Recent advances and application of doubled haploids in wheat breeding

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Genetic improvement to develop varieties with high yield potential and resistance/tolerance to abiotic and biotic stresses with acceptable end use qualities is the most viable and environment friendly option to increase wheat yield in a sustainable fashion. In vitro haploid production followed by chromosome doubling greatly enhances the production of complete homozygous wheat lines in a single generation and increases the precision and efficiency of selection process in wheat breeding. It also enables the detection of linkage and gene interactions, estimate genetic variance and the number of genes for quantitative characteristics, produce genetic translocations, substitutions and chromosome addition lines, and facilitate genetic transformation and mutation studies. Wheat cultivars developed from doubled haploids using anther-culture and maize induction systems have been released for cultivation in both developed and developing countries. In this review, the origin and production of haploids, techniques in anther-culture and wheat x maize wide crosses, and their application in wheat breeding are summarized.

Key words: Doubled haploid, wheat breeding, wheat yield.

INTRODUCTION

Wheat is the most widely grown cereal crop in the world and one of the central pillars of global food security. About 651 million tons of wheat was produced on 217 million hectares in 2010 with productivity level of 3 t/ha\(^1\) (FAO, 2012). After the quantum leap of the Green Revolution, wheat yields have been rising by only 1.1% per year, a level that falls far short of the demand of a population that is growing by 1.5% or more annually. According to some estimates, the global wheat production must increase at least by 1.6% annually to meet a projected yearly wheat demand of 760 million tons by 2020 (Rosegrat et al., 2001).

Genetic improvement to develop varieties with high yield potential and resistance/tolerance to abiotic and biotic stresses with acceptable end use qualities is the most viable and environment friendly option to increase wheat yield in a sustainable fashion. Such improvement of crops requires creation and introduction of genetic variation, inbreeding coupled with selection and extensive evaluation of breeding materials at multi-locations to identify adapted and stable genotypes with desirable agronomic traits. Variations could be created by sexual crosses (usually single, three-way, double or back crosses) and mutation breeding. Breeders have used different methods to fix and develop homozygous genotypes from such variations. Isolation of homozygous and homogeneous genotypes through conventional inbreeding methods (single seed descent, backcrossing and selfing in the field, plus in some cases, the use of off-season nurseries and shuttle breeding approaches) requires several cycles of inbreeding and selection, making it the most tedious, time consuming and expensive phase of any breeding program. Furthermore, in conventional plant breeding, truly homozygous lines are rare and most selection contains some heterozygous loci (Baenziger and Peterson, 1992; Baenziger and

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Recent advances in plant tissue culture and its related disciplines opened an avenue that greatly facilitated the haploidioploidy breeding scheme, which enables the extraction of instant homozygous lines/varieties from crop plants with any degree of heterogeneity in a single generation (Baenziger and DePauw, 2009; Wu et al., 2012). Haploid individuals are sporophytes with gametic chromosome number (Riley, 1974; Ouyang et al., 1973) and by doubling the haploid chromosome complements doubled haploids (DH) can instantly be produced. In this review, the origin and production of haploids, techniques in anther-culture and wheat x maize wide crosses, and their application in wheat breeding are summarized.

**DOUBLED HAPLOIDS: ORIGIN AND PRODUCTION**

All the cultivated wheat belongs to the genus *Triticum*, which in turn is divided into three major taxonomic groups: einkorn, emmer and dinkel by Schultz (1913). This classification was supported by the pioneering cytological study of Sakamura (1918), who found that Schultz’s three wheat groups also differ in their chromosome number; the einkorns are diploids (2n = 2x = 14), the emmers are tetraploids (2n = 4x = 28) and the dinkels are hexaploids (2n = 6x = 42), all with the basic chromosome number x = 7. Soon after, based on cytogenetic analysis, Kihara (1924) designated the genome formulae for the cultivated einkorn (*Triticum monococcum* L., 2n = 2x = 14), emmer (*Triticum turgidum* L. 2n = 4x= 28) and dinkel (*Triticum aestivum*, 2n = 6x = 42) as AA, AABB and AABBD, respectively.

Each group of wheat forms their own respective haploids. Haploids are sporophytes that contain gametic chromosome numbers (n). The haploids from einkorn, emmer and dinkel possess n = x = 7, n = 2x = 14, and n = 3x = 21chromosomes with genomic constitution of A, AB and ABD, respectively (Quisenberry and Reitz, 1967; Fehr, 1993; Folling and Olesen, 2002). Haploids can originate spontaneously in nature or as a result of various induction techniques. Spontaneous development of haploid plants has been known since 1922, when Blakeslee first described this phenomenon in *Datura stramonium* (Blakeslee et al., 1922). However, spontaneous occurrence is a rare event and therefore of limited practical value. Doubled haploid (DH) is a genotype produced when haploid cells undergo the process of chromosome doubling (Ouyang et al., 1973; Picard and De Buyser, 1973). Doubled haploid production requires induction of haploids and doubling of chromosomes (Snape, 1989; Raina, 1997).

**Haploid induction techniques**

The potential of haploidy for plant breeding arose in 1964 with the achievement of haploid embryo formation from *in vitro* culture of Datura anthers (Guha and Maheshwari, 1964), which was followed by successful *in vitro* haploid production in tobacco (Nitsch, 1969). Subsequently, wheat haploids have also been produced by anther culture (Ouyang et al., 1973), isolated microspore culture (Wei, 1982) and by using wide hybridization with *Hordeum bulbosum* and *Zea mays* (Barclay, 1975; Laurie and Bennett, 1986, 1988; Inagaki, 1997). Anther culture and wheat x maize wide cross systems are the two most commonly used induction methods in wheat.

**Anther culture**

The first success in regeneration of wheat (*T. aestivum*) plants through anther-culture was achieved in the early 1970s (Ouyang et al., 1973; Picard and De Buyser, 1973; Liang et al., 1987; Baenziger et al., 1989). Anther-culture exploits the fact that a certain proportion of pollen grains *in situ* are embryogenic. These pollen grains can develop into embryos only when they are placed on artificial medium. In anther culture systems, stress is of critical importance for blocking gametophytic development and for triggering pollen embryogenesis in competent microspores (Bueno et al., 1997; Touraev et al., 1997). The process of anther-culture begins in the selection of primary wheat spikes which contain anthers with pollen at the mid-late uninucleate stage of development (Ouyang et al., 1973; Picard and De Buyser, 1973; Liu et al., 2002). The stage of pollen development is very important as minor deviations can lead to major decreases in yield. Anthers are then aseptically dissected and cultured on induction medium such as CHB3, N6 and ½ MS, and incubated in darkness at 26 - 28°C for 4 to 6 weeks after which calli are transferred to a solid plant regeneration medium and incubated in a culture room with 25°C and 16 h day length for about 30 days. Green plantlets are transferred to culture tubes containing 20 ml of modified plantlet regeneration medium. After one to two months of hardening, vigorous seedlings were transplanted into pots with 2:1:1 soil : sand : peat : moss mixture and kept in a plastic house (Tawkaz, 2011).

Anther culture is used in many cereal breeding programmes and is more cost-effective than intergeneric crosses in the production of DHs (Snape et al., 1986; Pratap et al., 2006). However, anther culture is highly dependent on genotypes (Wehr and Zeller, 1990; Pratap et al., 2006, Khiabani et al., 2008; Grauda et al., 2010; El-Hennawy et al., 2011; Tawkaz, 2011). The procedure of anther-culture in wheat is illustrated in Figure 1. To counter the genetic effect, components of the system such as growth environment of donor plants, modification of the medium components, changing the physical state of the medium (liquid or solid), cold pretreatment of anthers before culturing, increasing the incubation temperature during the first few days of culture and...
subjecting anther explants to gamma irradiation have been investigated by different authors (Karimzadeh et al., 1995; Karsai et al., 1994; Xynias et al., 2001; Zamani et al., 2003; Shirdelmoghanloo et al., 2009; Tawkaz, 2011). Recently, Grauda et al. (2010) have reported increased DH production efficiency through the utilization of androgenic microspore culture (AMC) induction medium with copper. The positive influence of copper while obtaining DH plants in anther culture is expressed both in reducing the number of albino plants and in increased numbers of green plants-regenerants. These effects are related to improved survival of microspores during the different tissue culture stages and with the synchronization of the first microspore symmetric division (Wojnarowicz et al., 2002; Jacquard et al., 2009).

A further disadvantage of anther-culture in barley and wheat breeding is in the high rates of albino plants. In some bread wheat genotypes the frequency of albinism has been found to range from 20-50% with a mean of 30% of all regenerated plants (Abd El-Maksoud, 1992; Abd El-Maksoud et al., 1993; Tawkaz, 2011). Durum wheat (T. turgidum subsp. Durum), is very recalcitrant to anther-culture with low regeneration rate and very high frequency of albino plants (Cistué et al., 2006, 2009; Labbani et al., 2007). In an effort to improve this problem in durum wheat, Ayed et al. (2010) have applied different pre-treatments and found that cold treatment for five weeks (4°C) improved significantly the embryogenesis induction and green plant regeneration with green : albino plant ratio of about 75%. Mannitol pretreatment has also be found to be effective in improving the efficiency of anther-culture in durum wheat (Labbani et al., 2007; Ayed et al., 2010). Previously, Bueno et al. (1997) have reported the significant effect of starvation and heat shock on successful production of haploid plants in cork oak.

**Wheat x maize wide cross method of haploid induction**

Wide crosses have been utilized for the production of haploids for crop improvement and genetic studies (Baum et al., 1992). Bread wheat DH are produced by various intergeneric crosses viz., wheat x maize (Laurie and Reymondie, 1991; Inagaki and Mujeeb-Kazi, 1995; Inagaki, 2003) wheat x pearl millet (Inagaki and Mujeeb-Kazi, 1995; Amin et al., 2010), wheat x teosinte (Suenaga et al., 1997), wheat x barely (Barclay, 1975) and wheat x sorghum (Inagaki and Mujeeb-Kazi, 1995). The crossability of T. aestivum × Hordeum bulbosum depends on the wheat allelic composition for the K_r genes responsible for the incompatibility between these two species (Sitch and Snape, 1987). Production of haploids in wheat through wheat x maize crossing was reported successful without the development of albino plants (Sadasivaiah et al., 1999; Ushiyama et al., 2007) and insensitivity of maize to the action of crossability inhibitor (K_r) genes. Several reports demonstrated the success of doubled haploid plant production using maize pollen on hexaploid wheat (Suenga and Nakajima, 1989; Amrani et al., 1993; Niroula et al., 2007) but relatively few durum wheat genotypes show such crossability with
Figure 2. Procedures of wheat x maize wide cross method for doubled haploid wheat production: a = growing maize plants; b = pollinated spikes with maize pollen; c = obtained seed from crosses with maize; d = plant developing from rescued embryo; e = haploid plants under colchicine treatment (0.2%); f = chromosomes counting (Inagaki, 2003).

maize (Almouslem et al., 1998; David et al., 1999; Garcia-Llamas et al., 2004). Recently, Sourour et al. (2012) have reported the production of successful DH durum wheat genotypes using the wheat x maize system.

In wheat x maize system, the culture of wheat donor plants is carried out in climate chambers and in the greenhouse. One to two days before anthesis, the wheat florets are emasculated and two days later they are pollinated with fresh maize pollen. Cryopreserved (-80°C) maize pollen can also be successfully utilized for pollination of wheat florets. However, the efficiency of haploid formation was quite low as compared to fresh pollen (Inagaki, 1997). But this technique is quite advantageous where synchronization is a problem. Once the pollination is complete, the application of auxin is essential for the successful recovery of haploid wheat embryos (Suenga and Nakajima, 1989; Niroula et al., 2007). Recently, frequency of polyhaploid embryo formation has greatly improved through the manipulation of Dicamba alone or in combination with 2,4-dichlorophenoxyacetic acid (2,4-D) (Almouslem et al., 1998; O’Donoughue and Bennett, 1994; Garcia-Llamas et al., 2004; Ahmad and Chowdhry, 2005). Embryo rescue is essential to recover haploid plantlets from wheat x maize system (Sood et al., 2003; Suenga and Nakajima, 1989; Niroula et al., 2007; Zhang et al., 1996; Niroula and Bimb, 2009) after 14 to 20 days of post pollination. Excised embryos can be cultured on either full strength MS (Murshige and Skoog, 1962) or ½ MS or B5 basal medium (Gamborg et al., 1968) containing various modifications of organic supplements (Zenkler and Nitzsche, 1984; Zhang et al., 1996; Suenga et al., 1997; Campbell et al., 2000; Singh et al., 2004; Ayed et al., 2011) and can be grown in vitro for 3 to 5 weeks at 20 to 25°C and 16 h day length. Generally, seedlings are ready for transfer up to that period and need to be hardened for one week in growth chamber at the same environmental regime. Figure 2 illustrates the wheat x maize haploid
production system.

Chromosome doubling

Haploid plants are infertile as sexual fertility depends upon meiotic division of the diploid chromosome number. Spontaneous rates of chromosome doubling among plants derived from microspores of wheat are relatively low. In many experiments, only 15 to 20% of the plants obtained are capable of seed set by selfing without a treatment for chromosome doubling (Hansen et al., 1988; Navarro-Alvarez et al., 1994; Ouyang et al., 1994). To regain fertility, the number of wheat chromosomes need to be doubled following haploid embryo rescue and seedling formation. Colchicine is the most frequently used drug for chromosome doubling in plants (Ouyang et al., 1994; Soriano et al., 2007). The drug inhibits spindle function during mitosis and disturbs normal polar segregation of sister chromatids to form a restitution nucleus. Upon mitotic divisions of such affected cells, chromosome doubled chimera sectors are formed, which lead to partial fertility of the plant if they comprise sexual organs. Colchicine is traditionally administered to the young plants established in soil at the 3 to 5 tillering stages.

For the treatment of colchicine, the procedure proposed by Inagaki (1997) is very efficient. According to his method, roots of the haploid seedling are pruned leaving a zone of 2 to 3 cm and submerged in a 0.1% colchicine solution supplemented with 2% dimethyl sulfoxide and ca. 0.05% Tween 20 at 20°C for 5 h. After this treatment, the roots are washed free of residual colchicine and potted in peat soil. In a large scale production of doubled haploids, the colchicine treatment of individual plants after establishment in soil is expensive both in terms of chemical and labour costs. It causes high mortality rate and production of mixoploids or chimeric plants that leads to low seed production and therefore, an additional growth cycle for seed multiplication before evaluation in the field (Chen et al., 1994; Islam, 2010). Chromosome doubling techniques directly integrated into the haploid induction procedures may have a potential for more cost efficient chromosome doubling of haploids for future wheat breeding. Different methods for in vitro chromosome doubling of wheat have been proposed. Colchicine has been added directly to the anther culture induction medium at concentrations of about 0.2 g/l (500 M). Anthers were transferred to colchicine free medium after 72 h, resulting in a frequency of fertile plants up to about 70% (Barnabas et al., 1991; Navarro-Alvarez et al., 1994). Alternatively, Ouyang et al. (1994) cultured pollen calli on colchicine containing medium during regeneration and reported an average increase in the frequency of fertile plants from 17% in the control to 54% with the best in vitro treatment. Recently, however, it has been reported that high concentrations of colchicine have a toxic effect that reduces the number of embryos derived from the microspore culture (Barnabas et al., 1991; Navarro-Alvarez et al., 1994). Furthermore, the early chromosome doubling event may lead to a possible increased frequency of aneuploids among in vitro chromosome doubled wheat haploids.

Several microtubule depolymerising herbicides were also proved to be efficient for in vitro chromosome doubling of microspores. Zhao and Simmonds (1995) tested trifluralin and Hansen and Andersen (1996) trifluralin, oryzalin and amiprophos methyl in rapeseed. They achieved the mean rate of diploiddization of 60 to 65%, which is comparable with the application of colchicine. Most recently, Pintos et al. (2007, 2010) have reported other effective antimitotic agents such as charcoal and amino acid treatments in cork oak.

STABILITY AND AGRONOMIC PERFORMANCE OF DOUBLED HAPLOIDS

To be used in a breeding programme, DH plants have to be genetically stable with no aberrant genetic variation arising during the process. Therefore, it is important to determine if any genetic variation is introduced during the production of DH lines. Very limited studies have been conducted on this line. Suenaga and Nakajima (1993) evaluated 110 wheat DH lines derived from wheat x maize crosses and found that 15 DH lines were variable for 2 traits like extreme dwarfism, low seed fertility, alteration of spike type and strips. Similarly, Kammholz et al. (1998) also found that expected normal segregation pattern for 6 glutenin loci across the 7 crosses indicated that wheat x maize system is stable across the generations. Laurie and Snape (1990) assessed the agronomic performance of wheat doubled haploid lines derived from wheat x maize crosses. They compared the performance of various DH lines of Chinese Spring, Hope, and lines of a single chromosome substitution of Chinese Spring and their respective parents under field conditions. No significant variation was detected in either population of Chinese Spring DH lines and neither population differed significantly from its parent.

Kisana et al. (1993), compared wheat DHs produced through anther-culture and wheat x maize crosses, and found that anther-derived plants were cytologically unstable, whereas all the plants regenerated from wheat x maize crosses were stable. In contrast, in other experiments, where the extent of variation from intergeneric cross methods and anther culture methods were compared, there were no significant differences in agronomic characters between the methods of DH production (Henry et al., 1988; Bjomstad et al., 1993). In studies of comparing the agronomic performance of best wheat genotypes selected through DH, single seed descent (SSD) and pedigree methods, no significant differences in grain yield were found among any of the
populations when the parental varieties were closely related in their pedigrees (Abd et al., 1993; Inagaki et al., 1998). In two crosses with low coefficients of parentage and large progeny variation, grain yield of selected DH lines was significantly lower than grain yield of SSD and pedigree selected lines (Inagaki et al., 1998). Recently, El-Hennawy et al. (2011) have evaluated the agronomic performance of anther-culture derived doubled haploid wheat genotypes and identified genotypes which are highly stable and superior to the best checks in grain yield performance.

**FACTORS TO BE CONSIDERED IN DOUBLED HAPLOID WHEAT BREEDING**

For successful implementation of doubled haploid wheat breeding program, different factors such as the filial generation from which doubled haploids are made, the population size and the comparative advantage of DH with other conventional breeding methods need to be determined. Conventionally, in an effort to shorten the breeding cycle, most breeders prefer to produce DH from the F1 generation. Inducing homozygosity at such an earlier stage may limit the opportunity for recombination events which create potentially useful genetic variation for breeders. Snape and Simpson (1981) examined the theoretical and practical effects of linkage on traits of DH lines derived from F1, F2, F3 and intermated F2 (S3) generations of barley, and found a significant gain in the genetic variation for spike emergence time, height, grain number/spike and spikes/plant from delaying the production of DHs to the F2 generation due to the breakup of repulsion linkages and creation of new allelic configurations at unlinked loci (Snape and Simpson, 1981; Choo et al., 1985; Patel et al., 1985; Yonezawa et al., 1987). In contrast, Iyamabo and Hayes (1995) quantified the effects of an additional round of recombination when comparing F1 and F2 derived barley DH lines and showed that the additional round of recombination did not lead to large performance differences between the two populations.

Population size plays an important role in the success of any breeding program. Theoretically, if a hybrid has n pairs of independently segregating genes, the chance to select a particular homozygous genotype from the F2 population in a conventional breeding program is (1/2)^n, whereas in haploid breeding, it is (1/2)^n (Chu, 1982). Thus, the selection efficiency in haploid breeding is 2n times better than the conventional methods. Single seed descent (SSD) is similar to the doubled haploid method in that both methods provide rapid generation advancement for producing homozygous lines (Grafius, 1965). In doubled haploid breeding, there is only one opportunity for recombination if F1 plants are used as donors, while in SSD, recombination can occur in every generation of inbreeding (Grafius, 1965). Minimum population sizes depends on how many unlinked loci were to be fixed, for example, to be sure of fixing two unlinked loci, 16 DHs would be needed. To obtain five unlinked loci, a DH population of 203 would be needed. In the presence of linkage, minimum population sizes would have to increase (Yonezawa et al., 1987; Jansen, 1992) or alternatively, the production of DHs could be delayed in a generation. In general, to improve quantitatively inherited traits such as yield, it is highly important to increase the population size by nominating limited number of elite x elite crosses for DH production.

**APPLICATION OF DOUBLED HAPLOIDS**

The induction and regeneration of haploids followed by spontaneous or induced doubling of chromosomes are widely used techniques in advanced breeding programs of several agricultural species. In traditional plant breeding, it generally takes at least five generations after crossing before a sufficiently homozygous population is obtained, while DH produces a homozygous plant in one generation. Therefore, DH technology dramatically increases the speed of the inbred developmental processes by reducing several time-intensive generations of inbreeding and by making phenotyping and genotyping more predictive. Doubled haploid methods have been employed in wheat breeding programmes, and new wheat cultivars of DH origin have already been released in many countries such as China, France, Hungary and Canada (Hu et al., 1983; DeBuyser et al., 1987; DePauw et al., 2011). Wheat DH populations have been used also in the creation of molecular marker maps and quantitative trait locus (QTL) identifications (Chauhan and Khurana, 2011; Wu et al., 2012).

Due to complete homozygosity, the efficiency of selection for both qualitative and quantitative characters is increased since recessive alleles are fixed in one generation and directly expressed. Additionally, doubled haploids can be used in a recurrent selection scheme in which superior doubled haploids of one cycle represent parents for hybridization for the next cycle. Several cycles of crossing, doubled haploid production and selection are performed and gradual improvement of lines is expected due to the alternation of recombination and selection. Barkley and Chumley (2011) have demonstrated the advantages of a doubled haploid laboratory for Kansas wheat breeding program using economic model analysis (Figure 3). The graph is drawn assuming that the rate of change in yield potential is 150% greater with the use of DH, relative to the baseline scenario of the conventional breeding program. If a DH laboratory were to be built in 2011, a new variety could be released seven years later in 2018 with increased yield potential and/or the same level of genetic potential as varieties released by the conventional breeding program four years later in 2022. As indicated in Figure 3, the large discrete change in
2017 reflects the advantage due to the gain in time and the steeper slope trend indicates the enhanced rate of genetic gain.

DH systems are also the method of choice for mutant selection, due to the ease of selection and fixation of mutations and the desired recombinants, especially when quantitative traits are concerned (DePauw et al., 2011; Wu et al., 2012). Haploid technology has tremendous use for accelerating breeding technologies when combined with marker assisted selection (MAS). MAS, when combined with double haploidy, is a time saving method of performing backcross conversion to select an elite line with a particular trait. By combining molecular markers and DHs, it is possible to stack resistance genes. DH populations can be used as permanent mapping populations because they are stable and constant.

CONCLUSIONS

Doubled haploid breeding is developing more and more into a core technology in crop improvement. The DH technology platform offers a rapid mode of truly homozygous line production that helps 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 MAS, induced mutagenesis, and transgene technologies could also effectively expedite the wheat improvement programs. Direct incorporation of cloned genes at the haploid level following subsequent chromosome doubling may help accelerate stable integration of target gene(s) into wheat. Considering the cost and the fact that over-usage of doubled haploidy may reduce genetic variation in breeding germplasm, it is advisable to use DH for very limited and highly desirable crosses in wheat breeding program.

REFERENCES


Figure 3. Comparison of conventional and DH wheat variety development (Barkley and Chumley, 2011).


