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Review

# Use of Androgenesis in Haploid Breeding

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## Abstract

Haploids are plants with a gametophytic number of chromosomes in their sporophytes. Androgenesis occurs from asymmetric division of pollen grains into generative cells and vegetative cells, followed by re-entry of the vegetative cell during S-phase, which causes microspores progress into G2/M transition in culture. One of the most interesting features of haploids is the possibility to produce doubled haploid (DH) individuals. Doubled haploidy is extremely useful to plant breeders because it enables shortened breeding periods and efficiency in selection of useful recessive agronomic traits. Doubled-haploid technology is not only applicable to breeding, but also to transformation programs of desired genes. In addition to practical breeding programs, DH lines provide useful materials of fundamental genetics including exploitation of QTLs and genes conferred with various agronomic traits by establishing DH populations. This paper provides historical overviews on androgenesis and describes several mechanisms associated with pollen embryogenesis, including mode of actions in pollen embryogenesis, mechanisms of chromosome doubling and factors affecting androgenesis. We also discuss recent progress in application of haploids to breeding, genes associated with *in vitro* response and drawbacks to anther culture for application of doubled haploids in crop breeding.

**Keywords** : Androgenesis, Breeding, Doubled-haploid, Haploid, *in vitro*

## Introduction

Haploids are plants with gametophytic numbers of chromosomes in their sporophytes. Haploidy occurs naturally in the gametophytic phases of their ovules and pollen by androgenesis at a low frequency. Since the first spontaneous haploid was reported in Sea Island Cotton (Harland 1920, 1936), more than 71 species representing 39 genera in 16 families of angiosperms have been discovered (Kimber and Riley 1963). Haploidy can be obtained by modifying pollination methods *in vivo* such as interspecific/wide hybridization, chromosome elimination, and pollen treatment and by *in vitro* culture of immature male or female gametophytes (Braniste et al. 1984; Zhang et al. 1990; Reynolds 1997; Andersen 2005; Germaná 2006, 2007). Obtaining of diploids has been reported following interspecific hybridization from tetraploids and crosses between parents with different ploidy levels (Wedzony al. 2009; Dunwell 2010). Kasha and Kao (1970) discovered that haploids were induced by a process of selective chromosome elimination with crosses between *Hordeum* vulgare and *H. bulbosum*. Pollen treatment prior to pollination with various physical or chemical agents causes maternal haploids by uncoupling of the organelle and nuclear transmission genomes (Chat et al., 2003). The formation of maternal haploids (gynogenesis) via pollen irradiation has been reported in 17 species including wheat, apple and onion (Dunwell, 2010). Gynogenesis provides an alternative source for haploid production in species in which androgenesis is recalcitrant due to male sterility or dioeciously (Thomas et al. 2000; Bhat and Murthy 2007). Haploids can also be obtained via parthenogenesis and polyembryony. In parthenogenesis, the egg cell in the embryo sac develops into an embryo without involvement of the sperm nucleus (Kendall 1934; Nezhevenko and Shumnyi 1970; Bordeset al. 1997).

*In vitro* techniques for the production of haploids have played important roles in the fields of biotechnology and plant breeding in the past few decades. Guha and Maheswari (1964) first developed an anther culture technique for production of haploids through androgenesis in *Datura inoxia*. Successful recovery of haploid plants was then described in barley through *in vitro* culture of unfertilized ovaries (San Noeum 1976). Haploid production technologies have been applied to over 250 plant species, including *in vitro* culture of unfertilized ovules/ovaries in 21 angiosperm species (Wu 2003), (Maluszynski et al. 2003). Since the first production of *in vitro* haploid plants (Niizeki and Oono 1968), many studies have been carried out to

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investigate various aspects of rice anther cultures, including pollen ontogeny during culture (Guha et al. 1970; Iyer and Raina 1972).

### 1. Mode of action in androgenesis/gynogenesis

The fate of microspore development is affected by endogenous and exogenous factors such as the developmental stage of microspores, genotypes, species, and individuals in the same cultivar (Datta 2005; Smykal 2000; Wang et al. 2000). Androgenic alternatives to male gamete formation start from microsporogenesis and microgametogenesis. Male-derived haploid or doubled haploid individuals can originate via three routes, (1) gamete formation, egg fertilization without nuclear fusion, and dismantling of the maternal nucleus, (2) deviation of the vacuolated microspore or the young pollen grain towards embryogenesis or occasionally callogenesis followed by organogenesis, and (3) deviation of the meiocyte towards callogenesis, which may lead to haploids and doubled haploids, as well as heterozygous diploids (Figure. 1).

### 2. Chromosome doubling

Five major mechanisms for plant chromosome doubling have been proposed (Figure 2) (Jensen 1974; d'Amato 1984, 1989; Kasha 2005; Shim et al. 2006). 1) Spontaneous chromosome doubling, in which duplication occurs in response to external stressors such as duration of inductive conditions, temperature,

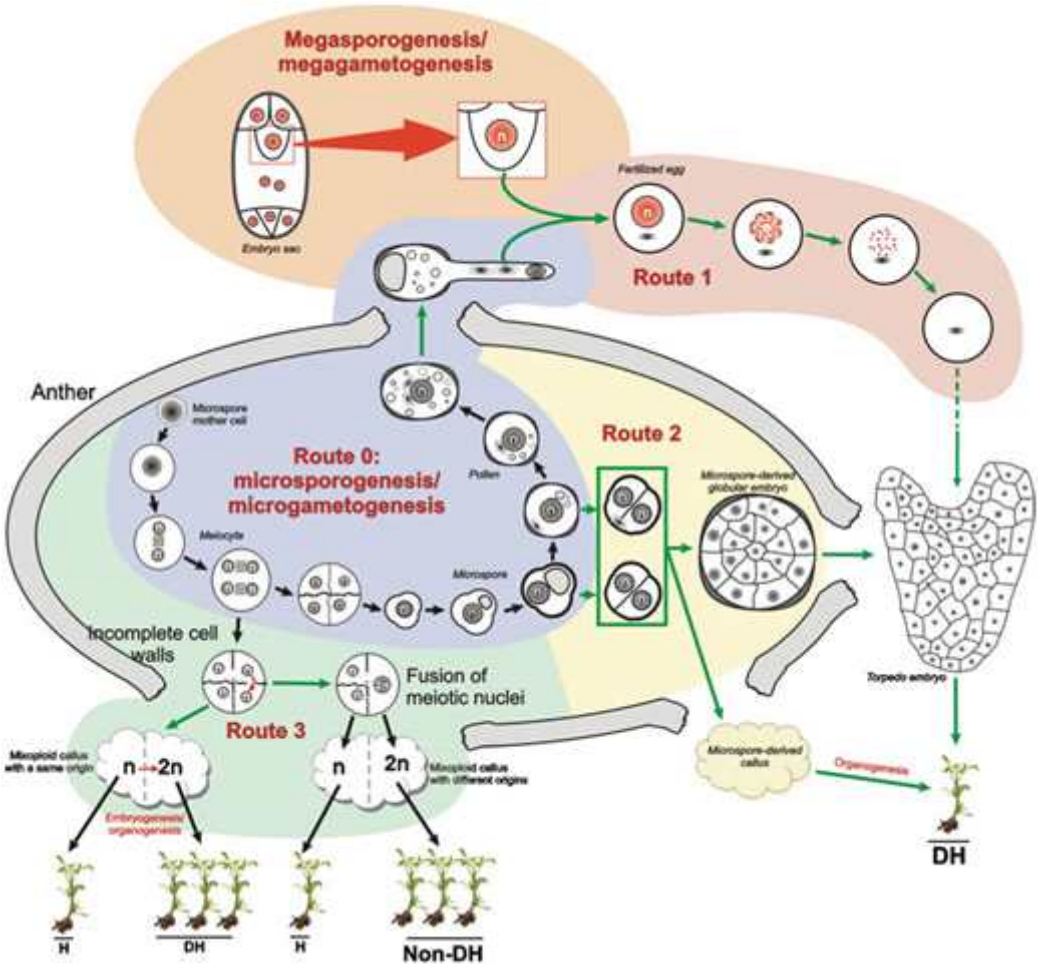


**Figure 1. Induction of microspore embryogenesis from microspores/pollen.**

Vacuolate microspores and young bicellular pollen can be deviated from the natural, gametophytic pathway (blue background) toward embryogenesis (light yellow background). Note the changes in cell size and shape, nuclear positioning, vacuole fragmentation, and plane of division (red dashed lines) between sensitive gametophytic stages and the first embryogenic stages(star-like and embryogenic microspore). Alternatively to embryogenesis, microspores may give rise to a callus (green background) capable of regenerating haploid/DH plants through organogenesis. Other microspores adopt a pollen-like development before dying (white to pink background), while many others directly arrest and/or die (pink background) (Source; Seguí-Simarro JM and Nuez F, 2008a).

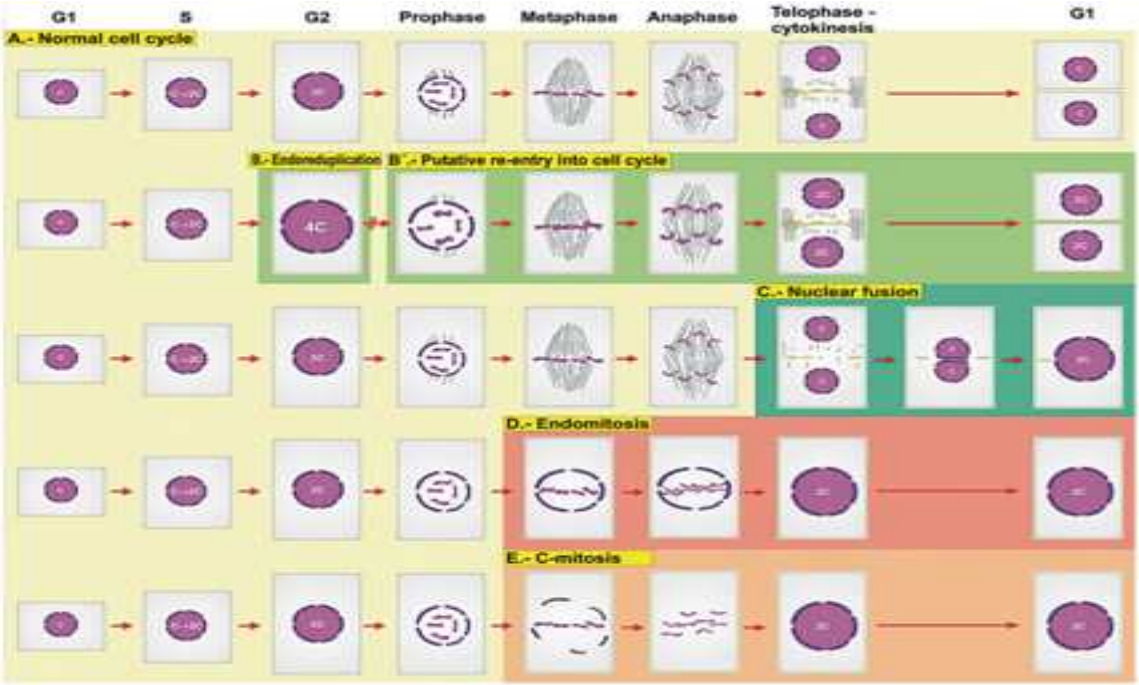


**Fig. 2. Diagram of alternatives for chromosome doubling compared with a normal cell cycle.**

(A) Normal cell cycle. (B) Endo-reduplication. (B) Putative pathway to re-enter the cell cycle with diplochromosomes after endo-reduplication. (C) Nuclear fusion after defective cytokinesis. (D) Endomitosis. (E) C-mitosis after mitotic blockage (Source, Source; Seguí-Simarro JM and Nuez F, 2008a).

or osmotic agent pretreatments (e.g., mannitol, colchicine and other antimitotic drugs). Plant hormones for *in vitro* cultures have also been directly related to DNA duplication events (Joubes and Chevalier 2000) in the microspore stage. 2) Endo-reduplication, in which DNA duplication occurs without mitosis in the absence of both mitotic spindle and nuclear envelope breakdown. Endo-reduplication is characterized by one or more extra rounds of chromatid duplication during the phase of DNA synthesis (S-phase) of the cell cycle. 3) Nuclear fusion, in which multiple nuclei merge or coalesce into a larger nucleus, resulting in mixing of both DNA contents. One of the reliable causes of nuclear fusion is parallel fusion of the interphasic nuclei, which consists of a normally-occurring karyokinesis and nuclear reassembly, followed by disrupted cytokinesis. This allows daughter nuclei to coalesce within the same cytoplasm and fuse into a single, larger nucleus with twice the chromosome number of the original nucleus. 4) C-mitosis, in which colchicine-induced collapse of the mitotic spindle and breakdown of the nuclear envelope occur. C-mitosis has been applied to an artificially-induced form of chromosome doubling produced by colchicine, whereby mitosis is blocked to differing extents depending on the dose of colchicine. At high doses, mitosis is stopped at metaphase (c-metaphase), while lower dosages allow sister chromatids to detach from each other. During this process, centromeres stay together longer due to the lower turnover rate of kinetochore microtubules, but they eventually separate, yielding doubled chromosomes. Among these, endo-reduplication is the most common way to increase ploidy during the normal life cycle of plants. Indeed, it is believed that 90% of the cases of doubling that occur in flowering plants are a result of endo-reduplication (d'Amato 1984).

### 3. Factors affecting pollen embryogenesis

Endogenous and exogenous factors influencing pollen embryogenesis have been well summarized by Ferrie and Caswell (2011), including genotypes, growth conditions and developmental stages of the donor plant, pretreatment of anthers, composition of culture medium (aminoacids, carbon source and hormones), photoperiod conditions, and the presence of somatic tissues in culture. The growth conditions influence the androgenic response by affecting the vigor and quality of donor plants (Jähne and Lörz 1995). Donor plant conditions play an important role in regeneration of embryos as well as response of microspores. The qualities of donor plants are affected by light intensity and wavelength, nutrition, photoperiod, and temperature. It has been reported that barley grown under a

growth chamber produced more DH green plants than donor plants grown under greenhouse conditions (Dahleen 1999). Studies on *in vitro* development of pollen microspore indicated that frequency of callus induction was accelerated by developmental stage of pollen. Chung and Sohn (1986) reported that the optimum developmental stage for anther culture is early to mid-uninucleate. Among the factors affecting anther culture response, temperature shock and the composition of the culture medium appear to be critical to plant regeneration (Xieet al. 1997). It has been suggested that cold pretreatment during callus induction delays pollen or anther wall senescence and increases symmetric divisions of pollen grains and the release of substances necessary for androgenesis, which primarily consist of aminoacids and heat shock proteins (Xie et al. 1997; Kiviharju and Pehu 1998). Although the mechanisms through which temperature shock act on anthers or microspores are not well understood, temperature shock seems to facilitate switching of normal gametophytic development to embryogenesis and nursing microspore in anther tissue (Zheng 2003). Chung and Sohn (1986) reported that the optimum pollen stage for rice anther culture is the early to mid-uninucleate stage and that cold shock of panicles at 8° to 12℃ for 8 to 15 days prior to inoculating anthers in the medium promoted callus induction and plant regeneration. The second critical factor affecting pollen response is media composition. The most commonly used basal media for anther culture are $N_6$ medium (Chu 1978), (modified) MS medium (Murashige and Skoog 1962), Nitsch and Nitsch (1969) medium and B5 medium (Gamborget al. 1968). It has been reported that increasing glutamine and decreasing ammonium nitrate enhance culture efficiency and embryo development in many cereals (Dattaet al. 1990). Chung and Sohn (1986) developed revised $N_6$ medium ($N_6$-$Y_1$), which reduced nitrogen source to half of $N_6$ and intensifying amino acid, L-glutamine for rice anther culture. Carbohydrates provide a source of energy and regulate the osmotic properties of the culture media. Sucrose is the most common carbon source used in anther culture, normally being present at levels of 2 to 4% (Reinert and Bajaj 1977). However, the equivalent osmotic effects in the medium, maltose have proven to be much effective than sucrose and other carbohydrates in inducing anther culture response (Bishnoi et al. 2000). High osmorality has been shown to increase green plants production and decrease the number of albino plants emerging in barley anther culture (Hoekstra et al. 1993). Auxins are essential plant growth regulators for the induction of calluses from anthers (Sohn et al. 1984, Zhu et al. 1998). IAA and NAA induce direct androgenesis, while 2,4-D accelerates cell proliferation and the formation of nonembryogenic calluses (Ball et al. 1993). A combination of 2 ppm NAA and 1 ppm kinetin and 10 ppm ABA showed better responses in both callus induction and green plant regeneration (Chung and Sohn, 1986).

## 4. Application of haploidy to breeding

One of the most interesting features of haploidy is the possibility to produce doubled haploid (DH) individuals. Breeders promptly recognized the advantages of DH technologies based on theoretical and practical aspects of plant biology and genetics (Forster and Thomas 2005). Double haploidy provides extremely useful tools for breeding programs (Chupeau et al. 1998; Dunwell 2010; Forster et al. 2007; Touraev et al. 2001). DH populations require only one inbreeding generation to induce homozygosity as opposed to the typical 7 to 8 generations required by non DH populations. Conventional methods performed to achieve homozygosity consist of carrying out several backcrosses, which is time-consuming and labor-intensive (Morrison and Evans 1987). Production of haploid and dihaploid plants has also been shown to be useful for providing access to recessive genes and biotechnological manipulations. In the context of plant breeding, DHs are essential to genetic mapping of complex traits such as yield or quality. DHs can also be applied in transgenic production to avoid heterozygotes and save time and resources in both homologous chromosomes. Moreover, they are very useful for basic investigations of linkage and estimation of recombination fractions. Although these studies can also be conducted conventionally using genetic crosses, DH populations have the advantage of generating homozygous lines simply by selfing. DH techniques have been well established in most economically important crops, including major cereals (Wedzony et al. 2009). These methods make genetic selection and screening of recessive mutants feasible and homozygous doubled haploid plants can be easily recovered by chromosome doubling of haploid plants.

## 5. Genes associated with *in vitro* response

Plant recoveries from cultured tissues have been continuously improved through factors affecting tissue culture response. However, both somatic and gametic plant regeneration have been shown to be influenced by genotype, physiological status of the donor plant, medium and the interactions among these factors (Guha-Mukherjee 1973; Niizeki 1983). In rice, indica varieties tend to be more recalcitrant than japonica varieties with respect to callus induction and plant regeneration. Genotypic differences in anther response (Guha-Mukherjee 1973) have retarded the use of anther culture for production

of indica varieties of rice. Additional extensive studies have been conducted to better understand the regulation of plant regeneration at the DNA level (for a review see Henry et al. 1994). It has been reported that *in vitro* plant tissue culture response and plant regeneration are regulated by nuclear genes (Henry et al. 1994; Taguchi-Shiobaraet al. 1997a). Many QTL analyses of tissue culture response-traits have been conducted using monocots such as barley (Bregitzer and Campbell 2001; Mano and Komatsuda T 2002), rice (Kwon et al. 2002; Taguchi-Shiobara 1999; Takeuchi et al. 2000) and maize (Murigneux et al. 1994). Five putative quantitative trait loci (QTL) controlling the regeneration ability of rice seed calluses were identified on chromosomes 1, 2 and 4 (Taguchi-Shiobara et al. 1997b). In rice anther culture, two QTLs for callus induction were identified on chromosomes 3 and 4, while two QTLs associated with green plant regeneration were detected on chromosomes 3 and 10 (Kwon et al. 2002). Bolibok and Rakoczy-Trojanowska (2006) evaluated the quantitative trait locus (qAGR-10) associated with the capacity for green plant regeneration located on chromosome 10 and found that it showed promise based on its consistencies. Moreover, molecular marker RZ400 linked to qAGR-10 was able to effectively identify genotypes with good (>10%) and poor (<3.0%) regenerability based on the marker genotypes in the 43 rice cultivars and two F2 populations (Kwon et al. 2002).

### 6. Drawbacks to anther culture

Dihaploid production through pollen embryogenesis is useful to plant breeding because it reduces the number of breeding cycles needed to generate homozygous lines. However there are several limitations to applications of anther culture to practical breeding including genotypic variance of the donor plant, gametic selection, albinism and somaclonal variations. The poor androgenic response of the recalcitrant genotypes limits utilization of this technique as a breeding tool in areas predominantly planted with this ecotype. Genotypic variance is also responsible for segregation distortions of progenies. Finally, partial and slight gametic selection has been reported (Guiderdoni 1991).

One of the major problems that must be overcome before anther or microspore culture is the occurrence of albinos amongst the regenerants (Torp and Andersen 2009). Gynogenesis is a possible alternative source for haploid production in plants, particularly in species in which androgenesis is recalcitrant or the level of albino regenerated plants is high (Bhat and Murthy 2007). Both desirable and deleterious variants have been reported in various phenotypes and agronomic traits (Cao et al. 1991; Oono 1985). Larkin et al. (1989) proposed several factors that could be a possible cause of somaclonal variation including chromosome number, physical and biochemical, amplification of genes, single gene mutation, mobilization of transposable elements and DNA methylation. A type of transposable element, *Tos 17*, which is activated during tissue culture, was reported as a potential cause of somaclonal variation in rice (Hirochika et al. 1996). The publication of draft sequences of japonica and indica rice, another type of DNA transposon, miniature inverted-repeat transposable element (MITE) called *mPing*, was also reported (Jiang et al. 2003). Activation of the *MITE* element, *mPing*, occurs with high frequency during anther culture (Kikuchi et al. 2003).

## Conclusion

In the last few decades, haploid breeding techniques have been widely applied in breeding programs of many crops. Anther culture is one of the haploid breeding techniques used to culture tissues of male gametophytes *in vitro* and obtain doubled haploid plantlets through androgenesis in a single generation, significantly reducing the time required for breeding programs. Doubled haploid techniques have recently been adopted in the generation of fine mapping popu1ations, transgenic developments and e1ucidation of the genes that confer agronomic traits. Although the application of haploid techniques was successfully launched in the breeding program of many crops, many others of interest are still recalcitrant, and the cellular, biochemical and molecular bases for the transformation of microspores are still poorly understood. Conversely, opponent gametophytic organs and unfertilized ovary/ovule culture is not sufficient to produce DH plants for breeding programs. However, there have recently been an increasing number of reports on gametic embryogenesis. Cheet al. (2011) suggested that application of gynogenesis for haploid plant production will facilitate production of plant species having male sterility or that are unresponsive to androgenesis and microspore culture. It is necessary to conduct future research programs aimed at elucidating pathways involved in mechanisms associated with microspore induction and developing creative approaches to improve efficiency of microspore culture for DH production.

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