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Review

Production and use of haploids and doubled haploid in maize breeding: A review

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Haploids and doubled haploids (DH) have become effective tools in maize genetics and breeding. The DH technology is replacing the conventional method of line development and speeding up the process of obtaining new varieties. Numerous maize DH based inbred lines and hybrids have been developed and released around the world. Temperate maize breeding programs have taken the lead in adopting the DH technology while in the tropical maize breeding uptake is still hampered by limited awareness of the potential and applicability of the technology. There are various methods for haploid induction, identification of putative haploids and chromosome doubling. However the effective and efficient large scale use of the DH technology depends on the most effective, sustainable and efficient methods of haploid induction, identification of putative haploids and chromosome doubling. The most commonly used method for haploid induction is *in vivo* haploid induction of maternal haploids through the use of inducer lines. The R1-nj marker system for haploid identification and chromosome doubling through the use of various concentrations of colchicine are considered the most common methods of identifying putative haploids and chromosome doubling respectively. Herein, they reviewed (i) the various procedures available for DH production, (ii) the methods for identifying putative maize haploids (iii) chromosome doubling methods (iv) the genetic basis of *in vivo* haploid induction in maize and (v) the use of the DH technology in maize breeding.

Key words: Haploids, doubled Haploids, haploid induction, chromosome doubling, identification of putative haploids.

INTRODUCTION

Maize is an important staple food crop for more than a billion people worldwide, it is vital in the livelihoods of people in Central America and Sub Saharan Africa. Being an important crop, there have been remarkable advances and achievements in maize research in terms of the development of new and better adapted varieties through the use of various technologies including the DH technology for inbred line development. Private and public breeding programs for temperate and tropical maize has swiftly taken up the DH technology due to the technical advances in the production of DH lines and the associated genetic, operational and economic advantages (Melchinger et al, 2005; Prigge et al, 2012; Smith et al, 2008). DH technology makes use of haploid plants to rapidly generate completely homozygous doubled haploids plants for plant breeding programs and genetic studies.

Haploid plants have the gametic chromosome number in their somatic cells, a characteristic that makes them of great importance to plant genetics and plant breeding (Don Palmer and Keller, 2005; Dunwell, 2010; Murovec and Bohanec, 2012). Haploids can occur spontaneously in nature or as a result of either cells and tissues culture (*in vitro*) or genetic induction (*in vivo*) (Dwivedi *etal*, 2015;

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Murovec and Bohanec, 2012). Spontaneous occurrence is however a very rare event that cannot be relied on for practical use. Although spontaneous occurrence of haploid plants has been reported since 1922 when the phenomenon was first described in *Datura stramonium* (Blakeslee *et al*, 1922), their potential and relevance for crop improvement was only realized after a breakthrough in the production of haploids from anther culture in *Datura* (Guha and Maheshwari, 1964). The production of DH plants that have doubled chromosome number of the haploids, after successful spontaneous or artificial chromosome doubling of haploid seeds or plants, has revolutionized plant breeding by speeding up the process of developing completely homozygous lines. DH plants have cells containing two gene sets that are exactly identical meaning they are completely homozygous at all loci (Murovec and Bohanec, 2012). In self-pollinated crops, DH can represent a new variety. For example, in wheat and barley DH varieties have been released directly after field trials while in cross pollinated plants like maize, where inbreeding depression is exhibited, DH plants have been used as parental inbred lines for hybrid breeding (Murovec and Bohanec, 2012; Veilleux and Flickinger, 2009).

The procedure for DH production involves the following steps: haploid induction, identification of putative haploids, chromosome doubling and the generation of DH seeds. Haploid induction by *in vitro* methods involving regeneration of plants from haploid cells or tissues are the most widely used methods in crop species that are not recalcitrant to *in vitro* haploid production (Morrison et al, 1991). Genetic induction which results in *in vivo* haploid induction through wide crosses (Melchinger et al. 2016a) or intra specific crosses to lines with specific genetic determinants (Inducer lines) (Coe, 1959; Kermicle, 1969), is an alternative method for haploid induction in some crop species. In maize, haploids can be produced via both *in vitro* (Ao Guangming, 1982; Tang et al, 2006; Truong-Andre and Demarly, 1984) and *in vivo* methods (Hu et al, 2016; Kelliher et al, 2017; Qiu et al., 2014; Rober etal, 2005; Zhang et al, 2008). A number of methods are also available in maize for identification of putative haploids after haploid induction (Chaikam et al, 2017; De La Fuente et al, 2017; Geiger HH, 1994; Jones et al, 2012; Melchinger et al, 2013; Mirdita etal, 2014; Rotarenco et al, 2007) and for chromosome doubling of the identified haploids (Kato and Geiger, 2002; Melchinger et al, 2016; Weber, 2014). The successful and efficient utilization of the DH technology in maize on a large scale depends on efficient, sustainable and effective methods of haploid induction, putative haploid identification and chromosome doubling.

In sub Saharan Africa, DH technology is increasingly becoming a popular and significant tool in maize breeding among the institutions of the national agricultural research systems (NARS) and the small- and medium-scale enterprise (SME) seed companies because of the establishment of the Maize Doubled Haploid Facility in Kiboko Kenya by CIMMYT in partnership with Kenya Agricultural and Livestock Research Organization (KALRO). However the adoption of the DH technology in tropical maize breeding programs is still lagging behind when compared to the adoption in temperate maize breeding programs. This is because there is no awareness of DH technology yet; an understanding of all the procedures involved in DH technology is still limited amongst young African plant breeding scholars. A review of the different procedures involved in maize DH technology will provide an up-to-date understanding of DH technology in maize based on past and current literature and will help in formulating further research questions on the subject. Herein, we review (i) the various procedures available for induction of haploids in DH production, (ii) the methods for identifying putative maize haploids (iii) chromosome doubling methods (iv) the genetic basis of *in vivo* haploid induction in maize and (v) the use of the DH technology in maize breeding.

HAPLOID INDUCTION METHODOLOGIES

In maize like in other higher plants, haploid and doubled haploid plants can occur spontaneously in nature or by either *in vitro* culture of immature male or female gametophytes, or can be induced by modified pollination methods *in viv*o (Büter, 1997; Don Palmer and Keller, 2005; Dunwell, 2010; Prasanna *et al*, 2012).

In vitro haploid induction

Successful *in vitro* gynogenesis in maize was reported in the 1980s by AoGuangming (1982) and Truong-Andre and Demarly (1984). They, reported successful induction of mature un-pollinated hybrid maize ovaries culture directly into haploid plantlets without the formation of callus after they were incubated on MS and N3 mediums. Tang et al. (2006) later reported the possibility and success of maize haploid induction by *in vitro* culture of pollinated maize ovaries. However the haploid induction frequency was rather lower than in *in vitro* androgenesis and in *in vivo* haploid induction. Generally *in vitro* gynogenesis in maize has not been extensively reported and the few available reports indicate that it is not an efficient method when compared to *in vitro* androgenesis and *in vivo* haploid induction and therefore it is less preferred.

Induction of haploids in maize by *in vitro* androgenesis through anther-microspore culture is more common and better preferred than *in vitro* gynogenesis (Zheng et al., 2003). Successful *in vitro* androgenesis in maize was first reported by the Chinese who cultured middle to late uninucleate stage microspores on MS basal medium with a 12-24% sucrose and obtained positive growth response frequency which was almost 1% (Genovesi and Collins, 1982). More reports on *in vitro* androgenesis with higher

response frequencies still came from the Chinese (Genovesi and Collins, 1982; Kuo et al, 1986) , Reports that later came from outside China still depended on responsive Chinese germplasm (Brettell et al, 1981; Genovesi and B. Collins, 1982). Later American germplasm which was responsive to *in vitro* gynogenesis was identified (Cowen et al, 1992). Although a lot of work has been reported on *in vitro* androgenesis in maize and efforts made to establish *in vitro* androgenesis in maize, it still remains a less desirable method mainly because it is very genotype-dependent with most genotypes not responsive. Furthermore, in responsive genotypes many important variables such as anther stage, anther pre-treatment and specific media components are necessary for successful *in vitro* androgenesis (Genovesi and Collins, 1982). The limitations of *in vitro* haploid induction techniques in maize coupled with their requirement to have a good laboratory and skilled staff makes them less efficient methods.

In vivo haploid induction

In vivo haploid induction which is a result of intra specific crosses to lines with specific genetic determinants (Inducer lines), is unique to maize (Hu et al, 2016; Kelliher et al, 2017). There are two approaches for *in vivo* haploid induction in maize resulting in paternal or maternal haploids respectively (Rober et al, 2005; Zhang et al, 2008). In maternal haploids, the genomes originate exclusively from the seed parent plant. Haploid induction in this case is caused by the pollinator parent. The opposite applies to the induction of paternal haploids, where the pollinator serves as genome donor and the female as the inducer (Kermicle, 1969).

Paternal haploids

In maize stocks of diverse origin, Chase (1963) observed an average of one paternal haploid case for every 80 maternal haploid case and a total haploid frequency which averaged about one per thousand seedlings (Chase, 1963). This haploid frequency was strongly influenced by the paternal, as well as by the maternal, parentage. Similar influences of parentage were observed for Stock 6. However in a case described by (Kermicle, 1969), the haploid induction was associated specifically with the maternally sex-limited expression of a single gene that induces predominantly paternal (androgenetic) rather than maternal (gynogenetic) haploids. The gene that forms the basis for paternal haploids in maize is a mutant gene ***ig1*** (indeterminate gametophyte) that occurred spontaneously in the inbred Wisconsin-23 (W23) (Kermicle, 1969). The mutant ***ig1*** gene located on chromosome 3 of the maize genome(Cowen et al, 1992; Kermicle, 1994), has influence on female gametophyte development. It conditions various incompletely penetrant irregularities in seed formation and can increase the frequency of haploids in its progeny (Kermicle, 1971). The ***ig1*** homozygous mutants are male sterile, have multiple embryological abnormalities, unusual fertilization events and consequent ploidy variation in the embryo and endosperm (Kermicle, 1994). Results of crossing males with or without the mutant ***ig1*** gene (***Ig1 Ig1*** or ***Ig1 ig1*** respectively) with females carrying the mutant ***ig1*** gene (***Ig1 ig1*** or ***ig1 ig1***) show that the mutant ***ig1*** of paternal origin does not affect the incidence of haploid induction (Kermicle, 1969). Therefore the haploid induction capacity of the mutant of ***ig*** is maternally sex-limited; the gene that incites the event is itself excluded from the nucleus of the embryo. Results from experiments involving different inbred lines as males also show that the general genetic composition but not the specific ***ig*** constitution of the male gametophyte, influences the incidence of paternal haploid induction (Kermicle, 1969). Haploidy induced because of the ***ig*** mutant may involve either substitution or hybridization of cytoplasms resulting in paternal haploids containing the cytoplasm of the inducer female plant and chromosomes from the donor (male) plant (Kermicle, 1969; Pollacsek, 1992).

In conjunction with paternal haploid induction, the ***ig*** gene can be used to get cytoplasmic male sterile lines, provide a rapid evaluation of the ability either for maintenance or restoration of male fertility and to transfer useful cytoplasm (Pollacsek, 1992). However, dependence on the genotype of the male parent, changes in the constitution of cytoplasm from the donor genotype and low haploid induction frequency makes the use of the ***ig*** mutant for paternal haploid induction a less attractive method for *in vivo* haploid induction in maize.

Maternal haploids

To produce maternal haploids, the haploid inducer line is used as the male parent while the source germplasm or donor is used as the female parent (Prasanna *et al.,* 2012). Induction is caused by the male parent and resulting maternal haploids carry both cytoplasm and chromosomes from the donor parent. Following the discovery of Stock 6 and the heritable nature of the haploid induction trait (Aman and Sarkar, 1978; Sarkar et al, 1972), an array of haploid inducer lines with improved haploid induction frequency, pollen production, disease resistance and plant vigor were developed through testing and selection(Rotarenco et al., 2010).

Mechanisms of in vivo maternal haploid induction

The exact mechanisms behind *in vivo* haploid induction are still not fully understood (Prigge et al, 2012b; Eder et al, 2002). Basically two hypotheses have been put forward to explain *in vivo* maternal haploid induction (Qiuet al, 2014). The first hypothesis is that one of the two sperm cells fails to fuse with an egg cell, but instead

triggers haploid embryogenesis while the second sperm cell fuses with the central cell and leads to a regular triploid endosperm. The second hypothesis is that one of the two sperm cells provided by the inducer is defective but yet able to fuse with the egg cell. During subsequent cell divisions, the inducer chromosomes degenerate and are stepwise eliminated from the primordial cells while the second sperm cell fuses with the central cell and leads to a regular triploid endosperm (Rober et al, 2005; Zhao et al 2013).

Progression of *in vivo* haploid induction in maize

The spontaneous occurrence of haploid maize in certain crosses was first observed by Emerson and Randolph in the 1930s (Chase, 1969). Thereafter a report on the spontaneous occurrence of haploids in maize at an induction rate of 0.1 % (which was too low for practical use by breeders), followed by the discovery of a genetic strain Stock 6 by (E. H. Coe, 1959), that, on selfing, produced a haploid frequency as high as 3.23%, laid up the foundation for use of inducer lines in enhancing haploid frequencies and hence *in vivo* haploid induction in maize (Couto et al, 2015; Lashermes and Beckert, 1988)

The first set of inducer lines and their improved version were developed from temperate germplasm and principally evaluated for HIR and agronomic performance under temperate climatic conditions (Prigge et al, 2012a). While temperate maize breeding programs were benefitting from the DH technology because of the development of improvement inducer lines, adoption of the DH technology in the tropical maize breeding programs was lagging behind due to a lack of tropical haploid inducers and reliable information on the performance of temperate inducers under tropical conditions. To boost the adoption of the DH technology in the tropics, temperate inducer lines were first evaluated for their haploid induction ability when crossed to a diverse set of tropical maize source germplasm under tropical conditions and the evaluation results indicated that the temperate inducers could be used in kick starting DH breeding in the tropics (Prigge et al, 2011). However, due to lack of synchrony between anthesis of inducers and silking of tropical source germplasm, poor vigor, poor pollen production, poor seed set, and high susceptibility to tropical maize diseases particularly leaf blight caused by *Excelohilumturcicum*, efficient and large-scale production of DH lines using temperate inducers was not possible (Prigge et al, 2012a). Using the temperate inducer lines that were evaluated for haploid induction under tropical conditions, a breeding program at the International Maize and Wheat Improvement Center (CIMMYT) aiming at developing haploid inducers with tropical adaptation was then initiated. From this breeding program, tropically adapted haploid inducer lines with an induction rate of up to 10% were then developed (Prigge et al, 2012a).

Identifying putative haploids

After pollinating the source germplasm with pollen of current inducers, a fraction of 8 to 10% of the developing seeds have a haploid embryo and the remaining fraction of about 90% of the kernels are diploid and therefore undesirable for production of DH lines as their embryo contains 50% each donor and inducer genomes (Boote et al, 2016; De La Fuente et al, 2017; Melchinger et al, 2013) At the adult stage, haploids can be distinguished from diploids based on plant characteristics (haploids have shorter stature, slender weak stems, erect and narrow leaves, and reduced growth rate) and phenotypic markers(Weber, 2014; Wu *et al,* 2014; Xu *et al.,* 2013). However, when producing DH lines, it is crucial to identify haploids at an early stage (seed or seedling stage), before chromosome doubling so as to save resources with respect to the use of chemicals, greenhouse space, field space, labor and expenses related to management of diploids in the field (Chaikam et al, 2016).

Several methods are available for identifying haploids such as use of flow cytometry, multispectral imaging and genetic marker systems (R1-nj (Navajo), high oil, red root, transgenic markers and herbicide resistance). Detection of haploids using the anthocyanin color marker of the R-Navajo gene is the most widely used method of identifying haploids and most of the currently used haploid inducers have the genetic constitution that is necessary for R1-Navajo (R1-nj) expression (Chaikam et al, 2015). The R1-nj allele of the R1 regulatory gene on chromosome 10 which regulates kernel anthocyanin biosynthesis, induces the expression of the Navajo phenotype (Greenblatt and Bock, 1967). The R1-nj allele together with other dominant pigment conditioning genes cause deep pigmentation of the (purple coloration) in the aleurone layer on the crown region of the endosperm and the scutellum of the embryo(Chase, 1969; Greenblatt and Bock, 1967). In a cross between source germplasm without anthocyanin coloration and a haploid inducer with the R1-nj marker system, the progeny display purple/red coloration on both the endosperm and diploid embryo, whereas the putative haploid kernels exhibit purple coloration on the endosperm, but not on the embryo, facilitating easy and quick visual identification of haploid kernels at the seed stage during in vivo haploid induction process in maize (Chaikam and Prasanna 2012).

Despite being widely used, the R1-nj marker system for haploid detection is very labor intensive and has so far not been amenable to automation. Furthermore, variable color intensity of the embryo marker hampers an unequivocal classification of seeds (Dang et al, 2012). The Navajo phenotype can be completely suppressed or poorly expressed in some germplasm due to either presence of dominant color inhibitor genes or segregation for R1-nj expression. Poor intensity of the R1-nj marker expression can result in high rates of misclassification in temperate flint germplasm (Röberet al, 2005; Melchinger et al, 2014) and tropical landraces (Prigge et al, 2011). Physiological factors such as high moisture content (Rotarencoet al, 2010) and the development of air pockets underneath the pericarp (Prigge et al, 2011) can also affect the efficiency and accuracy of R1-nj based

haploid identification. Another potential problem is the masking of R1-nj phenotype by natural anthocyanin coloration in the seed, especially in the pericarp, of maize landraces.

Due to the limitations of the R1-nj marker system, other marker systems have been proposed and evaluated for haploid identification. To compliment the R1-nj maker, two more anthocyanin marker systems based on stem and root coloration were combined with the R1-nj marker. The use of the purple/red stem and sheath color marker conditioned by the anthocyanin regulatory gene Pl for identification of haploids has been reported (Rober et al, 2005; Rotarenco et al, 2010). Chaikam et al. (2016) developed haploid inducer lines with triple anthocyanin color markers, including the expression of anthocyanin coloration in the seedling roots and leaf sheaths, in addition to the Navajo marker on the seed. The use of these three color marker inducers in the identification of haploids showed that the addition of the red root marker more accurately identified haploids among the germinating seedlings in germplasm that showed complete inhibition of the R1-nj marker. Moreover, it was revealed that anthocyanin accumulation in the roots of germinating seedlings is very rare compared with anthocyanin accumulation in the seed and leaf sheath tissues, meaning that the red root marker can serve as a highly complementary marker to R1-nj to enable effective identification of haploids.

Identification of haploids based on their oil content after pollinating the source germplasm with an inducer distinguished by a high oil content in the seeds is another method that is being used (Melchinger et al, 2013). This method was first proposed by Rotarenco et al. (2007), but not based on an inducer with increased oil content. Using a non-high oil inducer as proposed by Rotarenco et al. (2007) was not reliable because of the small differences in the mean oil content of the germplasm relative to the large phenotypic variation within each fraction (Melchinger et al, 2013). For this reason Melchinger et al. (2013) proposed the use of a high oil content inducer as essential for the successful identification of haploid seeds based on their oil content. Successful identification of haploid seeds based on oil content is dependent on the haploid induction rate (HIR) of the inducer, the difference in the mean oil content of haploid and diploid seeds, the phenotypic variance of oil content among seeds within each of these two seed fractions and the choice of an appropriate threshold for discriminating putative haploid seeds (Melchinger et al, 2013). This method shows great potential in increasing the efficiency of DH technology in maize because it is amenable to automated high-throughput screening and applicable to any maize germplasm worldwide and has lower false discover rates (FDR) and false negative rates (FNR) values (Melchinger et al, 2014; Melchinger et al, 2015; Melchinger et al, 2013).

A fully-automated high-throughput NMR screening system for maize haploid kernel identification based on oil content have also been developed(Liu et al, 2012; Wang et al, 2016). Automation has been tried on other platforms as well, automated NIR transmission spectroscopy for identifying haploid maize kernels has also been investigated (Jones *et al.,* 2012) where the reflection of haploidy in the embryo was used as the basis for identifying haploids. Results from this study showed that NIR spectroscopy can be successful in identifying haploids especially after sorting the seed according to genotype first. Another automated method that has been tried is an approach based on Videometer Lab 3 spectral imaging system (De La Fuente et al, 2017). Though still relatively new to maize, this method is said to have shown great success in other seed-based assays. The Videometer system is used for the automation of sorting of haploid seed for maize DH programs utilizing the R1-nj marker system in their *in vivo* induction program. It makes use of the existing inducers and marker system and is able to detect subtle coloration differences in maize kernels and use these differences to classify kernels. However, because of its dependency on the R1-nj marker system, this automated system is also affected by the expression of the R1-nj, modification of the expression of R1-nj could result in difficulties in visual and automated sorting.

Transgenic herbicide resistance as a physiological marker has also been tried in the identification of haploids seedlings. Geiger et al. (1994) investigated the usefulness of a transgenic herbicide (BASTA) resistance which is inherited as a monogenic dominant trait. By crossing homozygous resistant haploid inducers with sensitive donors, the resultant maternal haploids as well as spontaneously doubled maternal haploids were both sensitive to the herbicide BASTA while the sexual (F1) seedlings were heterozygous at the two loci and thus displayed BASTA resistance. These results clearly demonstrated the usefulness of BASTA resistance as a foolproof marker system to identify maternal haploids. BASTA resistance has the advantage of unambiguity and independence of the genetic background of the female parent (Geiger HH, 1994). However this method is labor intensive since the kernels have to be raised to the seedling stage before the resistance test can be applied. Another transgenic trait that has proved useful for identification of haploids is the transgenic Green Fluorescent Protein (GFP) marker (Chaikam *et al.,* 2017; Mirdita *et al,* 2014; Yu and Birchler, 2015) The transgenic dominant GFP marker gene that was introduced into a maize haploid inducer allows the identification of haploids in the early germination stage by visualizing the GFP expression of germinated kernels. Germinated diploid seeds will produce GFP fluorescence in emerged radicles and coleoptiles, but haploids will be GFP negative because of the lack of paternal GFP gene during hybridization with the haploid inducer(Yu and Birchler, 2015). This method proved to be useful in identifying

haploids from various commercial sweet corn hybrids, which have a genetic background that prevents haploid identification by other systems(Yu and Birchler, 2015). The use of transgenic in haploid identification has the major disadvantage of being not practical in some countries that do not allow transgenics. Furthermore the costs for development of transgenics and regulatory approval processes in some countries prevents an obstacle to the use of transgenics (Chaikam et al, 2017).

Use of herbicide resistance to select haploids exploits the sensitivity in donor and inducer plants to different levels of herbicide. For this method to work according to Tseng(2012), there should be a single dominant gene for sensitivity in maize to an existing herbicide. The haploid inducer genotype should carry this dominant gene for sensitivity in its homozygous state and be sensitive to the herbicide while the source genotype carries the homozygous recessive alleles for this gene and are tolerant to the herbicide. After pollination of the herbicide tolerant source genotype with the herbicide sensitive inducer genotype, the resultant progeny will either be haploid seed or heterozygous F1 seed which is diploid. If dominant herbicide sensitivity exists, hybrids between the two genotypes will be heterozygous and are expected to be more sensitive to the herbicide than the haploid plants because the heterozygotes got the dominant sensitivity allele from the inducer while the tolerant haploid plants only has a single recessive herbicide tolerance allele from the source genotype. This means that haploids survive herbicide treatment, which eliminates the undesirable heterozygotes. The herbicide resistance method for identifying haploids have been tried by Tseng (2012)on 2-3 leaf stage maize seedlings of different genotypes using the herbicide Laudis whose active ingredient is tembotrione (Santel, 2009). The results of this experiment showed herbicide tolerance in the F1 seedlings that were expected to be sensitive to the herbicide, meaning that in this case sensitivity against tembotrione was a recessive trait and is therefore not usable for the purpose of haploid selection. Similar results were found in another experiment with an inbred line which was confirmed to be sensitive to the herbicide nicosulfuron (Tseng, 2012). Though results from these two experiments showed that neither tembotrione, nor nicosulfuron-based herbicides are useful for the purpose of haploid selection, it should be noted that these two experiments only used two herbicides and a few genotypes. More herbicides can therefore be evaluated to identify those, to which dominant sensitivities may exist and tried on a wide array of genotypes for usefulness in identifying haploid plants. Furthermore by broadening the germplasm studied, potentially dominant sensitivities could yet be found for tembotrione and nicosulfuron.

Flow cytometry has also been used in the identification of haploids because of its accuracy, reliability, ease and speed of both sample preparation and result acquisition (Battistelli et al, 2013; Couto et al, 2013). It allows the effective identification of haploid seedlings among the induced progeny by simultaneously measuring and analyzing DNA content of cells as they flow in a sample stream through a beam of light (Dang et al., 2012; Marrone, 2009). Flow cytometry has been used as a tool to help determine the efficiency of the haploid-inducing method by identifying haploids and verify the success of chromosomal duplication (Battistelli et al., 2013). In a study to find the best method for identifying haploid seeds derived from tropical and subtropical waxy x QPM hybrids after haploid induction with different modern European inducer lines, many false positives were detected by flow cytometry among putative haploid seeds that were identified based on anthocyanin pigmentation (Dang et al., 2012).

In view of the different limitations of the several different methods used in identifying haploids, a novel method of haploid/diploid identification based on natural differences in seedling traits of haploids and diploids, was recently proposed by Chaikam *et al*. (2017). This method of using seedling traits particularly radicle length, coleoptile length and number of lateral seminal roots can be used in any induction cross independent of the genetic marker systems. Compared to the R1-nj marker system, use of seedling traits reduced false positives by several folds in the early identification of haploids in ten populations. Therefore the addition of seedling traits for haploid identification especially in populations that are not amenable to use of genetic markers, may improve the efficiency of DH line production by reducing the false positives (Chaikam et al., 2017).

Chromosome doubling

Chromosome doubling in seedlings or plants developing from haploid seeds is a crucial step in the development of DH lines (Dang et al, 2012). Once haploid induction has been successful, the next step would be to develop doubled haploids through chromosome doubling. Haploid plants are usually sterile because they contain only one set of chromosome and normal meiotic cell division to produce viable gametes cannot occur (Vanous et al, 2016). To restore fertility in haploids plants, the chromosomes have to be duplicated to produce a doubled haploid with two sets of chromosomes.

Chromosome doubling can occur spontaneously or be induced with chemicals (Weber, 2014). Spontaneous chromosome doubling may occur via somatic cell fusion, endoreduplication, endomitosis and possibly many other mechanisms (Testillano et al., 2004). In maize, spontaneous chromosome doubling occurs at a very low and unreliable frequency and is genotype specific (Wan et al, 1991). To increase the frequency of chromosome doubling in haploids to a level that ensures an efficient application of the DH technology to maize breeding, artificial chromosome doubling through the use of mitotic inhibitor chemicals is necessary (Prasanna et al., 2012;

Weber, 2014). These chemicals alter the regular mitosis in such a way that only a single cell with double the number of chromosomes results after mitosis. The most commonly used agent to induce chromosome doubling is colchicine (Häntzschel and Weber, 2010; Melchinger et al, 2016; Wan et al, 1991). Colchicine application is an integral part of the standard protocol for DH production in most maize breeding programs that have integrated the DH technology into their breeding (Melchinger et al, 2016). Colchicine disrupts mitosis by binding to tubulin, the protein subunit of microtubules, thus inhibiting the formation of microtubules and the polar migration of chromosomes, which results in a cell with a doubled chromosome number (Prasanna et al., 2012; Wan et al, 1991). Several reports that describe various colchicine treatments on haploid maize seedlings has shown that colchicine has a high success rate in chromosome doubling of maize(Jiang et al, 2017; P. Gayen, 1994; Wan et al., 1989). However colchicine is highly toxic, potentially carcinogenic and hazardous to the environment and therefore requires proper application, proper handling, correct storage and proper disposal (Melchinger et al., 2016). Some studies have also shown that although success rates with colchicine are high, colchicine has a low affinity for plant microtubules than for animal tubulins and therefore higher concentration of colchicine are needed (Morejohn et al., 1984; Morejohn and Fosket, 1984). Colchicine effects were also found to be highly genotype specific and colchicine treatments were affected by the growing conditions, for instance, rapidly growing vigorous haploid seedlings were more prone to colchicine injury (Bordes et al., 1997). Considering the negative side of using colchicine, alternative chromosome doubling treatments have been tried and some adopted as alternatives to colchicine in chromosome doubling.

Nitrous oxide gas has been tried and tested as an alternative to colchicine. A procedure was developed by Kato and Geiger (2002) where maize haploid seedlings obtained were treated with nitrous oxide gas (2 days at 600 kPa). Results showed that treatment at the six-leaf stage (flower primordia formation stage) significantly increased the occurrence of fertile sectors on both tassels and ears so that approximately half (44%) of the treated haploids produced kernels after self-pollination while in the control, only 11% of haploids produced selfed kernels owing to spontaneous chromosome doubling. However a strong genotypic effect on the occurrence of chromosome doubling after the treatment was observed. In practice this method has not been adopted because it requires large equipment (safe gas chambers)(Weber, 2014) and application of nitrous oxide gas *in vivo* to adult maize plants, which is hardly amenable for large scale application (Melchinger et al, 2016).

Procedures that utilize antimicrotubule herbicides that bind more specifically than colchicine to plant tubulin in vitro have also been developed and used for chromosome doubling (Melchinger et al, 2016; Wan et al., 1991). Some antimicrotubule herbicides have been shown to be as effective as colchicine in chromosome doubling (Melchinger et al, 2016; Wan et al, 1991). Wan et al.(1991) evaluated the ability of four antimicrotubule herbicides, amiprophosmethyl (APM), pronamide, oryzalin, and trifluralin to induce chromosome doubling in anther-derived, haploid maize callus. Results from this investigation showed that all the four herbicides could induce chromosome doubling but only APM and pronamide were useful agents for inducing chromosome doubling of anther-derived maize haploid callus at very low concentrations. APM and pronamide, were again found to be effective alternatives to colchicine (Melchinger et al, 2016). In a study by Melchinger et al. (2016) , the herbicides APM and pronamide outperformed all other alternative treatments tested and reached almost the same success rate as colchicine when they were applied to seedlings using the seedling soaking method (i.e. subsequently soaking the seedlings for 8 or 16 hours in the respective incubation solution after cutting 1 mm off the coleoptile).

Doubled haploid maize plants and plant components have also been developed using a chromosome doubling agent, cycloalkane, which is claimed to have low mammalian toxicity, low mortality rates and higher chromosome doubling rate in plants (Cui et al, 2013). Data on the success rate of the chromosome doubling agent cycloalkane is limited.

Genetic basis of haploid induction in maize

Haploid induction (HI) is determined mainly by the genetic constitution of the inducer line but also by that of the female line receiving inducer pollen (Chase, 1969). Haploid induction was found to be a highly heritable trait controlled by a large number of genes with additive effect and not significantly influenced by environment (Aman and Sarkar, 1978). A number of Quantitative Trait Locus (QTL) studies have been done with the aim of deciphering the genetic architecture of maternal haploid induction in maize. Barret et al. (2008) in a QTL mapping study using a cross between non-inducing and inducing lines, identified a major locus on maize chromosome 1 controlling *in vivo* induction of maternal haploids and named it ***gynogenesis inducer1*** ***(ggi1)***. In the same study, a genetic component analysis showed the presence of segregation distortion against the inducer at the ***ggi1*** locus, segregation resulting only from male deficiency and a correlation between the rate of segregation distortion and the level of maternal haploid induction. In addition, results from this study showed that the genotype of the pollen determined its capacity to induce the formation of a haploid female embryo, indicating gametophytic expression of the character with incomplete penetrance. Presence of the ***ggi1*** major QTL on maize chromosome 1 was also substantiated and

seven more QTLs were detected in a very comprehensive study with four biparental populations involving inducer lines CAUHOI and UH400, but in this study the QTL was called ***quantitative haploid induction rate 1 (qhir1)*** instead of***ggi1*** (Prigge et al, 2012b). This ***ggi1*** or ***qhir1*** QTL was found to have by far the strongest effect explaining in certain crosses up to 66% of the genotypic variance for haploid induction (Prigge et al, 2012b). It is thought to be mandatory for haploid induction ability and has been associated with poor transmission of inducer pollen leading to segregation distortion (Barret et al, 2008; Prigge, 2012). The other seven QTLs detected in a comprehensive study by Prigge et al. (2012b) were identified on five chromosomes and one of them, a QTL named ***qhir8*** was also found to be the second large effect QTL significantly affecting haploid induction. This ***qhir8*** QTL which was fine-mapped to a region of 789 kb on chromosome 9, was found to explain 20% of the genotypic variance and is important for enhancement of the inducing capacity of the first QTL ***ggi1***/***qhir1*** (Liu et al, 2015; Prigge, 2012)

Using a novel method for genome-wide association studies (GWAS) that allows detection of selective sweeps even under almost perfect confounding of population structure and trait expression as is the case with inducers and non-inducers, the previously detected ***qhir1*** QTL region was dissected into two closely linked genomic segments (named ***qhir11*** and ***qhir12***) relevant for HI expression (Hu et al, 2016). The first region ***qhir11*** comprises the 243 kb interval that was fine-mapped by Dong et al. (2013) and was presumed to be neither diagnostic for differentiating inducers and non-inducers nor effective for conditioning HI ability in maize (Hu et al, 2016; Nair et al, 2017). The second region, ***qhir12***, was found to have a haplotype allele common to all inducer lines used in the study but not found in any of the non-inducers also used in the study and three candidate genes involved in DNA or amino acid binding were detected in this region (Hu et al, 2016). Thus the ***qhir12*** region was proposed to be mandatory for haploid induction (Nair et al, 2017). However in a follow up study by Nair et al. (2017) it was revealed that only the ***qhir11*** sub-region has a significant effect on haploid induction ability, besides causing significant segregation distortion and kernel abortion, traits that are strongly associated with maternal haploid induction.

In other studies, it was established through fine mapping, genome sequencing, genetic complementation, and gene editing that haploid induction in maize is triggered by a 4 base pair frame-shift mutation in the gene coding for a pollen-specific phospholipase protein(Gilles et al, 2017; Kelliher et al, 2017; Liu et al, 2017). Little is known about functions of this phospholipase protein in haploid induction and therefore the molecular and cellular mechanisms linking this phospholipase activity to haploid induction require further investigations(Gilles et al., 2017; Liu et al., 2017). The Phospholipase gene family is common and present in rice, Arabidopsis, sorghum, and many other plants (Wang et al., 2012) and the high sequence similarity of the phospholipases from different plants may indicate certain conservation of their function. This therefore means that further investigations on the molecular and genetic mechanisms of phospholipase activity to haploid induction and its targeted disruption may allow establishing powerful haploid breeding tools in numerous crops(Gilles et al., 2017; Kelliher et al., 2017; Liu et al., 2017).

DH in maize breeding

Climate change, population growth and the need for nutrient enriched maize especially in the developing countries, necessitates the increase in efficiency of various maize breeding programs. In the world of plant breeding, efficiency of a breeding program is measured in terms of genetic gains over a period of time. Genetic gain is the predicted change in the mean value of a trait within a population that occurs with selection (Moose and Mumm, 2008). It is measured per cycle or per year. As expressed in the genetic gains equation (Moose and Mumm, 2008), the length of time necessary to complete a cycle of selection limits or enhances genetic gains. Length of time necessary to complete a breeding cycle is not only a function of how many generations are required to complete a selection cycle, but also how quickly the generations can be completed and how many generations can be completed per year (Moose and Mumm, 2008). Decreasing the length of time necessary to complete a breeding cycle enhances genetic gains and this is where the DH technology comes in.

The DH technology compared to the conventional breeding strategy in maize breeding has the major advantages of (i) reducing the time required to develop completely homozygous inbred lines (that can be used as parents in hybrid breeding) and testing the hybrids hence reducing the overall time required to release a new variety (ii) allowing maximum genetic variance between lines per se and testcross performance from the first generation and increasing selection gain as only additive variance are involved on the selection (iii) increasing the genetic gains by shortening breeding cycle, increasing selection gain and reducing expenses for selfing and maintenance breeding and also simplifying logistics (iv) perfectly fulfilling the DUS (distinctness, uniformity, stability) criteria for variety protection (v) increasing efficiency in marker-assisted selection, gene introgression, functional genomics, molecular cytogenetics, genetic engineering and stacking genes in lines and (vi) Improving the precision of genetic and mapping studies.

Considering the advantages of incorporating the DH technology in a breeding program, it makes sense to adopt this technology. However, the key question in

applying doubled haploid technology in maize breeding is the generation during which haploids should be induced (Bernardo, 2009). Different types of populations can be used to induce haploids that can be used as parental components for hybrid breeding, under the premise that they combine a high population mean with sufficient response to selection (Prigge, 2012). The use of F1 populations for induction of haploids is however a common practice (Prigge et al., 2012a; Smith et al, 2008) even though a number of studies have shown that the reduced recombination in DH lines may decrease the response to a single cycle (Riggs and Snape, 1977) or to multiple cycles of selection (Jannink and Abadie, 1999). Using an F1 population for haploid induction means that the DH lines are produced after only one meiosis (Bernardo, 2009) and have inherited larger blocks of parental chromosomes (because of fewer crossovers) than are DH progeny developed from F2 population or other population developed from successive generations of selfing (Smith et al., 2008). Based on results from a study by Bernardo (2009) to determine if for sustaining long-term response to selection, doubled haploids should be induced in F1 or F2 plants during maize inbred development, it is prudent to induce haploids from F2 plants rather than from F1 plants. Induction of DH lines among F2 plants would allow an increased amount of recombination in the resulting DH lines without substantially increasing the amount of time needed for generating inbred lines. In other studies the induction of haploids from open-pollinated and landrace populations to produce useful DH inbreds was demonstrated (Prigge et al, 2012a; Wilde et al., 2010). The use of open pollinated and landrace populations in developing DH lines allows access to and exploitation of the untapped broad genetic diversity of landraces and open pollinated varieties in research and breeding (Wilde et al, 2010).

Using DH technology in less obvious ways to increase the efficiency of selection rather than to produce a homozygous end product can increase genetic gains per unit of time but it should also be considered simultaneously with varietal development (Bordes et al, 2006; Griffing, 1975). The DH technology has advantage of producing lines that are directly usable as parents of potential hybrid cultivars at each cycle. Thus, if the genetic advance per unit of time is evaluated at the level of developed varieties even with the same or with a lower genetic advance in population improvement, the DH method appears to be the most efficient (Bordes et al., 2006).

In terms of agronomic performance, DH lines do not differ much from lines developed through the conventional line development methods. When DH lines were compared to lines developed through pedigree selection or single seed descent (SSD), no huge difference were found in terms of their per se value or their testcross performances (Marhic et al., 1998; Murigneux et al., 1993). DH lines produced from a broad-base population were found to be as good as those produced by SSD methods for grain yield, kernel moisture, plant height, and ear height and leaf length (Bordes et al., 2007). The testcross performance of DH lines derived from tropical adapted backcross populations was also found to be as good and competitive as the commercial hybrids developed through conventional pedigree methods (Beyene et al., 2012).

CONCLUSION

The DH technology in maize has proven to be very useful in modern plant breeding and genetics studies by accelerating the development of improved maize varieties. Substantial advances have been made in the development of methods for haploid induction, identification of putative haploids after *in vivo* haploid induction and the chromosome doubling in maize. With various methodologies available for the induction of haploids, only *in vivo* haploid induction through the use of inducer lines seems to be the most effective and efficient means of haploid induction in African maize breeding programs. More so because of the advances in the development of tropical inducer lines. A frameshift mutation in the gene encoding a sperm specific phospholipase in inducer lines results ina rare allele that is responsible for haploid induction. However little is known about the functions of this gene and this warrants further investigations on its role in haploid induction. Furthermore the gene involved in haploid induction is part of large gene family that is common and present in many other crop plants. Hence the need for further investigations on the gene so as to enhance breeding in maize and other crops through the use of the doubled haploid technology. Considering the negative impacts of using colchicine for chromosome doubling, it makes sense to consider safer alternatives to colchicine. The use of herbicides for chromosome doubling should be further investigated using tropical germplasm and a wider array of anti-microtubule herbicides that are common on the African market. For effective application of the DH technology in maize it is important to induce haploids in a population that would allow an increased amount of recombination in the resulting DH lines without substantially increasing the amount of time needed for generating inbred lines.

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