Chapter 10
Doubled Haploids

Ming-Tang Chang and Edward H. Coe, Jr

10.1 Introduction

Maize doubled haploid (DH) technology provides fixed, pure lines from a donor parent. Protocols for breeding of DH lines are available for over 250 crop species, and over 300 DH-derived cultivars have been developed in 12 species worldwide (Forster and Thomas, 2005). In maize, methods for inducing, selecting and doubling haploid plants are advanced and are in widespread use.

In a haploid plant, expression of positive or deleterious effects of genes for seed development, plant growth and function is unmasked, and plants that function effectively will have a better chance to grow to maturity and set seeds. Haploid plants that show good vigor in a natural environment will usually perform well as DH progenies under environmental stress. When doubled and brought to normal genetic balance, DH lines can be selected for agronomic traits, and testing can more accurately estimate yield potential and yield stability under different environments. The DH genome with its pure genetic makeup may still be challenged by environment in the absence of prior selective pressure. The characteristics of DH lines in theory are fixed and stable, and no further inbreeding depression should be observed from generation to generation, although spontaneous mutation or genomic changes caused by transposable elements cannot be avoided (Stadler 1951; Messing 2005).

Ming-Tang Chang
BASF Plant Science LLC, ExSeed Genetics, 2901 South Loop Dr. Bldg 3 Suite 3800, Ames, IA 50010, USA
Current address: 5333 Cervantes Dr., Ames, IA 50014-6914, USA
e-mail: mtcchang@hotmail.com

Edward H. Coe, Jr
University of Missouri, Curtis Hall, Columbia, MO 65211-7020, USA
e-mail: coee@missouri.edu

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10.2 History

Maize haploid studies started in the 1920s. Spontaneous parthenogenetic or androgenetic haploids are rare and are generally not noticeable in genetic and breeding studies (Randolph 1938, 1940; Randolph and Fisher 1939). In an article on genetic variation, Randolph (1932) notes the following: "Haploidy in maize was first reported by L.J. Stadler, and the writer, in papers presented before section 0, Amer. Assoc. Adv. Sci., Des Moines, Iowa, 1929." Accordingly, Stadler and Randolph were the first to describe maize haploids (Rober et al. 2005). East (1930) mentions studies by Stadler and unpublished studies by R.A. Emerson, and points out that homozygotes would arise through parthenogenesis and that DHs could eliminate the labor of producing homozygotes through long periods of self-fertilization. There were limited studies on maize haploidy until Chase began to develop pure elite DH lines for commercial hybrid application (Chase 1947, 1949b). He demonstrated practical application of DHs in maize breeding (Chase 1951), and in his breeding career developed very useful DH lines for commercial hybrid application. His first significant haploid-parented hybrid was DeKalb 640 (Forster and Thomas 2005), a double cross (B14×H2167/H2386×H2389) with three DH lines and a station inbred. H2167 was derived from the first cycle Iowa Stiff Stalk Synthetic of Sprague. H2386 and H2389 were sister lines, second cycle DH lines derived from W22xH225 cross. H225 was the ninth, H2167 the 127th, H2386 the 190th and H2389 the 192nd, DH lines produced. It was important in the eastern US market (Pennsylvania) for a number of years and was the first high-density tolerant hybrid of wide acceptance. DeKalb 640 also had a good market in Europe, southern France, northern Italy and the Danube Basin, under different designations. An interesting point is that Marcus Zuber once told Chase that when he was testing Mo17, the high performing single hybrid was Mo17 × N22. N22 was one of the Stiff Stalk Synthetic DH lines out of Chase's Ames program, and this line went out to Nebraska and was released by that station. This performance alerted Zuber to the potency of Mo17 with SSS lines, and when B73 came along, that line took the place of N22 (S.S. Chase, personal communication). Chase did breed many other useful lines for commercial hybrid production, such as popular hybrids DeKalb XL66 (1.9 million seed units) in 1967 and DeKalb XL64 (3.8 million units) in 1970 (Troyer 2004). Chase carried out many firsts in maize haploid breeding, including (1) the first successful selfing of a maize haploid (giving rise to HD-1, a sweet corn line out of Golden Cross Bantam) (Chase 1949b), (2) recognizing that even parthenogenetic rates as low as 1 per 1000 or 2000 were practical if good genetic screening stocks were used, (3) producing DH lines in quantity, (4) showing that the pollinator stock affected parthenogenetic rates (Chase 1952), (5) determining that chromosome doubling treatments were not necessary for success as the rate of natural doubling was high, and (6) suggesting that androgenesis can be used for direct transfer of maize cytoplasm (Chase 1963).

The Northrup King Seed Company had a late Mexican meal corn with the unique genetic traits of red collar, white chalky endosperm and purple-seeded floury flint type. This line had no practical use for Northrup King, but it was good genetic

material, and they gave it to Dr. Charles R. Burnham in 1941 for research purposes. Coe, a graduate student with Burnham, received the seeds for a study of pigments. For identification purposes, Coe designated it as Stock 6 in 1950. Based on subsequent studies, self-pollinated plants of Stock 6 yielded 1.97% haploids and Stock 6 selfs (haploid by sib) yielded 2.86% haploids, with an average haploid induction rate of 2.52% (Coe 1959). Coe developed several marker systems, such as *C1-I* and *R1-nj*, which his student, K.R. Sarkar, applied to facilitate identification of haploid seeds (Coe and Sarkar 1964; Sarkar and Coe 1966, 1971). Sarkar advanced studies of maize haploid induction after returning to India (Sarkar et al. 1972; Sarkar 1974; Mathur et al. 1976; Aman and Sarkar 1978). Kato (2002) derived DH progeny from four inbred lines and four hybrids crossed by Stock 6 marked with *R1-scm2*, doubled by the use of nitrous oxide gas.

A mutant gene, *ig1* or indeterminate gametophyte, can increase the frequency of androgenetic haploids in its progeny (Kermicle 1969, 1971; Lin 1981). The effect of *ig1* on seed development is a failure of normal differentiation of nuclei and cells of the female gametophyte, in which the number of cells that function as eggs is indeterminate. In addition to producing 6% poly-embryony, 7% hetero-fertilization, 45% elevated ploidy level of the endosperm and other rare anomalies, the *ig1* stock produces about 3% paternal origin haploid seeds (Kermicle 1969). Androgenesis in higher plants is the development of offspring with paternal chromosomes only, affording breeders and geneticists a means for direct transfer of cytoplasm from one strain to another (Chase 1951, 1963). The practical application of *ig1*/*ig1* stock in corn breeding is the conversion of an inbred line to its cytoplasmic male sterile form. The homozygous *ig1*/*ig1* cms W22 stock is used as female and is crossed by a normal inbred. The doubled androgenetic haploid plants are isogenic with the normal except that they carry male sterile cytoplasm.

Study of maize haploids at Krasnodar Agricultural Research Institute in 1969 in Russia was begun by M.V. Chumak and was continued by V.S. Shcherbak, O.A. Shatskaya and E.R. Zabirova. They used Chase PEM (purple embryo marker), Coe Stock 6 and several stocks received in 1982 from V.S. Tyrnov and A.N. Zavalishina from Saratov University as source materials for creating a new haploid inducer. From crosses among those materials and individual selection in their progenies, they were able to generate several new inducers under the general name EMK (Embryo Marker Krasnodarsky) or ZMK (Zarodyshevy Marker Krasnodarsky). EMK-1, with an induction rate of haploids from 6% to over 10%, was produced in 1991 (Shatskaya 2004). In Russia, many registered hybrids use DH lines as one of their parents. Hybrids using DH line Kr716, developed by Chumak, are Krasnodarsky 383MV (1998–2005), Krasnodarsky 384MV (2000–2005), Krasnodarsky 382MV (1992 to current) and ROSS 387MV (1994–2004). A hybrid using DH line Kr503-1 is Krasnodarsky 599MV (2006 to current). Hybrids using DH line Kr640-3 can yield very highly productive hybrids, including Krasnodarsky 290MV (2004 to current), Krasnodarsky 385MV (2005 to current), Krasnodarsky 291MV (2006 to current), Intercras 375 (2006 to current) and Intercras 405 (2006 to current). Several hybrids with Kr640-3 were registered this year in the Ukraine (E.R. Zabirova, personal communication). Line AT-1, developed in 1982 at Saratov University (Tyrnov 1997),

gave maternal haploids with a frequency of 90–100%. The frequency of haploids could be higher than 100% because of the occurrence of haploid twins and triplets. During the first 4 years there was a constant threat of losing this line because of its haploid nature, and diploid seeds were rare. In addition, this line was seriously affected by *Ustilago maydis* and could not be practically used. It was converted to a resistant form, in which the haploid induction rate of the newly selected line is about 2–3% (Tyrnov 1997). Further study revealed that early pollination of the AT-1 and AT-3 haploids by normal pollen grains resulted in 3.6% and 2.7% haploids, respectively, and late pollinations resulted in 78% and 75% haploids, respectively (Smolkina and Tyrnov 2003), showing the significant effects of handling on haploid frequency. Chalyk et al. (1994) created a new inducer line MHI (Moldovian Haploid Inducer) from parents of KMS (Korichnevy Marker Saratovsky) and ZMS. This inducer line has an induction rate of 6.5% on average (Chalyk 1999; Eder and Chalyk 2002). The Krasnodar Embryo Marker Synthetic or KEMS (possibly EMK-1) and the French induction line WS14 (W23ig/Stock 6) are the parents of the new induction line RWS (Rober 1999; Rober et al. 2005). Many other inducer lines have been created in different countries, including CAU (China Agricultural University) inducer 1 (Liu and Song 2000a), which was developed by selection of progenies from crosses between Stock 6 and BHO (Beijing High Oil Population) and has an induction rate of 5–6%, and UH400 (University of Hohenheim 400) (Melchinger et al. 2005). UH400 is an inbred line derived from KEMS by W. Schipprack in Melchinger's group at Hohenheim (D. Geiger, personal communication).

10.3 Methods

In corn, haploid methods have been well developed over the last 60 years. Currently there are several types of haploid induction methods, briefly described below.

10.3.1 Spontaneous Haploids

The frequency of spontaneous maize haploids is 0.05–0.1%. The majority are maternal in origin (parthenogenesis). Androgenetic haploids are rare and the rate is about 1 in every 100,000 seeds (Randolph and Fisher 1939; Chase 1949a). Frequencies of both vary according to background.

10.3.2 Genetic Induction

Certain genetic stocks or some unique genes can produce higher percentages of haploid seeds when used as either the male or female, such as A385, 38-11 (Chase 1949a), Stock 6 (Coe 1959), *ig1* (indeterminate gametophyte) gene (Kermicle 1969), and advanced strains derived in part from them, described above.

The mechanism for haploid induction is still not fully understood, although there is some evidence that chromosome elimination may be involved (Rober et al. 2005). In most crosses, Stock 6 can produce 2–3% maternal haploids, Krasnodar marker 6–8%, and MHI 5–6%. Homozygous *ig1* plants used as female can induce from 1–15% paternal haploids (Shatskaya et al. 1994a). Color marker genes have been incorporated into male inducer lines for purple leaf, sheath and plants (with dominant *A1*, *A2*, *B1* and *Pl1*), and for purple endosperm crown and purple plumule color (with dominant *A1*, *A2*, *Bz1*, *Bz2*, *C1*, *C2* and *R1-nj*), to facilitate identification of haploid seeds at the ear level. *R1-nj* is particularly useful because it provides both recessive and dominant marking when crossed as a male on an *r*1 parent. Hybrid seeds have purple endosperm crown and purple plumule, while haploid seeds have purple crown but no plumule color and can be clearly identified by eye. Morphological differences associated with haploidy include fewer, narrower and stiffer leaves with occasional white sectors; smaller plant with slower growth rate; smaller cell size; and smaller guard cells (Chase 1947, 1969; Coe and Sarkar 1964; Greenblatt and Bock 1967; Dankov et al. 1990; Han et al. 2006). The xenia effect from a high oil inducer line can immediately identify haploid seeds with 90% accuracy based on their oil content or embryo size (Chen 2003). The size of haploid embryos in this cross is much smaller than the hybrid seeds. On average from non-destructive single seed NMR (Nuclear Magnetic Resonance) measurement, the oil content of hybrid seeds is 5.26%, the oil content of self-contaminated seeds is 3.86% and the oil content of haploid seeds is 3.42% (Chen 2003). Toward selecting for higher rates from an inducer line, recessive seedling markers such as glossy or liguleless can be reliable aids for the determination of haploid frequencies. A female line carrying glossy or liguleless is crossed with a haploid inducer, and the seeds are planted in a sand bench to be screened for recessive seedlings. The percentage of recessives represents the haploid induction rate, so long as there is no self-contamination or chromosome loss.

10.3.3 Modifications in Handling

Haploid induction rate is affected mainly by genetics, but some studies have shown that changes of environments or handling affect rate of haploid induction. Factors such as delaying pollination to the afternoon, aging of silks, heat, etc. may change the haploidy rate (Rober et al. 2005; Zaharova 1955; Aman et al. 1981; Mathur et al. 1980; Smolkina and Tyrnov 2003).

10.3.4 Artificial Induction

Certain chemicals and radiation can induce haploid formation, such as maleic hydrazide (MH), 2,4-D, NAA-Na, GA_3, IAA, colchicine (Deanon 1957; Zhao and Gu 1984, 1988), trifluralin (Kato 1997), radiation (Mathur et al. 1976), Basagran and other herbicides (Dankov et al. 1997; Wan et al. 1991; Hansen and Andersen 1998).

Kato treated pre-flowering tassels with trifluralin to inhibit the second microspore mitotic division. Zhao and Gu (1988, 1984), Tu et al. (1994) and others used injection to deliver 40 mg/L MH + 2% DMSO + 0.1% colchicine solution into the cob of unfertilized ears, and were successful in obtaining DH lines.

10.3.5 Anther Culture, Embryo Culture and Microspore Culture

Anther culture, embryo culture and microspore culture have been used to generate haploid plantlets (Petolino and Jones 1986; Wan et al. 1991; Aulinger et al. 2003; Zheng et al. 2003; Barnabas 2003; Armstrong et al. 2004), but application is limited because it has low efficiency, is genotype dependent, is time consuming and involves technical demands. The Laboratory of Plant Cell and Tissue Culture (1975) of the Institute of Genetics, Academia Sinica, first reported their success in maize anther culture. Green shoots, leaves and roots emerged from callus and developed into plantlets. Examination of chromosome number in root tip cells showed that they were haploids with 10 chromosomes.

10.3.6 Wide Crosses and Chromosome Elimination

Remote or wide hybridization and chromosome elimination can generate DHs in some species. For example, maize pollen applied to wheat, oat or rice can induce unfertilized haploid embryo development (Zhou et al. 1979; Zenkteler and Nitzsche 1984; Laurie and Bennett 1986; Matzk and Mahn 1994; Bains et al. 1995; Berzonsky et al. 2003; Inagaki 2003; Rines 2003). This is a quite useful technique to develop doubled haploid lines. Because of incompatibility the maize chromosomes are rapidly eliminated during cell mitosis and leave only a haploid genome from the original parent. So far, there is no successful record to indicate that this method is applicable to maize, even though maize can be crossed with closely related relatives, such as gama grass and teosintes.

10.3.7 Apomixis (Parthenogenesis and/or Androgenesis)

Apomixis is a process of regenerating seeds or plantlets from unfertilized gametophytes. It has a genetic basis, and genes responsible for apomictic responses can be identified by monosomic or segment translocation methods. It is possible to introduce apomictic genes from gama grass (*Tripsacum*) into maize (Sokolov et al. 1998; Kindiger 1997, 1998, 2006; Kindiger and Sokolov 1998). Current study has identified a small fragment from gama grass chromosome 16L, transferred to maize chromosome 6L, as being responsible for apomixis.

10.4 Chromosome Doubling

Production of doubled haploids requires that progeny be derived from selected haploid plants. Doubling can occur spontaneously, and its rate can be enhanced by selection. Methods for artificial induction of doubling have been developed.

10.4.1 Spontaneous Doubling

Spontaneous doubling in haploid tassels produces fertile diploid sectors. It is evident that a small proportion of somatic haploid cells of a haploid plant are doubled spontaneously through somatic cell fusion, endoreduplication, endomitosis or some other mechanism (Jensen 1974; Testillano et al. 2004). Doubling at PMC (pollen mother cell) stage just before meiosis can yield a quartet with four normal pollen grains. Staining pollen grains from intact haploid anthers with iodine solution often displays a small proportion of blue round, light blue round and many transparent irregular aborted pollen grains. The dark blue round pollen grains presumably are starch-filled, normal pollen grains, but they are accompanied by much non-fertile pollen. Such anthers are not able to open naturally, and the few normal pollen grains are not able to release and to serve their normal function. Larger amounts of normal pollen grains inside the anther will help the anther to function, split and release pollen grains. It is possible to cut anthers in half or squeeze anthers to force normal pollen grains out to fertilize the silks. This method is tedious and laborious and it is not recommended as a practical exercise for large-scale breeding processes. The percentage of haploid tassels that shed normal pollen grains varies significantly, in the range 2.8–46%, and is genetically dependent (Shatskaya et al. 1994b; Liu and Song 2000b; Wei and Chen 2006; Han et al. 2006). Generally, many haploid tassels have only a few florets that can shed normal pollen grains, and only about 54% fertile tassels have shown a large sector of normal anthers. Spontaneous fertility restoration of the female inflorescence or the ears is in the range 25–94% (Chalyk et al. 1994; Liu and Song 2000b; Han et al. 2006), which is much higher than for the haploid tassel. Therefore, tassel fertility is the limiting factor for application in a DH breeding program. Using pollen grains collected from normal diploid plants to pollinate haploid ears, it is possible to determine the frequency of fertility restorations, seed set, size of doubling tissue and seed distribution of the haploid ears. The average seed set is about 25–30 seeds per ear, and seed distribution on the ear is either from a large cluster in a specific area or randomly distributed (Liu and Song 2000b; Han et al. 2006). This implies that spontaneous doubling events can be either single events leading to a large chimerical sector on the ear or multiple random events that form scattered seed set. Any normal egg with normal silks can be fertilized by a normal pollen grain and develops to a normal mature seed. The time and rate of spontaneous doubling of haploid cells are background dependent. Haploid plants that are derived from certain parent materials show a few early doubling events during mitosis and produce a large fertile sector or sectors with a fair

amount of normally shed pollen grains or a large cluster of seeds on the ear. Those materials in general have a higher rate of fertility restoration. Other parent materials show many late doubling events and produce many scattered normal pollen grains within underdeveloped anthers. Those anthers cannot open and release pollen grains naturally, but can be released by cutting or mechanical methods. Those materials in general have a lower rate of fertility restoration. Furthermore, haploid plants that are derived from some parent materials are quite stable and do not show any doubling events during mitosis. The latter require chemical treatment to induce chromosome doubling artificially (Sect. 10.4.3).

10.4.2 Selection for Spontaneous Doubling

Recycling of doubled haploid lines by using DH lines as source parents in the next cycle of selection will increase the frequency of spontaneous diploidization of the haploid tassel. Studies have shown that by recycling DHs for production of haploids, the fertility restoration rate increased from 9.4% to 33% (Chase 1952; Zabirova et al. 1993, 1996) or even to 43% (Shatskaya et al. 1994b).

10.4.3 Artificial Doubling

It is not necessary to apply chemical treatment to double the chromosome number if the spontaneous restoring rate of tassel fertility is over 20%. Generally, it is possible to increase the frequency of doubling in haploid tassels to 20–50% by treating haploid seedlings with 0.06–0.5% colchicine solution (Han et al. 2006). Colchicine response is genotype dependent. Nitrous oxide gas was applied by Kato (2002) to double haploids and derive progeny from haploids from four inbred lines and four hybrids.

10.5 Advantages

10.5.1 Genetic Homozygosity

DH lines provide genetic homozygosity in one generation. Because haploids carry only a single copy of every gene, any gene or genes that have deleterious effects for seed or plant development will have immediate genetic effects to depress or inhibit normal seed or plant development so these plants will be quickly eliminated at the haploid stage. This provides an efficient tool to eliminate unfavorable genes and to enrich favorable genes to improve the genetic pool rapidly. This resembles the process of natural selection but in a very rapid way to quickly fix favorable

genomic combinations, conserving many useful genes from a breeding perspective. Doubling of those favorable haploids will generate a DH line with 100% genetic homozygosity. This overcomes the slow process of continuous selfing over many generations to reach almost genetic homozygosity in a conventional breeding program. These DH lines will not show either genetic segregation or inbreeding depression in the following generations, except for the influence from spontaneous gene mutations or transpositions that may cause certain deleterious influences and segregation.

10.5.2 Genetic Enrichment

Studies show that recycling DH lines can quickly improve haploid frequency and fertility restoration (Chase 1952; Zabirova et al. 1993; Shatskaya et al. 1994a, b; Liu and Song 2000a). According to Chase (1952), the original Stiff Stalk Synthetic materials yielded 0.13% haploids and the haploid-derived DHs yielded 0.43% haploids. The haploid fertility restoration of the original Stiff Stalk Synthetic was 9.4% and the DH-derived haploids have increased frequency to 33%. It appears that selection favors genetic or germplasm enrichment for production of haploids and fertility restoration. If that is the case, then germplasm enrichment for yield, general vigor and agronomy of a corn plant can be achieved by applying random mating of high yielding DH lines as source materials for the next cycle of haploid selection. Recycling of selected DH lines through recurrent selection or any other breeding scheme is a fast and powerful way to achieve genetic enrichment of the inbred carrying more favorable alleles for yield, pest resistance, stress tolerance and general agronomic traits (Griffing 1975; Gallais 1988, 1989; Dietzmann and Wehr 1996; Bouchez and Gallais 2000; Chalyk and Rotarenco 2001).

10.5.3 Gamete Selection

Gamete selection (Stadler 1944) is a simple and powerful tool, the potential of which can be realized by DH technology applied to breeding. Selection at the gamete or haploid level is more effective than at the diploid level because the probability of obtaining any genotype that carries n favorable genes is 1 per 2^n individuals for DHs, and the chance is much higher than 1 per 4^n individuals for diploids (Schlegel 2003). Selection is performed at the gamete level, and DHs in a sense are derived from gametes. Using the DH method a gamete with excellent genetic make-up can quickly be fixed to become a homozygous individual. The formation of haploids is a random event, based on isozyme, recessive marker genes and genetic similarity studies (Chang 1992; Chalyk and Chebotar 2000; Seitz 2005). If the number of DH lines is high enough, then results from selection can be effective. DHs have maximum genetic variance in line per se and test-cross trials (Rober

et al. 2005). Studies of inbreeding cereals show that DH does not lead to any bias of genotypes in populations, and random DHs were even found to be comparable to selected lines produced by pedigree selection (Forster and Thomas 2005). The DH lines are good source materials for testing of their hybrid yield potential, level of genetic heterosis and yield stability. Results are more reliable than materials with various degrees of genetic segregation or levels of inbreeding. Generally, selected DH lines maintain high yield and outstanding agronomic traits constantly from generation to generation. In other words, their early selection results show high repeatability.

10.5.4 Gene Mutation

Maize haploids are good source materials for mutation study. It is possible to use haploids to estimate the spontaneous mutation rate of a specific gene locus. Since haploids only carry a single genome, the estimation of mutation rate should be more accurate and straightforward. In a haploid field with 50,000–100,000 haploids, it is not unusual to observe certain seedling mutants, such as liguleless, glossy, dwarf, brown midrib, albino and tassel seeds. Kernel mutants are more difficult to study, and can be enumerated only if the number of doubled haploids is high enough, such as 20,000—50,000 DH lines. Occasionally, a DH ear shows all waxy, or sugary or opaque seeds, proving that they are actually homozygous mutants. In microspore culture, it is very effective to generate mutants by treating microspores with chemical mutagens at the uninucleate stage, and this will generate pure elite mutant inbred lines (Szarejko 2003). Another application of the DH method is forward breeding to create new homozygous mutant lines in place of backcross conversion. For example, a waxy inbred can be crossed with a normal yellow dent inbred, and the hybrid seeds can then be crossed with an inducer. In theory, 50% of the DHs will be homozygous waxy inbred lines and 50% will be homozygous yellow dent lines. They are completely new lines, and may or may not be better than the original lines, but they are an alternative to converting traits by backcrossing.

10.5.5 Molecular Mapping Applications

Currently DH lines are routinely used to produce mapping populations for mapping simple genetic traits of agronomic importance, such as disease resistance and plant stature. DHs are ideal mapping materials for constructing a genetic linkage map and can be reconstructed and repeatedly sampled from time to time. Map construction is much easier using DH lines derived from hybrids of two pure homozygous parents. Using DH materials, genetic maps of barley, rice, wheat, rapeseed and pepper have been constructed. It is also possible to use marker-assisted selection (MAS) to identify the most promising DH for commercial line development and application.

DH lines are very effective tools for QTL (quantitative trait locus) analysis. Since DH lines are homozygous, data collected from multiple sites, seasons and years in replicated trials can be pooled and assessed for QTL analysis, such as yield, yield potential, general stress responses and yield stability under different environments (Jansen et al. 2003; Forster and Thomas 2005).

10.6 Future Perspectives

So far, the most effective method of haploid induction in maize is genetic induction. Most of the corn seed companies now have well-established haploid breeding programs and DH lines are routinely produced in reasonable numbers. Yet, the efficiency of certain steps for haploid seed production is so low that application of the DH method is limited: (1) the 3% haploid frequency is too low for efficient application, (2) marker systems are not always precise and efficient to screen for haploids, and (3) the chromosome doubling frequency is low, at about 10%. If haploid frequency could be increased to 12%, color marker intensity enhanced and doubling frequency increased to 30%, the efficiency of the DH method would be improved significantly. Then it would become a still more powerful tool to speed up the breeding program.

DHs are a short-cut way to generate immediate homozygous pure inbred lines. Currently, more than 20 crops are using DH methods to produce new cultivars or pure lines. Anther culture and microspore culture methods are very successful in tobacco, rapeseed, cauliflower, broccoli, Brussels sprouts, cabbage, barley, wheat, triticale, turnip, rape, mustard, flax and apple. Anther culture only is quite promising in eggplant, rice, pepper, rye, oat, ryegrass, potato, cork oak and poplar. The wide crossing method is applicable in wheat, oat, rice, barley, triticale and potato. Gynogenesis (ovary and flower culture) is routinely used in cucumber, onion, sugar beet and citrus breeding. Maize is the only crop that is using the genetic method for mass quantity haploid production. The major problems that limit application of the DH method in other species are technical difficulties, time consumption and high cost. Techniques are genotype dependent in most cases and restrict the application to only a few limited genotypes. In addition, there are many other problems that need to be overcome, such as inbreeding depression, embryo germination, chromosome doubling, polyploids, albinism, physiological weakness, regeneration and fertility (Forster and Thomas 2005).

Technique difficulty in chromosome doubling of haploids is one of the limiting factors that restrict the general application of the DH method to crop breeding. In maize, colchicine and those herbicides inhibiting microtubule formation in mitosis appear to work well in doubling the chromosome number (Wan et al. 1991). Colchicine is a toxic chemical and may enter the environment. Therefore, it may not be a good choice to use for a large-scale application. The herbicides are a better choice because they are degradable in the soil. There are many different methods to apply chemical treatments, including soaking, injecting, dripping and

spraying chemical solutions to cultured cells, tissue, seeds, plantlets, seedlings or plants (Jensen 1974). The results are quite different due to differences in procedures and methodologies. In maize, spontaneous fertility restoration of the tassel is useful but is variable. The rate of fertility restoration of the female inflorescence is high enough and doubling chromosome number of the female is less important. Chromosomal doubling is required only if the spontaneous doubling rate of the tassel is less than 20%. A doubling rate of the tassel higher than 20% is acceptable for practical and economic breeding application. Recycling selected DHs as source materials for next cycle haploid production increases the frequency of spontaneous doubling of the tassel and fertility restoration. In addition, recycling of selected DH lines through recurrent selection or other breeding schemes can satisfy objectives for maize population improvement.

The DHs have many advantages for application in basic genetic research, molecular studies and practical applications in plant breeding. Maize has a unique genetic system to generate routinely and randomly a large quantity of haploids. A reasonable amount of DHs are generated from the haploids and yield potential is evaluated in test crosses. Results indicate that the DH lines represent a random sample of gametes of the initial breeding population (Chang 1992; Seitz 2005). Grain yield comparison across all sets, years and locations of S2 lines, S3 lines and DH lines shows no significant advantage of any one method. In contrast, ranges of test-cross means were larger for the DH lines in all ten sets and the top yielding lines were obtained by the DH method in seven out of ten sets (Seitz 2005). This may not be a generally applicable rule, but it does indicate the possible advantages for DH application in maize breeding. Currently, most of the larger maize seed companies have a DH program and produce DH lines routinely for future commercial product development and applications. The forward breeding method can produce improved new DH lines carrying unique genes or genes of commercial importance. The future is filled with challenges and opportunities for the use of dihaploid technology in maize breeding.

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