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Invited Review Article

Haploid production technology in wheat and some selected higher plants

Saikat Kumar Basu[1], Madhuleema Datta[2], Manorma Sharma[3], and Ashwani Kumar[4]*

[1]Department of Biological Sciences, University of Lethbridge, Alberta, T1K 3M4; Canada; [2]Gurudas College, Kolkata Department of Microbiology, Gurudas College Road, Kolkata Kolkata, WB 700054 India; [3]Department of Botany, Vedic PG Girls College, University of Rajasthan Jaipur 302004, India; [4]Department of Botany, University of Rajasthan, Jaipur 302004, India

***Corresponding author: Akbiotechnology214@gmail.com**

Abstract

Haploid plants are very important in various realms of research disciplines such as plant biotechnology, molecular genetics and traditional plant breeding. They provide useful information regarding recombination and genetic control of chromosomal pairing. Haploidy expedites the breeding process thereby increasing the crop yield. Researchers have been working on the haploidy approach for more than half a century. Some crops have shown interesting results in producing haploid cultivars include bread wheat and other crops such as maize, oat and onion. This technique also has useful applications in genetic transformation for generating polyploidy wheat. Wheat cultivars developed from doubled haploid from both anther-culture and maize induction systems have been released for cultivation in all the major continents. Several techniques have been adapted for the production of haploid plants such as anther culture, isolated microspore culture some of which have been discussed in this review. With the ability to increase the yield of haploids in bread wheat and durum wheat, the haploidy technique may play an ever increasing role in basic cytogenetic, genetic, and genomic research as well as in applied plant breeding in several crop species in the not so distant future. This review aims to capture few of the great achievements being made in the field of haploid production technology in some selected crop/higher plant species and its implications to modern agriculture and in crop development programs.

Keywords: androgenesis, anther culture, double haploids, gynogeneis, haploids, isolated microspore culture
Abbreviation: 2,4-D: 2,4-dichlorophenoxy acetic acid; CPP: Cell Penetrating Peptide; DH: Double Haploid; IMC: Isolated Microspore Culture; MAS: Marker Assisted Selection; MAS: Marker Assisted Selection; MCS: Multi-cellular Structures; RIL: Recombinant Inbred Lines; QTL: Quantitative Trait Loci; SH: Synthetic Hexaploids

Introduction

Haploid plants have many uses in basic plant research disciplines such as cytogenetics, molecular genetics, crop evolution, plant biotechnology and traditional plant breeding (Chawla, 2002; Cuthbert et al., 2008; Touraev et al., 2009). Haploids provide an effective research tool for studies on induced mutagenesis and on genetic transformations (Folling and Olesen, 2002). A haploid is a common terminology used for all sporophytes, whether diploid or polyploid with the gametic chromosome number. In other words, a haploid plant derived from a diploid species is more appropriately termed a monoploid since it has only one set of chromosomes (*i.e.* one genome only) (Fehr, 1993; Quisenberry and Reitz, 1967). The monoploids being sporophytes, their somatic chromosome number is simply represented as 2*n* (as in the case of diploids and polyploids). However, 2*n* refers to somatic chromosome number; while, *x* represents the basic chromosome number (*i.e*. the chromosome number in one genome of a specific monoploid species) (Folling and Olesen, 2002). Hence, a haploid (monohaploid/monoploid) derived from diploid Einkorn wheat (*Triticum monococcum* L.) is indicated to have $2n = x = 7$ chromosomes (Taurev et al., 2009). However, a haploid derived from a polyploid species such as bread wheat (*T. aestivum* L., $2n = 6x = 42$; AABBDD) or durum wheat (*T. turgidum* L., $2n = 4x = 28$; AABB) is technically called polyhaploid. The polyhaploid of bread wheat has $2n = 3x = 21$ chromosomes with the genomic constitution ABD; whereas, the polyhaploid of durum wheat has $2n = 2x = 14$ chromosomes with the genomic constitution AB (Quisenberry and Reitz, 1967; Fehr, 1993; Folling and Olesen, 2002) (Fig. 1). Polyhaploids derived from polyploid species are further classified according to the nature of polyploidy of the parental species from which they are descended. A polyhaploid from an allopolyploid (like bread wheat) is termed *allopolyhaploid* ($2n = 3x = 21$); whereas one derived from an autopolyploid, such as the potato (*Solanum tuberosum* L., $2n = 4x = 48$), is called *autopolyhaploid* (Fehr, 1993). However, the term haploid is commonly used as a generic term in case of both diploid as well as polyploidy plant species (Allard, 1960). It is interesting to note that the terms haploid and polyhaploid are often used interchangeably for haploids generated from the polyploidy ancestral species (Allard, 1960; Fehr, 1993; Chawla, 2002). Since allopolyploid wheat is referred to as *amphidiploids* or *amphiploids*; their haploids can be termed *amphihaploids*. Thus, a haploid or allopolyhaploid derived from the durum wheat (AABB = 28) can also be referred as *amphihaploid* (AB = 14) due to their unique constitution of their chromosomes (Allard, 1960; Fehr, 1993; Quisenberry and Reitz, 1967; Touraev et al., 2009).

Doubled Haploids: What are they?

In very simple terms, a doubled haploid (DH) is a genotype produced when haploid cells undergo the process of chromosome doubling (Chawla, 2002). The DH populations, which are similar in genetics to Recombinant Inbred Lines (RILs) generated by single seed descent approach, have been applied for mapping Quantitative Trait Loci (QTLs) for several desirable characters (Muñoz-Amatriaín et al., 2008). Wheat cultivars developed from DH technology have been released for cultivation and have now turned out as dominant cultivars in several countries across the globe (Baenziger and DePauw, 2009; Touraev et al., 2009). It is in fact possible to track back on the evolutionary ladder and extract durum haploids AB and then retrieve tetraploid or disomic durum plants from them. Similarly, it is also possible to retrace the steps of evolution for bread wheat (Jauhar, 2007). Crosses of bread wheat with maize (*Zea mays* L.) has recently emerged as an efficient technology platform after the pioneering work of Laurie and Bennet (1987) as an important source for generating polyhaploids across all bread wheat germplasm/genotypes in which the hormonal influence of 2,4 dichlorophenoxy acetic acid (2,4-D) plays an essential critical role. Fairly recently; pearl millet [*Pennisetum glaucum* (L.) R.Br.] and *Tripsacum* spp. pollen sources also served an identical role in haploid production in maize (Touraev et al., 2009). The DH plants produced from monohaploids/ allohaploids represent pure bred lines. Since homozygous plants are produced in a single generation; the time period necessary for cultivar development could be efficiently reduced by 3-4 years. The selection efficiency can also be substantially improved DH production because the phenotype of the plant is not masked by dominance effects. The heritable traits encoded by recessive gene(s) could be efficiently detected and a small population of DH plants is necessary while screening for desirable recombinants (Snape et al., 1986). Microspore embryogenesis in anther/microspore cultures are the most commonly used methods to generate DHs (Maluszynski et al., 2003). Isolated Microspore Cultures (IMC) has several merits over other commonly available techniques (Touraev et al., 2001; 2009). Microspores can be isolated in greater amounts, providing large number potentially embryogenic single haploid cells. The IMC technique represents an individual cell (*n*), making single cell selection a reality and it also makes it possible to investigate the impacts of different media constituents on microspore performance directly (Touraev et al., 2001; 2009; Basu et al., 2010). A table summarizing important DH techniques used in selected species of higher plants is presented in table 1. Haploids generated from anther culture and IMC are regularly studied both for inter- and intra-genomic chromosomal pairing in their seed heads without any regular colchicine treatment or application of cross pollination. Recently researchers in Australia reattempted to generate sterile haploid plants to overcome major obstacles such as sufficient pollen production, in petal staining by pollens and self-pollination during the pollen transport in Sturt's desert pea [*Swainsona formosa* (G. Don) Joy Thomps.] (Zulksrnsin et al., 2002). The researchers from Kuwait successfully introduced this particular species and developed a protocol for the mass production of the selected clones using somatic embryogenesis approaches (Sudhersan and AboEl-Nil, 2002). In Iran, gynogenesis (development of plants from female gametophyte) has been adapted as a suitable method for haploid plant production. Important effects on the successful production of haploid plants in tobacco (*Nicotiana tabacum* L.) are the stage of microspore development, anther culture medium, pretreatment of anthers prior to placing them on the anther culture medium and the duration of time in the flowering cycle of the mother plants supplying anthers for the tissue culture process (Sudhersan and AboEl-Nil, 2002; Touraev et al., 2009). The DH production and the cross-pollinated DH-progenies could possibly be the most important source for variability in the population for rye (*Secale cereale* L.) breeding or selection. In case of DH production in cereals, for example barley (*Hordeum vulgare* L.) through anther (Fig. 2) and/or isolated microspore culture/IMC standard protocols are available (Clapman, 1973; Köhler and Wenzel, 1985; Hoekstra et al., 1992); while in others, such as triticale, new protocols and opportunities are currently being investigated (Tauarev et al., 2009). Previous studies on rye DH generation begun in the mid 1970s to the late 1990s and focused predominantly on the anther culture techniques only (Wenzel and Thomas, 1974; Thomas et al., 1975; Wenzel et al., 1977; Flehinghaus et al., 1991; Dainel, 1993; Flehinghaus-Roux et al., 1995). Earlier, Deimling et al. (1994) described the successful regeneration of fertile green plants from Isolated Microspore Culture (IMC) of semi-wild rye (*Secale vavilovii* Grossh) SC35. However, it is interesting to note that two different research groups, working at two different laboratories, Immonen and Anttila (1996) and Rakoczy-Trojanowska et al. (1997) simultaneously reported moderate success in the frequency of regeneration green plants from true rye (*Secale cereale* L.) only. Next, Wenzel et al. (1975) isolated microspores either directly from the spikes or from the pre-cultured anthers; but, unfortunately, they were unable to regenerate fertile green plants in either case. Later, Immonen and Anttila (2000) described rye anther culture in the liquid medium; followed by Guo and Pulli (2000) who successfully demonstrated relatively high callus induction rates and green plant regeneration frequencies from IMC of true rye (*Secale cereale* L.). Oat (*Avena sativa* L.) haploid and DH production through wide hybridization (Rines and Dahleen 1990; Matzk, 1996; Rines 2003; Sidhu et al., 2006) and anther cultures (Rines 1983; Kiviharju et al., 2000, 2005) have been reported. Rines et al. (1997) made initial progress in the development of anther culture method for oats. Later, Kiviharju et al. (2005) have improved the anther culture method by several adjustments to the older methods and reported up to 30 green plants per 100 anthers cultured for individual cross. However, in case of barley (*Hordeum vulgare* L.), Davies and Morton (1998) demonstrated higher frequencies of green regenerants per anther using IMC approach compared to the conventional anther culture techniques. A possible explanation for this could be the fact that individual microspore being suspended in liquid culture medium in IMC has sufficient and continuous supply of nutrients; whereas, microspores in the anther culture technique are exclusively dependent on the constant diffusion of available nutrients through the anther wall (Tauraev et al., 2009; Basu et al., 2010). Another challenge associated with IMC has been the problem of production of albino plantlets which often impacts breeding programs considerably by reducing the frequency of green plantlet generation (Tauraev et al., 2009). The albino production is mostly associated with the species, genotypes and the cultural conditions and sometimes difficult to eradicate.

Production approaches and technologies used in the breeding of some selected species of higher plants

For the production of haploids of durum wheat and bread wheat, it is important to first comprehend their genomic

Table 1. Summary of techniques used in Haploid productions.

Techniques for haploid production	Plant species	Years of development	References
Anther culture	*Nicotiana spp.*	1965-1969	Nistch,1969
Anther and microspore culture	*Hordeum Vulgare*	1973-2009	Clapman, 1973; Hoekstra et al., 1992; Touraev et al., 2009
Somatic embryogenesis	*Swainsona formosa*	2000-2002	Sudhersan and AboEl-Nil, 2002
Anther culture	*Avena sativa*	2000-2005	Kiviharju et al., 2000; 2005
Somatic embryogenesis	*Swainsona formosa*	2000-2002	Sudhersan and AboEl-Nil, 2002
Gynogenesis	*Allium cepa*	2006-2008	Alimousavi, 2006; Touraev et al., 2009
Maize based haploid induction	*Triticum turgidum*	2000-2008	Touraev et al., 2009
Anther culture in solid and liquid medium	*Secale cereale*	2000-2009	Touraev et al., 2009; Basu et al, 2010.
IMC	*Triticale*	2005-2009	Touraev et al., 2009; Basu et al, 2010.

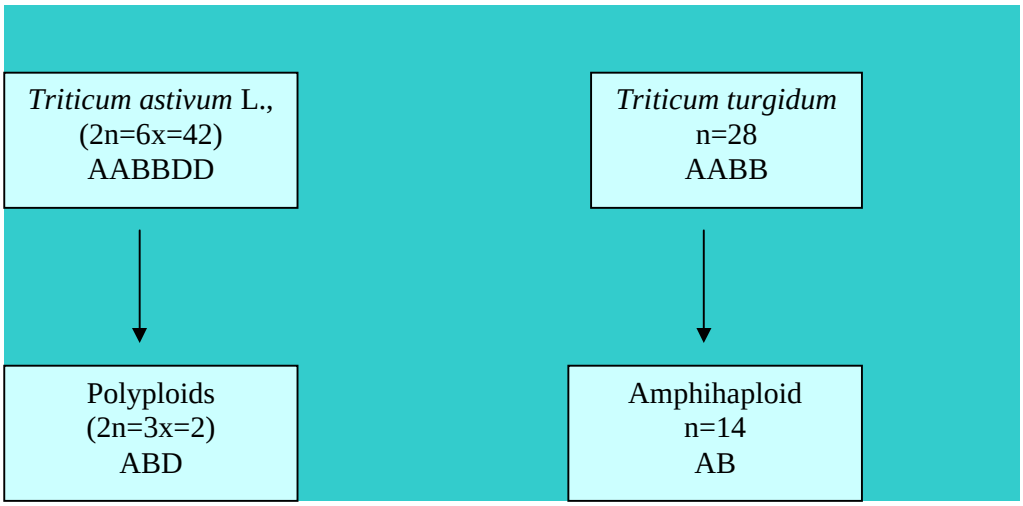

Fig 1. Formation of haploids in wheat**.**

constitution and genetically-enforced meiotic integrity. Cultivated wheat provided us with a classical model of evolution by the process of allopolyploidy. They are in fact the true breeding natural hybrids. Furthermore, bread wheat is an allohexaploid with three distinct genomes namely AA, BB and DD, collectively referred as the ABD genomes. If the extra chromosome is from the basic gametic set (*x*) of the concerned species then the plant is called a *disomic haploid* ($2n = x + 1$) since it exhibits disomy for an individual chromosome but will be monosomic for the remaining chromosomes. Genetically similar and evolutionarily related chromosomes are called *homeologues*. Because the homeologous chromosomes (*e.g.* chromosome 1A, 1B and 1D) are closely related and are capable of mutual pairing with one another; a specific homeologous pairing-suppresser gene called *Ph1* specifically restricts pairing among the homologous partners. Such a genetically-enforced pairing mechanism similar to diploid species confers regularity in meiotic cycle of the species and brings about reproductive stability to the polyploid wheat members (Touraev et al., 2009; Basu et al., 2010). Spikes on detached tillers of all durum cultivars and Synthetic Hexaploids (SH) were placed under water following hand-emasculation and the plants and finally pollinated after two days with fresh maize pollen (Mujeeb-Kazi et al., 1987,2002). The pollinated spikes were cultured in a nutrient solution containing 40 g/L sucrose, 100 mg/L 2,4-D, 8 mL/L sulfurous acid and kept in a growth chamber. To facilitate embryo formation and development, the growth chamber conditions were maintained at continuous 22.5 ^{o}C temperature, 12 hour day length and 60-70 % relative humidity. After 14-16 days of pollination the seed sets were removed from individual spike, counted, sterilized and dissected under a stereo-microscope to excise the newly-formed embryos. Subsequently, embryo formation frequencies were calculated for each durum genotype and its' related SH wheat derivative. There were about 5-7 spikes tested for individual entry. The embryo rescue, cold treatment, regeneration and transplantation procedures were similar to those of Mujeeb-Kazi et al. (1987). Using improve

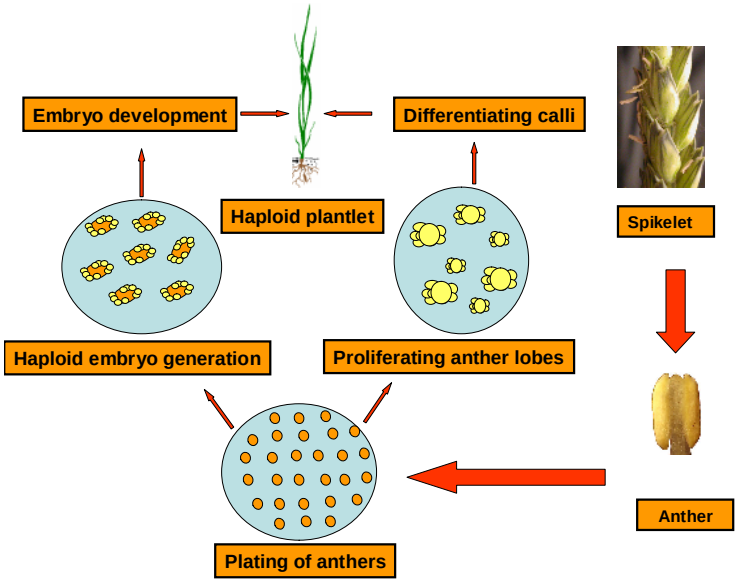

Fig 2. Schematic representation of the green plant regeneration following anther culture technique in cereals (wheat).

ed plant regeneration rates from oat anther culture some DH lines were obtained yielding comparatively higher than the commercial cultivars. DH lines of wheat have been used for selection of high molecular weight glutenin subunits (Rines 2003; Sidhu et al., 2006; Kiviharju et al., 2000, 2005; Touraev et al., 2009). Cytology protocols of Mujeeb-Kazi et al. (1994) allowed validation of the haploid status *i.e.* $n = 3x = 21$, (ABD) or, $n = 2x = 14$, (AB), and also of their meiotic associations on separate test plants for individual haploid plants. After three weeks of growth all haploid plants were colchicine treated as a root-treatment procedure (Mujeeb-Kazi and Riera-Lizarazu, 1996). Successful chromosome doubling was reported to be achieved from the seed setting on the colchicine-treated polyhaploid plants in producing haploid onion cultivars like 'Sefid- e- Kurdistan' and 'Sefid-e- Neishabour' (Touraev et al., 2009). Gynogenesis is extensively used in Iran for the production and release of high yielding and disease resistant cultivars/varieties of onion (*Allium cepa* L.) (Alimousavi, 2006). Iranian onion cultivars have good horticultural characters such as better storage/shelf life, bigger shape and resistance to thrips (*Thrips tabaci* Hind.) (Alimousavi, 2006). Polyamines (*e.g.* putrescine, spermidine, spermine, cadaverine and agmatine) are plant growth regulators that are involved in all biochemical and physiological growth and development process of higher plants (Kumar et al., 1997). Increasing polyamine biosynthesis has been reported to precede or accompany callogenesis under *in vitro* conditions (Ponchet et al., 1982) and organogenesis (Aribaud et al., 1994). Martinez et al. (2000) reported positive effects of polyamines (putrescine and spermidine) in case of *in vitro* gynogenesis in onion. Several variables have been scrutinized for their effect on haploid plant generation on *Nicotina* species from anther culture such as the particular stage of the microspore development, the anther culture medium and the pretreatments of anthers before placing them on the culture media and the specific stage of the flowering cycle during which the floral buds were procured for tissue culture work. Several *Nicotiana* species (namely, *N. glutinosa* L., *N. knightiana* Goods., N. *paniculata* L., *N. rustica* Schrank, *N. sylvestris* Speg. & Ccmes and *N. tabacum* L.) were produced primarily as haploid plants from their corresponding anther culture. Diploid plants were obtained from the anther cultures of *N. bonariensis* Lehm., *N. longiflora* Cav., *N. langsdorfii* Wein., *N. nudicaulis* Wat., *N. plumbaginifolia* Viv. and *N. stocktonii* Brand. Plants of undetermined ploidy level were obtained from *Nicotiana* too (Nistch, 1969). Anthers that were cultured at the optimal uninucleate/binucleate microspore stages generated higher frequency of plants. More plants were generated per anther from these cultures than from anthers cultured at other microspore stages. Anthers from floral buds collected from plants in flower for an extended period did not produce as many plants as those procured from plants in earlier stages of development. Low temperature anther pretreatment increased the number of plants produced under specific cultural conditions, but did not extend the number of species in which haploid plants were formed. The effect of culture medium varied with the species, length of flowering time and with when the anthers were procured and also on the pretreatment conditions (reviewed in Touraev et al., 2009). Recently *S. formosa* plants were successfully introduced to Kuwait from southern Australia. Haploid plantlets have been produced from pollen grains through somatic embryogenesis for the first time in this species (Sudhersan and AboEl-Nil, 2002). In cereal, double haploid production, for example in the case barley (*Hordeum vulgare* L.) using anther and microspore culture techniques are currently available (Clapman, 1973; Köhler and Wenzel, 1985; Hoekstra et al., 1992; Touraev et al., 2009). In rye (*Secale cereale* L.) anther culture in solid and liquid medium and in the Isolated Microspore Culture (IMC) have been successfully compared. Three weeks cold pretreatment of spikes and two days of mannitol pre-treatment of anthers maximized callus and green plant generation under both culture methods (Touraev et al., 2009; Basu et al., 2010). Intensity order of the culture methods in callus and green plant production was: IMC, anther culture in liquid medium and anther culture in solid medium. Genotype ability of embryogenesis followed the same pattern in both cultivation methods (reviewed in Touraev et al., 2009). It has been reported that the cold pretreatment (4 °C) in the dark for minimum 6 weeks was necessary to consistently achieve microspore growth transformed into Multi-cellular Structures

(MCS). Longer pre-treatments of up to 9 weeks were also investigated and reported to be positively correlated with the number of MCS generated (Barinova et al., 2004). Microspore culture media with pH 8.0 produced significantly more MCS bigger than eight cells in dimension in comparison to media with pH 5.8. The use of media conditioned by actively growing barley microspores significantly increased the numbers of MCS bigger than eight cells in dimension in sharp contrast to non-conditioned media. Green plants were successfully regenerated from cultures using conditioned medium only (Davies, 2003). However, in case of durum wheat, results indicate that haploid production is still a long way to go from having the protocol integrated into a durum wheat breeding program and to expedite the efficiency compared to bread wheat breeding (Mujeeb-Kazi et al., 2002). Limited success has been reported with high fertilization frequencies in a very small number of durum genotypes (O'Donoughue and Bennett, 1994). It is also encouraging that some high quality cultivars have responded to maize based haploid induction (Touraev et al., 2009). Hence, these cultivars could form a basis for exploitation in molecular mapping, cytogenetics and genetic transfer of genes of interest in future breeding programs where DH is considered advantageous by several researchers (Mujeeb-Kazi, 2005; Touraev et al., 2009; Basu et al., 2010). Durum wheat has inadequate genetic diversity for some important production limitations for which presently *Fusarium* head scab apparently is the priority. The D genome based SH wheat have good resistance, and it is plausible to transfer the *Aegilops tauschii* Coss. resistance into high quality durum wheat that are amenable to haploid generation (Almouslem et al., 1998). The strategy involves the *ph1c* Capelli genetic stock to initiate A and D genome interchanges via homoeologous recombination; thereby identifying the D introgression derivatives, next top-crossing with novel durum lines (positive for haploid induction) and achieving homozygosity of the translocated lines. This approach is adapted from Mujeeb-Kazi (2001) currently in use for several bread wheat germplasm/cultivars. The durum identified as being amenable to haploid induction are good candidates for use in development of molecular mapping populations where mostly F1 seed from resistant and susceptible crosses are efficiently used, having at least the trait susceptible durum cultivar(s) responsive to haploidy. Such populations are commonly generated for bread wheat and wheat researchers are optimistic that a similar approach may work out successfully for durum instead of the traditional method of generating recombinant inbred lines through single seed descent approach (Touraev et al., 2009; Basu et al., 2010).

Utilities of haploid cultivars

The haploidy technology has now been adapted in different plant breeding programs across all the major continents as the most commonly used approach for rapid crop development for transferring genes of interest, chromosomal segments or even complete chromosomes (Ceoloni and Jauhar, 2006; Baenziger and DePauw, 2009; Touraev et al., 2009). Induced haploidy facilitates the stabilization of heterozygous wheat source with gene(s) of interest. Advanced hybrid lines are then haploidized by crossing with maize following chromosome doubling for bringing in homozygosity for the introduced foreign gene(s) thereby stabilizing the newly reconstructed highly complex plant genome. This technique also has been very found to be extremely helpful in genetic transformation of polyploidy wheat (Touraev et al., 2009). Direct introduction of genes at the haploid state following chromosome doubling helps considerably in the stabilization of the newly generated synthetic transformants. The haploid technique was successfully employed to produce inbreds in maize in the 1940s and 1950s (Chase, 1951). Thus, before 1980, DH technique was applied only to a small number of plant species; however, in the past four decades huge volume of research has been successfully conducted all over the globe. Now this technique has been successfully applied to over more than 250 species (Maluszynski et al., 2003; Baenziger et al., 2006; Touraev et al., 2009; Basu et al., 2010). The technique is now commonly applied for rapid production of pure inbreds and for expediting plant breeding programs (Baenziger and DePauw, 2009). Interestingly, doubled haploidy is being exclusively applied by at least one US commercial seed company to generate new maize inbreds (Seitz, 2004; Touraev et al., 2009). Wheat cultivars developed from DHs from both anther-culture and maize induction systems have been released for cultivation in all major continents (Guzy-Wróbelska and Szarejko, 2003; Thomas et al., 2003; Humphreys et al., 2007 a,b; Touraev et al., 2009). DHs have been widely employed in barley breeding programs resulting in the release of several cultivars. (Muñoz- Amatriaín *et al.*, 2008). Androgenesis (development of plants from male gametophyte)-based DH-technique has been increasingly adapted to release barley varieties better adapted to Peruvian highlands where barley is grown as a major food crop (Gomez-Pando et al., 2009). Using improved plant regeneration rates from oat anther culture, Kiviharju et al. (2005) developed some DH lines that exhibited similar or higher yields compared to the currently available commercial varieties. The DH lines of wheat have been successfully utilized for selection of high molecular weight subunits of glutenin (Radovanovic and Cloutier, 2003).

Conclusion and future prospects

Haploid plants and haploid-derived homozygous lines are useful in several domains of basic research in the realms of classical plant genetics and cytogenetics, modern molecular genetics including induced mutagenesis, site-directed mutagenesis, genetic transformation research, genome mapping and assessing distant genome relationships, gene dosage effects, analysis of linkages, mechanisms of the genetic control of chromosomal pairing and in conventional plant breeding studies. The DH technology platform offers a rapid mode of truly homozygous line production that help to expedite crop breeding programs where homogeneity is an absolutely essential parameter for rapid crop development. Integration of the haploidy technology with other available biotechnological tools such as Marker Assisted Selection (MAS), induced mutagenesis, and transgene technologies could also effectively expedite the crop improvement programs running all across the globe. Thus direct incorporation of cloned genes at the haploid level following subsequent chromosome doubling may help accelerate stable integration of target gene(s) into wheat and several other crops and/or higher plant species. To be useful, however, it is important to note that an efficient and reliable method of haploid and DH production will be essential. Recent research has very clearly demonstrated that maize-induced chromosome elimination offers a very useful approach for rapid haploid plant production in bread wheat and durum wheat. However, further improvements in microspore culture could substantially bring in more changes in the not so distant future. It is interesting to note that the totipotent

nature of the haploid cell is being efficiently and effectively explored in different facets of modern biological and agricultural research disciplines. By efficiently utilizing DH populations, QTLs associated with yield and yield components have been successfully identified allowing marker-assisted breeding approaches to be employed in several major wheat improvement programs (Touraev et al., 2009). The haploidy technique has played an important role in practical plant breeding as can be seen in widely grown DH cultivars in all the major continents where some of them have earned the recognition of dominant cultivars.

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