 17: 365-380 (1993)

Chandler P.M. and M. Robertson. Gene expression regulated by abscisic acid and its relation to stress tolerance. Annu. Rev. Plant Physiol. Plant Mol. Biol. 45: 113-141 (1994)

Chasan R. Tracing tracheary element development. Plant Cell 6: 917-919 (1994)

Cho U.H. and K.J. Kasha. Relationship of senescence to androgenesis in barley (*Hordeum vulgare* L. cv. Klages). J. Plant Physiol. 139: 299-302 (1992)

Chomczynski P. and N. Sacchi. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem. 162: 156-159 (1987)

Cotter T.G. and M. Al-Rubeai. Cell death (apoptosis) in cell culture systems. Tibtech. 13: 150-155 (1995)

Davies W.J. and H.G. Jones (eds) In: Abscisic Acid physiology and biochemistry. Bio's scientific publishers limited (1991)

DeJong A.J., T. Hendriks, E.A. Meijer, M. Penning, F. LoSchiavo, M. Terzi, A. VanKammen and S.C. DeVries. Transient reduction in secreted 32 kD chitinase prevents somatic embryogenesis in the carrot (*Daucus carota* L.) variant *tsl1*. Dev. Genet. 16: 332-343 (1995)

DeJong A.J., R. Heidstra, H.P. Spaink, M.V. Hartog, E.A. Meijer, T. Hendriks, F. LoSchiavo, M. Terzi, T. Bisseling, A. VanKammen and S.C. DeVries. A carrot somatic embryo mutant is rescued by chitinase. Plant Cell 4: 425-433 (1993)

Elamrani A., J.P. Gaudillere and P. Raymond. Carbohydrate starvation is a major determinant of the loss of greening capacity in cotyledons of dark-grown sugar beet seedlings. Phys. Plant. 91: 56-64 (1994)

Fischer A., H. Saedler and G. Theissen. Restriction fragment length polymorphism-coupled domain-directed differential display: A highly efficient technique for expression analysis of multigene families. Proc. Natl. Acad. Sci. USA 92: 5331-5335 (1995)

Frisch S.M. and H. Francis. Disruption of epithelial cell-matrix interactions induces apoptosis. J. Cell Biol. 124: 619-626 (1994)

Frohman M.A., M.K. Dush and G.R. Martin. Rapid production of full-length cDNAs from rare transcripts: amplification using a single gen-specific oligonucleotide primer. Proc. Natl. Acad. Sci. USA 85: 8998-9002 (1988)

Genetics Computer Groups (1991), Program Manual for the GCG Package, Genetics Computer Groups, Madison

Goldberg R.B., T.P. Beals and P.M. Sanders. Anther development: basic principles and practical applications. Plant Cell 5: 1217-1229 (1993)

Greenberg J.T., A. Guo, D.F. Klessig and F.M.

Ausubel. Programmed cell death in plants: a pathogen-triggered response activated coordinately with multiple defense functions. Cell 77: 551-563 (1994)

Hoekstra S., M.H. VanZijderveld, J.D. Louwerse, F. Heidekamp and F. VanderMark. Anther and microspore culture of *Hordeum vulgare* L. cv. Igri. Plant Sci. 86: 89-96 (1992)

Hoekstra S., M.H. VanZijderveld, F. Heidekamp and F. VanderMark. Microspore culture of *Hordeum vulgare* L.: the influence of density and osmolarity. Plant Cell Rep. 12: 661-665 (1993)

Hoekstra S., S. VanBergen, I.R. VanBrouwershaven, R.A. Schilperoort and F. Heidekamp. The interaction of 2,4-D application and mannitol pretreatment in anther and microspore culture of *Hordeum vulgare* L. cv. Igri. J. Plant Phys. 148: 696-700 (1996)

Hoekstra S., I.R. VanBrouwershaven, S. VanBergen, R.A. Schilperoort and M. Wang. The role of mannitol, calcium and ABA during pretreatment in barley androgenesis. Submitted (1996)

Ignatius S.M.J., R.K. Chopra and S. Muthukrishnan. Effects of fungal infection and wounding on the expression of chitinases and ß-1,3 glucanases in near-isogenic lines of barley. Phys. Plant. 90: 584-592 (1994)

Koes R., E. Souer, A. VanHouwelingen, L. Mur, C. Spelt, F. Quattrocchio, J. Wing, B. Oppedijk, C. Ahmed, T. Maes, T. Gerats, P. Hoogeveen, M. Meesters, D. Kloos, J.N.M. Mol. Targeted gene inactivation in *Petunia* by PCR based selection of transposon insertion mutants. Proc. Nat. Acad. Sci. USA 92: 8149-8153 (1995)

Kragh K.M., S. Jacobsen, J.D. Mikkelsen and K.A. Nielsen. Purification and characterization of three chitinases and one ß-1,3-glucanase accumulating in the medium of cell suspension cultures of barley (*Hordeum vulgare* L.). Plant Sci. 76: 65-77 (1991)

Matousch J. and J. Tupy. The release and some properties of nuclease from various pollen species. J. Plant Physiol. 119: 169-178 (1985)

McCormick S. Male gametophyte development. Plant Cell 5: 1265-1275 (1993)

Mittler R. and E. Lam. Sacrifice in the face of foes: pathogen-induced programmed cell death in plants. Trends Microbiol., in press.

Mittler R., V. Shulaev and E. Lam. Coordinated activation of programmed cell death and defense mechanisms in trangenic tobacco plants expressing a bacterial proton pump. Plant Cell 7: 29-42 (1995)

Neelam A. and R. Sexton. Cellulase (endo ß-1,4 glucanase) and cell wall breakdown during anther development in the sweet pea (*Lathyrus odoratus* L.): isolation and characterization of partial cDNA clones. J. Plant Physiol. 146: 622-628 (1995)

Ojcius D.M., A. Zychlinsky, L.M. Zheng and J.D. Young. Ionophore-induced apoptosis: Role of DNA fragmentation and calcium fluxes. Exp. Cell Res. 197: 43-49 (1991)

Raff M.C. Social controls on cell survival and cell death. Nature 356: 397-400 (1992)

Salisbury F.B. and C.W. Ross (eds), Plant physiology. Wadsworth Publishing Company, Belmont California (1991)

Schmidt E.D.L., A.J. de Jong and S.C. de Vries. Signal molecules involved in plant embryogenesis. Plant Mol. Biol. 26: 1305-1313 (1993a)

Schmidt J., H. Roehrig, M. John, U. Wieneke, G. Stacey, C. Koncz and J. Schell. Alteration of plant growth and development by *Rhizobium nodA* and *nodB* genes involved in the synthesis of oligosaccharide signal molecules. Plant J. 4: 651-658 (1993b)

Scott R., R. Hodge, W. Paul and J. Draper. The molecular biology of anther differentiation. Plant Sci. 80: 167-191 (1991)

Siefert F., C. Langebartels, T. Boller and K. Grossmann. Are ethylene and 1-aminocyclopropane-1-carboxylic acid involved in the induction of chitinase and ß-1,3-glucanase activity in sunflower cell-suspension cultures? Planta 192: 431-440 (1994)

Skriver K. And J. Mundy. Gene expression in response to abscisic acid and osmotic stress. Plant Cell 2: 503-512 (1990)

Smart C.M. Gene expression during leaf senescence. New Phytol. 126: 419-448 (1994)

Smith P.K., R.I. Krohn, G.T. Hermanson, A.K. Mallia, F.H. Gartner, M.D. Provenzano, E.K. Fujimoto, N.M. Goeke, B.J. Olson and D.C. Klenk. Measurement of protein using bicinchoninic acid. Anal. Biochem. 150: 76-85 (1985)

Spaink H.P. and B.J.J. Lugtenberg. Role of rhizobial lipo-chitin oligosaccharide signal molecules in root nodule organogenesis. Plant Mol. Biol. 26: 1413-1422 (1994)

Spaink H.P., D.M. Sheeley, A.A.N. VanBrussel, J. Glushka, W.S. York, T. Tak, O. Geiger, E.P. Kennedy, V.N. Reinhold and B.J.J. Lugtenberg. A novel highly unsaturated fatty acid moiety of lipo-oligosaccharide signals determines host specificity of *Rhizobium*. Nature 354: 125-130 (1991)

Spaink H.P., H.M. Wijfjes, T.B. VanVliet, J.W. Kijne and B.J.J. Lugtenberg. Rhizobial lipo-oligosaccharide signals and their role in plant morphogenesis; are analogous lipophilic chitin derivatives produced by the plant? Austr. J. Plant Physiol. 20: 381-392 (1993)

Sunderland N., B. Huang and G.J. Hills. Disposition of pollen *in situ* and its relevance to anther/pollen culture. J. Exp. Bot. 35: 521-530 (1984)

VanLoon L.C. Pathogenesis-related proteins. Plant Mol. Biol. 4: 111-116 (1985)

Vaux D.L., G. Haecker and A. Strasser. An evolutionary perspective on apoptosis. Cell 76: 777-779 (1994)

Wang M., B. VanDuijn, R.M. VanderMeulen and F. Heidekamp. Effect of abscisic acid analogues on intracellular calcium level and gene expression in barley aleurone protoplasts. In: Karssen CM et al. (eds) Progress in plant growth regulation. Kluwer Academic Press, Dordrecht, Netherlands pp. 635-642

Wang M., S. Heimovaara-Dijkstra and B. VanDuijn. Modulation of germiantion of embryos isolated from dormant and nondormant barley grains by manipulation of endogenous abscisic acid. Planta 195: 586-592 (1995)

Chapter 7

General Discussion

In this chapter, first an overview of efficiencies during the development from microspore into plant will be described. The tempting question whether androgenesis follows the process of embryogenesis, is raised. Second a mechanism by which androgenesis might be induced is presented. Third, the major parameters in the assumed model are discussed, based on comparison with literature.

Efficiencies and embryogenesis

The efficiency of microspore derived plant production, determines the applicability of androgenesis in breeding. In chapter 1, 2, 3, 4 and 5, factors have been described that influence the plant production efficiency. In table 1, an overview of regeneration efficiencies of anther and microspore culture with the model variety of barley, cv. Igri, is presented. The differences found in regeneration efficiencies, can be explained by difference in the various parameters of the method used. Data from Table 1 reveal that the regeneration efficiency of barley androgenesis has been improved at least five-fold (see also chapter 3), and based on the results presented in chapter 5 a further improvement of about 40% can be expected. In conclusion, after comparison with literature data, the importance of the major parameters studied in the chapters 2 to 6, e.g. material selection including microspore developmental stage, pretreatment and growth regulator application, is confirmed.

With regard to the microspore developmental stage, the existence of a window for maximal plant production has been described in chapter 2. In fact, the required window of late uninucleate microspore turned out to be very small. It is probably linked with a specific stage in the cell cycle. Apparently cells only become competent, if the inducing trigger is applied before stages of final differentiation into mature pollen, with characteristics like starch deposition, mRNA accumulation (Goldberg et al., 1993), are commenced. Indeed,

Table 1: Regeneration efficiencies reported in literature for androgenesis in barley cv. Igri.
*suboptimal conditions

Reference(s)	n plants
anther culture	
Knudsen et al. 1989	2
Olsen 1987	4.6
Jähne et al. 1991	7.2
microspore culture	
Ziauddin et al. 1990	2.9
Olsen 1991	9.4
Kuhlman et al. 1991	10
Mordhorst et al. 1993	4.8
Cistué et al. 1995	17.4
Hoekstra et al. chapter 2	12.4
Hoekstra et al. chapter 3	50
Hoekstra et al. chapter 4	15.8*

Sangwan and Sangwan-Norreel (1987) hypothesize that in all androgenic species, starch accumulation only started at the late bicellular stage, wheras in the recalcitrant species accumulation occurred throughout pollen development. Especially as in barley a large part of the microspore population spontaneously diploidizes, the optimal stage might be different compared to other species, however, does not seem to be specific for barley. In tobacco, the application of the DNA replication inhibitor hydroxyurea during pretreatment, reveals accumulation of vegetative cells with the nucleus in S-phase which correlates with an increased embryo formation (Zarsky et al., 1992). Similar results are obtained by application of the microtubule reorganizer, colchicine (Zaki and Dickenson, 1991; Szakacs and Barnabas, 1995). These authors hypothesize symmetrical division as being required for embryo development. Considering the efficiency during the development of microspore into plant, in general high frequencies of first divisions are observed, although only a small fraction of the microspore population develops into plants. To date, in the model species *Brassica napus* cv. Topas at least 40% of the microspores undergoes a first division, but only 1% forms a plant (Custers et al., pers. comm.); in *Nicotiana sp.* upto 90% of the microspores start to divide, but the percentage that develops into plant has never been critically determined (Heberle-Bors et al., pers. comm.); in *Hordeum vulgare* cv. Igri 15% of the microspores progress until the first division three days after isolation, and about 10% of the isolated microspores develop into plants (Hoekstra et al., unpublished data). Apparently at most 10% of the microspores develop into a plant and this makes a hypothesis of many microspore subpopulations being embryogenic, possible. It would be very interesting to evaluate which microspores eventually develop into a plant. In collaboration with dr. T. Golds and prof.dr. H.U. Koop (University of Munich, Germany), a few cell tracking experiments were performed. Preliminary data revealed this to be extremely difficult. Although barley microspores could be embedded in

agarose (according to Golds et al., 1992) which was necessary for position fixation in a culture, the development upon isolation dramatically progressed within a few hours which hindered appropriate determination of the microspore morphology directly after isolation. Upon pretreatment in barley, a mixture of both asymmetric and symmetric division was observed and continuation of pollen maturation has never been observed under these conditions. In conclusion, cell tracking could not be commenced beyond the globular stage (about 16 days after microspore isolation) and could not be performed until and including the plantlet stage (Hoekstra, Golds and Koop, unpublished data). Sofar, no clear evidence for the requirement of symmetric divisions for embryogenic development has been demonstrated to my opinion, as the complete development from a single microspore into a plant has never been followed. Besides Touraev et al. (1995) show that upon symmetrical division, gametophytic development can be completed in tobacco, resulting in germination of pollen. These authors only recently observed similar to the situation in barley, both symmetrical and asymmetrical microspore mitoses and the formation of multicellular structures from the products of both types of division in tobacco (Touraev et al., in press).

Furthermore, the plane of the first division is supposed to be a first indication whether the development of microspore into plant indeed follows the embryogenic pathway. Comparison of the developmental pattern during gametophytic and zygotic embryogenesis shows, that the polarity of the zygote (Goldberg et al., 1994) strongly resembles the morphology of what is called an embryogenic microspore. The importance of an asymmetrical first division in zygotic embryogenesis has been emphasized by de Jong et al. (1993). West and Harada (1993) state that a transverse and asymmetric division of the zygote is not a prerequisite for embryogenic development in all plants and that there is no universal pattern of early cell cleavages. Pattern formation in the zygotic embryo of *Arabidopsis* depends largely on cell-cell communication (Jürgens, 1995). According to Meinke (1991) the initial division of the zygote is often transverse, but subsequent division patterns clearly differ between taxonomic groups. For the monocotyledonous species barley, early phases of zygotic embryogenesis are largely characterized by irregular patterns of early cell divisions (Sheridan, 1995). From our investigations we also got the impression that, with the exception of the first division, other cell divisions within the exine occur *at random*. However, a random plane of division in early gametophytic embryogenesis is also observed in *Brassica napus* (Telmer et al., 1995; Yeung et al., 1996). Latter authors find some differences in the morphology of zygotic and microspore-derived embryos and obtain the indication from their studies, that the tissues of the microspore-derived embryo are not always fully determined. Only after breakage of the exine, in both species a protoderm is formed, a key morphological feature of

embryogenesis in androgenesis. Moreover, from our microscopic observations no indication for indirect or secondary embryogenesis was found, since embryo-like structures as a whole acquired a smooth epidermal layer. These data do not elucidate the questions when the embryogenic development of the microspores is initiated and whether a phase specific for gametophytic embryogenesis, that is characterized by unorganized growth, preceeds embryogenesis. Based on the interpretation of both literature and experimental data, here the assumption is made that in microspores the first (a)symmetric division followed by an irregular pattern of divisions, is part of an early direct embryogenic pathway which takes place under specified *in vitro* conditions.

A model proposed for the induction of androgenesis

The importance of optimal pretreatment for maximal microspore-derived plant formation, has been demonstrated in various chapters: the time of pretreatment duration in chapter 2, the influence of the osmolality in chapter 3, the presence of anther tissue during pretreatment (chapter 4) and the concentrations of as well mannitol as calcium ions in chapter 5. Based on the results of chapter 4, 5 and 6, a model was generated by which the mechanism of androgenesis induction in barley might be explained. Key experimental data have been used to propose a mechanism for optimal plant production from androgenesis and that is visualized in Figure 1 and will be outlined below.

Note: In the description of the model two fonts are used in order to differentiate between experimental data and hypothesis: data from literature or from previous chapters of this thesis are printed in normal font, WHEREAS HYPOTHETICAL INFORMATION IS DISPLAYED IN SMALL CAPITAL.

Upon harvest and wounding, the anther is subjected to stress in several ways. First of all, cell-cell communication is interrupted, and the supply of nutrients from the vascular tissue has been cut off. Secondly, under optimal conditions for androgenesis the anther is subjected to a stress treatment: the anther is placed on a solution, containing mainly mannitol and calcium ions. The anther wall blocks the entry of these compounds into the locule containing microspores. Sensing the stress, the anther starts to break down the chlorophyll and to increase the ABA_i level. Around day -3 general stress-induced mRNAs, like "ABA responsive" (Skriver and Mundy, 1990) and chitinase (Collinge et al., 1993), are synthesized in the anther tissue. THEREUPON, protein and RNA are degraded, and between day -3 and -2 *de novo* ABA synthesis takes place. Under THE APPROPRIATE stress conditions in the presence of calcium ions, CA^{2+}-DEPENDENT ENDONUCLEASES BECOME ACTIVATED RESULTING IN NUCLEOSOMAL FRAGMENTATION, leading to programmed cell death or apoptosis. THE PRESENCE OF MANNITOL IN THE PRETREATMENT SOLUTION PREVENTS ENHANCED ANTHER CELL DEATH DUE TO AN INAPPROPRIATE OSMOTICAL LEVEL. As a result of programmed cell death, the ANTHER tissue gradually degrades

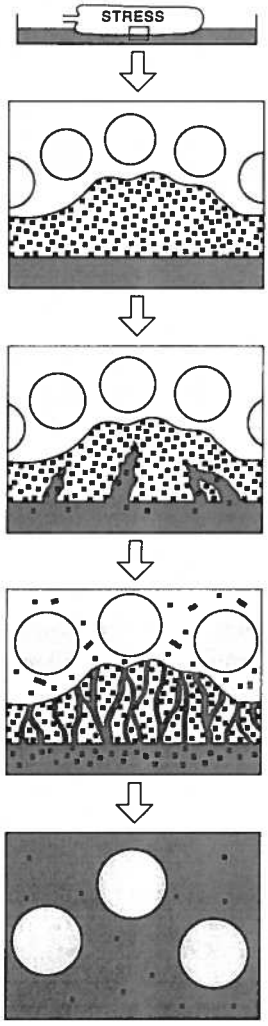


Figure 1: A schematic presentation of the mechanism proposed during anther pretreatment on mannitol with pre-medium leading to androgenesis in barley.
■ = ABA, ▬ =pretreatment solution, = microspore, □ = anther tissue

between day -3 and -1, ABA_i DISPERSES LITTLE BY LITTLE INTO THE EXTRACELLULAR SPACES AND THE PRETREATMENT SOLUTION, AND LIPO-OLIGO-SACCHARIDES (LOS) MIGHT BE FREED FROM THE CELL WALL BY CHITINASE ACTIVITY. An increase of the ABA_e level is already detectable 4 hours after the anthers are placed on the solution. DUE TO THE BREAK DOWN OF THE ANTHER TISSUE, THE MICROSPORES WITHIN THE ANTHER COME IN CONTACT WITH the gradually increasing ABA_e concentration TOGETHER WITH THE PRETREATMENT SOLUTION AND MAYBE LOS, STARTING FROM DAY -2. From that time point on, the microspores show expression of mRNAs from "ABA responsive" genes. A hypo-osmotical situation in 440 mOs/kg (by calcium ions and mannitol) is created resulting in fresh weight increase and enlargement of the microspores, and finally in the generation of embryogenic microspores.

The question now rises, what happens under suboptimal conditions for androgenesis, like the other three conditions (pre-medium, mannitol and water) analysed in chapter 5. In the presence of pre-medium, CALCIUM IONS PROMOTE PROGRAM MED CELL DEATH and ABA is gradually released into the solution until day -2. However, as no mannitol is present, NO APPROPRIATE OSMOTICAL ENVIRONMENT IS SENSED and during the last stages of apoptosis around day -1, STILL a rapid increase of ABA_e takes place. During pretreatment on water or mannitol, calcium ions are absent and a sudden extreme increase of

ABA_e occurs AS DURING ACCIDENTAL CELL DEATH. In the presence of mannitol a proper osmotical environment is created, and the rise of the ABA_e level is less extreme compared to pretreatment on water, but still far too high for optimal induction of androgenesis.
The microspores respond OPTIMALLY to a STIMULATORY BUT NON-TOXIC level of ABA_e, below 1000 pg/ml as in the environment of pre-medium with mannitol at 440 mOs/kg. Under these circumstances ABA SIGNAL TRANSDUCTION TAKES PLACE and significant production of specific mRNAs, like "ABA responsive" occurs in the microspores. THESE TRANSCRIPTS AND ACTIVATION OF OTHER GENES INVOLVED IN STRESS RESPONSE PROBABLY ARE REQUIRED FOR THE DEVELOPMENT INTO EMBRYOGENIC MICROSPORES.

A central part in the above described model for induction of androgenesis, is activation of programmed cell death in the anther tissue. Whether a cell will divide, differentiate or undergo apoptosis is determined by controls of the cell cycle, which govern the transitions of cell populations into and out of the proliferative state (Jacobs, 1992; King and Cidlowski, 1995; Meikrantz and Schlegel, 1995). Certain cell cycle proteins, like cyclin-dependent kinases have been suggested to control apoptosis (Meikrantz and Schlegel, 1995). Further testing of the occurrence of apoptosis during induction of embryogenesis would be worthwhile. For example, it would be interesting to know, whether Ca^{2+}-dependent endonuclease activity can be detected in the anther tissue (Mittler and Lam, 1995), or whether fragmentation of nuclei in cells from the anther wall can be observed during different pretreatment conditions (Earnshaw, 1995). Until very recently, a correlation between activation of programmed cell death and induction of embryogenesis has not been published. Havel and Durzan (1996) show that the process of apoptosis occurs during diploid parthenogenesis and early somatic embryogenesis of spruce.

In the presented model, apoptosis occurs in the anther tissue that surrounds the microspores. However, in addition apoptosis might take place in microspores. Only in the species *Brassica* and *Nicotiana,* microspores are generally pretreated without the presence of anther tissue, for development into a plant. In other species only very few if any plants are obtained when microspores are pretreated without anther tissue (Table 2). Moreover, pretreatment of isolated microspores more often results in plants, if the pollen developmental stage at pretreatment initiation is mid-binucleate (Kyo and Harada, 1986). Wei et al. (1986) report the same for barley, and mention that uninucleate pollen will die when pretreated without anther tissue.

Several explanations for the difference in capability of haploid plant production can be argued, without the presence of anther tissue during pretreatment, considering tobacco and rapeseed versus barley and rice. One reason might be that suboptimal regeneration conditions have been applied, comparable to the Kyo data that are improved by Touraev; however, after scrutinizing media and methods

used, this seems to be very unlikely.
Another reason might be that the concentration of a mitosis-related compound or signal (required for the induction of stress genes leading to embryogenic microspores), and that such molecules are not present in unicellular microspores and too low abundant or not as active in rice and barley bicellular pollen; it is plausible that the concentration or activity of such molecules is very dependent on the developmental stage of the starting material. Moreover, such molecules could be generated in the right molarity, if pretreatment takes place in the presence of the anther tissue (the developmental stage of the microspores progresses during pretreatment within the anther).
A third reason might be that an apoptosis-related compound or signal is too low abundant in isolated uninucleate microspores of rice and barley, and can be generated by apoptosis in the anther tissue both *in vitro* and *in vivo*. Such molecules could be supplied *in vivo* by the tapetal cells (these are present *in vivo* only till shortly after the first pollen mitosis; Goldberg et al., 1993). ABA could play a role in the model as a signal or by retardation of cell cycle completion to provide more time for the signal transduction (Müller et al., 1994). The nature of such (a) signal(s) might be diverse: it could also be a cell wall component stored during this specific developmental stage of the exine, or it could be a nucleotide. Uridine e.g. is found to act as a morphogen in plant roots (Smit et al., 1995).
A last reason that can be argued is that different species have a different mechanism for the induction of androgenesis, e.g. apoptosis is required in monocotyledonous crops, whereas this is not the case in dicotyledonous species.

Table 2: Plant production from isolated microspores pretreated without anther tissue presence in various species, with the developmental stage at isolation, plant production, and the corresponding references listed.
* 5-10% are mitotic or bicellular pollen

Species	Stage	embryos	plants	Reference(s)
Brassica	U/B*	many	many	Gland et al. 1988; Custers et al. 1994
Nicotiana	U/B*	many	many	Touraev et al. 1996
Nicotiana	U	0	0	Kyo and Harada 1986
	B	many	0	
Hordeum	U	0	0	Wei et al. 1986
	B	many	14	
Hordeum	U	0	0	Hoekstra et al. chapter 4 and unpublished data
Oryza	B	many	3	Ogawa et al. 1994

Pretreatments that induce androgenesis and a comparison with somatic embryogenesis

Activation of the cell death process is dependent on certain cellular conditions, and a specific level of stress is needed (Cotter and Al-Rubeai, 1995; Mittler et al., 1995). In chapter 5, the importance of relatively high calcium concentrations during pretreatment is demonstrated for barley. As already mentioned earlier, microspores are equally efficiently induced into the sporophytic pathway after cold pretreatment of barley spikes. The beneficial effect of increased calcium concentration during anther pretreatment at 25°C (chapter 5), if compared to cold pretreatment of spikes where no extra calcium ions are added, can be explained by a 15-fold increased influx of calcium at 4°C compared to 25°C (Monroy and Dhindsa, 1995).

Table 3: Various treatments that induce gametophytic embryogenesis in different species, with the the most important references.

Species	Inducer	Reference(s)
Nicotiana	starvation	Kyo and Harada 1986 Heberle-Bors 1989
	starvation plus heat	Touraev et al. 1996
Brassica	heat	Pechan et al. 1991 Custers et al. 1994
	gamma irradiation	MacDonald et al. 1988 Pechan and Keller 1989
	ethanol	Pechan and Keller 1989
	colchicine	Zhao et al. 1995
	anther dissection	Osolnik et al. 1993
Triticum	spike in water	Mejza et al. 1993
	colchicine	Szakacs and Barnabas 1995
	starvation plus ABA	Hu et al. 1995
	starvation plus heat	Touraev et al. in press
Zea	cold plus heat	Genovesi 1990
	proline on donor	Büter et al. 1991
Hordeum	cold	Huang and Sunderland 1982
	starvation	Roberts-Oehlschlager and Dunwell 1990 Hoekstra et al. chapter 3, 5
	2,4-D	Hoekstra et al. chapter 4
	LOS	Hoekstra et al. chapter 6

In all conditions required for induction of androgenesis mentioned throughout this thesis, single or multiple forms of stress are involved. During pretreatment, osmotic stress is evident. Further, both BAP and 2,4-D are used in combination with mannitol pretreatment, at very high concentrations (10^{-6} M or higher), exceeding physiological concentrations (chapter 2, 3 and 4). Moreover, in chapter 4 is demonstrated that by application of extremely high 2,4-D concentrations (10^{-4} M for 72 hours), also plant formation is possible without mannitol starvation. An overall comparison of treatments applied for induction of gametophytic embryogenesis in different species are listed in Table 3 and revealed that all treatments can be characterized by the term "stress". Further, the requirement of different stress treatments per species is obvious. On the contrary, recent experiments by Touraev et al. (1996 and in press) indicate that there might be a unified model for induction of androgenesis. If these inducing treatments for androgenesis are compared with treatments applied for induction of somatic embryogenesis (Table 4), a high degree of similarity can be observed.

Indeed, Dudits et al. (1991) proposed as a working hypothesis that the formation of embryos *in vitro* is a stress response. The method described by the group of Komamine (Osuga and Komamine, 1995), requires levels of $CaCl_2$ and mannitol that approach the concentrations used in chapter 5 (resp. 30 mM and 0.3M), except that the level of 2,4-D is 20 times lower. Masuda et al. (1995) use in the same carrot system the presence of 0.4 μM 2,4-D for 12 till 24 hours, which is a ten times reduced concentration compared to our results (chapter 4). Zimmerman (1993) mentioned that the time and concentration of 2,4-D required for induction depends on the nature of the explant and its competence for embryogenesis. The dose-response characteristics of growth regulators are

Table 4: Various treatments that induce somatic embryogenesis in *Daucus carota* with the most important references.
* in *Trifolium sp.*, *Linum sp.* and *Brassica napus*.

Inducer	Reference(s)
0.05 μM 2,4-D, 15 mM $CaCl_2$, 0.2 M mannitol	Osuga and Komamine 1995
12-24 hours 0.4 μM 2,4-D	Kamada et al. 1979
BAP*	Merkle 1990
cold treatment donor	Krul 1993
plasmolysis	Wetherell 1984
wounding, pH	Smith and Krikorian 1990a, b
heavy metals	Kiyosue et al 1990 and 1992 Roustan 1989

likely to result from sensitivity variation among the individual cells that constitute the responding tissue. In addition, biotime may be passing at different rates among individual cells or tissues of a single plant (Bradford and Trewavas, 1994). It is remarkable that somatic embryogenesis can also be induced by BAP in clovers, flax and oilseed rape (Merkle 1990). Besides, growth regulators are not necessary for induction in carrot as mechanically wounded mature zygotic embryos respond in hormone-free medium under specific pH conditions (Smith and Krikorian 1990a and b). The alternative pretreatment in barley androgenesis is cold incubation of spikes, and in carrot somatic embryogenesis is enhanced by cold pretreatment of stock plants (Krul, 1993). Further typical stress treatments like heavy metals also induce somatic embryogenesis (Kiyosue et al., 1990, 1992; Roustan, 1989). Finally it is worth to mention that pseudomeiosis is related to the acquisition of totipotency *in vitro* (Terzi and LoSchiavo, 1990). Their results provide another indication for the existence of a general mechanism for induction of both gametophytic and somatic embryogenesis.

Various stress treatments leading to one pathway or one response?

The question is raised, whether all these different stress treatments listed in Table 3 and 4, can activate expression of (the same) genes required for the same cellular response: induction of embryogenesis. In Table 5, a few examples are presented of a transcript or protein that is induced under a variety of environmental stimuli used for androgenesis induction. Data from Table 5 indicate further that there is a variety of transcripts or proteins induced under apparently similar conditions.

Below, molecular mechanisms are outlined that contribute to a general stress response according to Ruis and Schueller (1995), together with other relevant literature data. Some proteins already present in cells not pre-exposed

Table 5: Examples of a transcript or protein that is induced under different environmental conditions.

Stress type	References
carbon starvation, highT	Tassi et al. 1993
water, lowT, highT, ABA, NaCl	Pareek et al. 1995
lowT, ABA, osmoticum	Wang et al. 1995
carbon starvation, highT, heavy metals	Hsieh et al. 1995
water, ABA, salt,drought	Ishitani et al. 1995
water, lowT, ABA	Lång et al. 1992
highT, ABA, heavy metals, ethylene, 2,4-D	Czarnecka et al. 1988
lowT, ostmicum, dehydration	Holappa and Walker-Simmons 1995

to stress conditions, like protein kinases, will be activated by stress signals, as immediate or early response in the stress signal transduction pathway. For example from the wheat abscisic acid-responsive protein kinase, PKABA1, is suggested that it may be part of general environmental stress responses. Latter gene shares homology with yeast SNF1 protein kinases that are responsive to nutrient stress (Holappa and Walker-Simmons, 1995) indicating possible eukaryotic conservation of a general stress response. According to Ruis and Schueller (1995), such immediate or early responses initiate delayed or late responses, e.g. the synthesis of heat shock proteins (HSP). These delayed responses will protect cells more permanently and effectively by allowing an adaptation to persistent stress. Specific gene control elements and stress-activated transcription factors, like heat shock factors (HSF) binding to them, are necessary for this production of protective proteins. T h e investigations of Pareek and co-workers (1995) on HSP, also called stress proteins, are a good example for support of the model of androgenesis induction presented in this thesis. These authors observed the accumulation of two HMW HSPs of 90 and 104 kDa, when rice plants were subjected to high- and low-temperature stress, salinity, water stress, and exogenous abscisic acid application. Moreover in response to high-temperature stress, the same polypeptides were found in wheat, sorghum, pea, maize, mustard and yeast. These authors suggest that both proteins are conserved stress proteins. Indeed, conservation of the nucleotide sequence of both HSP genes and their transcriptional activators, HSF genes, has well been characterized in at least certain cases (Czarnecka-Verner et al. 1995; Vierling, 1994). Different stress conditions according to Ruis and Schueller (1995), might trigger a general stress response by creating the same intracellular signal, like alterations in cytoskeletal structures. In the moss *Funaria hygrometrica*, a similar disturbance of polar growth is observed upon exposure to either the heavy metal lead or colchicine (Basile et al., 1995). Different parts of the described general stress response mechanism can be recognized in the data available for the androgenesis model *Brassica napus*: induction is accompanied by accumulation of 70kDa HSP (Cordewener et al., 1995) and changes in the cytoskeleton, i.e. disappearance of microtubular arrays, are observed upon heat treatment (Hause et al., 1993). By application of the microtubule disruptor colchicine, Zhao et al. (1996) show that without heat treatment, rapeseed microspores can also complete the sporophytic pathway. These data indicate (only) a general stress response during induction of embryogenesis.

Activation of *hsp* genes may simply reflect a normal stress response, but it is also possible that specific HSPs are directly associated with the process of induction of androgenesis. The latter possibility is not unlikely, since over the last few years it has become clear that many *hsp* genes, in animals as well as in plants, are not only induced by heat or sometimes by other stress factors, but are also developmentally regulated, primarily

during gametogenesis and embryogenesis (Atkinson et al., 1993; Marrs et al., 1993; Zarsky et al., 1995). An alfalfa LMW *hsp* gene is transcriptionally activated in early somatic embryos (Györgyey et al., 1991). Using this alfalfa cDNA clone, a tobacco homologue was isolated and its expression pattern studied in tobacco. Northern analysis reveals that the transcript is detected in both early microspore-derived and zygotic embryos, expression is activated at normal temperature during the dehydration phase of *in situ* pollen development, and a dramatic increase of the transcript level is found upon *in vitro* starvation leading to embryogenic microspores (Zarksy et al., 1995).

Further, the compound 2.4-D also induces accumulation of HSPs (Czarnecka et al., 1988). Genes that are induced upon 2,4-D treatment have been isolated. Droog et al. (1993) demonstrate that the encoded proteins show homology with glutathione S-transferases. Such 2,4-D-inducible glutathione S-transferases are not related to auxin activity or to changes in the endogenous auxin levels (Flury et al., 1995). Functions proposed for the protein by Droog et al. (1993) are protection against damaging effects of oxidative reactions or induction of plant defense genes. Besides, a glutathione S-transferase gene is found to be induced during senescence (Smart et al., 1995). All data available on 2,4-D induced proteins, point to a role in certain types of stress.

After application of 2,4-D to an alfalfa microcallus suspension, the level of expression of the cdc2 gene was significantly increased (Dudits et al., 1991). The cdc2 encodes a protein kinase, p34, which is assumed to be a key regulatory element in the cell cycle control (Nagl, 1995). The Arabidopsis cdc2a gene expression is not always coupled with cell proliferation, but may reflect a state of competence to divide and is induced upon 2,4-D treatment (Hemerly et al., 1993). Comparable accumulation of the cdc2 transcript, is detected when LOS are applied (Spaink et al., 1994). Such chitin derivatives also occur in secondary plant cell walls of various plant species (Benhamou and Asselin, 1989; Spaink et al., 1993). Expression of some plant chitinases is correlated with plant development, indicating also that chitin-like molecules occur in uninfected plants and could play a role in plant development (Spaink et al., 1994). It would be interesting to investigate which molecule specifically is required for induction of barley androgenesis, as a mixture of four different molecules is applied in the LOS experiments described in chapter 6. Chitin-like molecules could be rather generally involved in induction of embryogenesis, as these molecules even might play a role during embryogenesis in vertebrates (Spaink et al., 1994).

Genes involved in the induction of embryogenesis

From the text above, it is clear that sofar aspecific, general stress responsive genes and their products are found to be associated with induction of embryogenesis. Little is known about specific

genes involved in the induction of and during early embryogenesis, as analysis is hampered by apparent material availability. One gene that produces a lipid transfer protein (EP2) has been particularly useful as a marker for epidermal cell differentiation during embryogenesis (Sterk et al., 1991). In two other studies genes are isolated about 3 days after transfer to auxin free medium and both studies assume that the gene products play an important role as a cell wall protein (Dudits et al., 1991; Sato et al., 1995). Further, a cDNA differentially expressed in carrot hypocotyls after 10 hours of 2,4-D treatment, shows no homology with the EMBL nucleotide sequence data base (Nagata et al., 1993). Recently PCR methods are used such as mRNA differential display (Liang and Pardee, 1992) especially suitable for small amounts of tissue aiming at identification of active genes. Using this method, Momiyama et al. (1995) detected differential expression (of unknown function) in early stages of eggplant embryogenesis. Recently Schmidt et al. (in preparation) isolated a serine kinase gene, that is correlated with very early stages of somatic embryogenesis in *Daucus carota*. Specifically on molecular analysis of gametophytic embryogenesis, sofar only one publication appeared. In wheat, two embryoid-abundant genes of unknown function are detected which activity starts 2 days after isolation (Reynolds et al., 1992). Very recently a first confirmation of part of the model is found in the results of Reynolds et al. (in press). His data show accumulation of an ABA-responsive, early cysteine-labeled metallothionein transcript during the first 24 to 48 hours of wheat anther culture. The transcript is absent in freshly isolated anther tissue, but is also present in developing zygotic embryos. He further finds ABA biosynthesis a requirement for the ability of microspores to form embryoids.

The importance of the cell wall

Studies aiming at detection of morphological differences between embryogenic and non-embryogenic tissue reveal the importance of cell-cell communication. Once the embryogenic nature is established, characteristics of embryogenic cells are a closer adherence and a higher number of plasmodesmata (Emons et al., 1992; Kikuchi et al., 1995). Plasmolysis of explant cells is shown to enhance induction of somatic embryogenesis in carrot. These factors are presumed to alter the epigenetic state of cells, and may be related in their ability to disrupt the cell-cell interactions required to maintain coordinated patterns of development (Merkle, 1990). Comparison with the situation during the early stage of zygotic embryogenesis shows a barrier between the inner ovule cell layer and the embryo sac which prevents the direct transfer of material between these compartments (disruption of cell-cell interaction) (Goldberg et al., 1994). Other factors found to cause enhancement of embryogenesis induction, are arabino-galactan-proteins (AGP) (Kreuger et al., 1995; Egersdotter and VonArnold, 1995). AGPs are proteo-

glycans occurring on cell membranes and are supposed to play a role in a.o. pathogen defense (Kreuger and VanHolst, in press). Recently is shown, that AGPs identify those cells of the coleoptile which are committed to programmed cell death (Schindler et al., 1995). Based on the model presented in this chapter, these data could be in agreement and support the importance of the cell wall. In the Fucus zygote it is shown that different cell fates may be imprinted into the cell wall (Berger et al., 1994).

It might be that cells *in vitro* have the potential to produce putative maternal or gametophytic factors under the proper conditions. According to Goldberg et al. (1994) literature data suggest that embryo morphogenesis and cell specification events are directed primarily by the expression of the zygotic genome after the fertilization process, which would significantly differ from animals in which maternally supplied factors influence the patterns of early embryo development. However, with the involvement of programmed cell death during *in vitro* embryogenesis, putative apoptosis-derived compounds, factors from the cell wall and cell-cell communication might mimick maternal factors occurring during zygotic embryogenesis.

Concluding Remarks

The aim of this thesis was two-fold, as is described in chapter 1, a "direct-breeding-applicable" part and a fundamental part. Parameters which are directly applicable in breeding for the production of doubled haploids in barley are presented in chapter 2, 3, 4 and 6. Further, the fundamental part focussed on analysis of the anther pretreatment (chapter 5 and 6), and resulted in the model which is described above in detail. The model presented provides two valuable tools for optimization of androgenesis. Both screening for the occurrence of apoptosis and determination of the extracellular ABA concentration during pretreatment are fast and efficient methods which can be performed within a few days in small amounts of tissue, further enabling improvement of androgenesis-derived plants in breeding.

A research lead for the future is evaluation of the fit of the pretreatment model for a variety of species in androgenesis as well as somatic embryogenesis.

References

Atkinson B.G., M.B. Raizada, R.A. Buchard, J.R.H. Frappier and D.B. Walden. The independent stage-specific expression of the 18kDa heat shock protein genes during microsporogenesis in *Zea mays* L. Dev. Genet. 14: 15-26 (1993)

Basile A., S. Giordano, V. Spagnuolo, F. Alfano and R. Castaldo Cobianchi. Effect of lead and colchicine on morphogenesis in protonemata of the moss *Funaria hygrometrica*. Ann. Bot. 76: 597-606 (1995)

Benhamou N. and A. Asselin. Attempted localization of a substrate for chitinases in plant cells reveals abundant *N*-acetyl-D-glucosamine residues in secondary walls. Bio. Cell 67: 341-350 (1989)

Berger Science 263: 1421-1423 (1994)

Bradford K.J. and A.J. Trewavas. Sensitivity thresholds and variable time scales in plant hormone action. Plant Phys. 105: 1029-1036 (1994)

Büter B., J.E. Schmid and P. Stamp. Effects of L-proline and post-plating temperature treatment on maize (*Zea mays* L.) anther culture. Plant Cell Rep. 10: 325 (1991)

Collinge D.B., K.M. Kragh, J.D. Mikkelsen, K.K. Nielsen, U. Rasmussen and K. Vad. Plant chitinases. Plant J. 3: 31-40 (1993)

Cotter T.G. and M. Al-Rubeai. Cell death (apoptosis) in cell culture systems. Tibtech. 13: 150-155 (1995)

Cordewener J.H.G., G. Hause, E. Goergen, R. Busink, B. Hause, J.J.M. Dons, A.A.M. VanLammeren, M.M. VanLookeren Campagne and P. Pechan. Changes in synthesis and localization of members of the 70-kDa class of heat shock proteins accompany the induction of embryogenesis in *Brassica napus* L. microspores. Planta 196: 747-755 (1995)

Custers J.B.M., J.H.G. Cordewener, Y. Noellen, J.J.M. Dons and M.M. VanLookeren Campagne. Temperature controls both gametophytic and sporophytic development in microspore cultures of *Brassica napus*. Plant Cell Rep. 13: 267-271 (1994)

Czarnecka-Verner E., C.X. Yuan, P.C. Fox and W.B. Gurley. Isolation and characterization of six heat shock transcription factor cDNA clones from soybean. Plant Mol. Biol. 29: 37-51 (1995)

Czarnecke E., R.T. Nagao, J.L. Key and W.B. Gurley. Characterization of *Gmhsp26-A*, a stress gene encoding a divergent heat shock protein of soybean: heavy-metal-induced inhibition of intron processing. Mol. Cell Biol. 8: 1113-1122 (1988)

DeJong A.J., R. Heidstra, H.P. Spaink, M.V. Hartog, E.A. Meijer, T. Hendriks, F. LoSchiavo, M. Terzi, T. Bisseling, A. VanKammen and S.C. DeVries. A carrot somatic embryo mutant is rescued by chitinase. Plant Cell 4: 425-433 (1993)

Droog F.N.J., P.J.J. Hooykaas, K.R. Libbenga and E.J. VanderZaal. Proteins encoded by an auxin-regulated gene family of tobacco share limited but significant homology with glutathione S-transferases and one member indeed shows *in vitro* GST activity. Plant Mol. Biol. 21: 965-972 (1993)

Dudits D., L. Boegre and J. Gyoergyey. Molecular and cellular approaches to the analysis of plant embryog development from somatic cells *in vitro*. J. Cell Sci. 99: 475-484 (1991)

Earnshaw W.C. Nuclear changes in apoptosis. Curr. Opinion Cell Biol. 7: 337-343 (1995)

Egersdotter U. And S. VonArnold. **Importance of arabinogalactan proteins for the development of somatic embryos of Norway spruce (*Picea abies*). Physiol. Plant. 93: 334-345 (1995)**

Emons A.M.C., J.W. Vos and H. Kieft. A freeze fracture analysis of the surface of emrbyogenic and non-embryogenic suspension cells of *Daucus carota*. Plant Sci. 87: 85-97 (1992)

Flury T., D. Adam and K. Kreuz. A 2,4-D-inducible glutathione S-transferase from soybean (*Glycine max*). Purification, characterisation and induction. Phys. Plant. 94: 312-318 (1995)

Genovesi A.D. Maize (*Zea mays* L.): *In vitro* production of haploids. In: Biotechnology in agriculture and forestry 12. Haploids in crop improvement I. Bajaj Y.P.S. ed., Springer Verlag, pp.176-203 (1990)

Gland A., R. Lichter and H.G. Schweiger. Genetic and exogenous factors affecting embryogenesis in isolated microspore cultures of *Brassica napus* L. J. Plant Physiol. 132: 613-617 (1988)

Goldberg R.B., T.B. Beals and P.M. Sanders. Anther development: basic principles and practical applications. Plant Cell 5: 1217-1229 (1993)

Goldberg R.B., G. DePaiva and R. Yadegari. Plant embryogenesis: zygote to seed. Science 266: 605-614 (1994)

Goldberg R.B., P.M. Sanders and T.P. Beals. A novel cell-ablation strategy for studying plant development. Phil. Trans. R. Soc. Lond. B 350: 5-17 (1995)

Golds T.J., J. Babczinsky, G. Rauscher and H.U. Koop. Computer-controlled tracking of single cell development in *Nicotiana tabacum* L. and *Hordeum vulgare* L. Protoplasts embedded in agarose/alginate films. J. Plant Physiol. 140: 582-587 (1992)

Györgyey J. A. Gartner, K. Nemeth, Z. Magyar, H. Hirt, E. Heberle-Bors and D. Dudits. Alfalfa heat shock genes are differentially expressed during somatic embryogenesis. Plant Mol. Biol. 16: 999-1007 (1991)

Hause B., G. Hause, P. Pechan and A.A.M. VanLammeren. Cytoskeletal changes and induction of embryogenesis in microspore and pollen cultures of *Brassica napus* L. Cell Biol. Int.

17: 153-168 (1993)
Havel L. and D.J. Durzan. Apoptosis during parthenogenesis and early somatic embryogenesis of norway spruce. Int. J. Plant Sci. 157: 8-16 (1996)
Heberle-Bors E. Isolated pollen culture in tobacco: plant reproductive development in a nutshell. Sex. Plant Reprod. 2: 1-10 (1989)
Hemerly A.S., P. Ferreira P., J. DeAlmeida Engler, M. VanMontagu, G. Engler and D. Inze. *Cdc2a* expression in *Arabidopsis* is linked with competence for cell division. Plant Cell 5: 1711-1723 (1993)
Holappa L.D. and M.K. Walker-Simmons. The wheat abscisic acid-responsive protien kinase mRNA, PKABA1, is up-regulated by dehydration, cold temperature, and osmotic stress. Plant Physiol. 108: 1203-1210 (1995)
Hsieh H.M., W.K. Liu and P.C. Huang. A novell stress-inducible metallothionein-like gene from rice. Plant Mol. Biol. 28: 381-389 (1995)
Hu T.C., A. Ziauddin, E. Simion and K.J. Kasha. Isolated microspore culture of wheat (*Triticum aestivum* L.) in a defined media I. Effects of pretreatment, isolation methods, and hormones. In Vitro Cell Dev. Biol. 31: 79-83 (1995)
Huang B. and N. Sunderland. Temperature-stress in barley anther culture. Ann Bot 49: 77-88 (1982)
Ishitani M., T. Nakamura, S.Y. Han and T. Takabe. Expression of the betaine aldehyde dehydrogenase gene in barley in response to osmotic stress and abscisic acid. Plant Mol. Biol. 27: 307-315 (1995)
Jacobs T. Control of the cell cycle. Dev. Biol. 153: 1-15 (1992)
Jähne, A., P.A. Lazzeri, M. Jäger-Gussen and H. Lörz. Plant regeneration from embryogenic cell suspension derived from anther cultures of barley (*Hordeum vulgare* L.). Theor. Appl. Genet. 82: 74-80 (1991)
Jürgens G. Axis formation in plant embryogenesis: cues and clues. Cell 81: 467-470 (1995)
Kamada H. and H. Harada. Studies on the organogenesis in carrot tissue cultures I. Effects of growth regulators on somatic embryogeneis and root formation. Z. Pflanzenphysiol. 91: 255-266 (1979)
Kikuchi A., S. Satoh, N. Nakamura and T. Fujii. Differences in pectic polysaccharides between carrot embryogenic and non-embryogenic calli. Plant Cell Rep. 14: 279-284 (1995)
King K.L. and J.A. Cidlowski. Cell cycle and apoptosis: common pathways to life and death. J. Cell. Biochem. 58: 175-180 (1995)
Kiyosue T., S. Satoh, H. Kamada and H. Harada. Immunological detection of an embryogenic protein (ECP31) during stress-induced somatic embryogenesis in carrot. Can. J. Bot. 70: 651-653 (1992)
Kiyosue T., K. Takano, H. Kamada and H. Harada. Induction of somatic embryogenesis in carrot by heavy metal ions. Can. J. Bot. 68: 2301-2303 (1990)
Knudsen S., I.K. Due and S.B. Andersen. Components of response in barley anther culture. Plant Breed. 103: 241-246 (1989)
Kreuger M. and G.J. VanHolst. Arabinogalactan-proteins and plant differentiation. Plant Mol. Biol., in press.
Kreuger M., E. Postma, Y. Brouwer and G.J. VanHolst. Somatic embryogenesis of *Cyclamen persicum* in liquid medium. Physiol. Plant. 94: 605-612 (1995)
Krul W.R. Enhancement and repression of somatic embryogenesis in cell cultures of carrot by cold pretreatment of stock plants. Plant Cell Tiss. Org. Cult. 32: 271-276 (1993)
Kuhlmann U., B. Foroughi-Wehr, A. Graner and G. Wenzel. Improved culture system for microspores of barley to become a target for DNA uptake. Plant Breeding 107: 165-168 (1991)
Kyo M. and H. Harada. Control of the developmental pathway of tobacco pollen *in vitro*. Planta 168: 427-432 (1986)
Lång V., E. Mantfyla, B. Welin, B. Sundberg and E.T. Palva. Alternations in water status, endogenous abscisic acid content, and expression of rab 18 gene during the development of freezing tolerance in *Arabidopsis thaliana*. Plant Physiol. 104: 1341-1349 (1994)
Liang P. and A.B. Pardee. Differential display of eukaryotic messenger RNA by means of the polymerase chain reaction. Science 257: 967-971 (1992)
MacDonald M.V., M.A. Hadwiger, F.N. Aslam and D.S. Ingram. The enhancement of anther culture efficiency in *Brassica napus* ssp. *oleifera*

Metzg. (Sinsk.) using low doses of gamma irradiation. New Phytol. 110: 101- (1988)

Marrs K.A., E.S. Casey, S.A. Capitant, R.A. Bouchard, P.S. Dietrich, I.J. Mettler and R.M. Sinibaldi. Characterization of two maize HSP90 heat shock protein genes: expression during heat shock, embryogenesis, and pollen development. Dev. Genet. 14: 27-41 (1993)

Masuda et al J. Pl. Physiol. 145 531-534 (1995) is reference Kamada et al. In Vitro Cell Dev. Biol. 25 (89) 1163-1166)

Meikrantz W. and R. Schlegel. Apoptosis and the cell cycle. J. Cell. Biochem. 58: 160-174 (1995)

Meinke D.W. Perspectives on genetic analysis of plant embryogenesis. Plant Cell 3: 857-866 (1991)

Mejza S.J., V. Morgant, D.E. DiBona and J.R. Wong. Plant regeneration from isolated microspores of *Triticum aestivum*. Plant Cell Rep. 12: 149 (1993)

Merkle S.A., W.A. Parrott and E.G. Williams. Applications of somatic embryogenesis and embryo cloning. In: Plant tissue culture: applications and limitations, ed. S.S. Bhojwani, Elsevier pp. 67-101 (1990)

Mittler R. and E. Lam. Identification, characterization, and purification of a tobacco endonuclease activity induced upon hypersensitive response cell death. Plant Cell 7: 1951-1962 (1995)

Mittler R., V. Shulaev and E. Lam. Coordinated activation of programmed cell death and defense mechanisms in trangenic tobacco plants expressing a bacterial proton pump. Plant Cell 7: 29-42 (1995)

Momiyama T., Afele J.C., Saito T., Kayano T., Tabei Y., Takaiwa F., Takayanagi K., Nishimura S. Differential display identifies developmentally regulated genes during somatic embryogenesis in eggplant (*Solanum melongena* L.). Biochem. Biophys. Res. Comm. 213: 376-382 (1995)

Monroy A.F. and R.S. Dhindsa. Low-temperature signal transduction: induction of cold acclimation-specific genes of alfalfa by calcium at 25°C Plant Cell 7: 321-331 (1995)

Mordhorst A.P. and H. Lörz. Embryogenesis and development of isolated barley (*Hordeum vulgare* L.) microspores are influenced by the amount and composition of nitrogen sources in culture media. J. Plant Physiol. 142: 485-492 (1993)

Müller M.L., P.W. Barlow and P.E. Pilet. Effect of abscisic acid on the cell cycle in the growing maize root. Planta 195: 10-16 (1994)

Nagata R., Wada M., Satoh S., Kamada H., Harada H. A cDNA differentially expressed between embryogenic and nonembryogenic carrot (*Daucus carota* L.) cells. Plant Physiol. 103: 102 (1993)

Nagl W. Cdc2-kinases, cyclins, and the switch from proliferation to polyploidization. Protoplasma 188: 143-150 (1995)

Ogawa T., H. Fukuoka and Y. Ohkawa. Induction of cell division of isolated pollen grains by sugar starvation in rice. Breed. Sci. 44: 75-77 (1994)

Olsen F.L. Induction of micropsore embryogenesis in cultured anthers of *Hordeum vulgare*. The effects of ammonium nitrate, glutamine and asparagine as nitorgen sources. Carlsberg Res. Commun. 52: 393-404 (1987)

Olsen F.L. Isolation and cultivation of embryogenic microspores from barley (*Hordeum vulgare* L.). Hereditas 115: 255-266 (1991)

Osolnik B., B. Bohanec and S. Jelaska. Stimulations of androgenesis in white cabbage (*Brassica oleracea* var. *capitata*) anthers by low temperature and anther dissection. Plant Cell Tiss. Org. Cult. 32: 241(1993)

Osuga K. and A. Komamine. Synchronization of somatic embryogenesis from carrot cells at high frequency as a basis for the mass production of embryos. Plant Cell Tiss. Org. Cult. 39: 125-135 (1995)

Pareek A., S.L. Singla and A. Grover. Immunological evidence for accumulation of two high-molecular-weight (104 and 90 kDa) HSPs in response to different stresses in rice and in response to high temperature stress in diverse plant genera. Plant Mol. Biol. 29: 293-3-1 (1995)

Pechan P.M., D. Bartels, D.C.W. Brown and J. Schell. Messenger-RNA and protein changes associated with induction of *Brassica* microspore embryogenesis. Planta 184: 161-165 (1991)

Pechan P.M. and W.A. Keller. Induction of microspore embryogenesis in *Brassica napus* L. by gamma irradiation and ethanol stress. In Vitro Cell. Dev. Biol. 25: 1073-74 (1989)

Reynolds T.L. and R.L. Crawford. Expression of

an abscisic acid-responsive, early cyteine-labeled metallothionein gene during pollen embryogenesis in bread wheat (*Triticum aestivum*). Plant Mol. Biol. in press.

Reynolds T.L. abd S.L. Kitto. Identification of embryoid-abundant genes that are temporally expressed during pollen embryogenesis in wherat anther cultures. Plant Physiol. 100: 1744-1750 (1992)

Roberts-Oehlschlager S.L. and J.M Dunwell. Barley anther culture: pretreatment on mannitol stimulates production of microspore-derived embryos. Plant Cell, Tiss. Org. Cult. 20: 235-240 (1990)

Roustan J.P., A. Latche and J. Fallot. Stimulation of *Daucus carota* somatic embryogenesis by inhibitors of ethylene synthesis: cobalt and nickel. Plant Cell Rep. 8: 182-185 (1989)

Ruis H. and C. Schueller. Stress signalling in yeast. Bioessays 17: 959-965 (1995)

Sangwan R.S. and B.S. Sangwan-Norreel. Ultrastructural cytology of plastids in pollen grains of certain androgenic and nonandrogenic plants. Protoplasma 138: 11-22 (1987)

Sato S., T. Toya, R. Kawahara, R.F. Whittier, H. Fukuda and A. Komamine. Isolation of a corrot gene expressed specifically during early-stage somatic embryogenesis. Plant Mol. Biol. 28: 39-46 (1995)

Schindler T., R. Bergfeld and P. Schopfer. Arabinogalactan proteins in maize coleoptiles: developmental relationship to cell death during xylem differentiation but not to extension growth. Plant J. 7: 25-36 (1995)

Sheridan W.F. Genes and embryo morphogenesis in angiosperms. Dev. Genet. 16: 291-297 (1995)

Skriver K. And J. Mundy. Gene expression in response to abscisic acid and osmotic stress. Plant Cell 2: 503-512 (1990)

Smart C.M., S.E. Hosken, H. Thomas, J.A. Greaves, B.G. Blair and W. Schuch. The timing of maize leaf senescence and characterisation of senescence-related cDNAs. Phys. Plant. 93: 673-682 (1995)

Smit G., C.C. DeKoster, J. Schripsema, H.P. Spaink, A.A. VanBrussel and J.W. Kijne. Uridine, a cell division factor in pea roots. Plant Mol. Biol. 29: 869-873 (1995)

Smith D.L. and A. D. Krikorian. Somatic embryogenesis of carrot in hormone-free medium: external pH control over morphogenesis. Am. J. Bot. 77: 1634-1647 (1990)

Smith D.L. and A. D. Krikorian. Somatic proembryo production from excised, wounded zygotic carrot embryos on hormone-free medium: evaluation of the effects of pH, ethylene and activated charcoal. Plant Cell Rep. 9: 34-37 (1990)

Spaink H.P. and B.J.J. Lugtenberg. Role of rhizobial lipo-chitin oligosaccharide signal molecules in root nodule organogenesis. Plant Mol. Biol. 26: 1413-1422 (1994)

Spaink H.P., H.M. Wijfjes, T.B. VanVliet, J.W. Kijne and B.J.J. Lugtenberg. Rhizobial lipo-oligosaccharide signals and their role in plant morphogenesis; are analogous lipophilic chitin derivatives produced by the plant? Austr. J. Plant Physiol. 20: 381-392 (1993)

Sterk P., H. Booij, G.A. Schellekens, A. VanKammen and S.C. DeVries. Cell-specific expression of the carrot EP2 lipid transfer protein gene. Plant Cell 3: 907-921 (1991)

Szakács E. and B. Barnabás. The effect of colchicine treatment on microspore division and microspore-derived embryo differentiation in wheat (*Triticum aestivum* L.) anther culture. Euphytica 83: 209-213 (1995)

Tassi F., E. Maestri, F.M. Restivo and N. Marmiroli. The effects of carbon starvation on cellular metabolism and protein and RNA synthesis in *Gerbera* callus cultures. Plant Sci. 83: 127 (1993)

Telmer C.A., W. Newcomb and D.H. Simmonds. Cellular changes during heat shock induction and embryo development of cultured microspores of *Brassica napus* cv. Topas. Protoplasma 185: 106-112 (1995)

Terzi M. and F. LoSchiavo. Somatic embryogenesis. In: Plant tissue culture: applications and limitations, ed S.S. Bhojwani. Elsevier pp. 54-66 (1990)

Toureav A., A, Ilham, O. Vicente and E. Heberle-Bors. Stress-induced microspore embryogenesis in tobacco: an optimized system for molecular studies. Plant Cell Rep. 15: 561-565 (1996)

Touraev A., F. Lezin, E. Heberle-Bors and O. Vicente. Maintenance of gametophytic

development after symmetrical division in tobacco microspore culture. Sex. Plant Reprod. 8: 70-76 (1995)

Vierling E. The roles of heat shock proteins in plants. Annu. Rev. Plant Physiol. Plant Mol. Biol. 42: 579-620 (1994)

Wang H., R. Dastla, F. Georges, M. Loewen and A.J. Cutler. Promoters from *kin1* and *cor6.6*, two homologous *Arabidopsis thaliana* genes: transcriptional regulation and gene expression induced by low temperature, ABA, osmoticum and dehydration. Plant Mol. Biol. 28: 605-617 (1995)

Wei Z.M., M. Kyo and H. Harada. Callus formation and plant regeneration through direct culture of isolated pollen of *Hordeum vulgare* cv. Sabarlis. Theor. Appl. Genet. 72: 252-255 (1986)

West and Harada Plant Cell 5: 1361-1369 (1993)

Wetherell D.F. Enhanced adventive embryogenesis resulting from plasmolysis of cultured wild carrot cells. Plant Cell Tiss. Org. Cult. 3: 221-227 (1984)

Yeung E.C., M.H. Rahman and T.A. Thorpe. Comparative development of zygotic and microspore-derived embryos in *Brassica napus* L. Cv. Topas. I. Histodifferentiation. Int. J. Plant Sci. 157: 27-39 (1996)

Zaki M. and H. Dickinson. Modification of cell development *in vitro*: the effect of colchcine on anther and isolated microspore culture in *Brassica napus*. Plant Cell Tiss. Org. Cult. 40: 255 (1995)

Zaki M.A.M. and H.G. Dickenson. Microspore-derived emrbyos in *Brassica*: the significance of division symmetry in pollen mitosis I to embryogenic development. Sex. Plant Reprod. 4: 48-55 (1991)

Zarsky V., D. Garrido, N. Eller, J. Tupy, O. Vicente, F. Schöffl and E. Heberle-Bors. The expression of a small heat shock gene is activated during induction of tobacco pollen embryogenesis by starvation. Plant Cell Env. 18: 139-147 (1995)

Zarsky V., D. Garrido, L. Rihova, J. Tupy, O. Vicente and E. Heberle-Bors. Derepression of the cell cycle by starvation is involved in the induction of tobacco pollen embryogenesis. Sex. Plant Reprod. 5: 189-194 (1992)

Zhao J.P., D.H. Simmonds and W. Newcomb. Induction of embryogenesis with colchicine instead of heat in microposres of *Brassica napus* L. cv. Topas. Planta 198: 433-439 (1996)

Ziauddin A., E. Simion, and K.J. Kasha. Improved plant regeneration from shed microspore culture in barley (*Hordeum vulgare* L.) cv. Igri. Plant Cell Rep. 9: 69-72 (1990)

Zimmerman L. Somatic embryogenesis: A model for early development in higher plants. Plant Cell 5: 1411-1423 (1993)

Summery

The research described in this thesis deals with the optimization of androgenesis, a method by which (doubled) haploid plants from immature pollen or microspores are created. In addition, physiological and molecular aspects of androgenesis induction were studied. The method was performed with the crop barley (*Hordeum vulgare* L.) and in all chapters mainly the model cultivar Igri was used. Haploid plants contain half the genetic information of what is generally present in a (diploid) plant. Such haploid plants are an important tool in plant breeding for the development of new varieties with desired traits. Often, it can be rather complicated to determine the presence of a trait in a certain plant. Desired traits, like many disease resistances, are encoded by multiple genes that differ in level of dominance. The use of haploid plants enables a.o. to verify the presence of these problematic polygenic resistances, what is called fixation of traits. By integration of androgenesis-derived plants in breeding, the quality and the efficiency for the development of new varieties can be improved.

In chapter 1 the importance of a reproducible method for the production of doubled haploid is outlined. Furthermore, the state of the art in the literature and the lack of knowledge in this field are indicated. Based on these data and from the results presented in this thesis, three preconditions are formulated for an efficient induction of microspore-derived plant production: (i) the explant should be in the late-uninucleate microspore developmental stage, (ii) a pretreatment of anthers should be applied and (iii) growth regulators should be added into the culture medium. These preconditions are the key factors that are investigated in the following chapters.

In chapter 2 the basic method is described, in which material is used grown under controlled environmental conditions. The influence of four parameters on plant regeneration, namely (i) the microspore developmental stage, (ii) the duration of pretreatment, (iii) the composition of media and (iiii) the application of aeration, is tested. Optimal regeneration frequencies were obtained, if (i) at least 50% of the microspores were in the mid-late to late uninucleate stage at harvest of the anthers, if (ii) anthers were pretreated on a mannitol containing solution for 4 days, and if (iii) oxygen was supplied regularly to the cultures. Addition of vitamines and caseine hydrolysate to the culture medium stimulated regeneration only in microspore culture. Little if any effect was observed in anther culture. Microspore culture was at least five times more efficient in plant production than anther culture, resulting in 12,4 green plants per anther.

In chapter 3 studies on further optimization of the method for microspore culture is presented. The importance of the osmolality during pretreatment and in culture was demonstrated. Further, it is described how an embryogenic subpopulation of microspores can be recognized. Combination of optimal osmolality with adjustment of the microspore population with regards to homogenity, by standardizing the density of embryogenic microspores, resulted in 50 green plants per anther.

In chapter 4 the effect of varying the growth regulator concentration in anther and microspore cultures is shown. In stead of the generally applied cytokinine called benzylaminopurine (BAP), the synthetic auxine 2,4-dichlorophenoxy acetic acid (2,4-D) was used. Both a 2,4-D concentration of 10^{-6} M continuously present in the culture medium, and a higher concentration present for a shortened period of time (10^{-4} M for 1 hour), resulted in similar regeneration frequencies. The values observed were comparable to those for the regeneration obtained in BAP-derived cultures (see chapter 2 and 3). Production of plants in the absence of pretreatment also was possible, although at lower frequencies. It is concluded, that application of 10^{-4} M 2,4-D for 1 hour might be a more general applicable regeneration method, since barley microspores as well as small clusters of *Daucus carota* cells in suspension culture produce plants under such inductive conditions.

Chapter 5 and 6 deal with the pretreatment conditions for the anther tissue. In chapter 5 the influence of mannitol and calcium ion concentrations during pretreatment on regeneration frequency is presented. Optimal results were obtained by anther pretreatment on pre-medium containing 30 mM $CaCl_2$ dissolved in mannitol upto an osmolality of 440 mOs/kg. During pretreatment the concentration of abscisic acid (ABA), a stress-related hormone, was determined. A peak of endogenous ABA after 24 hours of pretreatment was found, which does not correlate with induction of androgenesis. The level of exogenous ABA however, proved to be important and was furhter analysed by either removal of pretreatment solution or application of the ABA biosynthesis inhibitor fluridone. The results show that for optimal development of plants the exogenous ABA concentration should be at a specific level between 24 and 48 hours after initiation of pretreatment.

In chapter 6 the question is addressed whether senescense is involved during pretreatment, and characteristics thereof are determined. It appeared that not the process of senescense, but programmed cell death or apoptosis took place. Based on expression patterns generated by domain directed differential display for "ABA responsive" genes, chitinase genes and glucanase genes, a model for induction of androgenesis in pretreated anthers is proposed. In the model, the process of calcium induced apoptosis plays a central role. Apoptosis is induced in the presence of calcium ions by calcium-depending nucleases. Since no necrotic cell death occurs, the exogenous ABA concentration increases little by

little during pretreatment. A stimulating, but non-toxic concentration of exogenous ABA induces the expression of specific "ABA responsive" genes in the microspores, resulting in the generation of embryogenic microspores at the end of anther pretreatment. Besides an observed stimulating effect of lipo-oligosaccharide (LOS) addition during pretreatment on plant production, is integrated in the described model.

In the last chapter the model of androgenesis induction is discussed in more detail. Aspects like the microspore developmental stage, the presence of the anther wall and various stress-inducing agents are reviewed. A comparison is made for induction of embryogenesis derived from either gametes or somatic cells. Moreover, efficiencies during barley androgenesis are compared with *Brassica napus* and *Nicotiana tabacum*. From this information, the embryogenic microspore type and the question whether the development of microspore into plant follows the embryogenic route, is discussed and compared with the process *in planta*, zygotic embryogenesis.

Samenvatting

Dit proefschrift beschrijft het optimaliseren van androgenese, een methode voor het genereren van (verdubbelde) haploide planten uit onrijpe stuifmeelkorrels, die microsporen worden genoemd. Daarnaast heeft een studie van fysiologische en moleculaire aspecten van androgenese plaats gevonden. Deze methode is toegepast bij het gewas gerst (*Hordeum vulgare* L.) en in alle hoofdstukken is voornamelijk de model cultivar Igri gebruikt. Haploide planten bevatten de helft van alle genetische informatie, die normaal gesproken in een (diploide) plant aanwezig is. Dergelijke planten zijn een belangrijk hulpmiddel in de plantenveredeling voor de ontwikkeling van nieuwe rassen met gewenste eigenschappen. In veel gevallen is het niet eenvoudig om vast te stellen of een plant bepaalde nuttige eigenschappen bezit. Gewenste eigenschappen, zoals bij voorbeeld vele soorten van ziekte-resistentie, worden gecodeerd door meerdere genen die elk verschillende niveaus van dominantie kunnen bezitten. In haploide planten is het o.a. mogelijk om de aanwezigheid van deze lastige zogenaamde polygene resistenties aan te tonen. Dit wordt het fixeren van eigenschappen genoemd. Door integratie van via androgenese verkregen planten in de veredeling, kan de kwaliteit en de efficientie bij het ontwikkelen van nieuwe rassen worden verbeterd.

In hoofdstuk 1 wordt het belang van een toepasbare methode voor produktie van verdubbelde haploiden nader toegelicht. Voorts zijn in dit hoofdstuk naast de stand van zaken in de literatuur tevens de lacunes in kennis op dit vakgebiek aangegeven. Gebleken is dat voor inductie van plant produktie via microsporen, aan een drietal voorwaarden voldaan moet worden: (i) het uitgangsmateriaal moet in het laat-uninucleate microsporen ontwikkelings-stadium zijn, (ii) er moet een voorbehandeling toegepast worden, en (iii) er moeten groeiregulatoren in het cultuur medium worden toegediend. Deze voorwaarden zijn in de volgende hoofdstukken nader beschreven.

In hoofdstuk 2 wordt de basis methode beschreven, waarbij wordt gebruik gemaakt van antheren die afkomstig zijn van planten die zijn opgegroeid onder geconditioneerde omstandigheden. De invloed van een aantal factoren op regeneratie van planten, namelijk (i) het microsporen ontwikkelingsstadium in de anther, (ii) de lengte van voorbehandeling van de anther, (iii) de samenstelling van media en (iiii) gebruik van aeratie, werden uitgetest. De beste regeneratie frequenties werden verkregen, als tenminste 50% van de microsporen in het mid-laat tot laat uninucleate stadium was, als antheren gedurende 4 dagen op een mannitol bevattende oplossing voor-

behandeld waren en als regelmatig zuurstof aan de cultures werd toegevoegd. Toevoeging van vitamines en caseine hydrolysaat aan het cultuurmedium heeft alleen een positief effect op microsporen cultuur. Weinig of geen effect werd waargenomen voor antheren cultuur. Bij toepassing van microsporen cultuur in plaats van antheren cultuur, werden minimaal vijf keer zo veel planten verkregen, resulterend in 12,4 groene planten per anther.

In hoofdstuk 3 wordt het onderzoek behandeld dat tot doel had de methode van microsporen cultuur verder te optimaliseren. Aangetoond werd dat de osmolaliteit tijdens de voorbehandeling en in het kweekmedium van belang was. Verder werd vastgesteld hoe een embryogene subpopulatie microsporen kan worden herkend. In combinatie met een optimale osmolaliteit, resulteerde standaardisatie van de dichtheid van de subpopulatie embryogene microsporen, in 50 groene planten per anther.

In hoofdstuk 4 wordt het effect van het varieren van de groeistof concentratie in microsporen cultures getoond. In plaats van het algemeen gebruikte cytokinine, dat benzylaminopurine (BAP) genoemd wordt, werd het synthetische auxine 2,4-dichlorophenoxy acetic acid (2,4-D) toegediend. Aangetoond werd dat zowel een continue aanwezigheid van concentratie 2,4-D (10^{-6} M) in het kweekmedium, als een hogere concentratie, maar dan gedurende een kortere tijd (10^{-4} M gedurende 1 uur), resulteerde in dezelfde regeneratie frequenties. Er werden waarden gevonden die vergelijkbaar zijn met waarden verkregen uit kweken met BAP (zie hoofdstuk 2 en 3). In afwezigheid van een voorbehandeling van antheren is het eveneens mogelijk om, weliswaar in lagere frequenties, plant vorming te induceren. Geconcludeerd is, dat toepassing van 10^{-4} M 2,4-D gedurende 1 uur kennelijk een meer algemeen toepasbare methode is voor regeneratie, aangezien deze conditie zowel bij gerst microsporen als bij kleine clusters van *Daucus carota* cellen in suspensiekweken, leidt tot goede plantvorming.

Hoofdstuk 5 en 6 handelen over de voorbehandelings condities van antheren. In hoofdstuk 5 wordt de invloed van mannitol en calcium ion concentraties tijdens de voorbehandeling op regeneratie frequentie getoond. De beste resultaten werden verkregen door antheren een voorbehandeling te geven op pre-medium dat 30 mM $CaCl_2$ bevatte en was opgelost in mannitol tot een osmolaliteit van 440 mOs/kg. Er werd tevens nagegaan in hoeverre de concentratie van abscisine zuur (ABA), een stress-gerelateerd hormoon, een rol speelt tijdens de voorbehandeling. Een piek van endogeen ABA na de eerste 24 uren van voorbehandeling werd waargenomen. Deze was niet gecorreleerd met de inductie van androgenese. Met behulp van het wegvangen van exogeen ABA of het toedienen van de biosynthese remmer fluridone, werd geconstateerd dat het niveau van exogeen ABA tussen 24 en 48 uur na het begin van de voorbehandeling, een specifiek niveau moet hebben voor optimale ontwikkeling van planten.

In hoofdstuk 6 is gekeken of er sprake is van een verouderingsproces tijdens de

voorbehandeling van antheren en zijn de daarvoor karakteristieke kenmerken bestudeerd. Uit deze studie bleek dat er geen veroudering, maar geprogrammeerde cel dood, ook wel apoptosis genoemd, plaats vond. Op basis van expressie patronen verkregen met domein specifieke differential display's voor "ABA responsieve" genen, chitinase genen en glucanase genen, is een model voor inductie van androgenese voorgesteld. Een centrale rol in dit model speelt het proces van apoptosis. In aanwezigheid van calcium ionen wordt apoptosis geinduceerd m.b.v. calcium-afhankelijke nucleases. Doordat er geen necrotische celdood plaats vindt, stijgt het gehalte van exogeen ABA geleidelijk tijdens de voorbehandeling. Een stimulerende, maar niet-toxische concentratie van exogeen ABA initieert de expressie van specifieke 'ABA responsieve' genen in microsporen, dat uiteindelijk leidt tot het ontstaan van embryogene microsporen aan het einde van de antheren voorbehandeling. Bovendien is een waargenomen stimulerend effect van toediening van lipo-oligosacchariden (LOS) tijdens de voorbehandeling op plant produktie, verwerkt in het beschreven model.

In het laatste hoofdstuk wordt het model van inductie van androgenese, uitgebreid bediscussieerd. Aspecten als het microsporen ontwikkelingsstadium de aanwezigheid van de antherewand en verschillende stress-inducerende agentia worden besproken. Tevens wordt een vergelijking gemaakt voor inductie van embryogenese vanuit gameten en somatische cellen. Daarnaast worden efficienties tijdens gerst androgenese vergeleken met de andere modelgewassen *Brassica napus* en *Nicotiana tabacum*. Aan de hand van deze informatie wordt het embryogene type microspore en het optreden van het proces van embryogenese in de ontwikkeling van microsporen tot plant, bediscussieerd en vergeleken met het proces dat plaats vindt aan de plant, de zygotische embryogenese.

Curriculum vitae

Sietske Hoekstra werd geboren op 30 juli 1963 te Amsterdam. In 1981 behaalde ze haar Gymnasium-β diploma aan het Zaanlands Lyceum te Zaandam, waarna ze in hetzelfde jaar begon met de studie biologie aan de Vrije Universiteit te Amsterdam. Tijdens haar studie werkte ze bij de vakgroep Moleculaire Genetica aan somatische cybridisatie en *Agrobacterium tumefaciens* transformatie in *Lycopersicon esculentum*. Hierna onderzocht zij de invloed van ectomycorrhizae bij *Tilia sp.* in de stad Amsterdam in een samenwerkingsproject tussen de vakgroepen Fytopathologie en Bosteelt, beide te Wageningen. Vervolgens werkte zij in het Fytopathologisch Laboratorium te Baarn aan de *in vitro* interactie tussen *Lycopersicon esculentum* en *Verticillium albo-atrum*. Zij heeft haar hoofdvak Moleculaire Genetica in Amsterdam afgerond met contract research naar somatische regeneratie en cybridisatie in *Brassica oleracea*; hiervoor vond een stage plaats bij prof.dr. K. Glimelius te Uppsala (Zweden). In augustus 1987 verkreeg zij de titel doctorandus en tegelijkertijd onderwijsbevoegdheid voor biologie.

Het *Brassica* onderzoek heeft zij als research medewerker van de afdeling Biotechnologie van Zaadunie B.V., te Enkhuizen voortgezet en CMS overdracht naar *Brassica oleracea* middels somatische cybridisatie bewerkstelligt.

Vanaf april 1989 was zij in dienst bij de afdeling Planten Biotechnologie van TNO-Voeding te Leiden. Zij is aldaar begonnen als research medewerker en verrichtte werkzaamheden in het kader van het ABIN-project (Adaptation of Barley for Industrial Needs) binnen het thema regeneratie en transformatie. De samenwerking en een stage bij het Carlsberg Research Laboratory (Denemarken) mogen hier niet onvermeld blijven. In 1992, 1993 en de eerste helft van 1994 heeft zij zich bezig gehouden met contract research en het samenwerkingsproject tussen TNO en de RUL op het gebied van transformatie en de moleculaire analyse van embryogenese in *Oryza sativa* en *Hordeum vulgare*. In het kader daarvan heeft zij onderzoek verricht in de vakgroep van prof.dr. H.U. Koop in München (Duitsland). In 1993 en 1994 heeft zij bovendien de functie marktontwikkelaar bekleed.

Vanaf juli 1994 was zij in dezelfde TNO afdeling werkzaam als produktmanager van het produkt 'Gewasverbetering'.

Sinds april 1996 is zij werkzaam als lijnmanager 'Gewas Transformatie' bij Mogen International N.V. te Leiden.

Publications

Articles

Hoekstra S., M.H. VanZijderveld, J.D. Louwerse, F. Heidekamp and F. VanderMark. Anther and microspore culture of *Hordeum vulgare* L. cv. Igri. Plant Science 86: 89-96 (1992)

Hoekstra S., M.H. VanZijderveld, F. Heidekamp and F. VanderMark. Microspore culture of *Hordeum vulgare* L.: the influence of density and osmolarity. Plant Cell Reports 12: 661-665 (1993)

Hoekstra S., M. VanZijderveld, S. VanBergen, F. VanderMark and F. Heidekamp. Genetic modification of barley for end use quality. In: Improvement of cereal quality by genetic engineering. eds. R.J. Henry and J.A. Ronalds, Plenum Press, pp. 139-144 (1994)

Hoekstra S., S. VanBergen, I.R. VanBrouwershaven, R.A. Schilperoort and F. Heidekamp. The interaction of 2,4-D application and mannitol pretreatment in anther and microspore culture of *Hordeum vulgare* L. cv. Igri. J. Plant Physiol. 25: ? (1996)

Hoekstra S., I.R. VanBrouwershaven, S. VanBergen, R.A. Schilperoort and M. Wang. Androgenesis in *Hordeum vulgare* L.: The role of ABA during anther pretreatment, on a solution containing mannitol and calcium. Manuscript submitted.

Hoekstra S., M. VanderHeijden, B. Oppedijk, S. VanBergen, F. Heidekamp and R.A. Schilperoort. Androgenesis in *Hordeum vulgare* L.: Apoptosis and the effect of lipo-oligosaccharides during anther pretreatment. Manuscript in preparation.

Patents

Hoekstra S., A.J. Kool, M.G. Nootebos and M.M.C. Tan. Improvements in or relating to organic systems: Method for producing CMS *Brassica oleracea* by protoplast fusion. Patent Application, filed in several countries e.g. GB 2211205B, FR2628601A, US5254802A.

Reports and abstracts

Broerse J., K. Kingma, S. Hoekstra, F. Heidekamp, P. Sluimer, T. VandeSande and J. Bunders. Biotechnology and cereal production for developing countries. European Parliament, Directorate General for Research, the STOA Programme, pp. 37-51 (1994)

Hoekstra S., M.H. VanZijderveld, N.J. Ruys, F. Heidekamp and F. VanderMark. Microspore-derived cellsuspensions and protoplast isolation from barley. Proceedings of Embo workshop "Molecular basis of plant embryogenesis", Doorwerth, Netherlands June 12-19 (1990) and of 7th IAPTC Symposium, Amsterdam, Netherlands June 24-29 (1990) pp. A1-62

Hoekstra S., M.H. VanZijderveld and F. VanderMark. Barley cellsuspension cultures derived from isolated microspores and their ploidy level. Abstract of 8th International Protoplast Symposium, Uppsala, Sweden June 16-20 (1991) pp. 216

Hoekstra S., M.H. VanZijderveld, J.D. Louwerse, F.L. Olsen, F. Heidekamp and F. VanderMark. Anther and microspore culture of *Hordeum vulgare* cv. Igri. Acta Botanica Neerlandica (1992)

Hoekstra S., M.H. VanZijderveld and F. VanderMark. Microspore culture of *Hordeum vulgare* L. Abstract of 13th Eucarpia Congress, Angers, France July 6-11 (1992) pp. 167

Hoekstra S., M.H. VanZijderveld, F. Heidekamp and F. VanderMark. Microspore culture of *Hordeum vulgare* L. Abstract of Gordon Conference, Plant Cell and Tissue Culture, Wolfeboro, NH, USA June 13-18 (1993) pp. A-16

Hoekstra S. Basic and applied aspects of the microspore culture system in *Hordeum vulgare* L. Workshop "Gametic embryogenesis" Ås, Norway June 24-27 (1993)

Hoekstra S., I. VanBrouwershaven, S. VanBergen and F. Heidekamp. Induction of differentiation in microspores of *Hordeum vulgare* L. Abstracts 8th International Congress

of Plant Tissue and Cell Culture, Firenze, Italy, June 12-17, (1994) pp. S3-44

Hoekstra S., M. VanderHeijden, B.O. Oppedijk, I.R. VanBrouwershaven, S. VanBergen, F. Heidekamp and R.A. Schilperoort. An Apoptosis-based model for the induction of androgenesis in *Hordeum vulgare* L. Society for In Vitro Biology, June 22-27 (1996)

Nawoord

Graag wil ik van deze gelegenheid gebruik maken om een aantal mensen erkentelijk te zijn.

De mensen die het TNO- lab op de 2e bevolkten. Speciaal wil ik noemen: Marion met wie ik samen heel wat 'supers' bekeken heb en enorme hoeveelheden suspensies versjouwd en overgezet heb, Jeanine met wie ik samen vele discussies had 'zou jij dit nou een late of een bi noemen' en met wie ik vele microsporen blauw en soms ook aan barrels geschoten heb, Frits voor zijn enthousiasme en zeer gewaardeerde kritische noot, en Jan V. die voor continue goede kwaliteit donoren zorgde en samen met San de thripsen te lijf ging.

Lok who gave me an introduction in androgenesis by transforming aleuron protoplasts in the middle of the night, and his colleagues Preben and Søren of Carlsberg.

Anke en Sas als de harde en gezellige kern van de rijstgroep: eerst als buren op de 2e en later voordeurdeler op de begane grond; bij Harry zal ik toch vaak terug moeten denken aan 'hoe zat het nou ook alweer met die ELS'...

En natuurlijk San (we hebben heel wat meegemaakt), Ilse (zóóó snel), Berry (waar een boor al niet goed voor is), Maurice (die éne gel) van de microsporen gerstclub, en de enerverende samenwerking met Mei. En uiteraard vele anderen, die het leven in het Clusius tot een aangenaam toeven maakten.

Naast 'het Leidse', heb ik de afgelopen jaren met veel plezier mijn contacten met de industrie onderhouden, die mij mijn theoriën over haploiden in praktijk laten zien.

De charme van het internationale netwerk met mensen als Erwin, Alisher, Bernd, Yves, Eva, Swapan, Masaharu, Şule, Hans Ulrich, Sasha, Ken, Debra, is een onmeetbare stimulans voor het onderzoek.

Graag wil ik mijn ouders bedanken voor hun eeuwige bereidheid om te helpen en de vele vrijdagen dat jullie op Nils pasten, zodat ik de handen vrij had om te schrijven.

Tot slot wil ik vooral Uli, maar ook Nils bedanken voor het koffie met iets lekkers brengen in de vele "labweekenden". Jullie gezelschap, steun, en relativeringsvermogen was onmisbaar om deze vrijetijdsbesteding zo af te kunnen ronden.....

Stellingen

1. Integratie van androgenese in de plantenveredeling wordt belemmerd door onderschatting van complexiteit van het biologische proces en daardoor ook van de methodiek.

2. 2,4-D is een stof, die embryogenese induceert in plantecellen, mits het in een niet-physiologische concentratie en bepaalde tijdsduur toegepast wordt.
Dit proefschrift.

3. Geprogrammeerde celdood of apoptosis ten gevolge van specifieke stress condities, is nodig voor inductie van *in vitro* embryogenese.
Dit proefschrift.

4. De conclusie van Zaki en Dickenson (1991) en Szakacs en Barnabas (1995), dat een symmetrische eerste deling voor de ontwikkeling van microspore tot plant in *Brassica napus* van belang is, is niet onomstotelijk bewezen.
Zaki M.A.M. and H.G. Dickenson. Sex. Plant Reprod. 4: 48-55 (1991)
Szakács E. and B. Barnabás. Euphytica 83: 209-213 (1995)

5. Microsporen transformatie in combinatie met pollen maturatie zal veel arbeidsintensieve weefselkweek overbodig maken.
Alwen A., N. Eller, M. Kastler, R.M. Benito Moreno and E. Heberle-Bors. Phys. Plant. 79: 194-196 (1990)

6. Voor het produceren van stabiel getransformeerde planten is toepassing van DNA overdracht m.b.v. *Agrobacterium tumefaciens* te prefereren boven het deeltjesgeweer.
Hiei Y., S. Ohta, T. Komari and T. Kumashiro. Plant J. 6: 271-282 (1994)
Christou P., T.L. Ford and M. Kofron. Biotechnology 9: 957-962 (1991)

7. Het aantal niet-detecteerbare differentials, die gegenereerd zijn met mRNA differential display middels PCR (Liang en Pardee, 1992) kan verlaagd worden, wanneer *in situ* hybridisatie als detectie methode gebruikt wordt.
Liang P. and A.B. Pardee. Science 257: 967-971 (1992)

8. *In situ* hybridatie experimenten waarbij laag-moleculaire stoffen als IAA subcellulair worden gelocaliseerd, zijn zeer discutabel.
Ohmiya A. And T. Hayashi. Phys. Plant. 85: 439-445

9. Niet iedere persoon die affiniteit toont voor weefselkweek is per definitie 'a person skilled in the art'.
T60/89 (Harvard) O. J. EPO 268 (1992)

10. Educatie van vandaag is van essentieel belang voor een florerende economie in de toekomst.

11. Effectief omgaan met de overvloed van data, beschikbaar via electronische netwerken, vraagt op termijn een aanpassing in de werkwijze.

12. Een onderzoeker is een eigenwijze doorzetter, die lang niet altijd open staat voor kritiek.

Stellingen behorend bij het proefschrift: 'Androgenesis in *Hordeum vulgare* L.' door Sietske Hoekstra, Leiden, 1 juli 1996.