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Identification of SNP Markers Associated to Protein and Oil Content in Doubled Haploid Maize Lines Derived from Subtropical Breeding Populations

Juan P. Valenzuela-Apodaca[1] · Abraham Cruz-Mendívil[2] · Grethel P. Gaytán-Pinzón[1] · Hervey Rodríguez-González[1] · Luis A. Peinado-Fuentes[3] · Eduardo Sandoval-Castro[1] · Carlos L. Calderón-Vázquez[1]

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Abstract

Genes related to oil and protein accumulation have been identified in maize, however, many loci underlying the variation in these traits are still unidentified. In this study, 49 doubled haploids (DH) lines derived from subtropical maize recombinant populations were genotyped through DArTSeq technology, resulting in 29,398 single nucleotide polymorphisms (SNPs), of which 20,202 markers with minor allele frequency > 0.05 were kept. Oil content in DH lines ranged from 4 to 8.3%, whereas protein content ranged from 8.44 to 15.63%. A genome-wide association study (GWAS) was conducted to detect significant SNPs associated with oil and protein content. The physical position of SNPs was mapped and a 100-kb window surrounding the significant SNPs was scanned to identify genes associated with the phenotype. Three significant SNPs were close to genes related to oil accumulation, and one was close to a gene related to protein accumulation. The favorable alleles for the SNPs 5589069 (A/G), 100156460 (C/T), and 2412207 (G/T) were present in 70.9%, 35.4%, and 22.5% of the DH lines with high oil (> 6%) content (HOC), respectively. The favorable allele (A/G) for SNP 5586538 was present in 14.63% of the DH lines with high protein (> 10%) content (HPC). Once the favorable alleles were detected, a genotyping validation by High-Resolution Melting (HRM) technique was performed, showing similarity rates with DArTSeq between 56% and 78.9% for the assayed SNPs. These novel SNPs could be useful for further marker-assisted breeding of HOC or HPC in subtropical maize.

Keywords Breeding · Doubled haploids · Genotyping · Oil content · Protein content

Abbreviations

BWP	Bajio white population
BYP	Bajio yellow population
DH	Doubled haploids
EIF	Elongation Initiation Factor
GLM	General linear model
GWAS	Genome wide association study
HOC	High oil content
HPC	High protein content
HRM	High resolution melting
K	Kinship
MAF	Minor allele frequency
MAS	Marker assisted selection
MLM	Mixed linear model
NGS	Next generation sequencing
NWP	Northwest white population
NYP	Northwest yellow population
PCA	Principal component analysis
Q	Population structure
SNP	Single nucleotide polymorphisms

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Juan P. Valenzuela-Apodaca and Abraham Cruz-Mendívil contributed equally to this work.

✉ Carlos L. Calderón-Vázquez
ccalderon@ipn.mx

[1] Instituto Politécnico Nacional, Centro Interdisciplinario de Investigación para el Desarrollo Integral Regional (CIIDIR) Unidad Sinaloa, Blvd Juan de Dios Bátiz Paredes #250, CP 81101 Guasave, Sinaloa, México

[2] CONACYT-Instituto Politécnico Nacional, CIIDIR Unidad Sinaloa, Guasave, México

[3] Instituto Nacional de Investigaciones Forestales, Agrícolas y Pecuarias (INIFAP), Campo Experimental Valle Del Fuerte, Guasave, México

Introduction

Maize (*Zea mays* L.) is the most produced and consumed cereal in Mexico, with a production of 27.4 million tons per year (FAOSTAT 2022) and a per capita consumption of 79.5 and 56.7 kg per year in rural and urban areas, respectively (Vázquez-Carrillo et al. 2015). Maize oil is known for its high content of linoleic acid (> 50%), the only essential fatty acid for humans (Serna-Saldivar 2010). In general, maize kernel contains between 2 and 6% of oil and the fatty acids composition is also variable; as the oil content increases, the degree of unsaturation decreases (Olmos et al. 2018). Several studies have improved the oil content in maize by recurrent selection; a cyclical procedure designed to accumulate the frequency of favorable alleles for the desired traits (Hallauer 2007). The first breeding program for high oil content (HOC) maize was initiated in 1896 and was able to increase the oil content from 4.5% to 22% after 100 generations, with a simultaneous decrease in grain yield; therefore, those HOC lines were not commercial (Dudley and Lambert 2010).

A maize breeding program including white and yellow germplasm, adapted to subtropical environments in Mexico (Northwest and Bajio regions) was established in 2004 to obtain HOC maize with competitive grain yield (Preciado-Ortiz et al. 2013). The oil content in the subtropical maize populations increased from 4 to 8% after 8 cycles of recurrent selection, accompanied by increases in oleic acid (1 mg/g per cycle) and in grain yield (0.1 ton/ha per cycle) (Preciado-Ortiz et al. 2013; Ortega-Corona et al. 2015). To accelerate the maize breeding program, doubled haploid (DH) lines were obtained from selected HOC individuals, and these were genotyped by DArTSeq technology, producing a total of 35,770 single nucleotide polymorphism (SNP) markers that were used to analyze their genetic diversity and structure (Ríos-Sandoval 2018). The values of genetic diversity indexes suggested that the identified SNPs were reasonably polymorphic (PIC = 0.48 ± 0.08) and that the DH lines were nearly homozygotes (Ho = 0.05 ± 0.06); also, the population structure analysis revealed that DH lines were first separated by kernel color (white and yellow) and then by region of origin (Northwest and Bajio) (Ríos-Sandoval 2017).

Genome-Wide Association Study (GWAS) has become the leading method to identify genes underlying agriculturally important traits, including SNP loci associated with HOC. For instance, Li et al. (2013) examined the genetic architecture of oil biosynthesis in maize through a GWAS. They analyzed 368 maize inbred lines derived from tempered and tropical germplasm, including HOC and identified 26 SNP associated with kernel oil concentration and fatty acid composition, which allowed to find favorable alleles in five genes associated with fatty acids biosynthesis: *FAD2, ACP, LACS, WRI1a* and *COPII*.

Several association studies with small samples number and low genetic variability have been performed successfully when a high statistical power was used to avoid false positives (type I errors). For example, Xu et al. (2014) used a Mixed Linear Model (MLM) that avoids the probability of spurious association by using Kinship (K) analysis, Principal Component Analysis (PCA), and population structure (Q) in 20 rice accessions from China, detecting 23 QTLs for 10 traits. Huang et al. (2013) carried out association analyses for chilling tolerance in 125 tropical, subtropical, and temperate maize inbred lines at the germination and seedling stages, using the maize SNP50K BeadChip and six association models, including General Linear Model (GLM), GLM + Q, GLM + PCA, MLM (without complement), MLM Q + K, and MLM PCA + K, to assess their suitability for false-positive correction, demonstrating through quantile–quantile tests that the behavior of markers is influenced by the presence of PCA and Q, obtaining more accurate associations with an MLM. Previously mentioned studies have shown the relevance of using an MLM in association analysis due to the higher statistical power provided by including PCA, K, and Q analysis. For this reason, in the current study, a GWAS with an MLM (K + PCA) was carried out with 49 HOC maize lines derived from subtropical populations to identify SNPs associated with oil and protein content.

Genotyping protocols have shown great power in detecting new alleles for breeding programs; however, validation methods are needed to increase reliability. High-Resolution Melting (HRM) is a technique based on comparing DNA fusion curves (Martino et al. 2010). Naidoo et al. (2013) used HRM to detect a mutation in the *Ipa1-1* gene that reduces the phytic acid content in maize, showing that HRM allowed to successfully differentiate the homozygous dominant (wild type), homozygous recessive (mutant), and heterozygous genotypes.

Despite the importance of maize production in subtropical climates worldwide, almost all knowledge about oil accumulation in maize kernels through recurrent selection has been generated in lines adapted to temperate regions. Under these circumstances, the maize breeding program reported by Preciado-Ortiz et al. (2013) is one of the pioneers of using subtropical maize with HOC. The aims of this work were: 1) discover new SNPs associated with oil and protein content in subtropical maize populations through GWAS, 2) validate DArTSeq genotyping results through HRM to obtain accurate information about the presence of SNPs and their corresponding alleles, and 3) create subtropical maize crosses based on on genetic distance and favorable alleles for oil and protein content.

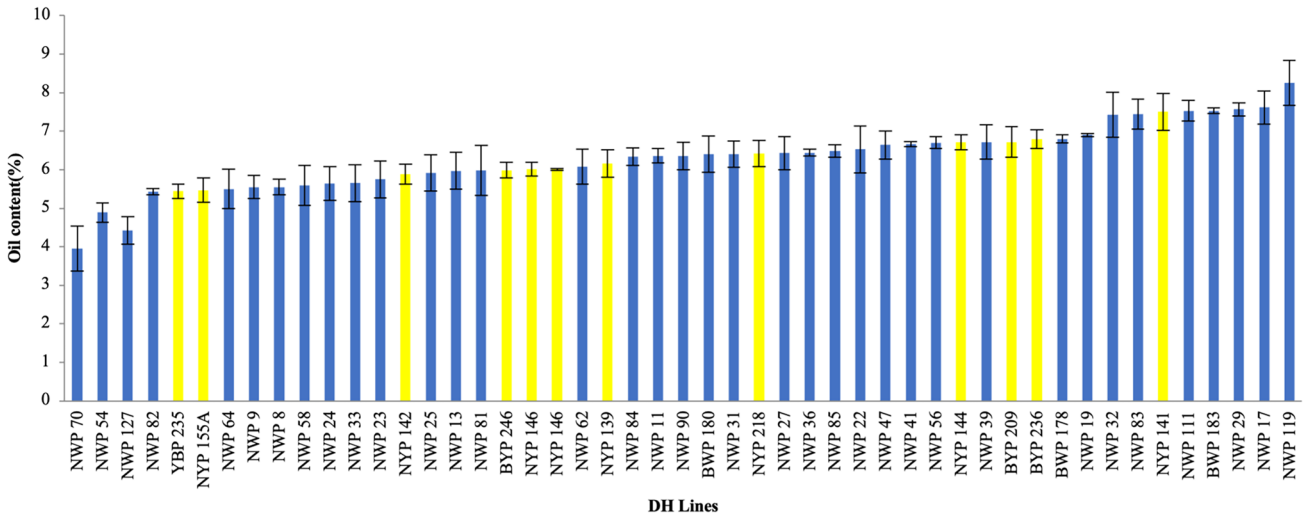

Fig. 1 Oil content in DH maize lines determined by Soxhlet method. Error bars indicate standard deviation. NWP: Northwest White Population, BWP: Bajio White Population, NYP: Northwest Yellow Population, BYP: Bajio Yellow Population. Yellow and blue bars represent yellow and white maize lines, respectively

Results

Oil and Protein Content

Oil content values oscillated between 3.96 and 8.26% for white lines and between 5.44 and 7.50% for yellow lines (Fig. 1). NWP-119, NWP-17, NWP-29, BWP-183, NWP-111, NWP-83, and NWP-32 are the white DH lines with higher oil content, with values ranging from 7.43 to 8.26%. BYP 209, BYP 236, and NYP 141 had the higher oil content for yellow lines, with 6.09, 6.79, and 7.5%, respectively.

The protein content values ranged from 8.44 to 15.63%. Remarkably, all yellow lines presented protein values above 10% and the highest values were observed in lines BYP-235 and NYP-144 (Fig. 2). Among the white lines, NWP-22 and NWP-111 presented the highest values for protein content (13.62 and 14.18%, respectively).

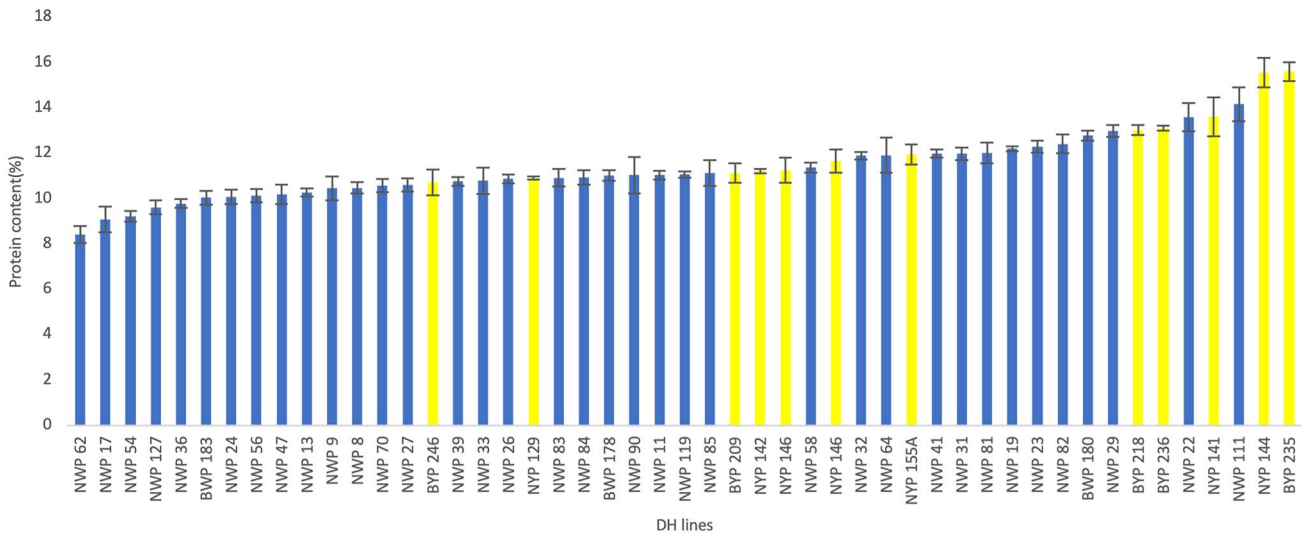

Fig. 2 Protein content in DH maize lines by Kjeldahl method. Error bars indicate the standard deviation. NWP: Northwest White Population, BWP: Bajio White Population, NYP: Northwest Yellow Population, BYP: Bajio Yellow Population. Yellow and blue bars represent yellow and white maize lines, respectively

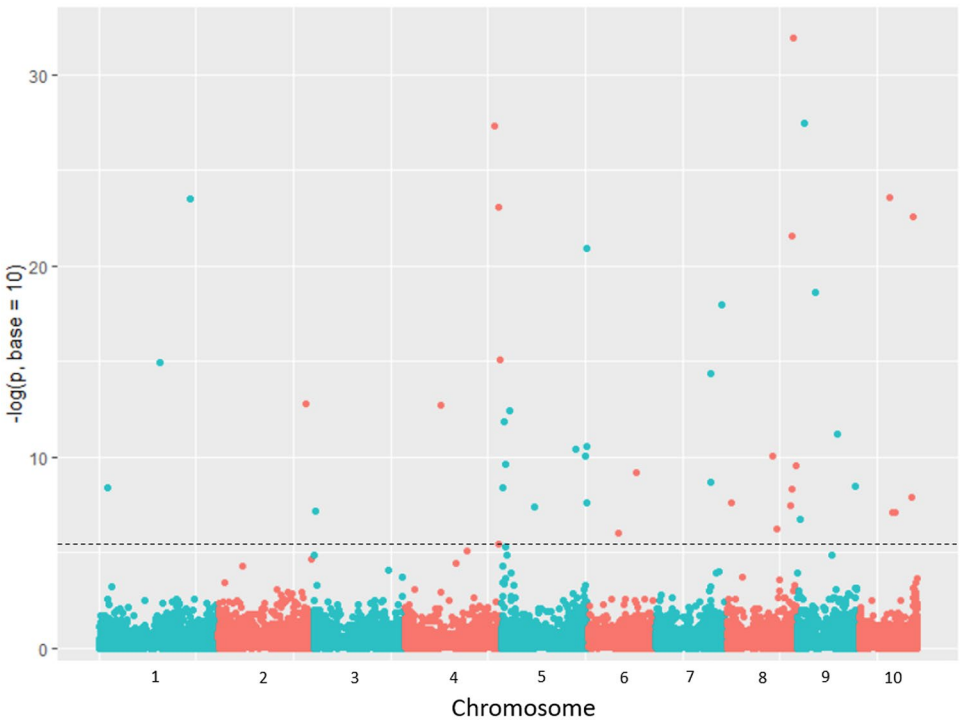

Fig. 3 Manhattan plot resulting from the GWAS for oil content. Analysis was performed with 20,202 SNPs in 10 chromosomes, and the p-value was adjusted at $1.7 \times 10^{-6.23}$ by Bonferroni method ($\alpha = 0.05$). The X-axis shows the maize chromosomes, and the Y-axis shows the –log10(p-values). The dots above the horizontal dashed line (threshold) are significant SNPs

GWAS

GWAS using MLM allowed us to identify 43 SNPs associated with oil content (Fig. 3) and two SNPs associated with protein content (Fig. 4). In order to identify genes associated with either oil or protein content, 69 bp reads from DArTSeq containing the significant SNP were blasted against the B73_v4 reference genome. All the SNPs that were close (100 kb window) or within a gene with any function related to protein or oil content were selected for further analysis (Table 1). The BLAST search detected three SNPs close to genes related to lipid metabolism, one in chromosome

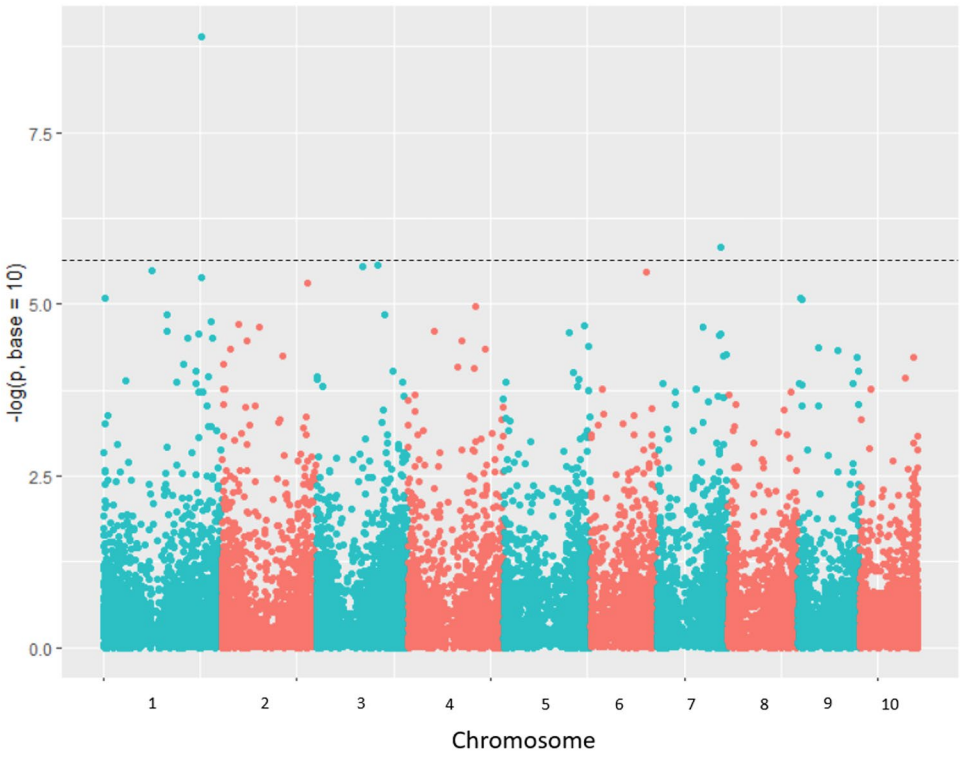

Fig. 4 Manhattan plot resulting from the GWAS for protein content. Analysis was performed with 20,202 SNPs in 10 chromosomes, and the p-value was adjusted at $1.7 \times 10^{-6.23}$ by Bonferroni method $\alpha = 0.05$. The X-axis shows the maize chromosomes, and the Y-axis shows the –log10(p-values). The dots above the horizontal dashed line (threshold) are significant SNPs

Table 1 Related genes associated with oil and protein content in maize DH lines

SNP ID	Chr	Chr. position	Gene ID	Gene description
Oil				
4775970	1	157404231	Zm00001d030725	Probable mediator of RNA polymerase II transcription subunit 37c
4777470	1	233340461	Zm00001d028827	Exonuclease DPD1 chloroplastic/mitochondrial
100145094	2	6822163	GRMZM2G113781	LysM domain containing protein
7060442	3	163187486	Zm00001d042357/LOC109945139/Zm00001d042359/LOC103652329	Protein XRI1/Uncharacterized/putative RING zinc finger domain superfamily protein/Uncharacterized
2472462	3	4165384	Zm00001d039435	Uncharacterized
4767429	4	240478472	Zm00001d053752	Uncharacterized
2616827	5	23460405	Zm00001d013869	SAUR56-auxin-responsive SAUR family member
9714119	5	190321766	Zm00001d017249	Ammonium transporter2
7061313	5	219137206	Zm00001d018359	Serinc-domain containing serine and sphingolipid biosynthesis protein
2562817	5	219715013	Zm00001d018384	DNA replication licensing factor MCM5
*5589069	7	141044791	Zm00001d021040	FATTY ACID EXPORT 1 chloroplastic
4768294	7	171486909	Zm00001d022164/GRMZM2G086856	Thioredoxin-like protein 4B/uncharacterized
4584964	8	166790610	Zm00001d012031	Hypothetical protein
*100156460	8	167539498	Zm00001d012081/Zm00001d012063	Protease inhibitor—seed storage—LTP family/cold and drought-regulated protein CORA
*2412207	9	18542642	Zm00001d045304	HXXXD-type acyl-transferase family protein
100127878	9	103214493	Zm00001d046717/Zm00001d046716	Brefeldin A-inhibited guanine nucleotide-exchange protein / RNA-binding protein 38
100099342	9	140912532	Zm00001d047753/Zm00001d047754/Zm00001d047755	Putative RNA binding protein ARP1/organic anion transporter/Arogenate dehydratase prephenate dehydratase 2 chloroplastic
100095877	9	47613869	Zm00001d0459631	Uncharacterized
100143021	9	102074832	Zm00001d046678	Uncharacterized
4593245	10	140866157	Zm00001d026203	MYB-related protein Zm1-like

Table 1 (continued)

SNP ID	Chr	Chr. position	Gene ID	Gene description
Protein				
*5586538	7	163521488	Zm00001d021815/Zm00001d021817/GRMZM2G434277	Elongation initiation factor/Putative protein phosphatase 2c 1/Protein STRUBBELIG-RECEPTOR FAMILY 1
2441790	1	255016427	Zm00001d033221/Zm00001d033222	Ubiquitin carboxyl-terminal hydrolase family protein/viviparous14

Gene IDs were collected at NCBI

*Function related to oil or protein content

7 close to a gene encoding a FATTY ACID EXPORT 1 (FAX1) protein (Maizegdb ID: Zm00001d021040), one in chromosome 9 in a gene encoding an HXXXD-type Acyl-transferase family protein (Maizegdb ID: 109942418, missense mutation from alanine to serine), and one in chromosome 8 close to a gene encoding a protease inhibitor/seed storage/lipid transfer protein (LTP) family protein (Maizegdb ID: Zm00001d012081). Finally, one SNP associated with protein content was in chromosome 7, close to a gene encoding an elongation initiation factor (EIF) protein (Maizegdb ID: 100217157).

The remaining significant SNPs were close to genes coding for transcription factors or uncharacterized, hypothetical or putative proteins. These genes might not be directly involved in oil and protein accumulation, but future studies could reveal new relationships between these and the accumulation of oil and protein in maize kernels.

SNP Confirmation

To confirm the presence of three SNPs associated with oil content and one SNP associated with protein content (Table 2), the 49 DH maize lines were genotyped through HRM, with the advantage of revealing the missing genotypes in DArTSeq data . Those alleles that were only present in lines with > 6% oil and > 10% protein content were defined as favorable alleles (Table 3). The favorable alleles of SNPs 5589069 (A/G), 100156460 (C/T), and 2412207 (G/T) were present in 70.9%, 35.4%, and 22.5% of the DH lines with high oil content, respectively; and were absent in the DH lines with regular oil content except for the SNP 2412207 in the NWP-127 line. On the other hand, the favorable allele (A/G) for the SNP 5586538 was present in 14.6% of the DH lines with high protein content and was absent in all the lines with normal protein content. A comparison between DArTSeq and HRM genotyping results was then performed, showing similarity rates between 56 and 78.9% (Table 4).

Discussion

HOC maize lines have been successfully developed through breeding and selection including Illinois High-oil, Alexho Synthetic, and Beijing High-oil populations, with 20%, 21%, and 15.5% of oil, respectively. However, the seed size and weight of those lines were significantly reduced (Larkins et al. 2017). The main goal of the maize breeding program described by Preciado-Ortiz et al. (2013) was to obtain HOC lines without grain yield losses. Seven breeding cycles of half-sib recurrent selection in white and yellow subtropical populations were performed, obtaining increases of 41–60% and 33–55% in oil content for yellow and white populations,

Table 2 Primers sequence for each associated SNP

SNP ID	Chr	Position	Forward	Reverse	Length (F/R)	Tm(F/R)	%GC(F/R)	Product length
5589069	7	141,044,791	gtctgcattcacgcacattg	cccttcttatgttcttccctttg	20/23	61.3/58.5	50/43.5	111 bp
100156460	8	167,539,498	actcaatgtcgaacgctacg	agcgctagatttggagtgagag	20/22	59/60.2	50/50	99 bp
2412207	9	18,542,642	agggttcaaggtgttcatcg	acagcagctcctgaatttcc	20/20	60/59.4	50/50	74 bp
5586538	7	163,521,488	catacgttcacgcatgtgac	cgacaagaagtcgattgagg	20/20	58.6/58.4	50/50	105 bp

SNP Single Nucleotide Polymorphism

Table 3 Favorable alleles for SNPs associated with oil and protein content

SNP ID	Trait	SNP	Favorable allele
5589069	Oil	G > A	G/A
100156460	Oil	C > T	C/T
2412207	Oil	G > T	G/T
5586538	Protein	A > G	A/G

respectively, along with a slight but significant increase in grain yield. In the present study, 49 DH lines derived from these improved populations were characterized. Oil content in seeds ranged from 3.9 to 8.3%, with an average of 6.3% (Fig. 1). According to Lambert (2000), maize kernels with oil above 6% can be considered as HOC maize.

Regular maize kernels contain between 8.5 to 10.5% of protein (Silva et al. 2005), whereas the DH lines analyzed in this study contain from 8.4 to 15.6% of protein, with an average of 11.4%. Remarkably, all yellow lines and 65% of the white lines presented protein contents above 10%. This suggests that although the breeding program reported by Preciado-Ortiz et al. (2013) was focused on oil content improvement, the protein accumulation in maize kernel was affected positively by the recurrent selection cycles. Okporie and Oselebe (2007) reported that a correlation exists between the increase in oil and protein content. After one cycle of recurrent selection in maize, Okporie et al. (2013) reported a decrease in endosperm weight and an increase in pericarp and germ weight, resulting in concurrent increases in oil (from 2.4 to 3.8%) and protein (from 8.7 to 10.8%) content. Based on this background, the DH lines of this study have the potential to create hybrids with improved protein content.

Large-scale GWAS using inbred lines derived from genetically unrelated individuals from different environments, locations, and progenitors, have provided new opportunities to understand the genetic architecture of complex quantitative traits such as oil and protein content in maize kernels. Using a GWAS, Li et al. (2013) identified 26 loci associated with oil concentration in maize kernel that could explain up to 83% of the phenotypic variation. In the present study, three genes related with lipid metabolism were located within the 100 kb flanking region on both sides of the SNPs associated with oil content. The first marker (SNP 5589069) was detected on chromosome 7, positioned close to a gene encoding a FAX1 protein (Maizegdb ID: Zm00001d021040). FAX1 protein transports fatty acids from plastids and participates in lipid accumulation in plants. Li et al. (2015) described *FAX1* mutants in *Arabidopsis thaliana* and clearly demonstrate that FAX1 mediates fatty acids export from plastids. Fatty acids are first synthesized from acetyl-CoA in the plastid through reactions catalyzed by acetyl-CoA carboxylase and then the fatty acid synthase complex is exported to cytosol. Li et al.

Table 4 Comparison between DArTSeq and HRM for SNP presence in DH lines

	5589069 (G/A)	DArTSeq	100156460 (C/T)	DArTSeq	2412207 (G/T)	DArTSeq	5586538 (A/G)	DArTSeq
DH lines								
NWP 81	AA	N	TT	TT	TT	TT	GG	GG
NWP 58	GA	N	TT	N	TT	N	GG	GG
BYP 236	GG	GG	CC	N	TT	N	GG	GG
NWP 24	GA	AA	TT	N	TT	N	GG	GG
NWP 36	GA	AA	TT	N	TT	N	GG	N
NWP 33	GA	AA	TT	TT	TT	TT	GG	GG
NWP 47	GA	GG	CC	TT	TT	TT	AA	N
NWP 31	GG	GG	TT	TT	TT	TT	GG	GG
NWP 70	AA	AA	TT	TT	TT	TT	AA	AA
NWP 64	AA	AA	TT	TT	TT	TT	GG	N
NWP 22	GA	AA	CC	TT	TT	TT	GG	AA
NWP 39	GG	GG	CC	N	GG	TT	AA	GG
NWP 209	GG	GG	TT	TT	TT	TT	GG	N
NWP 27	GA	N	CC	N	TT	N	GG	GG
NWP 62	GA	AA	TT	N	TT	N	GG	AA
NYP 146	AA	N	TT	TT	TT	TT	GG	N
BWP 180	GG	AA	TT	N	TT	GG	GG	GG
NWP 84	AA	N	TT	N	TT	N	GG	N
NWP 111	GA	GA	TT	N	TT	N	GG	N
NWP 9	AA	N	TT	TT	TT	TT	AA	GG
NYP 144	GG	N	CC	N	TT	N	GG	GG
NWP 13	AA	N	CT	TT	TT	TT	AA	GG
NWP 8	AA	GG	TT	N	TT	N	GG	GG
NYP 139	GA	N	TT	TT	TT	TT	GG	GG
NWP 90	GG	GG	TT	N	TT	N	AA	N
NYP 155a	GA	N	TT	N	TT	N	GG	GG
NWP 59	GA	N	TT	N	TT	N	GG	N
NWP 56	GG	GG	CT	CC	TT	TT	GG	N
NWP 48	AA	N	CT	N	TT	N	GG	N
BYP 218	AA	AA	TT	N	TT	N	GG	N
NYP 142	GA	N	TT	N	TT	N	GG	GG
BYP 246	GA	N	TT	N	TT	TT	GG	N
NWP 85	GG	GG	TT	TT	TT	TT	GG	GG
NWP 26	AA	AA	TT	N	TT	N	GG	N
NWP 183	GG	GG	CC	N	TT	N	GG	GG
NWP 127	AA	AA	TT	TT	GG	TT	GG	AA
NWP 17	GA	AA	TT	TT	GG	TT	GG	N
NWP 29	GA	N	CT	N	GG	N	GG	N
NWP 119	GA	AA	CT	N	GG	N	GG	GG
NWP 11	GA	N	TT	N	TT	N	GG	N
NWP 32	GG	N	TT	N	GG	N	GG	GG
NWP 41	AA	N	TT	N	GG	N	GG	GG
NWP 19	AA	N	TT	N	TT	N	GG	N
NWP 83	AA	N	TT	N	GG	N	GG	N
NYP 141 DH 9	AA	AA	TT	TT	TT	TT	GG	GG
NYP 146 DH 19	AA	N	TT	TT	TT	TT	GG	GG
BWP 178	GG	GG	CC	CC	TT	N	GG	GG
Similarity		56%		73.3%		78.9%		76%

"N" represent missing data

HRM High Resolution Melting, *SNP* Single Nucleotide Polymorphism, *NWP* Northwest White Population, *NYP* Northwest Yellow Population, *BWP* Bajio White Population, *BYP* Bajio Yellow Population

(2015) isolated a FAX1 protein that is inserted into the chloroplast inner envelope by the α-helical membrane-spanning domains. Detailed phenotypic and ultrastructural analyses of *FAX1* mutants in Arabidopsis showed that FAX1 function is crucial for biomass production, male fertility, and synthesis of fatty acid-derived compounds, such as lipids, ketone waxes or pollen cell wall material. In addition, endoplasmic reticulum-derived lipids decreased when *FAX1* was mutated (Li et al. 2015).

The second marker (SNP 100156460) associated with oil content was found in chromosome 8, close to a gene encoding a protease inhibitor/seed storage/LTP family protein (Maizegdb ID: Zm00001d012081). LTPs are small, basic proteins characterized by a tunnel-like hydrophobic cavity, capable of transporting various lipid molecules between lipid bilayers (Edstam et al. 2011).

LTPs enhance the bidirectional transfer of lipids between membranes by binding the hydrophobic cavity of acyl chains, facilitating the extraction of the lipids when LTPs interact with the membrane surface (Kader 1996). In vitro studies showed that LTPs can facilitate the inter-membrane exchange and transfer of several molecules including phospholipids, glycolipids, steroids, acyl-CoAs, and fatty acids (Wei and Zhong 2014).

The third marker (SNP 2412207) associated with oil content was found in chromosome 9, causing a missense mutation in a gene encoding an HXXXD-type acyltransferase family protein (Maizegdb ID: 109942418). Remarkably, this SNP showed the lowest P-value (2.50×10^{-37}) in the GWAS for oil content. HXXXD-type acyltransferase protein genes are part of QTLs related to seed yield, oil unsaturation degree, and the synthesis of linoleic, linolenic, oleic, palmitic, and stearic acids in *Linum usitatissimum* (Kumar et al. 2015). The missense mutation from alanine to serine could cause a modification in the secondary and tertiary structure of the HXXXD-type acyltransferase protein, which could be verified by further in silico analysis of protein structure.

Regarding the protein content trait, the SNP 5586538 was detected in chromosome 7, close to a gene encoding an EIF protein (Maizegdb ID: 100217157). The *EIF* gene codes for a lysine-rich protein that binds aminoacyl-tRNAs to the ribosome, making it a potential contributor to the increased lysine content in W64A*o2* mutant maize (Habben et al. 1995). Also, the overexpression of *EF-1α* in W64A endosperm was significantly associated with the total lysine content (Habben et al. 1995). Bantte and Prasanna (2004) analyzed the EF-1α content in the endosperm of several QPM lines, considering this protein as an indicator of lysine content in the endosperm proteins, finding significant differences in the *EF-1α* levels of the QPM and normal maize genotypes. Therefore, the SNP-5586538 dentified in this study could be used to develop subtropical maize hybrids with high protein and lysine content.

Traditional genotyping by melting analysis relies on the use of labeled probes that allows the detection of variants under the probe, but not outside the probe region, in contrast to HRM which allows high-throughput identification of variants in any region of interest without sequencing (Farrar and Wittert 2017). In this study, SNPs markers were validated using primers that flanked the SNP region, unlike conventional methods that identify variants within a PCR product, requiring a separation of the mixture through a gel or other matrix (Farrar and Wittert 2017).

HRM is more sensitive than denaturing high pressure liquid chromatography (Çelebi and Özdağ 2014) and Sanger sequencing (Gorniak et al. 2016). On the other hand, NGS has similar sensitivity and produces great amounts of DNA sequence, but HRM is faster, less expensive, and easier to use for specific targets (Hinrichs et al. 2015). For example, Chen et al. (2021) developed a set of 148 InDel markers with a high level of polymorphism utilizing the genome sequence of *Brassica rapa*. These polymorphic InDels were easily detectable at a cost-effective by HRM, making it an invaluable tool in genetic analysis and in breeding programs (Chen et al. 2021). Raizada and Souframanien (2019) used RNA-Seq in wild accessions of blackgram (*Vigna mungo*) to develop a large set of SNP markers and used HRM to validate the results. Seventy-one SNPs were amplified through HRM, and 57 SNPs (78%) were validated as homozygous by HRM assay in agreement with the RNA-Seq results (Raizada and Souframanien 2019). Li et al. (2012) also sequenced the transcriptomes of diverse alfalfa germplasm, identifying 872,384 SNPs among 27 genotypes, obtaining a validation rate of 91% for the predicted SNPs using HRM analysis. In this study, a comparison between DArTSeq and HRM was performed, showing similarity rates between 73.3 and 78.9% in three assayed SNPs (100156460, 2412207, and 5586538), whereas the fourth SNP (5589069) showed a 56% of similarity due to the presence of heterozygotes in DArTSeq. HRM allowed the detection of genotypes in lines where DArTSeq showed missing data, obtaining 100% of the data through an accurate technique. The above-mentioned studies together with the present work support that HRM is a viable technique for SNP genotyping that could be applied in MAS of plant crops.

Compared to traditional breeding, MAS breeding has the advantage of reducing the effort and time required in large-scale field tests (Jiao et al. 2012). DH lines with more than one favorable allele for oil content (Table 4) more frequently presented an oil content higher than 6% (Fig. 1). Thus, confirming that these SNPs should be a priority for MAS for oil content improvement in subtropical maize. Currently, the single-crosses NWP-85 X BWP-183, NWP-11 X BWP-183, and NYP-142 X BYP-236 that come from DH lines with HOC are being evaluated within the breeding program using the favorable alleles found in this study and exhibit potential to be commercialized soon.

Zhang et al. (2020) found SNPs and QTLs associated with kernel weight and 27.8–66.7% of the superior lines showed the presence of favorable alleles. In this study, the favorable alleles for oil and protein content were present in 22.5–70.9% of the lines with HOC, and in 14.6% of the lines with high protein content. These findings suggest that the integration of additional favorable alleles in these lines through MAS, could improve the oil and protein content in future maize crosses.

In conclusion, a GWAS for oil and protein content was performed with 20,202 markers in 49 subtropical maize DH lines, allowing the identification of 43 and two associated SNPs with oil and protein content, respectively. These SNPs were close to three genes (*FAX1*, *Protease inhibitor/seed storage/LTP family*, and *HXXXD-type acyl-transferase family protein*) related to oil content, and one gene (*Elongation Initiation Factor*) related to protein content in maize kernel. HRM is a highly accurate technique for SNP genotyping that allowed us to validate the DArTSeq results, and that could be also useful for the genotyping of new maize DH lines in a faster and inexpensive way. The directed crossing scheme (Table 5) based on favorable alleles for high oil and protein content, and genetic distance between DH lines, is currently under evaluation and will allow us to validate the GWAS results once the F2 seeds have been phenotyped.

Table 5 Crosses scheme based on favorable alleles detected by HRM, genetic distance, protein and oil content

SNP	Genetic Distance	%Oil	%Protein
2412207 - oil			
NWP 17 X NWP 29	0.17	7.62 X 7.57	-
NWP 17 X NWP 83	0.19	7.62 X 7.44	-
NWP 17 X NWP 141	0.19	7.62 X 7.45	-
NWP 17 X NWP 47	0.21	7.63 X 6.64	-
NWP 29 X NWP 47	0.2	7.57 X 6.64	-
100156460 - oil			
BWP 183 X NWP 56	0.22	7.53 X 6.7	-
BWP 183 X NWP 27	0.21	7.53 X 6.43	-
BWP 183 X NWP 39	0.21	7.53 X 6.72	-
BWP 183 X NWP 22	0.22	7.53 X 6.53	-
BWP 183 X NWP 47	0.22	7.53 X 6.64	-
NWP 56 X NWP 27	0.2	6.7 X 6.43	-
NYP 144 X BYP 236	0.22	6.71 X 6.79	-
NWP 27 X NWP 22	0.2	6.43 X 6.53	-
5589069 - oil			
BYP 236 X NYP 144	0.22	6.79 X 6.71	-
NWP 31 X BWP 180	0.21	6.72 X 6.4	-
NWP 31 X BWP 183	0.22	6.41 X 6.8	-
NWP 39 X BWP 180	0.21	6.72 X 6.4	-
NWP 39 X BWP 183	0.21	6.72 X 6.8	-
NWP 39 X NWP 32	0.2	6.72 X 7.43	-
BYP 209 X NYP 144	0.22	6.72 X 6.71	-
BWP 180 X NWP 90	0.21	6.4 X 6.36	-
BWP 180 X NWP 56	0.21	6.4 X 6.7	-
BWP 180 X NWP 32	0.22	6.4 X 7.43	-
NWP 90 X BWP 183	0.22	6.36 X 6.8	-
NWP 90 X NWP 32	0.2	6.36 X 7.43	-
NWP 56 X NWP 85	0.2	6.7 X 6.45	-
NWP 56 X BWP 183	0.22	6.7 X 6.8	-
NWP 85 X BWP 183	0.21	6.45 X 6.8	-
NWP 85 X NWP 32	0.2	6.45 X 7.43	-
BWP 183 X NWP 32	0.22	6.8 X 7.43	-
5586538 - protein			
NWP 9 X NWP 90	0.19	-	10.43 X 11.06
NWP 9 X NWP 47	0.2	-	10.43 X 10.2
NWP 70 X NWP 47	0.19	-	10.59 X 10.2
NYP 155A X NYP 144	0.2	-	11.97 X 15.58

HRM High Resolution Melting

Methods

Plant Materials

A total of 49 DH lines were obtained after nine cycles of recurrent selection for HOC in four subtropical maize populations whose genetic background and adaptation were previously reported by Ortega-Corona et al. (2015) and were distributed as follows: five from Bajio Yellow Population (BYP), seven from Northwest Yellow Population (NYP), three from Bajio White Population (BWP), and 34 from Northwest White Population (NWP). To increase the amount of seed per line, these were grown and self-pollinated at INIFAP "Valle del Fuerte" experimental station in Sinaloa, Mexico, during the autumn–winter cycle of 2015–2016.

Oil Content

The oil content in maize kernel was evaluated according to Soxhlet method (Cela et al. 2002). Five grams of maize flour were placed in coffee filters inside the Soxhlet extractor chamber, 70 mL of hexane was boiled at 69 °C in a flask, and the condensing the vapors fell drop by drop on the sample, extracting the soluble analytes. When the level of the condensed solvent in the chamber reaches the top of the lateral siphon, the hexane with dissolved analytes rises through the siphon and returns to the boiling flask, repeating the process until the extraction of analytes from the sample is completed (6 h). All the samples were evaluated by triplicate and the oil content was obtained by the following formula: % oil = (M2-M1/M) *100, where M2 is the weight of the flask with oil, M1 is the weight of empty flask and M is the sample weight.

Protein Content

Protein content in maize kernel was evaluated according to Kjeldahl method (Bremner 1996). Maize flour samples of 100 mg were placed in distillation tubes, and 5 mL of H_2SO_4 and a Kajeltab Cu/3.5 catalyst pellet (FOSS) were added. Tubes were placed in the digester BD28s/BD50s SEAL Analytical (Mequon, WI) and incubated at 350 °C for 1.5 h. Then, the tubes were taken to a distillation process in the Kjeltec 8200 self-distiller equipment FOSS (Scandinavia, Sweden). Once the digestion was completed, the total titratable acidity was determined with 0.1 N HCl. To determine the total nitrogen in the samples, the following formula was used: % N = ((V1-V2) (N) (14.007) / mg sample) * 100, where: V1 was used ml of HCl in sample, V2 was used ml of HCl in blank, N was normality of HCl, 14.007 is a HCl equivalents. The protein content was obtained by the following formula: % protein = (%N) (6.25). The analysis was performed by triplicate.

SNP Calling

The DH maize lines were previously genotyped at SAGA-CIMMYT with DArTSeq technology and analyzed by Ríos-Sandoval (2018). A total of 35,770 SNP markers were used to identify their position in the reference genome of maize B73_v4, resulting in 29,398 genome-aligned markers. Then, aligned markers were filtered with minor allele frequency (MAF) > 0.05 using TASSEL 5.0 software (Bradbury et al. 2007), and 20,202 filtered SNPs were kept for further analysis.

Kinship and Principal Component Analysis

The variation in phenotypic data was modeled considering the molecular markers as a fixed effect, and the K matrix was carried out in TASSEL v5.0 to indicate the relatedness between lines. This model was initially proposed by Yu et al. (2006) and modified by Kang et al. (2008) and is defined as follows: $y = X\beta + \underline{Z}u + e$, where y is the phenotypic vector, X is the molecular markers matrix, β is the parameters vector (fixed effects), Z is an incidence vector associates with vector u of polygenic effects and e is the error vector.

PCA was carried out in TASSEL v5.0 to identify the effect of the markers, to form groups in the lines, and to eliminate the false positives. The PCA consisted in five dimensions that were compared to explain the genotypic variation. K matrix and PCA are useful to eliminate type I and II errors.

GWAS

GWAS were carried out in TASSEL v5.0 using the phenotypic data (oil and protein content), the 20,202 filtered SNPs, the K matrix and the PCA data. GWAS were performed using an MLM and the results were represented in Manhattan plots that showed the genomic position of each SNP and their significance values (P). The SNPs were considered as significantly associated with the phenotypic traits when they exceeded the threshold calculated using the Bonferroni met hod = -log(α/#SNPs) = -log(0.05)/20,202) = 5.6.

The associated SNPs were aligned against the maize reference genome B73_v4 in order to detect the exact marker position. A 100 kb sequence region flanking both sides of the marker was scanned to find the close genes associated with oil and protein content.

SNPs Confirmation by HRM

Genomic DNA was extracted from maize seeds using a standard protocol of CTAB/SARCOSYL (Stewart and Via 1993) with some modifications. Five seeds per line were pulverized on a TissueLyser II (Qiagen, Venlo, Netherlands) and placed in a 2 mL centrifuge tube to one quarter of its capacity. Then, 1 mL of CTAB/SARCOSYL solution was added and mixed for 1.5 h at room temperature. One mL of chloroform-phenol solution was added and mixed for 20 min at room temperature. Subsequently, the tubes were centrifuged at 1,400 × g for 30 min, the supernatant was recovered in a new tube and a half volume of isopropanol (-20 °C) was added, the tube was shaken by inversion, followed by incubation at -20 °C for 1 h and centrifugation for 10 min at 16,200 × g. The supernatant was decanted, and the pellet was washed three times by adding 1 mL of ethanol (70%) and centrifugation at 1,400 × g for 10 min. Then, the washed pellet was air-dried for 24 h at room temperature and finally resuspended in 50 µL of sterile deionized water. The DNA integrity was visualized by agarose (2%) electrophoresis and DNA concentration were determined using a NanoDrop 2000c (Thermo Scientific, Barrington, IL). The accepted DNA values were A260/A280 = 1.8–2.0, and A260/A230 = 1.5–2.0.

Based on the flanking regions of each SNP, specific primers were designed using the software Primer3 v.0.4.0 (http://bioinfo.ut.ee/primer3-0.4.0/primer3/) and synthesized by T4OLIGO company (Irapuato, Mexico), based on the following criteria: (1) 70–115 bp product size; (2) only one SNP from each amplified fragment; (3) ~ 20 bp primer length; (4) 30–40% GC content in each primer; and (5) 58–60 °C of Tm (Table 2).

The SNPs associated with oil and protein content were confirmed by HRM assay using a QuantStudio 7 Flex real-time PCR system (Applied Biosystems, Foster City, CA), using a total mix volume of 10 µL (5 µL of Melt Doctor HRM Master Mix, 0.5 µL of DNA (20 ng/ µL), 0.5 µL of forward primer (5 µM), 0.5 µL of reverse primer (5 µM), 3.5 µL of deionized water). The amplification conditions were 1 cycle at 94 °C for 4 min; 35 cycles at 94 °C for 30 s, 59 °C for 30 s, followed by melting at 60 °C to 95 °C with increase of 0.025 °C/s.

Once confirmed alleles presence in DH lines, favorable alleles, oil and protein content and genetic distance according to Ríos-Sandoval (2017) were selected to create a directed crossing scheme (Table 5).

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Author Contributions Juan P. Valenzuela-Apodaca worked in all objectives whitin the project and wrote the main manuscript text and prepared figures and tables. Abraham Cruz-Mendívil advised and worked in bioinformatics analysis and data analysys. Grethel P. Gaytán-Pinzón worked in agronomic evaluations and laboratory experiments. Hervey Rodríguez-González advised in protein content analysis. Luis A. Peinado-Fuentes advised and worked in agronomic evaluations and was the project funder. Eduardo Sandoval-Castro advised in popuation´s genetics experiments and data analysis. Carlos L. Calderón-Vázquez (corresponding author), advised and worked throughout the entire project. All authors reviewed the manuscript.

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Declarations

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