British Biotechnology Journal
8(3): 1-7, 2015, Article no.BBJ.18394
ISSN: 2231–2927

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Development of Maize Haploid Inducer Lines and Doubled Haploid Lines in Pakistan

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Authors' contributions

This work was carried out in collaboration between all authors. Author KK designed the study, wrote the protocol, contributed in breeding work, managed the literature searches and wrote the first draft of the manuscript. Authors MRD and MA supervised the project and provided technical advice as required. Author AH managed all the farm and breeding work. Author MAS managed the research part for seed colchicine treatment of the project. All the remaining Authors gave breeding contribution in the field. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/BBJ/2015/18394
Editor(s):
(1) Mahalingam Govindaraj , ICRISAT, Patancheru, India.
Reviewers:
(1) Anonymous, Telangana University, India.
(2) Ashraful Alam, Bangladesh Agricultural Research Institute (BARI), Dinajpur, Bangladesh.
(3) Anonymous, Brazil.
(4) Anonymous, Serbia.
Complete Peer review History: http://sciencedomain.org/review-history/10125

Original Research Article

Received 20th April 2015
Accepted 16th June 2015
Published 10th July 2015

ABSTRACT

The work on maize doubled haploid development has started at the Maize Research Station, Ayub Agricultural Research Institute, Faisalabad, Pakistan during 2010 in collaboration with University of Agriculture Faisalabad, Pakistan. The aim of current study was to develop locally adopted maize haploid inducer lines utilizing cheap and easy source of Stock6 and indeterminate gametophyte mutant ig1 gene lines imported from Maize genetic Coop Stock Centre. The lines were later utilized to produce doubled haploid inbred lines. The environmental conditions of Faisalabad are extreme in nature. In spring crop during pollination period temperature may reach up to 45°C. Therefore, locally adopted haploid inducers are needed. Best performing local inbred lines were screened having branched heavy tassel, bold seed, good pollen shedding ability and vigor. These lines were utilized as female donor parent while imported lines as recurrent pollen parent in back cross

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breeding program until BC4 generation. These lines were selfed twice until BC4F2 and evaluated for haploid induction rate (HIR). Lines with high HIR were further intercrossed to achieve maximum transgressive segregation. Mass selection for the adaptation traits was exercised for individual F2 plants followed by ear-to-row plantation of selected progeny. Four best haploid inducer lines with HIR up to 5% having very good tassel size, height and heat stress tolerance were selected in 2014. Haploid seeds collected in different induction crosses until 2012, were used for colchicine doubling treatment. The doubling percentage of the haploid plants was very low 0.15% and out of 1000 treated seedling 15 survived and only 5 reached up to maturity, where only one D0 cob was harvested. This was successfully selfed and was grown in three different locations for next two seasons and there was no segregation in the successive generations. Developing countries that cannot afford costly haploid inducer lines can work on Stock6 and other cheap sources available free of cost and can develop their own haploid inducer lines well adapted to their own climatic conditions.

Keywords: Haploid inducer lines; stock6; ig1 mutant; Faisalabad; AARI; UAF.

1. INTRODUCTION

DH production is a simple method to improve efficiency of maize breeding [1-3]. Compared to conventional breeding it takes a single year to develop an inbred line. Therefore, it is adopted worldwide in routine maize breeding programs. This technique seems solution to many problems like slow hybrid development, in Agriculture research sector of Punjab, Pakistan.

Area of spring crop in Punjab is 200 000 ha and average yield is about 7-8 t/ha. Total hybrid seed import is about 8,289 t while local seed production is only 2,218 t. This import burden on whole national economy costs about 30 Million US dollars [4]. Most of the hybrid seed demand is fulfilled by multinational companies. Government institutes adopt old conventional methods of developing inbred lines and have been unsuccessful in producing hybrids as demanded by the farmers. If government sector adopts doubled haploid technology it can solve many problems looking hurdle in hybrid maize development. Doubled haploid technology accelerates inbred line development. Evaluation of putative hybrids at the beginning of the selection process is possible. The maximum additive variance is available in doubled haploid lines with reduced masking effects caused by residual heterozygosity. Doubled haploid technology reduces the costs for nursery and maintenance of breeding work [5].

Temperature ranges 18 to 48°C during the spring season in Punjab (Country report, 12th Asian Maize Conference [4]. Before development of tropically adapted inducer lines (TAILs) by CIMMYT [6], all the existing haploid inducer lines were of temperate origin that would never be successful in extreme environmental conditions of Punjab, Pakistan. These inducers display poor vigor, poor pollen production, poor seed set, and high susceptibility to tropical maize diseases when grown in tropical region [7].

The tropicalized haploid inducers are now available for sharing with interested institutions for research or commercial use but under specific terms and conditions. Many developing countries of tropical area cannot afford the purchasing cost of temperate as well as tropicalized haploid inducers which are the pre-request of doubled haploid line development. Therefore, initially work was started for inducer line development. First haploid inducer line (Stock6) seemed cheap and easy source to serve the purpose [8,9]. Indeterminate gametophyte *ig* gene can induce both androgenesis and gynogenesis [10]. In a previous research work crossing two haploid-inducing lines, KMS and ZMS, resulted in the production of transgressive genotypes whose haploid-inducing capacity was more than two times higher than that of the parental lines [11] Novel genetic variation may be present in maize accessions that could be exploited to improve HIR in maize [6].

The aim of our work was 1) to create new locally adopted haploid inducers which would have high haploid induction rate than parental lines, 2) and to utilize them in doubled haploid line developmental program. This will open new horizons for hybrid maize breeding in the region.

2. MATERIALS AND METHODS

The exotic lines (Stock6, *ig1*; *R-nj*/ *ig1*; *R-nj* male sterile line) were imported from Maize genetic Coop Stock Centre in 2010 (Fig. 5 and 6). Stock6 possess *B1*; *Pl1* and *R-nj*, allowing haploids to be identified both at the stage of mature seeds and at the stage of vegetating plants. The *ig1* mutant with *R-nj* marker gene was introgressed into the M14 inbred line more suitable for environment of Pakistan. Ten best local inbred lines performing best under field conditions were selected, having (1) good branched tassel size (2) good pollen shedding ability (3) vigorous under field conditions. Maize is not native to the area therefore, the segregating generations from various origins were collected in 90`s and later utilized in inbred line developmental program by the Maize Research Station, Ayub Agriculture Research Institute, Pakistan.

Imported material being temperate and early flowering therefore was planted 15 days later than the local lines to achieve synchronization for crossing. Crosses were made between 10 inbred lines as female donor parent and an inducer hybrid (Stock6 x *ig1* homozygous male sterile) and stock6 as male recurrent parent in back cross breeding program until BC_4 generation. These lines were selfed twice and selected on the basis of high HIR (Table 1). The 5 best selected BC_4F_2 were again crossed in all possible combinations and selfed twice. After their evaluation for HIR and other adaptational traits 4 (four) best locally adopted inducers were finally selected (Table 2). Selection was exercised on the basis of high HIR along with adaptational traits like vigor, tassel size,(Fig. 7 and 8) anthocyanin coloration and heat tolerance under field condition, especially in the spring season when temperature may reach up to 48°C in the month of May. Mass selection for the adaptational traits was performed on individual F_2 plants followed by ear-to-row cultivation of selected progeny [6]. Maize is normally grown in two seasons in Faisalabad, Pakistan but to create third season (December till February) these crosses were also made by growing the material in plastic tunnels.

2.1 DH Lines Production

Step-1: Generation of haploid seed through induction crosses
Female (F_1 or F_2) x male (inducer)

Step-2: Screening of haploid kernels – based on embryo and endosperm color marker gene (*R1-nj*).

Step 3: Chromosome doubling of haploid kernels- followed doubling protocol of CIMMYT.

Step 4: Potting of these delicate treated seedlings into pots with low concentration fertilizer.

Step 5: self–pollination of each plant (D_o) that shed pollen to produce (D_1) doubled haploid line. All the steps were carried out following CIMMYT DH-line developmental protocol [7]. The doubled haploid plants were slefed and tested for next two seasons at three different locations. The plants exhibited uniformity, homozygosity and no segregation for the successive generations.

Table 1. Five best (BC_4F_2) generations with their pedigree, origin and HIR

Donor Parent	Origin	Recurrent Parent	(BC_4F_2) HIR %	Tassel Size
F-107 Line	Nigeria	Stock6 Inducer line	1-2	Good
F-204 Line	Mexico	Stock6 Inducer line	1-3	Good
F-297 Line	USA	Stock6 Inducer line	1-2	Good
F-165 Line	Thailand	Stock6 Inducer line	0-1	Good
F-168 Line	Mexico	Hybrid cross (Stock6 x *ig*/*ig* male sterile line)	6	50% (plants) tassel sterile. Fertile tassels also with some sterile spikelet

Table 2. Genotypes finally selected as haploid inducers with their pedigree and HIR (Figs. 1, 2, 3 and 4)

Female (BC_4F_2)	Male (BC_4F_2)	F2 with HIR %	Tassel size	Vigor
F-204 x stock 6	F-107 x stock6	5	good	Excellent
F-204 x stock 6	F-297 x stock6	4	good	Excellent
F-204 x stock 6	F-165 x stock6	3	good	good
F-168 x Hybrid cross (Stock6 x *ig*/*ig* male sterile line)	F-107 x stock6	6	Sterility in some spikelet.	Fair

Fig. 1. F-204 x stock 6// F-107 x stock6

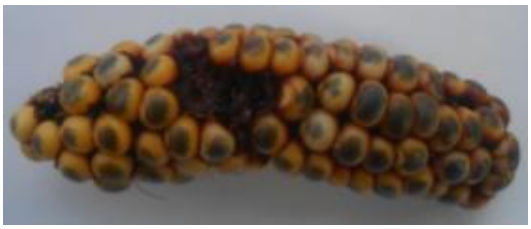

Fig. 2. F-204 x stock 6// F-297 x stock6

Fig. 3. F-204 x stock 6// F-165 x stock6

Fig. 4. F-168 x Hybrid cross (Stock6 x *ig*/*ig* male sterile line) // F-107 x stock6

3. RESULTS

All the above crosses were successfully obtained along with selfing and seed multiplication of imported lines. Haploid seed collected after induction crosses were used for colchicine doubling treatment according to CIMMYT protocol, in 2012. Out of 1000 treated seedling 150 survived and only 15 reached up to maturity, where most of the tassels were sterile and only few anthers shed pollen. Few plants only produced tassel and did not bear cob. Only few D_0 cobs were harvested. They were successfully selfed in the next season and first DH-line (Abdul Hameed 1, AH1) was reported in Pakistan.

4. DISCUSSION

Our first choice for cheap and easy access to haploid inducer was Stock6. On the basis of the first inducer of maternal haploids in maize (*Zea mays* L.), Stock 6 [8] a number of new inducer lines have been created [5,12-14]. The inducers possess dominant anthocyanin marker genes allowing haploids to be identified at different stages (dry seeds, seedlings and mature plants), and their haploid-inducing rate was significantly increased in comparison with the initial inducer (Stock 6), [15]. Both of the haploid inducers, ZMS and KMS, were created on the base of Stock 6 [8]. Although haploidy is natural process in maize but presence of this color marker has made detection of haploid plants easy at seed stage.

According to the conclusion made by breeders working on haploid inducer lines, induction of haploidy is not a limiting factor for DH line production in tropical maize, yet the development of well-adapted tropical inducers will be beneficial [6]. The haploid induction rate of temperate inducers was same in tropical environment but there were issues regarding vigor, seed set, pollen shedding ability and attack of diseases. Stock6 was very difficult to self under our field conditions. Some plant only developed tassel and other only cobs. Even problem of synchronization was there as cob appeared after complete pollen shed. The selfed seed developed for the first time were also with less purple pigment in aleurone (Fig. 6) but the marker gene expression was good in crossed seed. The second cycle of selection for high HIR and adaptational characters revealed higher HIR than the parental lines along with more adaptability. These results are in coincidence with previous studies [6,14,15]. It is possible to combine high HIR with adaptation to tropical climate and developed inducers with HIR of up to 10% and excellent agronomic characteristics under tropical lowland conditions. The high HIR than the parental lines is due to transgressive segregation and was also reported in a previous publication. Crossing two haploid-inducing lines, KMS and ZMS, resulted in the production of transgressive genotypes whose haploid-inducing capacity was more than two times higher than

that of the parental lines [11]. It was suggested that the parental lines KMS and ZMS differed from each other by two genes controlling haploid-inducing capacity. Transgressive genotypes carry both genes and are capable of inducing 7 to 9% of haploids. QTL mapping for in vivo haploid induction ability suggested that the trait is controlled by one or few major QTL and several small modifier QTL [16]. Screening random open-pollinated maize accessions, HIR of up to 3.0 % were observed, suggesting that novel genetic variation may be present in maize accessions that could be exploited to improve HIR in maize [6]. Our maize research station has maintained many inbreed lines, open pollinated varieties and many segregating generations. After their QTL analysis for HIR and identification of desired genotype, this germplasm can be a good source for future haploid inducer developmental programs of the region. Lines of our research station are also being exploited in Heat Tolerant Maize for Asia (HTMA) program by CIMMYT and proving best.

The indeterminate gametophyte mutant *ig1* line imported from Maize Genetic Coop Centre has same mutant as the stock W23 *ig* line. However, instead of being introgressed into the W23 inbred line, it was introgressed into the M14 inbred line homozygous for the *ig1* mutant (Fig 5). W23 is a Northern adapted inbred line and likely would not do well in Pakistan. M14 is a more vigorous inbred line and would likely grow better in Pakistan. Therefore, it seemed the better option. The cross between *ig* gene line x Stock 6 based on Ws14 a French induction line which is cross of W23*ig* x stock6. This cross also proved promising but only problem was with tassel sterility. Another haploid inducer in maize is the *indeterminate gametophyte* (*ig*) mutant [17,18]. The *ig* can induce both androgenesis and gynogenesis [10]. The *ig* gene manipulation procedure showes a fairly good level of haploid induction in backcross and appears to keep a promising genetic background. The *ig* gene does not appear to constitute a simple genetic system but is influenced by modifying genes. Now QTL analysis of *ig* gene is also available and revealed its presence on chromosome 3 [19]. Local material with same locus can be identified to overcome this problem of tassel sterility.

CIMMYT's Global Maize Program started in DH line development in 2007 and the involved processes have since been optimized. More than 4000 tropical DH lines have been produced between 2007 and 2010 [20] following the protocol described by Prigge and Melchinger [16]. This indicates that established protocols for in vivo DH line development can be readily applied to tropical maize breeding programs, although specific amendments owing to institutional and environmental conditions may be necessary. One of the obstacles during initiation of any DH production service involves reliable detection of haploids among the induction cross progeny [5]. In our study this wasn't the problem as marker gene expression was fairly good. But the mortality rate of the Colchicine treated seedlings was really high. Efforts must be taken to evaluate spontaneous doubling in our local germplasm under field conditions. Spontaneous haploid doubling technology and the cheap locally adopted haploid inducers can accelerate the hybrid maize development in public sector of the country.

Fig. 5. The *ig1* mutant with *R-nj* marker gene introgressed into the M14 inbred line (Please mention fig.5 inside the text)

Fig. 6 . Stock6 (*B1*; *Pl1* and *R-nj*), original imported stock6 seed on left side and lighter color seed, selfed in Pakistan, on right side

Fig. 7. Tassel of F-204 x stock 6// F-297 x stock6

Fig. 8 Tall and good tassel size F-204 x stock 6// F-107 x stock6

4. CONCLUSION

Countries of tropical region can exploit their own material for inducer line development. They can import stock6 from any reliable source and start their own inducer line developmental program. The new inducer lines developed will be with improved HIR and good adaptational characters. CIMMYT has provided a comprehensive protocol to develop DH-lines and following this will surely report several doubled haploid lines as first DH-line AH1 reported in Pakistan.

ACKNOWLEDGEMENTS

The authors of this manuscript are really grateful to Maize Genetic Coop Stock Centre for providing seed and information as and when required. The present work would not have been possible without their cooperation. Special thanks to Dr. B. M. Prasanna, (Director, Global Maize Program, CIMMYT) for encouraging, giving technical advice to our scientists and providing our scientist a training opportunity of doubled haploid technology in maize breeding, arranged by CIMMYT in 2012. Also special thanks to former director, Maize and Millet Research Institute Yusafwala, Sahiwal, Dr. Abdul Hameed, who encouraged and helped to initiate the project.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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Peer-review history:
The peer review history for this paper can be accessed here:
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