emergent
Life Sciences Research

Review Article

Scope of double haploid production for inbred development in tropical maize

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Abstract

Maize is a predominantly cross-pollinated crop. Maximum exploitation of yield potential can be achieved by harnessing heterosis through hybrid development. Inbred development for the purpose requires a lot of time. However, inherent mechanisms do exist in temperate maize for parthenogenetic development of haploid embryos in the seed parent due to the influence of haploid inducer gene(s) in the pollen. In contrast, the tropical maize neither has such haploid inducer gene(s) for gynogenic haploid induction nor responds better to *in vitro* androgenesis (anther culture). Introgression of haploid induction to tropical maize can open up avenues for gynogenic haploid production. In addition, the resulting introgressed haploid inducer lines may be amenable for *in vitro* anther culture for production of haploids and follow-up recovery of double haploids by colchicines treatment.

Keywords double haploids, haploid inducers, in vivo gynogenesis, in vitro androgenesis, maize (*Zea mays* L.)

Received: 30 January 2020
Accepted: 26 April 2020
Online: 2 May 2020

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Emer Life Sci Res (2020) 6(1): 21-27

E-ISSN: 2395-6658
P-ISSN: 2395-664X

DOI: https://doi.org/10.31783/elsr.2020.612127

Introduction

Globally, maize surpassed rice and wheat production [1]. Methods of breeding in maize include the ones based on mass selection and commercial hybrid production (single/double-cross) by using compatible inbreds. The highly adaptable inbreds with specific combining ability result in promising heterotic hybrids. In this context, double haploid breeding can offer quick development of complete homozygous double haploid lines from the wide gene pool or elite maize hybrids. This is not to be confused with di-haploids which are haploids of autotetraploid [2]. There are various methods of obtaining haploids, such as androgenesis and gynogenesis in an appropriate artificial medium, distant hybridization involving interspecific and intergeneric crosses, delayed pollination [3] and pollination with X-ray irradiated pollens. *In vitro* androgenesis also poses several problems e.g., genotype specificity, low embryogenesis and regeneration rate, segregation distortion, and the low frequency of chromosome doubling [4], though maize rarely produces albinos [5]. In contrast, the most usual technique is *in vivo* maternal haploid induction [6] by a suitable pollen parent [7]. However, genetic markers used in haploid detection are sometimes difficult to identify. It also adds to the complication by being specific to species and genotype.Maternal haploid production by intraspecific hybridization was first reported in 1959 and it seems to be a predominant way in maize [8-9]. Similarly, haploid production happens to be a usual phenomenon during early embryogenesis in *Hordeum vulgaris* x *Hordeumbulbosum* (L.) although the mechanism is not clear. In maize, maternal haploids produce ears with partial fertility and these are more viable than those derived by androgenesis. Consequently, the monoploids can be

converted to DHs at a large scale to serve as base materials for the selection of parental inbreds to constitute maize hybrids.

Anther culture response

The frequency of spontaneous (*in vivo*) haploid seed production is very low (usually one per thousand kernels) [3]. Haploids can be also produced either by *in vitro* androgenesis or in vitrogynogenesis. However, recovery of haploid plants can be largely improved by in vitro anther culture. Barnabás et al., [10] reported 50-90% callusing response and moderate regeneration response (20-30%) in the highly androgenic varieties [11]. The maternal haploids show higher viability than the paternal ones. Anther and microspore response depends on the genetic status of seed parents and their growth conditions [12].

Factors determining anther culture response

Genotype and culture conditions

Androgenesis in maize is influenced by numerous factors like genetic factors, colchicine pre-treatment, physiological conditions of the donor plant, pollen development stage, and embryo induction medium [13]. The genotypic influence of the paternal parent plays a crucial role in the gynogenic induction of haploids in the female parent. In a test of four unrelated pollinators, the most effective pollinator inbred‘38-11’ was 10 to 20 times more effective in inducing the spontaneous parthenogenesis than the poorest ‘A385’[14]. It is widely accepted that the uninucleate stage of microspores responds best to the culture forming embryogenic calli. A kind of abiotic stress initiates the development of pollens into embryo [15]. Embryos developed can lead to the direct regeneration of plantlets. However, the production of a higher number of embryos reduced the quality of the embryos [16]. The use of Whathman filter paper is reported to improve the shoot organogenesis and produced thicker and shorter roots as it induced water stress by osmosis [17].

Nitsch et al., [18] advised on growing the donor plants under optimal conditions while avoiding the pesticide application for the best androgenesis results. Haploid maize requires a lengthy culture duration and even then mostly results in undesirable clones. Anther culture in maize requires a long duration for embryogenesis as compared to other important cereals. However, the imposition of stress on the culture leads to the development of embryos into subsequent plants [19]. Cold pre-treatment gave better results when treated for 14 days at 8^{o}C as compared to 7 days [20]. Experiments have shown light regimes affecting callus induction and plant regeneration regarding their proportion and yield. It is believed that light modifies the levels and the response towards cytokinin, thus affecting shoot regeneration from calli [21]. Miao et al., [5] reported the formation of 80% embryogenic callus and induction of 20% organogenic mass in maize anther culture process. Anther response is reported to increase upon the addition of gametocides, though the gametocides are not available to the public [22].

Media and hormonal supplements

Media type plays an important role in the development of anthers to embryos. The N6 medium was initially prepared for rice anther culture and it also turned out to be better than MS media in maize [17]. High auxin concentration has been reported to increase the anther culture response in maize, but decrease the number of embryos induced [23]. Hosseini et al., [24] got embryogenic calli and recovered plantlet regeneration in maize anther culture using a modified YP-medium supplemented with 3mg/l Kn. Organic nitrogen added to culture media has been reported to be beneficial [25]. TIBA (2,3,5-triiodobenzoic acid: an anti-auxin) is also widely used for androgenesis owing to its positive effect on the proliferation of microspores [26]. The addition of cyanobacterial and microalgal biomass at the rate of 1 or 2g/l to the callus induction and regeneration medium improved the androgenic response and it was able to reduce the requirement of 2,4-D or even replace completely at the callusing phase [26]. Several conflicting results are available for the requirement of hormones, genotype [27], and the growth conditions of the donor plant [28]. The addition of activated charcoal (0.5%) greatly increases the anther culture response. It adsorbs the toxic components of the medium as well as anther exudates during culture although it interferes with the nutrient uptake. Thiamine and nicotinic acid are usually needed to elicit callusing response, whereas pyridoxine and

pantothenate seem to suppress [29]. Anthers responded better to the agarose medium rather than agar-based ones [25]. Moreover, liquid YP media with activated charcoal is also a better alternative to the agar-based culture of pollen grains [30].

Miao et al., [5] got 7% androgenic response in N6 basal medium with 2mg/l 2,4-D + 1mg/l Kn alongwith12% sucrose, 0.5% activated charcoal, and 500 mg/l casein hydrolysate. Similarly, Ku et al., [31], reported 13.1 % anther culture response using a Yu-pei (YP) basal medium with 0.5% activated charcoal, 500 mg/l lactalbumin hydrolysate, and 12% sucrose. Nitsch et al., [18] group used a cold pre-treatment of anthers for 7 days at 14°C and incubated the cultures under red fluorescent lights which produced 42 haploids out of 44 plants regenerated. For androgenesis, higher amount of sucrose(12%), inclusion of organic nitrogen(100mg/l proline, 400mg/l casein hydrolysate), 2,4-D(2mg/l) + Kn(1mg/l) combination, avoidance of pyridoxine or pantothenate and inositol seem to be appropriate for callusing response in maize anther culture. Hassan et al., [32] recovered ten direct haploid regenerants from a single crossM60 x M96 in N6 medium with 2,4-D (2mg/l) + Kn(1.5mg/l).

Identification of ploidy level

The mean stomata length of haploid and DH plants at early stages is a reliable marker for the detection of true haploids and DHs. The stomata through leaf 1 to leaf 8 stage have been observed to be smaller as compared to normal plants. Besides, R1-nj trait can be used to accurately detect haploids and DHs [33]. Another method is chromosome counting using root tips or other meristematic tissues, which is easy and fast to undertake during mitotic division [7]. Flow cytometry which measures nuclear DNA content [34] can be a direct approach. Melchinger et al., [35] has mentioned oil content as a biomolecular marker, though this process requires careful consideration of the genetic component of the parents as well as the offsprings [7]. Ribeiroet et al., [36] used chromosome duplication for the identification of haploids. The haploids identified by flow cytometry are treated for chromosome duplication and then transferred to a greenhouse for acclimatization. After acclimatization Flow Cytometry needs to be carried out again to check chromosome duplication.

Genetic basis for haploid induction response (HIR)

Kermicle [37] reported the occurrence of a spontaneous mutation termed as indeterminate gametophyte (*ig*) in the inbred Wisconsin-23 (W23). The crossing of the pollinator parent W23 (carrying *ig* allele) with the female parent with wild type *ig* allele results 3% haploid kernels (gynogenic) in the seed parent [25]. Thus, the pollen genotype is the determinant for the induction of haploid female embryos [38]. The indeterminate gametophyte (*ig*) is a haploid inducer gene in maize. Its presence in the pollinator stocks also improves in vitro androgenesis [39] through anther culture. Most of the tropical maize genetic stocks reveal poor anther culture response due to the lack of haploid inducer gene(s). The *ig* system makes use of the purple embryo marker (PEM) system to identify kernels with haploid (or doubled haploid) embryos. All the existing haploid inducer lines, being of temperate origin would never be successful in tropical conditions, displaying poor vigor, poor pollen production, poor seed set, and high susceptibility to tropical diseases [40]. The crossing of two haploid inducing lines, KMS and ZMS, resulted in the production of transgressive genotypes whose haploid-inducing capacity was more than two times than the above parental lines [41]. Besides, a haploid temperate inducer line (Stock6) can serve as an ideal donor of ig gene which can be introgressed to popular tropical maize inbred lines following back cross breeding [42]. Stock6 possess *B1*, *Pl1* and *R-nj* markers allowing identification of haploids both at the vegetative stage and at the mature seeds stage. The *ig1* mutant allele with *R1-nj* marker gene was introgressed into the tropical inbred line M14 suitable for the environment of Pakistan. *R1-nj* is a dominant marker that is commonly used in the identification of haploids due to their pigmentation properties. CIMMYT produced more than 4000 tropical DH lines [43] using a haploid inducer gene in pollen parent. Hybrids produce purple embryo whereas haploids do not show any color [44]. But, in some cases, *R1-nj* character may be masked by C1-1 'inhibitor' gene that inhibits anthocyanin production. To confirm inhibition, two gene-specific-markers C1-I InDel and C1-I SNP have been identified [45].

The androgenic response is reported to be controlled by various chromosomal segments [46]. Haploid induction in *Arabidopsis* is due to a centromere-specific histone 3 variant, CENH3 [47] which could be introgressed to other crop species including maize [48]. Chaikam [49] developed the second generation of tropically adapted inducer lines (2GTAILs) with more haploid inducing response (HIR) and good agronomic performance in both tropical and subtropical environments. The 2GTAILs were developed by the use of marker-assisted selection for the QTL '*qhir1*'. Dong et al., [50] confirmed that the QTL '*qhir1*' on chromosome 1 of elite tropical maize has positive effect on haploid induction, but showed strong selective disadvantage, as indicated by significant segregation distortion (SD). Xu et al., [51] pointed to a 450-kb region on the chromosome being responsible for SD. Prigge et al., [52] have found high SD for the haploid inducer QTL'*qhir1*', indicating non-transmittance of inducer gametes with that of haploid induction. Fine mapping of theqhir1 locus has narrowed down it to a 243 kb region flanked by markers X291 and X263 [50]. Besides, a gene'*ggi1*', is also present on the same above chromosome has role for in situ gynogenesis and also segregation distortion (SD). The position of the *ggi1*/*qhir1* QTL also proved that gynogenesis in maize after pollination is due to mutation [53].

A frame-shift mutation in pollen-specific phospholipase gene (MTL:Matrilineal) triggers gynogenic parthenocarpic haploid induction (6.7%) in maizeand the MTL knockout showed only 2% HIR.In contrast, the wild MTL protein of the pollen results in the reproductive success leading to normal zygote formation. MTL is highly conserved in cereals and may be useful in developing the haploid induction system. Liu et al., [54] used CRISPR/Cas9 gene-editing tool and discovered a 4bp insertion in the fourth exon of MTL in the inducer line 'CAU5' to cause the haploid inducing phenotype. Besides, a group of pollen-specific genes is reported to be over-expressed resulting in vivo haploid seed formation [55]. Prigge et al., [52] identified such large effect QTL *qhir1* and *qhir8* on Chromosome 1 and 9 respectively in two haploid inducer lines (CAUHOI: HIR=2% and UH400 :HIR=8%), which explained up to 66% and 20% of the genetic variance. On the other hand, Wu et al., [56] detected two haploid inducing QTLs on chromosome 1 (*qmhir1*) and chromosome 3 (*qmhir2*) from the maternal side, explaining 14.7% and 8.4% of the genetic variation, respectively.

Double haploid production using colchicines

In general, the haploid plants die after tillering and often show severe sterility at flowering [25] due to abnormal meiosis. To make the best utilization of these haploid plants, there is a need for chromosome doubling to convert them to the status of in breds. Double haploid technology was first suggested by Chase [57] in maize as the quickest and simpler way for the production of DHs. However, unlike rice and barley, the spontaneous doubling of a chromosome is severely low in maize (0.1-10%) as compared to rice [23]. Haploids produced either by *in vivo* gynogenesis due to the influence of pollen parent or in vitro pathway (gynogenesis or androgenesis) are amenable for double haploid production by colchicines treatment. The addition of colchicine at 250mg/l to the culture medium led to the recovery of 9.9 DH plants/100 anthers in a best responding genotype 'ETH-M 36' and it was shown to be 7 times more to that of non-treated control [58]. The frequency of first-generation DHs derived from colchicines treatment maybe 9.4%, but hybrids between such DHs can generate 2^{nd} generation DHs with very high frequency (33%) [3]. Colchicine treatment may lead to high mortality and abnormal growth of the seedling, and sometimes this also prevents self-pollination. Hence, colchicines application before regeneration [59], or inclusion of cold shock, or use of antimitotic drugs is preferred [10].

Conclusion and the future prospects

Double haploids derived from either gynogenetic or androgenetic pathway ensure the status of normal inbreds and also, some of these proved to be agronomically superior to the parental type. Maize is recalcitrant to *in vitro* androgenesis (anther culture). As compared to rice and wheat, progress in terms of *in vitro* androgenesis is very slow in maize plausibly due to the availability of inherent *in vivo* gynogenetic haploid induction, though at low frequency. Recently, a few researchers optimized the in vitro protocol of anther culture in maize for induction of embryogenetic haploids and follow-up high throughput production

of double haploids by colchicine treatment. The production of DH lines either from androgenesis or gynogenesis may be suitably maintained by controlled selfing and amenable for hybrid development in maize.

Acknowledgements

The authors highly acknowledge the contributions of different researchers included in the text as references.

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