



Today's Use of Haploids in Corn Plant Breeding

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Abstract

Haploids have become one of the most effective tools in maize breeding. This chapter discusses two approaches utilizing maize haploids that have assumed great importance in commercial corn breeding. They are (1) producing and doubling the chromosomes in maternal haploids to generate double haploids (instant inbreds) and (2) utilizing paternal haploids produced utilizing the *indeterminate gametophyte1* mutation to convert male-fertile lines into cytoplasmic male-sterile lines. Both approaches require only two generations, while traditional procedures require about seven to eight generations. These procedures have helped to make the breeding of corn more efficient and economical.

The characteristics of maize haploids, methods to produce and select them and double their chromosomes, and the advantages of utilizing maize haploids are discussed.



1. INTRODUCTION

Haploids, individuals with the gametic number of chromosomes in their somatic cells, have become one of the most effective tools in modern genetics and breeding. Haploids of true diploid species, such as maize (*Zea mays* L.), are termed “monoploids,” whereas haploids of polyploid species are termed “polyhaploids.” Even though polyploidy appears to have been present in the ancestry of maize, extensive restructuring of its genome has taken place, and its genome is currently generally considered to be that of a true diploid (Messing, 2009). Maternal (matroclinous, gynogenic, or gynogenetic) haploids contain a haploid genome from the female parent, whereas paternal (patroclinous, androgenic, or androgenetic) haploids contain a haploid genome from the male parent.

The first report of haploidy in a higher plant was in 1921 by A.D. Bergner in *Datura stramonium* (Blakeslee et al., 1922), and the first reports of haploid maize plants were made by Stadler and Randolph in 1929 (cited by Randolph, 1932).

This chapter discusses two approaches utilizing maize haploids that have assumed great importance in the commercial breeding of corn. These are (a) producing and doubling the chromosomes in maternal haploids to generate double haploids (DHs, “instant inbreds”) and (b) utilizing paternal haploids to convert male-fertile lines into cytoplasmic male-sterile lines.

Recent reviews on using maize maternal haploids to produce “instant inbreds” have been published (including Chang and Coe, 2009; Chase, 1969; Geiger, 2009; Geiger and Gordillo, 2009; Prigge and Melchinger,

2012). Also, two international symposia on haploids in higher plants (Davies and Hopwood, 1980; Kasha, 1974) and a review article by Forster and Thomas (2005) discuss doubled haploids in numerous plant species.



2. METHODS TO PRODUCE HAPLOIDS IN MAIZE

2.1. Spontaneous occurrence

Maternal haploids occur spontaneously in maize with an average frequency of approximately one per thousand, whereas paternal maize haploids occur spontaneously at a far lower frequency of about one per 80,000 (Chase, 1949). The frequencies vary widely in different genetic backgrounds.

2.2. Treatments reported to increase frequencies of haploids

Haploid induction rates have been reported to increase after certain treatments including wide-species hybridization, delayed pollinations, heat, and certain chemical treatments (summarized in Chang and Coe, 2009; Chase, 1969).

2.3. Anther culture and pollen culture

Haploids have been recovered in over 250 plant species from anther or pollen culture (Davies and Hopwood, 1980; Forster and Thomas, 2005; Kasha, 1974). Although these procedures have been very useful in many plant species, they have found limited usefulness in maize because they are inefficient, genotype-dependent, and time- and technically very demanding.

2.4. Genetic systems

Chase (1949) reported that certain genetic stocks produced higher frequencies of maternal haploids when crossed as female parents (WF9 × W22) or male parents (38-11), indicating that there was genetic control of haploid induction frequencies.

Coe (1959) reported that not only did a line of maize, stock 6, produce a high frequency of haploids (3.23% in self-pollinated progeny) but also the high frequency was largely attributed to the pollen of this stock. When stock 6 was used as a pollen parent, 2.29% of the progeny were maternal haploids in which the haploid genome was contributed by the female parent. Subsequently, several stocks were developed (discussed later) that had even higher maternal haploid-inducing frequencies when crossed as male parents. Stock 6 is in the ancestry of each of these inducer stocks.

Kermicle (1969) discovered the *ig1* (*indeterminate gametophyte1*) gene in maize that produces high frequencies of both maternal and paternal haploids when plants containing the *ig1* mutation are crossed as female parents. The paternal haploids are of great interest because they can be used to efficiently convert male-fertile lines into cytoplasmic male-sterile lines as will be described in [Section 5](#) of this review.



3. CHARACTERISTICS OF MAIZE HAPLOIDS

3.1. Phenotype

Haploid maize plants are smaller and less vigorous than their genetically equivalent diploids, and they resemble the morphology of the inbred line from which they were derived. In a detailed study, [Chase \(1964\)](#) reported that maize haploids develop about 1–2 days earlier and have 90% of the number of plant parts (e.g., leaves and ear rows), 72% of the linear dimensions, 55% of the area, and 37% of the volume (weight) of their diploid sibs. They often have mutant sectors on them (e.g., albino and necrotic) because recessive morphological mutations are expressed in the haploid plant ([Chase, 1969](#)). Haploid maize plants can easily be distinguished from their diploid counterparts because of their shorter stature, narrower leaves, and male sterility.

3.2. Fertility

Haploid maize plants are male-sterile unless diploid sectors are present in the plant. This is because the probability that all 10 chromosomes in a maize haploid meiotic cell will migrate to the same pole at anaphase I to produce a functional haploid gamete is 1 in 2^{10} (1 in 1024) if independent assortment of nonhomologous chromosomes occurs at anaphase I. However, the probability of forming a microspore with a complete haploid genome would actually be 1/512 because there are two poles to which the chromosomes can move at anaphase I. Because corn anthers with one or a few viable pollen grains fail to dehisce (shed), haploid maize plants are completely male-sterile except for sectors in which the chromosome number had doubled. Interestingly, [Zabirova et al. \(1993\)](#) found a donor line from which 33% of the induced haploids were diploids and male-fertile.

When maize haploids are crossed as female parents with haploid pollen from a diploid male, far more progeny are produced ([Chalyk, 1994](#); [Chase, 1969](#); [Geiger et al. 2006](#); [Weber and Alexander, 1972](#)). For example, [Chalyk](#)

(1994) reported that 96.6% of the haploids derived from a synthetic dent population produced ears with at least 1 kernel. The average number of kernels per ear was 27, and the highest number was 107. Geiger et al. (2006) reported even higher numbers of kernels per ear, with one population having an average of 80. Clearly, other mechanisms must be operating in the female. Possibly, the chromosomes are not distributed randomly to the two poles at anaphase I, restitution nuclei are sometimes formed in telophase I, diploid sectors are occasionally formed, or a combination of the previously mentioned is present. Unfortunately, analysis of meiosis in maize megagametophytes is very difficult, and few studies of it have been carried out.

3.3. Meiosis

McClintock (1933) analyzed meiosis in microsporocytes of maize haploids. She reported that extensive illegitimate pairing occurred between non-homologous chromosome segments at pachytene. These associations were indistinguishable from pairing between homologous chromosome segments, and no more than two chromosome segments were paired at any point. Similar illegitimate pairing has been observed in haploids of *Oenothera* (Catchside, 1932), *Nicotiana tabacum* (Lammerts, 1934), cultivated rye (Levan, 1942), cultivated sorghum (Brown, 1943), *Lolium perenne* (Meyers, 1944), *Antirrhinum majus* (Rieger, 1957), and others.

The illegitimate pairing is almost entirely lost by diakinesis, and very few paired chromosomes are observed at this stage. In maize, the frequency of diakinesis cells with one or more paired chromosomes was reported to be 29.6% (Ford, 1952), 50.9% (Ting, 1966), 15.25% (Snope, 1967), and 15.4% (Weber and Alexander, 1972) with most cells having only one pair. The associations could be either chiasmate or fortuitous achiasmate associations between nonhomologous chromosomes. The presence of bridge-like bivalent configurations at anaphase I in haploid maize microsporocytes was reported by Alexander (1964), Ford (1952), and Weber and Alexander (1972) suggesting that at least some of the associations of nonhomologous univalents in haploid maize might be chiasmate.

Reciprocal translocations have been recovered from maize haploids crossed as females by diploid males, indicating that at least some of the chromosome associations at diakinesis are indeed chiasmate (Alexander, 1964; Weber and Alexander, 1972). Because specific reciprocal translocations were found repeatedly with cytologically indistinguishable breakpoints, Weber and Alexander (1972) concluded that at least some of the translocations probably arose by crossing over between duplicated segments in

nonhomologous chromosomes. More recent work has demonstrated that duplicated nucleotide sequences are indeed present in nonhomologous maize chromosomes (Helentjaris et al., 1988) and others.

When haploid maize females were crossed with haploid pollen from diploid males, 1.3% of the progeny contained a reciprocal translocation (Weber and Alexander, 1972). Thus, a portion of the doubled haploids would be expected to be homozygous for a reciprocal translocation and have normal seed set. Doubled haploids that are translocation homozygotes could easily be recognized and eliminated from maize breeding programs in the following way. When plants homozygous for a reciprocal translocation are crossed with structurally normal maize plants, all of the F_1 progeny will be translocation heterozygotes. Translocation heterozygotes typically have ~50% aborted ovules, and the resultant ears with only half the normal number of kernels could be easily recognized and would likely be excluded from further breeding.



4. INDUCING AND DOUBLING MATERNAL HAPLOIDS TO PRODUCE DOUBLED HAPLOIDS (INSTANT INBREDS)

The doubled haploid-line technology has become an important tool in modern maize genetics and breeding. All major hybrid corn companies have extensive programs to produce maize haploids and double them to generate new inbreds, and the vast majority of new maize inbreds are currently being produced using this approach.

4.1. History

The value of doubled haploids for breeding and genetic studies has long been recognized (Blakeslee and Belling, 1924; East, 1930). In fact, East (1930) indicated that R. A. Emerson and L. J. Stadler endeavored to obtain and double maize haploids for several years prior to 1930. Chase recognized the potential of using haploids for maize genetics and breeding and carried out the pioneering work in this area starting in 1946 (Chase, 1969).

Although maternal maize haploids occur spontaneously with a frequency of about one per thousand progeny (Chase, 1969; Randolph, 1938, 1940), no selfed progeny had been obtained from the few haploid maize plants that had been recovered prior to Chase's pioneering studies (Chase, 1969). Chase (1951) demonstrated the practical application of doubled haploids in maize breeding, and he developed several lines from doubled haploids, some of which were used for the production of commercial maize hybrids.

Chase had many firsts in maize haploid breeding. He was the first to produce haploids in quantity, the first to observe of high rates of somatic chromosome doubling in haploids, the first to recover doubled haploids, the first to use embryo markers in dry seed to select kernels with haploid embryos, the first to produce doubled haploid lines that were used in successful commercial hybrids (e.g., DeKalb 640), and the first, with Sam Goodsell, to demonstrate cytoplasmic transfer through androgenesis. He stated, “little use has been made of the [doubled haploid] method in the development of homozygous diploids. Increased interest in and use of such radical techniques is likely in the future as the challenges of intensified commercial maize culture and of the highly competitive seed corn market necessitate an increasingly high degree of responsiveness on the part of the maize breeder” (Chase, 1969). This certainly was an accurate prediction.

Considerable progress has been made in three areas that have enabled the DH-line technology to flourish. These are (1) the development of efficient haploid-inducing lines, (2) the use of genetic markers to efficiently identify haploids, and (3) the development of efficient and effective chromosome-doubling techniques. Each of these will be discussed in subsequent sections.

4.2. Development of haploid-inducing lines

The frequency of spontaneous haploids observed by Chase (1969) (approximately one per thousand) was too low to be useful for commercial applications. A much higher overall frequency of 3.23% was produced by an inbred line, stock 6, described by Coe (1959). Preliminary tests indicated that the high frequency of haploids produced by this stock could largely be attributed to the pollen of this stock. This stock was of great importance for two reasons. First, it produced a high frequency of haploids, but more importantly, when it was crossed as a male parent onto female parents of a breeding population, the haploids recovered possessed a haploid genome that originated from the female parent. Therefore, stock 6 could be crossed as a male onto a breeding population, and numerous maternal haploids could be recovered each possessing a haploid genome from the breeding population. This phenomenon is termed “gynogenesis”: after fertilization of the ovum, the male nucleus is eliminated and the haploid (gynogenetic) individual possesses the maternal genome.

Several groups have developed haploid inducer stocks with induction rates that are considerably higher than stock 6. Stock 6 was an ancestor in each of these stocks. These groups are in India (Sarkar et al., 1994), the

former Soviet Union (Chalyk, 1994; Shatskaya et al., 1994; Tyrnov and Zavalishina, 1984), France (Bordes et al., 1997; Lashermes and Beckert, 1988), China (Liu and Song, 2000), and Germany (Deimling et al., 1997; Prigge et al., 2012; Röber et al., 2005).

RWS, a line developed at the University of Hohenheim, Stuttgart, Germany (Röber et al., 2005), is one of the most effective haploid-inducing lines. It was derived from a cross between KEMS (Shatskaya et al., 1994), a Russian inducer synthetic, and WS14, a French inducer line (Lashermes and Beckert, 1988). It has a haploid induction rate of about 8%, and this line and lines derived from it are widely used by the corn breeding industry. A sister line of RWS, RWK-76, was developed from the reciprocal cross (WS14 $\times$ KEMS) and has an induction rate of 9–10%. Because these lines and other inducer lines only shed moderate amounts of pollen and are not particularly vigorous, F_1 s between two inducer lines are often used for induction crosses, especially if they are to be used as pollen parents in isolation plots. Some F_1 s between inducer lines have induction rates that are comparable to those in the two parental lines.

When a haploid inducer line (such as RWS) is crossed as a male parent onto a diploid female, most kernels produced contain a diploid embryo and a triploid endosperm; however, a portion of the kernels have a haploid embryo and triploid endosperm. Such kernels germinate normally and grow into haploid plants.

There are two major ways that maternal haploid inducers might produce kernels with haploid embryos. First, an abnormal fertilization event might take place in which one sperm fertilizes the two polar nuclei and the other sperm fails to fertilize the egg of an embryo sac, producing a kernel that has a triploid endosperm and a haploid embryo. Second, the normal double fertilization events would take place, and then the chromosomes from the female parent are eliminated from the embryo after fertilization.

Evidence for the chromosome elimination hypothesis comes from a study by Wedzony et al. (2002) who analyzed embryos of the haploid inducer line, RWS, in the first 20 days after self-pollination. They found that in 8.9% of the embryos larger than 200 cells, micronuclei were present in the cytoplasm of every cell in suspensor part of embryos and in shoot primordia. Also, Zhang et al. (2008) analyzed a stock 6-derived inducer line, HZ11, that produced over 10% maternal haploids. They observed that lagging chromosomes and micronuclei were present in mitotic cells of ovules pollinated by HZ11, and over 56.4% of the radicals from kernels with purple aleurone and colorless embryos (kernels with presumptive haploid embryos)

were mixoploid ($2n=9-21$) and more than 45% of the cells were haploid in three crosses.

An experiment in progress in the author's lab also provides support for the chromosome elimination hypotheses. RWS (which is *Bm2*, *Lg1*, *Gl1*, *J1*, and *G1*) was crossed as a male parent onto a female parent that had recessive plant-expressed mutations on five of its chromosomes (*bm2*, *lg1*, *gl1*, *j1*, and *g1*). 1200 kernels were field-planted. Of the 1108 plants that grew, 121 expressed all five of the recessive mutations (maternal haploids). In addition, four exceptional plants were identified that expressed only one of the mutations (one was *bm2*, one was *lg1*, one was *gl1*, and one was *g1*). Each of these four exceptional plants had a morphology that was typical of a monosomic maize plant. Cytological analysis indicated that each of these exceptional plants was monosomic ($2n=19$). These exceptional plants could only have been produced if chromosome loss occurred after fertilization.

It is also important to recognize that some of the haploids produced by haploid inducers may contain a small portion of the paternal inducer genome. Fischer (2004) used microsatellite markers and observed that 1–2% of the maize haploids produced by RWS carry one or rarely several paternal chromosome segments.

Studies carried out by Deimling et al. (1997), Barret et al. (2008), and Prigge et al. (2012) each identified a major quantitative trait locus on maize chromosome 1 that is responsible for much of the haploid-producing activity of maize haploid-inducing lines.

In barley, when *Hordeum vulgare* is crossed with *Hordeum bulbosum*, the *H. bulbosum* chromosomes are eliminated several days after pollination independent of the crossing direction to produce haploids that contain the *H. bulbosum* chromosomes (Kasha and Kao, 1970). A centromere-specific type of histone protein, CENH3, is found in all centromeres of eukaryotic organisms (Black and Bassett, 2008). Sanei et al. (2011) found that the CENH3 was present on the *H. vulgare* chromosomes but not on the *H. bulbosum* chromosomes preceding elimination of the *H. bulbosum* chromosomes. A similar situation might exist for haploid-inducing strains of maize.

4.3. Genetic marker systems to identify maternal maize haploids

Genetic marker systems can be used in two ways to identify maternal maize haploids. First, plants homozygous recessive for a seedling-expressed morphological mutant, such as *glossy1* (*gl1*) on chromosome 7, can be crossed

as females by haploid-inducing stocks as male parents that carry the dominant alleles of the marker locus. The percent of seedling progeny that express the recessive phenotype are the percent of maternal haploid progeny. This procedure can be used to determine the haploid-producing efficiency of different inducers. *liguleless1* on chromosome 2 has also been used for this purpose.

Second, a genetic marker that is expressed in the embryo of a kernel can be used to identify kernels that have haploid embryos. The most effective and widely used mutant for identifying kernels of this type is *R1-nj*, the navajo allele of the “red color” gene, *R1*. This mutant was first used as a selectable marker in haploid induction studies by [Nanda and Chase \(1966\)](#) and [Greenblatt and Bock \(1967\)](#). When the dominant pigmentation genes *A1*, *A2*, *C1*, and *C2* are present, *R1-nj* produces a deep purple pigmentation on the crown (top, endosperm tissue) and also in the scutellum (embryonic tissue) of the kernel. The maize haploid inducer stocks that are currently in use typically are homozygous for these alleles.

When a maize haploid-inducing strain with *R1-nj*, *A1*, *A2*, *C1*, and *C2* is crossed as a male to commercial corn (nearly all are *r1/r1*), kernels with diploid embryos will have purple crowns and purple embryos, while kernels with haploid embryos will have purple crowns and colorless embryos. F_1 kernels with purple crowns and colorless embryos can be selected, and most of these will have haploid embryos. Also, F_1 kernels with colorless crowns and colorless embryos can be recognized and discarded; these are produced by contaminating pollen from the commercial corn that typically is *r1/r1*. Kernels with haploid embryos can be efficiently selected in this way. In addition, dominant alleles of color plant marker genes (*A1*, *A2*, *Bz1*, *Bz2*, *C1*, *C2*, and *R1-nj*) have been incorporated into many haploid-inducing strains to facilitate recognizing and removing diploid plants from the induction cross. Maternal haploids will lack purple plant pigmentation, whereas sibling diploids will have purple plant pigmentation.

The amount of embryo pigmentation varies in different genetic backgrounds, and in general, the amount of pigmentation is weaker in flint corn types than in dent corn types. In some crosses, the expression of the *R1-nj* allele may be too weak to be reliable, and in these cases, other approaches might need to be used. Because haploid maize seedlings grow more slowly than diploid sibling seedlings, one could enrich for haploids by growing seed of the inducer cross in sandbench plantings and selecting for smaller seedlings. Another possibility would be to plant all of the F_1 seed from the inducer cross in a field planting and select the maternal haploid plants on

the basis of their distinctive morphology. This requires extensive field space and is very inefficient. Kernel oil content can also be used to identify kernels with haploid embryos because embryos of haploid kernels are much smaller than embryos of diploid seeds. [Rotarenco et al. \(2007\)](#) determined that the percent of oil in pooled ground kernels with haploid embryos was lower than in kernels with diploid embryos in crosses with four inbreds and five hybrids. Therefore, single-seed analysis using wide-line NMR or NIR could be used to nondestructively determine the oil content to identify maize kernels with haploid embryos; however, the cost of these procedures would likely be impractical for use on a large scale. Still another approach would be to use herbicide resistance as a marker for maternal haploids. [Geiger et al. \(1994\)](#) introduced transgenic BASTA resistance into the haploid inducer line, WS 14, and treated leaves of germinating F₁ seedlings with BASTA. They were able to identify maternal haploids by their susceptibility to this herbicide. However, because BASTA resistance involves a transgenic factor, it may cause complications for a haploid breeding program. Another approach would be to incorporate a dominant morphological plant-expressed mutation into the inducer stock (such a purple root pigmentation) and select seedlings that do not express the dominant plant-expressed mutation. Also, a dominant maize mutation for yellow-green leaves ($Yg^*-N1582$) that was induced with EMS by Gerry Neuffer and characterized by Sachs and Stinard (2012) appears to be especially promising for selecting haploids. This mutation has no apparent deleterious effect on plants in the homozygous condition. A haploid inducer line that is $Yg^*-N1582/Yg^*-N1582$ could be crossed to a breeding population, and the resulting haploids would have green leaves and diploids would have yellow-green leaves.

4.4. Chromosome doubling

The chromosomes in the haploids need to be doubled to produce dihaploids. This can occur spontaneously or be induced with chemicals.

4.4.1 *Spontaneous doubling*

Tassels of maize haploids are completely male-sterile except for sectors in which chromosome doubling has taken place ([Jensen, 1974](#); [Testillano et al., 2004](#)). Anthers on a maize haploid do not dehisce if one or very few haploid pollen grains are present; however, if a diploid sector is present in the tassel, anthers in that sector will dehisce. The percentage of untreated haploid plants that shed viable pollen is genetically controlled and varies widely from 1.2% to 46% ([Shatskaya et al. 1994](#)). [Kleiber et al. \(2012\)](#) carried

out a very large-scale study and determined that the proportion of haploids with fertile sectors on untreated plants was 0–16.7% with an average of 2%, and there were significant genotypic differences among untreated populations. They concluded that if 5–10% of haploid plants had fertile sectors, they could be efficiently used for developing dihaploid lines, and artificial doubling treatments would not be necessary.

Chase (1969) reported that it was possible to self-pollinate 178 of 1001 maize haploids, and 105 of these set seed to produce homozygous diploid lines. Therefore, about 10% of the haploids produced viable seed. More recently, Geiger and Schönleben (2012) attempted to self-pollinate untreated haploids of nine different genetic backgrounds. Seven percent of 412 haploid plants were successfully selfed, and in one of the genetic backgrounds, 10 of 19 were successfully selfed.

Using doubled haploid lines as the parents in subsequent cycles of induction increases the frequency of spontaneous doubling rate in haploid tassels from 9.4% to as high as 43% (Chase, 1952; Shatskaya et al., 1994; Zabirowa et al., 1993).

4.4.2 Artificial doubling

The frequency of chromosome doubling in haploid tassels can be greatly increased using a chromosome-doubling agent. One widely used and highly effective chemical is colchicine, the alkaloid of the meadow saffron (*Colchicum autumnale* L.). This chemical binds to tubulin and destroys the mitotic spindle. Gayen et al. (1994) applied colchicine 2–3 days after germination at the coleoptile stage. The authors cut off the tip of the coleoptiles and immersed the whole kernel into a solution of 0.06% colchicine with 0.5% DMSO (dimethyl sulfoxide) for 12 h at 18 ° C. They obtained a chromosome-doubling rate of 18.05%. Deimling et al. (1997) modified this procedure and obtained a greater chromosome-doubling rate. Eder and Chalyk (2002) applied the procedure to a broad range of genotypes and achieved a success rate of 49%. Although colchicine is suitable for artificial chromosome doubling in large-scale DH programs, it is very costly, highly toxic, difficult, and expensive to discard, and treatments with it are very labor-intensive. Some commercial maize companies currently use colchicine and others are using proprietary chemical treatments for chromosome doubling. Kleiber et al. (2012) reported the results of a very large-scale experiment and found that 2% of untreated haploids had male-fertile sectors, while 42% of plants treated with a proprietary chemical treatment had fertile

sectors. It is also important to realize that the viability of kernels is often reduced after chromosome-doubling treatments.

Kato (2002) treated haploid plants at the six-leaf stage with nitrous oxide gas for 2 days at 600 kPa. This procedure proved to be highly effective; 44% of the treated haploid plants produced seed after self-fertilization, while 11% of the untreated control haploids produced seed. Because this procedure requires large equipment (safe gas chambers), it would not be adaptable to high-throughput applications. Furthermore, there was a strong influence of the donor genotype on the doubling rate.

Seedlings of haploid plants are usually treated with a doubling agent and then either transplanted directly into the field after treatment or grown in pots before being transplanted into the field.

Haploid plants with fertile sectors are self-pollinated if possible. Because female fertility of haploid plants is much higher than male fertility, a large portion of the self-pollinated haploids will produce seed.

4.5. Summary of procedures needed to produce and double maternal haploids to produce dihaploids (instant inbreds)

The procedures used to produce and double maternal haploids is summarized in Prigge and Melchinger (2012) and in a video produced by CIMMYT in 2010 and posted on YouTube titled “Doubled Haploids: A simple method to improve efficiency of maize.” These procedures are summarized as follows:

1. A haploid inducer, such as RWS, is crossed as a male onto female parents in a breeding population.
2. F_1 kernels with haploid embryos are selected, in most cases using the *R1-nj* allele.
3. The kernels with haploid embryos are germinated and treated with a chromosome-doubling agent such as 0.06% colchicine solution. They are then grown to maturity.
4. Haploid plants with male-fertile (doubled) sectors are selected and self-pollinated. Even if only a small amount of pollen is available, in many cases, seed will be produced after self-pollination because female fertility is much greater than male fertility in maize haploids.

4.6. Advantages of producing dihaploids from maternal haploids

First, it only takes two generations to produce new inbreds verses ~7–8 generations by conventional selfing.

Second, the new inbreds are completely homozygous; there is no residual heterozygosity as often is present after repeated backcrossing.

Third, very large numbers of dihaploids can be produced, and the cost to produce each dihaploid is less than producing inbreds by conventional selfing.

Fourth, deleterious and lethal mutations can be purged from the breeding population because haploids with lethal mutants would not survive and dihaploids with deleterious mutations would be weaker than those that do not have lethal mutations.

Fifth, because all individuals of a selfed dihaploid are genetically identical, they can be analyzed more effectively for traits of interest.

Sixth, no further breeding depression should occur because the doubled haploid lines are completely homozygous.



5. UTILIZING PATERNAL HAPLOIDS FOR CONVERTING MALE-FERTILE LINES INTO CYTOPLASMIC MALE-STERILE LINES

Cytoplasmic male sterility (CMS) is maternally inherited (all progeny have the phenotype of the female parent) and is caused by an alteration in the mitochondrial DNA. Elite inbred females are routinely crossed in hybrid production fields by unrelated male parents to produce F_1 hybrid seed that is then marketed. The elite inbred to be crossed as a female parent can be detasseled in the production field, a very labor-intensive and costly procedure, or it can be converted to CMS. Traditionally, to convert an elite inbred to CMS, the line would be crossed as a male parent to a line with a CMS, and the progeny would be backcrossed as female parents for 6–7 additional generations to produce a line that has almost all of the genome of the elite inbred and the CMS.

Chase (1951) suggested that androgenesis could be used to convert lines from male fertility to CMS much more efficiently than by backcrossing with a CMS line. Goodsell (1961) crossed female parents that possessed T CMS (Texas-sterile cytoplasm) with N6, an inbred line from the Nebraska Experiment Station. He recovered paternal haploids that contained the T CMS cytoplasm from the female parent and a haploid genome from the male parent. The haploid plants were crossed as female parents by pollen from N6 individuals and the progeny were male-sterile as expected. This work also demonstrated that the androgenetic sporophyte in maize does indeed carry the cytoplasm of the female parent.

Chase (1963) utilized a somewhat different approach. A tetraploid genetically marked stock with T CMS was developed, and several inbred lines were crossed as male parents onto this stock. One paternal diploid was recovered that contained the T-type CMS and a diploid genome from the male parent. This individual was partially male-fertile and was self-pollinated. Most progeny were completely male-fertile, but a few were partially male-fertile but phenotypically indistinguishable from the male-sterile siblings. He speculated that in the diploid gamete that fertilized the tetraploid, CMS male might have been heterozygous for factor(s) for partial male sterility.

5.1. The *indeterminate gametophyte1 (ig1)* mutation produces a high frequency of paternal haploids

Paternal haploids produced by the *ig1* mutation can be used to efficiently convert male-fertile lines into CMS lines that can then be used as female parents in hybrid production fields.

5.1.1 Discovery

Kermicle (1969) discovered a spontaneous recessive mutation, *ig1*, in the inbred, W23. He reported that 2.35% of the progeny of *ig1/ig1* females $\times$ *Ig1/Ig1* or *Ig1/ig1* male parents were paternal haploids. This frequency is far higher than the spontaneous rate of about one per 80,000 (Chase, 1963). He also reported that the frequencies of both maternal and paternal haploids produced by *Ig1/ig1* heterozygous female parents were approximately half of those produced by *ig1/ig1* homozygotes when adjusted for the reduced viability of seeds inheriting *ig1*. No paternal haploids were recovered from *Ig1/Ig1* females crossed by *ig1/ig1* or *Ig1/ig1* males. These observations indicate that *ig1* exerts its influence in the female gametophyte.

Kermicle named the gene *ig1* because the number of eggs and of polar nuclei in the central cell is not one and two as in normal embryo sacs but is indeterminate.

In addition, 0.65% maternal haploids were recovered from *ig1/ig1* females. Thus, the total frequency of haploids produced by *ig1/ig1* females was 3.0%, and most of these were paternal haploids. Also, 0.17% maternal haploids were recovered from *Ig1/Ig1* females, a frequency similar to that reported by Chase (1969) (0.1%).

5.1.2 Other effects of the *ig1* mutation

The phenotypic effects of the *indeterminate gametophyte1* gene in the inbred, W23, were described by [Kermicle \(1971\)](#) as follows. Homozygous recessive plants are completely or almost completely male-sterile. Heterozygous male parents transmit nearly equal numbers of gametes with recessive and dominant alleles to their progeny; however, female parents transmit fewer recessive alleles (35.5%) than dominant alleles to their progeny. Ears borne on homozygous recessive plants are highly abnormal with no more than half normal seeds. 6% of the seeds exhibit polyembryony, 7% have heterofertilization, and 45% have a higher level of ploidy in their endosperm in addition to other less frequent abnormalities. Heterozygous females have about half the frequencies of these abnormalities.

Lin found that there were multiple embryological abnormalities in developing embryo sacs ([Lin, 1981](#)) and in mature embryo sacs ([Lin, 1978](#)) that possessed the *ig1* mutation. He reported that megasporogenesis and embryo sac development through the second embryo sac division were not detectably different from those in *Ig1* embryo sacs. However, subsequent development deviated in many ways. All nuclei divided a third division in some embryo sacs, and in others, eight nuclei were present prior to cellularization, but four nuclei were not present at each end of the embryo sac as in normal development. In some, a fourth mitotic cycle took place, and there were additional changes. [Lin \(1978\)](#) also analyzed 62 mature *ig* female gametophytes. Only two of them had typical wild-type features, and the others had various types of abnormalities including asynchronous division, nuclei migrating away from the poles prematurely before cellularization, more than three mitoses after meiosis, and about 10% of the *ig* embryo sacs degenerated before maturity.

5.1.3 Mapping and cloning of the *ig1* mutation

[Kermicle and Demopulos-Rodriguez \(1980\)](#) determined that the location of the *ig1* gene was proximal to the *lg2* (*liguleless2*) locus on chromosome 3 in maize.

The *ig1* gene was cloned by [Evans \(2007\)](#) by positional cloning on the genome sequence of the orthologous region in rice. *Ig1* encodes a lateral organ boundaries domain protein with a high similarity to *asymmetric leaves2* of *Arabidopsis thaliana*. He also reported that homozygous *ig1* mutants have abnormal leaf morphology in addition to the abnormal embryo sac development.

5.1.4 Genetic marker systems to identify paternal maize haploid frequencies

Crosses like those to identify maternal haploids can be used, except that the parent carrying the recessive genetic marker would be the male parent. Male parents that are homozygous for a recessive seedling-expressed morphological mutation such as *gl1* can be crossed to a female parent that are *Gl1/Gl1*, and the percent of the seedling progeny that express the recessive glossy leaf phenotype are the percent paternal haploids. This procedure can be used to determine the paternal haploid-inducing efficiencies of different *ig1* inducers (Schneerman et al., 2000). In this and other similar studies, we found that an *ig1/ig1* stock that was originally provided to our lab by David Alvey was producing the highest frequency of paternal haploids.

Also, all or nearly all of the *ig1* stocks available are homozygous for the *R1-nj* gene and have the other necessary factors for anthocyanin pigmentation in the seed. To identify paternal haploids, one would cross the *ig1/ig1* inducer stock that is also *R1-nj/R1-nj* as a female parent by commercial corn (is typically *r1/r1*), and kernels with colorless embryos and purple crowns would be selected. Most of these will be paternal haploids.

5.1.5 Using a *ig1/ig1* stock that also carries a CMS to convert male-fertile lines to CMS lines

The following approach can be used to introduce a CMS into an elite inbred to be used as a female parent in a production field. An *ig1/ig1* female with a CMS would be crossed as a female parent by pollen from the elite inbred. The paternal haploids can be selected. These have a haploid genome from the elite male parent and male-sterile cytoplasm from the female parent. The paternal haploids can simply be pollinated with pollen from the elite inbred because nearly all haploid plants crossed as female parents produce some seed. The seed produced will have the diploid genome from the elite inbred plus the CMS cytoplasm from the *ig1/ig1* CMS female. This requires only two generations versus 6–8 generations to convert a male-fertile line to a CMS line by conventional backcrossing to a CMS female. Furthermore, the newly developed dihaploid line will have 100% of the genome of the elite inbred line.

5.1.6 Introducing CMS into an *ig1/ig1* line

Let us assume that you have identified an *ig1/ig1* line into which you would like to introduce a CMS. You immediately face the following dilemma. In most genetic backgrounds, *ig1/ig1* plants are male-sterile and *Ig1/ig1* and *Ig1/ig1* plants are male-fertile. Normally, *ig1/ig1* (male-sterile) plants would

be pollinated by *Ig1/ig1* (male-fertile) males. *ig1/ig1* progeny can be identified because they are male-sterile. Because CMS also makes plants male-sterile, we cannot select *ig1/ig1* plants from this cross if the plants contain a CMS.

Solutions to this dilemma are as follows. First, Kindiger and Hamann (1993) developed a protocol utilizing B–A translocations in maize that could be used to produce large numbers of *ig1/ig1* in various CMS cytoplasms. This author has worked extensively with stocks they produced for this procedure, and the stocks do not perform as described by them. Numerous small plants are produced; however, the small plants are not haploids. The small plants have the morphology of monosomic-3 plants or plants hypoploid for the long arm of chromosome 3. Chromosome counts were carried out on several of the smaller plants, and they each had 20 chromosomes. Second, *ig1/ig1* CMS lines with a nuclear restorer for the CMS can be crossed as male parents to plants with a CMS for several generations to produce CMS plants that are all *ig1/ig1*. Third, *ig1/ig1* plants in certain genetic backgrounds (W64a, A158, and the *ig1/ig1* line previously mentioned that was provided by David Alvey) are male-fertile. *ig1/ig1* in these genetic backgrounds can be crossed as males to plants with a CMS for several generations to produce CMS plants that are all *ig1/ig1*.

5.1.7 Using *ig1/ig1* lines with a CMS to convert male-fertile lines into male-sterile CMS lines

Different male-sterile cytoplasms have been introduced into *ig1/ig1* lines that are also *R1-nj/R1-nj*. These can be crossed as female parents by commercial inbreds (which are almost always *r1/r1*) to be converted to CMS. F₁ kernels that have colorless embryos and colored crowns can be selected, grown, and crossed with pollen of the original inbred, and seed produced will contain a diploid genome from the elite inbred and the CMS from the *ig1/ig1* stock. However, in some crosses, the expression of anthocyanin in the kernel may be too weak to accurately identify the kernels with haploid embryos. In these cases, one could even plant the F₁ kernels directly into the field and identify the paternal haploids by their distinctive morphology. The paternal haploids will be smaller and have the characteristic morphology of the elite inbred, while maternal haploids will be smaller and have the morphology of the *ig1/ig1* CMS inducer line. Because only one or a few paternal haploids need to be recovered and crossed as female parents with pollen from the elite inbred, this approach is feasible if necessary. In addition, plant color genes could be introduced into the inducer strain, and then paternal haploids would have green stalks and maternal haploids would have colored stalks.

5.1.8 Advantages of using paternal haploids generated by the *ig1/ig1* system for cytoplasmic conversions

1. Only two generations are needed to introduce a CMS into an elite inbred line, saving considerable time and cost.
2. The inbred lines produced with the CMS have 100% of the genome of the inbred line.
3. Chromosome doubling is not required because the paternal haploids can simply be crossed as female parents by the original elite inbred.



6. COMPARING THE MATERNAL HAPLOID AND PATERNAL HAPLOID SYSTEMS

6.1. Maternal haploid system utilizing haploid inducers such as RWS

1. Inducer lines are crossed as *male* parents to plants in a breeding population.
2. Maternal haploids are recovered on the female parent.
3. Maternal haploids are treated with a chromosome-doubling agent, and pollen borne on fertile (diploid) sectors is used to self-pollinate the plant. The treated maternal haploids are usually transplanted into the field.
4. Very large numbers of maternal haploids need to be recovered and doubled for commercial applications.

6.2. Paternal haploid system employing *ig1*

1. An *ig1/ig1* CMS inducer stock is crossed as a *female* parent by an elite inbred male. Maternal and paternal haploids are recovered on the *ig1/ig1* CMS stock.
2. Paternal haploids are selected and crossed as females by pollen of the elite inbred to produce diploids with the elite genome from the male parent and the CMS from the female parent.
3. Chromosome doubling is not required. The seed is planted directly into the field (seedlings do not need to be transplanted).
4. Very few paternal haploids need to be recovered and crossed.



7. CONCLUDING STATEMENT

The use of doubled maternal haploids to produce maize inbreds, and the use of paternal haploids to convert male-fertile lines to CMS lines, has helped to make the breeding of maize more efficient and economical. Continued refinements with these approaches will be expected in the future.

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