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Original scientific article

DEVELOPMENT OF LATE TEMPERATE *IN VIVO* HAPLOID INDUCERS

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In vivo doubled haploid technique has been widely used in advanced maize breeding programs due to cost, labor and time advantages and increase in efficiency. However, the number of available inducer lines in the world is sufficient. Six BC_1 breeding populations including RWS and RWK-76 haploid inducer lines and late temperate ADK-451, ADK-737 and ADK-455 lines were developed by Sakarya Maize Research Institute (MRI) in Turkey. The RWS and RWK-76 haploid inducer lines were used as donors. Pedigree method was employed to develop the inducer lines. Anthocyanin coloration of plant, tassel length, branch number of tassel, plant height, days to flowering, embryo-endosperm colorfulness and haploid induction rate (HIR) were determined. The genotypes with the best characteristics were selected. The families from BC_1F_3 to BC_1F_7 were hybridized to liguleless line to determine the HIR and families with HIR over 8% were selected from BC_1 populations. The HIR, plant height and days to tassel flowering values of in-1021 and in-1076 candidate haploid inducer lines were 10.5 and 12.3%, 195 and 200 cm, and 69 and 68 days, respectively. The HIR value of RWS donor haploid inducer ranged from 8.9 to 11.3% and for RWK-76 from 7.3 to 9.8%. Simple Sequence Repeats (SSRs) markers were used to identify genetic similarity between late temperate haploid inducer lines and donors. The similarity rates of in-1021 and in-1076 inducer lines to the RWS donor were 38 and 15%, and to the RWK-76 donor were 23 and 27%. The similarity rate between the two candidate inducer lines was 30%. The results indicated that the late temperate haploid inducer lines developed will increase the efficiency of maize breeding.

Keywords: Genetic similarity, haploid inducer line, haploid induction rate, liguleless line, pedigree method.

INTRODUCTION

In vivo doubled haploid technique has been widely used in advanced maize breeding programs due to being fast and high efficiency. Pollens of maize inducer genotypes are used to pollinate source germplasm from DH lines in *in vivo* induction of maternal haploids (PRIGGE *et*

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al., 2012). The haploid inducers are specialized genetic stocks which, when crossed to a diploid (normal) maize plant, result in progeny kernels in an ear with segregation for diploid (2n) kernels and certain fraction of haploid (n) kernels due to anomalous fertilization. Kernels with a haploid embryo have a regular triploid (3n) endosperm, and therefore, are capable of germination similar to the kernels with a diploid embryo (COE and SARKAR, 1964). Studies conducted to segregate generations derived from crosses between inducer and non-inducer parents revealed the continuous variation for haploid induction associated traits (SARKAR and COE, 1966; LASHERMES and BECKERT, 1988). In addition, the *in vivo* haploid induction trait is defined under polygenic control. The researchers reported that the haploid induction trait of Stock 6 inducer line had a dominant character with nuclear determination and was controlled by a few major genes.

The most efficient haploid identification marker is the 'red crown' or 'navajo' kernel trait encoded by the dominant mutant allele *R1-nj* of the 'red color' gene *R1*. In the presence of the dominant pigmentation genes *A1* or *A2* and *C2*, *R1-nj* causes deep pigmentation of the aleurone (endosperm tissue) in the crown (top) of a kernel and conditions the pigment in the scutellum (embryo tissue). The extent and intensity of pigmentation may vary depending on the genetic background of a particular donor and inducer (GEIGER, 2009).

When an inducer line is crossed (as male parent) to a source germplasm (as female parent) which does not have the anthocyanin color markers, all the hybrid kernels are expected to express the Navajo phenotype in the endosperm and in the embryo as *R1-nj* is dominant over the colorless *r1* allele. However, four different types of coloring occur in the kernel. Thus, the differential expression of *R1-nj* facilitates identification of maternal haploids from the diploid kernels. The putative haploid kernel does not have any color in embryo, while the endosperm has. Maternal haploids usually occur at a frequency between 6 and 10% when the haploid inducers with a high HIR are used in the induction cross (CHAIKAM and PRASANNA, 2012a).

New haploid inducer lines were developed using the CAU3/S23 and CAU5/S23 breeding populations. The BC_1F_1 to BC_1F_4 generations were selected by using molecular marker assisted with the *qhir1* and *qhir8*. Finally, the haploid inducer lines CS1, CS2 and CS3 were developed. The CS1 and CS2 from the CAU3/S23 population had a HIR of about 8.2-13.25% and 11.54-15.54% while CAU3 and CAU5 had a HIR of 11.22 and 11.89%, respectively. The HIR of CS3 derived from the CAU5/S23 population was about 8.14-12.28% (CHEN *et al*., 2020).

The possibility of additional color markers, especially those expressed in root and stem, for reliable identification of maternal haploids have been investigated by ROTARENCO *et al*. (2010). COE (1959) identified *Pl1* (*Purple1*) and *B1* (*Booster1*) genes, which can impart purple or red color to the plant tissues. The *Pl1* (*Purple1*) gene conditions sunlight-independent purple pigmentation in plant tissues, and *B1* (*Booster1*) conditions sunlight-dependent purple pigmentation in most of the above-ground plant tissues. However, some restrictions have been reported for *B1* and *Pl1* color markers by CHAIKAM and PRASANNA (2012a). Many of source materials contain *B1* and *Pl1* genes. The haploid plants in such source populations, also express coloration in the roots and stems; thus, reliable identification of such haploid plants may not be possible. Expression of the *B1* and *Pl1* genes are affected by plant growth conditions, especially sunlight and temperature. For example, the highest purple pigment accumulation occurs under low temperatures.

Identification of the haploid inducer lines using HIR requires suitable testers that allow unambiguous identification of haploids at the early seedling or kernel stage. Most commonly used testers possess are recessive genes like liguleless and glossy. When the testcross seeds from the cross of liguleless × inducer or glossy × inducer are germinated, only the haploid seedlings exhibit the glossy or liguleless phenotypes. The assays can be conducted on seedlings at three to four leaf stage in a greenhouse. The *R1-nj* anthocyanin marker system can be used for the assessment of HIR in inducer lines that incorporate *R1-nj*. In order to be provide an efficient system, the testers should not possess inhibitor genes and sufficiently express *R1-nj* under different environments. The inducers can be crossed to such testers and the HIR can be determined based on *R1-nj* expression (CHAIKAM and PRASANNA, 2012b).

The haploid inducer lines used to implement *in vivo* maternal haploid technique in maize breeding are imported by public and private sectors in Turkey. Most of the inducer lines used in *in vivo* haploid technique were adapted to the temperate zone. The inducer lines used in *in vivo* haploid technique are the members of early maturity group (FAO 400-450), have short plant height and poor pollen yield and other morphological characteristics compared to late temperate germplasms. Therefore, this study was conducted to transfer haploid induction and the *R1-nj* marker of the early haploid inducers to local inbred maize lines in Maize Research Institute in Sakarya, Turkey.

MATERIALS AND METHODS

The breeding programs have been initiated in 2011 to transfer haploid induction characteristics and *R1-nj* marker of early haploid inducer lines to local inbred maize lines in MRI. The ADK-451, ADK-737 and ADK-455 elite inbred lines (female parents) obtained from MRI were crossed to haploid inducer lines RWS and RWK-76 (male parents). The BC_1 populations planted were selected based on, tassel length, number of branches, plant height, days to flowering, embryo-endosperm colorfulness, anthocyanin coloration of plant and HIR. The generations between F_3 to F_7 were planted in ear-to-row and progenies were selected considering agronomic traits (Figure 1).

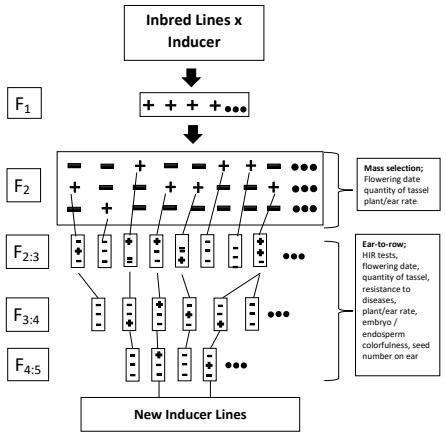

Figure 1. Breeding scheme of new inducer lines (PRIGGE *et al.*, 2012)

Total of 541 BC_1 families were used in the first year of the study. Fertilizers were applied based on the requirements of the maize, weed control was performed as needed and plants were watered using drip irrigation. Each material had an ear, which was planted in a single row. Of the starting materials, 324 were in F_4, 171 were in F_3 and 46 were in F_2. Total of 659 rows of candidate haploid inducer lines were self-pollinated in 2017 and 725 rows were self-pollinated in 2018 (Table 1). The number of inbreeding on each row was at least 4 or 6. Weak, diseased and abnormal plants were discarded by phenotypic selection throughout the study. The generations from F_3 to F_7 were planted in ear-to-row and were selected according to purple color of plant and kernel, late flowering, good plant vigor, and high seed set.

Table 1. Distribution of breeding material by years

Years	Number of candidate inducer lines sown	Number of inbreeding rows	Number of selected inducer lines rows at harvest
2016	541	515	283
2017	659	545	372
2018	725	497	371
Total	1925	1557	1026

Recessive morphological mutations were used in the development of haploid inducer lines. The genotype with liguleless feature was one of recessive morphological mutations. When a non-inducer maize line carrying ligule *LG* allele and a maize line carrying mutant *lg* allele are hybridized, the offspring *lg* allele is covered by the dominant *LG* allele and the offspring occurs in the *LG* phenotype. However, when an inducer line carrying the *LG* dominant allele and a maize line carrying the mutant *lg* allele are hybridized, haploid offspring appears in the *lg* recessive phenotype since the haploid offspring only contains the chromosomes of the mutant *lg* allele.

The 202B (*lg1*) liguleless line was used as a tester to determine the HIR of candidate haploid inducer lines. Each generation, selected from F_3 to F_7, was crossed with the liguleless line. Selected candidate inducer lines were used as male parent and liguleless line was used as female parent. The bulked pollen from a minimum of five plants was used to cross 36 candidate inducer lines to liguleless line in 2016, 58 in 2017 and 137 in 2018. Each candidate inducer line was hybridized with at least 5 ears of liguleless line. One hundred testcross seeds were planted in styroform trays with three replicates to evaluate the testcross progeny for haploid. The haploids of progeny were characterized by missing ligule and auricle (Photo 1). The HIR was assessed when the testcross progeny was 3-4 leaf stage of seedlings (Photo 2). The candidate haploid inducers with a HIR of 8% or more were selected for further processes.

The selection was carried out based on i) anthocyanin coloration of plant (ACP), ii) tassel length (length of main axis above the lowest side branch) (TL), iii) branch number of tassel (BNT), iv) plant height (PH), v) days to tassel flowering (DTF) and vi) intensity of purple embryo-endosperm coloration (PEC) (Photo 3). All visually traits were scored between 1 and 5, in which 1 represents the poor and 5 represents the excellent. The candidate haploid inducers with scale value of 3 to 5 were chosen for the next generation of genotypes.

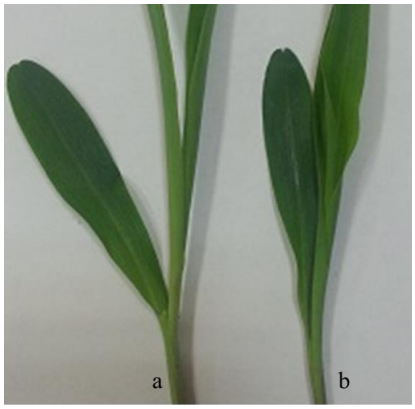

Photo 1. a) Seedling with ligule and auricle, b) Haploid seedling with liguleless and non-auricle.

Photo 2. Growing of haploid seedlings in the greenhouse and determining the phenotype of liguleless in 3-4 leaves stage.

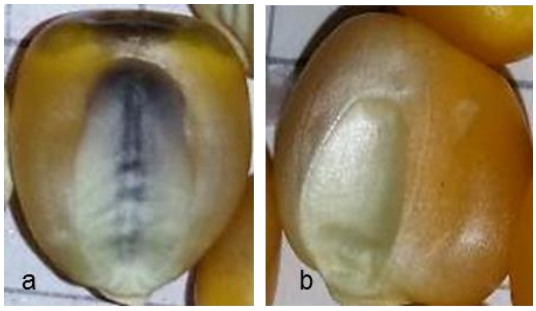

Photo 3. a) Appearance of color marker in embryo and endosperm (*R1-nj*), b) Lack of color in embryo and endosperm (*r1*)

The RWS and RWK-76 haploid inducer lines were purchased from the University of Hohenheim, Germany under the license agreement. The agreement stated that similarity between candidate inducer lines and the RWS and RWK-76 inducer lines used as donors should at least 87.5%. The agreement clearly indicated that genetic similarity between the new inducer lines obtained and the haploid inducer lines used as donor should be revealed. SSRs markers were used to identify the genetic similarity between candidate haploid inducer lines and donors. The SSRs markers were selected from the MaizeGDB database (http://www.maizegdb.org) to provide good coverage of the entire genome. Thirty SSR primers were identified and showed polymorphism. The genetic similarity of selected candidate lines with the highest HIR was compared with RWS and RWK-76 donors.

DNA isolation of 80 candidate inducer lines along with RWS and RWK-76 inducer donors was carried out in 2018. Capillary electrophoresis (Bioptic Qsep100) was used for PCR analysis. Allele sizes were determined using Q-Viewer (Version 2.0) of the same device.

Population structure was determined using STRUCTURE 2.3.4 software. Length of burning period at 10000 and number of MCMC reps after burning at 10000 were operated in the STRUCTURE software.

DNAs were extracted using CTAB method as explained by SAGHAI-MAROOF *et al*. (1984). Lyophilized tissue of 100-150 mg were homogenized by TissueLyser (Qiagen, Germany) and incubated at 65ºC for 90 min in CTAB buffer (1% CTAB; 1M Tris-HCl, pH 7.5; 0.5M EDTA, pH 8.0; 0.5M NaCl; 14M β-mercaptoethanol). After centrifugation of Chloroform-octanol (24:1), the supernatant was transferred into a new tube and cold isopropanol was added. The DNA was washed and precipitated with ethanol and resuspended in PCR grade water. The quality and quantity of isolated DNAs were measured by using a NanoDrop Spectrophotometer (Thermo Scientific, USA).

After diluting the DNAs, PCR was carried out with 3 µl of DNA (50 ng ul^{-1}), 0.03 µl of 100 µM dNTP, 1.5 µl of 5 X PCR buffer with $MgCl_2$ and 0.5 µl of 10 µM of each primer with 0.08 µl of 5 units/µl Taq DNA polymerase (Roche, Germany). The reactions were incubated at 94°C for 10 min. After 35 amplification cycles (2 min at 94°C, 30 s at 50-60°C, and 1 min at 72°C), final extension at 72°C for 7 min before cooling at 4°C were performed. After the amplifications, PCR products were separated by capillary electrophoresis in a QSEP100 DNA fragment analyzer (BiOptic, Inc., Taiwan).

For each SSR marker used in the study, the polymorphism information content (PIC) was determined according to SMITH *et al*. (1997). The PIC calculated with this method is the synonymous with the term of "gene diversity" (WEIR, 1996). In this method, the first (1) and none (0) bands of polymorphic bands were determined. Then, the individual frequencies of these bands were calculated. In addition, the average number of alleles for each amplified microsatellite, gene diversity (expected heterozygosity; He) was also determined (NEI, 1978; MORGANTE *et al*., 1994).

Genetic analysis was carried out using the method explained by ŞELLI *et al*. (2007). In this method, genetic parameters including number of alleles per all locus, allele frequency, expected and observed heterozygosity rate, and null allele frequency were determined. Probability of identity (PI) was determined using IDENTITY 1.0 (WAGNER and SEFC, 1999) software. Microsat (MINCH *et al*., 1995) software was employed to calculate the similarity index. The distance

matrix and dendrogram of the genotypes were created and displayed with the NTSYS-pc software using the unweighted pair group method with arithmetic mean (UPGMA), which is a simple agglomerative hierarchical clustering method.

RESULTS

Obtaining candidate haploid inducer lines and assessment of HIR

The seeds were selected each year based on *R1-nj* marker expression in kernel after the harvest. The appearance of anthocyanin color marker in the seeds varied from light to dark depending on the genotypes, while dark color seeds were particular selected. Anthocyanin coloration of plant and seed scores ranged from 1 to 5 (1 = week color and 5= excellent dark color) (Photo 4 and 5). Anthocyanin coloration of plant from 3 to 5 was selected. Ears were chosen considering the seed set, ear aspect, *R1-nj* marker expression and diseases at harvest.

Photo 4. Selection of candidate inducer lines according to R_1*-nj* color marker in kernel.

Photo 5. Differences of anthocyanin color marker in plant.

Photo 6. The ears of in-1021 and in-1076 haploid inducer lines and *R_1-nj* marker expression.

The HIR was determined for BC_1 families crossed with tester liguleless line and in families with 300 or more seeds. Families with low seeds were crossed to liguleless once more in the next year. The HIR of F_4, F_5 and F_6, which are the generations of BC_1 families obtained by testcross with liguleless tester, ranged from 4.7 to 16.2%, from 4.0 to 15.8 and from 3.1 to 14.3%. The HIR of RWS and RWK-76 haploid inducer donors in this study were 8.9-11.3 and 7.3-9.8%, respectively (Table 2). The candidate haploid inducers with HIR of 8% and higher were selected and their generation continued.

Table 2. BC_1 families crossed with tester liguleless line, total seed count range of each family (TSEF) and donor haploid inducer lines of HIR in 2016, 2017 and 2018 at Sakarya location

BC Families	Tester	Number of families	TSEF[a]	Range of HIR %
BC_1F_4	202B	36	300-407	4.7-16.2
BC_1F_5	202B	58	321-498	4.0-15.8
BC_1F_6	202B	80	386-1572	3.1-14.3
RWS[b]	202B		379-457	8.9-11.3
RWK-76[b]	202B		402-517	7.3-9.8

[a] Families with less than 300 seeds were excluded

[b] Three-year data for donor haploid inducer lines

PH of $BC_1F_{6:7}$ generation varied between 110 and 250 cm and mean plant height was 189.8 cm. The mean PH of $BC_1F_{6:7}$ families were 56.9% higher than the RWS donor and 69.5% than the RWK-76 donor. DTF of $BC_1F_{6:7}$ families were between 65 and 73 days. Considering the mean flowering days, the $BC_1F_{6:7}$ families bloomed 10.3 days later than the donors due to the selection based on the late maturity. The ADK-451, ADK-737 and ADK-455 elite inbred lines were used as the major parents while developing the BC_1 families. The DTF of ADK-451, ADK-737 and ADK-455 lines were 74, 70 and 77 days, and plant heights were 252.3, 217.7 and 255.7 cm, respectively (Table 3).

TL and BNT for $BC_1F_{6:7}$ families were ranged from 2.5 to 4.2 cm and from 2.7 to 3.8, respectively. Families scored 3 or more for intensity of purple embryo-endosperm coloration (PEC) and anthocyanin coloration of plant (ACP) traits were selected during inbreeding

generations. The PEC and ACP scores of the $BC_1F_{6:7}$ families varied between 3.3-5 and 3.1-5, respectively (Table 3).

Table 3. Days to tassel flowering (DTF), plant height (PH), tassel length (TL), branch number of tassel (BNT), anthocyanin coloration of plant (ACP), intensity of purple embryo-endosperm coloration (PEC) on kernel, and haploid induction rates (HIR) of donor haploid inducers (RWS and RWK-76), 2 temperate inducer candidates and mean of $F_{6:7}$ families at Sakarya location

No	Pedigree	DTF	PH cm	TL[a]	BNT[a]	ACP[a]	PEC[a]	HIR[b]
		Days		1-5	1-5	1-5	1-5	%
in-1021	ADP.KAY.333 1.1.1.3.1-1	69.0	195.0	3.7	3.3	4.0	4.2	10.5
in-1076	ADP.KAY.354 1.1.2.1.1-1	68.0	200.0	3.6	3.5	4.0	4.4	12.3
Mean of $F_{6:7}$	ADP.KAY	68.3	189.8	3.1	3.2	3.8	3.7	8.1
Max. of $F_{6:7}$	ADP.KAY	73.0	250.0	4.2	3.8	5.0	5.0	14.3
Min. of $F_{6:7}$	ADP.KAY	65.0	110.0	2.5	2.7	3.1	3.3	3.1
RWS		58.0	108.0	2.7	1.9	4.0	3.7	9.1
RWK-76		58.0	132.0	2.6	2.1	2.0	3.4	7.3
ADK-451		74.0	252.3	4.0	4.0	-	-	-
ADK-737		70.0	217.7	4.0	5.0	-	-	-
ADK-455		77.0	255.7	4.0	5.0	-	-	-

[a] Scored on a scale from 1 to 5 (1 = poor and 5 = excellent)

[b] Identified from seeds produced by pollinating the liguleless tester with inducer pollen at Sakarya location

Genetic diversity between candidate inducer lines and donors

The license agreement of RWS and RWK-76 haploid inducer lines stated that the similarity between the new haploid inducer lines obtained by using RWS and RWK-76 lines as donors should be at least 87.5%. Therefore, the genetic similarity between the new haploid inducer lines obtained and the inducer lines used as donors has been determined. The bar plot showed that the population have 2 subgroups (Figure2). Cluster 1 (red) contained 50 individuals and cluster 2 (green) contained 32 individuals. Candidate line 41 (in-1021) appeared as a green bar and candidate line 52 (in-1076) appeared as a red bar. Donor inducer lines 81 (RWS) and 82 (RWK-76) were also identified as green bars. The RWS and RWK-76 inducer lines were already improved as the sister lines (GEIGER, 2009) (Figure 2).

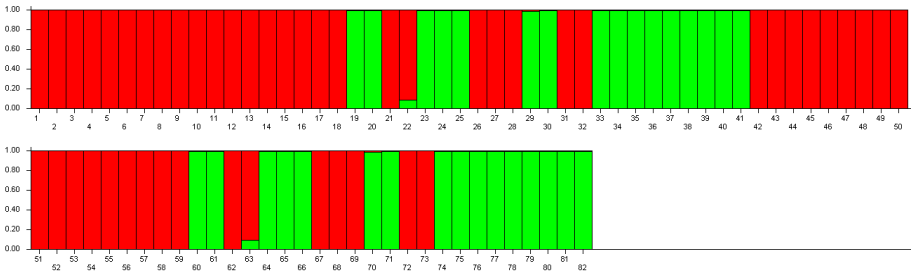

Figure 2. Bar plot shows subgroups of the population.

The average number of alleles was 4.7 and mean of polymorphism information content (PIC) was determined as 0.59. Clustering analysis was carried out using Past 3.20 software. Dendrogram was created using neighbor joining clustering and Euclidean similarity index (Figure 3). Two main groups were identified on the dendrogram. Similarity index was created by using Euclidean similarity index.

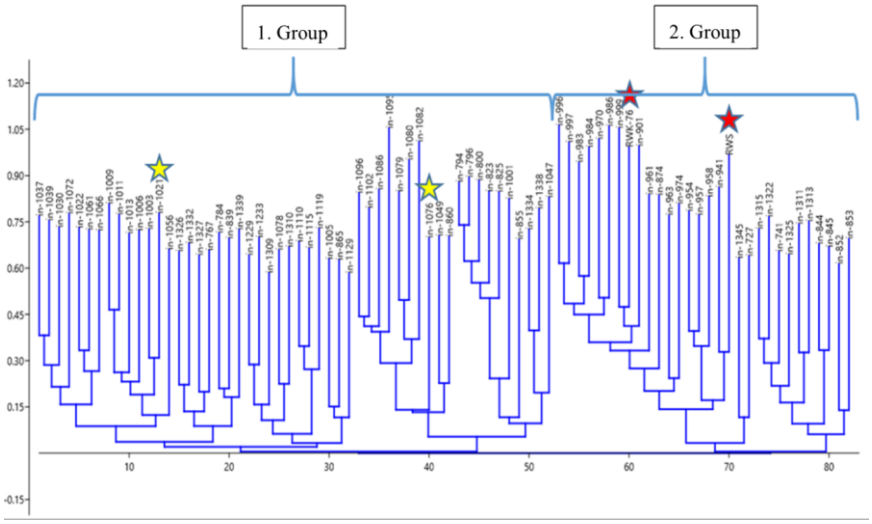

Figure 3. Dendrogram of 80 candidate inducers and donor lines generated by SSR analysis

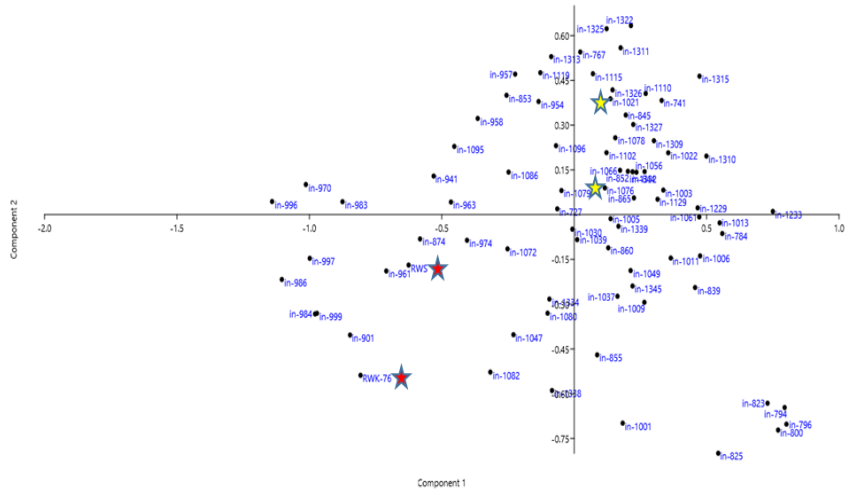

Figure 4. Scatter plot graph of SSR analysis using principal component analysis

The similarity between candidate inducer line in-1021 and RWK-76 and RWS donors was 23 and 38%, and the similarity index for in-1076 was 27 and 15%, respectively. The in-1021 and in-1076 candidate inducer lines were located in separate clusters from the RWS and RWK-76 donor inducer lines (Figure 3). The similarity indexes recorded for the candidate haploid inducer lines were significantly lower than the similarity index (87.5%) stated in the license agreement. The scatter plot graph was generated by using principal component analysis (PCA). The PCA also indicated that candidate haploid inducer lines (in-1021 and in-1076) were located in different regions from RWS and RWK-76 donor haploid inducers (Figure 4).

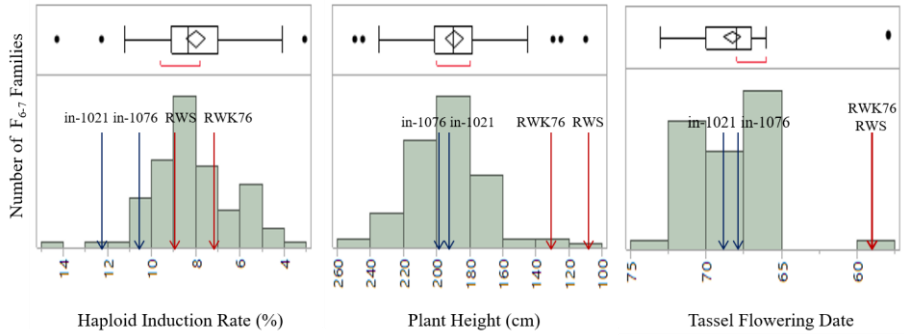

Figure 5. Comparison of candidate lines with donors in terms of haploid induction rate, plant height and tassel flowering date traits.

DISCUSSION

HIR distribution of 47_1F_2 plants obtained from the hybrid DH99 (non-inducing parent) x PK6 (male parent with 6% induction rate) were evaluated. Genotypes that showed a higher HIR than the haploid inducer line (PK6) emerged in the population (BARRET *et al*., 2008). The HIR assessment study performed using the liguleless tester in BC_1 generations indicated the existence of included families with lower and higher HIR than donor haploid inducer lines. These results are similar to those reported by BARRET *et al*. (2008). The DTF of $BC_1F_{6:7}$ families were later than the donor lines. The average PH of candidate lines was higher than the donor lines and HIR of the candidate lines ranged from 3.1 to 14.3% (Table 3). In addition, the number of seeds obtained from hybridization with liguleless also increased as the generation progressed (Table 2).

The in-1021 and in-1076 haploid inducer lines were selected considering all the traits of $BC_1F_{6:7}$ families included in the study (Photo 6). The HIRs of in-1021 and in-1076 late temperate haploid inducer lines were 10.5 and 12.3%, PH were 195 and 200 cm, and DTF were 69 and 68 days, respectively. The HIRs of RWS and RWK-76 donor haploid inducers were 9.1 and 7.3%, DTF of both donors was 58 days, and PH were 108 and 132 cm, respectively (Table 3 and Figure 5). Tropic and temperate haploid inducer lines were developed in different countries. The HIR values of haploid inducer lines were reported as 7.2-14.5% by Rotarenco et al. (2010), 1.3-10.2% by PRIGGE *et al*. (2012), 6.4-13.1% by CHAIKAM *et al*. (2018) and 8.2-15.54% by CHEN *et al*. (2020).

Genetic similarity between late temperate haploid inducer lines and donors were identified using SSRs markers. The similarity rates of the in-1021 and in-1076 inducer lines with the RWS donor were 38 and 15%, and with the RWK-76 donor were 23 and 27%, respectively. The similarity rate between the two candidate haploid inducer lines was 30%.

The in-1021 and in-1076 late temperate haploid inducer lines can be used individually for induction cross, in addition, in-1021 × in-1076 inducer hybrid can be also used for haploid induction. We have applied to register the in-1021 as ADAIL-1 and the in-1076 as ADAIL-2. Following the registration both will be integrated to public and private sector maize breeding programs. The haploid inducer lines developed will be used to increase the efficiency of *in vivo* maternal haploid technique applications in national maize breeding programs.

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RAZVOJ *IN VIVO* HAPLOID INDUKTORA ZA UMERENO PODRUČJE

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Izvod

In vivo tehnika duplih haploida se široko koristi u naprednim programima oplemenjivanja kukuruza brojnih prednosti (smanjenja troškova, radne snage i vremena i povećanja efikasnosti). Međutim, broj dostupnih linija induktora u svetu je dovoljan. Šest BC_1 oplemenjivačkih populacija, uključujući linije haploidnih induktora RWS i RWK-76 i kasnije linije umerenog područja ADK-451, ADK-737 i ADK-455, razvijene su od strane Instituta za istraživanje kukuruza Sakarja (MRI) u Turskoj. Kao donori korišćeni su linije haploidni induktori RWS i RWK-76. Za razvoj linija induktora korišćena je pedigre metoda. Utvrđeni su antocijaninska obojenost biljke, dužina metlice, broj grana metlice, visina biljke, dani do cvetanja, obojenost embriona-endosperma i stopa haploidne indukcije (HIR). Odabrani su genotipovi sa najboljim karakteristikama. Familije od BC1F3 do BC1F7 hibridizovane su u liniju bez ligula da bi se odredio HIR, a familije sa HIR preko 8% odabrane su iz populacije BC1. Vrednosti HIR, visine biljaka i dana do cvetanja metlica kod linija-1021 i 1076 kandidata za haploidne induktorske linije iznosile su 10,5 i 12,3%, 195 i 200 cm, odnosno 69 i 68 dana. Vrednost HIR za haploidni induktor donora RWS kretala se od 8,9 do 11,3%, a za RWK-76 od 7,3 do 9,8%. SSR markeri korišćeni su za utvrđivanje genetske sličnosti između kasnijih linija haploidnih induktora umerenih područja i donora. Stope sličnosti linija induktora in-1021 i in-1076 sa davaocem RWS bile su 38 i 15%, a sa davaocem RWK-76 23 i 27%. Stopa sličnosti između dve potencijalne induktorske linije iznosila je 30%. Rezultati su ukazali da će kasne linije haploidnih induktora umerenih područja povećati efikasnost oplemenjivanja kukuruza.

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