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IN-VIVO DOUBLED HAPLOID TECHNOLOGY IN MAIZE BREEDING

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ABSTRACT

This review of literature is aimed to summarize in-vivo maternal haploid inducer based doubled haploid technology in maize breeding. The three crucial and important steps in this method include the induction of haploids, their identification and subsequent duplication of these haploids to produce doubled haploids lines. With the advent of haploid inducer lines, the production and detection of haploids are possible in the dormant seed stage. The percentage of spontaneous duplication in these haploid plants is very low and germplasm specific. Therefore, the use of artificial duplicating agents is essential to increase the percentage of chromosomal duplication. The cost of developing the DH line can be reduced with the detection of early doubled haploids and the germplasm that supports spontaneous duplication. These developments can replace the long laboratory procedures used for the development of the DH line.

Keywords: Doubled haploid, Zea mays, chromosomal duplication, inbred lines, maize breeding

INTRODUCTION

The development and production of doubled haploid inbred lines are very popular in maize breeding **[1].** The main feature of this technique is the reduced time span to develop an inbred line. This technique just requires two selfing cycles, in contrast to the traditional method of 6-7 repeated self breeding for line development **[2].** Doubled haploid technology can be utilized in any breeding program either in the field or in the laboratory. Both in-vivo and in-vitro techniques can produce doubled haploids **[3].** In field, haploid inducer mediated doubled haploid production is a popular technique. In the laboratory, microspore and ovary culture techniques are used to produce haploids. But the in-vivo technique is the best for large scale maternal haploid production and DH-lines development **[4].** In-vivo haploid induction in maize can be of two different types either maternal or paternal. If the haploid is induced from male parent the haploid produced will be maternal **[5].** The pollinator parent performs the duty of haploid induction while, the female parent donates all the genome to the haploid individual. The male inducer parental genome is not present in the haploid individual. On the other hand, in paternal haploids the donor parent is a male pollinator **[6]**.

History

Maize sporophytes with gametic chromosome number were observed by many scientists. In normal progeny they observed a frequency of one out of one thousand to two thousand in the field **[7].** These haploids were not used for doubled haploid development instead, only preferred for genetic and breeding studies **[8, 9].** The first study of commercial maize hybrid development, utilizing these spontaneous monoploids initiated at the Iowa state college (1947 to 1953). It also started at Illinois from 1954 to 1966 **[10]**. Stadler in 1940 **[11]** for the first time reported doubled haploid maize. The first successful doubling and development of doubled haploid line was reported in 1948 by Chase **[12]**. This line was derived from a sweet corn hybrid Golden Cross Bantam. After successful self pollination several viable seeds were harvested. This first monoploid-originated homozygous diploid maize inbred line was named ‘HD no 1’.

DH-lines development through haploid inducers

The in-vivo maternal haploid inducer based doubled haploid technology in maize was first time suggested by Chase in 1952 **[13].** The producer is based on the induction of haploid and comprises of three steps: (i) Haploid seeds production through crossing of haploid inducer line as a male parent with another desirable maize genotype as female parent (ii) identification haploid kernels from the diploid kernels in the F_1 cob; and

(iii) haploid seedling chromosomal doubling and production of DH-lines **[14].**

Haploid inducer line

The main use of haploid inducer lines is to produce haploid seeds in the F_1 cob. These genotypes are called maternal or paternal haploid inducers. When the haploid inducer lines are used as male pollen parent and induce haploidy in the F_1 generation they are called maternal haploid inducer lines. On the other hand when they are used as female parent and induce haploidy in the F_1 generation they are called paternal haploid inducer lines. The first maternal haploid inducer reported in the history of the maize breeding was Stock6 having almost 0-2% haploid induction range **[15].**This discovery was a gateway to novel inducers with increased induction rate up to 10% **[16]** . The haploid induction rate has increased in the modern inducers from below 2% to 10% **[17].** All present inducers are developed through this parent. However, all these are of temperate origin and not suitable for tropical climate. TAIL is the first tropically adopted maternal haploid inducer by CIMMYT with a range of haploid induction ability i.e., 8-10%.

Identification of haploid

Maize haploids can be identified in the field and in dormant seed stage through different methods. Different genetic markers and haploid plant characteristics are used for identification.

Haploid identification at dormant seed stage

After induction cross with the haploid inducer, a certain percentage of haploid kernels appear in the F_1 cob. These maternal haploid seeds are detected through the presence of the *R1-nj* color marker gene **[18].** According to this color marker detection system a certain criteria is used to identify maternal haploid kernels. The haploid kernels can be distinguished from diploid kernels by the presence or absence of purple color in embryo outer layer scutellum. In the inducer line's kernels the purple color appears in both outermost layers of the maize endosperm and embryo. While, the female source populations used in the induction cross do not possess any anothocyanin coloration in these layers. The inducer genotypes are equipped with dominant purple color marker gene (R1-*nj*) that produce purple colored aleurone and scutellum of the F_1 seed. The both type of seed i.e., haploid and diploid have purple color in the aleurone. The scutellum of diploid seed is colored while, colorless in the case of haploid kernel **[19].** In this way R1-*nj* dominant color marker system helps in the differentiation of haploid kernels from the diploid kernels. Out-crossed or selfed seeds lack the purple colored aleurone and scutellum. Different factors like environment and genetic background of inducer or female parent etc effect the expression of the R1-*nj* color marker **[20, 21, 22].** The source or donor material with dominant anthocyanin inhibitor genes such as C1-*I*, C2-*Idf*, and In1-*D* can make the R1-*nj* color marker system ineffective **[23]. T**he possibility of additional color markers, especially those expressed in root and stem, for reliable identification of maternal haploids are explored by many workers **[86]**. Two such genes that can impart purple or red color to the plant tissues are Pl1 (Purple1), which conditions sunlight-independent purple pigmentation in plant tissues, and B1 (Booster1), which conditions sunlight-dependent purple pigmentation in most of the above-ground plant tissues [87].

There are some restrictions of B1 and Pl1 color markers. Many source materials contain B1 and Pl1 genes. In such source populations, haploid plants also express coloration in the root and stem, making it almost impossible to reliably identify haploid plants. Expression of the B1 and Pl1 genes are affected by plant growth conditions, especially sunlight and temperature. It was observed that purple pigments accumulate best under low temperatures **[85]**.

Haploid plants identification

The haploid plants are reported with narrow leaves with mutant stripes Chase in 1964 [**24**]. Their different parts are less in number than normal diploid plants, e.g. leaves, kernel rows in an ear, etc. Haploid plants developed multiple cobs because of sterility present in them. The apparent feature of monoploids is sterility [**25**]. The florets failed to shed anthers. The florets in few plants may shed anthers but fail to desiccate pollen. The roots, stems, leaves or any part of the haploid plants lack purple pigmentation while, the diploids/ Hybrids are with highly fertile tassel and ear inflorescences. They show hybrid vigor and lack sectoral sterility in both tassel and ear inflorescences [**26].** The purple pigmentation appears in any part of the diploid plants. Spontaneously doubled haploids plants are with highly fertile tassel and ear inflorescences, but they lack hybrid vigor **[27],** and lack of purple pigmentation in the plant tissue.

The chromosomal count is a more valid diagnostic test of haploids. Maize haploids have ten chromosomes as compare to normal diploids with twenty chromosomes per cell. The haploid cell contains one-half of the amounts of nucleus as compared to the diploid cell that's why the size differences between haploid and diploid plants can be justified. The difference in length and volume between haploid and diploid plants is present as the haploid plants are about 70% the height of diploid plants. Similarly, their leaf area is 56% of diploid individuals and volume is 35% to that of their diploid siblings **[28**].

Theories behind haploid induction

In the process of double fertilization in flowering plants, male parent contributes two sperm cells. One of these fertilizes the egg cell to form a diploid zygote. This later develops into the embryo (2n). The other fertilizes the two polar nuclei to form a triploid cell. This cell later develops into the endosperm (3n). When we use haploid inducer line as a male parent one of the two sperm cells behave normally and fertilizes the two polar nuclei. This results into (3n) endosperm. The certain fractions of the sperm cell do not behave normally. They either fail to fertilize the egg cell or after fertilization their chromosomes eliminates resulting in haploid embryo (1n). As the inducer purple colored genome is not present in the embryo cell therefore, it is colorless and can be visually identified. These kernels are with normal (3n) endosperm therefore, are capable of germinating normally, similar to normal diploid kernels **[29].**

There are two theories behind this phenomenon of haploid induction. One hypothesis is that out of two sperms of male gametophyte one is slow in speed. This sperm can trigger the fertilization process, but unable to fuse with the egg cell. This failure results in haploid embryo. The second sperm fuses with the central cell and gave rise to 3n endosperm **[30].** The second hypothesis of chromosome elimination states that one of the two sperm cells is defective. This defective sperm cell of inducer fuses with the egg cell at the time of double fertilization. But at the time of cell division, the chromosomes of the defective sperms deteriorate and get eliminated from the growing tissues **[31].**

Chromosomal doubling of haploid kernels

i) Artificial chromosomal doubling

Haploid plants donot produce normal gametes therefore haploidy and sterility in these plants go side by side **[32].** The duplication of haploid chromosomes is essential to attain fertile gametes and homozygous inbred lines. Although doubled haploid inbred line development is a simple producer, but practically it has not been so easy to double the haploid chromosome numbers. This haploid chromosomal doubling can occur naturally/ spontaneously but its occurrence is very low. Therefore, different artificial doubling agents are used to double these haploid chromosome numbers. Colchicine has been predominantly the artificial chromosome doubling agent of choice in corn. It is the most widely used chromosome doubling agent in the world. It binds to the spindle forming protein and disturbs chromosomal movement which results in their duplication **[33].** After induction cross the maternal haploids produced contain only half the number of maternal chromosomes. Their duplication results in doubled haploid lines. For this genome duplication a very efficiently reliable method is required which must be cost effective **[34].** For the germplsm where spontaneous chromosomal duplication is non-existing or very low this artificial duplication is necessary **[35].** This is done through the utilization of colchicine chemical. It is a yellow colored poisonous alkaloid obtained from a plant. The chemical influences on cell division of a plant and inhibits mitosis **[36]**. The spindle fibers are affected by this chemical witch disturbs the chromosomal alignment and disrupts the separation of these chromosomes.

ii) Spontaneous Chromosome Doubling

Spontaneous chromosome doubling rates are different for male and female inflorescence. They range from 2.8 to 46 percent for male inflorescence **[37, 38, 39]** and from 25 to 94 percent for female inflorescence **[40, 41, 42]** .These rates are germplasm specific. Spontaneous chromosome doubling rates range from 0 to 10 percent and is mostly credited to sterile male and female inflorescences **[43, 44, 45].** Due to lack of fertility the spontaneous duplication range lies from 0 – 10% **[46, 47, 48, 49]** Spontaneous chromosome doubling of haploid plant in case of the female inflorescence and seed development is reported to range from 25 to 94 percent, through different investigations **[50, 51, 52]**. Many mechanisms are reported in spontaneous chromosome doubling in haploid maize plants, including somatic cell fusion, endoreduplication, endomitosis, etc**.** **[53, 54].** Protoplasts of sterile haploid maize plant cells fuse to form fertile diploid cells. The process of somatic cell fussion starts with cell wall digestion. The cell walls of two neighboring somatic cells get digested by cellulase enzyme. The two different protoplasts and nuclei fuses with each other. After their fusion again hormones are released and cell wall reappears around the two fused cells, which now becomes a single cell. This single cell is with doubled chromosome number and is now diploid. This process of endoreduplication/ endomitosis occurs in the absence or decline in the process of mitosis [**55**]**.** If the mitosis do not start after 10 to 14 days after pollination this process begins **[56].** Endoreduplication is characterized by having repeated cycles of alternating DNA replication and non-replication. The chromatid do not separate, nucleus and cytoplasm do not divide **[57].** It was verified through flow cytometry that entire genome doubles during this process of endoreduplication [**58**]**.** It also plays key role in the spontaneous chromosome doubling.

Spontaneous development of doubled haploid from haploid embryo is natural in maize generations. Spontaneous chromosome doubling is a natural process in maize which can be exploited to replace artificial chromosomal doubling through toxic chemicals. This process is genotype specific and range between 0-10 percent **[59, 60].** The breeder's goal should be to identify such genotypes to exploit this natural process of duplication and avoid these harmful chemical doubling agents to reduce the cost of production and lengthy laboratory procedures. Penghao in 2014 **[61]** reported a special type of doubled haploid called early duplicated haploid (EH) that was directly generated by haploid induction in vivo. They found 83 EH plants induced by the Zhengdan958 hybrid, 55 families of their F2: 3 population and the parental lines. Through flow cytometry and SSR analysis all of these were confirmed as doubled haploids. EH plants were also detected in other genotypes with a frequency of 1-3.5%. Because the EH lines showed total fertility and were obtained from induction directly in a single step, they could be used in DH reproduction as a new breeding strategy. According to their observations, spontaneous duplication in these EH is likely to occur during the development of the embryo when haploid induction occurs.

Doubled haploid confirmation

Validation of maize doubled haploid inbred lines is not as difficult as in other plant species. Inbred lines can easily be distinguished due to their homozygosity and lack of segregation in the next generation. The developed DH-lines are sown in the next season to check and confirm the homozygosity. All the plants within a line are screened for uniformity of phenotypic traits such as plant height, ear height, anther color, silk color and overall appearance. The plants are also compared with their parental lines. After field evaluation and data comparison homozygosity within a line is confirmed [**62**]. Doubled haploids can also be analyzed and characterized by using microsatellites molecular markers and flow cytometry techniques. Haploid, diploid, and mixoploid individuals can be detected using flow cytometry. The microsatellites markers are also efficient for confirming and identifying homozygous individuals **[63].**Enzymatic mismatch cleavage has been applied for mutation discovery in many plant species. The same methods provide an efficient system to screen for the production of DH material without the need of specialized equipment and can be more efficient than SSR based screening **[64].** Stomata guard cell measurement is an easy, non-destructive, early screening method that may lead to a greater efficiency in DH production systems and optimization of resource allocation for space and labour **[65].**

Advantages and its utilization in breeding programs

Doubled haploid technique produce 100 homozygous fixed inbred lines in few breeding seasons [**66, 67, 68].** This technique also allows best possible trait combinations in a single DH-material in a reduced time span [**69**]. In research, DH lines are a valuable tool in trait association studies, marker-assisted selection and functional genomics. The doubled haploid technology has provided many quantitative genetic and economic advantages over traditional method of the line development **[70, 71, 72].** The additive variance is prevailing in DH-lines and their crosses can be early evaluation at the early stages of any selection scheme. This DH-line production technique is economically profitable by reduces the expanses of the nursery maintenance, logistic and breeding work [**73].** The DH lines have short breeding cycle. The early exploitation of genetic variance is possible. Logistic operations are very easy. These qualities along with the ability of plant variety protection make this technique superior than any other line developmental method [**74**]. Inbred lines are developed and selected with some desirable traits for the production of hybrids in maize [**75].** The DH technology has not only accelerated the line development, but also helped in recognition and utilization of the elite inbred lines. In recent years a large number of doubled haploid inbred lines are produced to constitute maize hybrids [**76, 77, 78**]. CIMMYT has developed 4000 maternal DH-lines till the end of last decade [**79**]. The DH technology has enhanced “forward breeding” **[80, 81-84].** This method of line development is more efficient than traditional method of inbred line development.

CONCLUSION

The research efforts of a whole century are summarized in the success of in-vivo DH-line developmental technique. This technique is an asset and breeder’s heritage which should be utilized to overcome future challenges. Efforts are needed to exploit natural chromosomal doubling of the haploids to make it more environments friendly. This will not only simplify this technique but will turn it more cost effective.

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