Insights into a Key Developmental Switch and Its Importance for Efficient Plant Breeding

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Sexual reproduction of plants starts with the formation of gametes and a 2-fold reduction in the number of chromosomes (ploidy) of these cells. Fertilization, the union of sperm and egg cell restores the ploidy level to its normal value again. During the formation of pollen grains, the structures that contain the male gametes, the reduction in the number of chromosomes happens when the vegetative cells undergo meiosis and give rise to microspores. In a diploid plant with 2*n* chromosomes the microspores are haploid with *n* chromosomes. Microspores normally develop into pollen grains, but as a result of an unusual event, microspores can also undergo a developmental process that leads to the formation of a haploid embryo. This androgenesis pathway is also called microspore embryogenesis or regeneration. During androgenesis the haploid microspore divides and develops into an embryo and subsequently into a new haploid plant. Haploid plants are not fertile because they cannot make gametes again since this would require another halving of the chromosome number. If chromosome doubling occurs at some stage during androgenesis, the regenerated plants from these microspores are completely homozygous (doubled haploid) fertile individuals. Such doubled haploid plants from haploid microspores provide excellent material for research, plant breeding, and plant transformation. However, androgenesis is not a naturally occurring event in angiosperms and only seems to be induced as a result of certain chemical and physical stimuli.

Since androgenesis involves the control and reprogramming of developmental switches, it provides opportunities to investigate key elements in developmental control. Moreover, via androgenesis, fertile homozygous progeny from a heterozygous parent can be obtained in a single generation, thus significantly reducing time required in breeding programs and providing a major advantage in preparing F_1 hybrid seeds as well. Both the possibilities for studying basic processes in plant development and the economic importance have motivated numerous research groups to investigate androgenesis in different crops. Here, the basic steps in androgenesis induction protocols, different processes and the signals involved in reprogramming of the pollen development pathway, the possible molecular markers, and the evolutionary perspective are considered.

LABORATORY METHODS FOR INDUCING ANDROGENESIS

The induction of androgenesis has been tested in a significant number of economically important plants. In monocotyledonous plant species such as wheat, rice, maize, barley, rye, and sorghum, different protocols for anther/microspore regeneration have been investigated. For the dicots, many reports concern *Brassica* sp., and some other plant species such as *Datura* sp., *Nicotiana* sp., potato, apple, and sunflower. Most protocols for androgenesis induction/microspore regeneration for breeding purposes have been developed by trial and error. In general, such protocols consist of different phases. As a typical example, the barley microspore regeneration protocol can be divided into the following major steps as shown in Figure 1: (a) pretreatment of the plant material: the right developmental stages of anthers are used for a stress pretreatment for approximately 4 d (Fig. 1, A–D); (b) microspore culture: microspore isolation from pretreated anthers and subsequent microspore culture (Fig. 1D). After 4 d, cell division can be observed (Fig. 1E), and after 14 d, formation of multicellular structures takes place (Fig. 1F); and (c) development of multicellular structures into embryos or embryo-like structures (Fig. 1G) and further development into young plants (Fig. 1H), which requires an additional 21 d.

Depending on the plant species, and even the plant variety, many variations of the basic protocol exist. The variations can be found in the type of plant material used for the pretreatment (e.g. whole flowers, isolated anthers, or isolated microspores), the type of stress pretreatment (e.g. temperature, starvation, or osmotic stress), and the type of cultures (e.g. microspore culture or anther culture). However, these protocols always follow the same basic scheme as summarized in Figure 1. The main bottlenecks for the practical approach are the quality of the donor material, the type of pretreatment, recognition of the occurrence of the developmental switch, the right culture conditions for the induction of embryogene-

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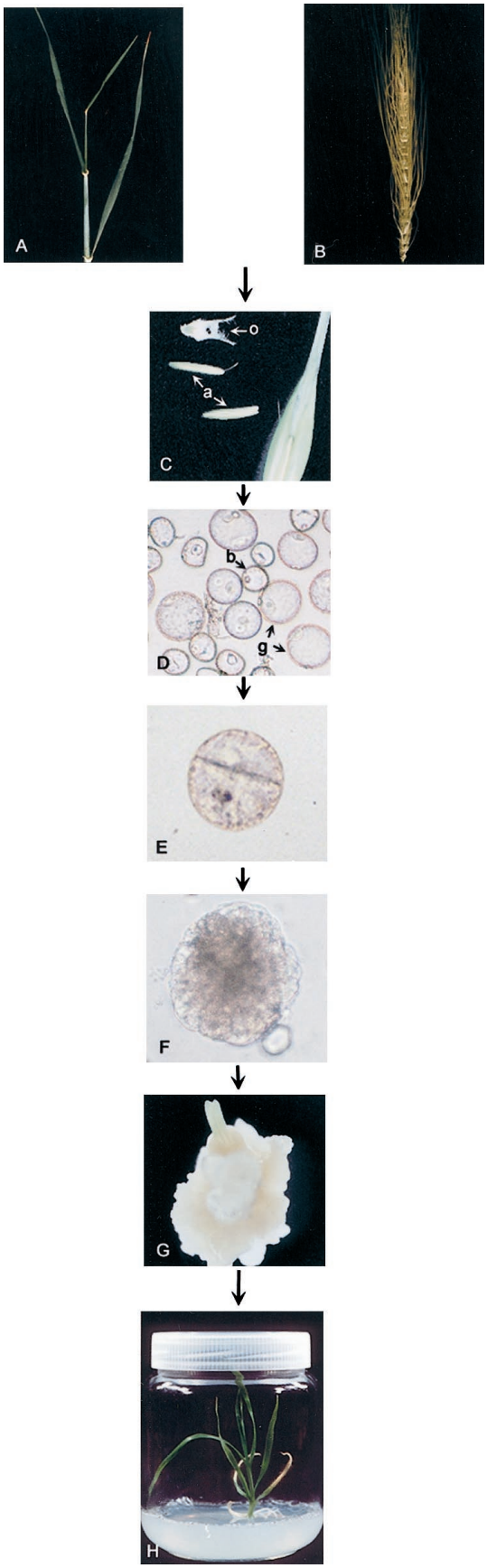

sis, and subsequent embryo development, germination, and outgrowth to a new plant.

Donor Material

The results of microspore regeneration experiments are highly dependent on the variety used, the growth condition of the plants, and the quality of the donor material. The conditioning of the plant growth is important, as exemplified by the significant effects of season on regeneration efficiency (Foroughi-Wehr and Mix, 1979). The natural flowering conditions (light intensity, day-length, temperature regime, humidity, etc.) are normally the best environment for donor plants to produce anthers to be used in successful regeneration experiments. Any infection or stress to the donor plants will lead to less success or complete failure for induction of androgenesis and further regeneration. The age of the plant material can also highly influence the regeneration efficiency. For example, in barley, the first five spikes show 15% to 20% higher regeneration efficiency than the later spikes. However, in the tree *Aesculus carnea* the older trees (60 and 100 years) give a higher success rate of androgenic anthers (approximately 20%) than the younger trees (20–40 years; approximately 9%) (Marinkovic and Radojevic, 1992). Besides plant age, the microspore developmental stage that is for the best regeneration varies between different species. For barley, the microspores should be at the late uninucleate stage (Hoekstra et al., 1997, and refs. therein), whereas in rye the best regeneration efficiency is obtained from microspores at the starch granule stage (Immonen and Anttila, 1998).

Variety-dependent success rates are well known. Different and sometimes closely related cultivars show different responsiveness to the same regeneration protocol. For example, the barley cv Igri has a regeneration efficiency 8.5%, whereas with the same regeneration protocol in the cv Digger only 0.85% regeneration efficiency was obtained (Van Bergen et al., 1999).

Pretreatments/Induction of the Developmental Switch

Once the donor material containing the microspores has been selected, microspore development

Figure 1. Scheme of microspore regeneration in barley. A, Developing spike within ensheathed leaves, original size is approximately 10 cm; B, isolated spike, original size approximately 9 to 10 cm; C, dissected flower with ovary (o) and anthers (a), original flower size is approximately 1 cm, original anther size is approximately 0.4 cm; D, isolated microspores after 4 d of anther pretreatment with mannitol, the enlarged embryogenic microspore (g) with red interference (diameter approximately 60 μm) and nonembryogenic microspore (b) with blue/black interference (diameter approximately 20–30 μm); E, dividing microspore, diameter approximately 70 μm; F, multicellular structure derived from microspore, diameter approximately 0.2 cm; G, formation of a young plant from an embryo-like structure derived from a multicellular structure, diameter approximately 0.4 cm; H, young regenerated barley plant in culture medium.

has to be switched from the gametophytic pathway to a sporophytic development. Induction of this switch requires specific pretreatment conditions, which usually consist of the application of different stresses to the plant material. The pretreatment can be applied at different levels of explants, such as intact flowers (e.g. for barley complete spikes), isolated anthers, or isolated microspores. With regard to different explants, the type, levels, and duration of pretreatments are different, and the regeneration efficiencies vary as well. The pretreatment of barley can be a cold treatment of spikes or a pretreatment in a mannitol solution of anthers. These different treatments result in different regeneration efficiencies (cold pretreatment of spikes results in a 1% regeneration efficiency, whereas pretreatment of anthers with a combination of starvation and osmotic stress results in a 8.5% regeneration efficiency). When the pretreatment was applied to isolated barley microspores, no plants could be obtained by regeneration. However, mannitol pretreatment of isolated tobacco microspores (Touraev et al., 1997) or heat shock pretreatment of freshly isolated *Brassica napus* microspores (Custers et al., 1996) have proven to be successful methods for induction of microspore regeneration.

Cell Culture and Embryogenesis

After the pretreatment, the microspores are cultured in a specific culture medium where cell division and differentiation occur. It has been suggested that in this phase the doubling of chromosomes (forming a doubled haploid) is taking place due to aberrations in the mitosis in callus formation or due to nuclear fusion in early division. The type of culture medium, the cell density, the application of plant growth regulators, and conditioned media all affect the regeneration efficiency (e.g. Hoekstra et al., 1997, and refs. therein; Manninen, 1997). At this stage of androgenesis, the induction of cell division is a bottleneck with a variety of possible solutions for different species and cultivars. Once a multicellular structure is formed, two different developments can occur. In the first, the structure develops into an embryo-like structure (Fig. 1, F and G) and via an in vitro zygotic-like embryo development pathway, a plant is formed. In the second, the structure will give rise to a callus from which secondary embryogenesis can be induced to form plants. We found that the number of structures showing cell division is much higher than the number of embryo's formed (S. Van Bergen and M. Wang, unpublished data). So, the pathway to undifferentiated callus formation is more likely to occur than the route to embryo formation. Culture condition variations, especially hormones and sugars, have important effects on these two separate pathways (e.g. Hoekstra et al., 1997, and refs. therein; Manninen, 1997).

Once an embryo is formed from the microspore-derived multicellular structure, this embryo will usually be transferred to a solid culture medium for induction of embryo growth (Fig. 1, G and H).

MORPHOLOGICAL CHANGES DURING REPROGRAMMING OF POLLEN DEVELOPMENT

In the three major steps discussed so far (pretreatment processes, microspore culture into multicellular structures, development of embryos) for microspore regeneration, the first step is key for the determination of the efficiency of regeneration (observed as cell division and differentiation). Early markers for induction of androgenesis, being morphological, biochemical, or molecular, and more information about the molecular basis of stress-induced cell division are essential for both understanding the mechanism of androgenesis and breeding applications. Only understanding of the androgenesis process and unequivocal markers will enable us to control microspore regeneration in a species-independent manner to significantly reduce the time span of a wide variety of breeding programs.

From the protocols it can be concluded that for the production of doubled haploid plants from microspores via androgenesis, a specific developmental stage of the microspores in combination with a stress treatment of anthers and microspores cultured at a specific density is essential (e.g. Heberle-Bors, 1989; Hoekstra et al., 1997, and refs. therein). These conditions induce a reprogramming of the pollen development that is barely understood. To gain a better understanding of the processes and working mechanisms involved in the control of this developmental switch, studies on the morphology and cell biological aspects of anthers and microspores during androgenesis were carried out. These studies not only resulted in useful parameters for protocol design but also show the complexity of the process and, moreover, the involvement of different cell types present in anther tissue.

Microspore Morphology

During pretreatment, part of the population of microspores changes into cells that are called embryogenic microspores. These microspores have the potential to complete sporophytic development. The embryogenic microspores can be recognized microscopically by their increased size (swollen to 50–60 μm in diameter for barley; Fig. 1D), whereas nonembryogenic microspores (the microspores that develop either into pollen or enter a cell death pathway) have a much smaller size (30 μm in diameter for barley; Fig. 1D). Other parameters vary for different varieties. For instance barley embryogenic microspores have a red interference color of the exine wall (Fig. 1D), whereas the nonembryogenic microspores can

be recognized microscopically by their blue/black interference color of the exine wall (Fig. 1D). After successful pretreatment, the swollen microspores form an extremely large vacuole that pushes the nucleus to the periphery of the cell close to the cell wall (Fig. 2).

Pretreatment-Induced Changes in Anther Tissue

During successful pretreatment of whole anthers, changes in cell wall properties of anther tissue are observed (Sunderland et al., 1984). The developing barley microspores (or mature pollen grains) are localized within the anther loculus (Fig. 2, L), the wall of which consists of three layers of cells (Fig. 3A). The loculus is lined by a layer of nutritive cells, the tapetum (Fig. 3A, T). The cells in the middle layer of the loculus wall contain chlorophyll. A rapid loss of chlorophyll and degeneration of tapetum and anther wall cells were observed in stress pretreated anthers (Hoekstra, 1996; Fig. 3B). After 4 d of pretreatment, the tapetal cells have disappeared, and the innermost layer of loculus cells is crushed (Fig. 3B).

The death of anther tapetal cells is much more rapid during pretreatment conditions than during non-pretreatment conditions (Wang et al., 1999). The tapetum is a specialized cell layer between the sporogenous tissue and the anther wall, which functions as a source of nutrients for developing pollen grains (e.g. Pacini et al., 1985). The ultimate fate of these cells is death when pollen grains mature. During stress pretreatment, these type of cells are apparently most sensitive to stress and enter a cell death pathway. The death of these cells may release signals that are able to trigger susceptible microspores to enter a sporophytic development pathway (dedifferentiation and division). The degradation products of cell wall components from the tapetal cells could be considered as signal molecules in this process.

SIGNALS AND SIGNAL TRANSDUCTION DURING ANDROGENESIS

Complete control over androgenesis in a species-independent protocol obviously requires full understanding of the signals and signal transduction involved in the reprogramming of pollen development. From the above it may be concluded that essential signal molecules may be produced by different tissues in the pretreated anther as well as by the non-sporophytic microspores in a cell density-dependent way. As pretreatment involves the application of stresses, the stress hormone abscisic acid (ABA) and its signal transduction pathways are mainly considered as signaling factors of importance. Although no other signaling molecules strongly involved in the regulation of androgenesis have been reported, new factors still may be found in e.g. conditioned medium of microspore cultures.

Role of ABA

As the pretreatments applied to anthers and microspores are stress treatments generally associated with ABA signaling, a role for this stress hormone in the reprogramming of pollen development has been postulated. As early as 1980, Imamura and Harada proposed that a specific level of ABA was required for induction of androgenesis because they found a peak in endogenous ABA level after 24 h of mannitol pretreatment in tobacco anthers. Similarly, in barley anthers, the starvation and mannitol stress pretreatment induces a peak in ABA levels within 24 h (Van Bergen et al., 1999). Results showing that ABA addition in combination with suboptimal pretreatment

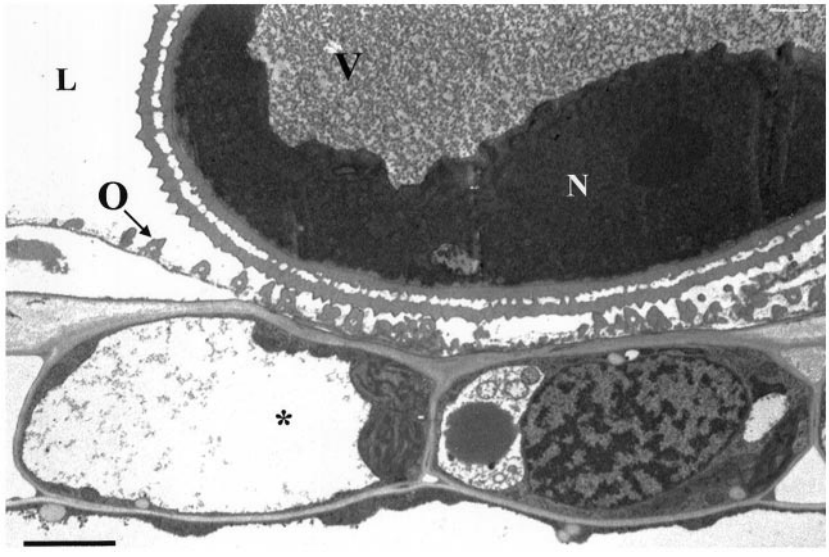

Figure 2. Transmission electron microscope photograph of ultrastructure of a pretreated microspore within an anther loculus (L). Part of the microspore with large vacuole (V) pushing the nucleus (N) to the periphery of the cell can be seen. For the orientation of the tissue, the orbicules (O) are indicated. Asterisk indicates an anther wall cell. Bar = 0.7 μm.

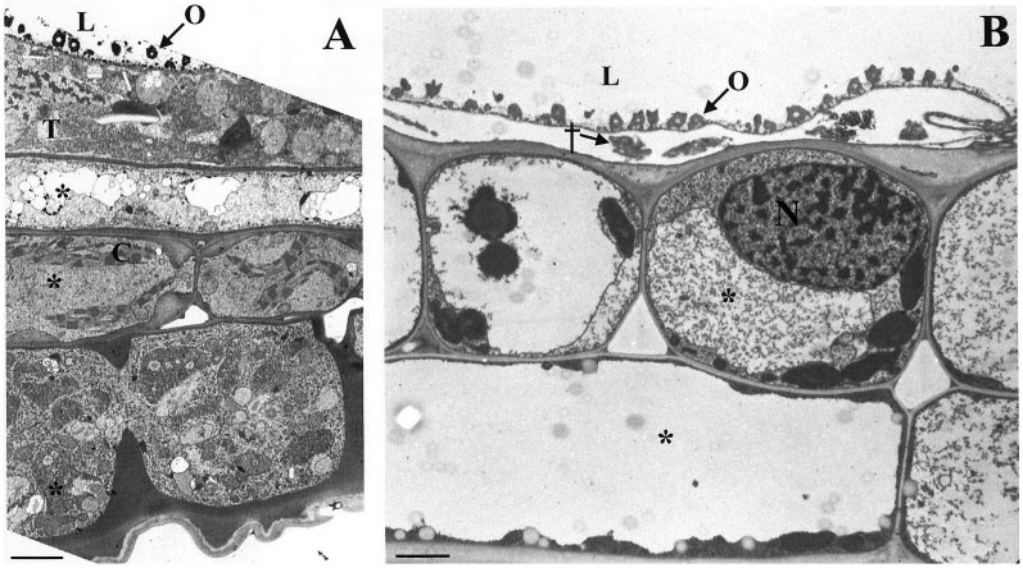

Figure 3. Transmission electron microscope photograph of ultrastructure of developing (A) and pretreated (B) anther wall cells (as indicated by asterisks). L indicates the anther loculus in which the microspores are localized. For the orientation of the tissue, the orbicules (O) are indicated. Barley anthers have four loculi with a central vascular region. Each loculus is composed of three layers of sterile anther wall cells (A, asterisks), which surround a single layer of tapetal cells (T). The tapetal cell layer separates the wall layers from the interior-most layer of microspores. The wall cells provide structural support for the anther loculus. Chlorophyll (C) can be seen in the middle layer of developing anther wall cells and the majority of this chlorophyll is lost after 4 d of mannitol pretreatment (B). In this pretreated anther, tapetal cells are lost and the inner-most layer of anther wall cell layer is crushed and only the rest of debris can be seen (see † in B). The cell content (asterisks in B) is also lost in pretreated anther wall cells. A, Bar = 2 μm. B, Bar = 1 μm.

restores regeneration efficiency (Van Bergen et al., 1999) and that fluridone inhibition of de novo ABA synthesis reduces regeneration efficiency (Hoekstra et al., 1997) strongly indicate a role for ABA in androgenesis. In addition, positive effects of ABA addition during pretreatment were shown for wheat androgenesis (Hu et al., 1995) and tobacco androgenesis (Kyo and Harada, 1986). Furthermore, endogenous ABA levels are important for morphogenic competence, and ABA application enhances somatic embryogenesis, whereas the inhibition of ABA synthesis in donor plants causes the loss of capacity for embryogenesis in *Pennisetum* sp. (Rajasekaran et al., 1987). The difference in regeneration efficiency of the barley cv Igri and cv Digger (high and low efficiency, respectively) is also reflected in different pretreatment-induced ABA peak levels in these cultivars (high followed by slow decrease and low followed by sharp decrease for cv Igri and cv Digger, respectively) (Van Bergen et al., 1999).

It was recently demonstrated in barley that there is a strong correlation between stress-induced ABA production and microspore viability and that addition of ABA to suboptimal pretreatment levels enhanced microspore viability (Van Bergen et al., 1999; Wang et al., 1999) and repressed pollen tube formation (S. Van Bergen and M. Wang, unpublished data). It is likely that by preventing cell death, the stress-induced increase in ABA affects the regeneration efficiency positively by increasing the number of viable microspores available for androgenesis. Moreover, the effect of ABA was not only a viability increase, but also a reduction in the number of binucleate microspores. These binucleate microspores show DNA degradation and are likely to be in the process of dying (Van Bergen et al., 1999).

It is likely that the effects of ABA are on at least two different processes: (a) preventing the death of microspores, thus increasing the number of viable microspores during pretreatment and (b) repressing further development of microspores into mature pollen.

As the experiments involving ABA measurements during pretreatment were carried out in anther tissue, it is not clear which cells produce the stress-induced ABA. Further investigations may reveal the ABA origin and a possible role for specific anther cells in the induction of the developmental switch.

ABA Signaling

Although the involvement of ABA in microspore regeneration is apparent, the involvement of ABA signal transduction components and ABA-induced genes is scarcely reported. Reynolds and Crawford (1996) demonstrated that there was a direct and positive correlation with an increase of ABA and expres-

sion of an early metallothionein gene in developing pollen embryoids.

As the mitogen-activated protein (MAP) kinase signaling cascade is associated with abiotic stresses, ABA signaling, cell cycle, and growth control in plants (e.g. Hirt, 2000), it is tempting to speculate that the stress-induced ABA signaling may activate a MAP kinase cascade inducing cell division and differentiation in microspores. A first indication for MAP kinase involvement in microspore development was reported by Préstamo et al. (1999) who demonstrated an increased expression and cellular distribution of a MAP kinase in strongly vacuolated pepper microspores. These microspores do not enter androgenesis but are at the verge of a mitotic division just before the developmental switch could be made. Stress-induced MAP kinase activation might induce equal cell division instead of asymmetric cell division at this point.

GENE EXPRESSION AND MARKER GENES FOR ANDROGENESIS

To understand the mechanism of the androgenic pathway and to find specific markers, it is important to identify genes that are developmentally specific for androgenesis, i.e. the developmental switch from the gametophytic to the sporophytic pathway. There are many reports on gene expression related to natural microspore development, zygotic, or somatic embryogenesis. These will not be considered here as the major bottleneck in microspore regeneration is the induction of the developmental switch into androgenesis. To date there is only limited information concerning this developmental switch in microspore regeneration.

During induction of androgenesis, there is a requirement for altered synthesis and accumulation of mRNAs and proteins in embryogenic microspores, leading to a sporophytic type of cell division. Raghavan (1981) demonstrated that the synthesis of de novo mRNA in *Hyoscyamus niger* androgenic microspores occurred during the 1st h of culture, and Pechan et al. (1991) identified mRNAs and proteins that appear to be associated with embryogenesis of *Brassica* sp. microspores. Up to now, only a few genes have been isolated that are specifically expressed during the early stage or switch of microspore embryogenesis (Touraev et al., 1997). Reynolds and Crawford (1996) identified an ABA-responsive gene that is expressed in the early stage of wheat microspore embryogenesis. In *Brassica* sp. microspores, a high expression of seed storage napin genes, and synthesis of a specific heat shock protein are correlated with embryogenic induction (Boutilier et al., 1994; Custers et al., 1996). It has been demonstrated that a peroxiredoxin anti-oxidant is predominately expressed in anther-derived cells that show embryogenic potential (Stirn et al., 1995).

More recently, using differential screening of barley cDNA libraries, three cDNAs have been identified that might be markers for early microspore embryogenesis (Virnten et al., 1999). One of them has homology to lipid transfer proteins. In carrot, a lipid transfer protein cDNA EP2 (embryogenesis protein 2) has been reported as a marker for embryogenic potential in somatic cell suspension (Sterk et al., 1991).

It is clear that the identification of genes involved in microspore embryogenesis is still in its infancy. More genes specific for the early processes in the microspore regeneration process need to be identified and their function established. For this, model systems that are well characterized at the molecular level and the regeneration protocol level are needed. Although the Arabidopsis system is powerful for gene identification and studying gene function, the bottleneck of using this system is the lack of an efficient Arabidopsis microspore regeneration protocol.

EVOLUTIONARY POINT OF VIEW: COULD MOSSES AND FERNS SHOW THE WAY TO A UNIVERSAL PROTOCOL?

In the life cycle of plants, the diploid phase that produces spores is called the sporophyte, whereas the haploid phase that produces the gametes is called the gametophyte. In an evolutionary development perspective from algae to flowering plants, the prominence in the appearance of the plant shifted from the gametophyte to the sporophyte. In mosses, the haploid gametophyte is the dominant structure, whereas in the monocots and dicots, the gametophytes are reduced to extremely small structures, embryo sac and germinated pollen, embedded in the highly dominant sporophyte. In mosses and ferns the gametophyte is formed from the haploid spores. In both mosses and ferns the spores divide to form a multicellular structure called protonema. In mosses the protonema develops further into plants consisting of leave and stem structures, whereas in the ferns the protonema remains small with gameteforming structures.

The spore development in mosses and ferns shows clear parallels with the androgenesis process. In both cases the haploid spore produced by the sporophyte divides mitotically and develops into a (haploid) multicellular structure, and both processes start with an increase of the cell volume. So we may hypothesize that during the evolution of plants, the spore development pathway into multicellular structures was greatly shortened in favor of direct gamete formation, but that this pathway is still present and can be activated as is shown in androgenesis. If so, the available knowledge about spore germination and development of mosses and ferns may be very useful to understand and control androgenesis in monocots and dicots plants. In addition, mosses and ferns may

provide valuable model systems for research on developmental switches involved in androgenesis.

Several mosses are currently studied as model systems for plant development and reproduction, and mutants with defects in spore development and protonema formation are available (for reviews, see Cove and Knight, 1993; Cove et al., 1997). In addition, external factors affecting spore germination have been studied for a long time. Light, calcium, auxin, and cytokinin all play a role in the initial phases in the formation of the multicellular structure of *Physcomitrella* sp. (Cove and Knight, 1993). In spore germination of different mosses and ferns a stimulating effect of red light, low pH, gibberellins, nitrate, and low temperature has been reported (e.g. Haas et al., 1992; Whittier and Moyroud, 1993). In the light of the above these factors may also be considered in the development of microspore regeneration protocols.

CONCLUSIONS

Although the first observations of androgenesis in barley were made in the 1930s, protocols that can be applied to breeding programs have only recently become available. Progress in understanding and controlling androgenesis has been slow. Most economically relevant crops are still recalcitrant, and a universal species-independent protocol for androgenesis induction is not within sight. In the last few years some progress was made in identifying genes that may be markers for androgenesis, and the roles for ABA and anther tissue have become clear. However, an integration of different essential disciplines (i.e. molecular biology, signal transduction research, cell biology, and practical experience in androgenesis protocol design) in a single suitable model system is lacking. Modern genomics and proteomics technologies are promising in this respect and will be useful if a model system is available that can be fully controlled. Hence, in the coming years our progress in understanding and controlling androgenesis will depend largely on combining the different new technologies with the available practical expertise from the trial and error androgenesis protocol design. In addition, mosses and ferns may provide an alternative model for research into the secrets of androgenesis.

ACKNOWLEDGMENTS

We thank Gerda Lamers and Wessel de Priester for kindly providing the electron microscopical photographs and Christa Testerink, Martien Caspers, and Sylvia de Pater for critical comments on the manuscript.

Received May 10, 2000; accepted June 6, 2000.

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