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Breeding Doubled Haploid Maize Inbred Lines for Methionine and Lysine Amino Acid Composition

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Abstract

Maize protein quality is deficient due to lower lysine and methionine essential amino acid content. Therefore, developing high methionine and lysine hybrids is of importance in maize. In this study, 64 maize source populations were crossed to RWS x RWK-76 haploid inducer genotype in Sakarya, Turkey to produce haploids in 2016 and 2017. Putative haploids were planted in a greenhouse in 2017-2018 winter season in Antalya, Turkey to produce D_0 lines. 115 D_1 lines were selected and planted in 2018 summer field season in Sakarya for further investigation. A total of 65 D_1 doubled haploid (DH) inbred lines were developed after final evaluations. While the majority of the developed lines gave higher values for methionine and lysine content, Ant-QPMDH-39 and Ant-QPMDH-42 lines were observed to have higher amino acid content when compared to parental lines. Statistics related to haploid induction rate, germination rate, misclassification rate and doubled haploid line rate revealed that effect of source populations cannot be ignored in DH line development. Our results showed that DH technology is efficient in line development in terms of special traits in maize in a short breeding time.

Introduction

Maize protein fractions are classified into five groups. The first group consists of albumins, globulins and soluble nitrogen, which constitutes 6.6% of the total protein. The second group is zein (alpha, beta, delta, and gamma) and contains 48.7% of the total protein. The third group is zein-like protein fraction, accounting for 14% of the total protein. The fourth group is classified as glutelin (17%) and the fifth group as glutelin-like (9.2%) (Bjarnason and Vasal, 1992).

Zein and zein-like protein fractions (62.7% in total) are insufficient for mammalian nutrition in terms of essential amino acids, especially lysine. Osborne and Mendel (1914) long years ago pointed out that the nutrient quality (protein quality) of maize could increase if the amount of zein decreased. Later the studies showed that high lysine and tryptophan maize germplasm had lower amount of zein content (Gibbon and Larkins, 2005).

High lysine maize, often referred to as Quality Protein Maize (QPM) carries a natural recessive *opaque2* gene which has been widely studied, both inbred lines and hybrids developed and released (Vasal et al., 1993; Vivek et al., 2008). QPM hybrids have high feeding quality for poultry and animals, as well as for humans, especially in Asian, African and Latin American countries. High methionine feed is also required by the poultry sector and is less known when compared with QPM. Breeding studies and mechanisms underlying methionine composition in maize have been studied (Olsen at al., 2003; Scott et al. 2008; Philips et al. 2008; Carena and Dong, 2017).

Although various breeding methods are used to develop parental inbred lines, the pedigree method is generally used requiring at least six to seven years to develop a line (Hallauer and Miranda 1981). An alternative approach is *in-vivo* maternal haploid or double haploid (DH) technique which can result in inbred lines in 1-2 years. In addition to obtaining inbred lines, there are quantitative genetic, operational, logistic and economic advantages of the doubled haploid technique (Nei, 1963; Melchinger et al. 2005; Rober et al. 2005; Seitz 2005; Smith et al. 2008; Geiger, 2009). In commercial maize breeding, doubled haploids are preferred to conventional breeding (Geiger and Gordillo, 2009; Prigge and Melchinger, 2012).

In the DH method, source populations are crossed with specific inducers that have an ability to produce small numbers of haploid embryos. There are several tropical and temperate adapted maize inducer lines. RWS, RWK-76 (Rober et al., 2005), MHI (Chalyk, 1999) and PHI inducer series (Rotarenco et al., 2010) are reported to produce more than 6% of haploid induction rate (HIR).

An effective pleiotropic haploid morphological marker is the 'red crown' or 'navajo' grain characteristic of the R1-nj dominant mutant allele of the R1 gene. Purple colored endosperm (no color in embryo) kernels which carries R1-nj allel are selected from crosses among source population x inducer lines and then subjected to artificial chromosome doubling for generating diploid embryo. Colchicine, a dangerous chemical is widely used in chromosome doubling stage, other substances can be used that are less hazardous (Geiger and Gordillo, 2009). After chromosome doubling, the plants are transferred either to greenhouse or field to produce DH lines.

The *in-vivo* maternal haploid technique has been used in many studies. Seitz (2005) reported that the performance of lines developed by the DH method and the performance of the lines developed by conventional method were similar. Beyene et al. (2011), showed that double haploid lines have good agronomic and agricultural properties as much as classical pedigree breeding and therefore they recommended the *in-vivo* DH technique due to time saving. In a study of quality protein in maize, Dang (2010) used DH technique to increase the content of lysine in waxy maize. In the study of Dang (2010), RWS, RWK 76 and RWS x RWK 76 inducer genotypes and waxy x QPM (lysine-rich) F1 hybrids were crossed and as a result of studies, high lysine waxy maize DH lines were developed. Although this study showed that QPM lines can be developed by DH technology, a limited number of studies have been conducted to improve grain protein quality by *in-vivo* doubled haploid technique. We could not find any published DH studies focusing on both methionine and lysine.

The objectives of this study were (i) to generate knowledge from DH techology regarding to methionine and lysine and (ii) to develop high methionine and lysine DH maize lines to be used for improved amino acid composition.

Materials and Methods

Production of haploids

The *invivo* maternal haploid induction technique was used for production of haploids in maize. Two breeding populations, HQPSCB (Pool 33 QPM (Early) / 2 * BSCB1 (R) C11) and HQPSSS (Pool 33 QPM (Early) / 2 * BSSS (R) C11), were used as donor sources in 2016. These high lysine populations were developed from crosses among CIMMYT 33 QPM and synthetic temperate populations (Zehr and Hamaker 1995). Two high lysine, three high methionine and three high yielding normal endosperm lines were crossed in a 8 x 8 full diallel mating design in 2017 to produce 56 F1 source populations to be used for DH line development. Besides, six F1s which were produced from crosses between high methionine and normal endosperm lines also used as donors. Totally, 64 source populations were used in the study. The RWS x RWK-76 inducer genotype was used as the male parent during hybridiziations.

Haploid seed identification

The method of selection of haploid seeds with the help of color marker described by Rober et al. (2005) and CIMMYT (2010) was used for haploid seed identification. Seeds from crosses among source population x inducer lines were subjected to haploid seed identification. Haploid seed selection was done visually by selecting of purple colored endosperm kernel marker that carried the R1-nj allele. Total number of seed of crosses (TNS), number of haploid seed (NHS) and haploid induction rate (HIR), for each cross were determined. Below equation was used to calculate HIR

HIR (%)= NHS/TNSx100.

Artificial Chromosome doubling

Haploid plants contain a single set of chromosomes (n) and thus are non-fertile. Genome doubling with a doubling agent, generally colchicine, is required to obtain a homozygous, fertile, diploid (2n) plant. Chromosome doubling of the haploids was done according to modified Deimling et al. (1997). Accordingly, putative haploid seeds were kept in an atmosphere controlled germination room at 26 ^{0}C for 3 days with 70% moisture. The ideal coleoptile length of seedlings for colchicine application is 1-2 cm and therefore longer coleoptiles were shortened to 2 cm before colchicine application. Haploid seedlings were then exposed to a 0.04% colchicine and 0.5% DSMO (dimethylsulfoxid) solution at 18 °C for 12 hours. After colchicine treatment, the seedlings were washed with tap water for 20 minutes and then kept in the climate chamber at a humidity of 80% until seedlings reached the 2-3 leaves stage. After this stage plants were transferred to a greenhouse for producing first generation doubled haploid (D_0) lines. Germination rate (GR) was determined by dividing the number of germinated seeds to the number total of seeds sown. Since some seedlings had died during growing period in climate room, the rate of seedlings transferred to greenhouse (RSTGH) from each genotype was calculated by dividing number of healthy seedlings to the total number of seedlings.

Table 1 - Data of source populations obtained from doubled haploid technique applications

Source population	TNS (No)	NHS (No)	HIR (%)	GR (%)	RSTGH (%)	MCR (%)	NSP (No)	NE (No)	NDHE (No)	DHLR (%)
L1 x L2	3089	121	3.92	96	88.4	14.3	89	86	0	0.0
L1 x M1	1693	130	7.68	96	90.8	17.0	95	93	1	1.1
L1 x M2	1177	206	17.50	99	86.4	17.3	137	134	0	0.0
L1 x M3	1997	26	1.30	88	57.7	37.5	7	7	0	0.0
L1 x A1	1082	80	7.39	100	81.3	25.5	45	44	0	0.0
L1 x S1	2961	57	1.93	84	61.4	21.7	20	19	0	0.0
L1 x S2	5283	164	3.10	99	86.0	17.0	105	105	1	1.0
L2 x L1	5118	125	2.44	98	85.6	23.3	79	80	1	1.3
L2 x M1	2618	83	3.17	100	95.2	14.1	59	57	0	0.0
L2 x M2	1844	171	9.27	97	72.5	27.6	100	97	1	1.0
L2 x M3	4147	157	3.79	90	80.9	23.7	80	80	3	3.8
L2 x A1	3225	34	1.05	97	61.8	27.3	10	10	0	0.0
L2 x S1	3856	108	2.80	94	82.4	28.2	60	58	0	0.0
L1 x S2	4963	320	6.45	97	72.2	20.1	180	176	4	2.3
M1 x L1	2829	268	9.47	98	60.4	35.0	144	126	2	1.6
M1 x L2	3265	117	3.58	92	73.5	34.8	61	60	0	0.0
M1 x M2	1864	75	4.02	95	73.3	44.8	26	25	0	0.0
M1 x M3	1708	33	1.93	85	60.6	30.8	9	8	1	12.5
M1 x A1	3304	90	2.72	91	63.3	41.4	24	23	2	8.7
M1 x S1	942	27	2.87	85	81.5	31.6	17	15	0	0.0
M1 x S2	2852	71	2.49	92	74.6	14.3	32	28	3	10.7
M2 x L1	2482	144	5.80	97	89.6	6.7	95	94	2	2.1
M2 x L2	1907	46	2.41	85	50.0	30.8	11	10	1	10.0
M2 x M1	982	39	3.97	79	61.5	25.0	12	13	1	7.7
M2 x M3	1966	28	1.42	89	85.7	0.0	7	7	1	14.3
M2 x A1	1066	41	3.85	80	65.9	35.7	11	10	0	0.0
M2 x S1	1730	44	2.54	75	70.5	23.8	14	14	0	0.0
M2 x S2	3173	137	4.32	93	82.5	42.7	66	64	3	4.7
M3 x L1	1866	21	1.13	95	81.0	40.0	7	7	0	0.0
M3 x L2	3502	65	1.86	92	86.2	39.5	31	30	0	0.0
M3 x M1	1382	35	2.53	89	65.7	26.7	13	13	1	7.7
M3 x M2	1297	29	2.24	86	69.0	27.3	11	11	1	9.1
M3 x A1	2280	63	2.76	97	74.6	42.9	12	12	2	16.7
M3 x S1	3748	59	1.57	98	79.7	21.9	22	21	3	14.3
M3 x S2	222	38	17.12	89	86.8	23.1	10	10	1	10.0
A1 x L1	1470	33	2.24	91	75.8	26.3	13	13	0	0.0
A1 x L2	1583	43	2.72	88	76.7	9.1	12	12	2	16.7
A1 x M1	1795	41	2.28	88	68.3	16.7	9	8	2	25.0
A1 x M2	3362	84	2.50	89	82.1	35.6	30	30	0	0.0
A1 x M3	1732	11	0.64	91	72.7	0.0	1	1	0	0.0
A1 x S1	6405	60	0.94	90	73.3	13.0	20	20	6	30.0
A1 x S2	4548	29	0.64	83	82.8	15.0	15	15	3	20.0
S1 x L1	4012	60	1.50	98	90.0	12.5	31	33	4	12.1
S1 x L2	5550	54	0.97	100	90.7	10.0	22	19	3	15.8
S1 x M1	8258	212	2.57	96	77.8	11.6	114	113	12	10.6
S1 x M2	5072	61	1.20	92	75.4	22.2	28	27	3	11.1
S1 x M3	5017	57	1.14	88	75.4	5.1	32	30	5	16.7
S1 x A1	6518	55	0.84	96	81.8	12.2	30	30	6	20.0
S1 x S2	4475	90	2.01	94	95.6	9.6	71	71	3	4.2
S2 x L1	3604	54	1.50	83	75.9	17.5	35	35	2	5.7
S2 x L2	5041	176	3.49	94	85.2	24.1	98	94	5	5.3
S2 x M1	5714	261	4.57	97	73.9	19.2	143	140	1	0.7
S2 x M2	3581	85	2.37	91	71.8	18.2	33	33	2	6.1
S2 x M3	5178	37	0.71	92	78.4	33.3	10	10	1	10.0
S2 x A1	4603	45	0.98	82	55.6	4.8	18	18	4	22.2
S2 x S1	6129	82	1.34	95	82.9	7.1	48	48	11	22.9
Ant- QPM-17 x ANT-QPM-8	3509	174	4.96	89	83.3	38.2	96	46	1	2.2
Ant- QPM-10 x ANT-QPM-11	3871	28	0.72	86	92.9	38.5	10	10	0	0.0
Ant- QPM-11 x ANT-QPM-16	5510	60	1.09	90	76.7	20.0	26	25	2	8.0
Ant- QPM-8 x ANT-QPM-11	4630	96	2.07	89	76.0	31.0	36	39	1	2.6
Ant- QPM-8 x ANT-QPM-10	4437	89	2.01	92	78.7	34.0	43	41	1	2.4
Ant- QPM-17 x ANT-QPM-16	5234	301	5.75	95	83.7	44.8	165	159	0	0.0
Sum	208.258	5.630	-	-	-	-	2890	2767	115	-
Mean	-	-	3.33	92	77	23.6	-	-	-	6.6

TNS: Total number of seed obtained from crosses, NHS: number of haploid seed, HIR: haploid induction rate, GR: germination rate, RSTGH: the rate of seedlings transferred to greenhouse, MCR: misclassificaton rate (MCR), NSP: number of selfed plants,NDHE: number of doubled haploid ears, DHLR: doubled haploid line rate

Investigation of D_0 lines

Seedlings were grown in a greenhouse during 2017 (2 populations) and 2018 (62 populations) winter season (January to June) to produce D0 lines. Before flowering, the number of purple colored plants, or false positives (misclassified) which are not doubled haploids, were counted. Misclassification rate (MCR, %) for each entry was calculated according to the below formula described by Kebede et al. (2011).

MCR (%)= (Number of purple colored plants / Number of putative haploid plants)x100

Selfing was carried out using the technique applied by Russel and Eberhart (1975). In the process of selfing, the ears of the selected lines were closed with paper bags and thus pollination was prevented. The tassels of the same plants were isolated by paper bags when pollen started to dehisce. When the ear silks were out and ready to receive pollen, collected pollen in the paper bags of the same plants were poured carefully on ear and the selfed ears were kept with pollen bags until harvest.

Developing of D_1 lines and kernel quality analysis

D_0 plants were harvested in June in 2018. Immediately after harvests the selected D_1 lines were sown in Sakarya, Turkey due to its better ecological conditions to Antalya where drought and heat stress often occur. During D_1 line production season, traits such as flowering time (days), plant height (cm), the first ear height (cm), ear length (cm), ear diameter (mm), the number of kernel per ear (number) and thousand kernel weight were observed. D_1 line ears were harvested in October 2018 and seed were subjected for kernel protein (%), starch (%), oil (%), lysine (g/100g) and methionine (g/100g) content. Protein content was determined by the Dumas classical method (AOAC International, 2002). Starch content was determined according to ICC (1994). The amount of kernel oil content was determined by Soxhlet Method (AOAC, 2000). Liquid Chromatography Triple Quadrupole Mass Spectrometry (LC-MS/MS) was used for quantifying the methionine and lysine amino acid analysis. Before analysis maize samples were hydrolised based on the method of Chan and Matanjun (2017) which was modified according to Faountoukakis and Lahm (1998). 0.2 g of the sample homogenized according to this method was weighed into a solution of 10 mL of 6 N HCl (containing 0.02% of phenol). The mixture was mixed by vortexing in a tightly sealed test tube for 5 minutes and then stored in an oven at 110° C for 24 hours to complete the hydrolysis. The cooled mixture at room temperature was filtered through a 0.45 µm PTFE membrane and then injected into the LC-MS / MS device. Total lysine and methionine values were calculated as content (g / 100 g, dry matter). Protein quality indexes (lysine/protein and methionine/protein) were calculated for both lysine and methionine.

Results and discussion

Source populations from DH technique, D_0 characterization

According to the data, both F_1 seed (population x inducer) and haploid seed were taken in all combinations used in the study (Table 1). A total of 208258 hybrid seeds were taken, of which 5630 seed were selected as haploid. The haploid induction rates (HIR) ranged from 0.64% (A1 x S2) to 17.50% (L1 x M2) and mean HIR value was 3.33% (Table 1). Generally, HIR was reported to be in between 6-14% (Coe, 1959; Geiger and Schönleben, 2011). Rober et al. (2005) found 8% HIR value in RWK-76 inducer line, while, Geiger and Gordillo (2009) reported approximately 9-10% HIR for RWS X RWK-76 hybrid inducer. Rober et al. (2005) stated that environmental factors, the haploid production methods and hybridization time may have potential to affect the amount of haploid seed production. Our data of HIR values obtained from different source populations (donors) suggest that effect of donor genotype may not be ignored in HIR value. Kebede et al. (2011) crossed 10 source populations with KWS x UH400 haploid inducer and they reported that HIR ranged from 2.90% to 6.74% in their study. Our findings related to HIR is consistent with this literature and it is thought that source populations affect the HIR as much as inducer genotype.

The selected haploid seeds were germinated at 26 °C and 70% humidity for 3 days in a dark room and a very successful germination rate (92%) was achieved in this stage. After applying the colchicine solution for chromosome doubling, the seedlings were sown in the seedling starter trays. Approximately after 2 weeks, healthy and good looking 4404 seedlings (D_0 plants) transferred to the greenhouse. Rate of seedlings transferred to the greenhouse (RSTGH) was calculated as 77% (Table 1).

D_0 plants in greenhouse were selfed in April and plants were harvested in mid-June 2018. Misclassification rate (MCR, %) values for each source population is given in Table 1. According to the results, MRC values were changed between 0.0 % (M2 x M3 and A1 x M3) and 44.8 (M1 x M2 and Ant-QPM-17 x ANT-QPM-16). The mean MRC value was 23.6%. MCR value is an indication of non-haploid seed rate in a population. Figure 1 shows the images of misclassified maize germplasm and true doubled haploid yellow and white seeded (D_0)

Table 2 - Traits observed in D_1 lines

No	Adı	AD (day)	SD (day)	PH (cm)	EL (cm)	ED (cm)	KPE (number)	TKW (g)
1	Ant-QPMDH-2	63	65	250	14.5	4.6	324	336.5
2	Ant-QPMDH-3	62	64	235	20.0	4.3	182	427.5
3	Ant-QPMDH-4	65	67	230	15.0	3.2	377	228.5
4	Ant-QPMDH-5	65	67	230	13.0	3.2	392	255.5
5	Ant-QPMDH-6	64	66	260	12.1	3.9	320	270.0
6	Ant-QPMDH-7	66	68	210	13.5	3.5	341	271.0
7	Ant-QPMDH-8	64	66	150	10.5	2.3	155	250.0
8	Ant-QPMDH-9	63	65	220	13.5	3.6	230	290.5
9	Ant-QPMDH-10	65	67	275	16.5	3.9	425	278.5
10	Ant-QPMDH-11	65	67	205	19.5	3.6	130	295.0
11	Ant-QPMDH-12	68	70	240	14.0	3.5	280	214.0
12	Ant-QPMDH-13	65	67	240	16.0	4.3	429	332.0
13	Ant-QPMDH-14	65	67	220	15.2	3.2	314	296.5
14	Ant-QPMDH-15	66	68	210	10.0	4.3	264	270.0
15	Ant-QPMDH-16	66	68	225	8.0	4.6	56	378.0
16	Ant-QPMDH-17	64	66	245	19.0	4.6	594	302.5
17	Ant-QPMDH-18	65	67	245	17.5	4	313	286.0
18	Ant-QPMDH-19	63	65	175	8.0	3.7	184	263.0
19	Ant-QPMDH-20	68	69	250	17.1	4.1	396	264.0
20	Ant-QPMDH-21	67	69	220	17.0	3.6	462	341.0
21	Ant-QPMDH-22	67	69	195	14.0	3.5	240	285.0
22	Ant-QPMDH-23	67	69	220	12.0	4	264	375.0
23	Ant-QPMDH-24	65	67	210	16.0	3	121	297.5
24	Ant-QPMDH-25	68	70	220	18.0	3.5	373	181.0
25	Ant-QPMDH-26	65	67	205	7.5.0	3.2	95	215.0
26	Ant-QPMDH-27	68	70	250	18.6	4.1	370	315.0
27	Ant-QPMDH-28	69	71	220	16.2	4.2	498	312.5
28	Ant-QPMDH-29	68	69	215	15.0	4.4	464	367.0
29	Ant-QPMDH-30	58	60	260	15.1	4.2	361	306.0
30	Ant-QPMDH-31	68	70	270	19.0	4	430	334.5
31	Ant-QPMDH-32	65	67	230	13.0	4.4	144	350.0
32	Ant-QPMDH-33	64	66	260	17.0	4	305	296.5
33	Ant-QPMDH-34	66	68	220	16.5	4.3	351	310.0
34	Ant-QPMDH-35	70	72	255	20.0	4.5	282	396.5
35	Ant-QPMDH-36	65	67	240	11.0	4.5	343	219.5
36	Ant-QPMDH-37	66	68	250	18.2	4.7	492	314.5
37	Ant-QPMDH-38	66	68	235	19.0	4.2	474	297.0
38	Ant-QPMDH-39	67	69	235	16.0	4.2	426	290.5
39	Ant-QPMDH-40	69	71	245	16.3	4.2	410	290.5
40	Ant-QPMDH-41	65	67	230	19.4	4.1	397	357.5
41	Ant-QPMDH-42	64	66	245	14.0	4.6	472	286.0
42	Ant-QPMDH-43	69	71	280	13.5	3.8	306	284.0
43	Ant-QPMDH-44	65	67	195	10.0	3.3	252	177.0
44	Ant-QPMDH-45	67	69	240	15.8	3.9	470	319.0
45	Ant-QPMDH-46	70	72	245	13.0	4.1	150	384.0
46	Ant-QPMDH-47	68	70	270	16.0	3.2	198	290.0
47	Ant-QPMDH-48	69	71	265	17.5	3.7	286	287.0
48	Ant-QPMDH-49	67	69	250	12.1	3.3	250	269.0
49	Ant-QPMDH-50	65	67	265	13.0	4.4	278	377.0
50	Ant-QPMDH-51	65	67	230	13.5	4.2	432	184.0
51	Ant-QPMDH-52	69	71	225	15.0	3.5	292	178.5
52	Ant-QPMDH-53	69	71	235	13.5	3.8	308	278.0
53	Ant-QPMDH-54	67	69	230	16.5	4.2	382	301.5
54	Ant-QPMDH-55	65	67	250	16.5	4.1	294	279.0
55	Ant-QPMDH-56	67	69	260	19.0	4.7	467	343.0
56	Ant-QPMDH-57	65	67	260	18.0	4.4	270	314.0
57	Ant-QPMDH-58	68	70	280	18.0	4.5	371	312.5
58	Ant-QPMDH-59	71	73	180	16.0	3.9	440	257.0
59	Ant-QPMDH-60	67	69	240	13.5	4.5	292	299.5
60	Ant-QPMDH-61	66	68	260	16.5	4.5	250	372.0
61	Ant-QPMDH-62	65	67	260	16.0	4.1	420	336.0
62	Ant-QPMDH-63	64	66	190	14.0	3.6	460	244.0
63	Ant-QPMDH-64	65	67	255	20.0	4.6	517	304.0
64	Ant-QPMDH-65	64	66	195	13.0	4.1	278	263.0
	Mean	66	68	234	15.2	3.9	330	295.3

AD: anthesis day, SD: silking day, PH: plant height, EL: ear length, ED: ear diameter, KPE: kernel per ear TKW :thousand kernel weight

lines. As it can be seen from the figure, misclassified seed progeny (D_0) segregated in terms of endosperm color.

A reason for using in vivo maternal haploid technique in maize breeding is that this method is simple and fast for producing DHs. The seed morphological color pigment marker (R1-nj) facilitates rapid selection of haploid seeds after hybridization. However, as in our study, this morphological marker was ambiguous in some genotypes. Since our source material was composed of both yellow and white grained maize, the purple color marker was often less easily detected in the endosperm. However, in some cases, the purple color in embryo was not clear, but when the embryo was cut and examined the purple pigmentation could be observed. As a matter of fact, in some new releasing inducers, the color marker has been transferred into a root morphological marker, and after the germination process colored roots can be visualized more easily and therefore the MCR ratio was lower in these inducers (Chaikam et al., 2016). Therefore, we suggest to use of inducers that have both endosperm and root color markers.

Fig. 1 -Yellow grained (top), misclassified seed material (center) and white grained (bottom) D_0 lines

A total of 2890 D_0 plants (NSP) were selfed in the greenhouse during April 2018. As a result, 2767 selfed ears (NE) with adequate seed were obtained. Ears subjected to selections and 115 D_0 DH lines (NDHE) which had no color marker were selected. Doubled haploid line rate (DHLR) of the source populations was ranged from 0.0 % to 30 % with 6.6 % experiment average.

Agronomic and Agro-morphological traits, grain quality composition of D_1 lines

The 115 D_0 lines were grown and selfed to produce D_1 DH lines in Sakarya MAE during the 2018 summer maize growing season. Totally, 64 D_1 lines were selected based on field and ear observations. Some morphological and agronomic traits of these lines are given in Table 2. As the line (Ant-QPMDH-1) was developed in 2016 and 2017, no data was received for this line but included in the quality analysis. Accordingly, it can be said that the lines were developed from many different combinations. Furthermore, it can be seen that these lines have a variation according to the observations given in Table 2.

Kernel starch (%), protein (%), oil (%), lysine (g/100g) and methionine (g/100g) composition values are presented in Table 3. The difference between DH lines were found to be statistically significant ($p < 0.01$). Accordingly, starch values ranged from 60.9% (Ant-QPMDH-11) to 70.8% (Ant-QPMDH-37). The average of the trial was 67.4% and thus 38 lines were above the average. Ant-QPMDH-37, Ant-QPMDH-59, Ant-QPMDH-58, Ant-QPMDH-64, Ant-QPMDH-17 and Ant-QPMDH-62 lines gave starch values of 70% or more. Protein values ranged from 9.4% (Ant-QPMDH17 and Ant-QPMDH-22) to 14.7% (Ant-QPMDH-62). The average of the experiment was 11.4% and 32 lines gave this result and above. Ant-QPMDH-62, Ant-QPMDH-16, Ant-QPMDH-54, Ant-QPMDH-36 and Ant-QPMDH-63 lines were noted with a protein value of 13% or more. The oil values ranged from 2.8% (Ant-QPMDH-3) to 5.8% (Ant-QPMDH-3), while the trial average was 4.1%. 36 lines were equivalent to or higher than this value. Ant-QPMDH-54, Ant-QPMDH-25, AntQPMDH-56,

Table 3 - Starch (%), Protein (%), oil (%), lysine (g/100g) and methionine (g/100g) values of the D_1 DH lines and parental inbred lines

No	Inbred line	Starch (%)		Protein (%)		Oil (%)		lysine (g/100g)		methionine (g/100g)	
1	Ant-QPMDH-2	65.0	yB	10.6	ru	3.4	CD	0.297	an	0.205	ae
2	Ant-QPMDH-3	63.8	CD	12.0	dj	2.8	E	0.280	gr	0.187	al
3	Ant-QPMDH-4	67.0	rv	11.1	ks	2.9	E	0.306	af	0.193	ak
4	Ant-QPMDH-5	66.0	uy	11.0	ls	3.5	AC	0.287	cq	0.196	aj
5	Ant-QPMDH-6	67.1	qu	11.4	jp	2.9	E	0.301	ak	0.196	aj
6	Ant-QPMDH-7	64.5	AC	10.9	ls	2.9	E	0.303	aı	0.196	aj
7	Ant-QPMDH-8	69.9	ae	10.9	ms	4.3	kl	0.285	cq	0.202	ag
8	Ant-QPMDH-9	68.3	ıp	10.1	tv	4.5	fı	0.305	ah	0.210	ab
9	Ant-QPMDH-10	67.9	ms	11.3	jq	4.6	ef	0.284	dr	0.190	al
10	Ant-QPMDH-11	60.9	F	11.6	gm	4.0	nr	0.307	ae	0.199	aı
11	Ant-QPMDH-12	69.1	dl	11.2	ks	3.8	tv	0.290	bp	0.203	af
12	Ant-QPMDH-13	68.2	jr	12.3	ce	4.7	e	0.299	am	0.195	aj
13	Ant-QPMDH-14	66.1	tx	11.1	ks	4.5	fı	0.291	bp	0.188	al
14	Ant-QPMDH-15	64.2	AD	11.4	jo	4.4	ık	0.286	cq	0.193	ak
15	Ant-QPMDH-16	61.6	EF	13.8	b	4.1	mp	0.301	ak	0.208	ac
16	Ant-QPMDH-17	70.1	ad	9.4	w	4.3	jl	0.277	ır	0.185	bl
17	Ant-QPMDH-18	68.1	kr	10.9	ns	4.0	nq	0.301	al	0.202	ag
18	Ant-QPMDH-19	68.0	ls	10.0	uv	3.7	vx	0.276	jr	0.171	jl
19	Ant-QPMDH-20	62.2	E	11.9	dj	4.1	mn	0.287	cq	0.198	aı
20	Ant-QPMDH-21	65.2	xA	10.5	su	4.2	lm	0.270	or	0.181	el
21	Ant-QPMDH-22	68.5	go	9.4	w	4.5	fı	0.295	ao	0.199	ah
22	Ant-QPMDH-23	68.9	eo	10.9	ms	3.9	pu	0.287	cq	0.206	ae
23	Ant-QPMDH-24	68.1	jr	11.6	fl	5.0	cd	0.292	bp	0.195	aj
24	Ant-QPMDH-25	64.8	zC	11.4	jo	5.2	b	0.281	fr	0.194	aj
25	Ant-QPMDH-26	64.9	zC	12.8	c	3.4	BD	0.277	jr	0.195	aj
26	Ant-QPMDH-27	68.3	jq	11.1	ks	3.7	vy	0.290	bp	0.199	ah
27	Ant-QPMDH-28	68.4	ho	9.8	vw	4.7	ef	0.279	hr	0.186	bl
28	Ant-QPMDH-29	69.5	bh	10.2	tv	4.9	d	0.291	bp	0.201	ah
29	Ant-QPMDH-30	66.4	tw	11.4	jo	3.6	xA	0.273	mr	0.170	kl
30	Ant-QPMDH-31	67.8	os	11.0	ls	4.1	mo	0.282	er	0.181	dl
31	Ant-QPMDH-32	66.0	vy	11.5	hn	4.0	os	0.263	qr	0.179	fl
32	Ant-QPMDH-33	69.1	dl	11.4	jp	4.1	mp	0.268	pr	0.192	al
33	Ant-QPMDH-34	66.6	tw	10.6	pu	4.5	eh	0.278	ır	0.195	aj
34	Ant-QPMDH-35	66.9	sw	11.2	jr	4.3	kl	0.302	aj	0.208	ac
35	Ant-QPMDH-36	64.7	AC	13.5	b	3.5	AC	0.279	hr	0.173	ıl
36	Ant-QPMDH-37	70.8	a	11.1	ks	4.4	ık	0.293	ap	0.203	af
37	Ant-QPMDH-38	69.3	cj	11.4	jp	4.0	nq	0.283	dr	0.195	aj
38	Ant-QPMDH-39	69.2	ck	11.1	ks	4.1	mn	0.309	ad	0.213	a
39	Ant-QPMDH-40	69.5	bg	11.4	jp	4.1	mp	0.294	ap	0.207	ac
40	Ant-QPMDH-41	68.5	go	10.9	ns	4.5	fı	0.305	ah	0.211	a
41	Ant-QPMDH-42	65.9	wz	12.1	dı	3.5	zB	0.318	a	0.207	ad
42	Ant-QPMDH-43	68.1	kr	12.2	cg	4.7	e	0.290	bp	0.207	ac
43	Ant-QPMDH-44	69.0	dn	11.2	ks	3.5	AC	0.300	al	0.203	af
44	Ant-QPMDH-45	68.1	kr	10.8	ot	4.3	jk	0.308	ae	0.190	al
45	Ant-QPMDH-46	63.3	D	12.4	cd	4.4	hk	0.283	dr	0.198	aı
46	Ant-QPMDH-47	67.8	ns	11.3	jr	3.3	D	0.285	cq	0.199	ah
47	Ant-QPMDH-48	69.0	dn	11.7	ek	3.6	wz	0.297	an	0.191	al
48	Ant-QPMDH-49	67.2	pt	11.6	hn	4.3	kl	0.293	ap	0.183	cl
49	Ant-QPMDH-50	66.6	tw	9.9	vw	3.8	uv	0.288	cq	0.201	ah
50	Ant-QPMDH-51	67.1	rv	12.3	cf	4.7	e	0.311	ac	0.209	ab
51	Ant-QPMDH-52	64.4	AC	11.4	jo	4.6	ef	0.299	an	0.194	aj
52	Ant-QPMDH-53	68.9	fo	12.2	dh	3.9	ru	0.316	ab	0.201	ah
53	Ant-QPMDH-54	69.0	dm	13.7	b	5.8	a	0.306	ag	0.203	af
54	Ant-QPMDH-55	65.1	xA	11.1	ks	4.1	mp	0.300	al	0.212	a
55	Ant-QPMDH-56	69.4	bı	12.3	ce	5.0	c	0.274	lr	0.183	cl
56	Ant-QPMDH-57	69.2	dl	10.6	qu	5.0	cd	0.291	bp	0.192	al
57	Ant-QPMDH-58	70.3	ac	11.5	hn	3.5	yA	0.273	nr	0.192	ak
58	Ant-QPMDH-59	70.4	ab	11.1	ks	4.6	eg	0.284	dr	0.186	bl
59	Ant-QPMDH-60	68.8	go	11.5	hn	3.9	qu	0.298	an	0.188	al
60	Ant-QPMDH-61	69.7	af	11.2	ks	4.5	gj	0.280	gr	0.198	aı
61	Ant-QPMDH-62	70.0	ae	14.7	a	3.9	pt	0.292	bp	0.201	ag
62	Ant-QPMDH-63	64.0	BD	13.5	b	4.5	fh	0.276	kr	0.185	bl
63	Ant-QPMDH-64	70.1	ad	12.1	dı	3.8	su	0.286	cq	0.184	bl
64	Ant-QPMDH-65	69.7	bf	11.6	gm	3.7	vw	0.285	cq	0.178	gl
65	Ant-QPMDH-1¥	69.1	dm	11.2	ks	3.6	wA	0.302	aj	0.186	al
66	L1‡							0.289	cq	0.185	bl
67	L2‡							0.294	ao	0.178	fl
68	M1‡							0.294	ap	0.199	ah
69	M2‡							0.294	ap	0.196	aj
70	M3‡							0.277	jr	0.200	ah
71	A1‡							0.275	kr	0.176	hl
72	S1‡							0.258	r	0.168	l
73	S2‡							0.274	mr	0.179	fl
	Mean	67.4		11.4		4.1		0.289		0.194	
	CV (%)	0.71		2.50		1.54		3.63		5.16	
	Genotype	**		**		**		**		**	

¥: DH line which was developed from 2016-2017 years studies , ‡: parental inbred lines

** Significant at the 0.01 probability level

Means with the same letter in the same column are not statistically different.

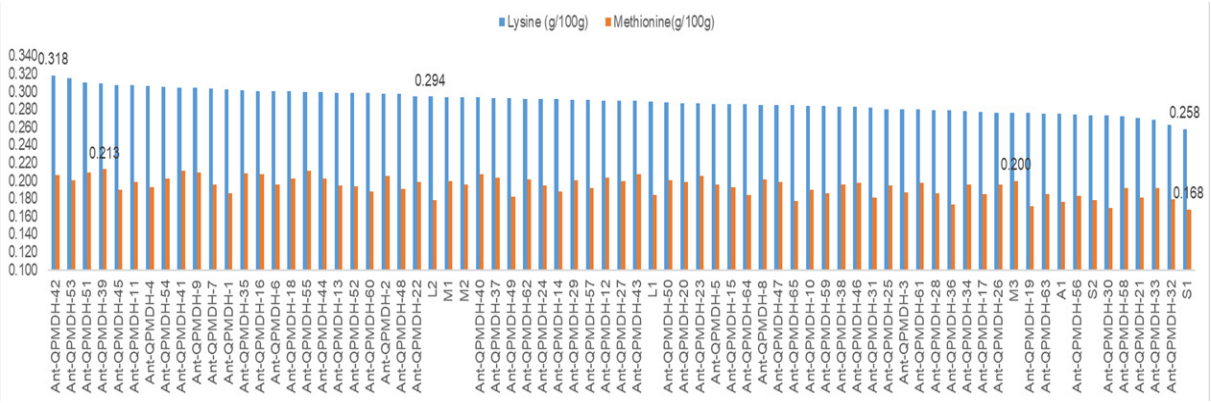

Fig. 2 -Lysine and methionine amino acid content of the developed D_1 lines and parental inbred lines
***: The labels on the columns are given to emphasize the lysine and methionine status of some specific lines**

Fig. 3 -Images of some developed D_1 lines with different types, colors and sizes

Ant-QPMDH-57, and Ant-QPMDH-24 lines gave the highest results with 5% or greater oil values.

Lysine and methionine grain composition

Developed 65 D_1 lines 8 parental lines total 73 lines were subjected to lysine and methionine composition analysis and the results are presented in Table 3. Also, these results are shown graphically in Figure 2. Accordingly, both lysine and methionine were found to be statistically significant ($p < 0.01$). Grain lysine values varied between 0.258 g / 100 g (S2) and 0.318 g / 100 g (Ant-QPMDH-42). The mean of the experiment was 0.289 g / 100 g and 39 lines were equivalent or higher than the experiment mean. Since L1 and L2 lines are high lysine parental lines used for the study, the mean of these two high lysine lines (0.292 g / 100 g) was used for evaluating developed lines in terms of lysine. 32 lines were found equivalent to or higher than this lysine mean. The first three lines of lysine were Ant-QPMDH-42, Ant-QPMDH-53 and AntQPMDH-51. The methionine values ranged from 0.168 g / 100 g (S1) to 0.213 g / 100 g (Ant-QPMDH-39), with a mean of 0.194 g / 100 g. In the study, 3 lines of methionine (M1, M2 and M3) were crossed into hybridization and the methionine average of these 3 lines was 0.198 g / 100 g. Therefore, the lines that give the same or higher values are considered as high methionine lines. AntQPMDH-39, Ant-QPMDH-55 and Ant-QPMDH-41 lines were the first three lines to give the highest values. As shown in Figure 2, lines with normal endosperm (high yielding) (A1, S1 and S2) yielded the lowest results in both amino acid and methionine levels. On the other hand, the AntQPMDH-39 line gave high results for both methionine and lysine. Similarly, the Ant-QPMDH-42 line is at the forefront of both lysine and methionine.

Figures 3 show the images of some D_1 lines obtained in different types and colors. The 65 D_1 lines developed within the scope of the study will be used in the future to develop high amino acid maize hybrids.

In conclusion, *in vivo* maternal haploid technique was used to obtain quality maize inbred lines in a short time. As a result of intensive studies, 65 D_1 inbred maize lines were developed. While the majority of the lines developed were high in methionine and lysine levels, especially the Ant-QPMDH-39 and Ant-QPMDH-42 lines gave high results for both methionine and lysine. These lines, which were developed in 2 years, will be evaluated in the breeding activities in the future periods to develop hybrids with high amino acid composition.

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References

AOAC International, 2002. Crude protein in cereal grains and oilseeds Official methods of analysis of AOAC International AOAC International, Washington, DC.

AOAC, 2000. Official Method 920.39 17th Ed., AOAC International Gaithersburg, MD, Official Method 920.39.

Beyene Y, Mugo S, Pillay K, Tefera T, Ajanga S, Njoka S, Karaya H, Gakunga J, 2011. Testcross performance of doubled haploid maize lines derived from tropical adapted backcross populations, Maydica 56 (4): 351-358.

Bjarnason M and Vasal SK, 1992. Breeding of quality protein maize (QPM), Theor Appl Genet 111: 888-897.

Carena MJ and Dong N, 2017. The ND EarlyQPM program: developing the next generation of healthier maize (Zea mays L.) products, Euphytica 213:150, DOI 10.1007/s10681-017-1937-x

Chaikam V, Martinez L, Melchinger AE, Schipprack W, Boddupalli PM, 2016. Development and validation of red root marker-based haploid inducers in maize, Crop Science 56: 1678–1688.

Chalyk ST, 1999. Creating new haploid inducing-lines of maize, Maize Genetic Cooperation newsletter. 73:53-54.

Chan PT and Matanjun P, 2017. Chemical composition and physicochemical properties of tropical red seaweed, Gracilaria changii, Food Chemistry, 221: 302-310.

CIMMYT, 2010. Doubled Haploids: A simple method to improve efficiency of maize breeding". https://www.youtube.com/watch?v=V2jOEuZjjrg, Accesseed in: May 23th, 2017.

Coe EH and Sarkar KR, 1964. The detection of haploids in maize J Hered, 55: 231–233.

Coe EH, 1959. A line of maize with high haploid frequency Am Nat, 93: 381–382.

Dang NC, 2010. Improvement of protein quality in waxy maize (Zea mays L.) by doubled haploid and marker assisted selection techniques, Agronomy and Plant Breeding, Institute of Plant, Animal and Agroecosystem, Agricultural and Food Science Dept, ETHZ, Switzerland.

Deimling S, Röber F, Geiger HH, 1997. Methodik und Genetik der in-vivo-Haploideninduktion bei Mais, Vortr Pflanzenzüchtg. 38: 203-224.

Faountoulakis M and Lahm HW, 1998. Hydrolysis and amino acid composition analysis of proteins, J chromatogr A, 826 (2), 109-134.

Geiger HH and Gordillo GA, 2009. Doubled haploids in hybrid maize breeding, Maydica, 54: 485-499.

Geiger HH and Schönleben M, 2011. Incidence of male fertility in haploid elite dent maize germplasm, Maize Genet. Coop. Newsletter, vol: 85.

Geiger HH, 2009. Doubled haploids. Maize Handbook - Volume II: Genetics and Genomics, Ed: Bennetzen JL., Hake, S., Springer Science and Business Media, New York.

Gibbon BC and Larkins BA, 2005. Molecular genetic approaches to developing quality protein maize, Trends in Genetics, 21:227-233.

Hallauer A.R and J.B. Miranda, 1981. Quantitive genetics in maize breeding, Iowa State University Press/ Ames. USA

ICC, 1994. Determination of starch content by hydrochloric acid dissolution. International Association for Cereal Science and Technology. Method No: 123/1

Kebede AZ, Dhillon BS, Schipprack W, Araus JL, Banziger M, Semagn K, Alvarado G, Melchinger AE, 2011. Effect of source germplasm and season on the in vivo haploid induction rate in tropical maize Euphytica, 180:219–226

Melchinger AE, Longin CFH, Utz HF, Reif JC, 2005. Hybrid maize breeding with doubled haploid lines: Quantitative genetic and selection theory for optimum allocation of resources Proc. of the 41st Annual Illinois Corn Breeders School 2005, Urbana-Champaign, IL. 7–8 Mar. 2005. Univ. of Illinois at Urbana-Champaign.

Nei M, 1963. The efficiency of haploid methods of

plant breeding. Heredity 18:95–100.
Olsen MS, Krone TL and Phillips RL, 2003. BSSS53 as a donor source for increased whole-kernel methionine in maize: Selection and evaluation of high-methionine inbreds and hybrids. Crop Sci 43:1634–1642.
Osborne TB and Mendel LB, 1914. Amino-acids in nutrition and growth. J Am Coll Nutr, 12 (5): 484-485.
Philips RL, Suresh J, Olsen M, Krone T, 2008. Registration of high-methionin versions of maize inbreds A 632, B73 and Mo17. J Plant Reg 2(3) : 243-245
Prigge V and Melchinger AE, 2012. Production of haploids and doubled haploids in maize. In: Loyola- Vargas VM, Ochoa-Alejo N (eds.) Plant Cell Culture Protocols Third edition. Humana Press - Springer Verlag, Totowa, New Jersey.
Rober FK, Gordillo GA, Geiger HH, 2005. In vivo haploid induction in maize – performance of new inducers and significance for doubled haploid lines in hybrid breeding Maydica 50:275–283.
Rotarenco V.A, Dicu G, State, Fuia S, 2010. New inducers of maternal haploids in maize. Maize Genet. Coop. News lett. 84 1521–22.
Russel W and Eberhart SA, 1975. Hybrid performance of selected maize lines from reciprocal recurrent and testcross selection programmes. Crop Sci, 15: 1-4.
Scott PM, Darrigues A, Timothy SS, Lamkey K, 2008. Recurrent selection to alter grain methionine concentration and improve nutritional value of maize. Crop Sci 48.1705–1713.
Seitz G, 2005. The use of doubled haploids in corn breeding. p. 1–7. In: Proc. Of the 41st Annual Illinois Corn Breeders' School 2005 Urbana-Champaign, Illinois, USA.
Smith JSC, Hussai, T, Jones ES, Graham G, Podlich D, Wal, S, Williams M, 2008. Use of doubled haploids in maize breeding: implications for intellectual property protection and genetic diversity in hybrid crops. Mol Breed 22:51–59.
Vasal SK, Srinivasan G, Pandy S, Gonzalez CF, Crossa J, Beck DL, 1993. Heterosis and combining ability of CIMMYT's quality protein maize germplasm I: Lowland tropical. Crop Sci 33:46–51
Vivek BS, Krivanek AF, Palacios N, Afriyie ST, Diallo AO, 2008. Breeding quality protein maize (QPM): protocols for developing QPM cultivars Mexico, D.F: CIMMYT.
Zehr BE and Hamaker BR, 1995. HQPSSS and HQPSCB maize germplasm. Crop Sci 35(6):1720