Androgenic haploids: Factors controlling development and its application in crop improvement

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Androgenesis in flowering plants is a unique biological process. It provides an understanding of the biological basis of single-cell microspore embryogenesis to the production of a dihaploid plant. This system provides an unparalleled opportunity to shorten the breeding cycle and fix agronomic traits in the homozygous state, such as recessive genes for disease resistance. The most desirable dihaploid variation in all the major crops including rice, wheat, barley, maize, rape, cotton, sunflower, coffee, etc. has already been developed and utilized in modern crop breeding. Many known and a few unknown factors are involved in such development. A few noteworthy factors are donor plants, genotypic variation, media composition, and handling of cultures, which may have a greater influence on the response of androgenesis. A further opportunity has arisen to use a pollen-specific gene, promoter and transgenic dihaploid (homozygous), gene expression, proteomics, translational regulation and post-translational modification of genes to widen the scope of crop improvement. The homozygous (isogenic) lines will provide unique genetic material for mapping populations for use in functional genomics and molecular breeding.

Keywords: Androgenesis, albinism, dihaploids, genomics, transgenesis.

MALE reproductive processes take place in the stamens in flowering plants. The diploid cells undergo meiosis and produce haploid male spores or microspores. In general, microspores divide mitotically and differentiate into multicellular male gametophytes or pollen grains. The principle of androgenesis is to arrest the development of the pollen grains (male gametophytes) and to force them towards a somatic pathway (Figure 1). *In vitro* androgenesis can be achieved from the microspores, leading to the formation of haploids either by direct embryogenesis or via callus formation. The callus-derived plants are generally undesirable as they exhibit genetic variation and polysomy. Anther culture is the main technique for haploid induction in crop improvement. Culture of whole or parts of inflorescences has helped simplify the technique[1–4]. Another alternative is to culture isolated or shed microspores. However the reports on isolated microspore culture are rather limited[5–9]; in majority cases, the *in vitro* response of microspores is observed within the anthers.

Since the beginning of modern plant breeding practices, intensive efforts have been made to speed up the production of homozygous lines, which normally requires at least six inbreeding generations. The starting material for the production of homozygous lines in just one generation is the haploid gametes. From the time of accidental but immensely valuable discovery of androgenic haploidy in 1964 by Guha and Maheshwari[10] and production of rice haploids in 1968 by Niizeki and Oono[11], impressive advances have been made in several laboratories throughout the world. The main advantage of using haploids is the rapid and complete homozygosity of the offspring, which allows an easy selection of phenotypes for quantitative characters.

As it is impossible to cover the entire work on advances in research on haploidy in crops, I have considered summarizing some important factors that influence the process of androgenesis and give some examples from major crops and from our own research dealing with androgenesis in cereals.

Donor plants

The unknown quality of donor plants decisively influences androgenesis. The sample of microspores, the release of microspores from the anther and their subsequent divisions leading to plant regeneration often depend on the conditions under which the donor plants grow in a particular environment. Donor plants of wheat and barley grown during October–December provided an excellent microspore response (personal experience during my work at Grünbach, Germany, 1985–86). In the rice crop, plants grown during the dry season have provided the best microspore response (personal experience at IRRI during 1993–2000). Under optimized conditions of a phytotron with controlled light, temperature, and humidity, which enable plants to maintain a healthy growth with disease and pest-free status, rice, wheat, and barley plants yield a high degree of success of anther culture response with reproducible results.

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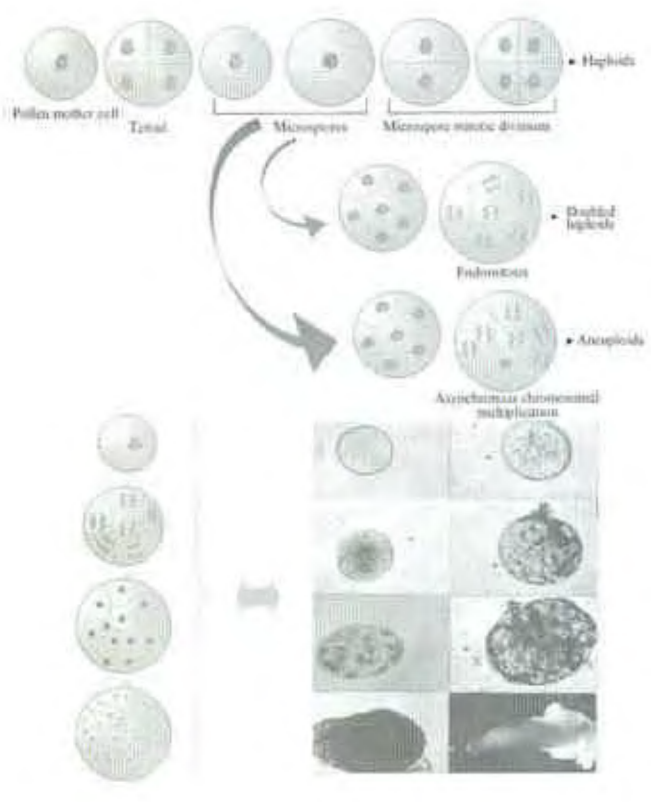

Figure 1. Schematic representation of microspore androgenesis.

Genotype response and environmental effect

A genotype grown in a particular environment plays an important role in androgenic response. Many crop genotypes are quite recalcitrant in their *in vitro* response. Several studies indicate that such a response is influenced by gene combinations, which will be discussed later. A few detailed studies have been made on the genetic control of microspore response of wheat[12], barley[13], rice[14–16], and maize[17–19] (Figure 2).

Microspore stage

In most cases, the early uni- to mid-uninucleate stage of microspores is the most suitable for androgenic response[4,6,8,20,21]. The anthers of maize containing microspores in the late uni- to early-binucleate stage have been found to be most responsive[22]. In dicot species, unicellular to early bi-cellular pollen stage is suitable for microspore embryogenesis (e.g. *Brassica napus*)[23].

Preculture treatment

The induction of microspores to sporophytic instead of gametophytic pathway is strongly influenced by some kind of stress treatment of the anthers before culture. The response to chilling or heat treatment is also genotype-dependent. However, a temperature shock has been reported to improve the androgenetic response in many plant species[24,25]. Starvation of anther culture in sugar-free medium before release of microspores induces better anther culture response[26]. Nevertheless, such procedures have to be optimized for each plant species. For example, some indica rice cultivars do not require any cold or hot treatment prior to culture to induce androgenesis (unpublished data), whereas 10 days, cold treatment at 8°C for wheat, and 8–28 days, cold treatment at 4°C for some genotypes of barley are very useful. However, genotype is the most critical factor in obtaining good microsporogenesis irrespective of cultivars/varieties used under certain culture conditions[27].

Culture media

The nutrient medium not only provides nutrition to the microspores but also directs the pathway of embryo development. It is critical to change the composition of the media or replenish them to keep the balance of micronutrients and maintain the pH. The pH of the media, particularly liquid media, changes dramatically with time at the onset of embryo development[7].

Two familiar basal media, the chemically defined N6 medium[28] and the MS medium[29], have been generally used with modifications. Anthers (*ca.* 30) were floated on the surface of 10-ml aliquots of media in 50-mm Sterilin plastic dishes or 10–15 anthers were cultured in 24 wells containing 1.5-ml of the media. It has been established that the nitrogen composition of the culture medium plays a significant role in androgenesis[30]. Increasing glutamine and decreasing ammonium nitrate enhance embryo development in many cereal species[6,21,27,31–33]. Higher concentration of sucrose showed better microspore-embryogenesis responses in wheat[6]. Addition of Ficol in the liquid medium improved plant regeneration of barley[34]. Further modification of the media and use of Ficol 400 were shown to promote the rate of haploid induction in barley and wheat[6,7,35]. The use of maltose instead of sucrose has dramatically enhanced embryo induction and plant regeneration in cereals. However, the concentration needs to be suitably adjusted for each crop. Since the use of maltose in the liquid medium of barley by Hunter[31], several groups to improve cereal androgenesis, protoplast culture, and plant regeneration have advocated the advantage of using maltose. The use of abscisic acid in the media[36] or use of potato media[37] could induce greater incidence of androgenesis in rice than standard media. Osmotic pressure of the medium may play an important role in the maturation of microspore-derived embryos.

Methods

The procedures for treating microspores in the medium for inducing androgenesis can be broadly classified into

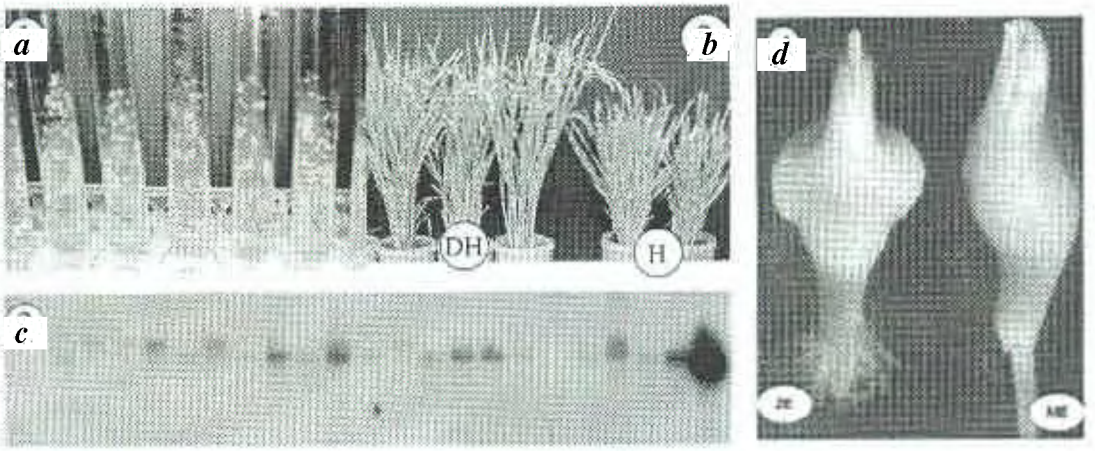

Figure 2. Anther culture (AC) derived haploid and diploid plants, microspore embryogenesis and molecular analysis of AC derived plants. ***a***, Anther culture of rice; ***b***, Haploid sterile (H) and dihaploid fertile (DH) rice plants; ***c***, Molecular analysis (Southern) of DH rice plants showing integration of transgenes; ***d***, Zygoic (ZE) and microspore derived embryos (ME) of maize germinating to normal bipolar structures.

four categories. The simplest one is to culture the inflorescences containing mid- to uninucleate microspores in the liquid or solid medium. A few such reports, including on barley[1,4], indicate that direct embryos can be obtained from the microspores originated from the anthers of the spikelets. The second method refers to the shed pollen culture, in which anthers are cultured on the liquid/agar medium. After a few days, microspores are shed freely from the anthers and they divide and develop further in major cereals. Often such microspores start division within the anthers even before they are released. On the top of the liquid medium containing Ficol, such embryos float and often produce direct plant regeneration[6–8,33–35]. The third method involves mechanical isolation of the microspores, which can be cultured as protoplasts. However, the response of plant regeneration is limited in this procedure[9,22,38–41]. The fourth method is to culture the anther on the surface of the agar medium. Orientation of anthers is not important in regulating embryo development. Anthers may be removed by hand individually or by using a suction pump and plated on the agar medium[42].

Ploidy level

The haploid set of chromosomes in microspore-derived plants becomes spontaneously doubled under culture conditions. However, the percentage of doubling varies among the crops, including different genotypes of a cultivar. So far, barley showed the highest incidence of spontaneous doubling (up to 87%)[38], followed by rice (up to 72%)[43], wheat (up to 50%)[44], and maize (6.3% and quite unpredictable)[45]. In general, colchicine is used for chromosome doubling at the whole-plant level for barley, rice, and maize. Sometimes, colchicine is used in the medium for chromosome doubling in wheat[21]. Colchicine employed in the medium before the first microspore mitosis can contribute to a significant increase in gametophytic chromosome number.

The microspore undergoes its first mitotic division, followed by endomitosis or nuclear fusion, resulting in dihaploid plants[20,33,46,47] (Figure 1). Direct microspore embryogenesis leading to fertile plant regeneration in barley and wheat is obviously influenced by the specific pathway combining with spontaneous doubling of the chromosomes. Microspore-derived calli and embryoids often show aneuploids, dihaploids, and polyploids[48].

Plant regeneration

Many factors are involved in obtaining regeneration of fertile green plants from cultured microspores (Figure 2). The kind of nutrient media, genotype, culture vessels, condition of donor plants, carbohydrate sources, phytohormones, reduced nitrogen (glutamine), and handling of cultures are some of the important factors that influence microspore embryogenesis. Individual factors become critical in the induction of microspore embryogenesis, such as co-culture of the ovary or ovary-conditioned media for wheat[6]. However, such factors may not influence genotypes having an inherent potential for high-frequency plant regeneration, such as *in vitro* friendly barley genotype 'igri', which yields 50 green plants per cultured anther[38]. However, it is to be emphasized that one noteworthy concept of embryogenic culture developed from immature embryos popularized by Indra Vasil which is very helpful in looking forward to the objective of cell culture development[49]. Many changes occur in the culture from day 1 to subsequent subculture. A thorough observation of the cultures, from induction to embryo development is critical. Replenishing the media to avoid depletion of some essential micronutrients and balancing the pH often helps in the conditioning of the cultures and their further develop-

ment. Embryo maturation is another critical stage, as the developing cereal embryos must be transferred to a regeneration medium at the right time, lowering the carbohydrate concentration and increasing relative cytokinins levels to auxins. A simple regeneration medium works very well for wheat and barley as is evident from the germination of encapsulated microspore-derived embryos of barley[50] and wheat[33,35].

Molecular understanding of albinism

Albinism is a common feature of microspore-derived plantlets. What factors affect the extent of albinism? Genetic background of the donor plants is an important factor. Cold pretreatment in general and the use of Ficol in the liquid medium may delay or arrest nuclear synchronization and help in producing green plants. It is evident that albino rice plants devoid of 23S and 16S rRNA[51] and albino barley plants do not contain mature chloroplasts[52]. In general, albino plants (e.g. wheat, barley, and rice) contain deleted forms of the plastid genome[53–55]. The size and location of the deletions differ among plants. The results indicated that some albino plants lack the region coding the *rbcL* gene in the plastid genome. However, a more detailed study is required to elucidate the actual cause of albinism. The use of a modified medium containing barley starch-melibiose has resulted in considerably fewer albino barley plants[56] and cold treatment also favoured more DH-green plants[6–8,31].

Haploid artificial seeds

Artificial or synthetic seeds consist of somatic embryos in a protective coating (calcium alginate). The main purpose is to utilize the somatic embryos efficiently for conversion of plants. Production of perfect somatic embryos is a prerequisite for the development of artificial seeds. Calcium alginate made from brown algae is used for the gel encapsulation system. The selected embryos were mixed with sodium alginate, single embryos were dropped into a bath of calcium salts, resulting in single somatic embryos encased in a clear, hydrated bead. The rigidity of the gel beads protects the fragile embryo during handling. The capsule gel can also potentially serve as a reservoir of nutrients just like an artificial endosperm. Microspore-derived artificial seeds of barley and wheat were developed and germinated to normal plants after storing them in a cold room[35,50]. Somatic embryogenesis has been reported in nearly all-major monocot and dicot species and a few gymnosperms[49,56–58].

Marker-assisted selection of dihaploids

Biotechnological tools complement breeding programmes in many ways, one of which is to be able to identify target genes (or mapped gene of agronomic importance) with the assistance of DNA markers, a process called marker-assisted selection or MAS[59].

Anther culturability is a quantitative trait controlled by nuclear-encoded genes[60–62]. However, earlier genetic studies on haploidy merely determined whether there are differences in response among varieties, and whether the traits such as callus induction and plant regeneration are heritable. With the development of MAS system, these characteristics can now be detected at the molecular level.

Quantitative trait loci responsible for culturability of anthers were surveyed and analysed with the molecular map constructed from a population resulting from anther culture of a DH line[63,64]. Parameters for four traits were callus induction, green plant differentiation frequency, albino plant differentiation frequency and green plantlet yield frequency. All four traits displayed continuous distribution among the DH lines. For callus induction frequency, five QTLs were identified on chromosomes 6, 7, 8, 10 and 12. Two QTLs for green plantlet differentiation frequency were located on chromosomes 1 and 9 whereas there was a major QTL for albino plantlet differentiation on chromosome 9. No independent QTL was found for green plantlet yield frequency. These results may be useful in the selection of parents with high response to anther culture for rice haploid breeding and in the establishment of permanent DH populations for molecular mapping.

To clarify the association between chromosomal regions showing distorted segregation and anther culturability, the anther culturability of DH lines derived from a Japonica/Indica cross having distorted segregation on chromosomes 1, 3, 7, 10 and 11 was examined[62]. One region on chromosome 1 was found to control callus formation from microspores, and another region on chromosome 10 appeared to control the ratio of green to albino regenerated plants. In both regions, the Nipponbare (Japonica parent) allele had a positive effect. Three regions on chromosomes 3, 7, and 11, however, showed no significant effect on anther culturability.

Likewise, using recombinant inbred lines from a cross between Milyang 23 and Gihobleo, QTL associated with green plant regeneration located on chromosomes 3 and 10 were mapped[65,66]. The QTL on chromosome 10 was detected repeatedly using three AC methods and was tightly linked to three markers. One of these three markers, RZ400, was able to effectively identify genotypes with good (>10%) and poor (<3%) regenerability based on the cultivars and two F2 populations. This marker enables the screening of rice germplasm for anther culturability and introgression into elite lines in breeding programmes.

The growth and development of plants from callus cultures is influenced by genes controlling the production of certain enzymes essential for the metabolism of differentiating cells, tissues and organs. Peroxidases are metalloprotein enzymes containing porphyrin-bound iron and are found to be associated with many physiological processes including morphogenesis. The amount of peroxidase present in four

indica cultivars and all possible F1 combinations was quantified[67]. These authors found that calli with high regeneration capacity showed high values of peroxidases while those with low amounts gave only albinos or predominant albinos with few green plants, suggesting the role played by peroxidases in morphogenesis of anther calli. By employing isozyme markers like peroxidase, the embrogenic as well as regeneration potentials of calli can be identified and utilized for selecting high regenerating calli.

Dihaploids in genomics

Genomics implies DNA sequencing, the routine use of DNA microarray technology to analyse the gene expression profile at the mRNA level, and improved informatic tools to organize and analyse such data[68].

Doubled haploid (DH) lines are useful for genetic analysis, particularly quantitative traits[69]. QTLs affect some important agronomic traits in cultivated rice. QTL studies have been facilitated by the development of molecular markers using segregating populations, F2 or backcross populations. However, these studies are difficult to replicate to obtain accurate phenotypic values for precise QTL mapping. The use of recombinant inbred lines (RILs) provides many advantages in QTL studies but it will take a long time to develop such populations. Recently, many studies have employed DH populations to construct genetic maps and locate QTLs. Because DH lines are homozygous, they can be propagated without further segregation. This characteristic feature allows for the precise measurement of quantitative traits by repeated trials and for a reduction in the environmental component of the total phenotypic variance[70]. One caution though in the use of anther cultured (AC)-derived materials is the possible distorted segregation of RFLP-markers derived DH populations. Yamagishi and colleagues[62] observed that ten and eleven of the 50 markers in two AC-derived populations showed distorted segregation ratios from the theoretical ratio of 1 : 1. Parental alleles were not randomly transmitted from the F1 plant to the AC-derived plants. Additionally, the segregation ratios of seven and six RFLP markers, respectively, were distorted both from the 1 : 1 ratios and from the observed ratios in the F2 population[62]. The chromosomal regions involving these markers were on chromosomes 1, 3, 7, 10, 11 and 12. The percentage of the markers showing segregation distortion in the AC-derived populations was similar to that in the F2 population. Thus, distortion in segregation does not appear to be a major drawback in the use of AC populations for rice breeding and genetics. The importance of doubled haploid populations in the study of quantitative traits is confirmed by Chen *et al.*[71] demonstrating that most gametoclonal variations among DH plants involve quantitative traits and the frequency of distinct variations is not high. Biochemical and molecular analysis proved high degree of genetic stability of gametoclones concluding that although AC may, to some extent, modify the performance of microspore-derived plants, it will not dramatically affect their utilization in plant breeding and genetic engineering programmes.

A good deal of research involving dihaploids in the study QTLs can be found in the literature. One such trait is tiller angle, which has great significance in the high yield breeding of rice; too small tiller angle reduces the resistance to disease while big tiller angle is undesirable for high yield[72]. Based on the constructed linkage map of a DH population from a female parent, which has a spreading plant type and a male parent having a compact plant type, two major QTLs were detected on chromosomes 9 and 11, and one minor QTL on chromosome 9. Similar studies using doubled haploids have been used in the study of QTLs for length of top internodes, plant height and days to heading[73], ratooning ability and grain yield traits[74], and cold tolerance of seedlings[75].

Employment of molecular genetic markers is particularly useful as an alternative strategy to phenotype selection for rice root traits. Breeding varieties with increased root penetration ability through hardpans and other root traits is difficult since screening numerous genotypes under field conditions is laborious and time-consuming. Further, soil compaction being not uniform and inconsistent throughout rice fields makes evaluation of root traits difficult[76]. For studies on QTLs for rice root characteristics such as root vitality[77], constitutive root morphology such as deep root morphology and root thickness[78], osmotic adjustment, root penetration index, basal root thickness, penetrated root thickness, root pulling force, total root dry weight, penetrated root dry weight and penetrated root length[79], doubled haploid populations have been used.

A DH population is a kind of permanently stable population. Its genetic structure is fixed so it can be grown at different times and locations for detecting QTLs and evaluating the interactions between genotypes and environment, i.e. the phenotypic expression level of QTLs in different environments. This technique has been applied in identification of 22 QTLs for six agronomic traits of rice in three different locations (environments). QTLs for spikelets and grains per panicle were common across environment, while traits like heading date and plant height were more sensitive to environment[70].

Doubled haploid rice populations have also been used in the QTL studies on rice grain quality[80], grain shape[81], paste viscosity characteristic[82], aromatic traits[83], and brown planthopper resistance[81].

Accumulation and fixation of marker genes using genetic male sterile composite crosses and later on employing anther culture technique was done by Suh and Song[84]. The dihaploid plants induced from the AC of the composite crossed plants showed the segregation ratio for male sterility as well as five or six marker genes generated through this method.

Aneuploids (plants with extra chromosomes in addition to the normal haploid chromosome complement) are useful

for genetic research; for example, for investigating genic imbalance caused by extra chromosomes at the haploid and diploid levels and for studying chromosome behaviour in meiosis and rice genome construction[85]. It has been difficult to produce aneuploids in rice, but through anther culture, haploid plants with one extra chromosome ($n + 1$) have been obtained. Like-wise, aneuploids and tetrasomics have been derived from anther culture of trisomic rice plants[85,86]. These aneuploids could be used to assign DNA markers to individual chromosomes. Meiotic behaviour and morphological features of auto-pentaploid rice plant derived from anther culture have also been investigated for genetic and cytological studies[87].

The new tools of marker assisted breeding such as Restriction Fragment Length Polymorphisms (RFLP) and Random Amplified Polymorphic DNA (RAPD) have been used very effectively in combination with DH-lines for molecular genome analysis for major crop species. RFLP-markers for different resistance genes have been identified in barley[88] including the resistance gene *ym4* (ref. 89). Furthermore, concerning *ym4* an isozyme as well as different RAPD markers including the very tightly linked marker OP-Z04H660 is known. However, this primer exhibiting an additional band of about 660 bp in susceptible lines shows a quite complex banding pattern. Furthermore, as OP-Z04H660 is inherited in a dominant manner and linked to the resistance allele in repulsion phase, it does not facilitate the identification of heterozygous susceptible plants in F_2 which will segregate resistant plants in the offspring. Therefore, although OP-Z04H660 has to be considered well suited for marker-assisted selection in DH lines, experiments were conducted in order to convert it in a more specific marker discriminating between homozygous and heterozygous genotypes.

Genes controlling androgenesis and pollen-specific genes

Regeneration of cultured microspores of crops is a heritable characteristic. Genetic determination of *in vitro* regenerability appears complex. Additive gene effects explain most of the variations observed in different genotypes but cytoplasmic influences and non-additive gene actions also play significant roles[90]. Most cereal genotypes are recalcitrant in androgenesis with a few exceptions. This suggests that the genetic factors govern the potential degree of *in vitro* androgenesis. Several groups were working in maize to map the genes affecting a high response to androgenesis, using RFLP analysis of the microspore-derived cultures of several F_1 hybrids. Certain regions of the chromosomes appear to be associated with the formation of embryo-like structures[91]. All limited attempts determining gene number have concluded that only a few genes are involved[91–94]. Further studies are required to finely map the regions that are highly associated with the anther culture response. Many DH lines, at least in barley, rice, and wheat, are available. Genetic and molecular maps including quantitative trait loci of major cereals (rice is in the advanced level) are becoming available. More precise location of such genes and their cloning could lead to further use in cereal breeding. The most intensively studied pollen-specific gene is *Zm-13* from maize, which is shown to be expressed in the late stages of microspore development[95]. A similar gene, *PS1,* has been cloned from rice and is shown to be expressed in rice[96].

Transgenesis

Dividing microspores can be used to develop an embryogenic cell suspension that is eventually used as a source of totipotent protoplast for genetic transformation. The first homozygous transgenic indica rice was reported to use such haploid microspore culture[97]. A few more reports are now available in rice[98] and barley[99]. Microinjection could be a powerful tool for introducing DNA into the nucleus of a potential microspore. Transgenic plants could be obtained as shown in *Brassica*[100]. However, except for transgene expression, this method did not produce any transgenic cereals. The present author, along with Gunther Neuhaus, German Spangenberg, Karabi Datta, and Ingo Potrykus at ETH-Zürich, Switzerland, worked intensively for 3 years using thousands of potential dividing microspores of cereals without success. However, rice androgenesis has been successfully exploited through anther culture of primary transgenic plants, thus attaining homozygosity of the transgene locus in one generation[101,102] (Figure 2). The biolistic system has been used to produce fertile transgenic barley plants using microspore cultures[99].

Protein synthesis during microspore embryogenesis

Biochemical analyses have been reported in several plant species like barley, wheat, rape, rice, etc. aiming to identify the markers for embryogenesis[103]. Six proteins were found differentially expressed during the later stage of pollen embryo development[104]. High throughput protein sequence analysis (proteomics) using mass spectrometry may provide new insights into protein profiling linked with the coding genes. Efficient microspore embryogenesis in *Brassica napus* makes it possible to study gene expression using mRNA differential display PCR (DD-PCR) and microarray. DD-PCR is a sensitive technique that can distinguish differentially expressed multiple gene families. Several laboratories are now involved in identifying those genes including transcription factor genes regulating microspore embryogenesis.

Applications

Androgenesis provides the most commonly used method for the doubled-haploid production that was eventually

applied in breeding and crop improvement. Today, many improved DH cultivars have been reported with several improved agronomic characteristics. Many improved crops including rice varieties, especially salt-tolerant ones have been developed[105–109], along with the development of other improved cereals such as barley[110], wheat[44], maize[111] and rape[23,108].

Conclusion

Homozygous lines are of utmost importance in breeding programmes. Androgenesis supports the development of such valuable DH lines. Recent developments in functional genomics, such as the fine mapping of DH populations, will help elucidate the genes that confer agronomic characters as well as *in vitro* responses. We should be able to use these genes in improving tissue culture regenerability of elite desirable cultivars along with the novel traits. The ability to transform and regenerate plants represents the most powerful tool and advancement in plant biotechnology. This process also provides identification and greater use of recessive genes for resistance. Transgenic homozygous crops are now available using this system. Homozygous lines provide uniform agronomic characters that can follow the stringent regulations required for registration under biosafety regulations of National Programmes. Dihaploids combining with value added transgenes or using MAS favour the development of new tailor made improved crops.

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