

Research Article

Identification of molecular markers associated with kernel iron and zinc concentrations in groundnut (*Arachis hypogaea* L.)

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Abstract

Micronutrient malnutrition affects more than one-half of the world's population, especially women and pre-school children. Poor consumers in developing countries acquire roughly one-half of their total iron intakes and a higher percentage of zinc intakes from staple foods. Bio-fortification, an approach of breeding crop plants for higher micronutrient concentration, is a cost effective and sustainable solution for tackling the micronutrient deficiencies. Developing countries, where micronutrient deficiencies are widespread, contribute world's maximum peanut area and production. Thus, peanut can contribute significantly towards reduction of protein-energy and micronutrient malnutrition. With the advent of molecular markers, by using segregating populations for the trait of interest for breeders, it has now become effective to map genes or Quantitative Traits Loci (QTLs) and identify valuable alleles for the corresponding traits. In the present study, an $F_{2:3}$ mapping population derived from the cross ICGV 06099 $\times$ ICGV 93468 was genotyped using 33 polymorphic SSR markers to identify the putative genomic regions associated with the kernel iron and zinc concentrations in groundnut using Single Marker Analysis (SMA). Results revealed that three markers viz., SEQ1B09 (0.23 %), IPAHM245 (2.19 %) and SEQ9G05 (6.34 %) showed significant association with the kernel iron and three markers viz., GM2638 (1.75 %), IPAHM245 (2.25 %) and SEQ9G05 (6.01 %) showed significant association with kernel zinc concentration. Significant positive association (0.549) was observed between kernel iron and zinc concentrations. Validation of these markers in an alternate $F_{2:3}$ population derived from the cross ICGV 06040 $\times$ ICGV 87141 also showed strong association of these markers with the traits of interest.

Key words

Groundnut, kernel iron and zinc concentrations, phenotyping, single marker analysis, SSR markers

Introduction

Micronutrient malnutrition affects more than one-half of the world's population, especially women and pre-school children (UNSCN, 2004). Thus, to fight against the hidden hunger and to cut down the mortality rate due to poor diet, we require additional technologies and approaches to improve nutritional status, which is an important determinant of these mortalities (Nestel *et al.* 2006). Breeding crop plants for higher micronutrient concentration, an approach termed as bio-fortification has become an active goal of plant breeding programs during recent times. Being a quantitative trait the expression of these traits is affected not only by large number of genes governing them but also by environmental effects. Molecular markers offer great scope for improving the efficiency of conventional plant breeding. With the advent of molecular markers, by using segregating populations for the trait of interest for breeders, it has become possible to map the genes or Quantitative Traits Loci (QTLs) and identify valuable alleles for the corresponding traits (Ender *et al.* 2008). Markers reduce the time required to develop new genotypes with desirable traits by screening in the early stage itself, instead of waiting until harvest (Davis *et al.* 2006). Thus in the present study, an attempt was made to identify the linked markers associated with the kernel iron

and zinc concentrations in groundnut using $F_{2:3}$ population of a cross ICGV 06099 $\times$ ICGV 93468 using SSR markers.

Materials and methods

One hundred eighty four individuals were developed by crossing parents contrasting for kernel iron and zinc concentrations viz. ICGV 06099 and ICGV 93468. Entries were evaluated using alpha-lattice design during rainy, 2014 and the spacing adopted was 30 $\times$ 10cm with a row length of 1m. Biochemical analysis for kernel iron and zinc concentrations was carried out at the Charles Renard Analytical laboratory (CRAL) at ICRISAT using Inductively Coupled Plasma Optical Emission Spectrometry ICP-OES method (Wheal *et al.*, 2011).

After harvesting, shelling was done manually using hands ensuring no metal contamination while sample preparation. Shelled kernels were cleaned to make them free from dirt, if any. The cleaned kernels were collected in packets and the kernel iron and zinc concentrations were measured using Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES). About 0.2-0.3 g of oven-dried ground sample was weighed and transferred into labelled tube. To this, 1.5-2.0 ml of nitric acid was added followed by addition of 0.5 ml

Hydrogen Peroxide (H_2O_2). The tube was then closed and allowed to stand overnight at room temperature, after which the contents were transferred to heating blocks set at $80^{\circ}C$. The cap was loosened to allow release of pressure during the reaction process. After 30-minutes of reaction initiation, the temperature of heating block was increased to $125^{\circ}C$ and heat digestion was continued for another 2-hours. The sample tubes were cooled and the volume was made up to 25 ml using distilled water. The tubes were vortexed for 1 to 2 minutes and the supernatant was collected and used for estimating iron and zinc concentration through ICP-OES. However Aluminium estimation was not carried out ensuring no interference with available iron and zinc concentration in groundnut.

DNA isolations from F_2 population were carried out using CTAB method (Mace, 2003) from young and tender leaves of groundnut. PCR reactions were conducted in 96 and 384-well plates in a GeneAmp PCR system PE 9700 (Applied Biosystem, USA) DNA thermal cycler in volumes of 5 μ l. Reaction conditions consist of initial denaturation for 5 min at $94^{\circ}C$ (to minimize primer - dimer formation and to activate the *Taq* polymerase), subsequently 10 cycles of denaturation for 15 seconds at $94^{\circ}C$, annealing at $61^{\circ}C$ to $52^{\circ}C$ for 20 seconds, the annealing temperature for each cycle is reduced by $1^{\circ}C$ and extension at $72^{\circ}C$ for 30 seconds followed by 40 cycles of denaturation at $94^{\circ}C$ for 10 seconds, annealing at $54^{\circ}C$ for 30 seconds and extension at $72^{\circ}C$ for 30 seconds followed by final extension at $72^{\circ}C$ for 20 min. PCR amplification was checked on 1.2% agarose gels and PCR products of direct labelled primers and M_{13} tailed primers were separated by capillary electrophoresis on an ABI3730xl sequencer and their sizes were determined using GeneMapper[®] Version 4.0 software (Applied Biosystems, USA) and PCR products of unlabelled primers were separated on Agarose gels.

Single Marker Analysis (SMA) was done with regression analysis (Srivastava *et al.* 2007) using STATISTICA 4.5 software to find the association between molecular markers and kernel iron and zinc concentrations. Polymorphic markers which showed significant association with kernel iron and zinc concentrations were validated in another $F_{2:3}$ mapping population of cross ICGV 06040 $\times$ ICGV 87141 in groundnut.

Results and discussion

Unlike other crops, groundnut kernels contain more zinc compared to iron (Upadhyaya *et al.*, 2012). The mean kernel iron concentration of the parents, ICGV 06099 and ICGV 93468 was 52.5 mg/kg and 37.3 mg/kg, respectively and mean zinc concentration was 79.5 mg/kg and 65.0 mg/kg, respectively. For the mapping population, the

kernel iron concentration ranged from 31.8-61.4 mg/kg with a mean value of 44.8 mg/kg, and the kernel zinc concentration from 59.6-90.4 mg/kg with a mean value of 76.7 mg/kg (Table 1). A few lines with kernel iron and zinc concentration higher than the better parent ICGV 06099 were identified in the mapping population. This indicated the presence of transgressive segregants in the mapping population for both traits (Gande *et al.* 2014).

The most basic way of determining whether an association exists between a molecular marker and a trait is to do single marker analysis. It will help in identifying significant association between markers and the trait of interest on individual marker basis by providing information on the amount of phenotypic variation contributed by a particular marker towards the trait of interest. More the contribution of marker to phenotypic variation, stronger will be the association between the marker and the trait targeted.

To identify polymorphic markers, parental polymorphism survey was performed between ICGV 06099 (high in kernel iron and zinc) and ICGV 93468 (low in kernel iron and zinc) using 200 SSR markers. The results revealed that 33 SSR markers (Table 2) (16.5 %) were polymorphic, 143 markers (71.5 %) were mono-morphic and remaining 24 markers (12 %) were not amplified among the parents. Subsequently, the thirty three polymorphic SSR markers were used for single marker analysis to identify the markers specifically associated with kernel iron and zinc concentrations in $F_{2:3}$ population of cross ICGV 06099 $\times$ ICGV 93468. Results revealed that three markers viz., IPAHM245 (lgA06), SEQ1B09 (lgA02) and SEQ9G05 (lgB05) showed significant association with a phenotypic variation of 2.19, 0.23 and 6.24 % (Table 3), respectively for kernel iron concentration, while three other markers viz. GM2638 (lgA04), IPAHM245 (lgA06) and SEQ9G05 (lgB05) showed significant association with a phenotypic variation of 1.75, 2.25 and 6.01 %, respectively for kernel zinc concentration in the $F_{2:3}$ mapping population. Earlier Berhanu *et al.* (2013) and Gande *et al.* (2014) identified the association of markers with grain iron and zinc concentrations in rice using single marker analysis. Among the identified markers, two markers viz., IPAHM245 (Cuc *et al.* 2008) and SEQ9G05 (Ferguson *et al.* 2004) were associated with both kernel iron and zinc concentration in groundnut suggesting the presence of QTLs governing kernel iron and zinc concentrations on the same location of the chromosome. This indicated that the two traits viz. kernel iron and zinc concentrations might be co-segregating with each other. So these two markers can be used for further studies to identify the exact genomic regions (QTLs) or genes

associated with kernel iron and zinc concentrations in groundnut.

Validation of putative markers is required to confirm the reproducibility of selected markers for marker aided breeding program (Miklas, 2007). So the markers which were found significant on $F_{2:3}$ population of cross ICGV 06099 $\times$ ICGV 93468 were validated on alternate $F_{2:3}$ population derived from the cross ICGV 06040 $\times$ ICGV 87141. Lines with polymorphic bands similar to the high iron and zinc parent ICGV 06099 were identified using markers and subjected to biochemical analysis to estimate their kernel iron and zinc concentration. The results revealed that most of the entries were having higher iron and zinc concentrations in their kernels (Table 4) suggesting that all the four markers which were found significant on the genotyping population were actually linked to the traits of interest. Hence, these markers can be efficiently utilised in marker aided breeding programmes aimed at improvement of these two micronutrient concentrations in groundnut kernels. Correlation studies revealed, a positive significant association between kernel iron and zinc concentration.

Conclusion

The present study enabled identification of the linked markers associated with kernel iron and zinc concentration in groundnut. Though the identified markers had shown low phenotypic variation percentage towards trait, the present work can be considered as a stepping stone to further map and to identify actual genomic regions (QTLs) governing the trait. Further there is need to attain large number of polymorphic markers to cover all the linkage groups present in groundnut to construct linkage map.

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Table 1. Estimates of kernel iron and zinc concentrations among parents and F_{2:3} population of cross ICGV 06099 × ICGV 93468

Characters	Parents				F _{2:3} Population	
	ICGV 06099		ICGV 93468		Mean	Range
	Mean	Range	Mean	Range		
Kernel iron concentration (mg/kg)	52.5	45.3 - 57.7	37.3	34.1 - 39.5	44.8	31.8 - 61.4
Kernel zinc concentration (mg/kg)	79.5	71.4 - 85.4	65.0	62.1 - 68.4	76.7	59.6 - 90.4

Table 2. List of markers found polymorphic in the study along with their sequence information

S. No.	Marker	Sequence		Linkage Group	Position (cM)	Size (bp)	References
		Forward	Reverse				
1	GM1954	GAGGAGTGTGAGGTTCTGACG	TGGTTCATTGCATTTGCATAC	A03	114.43	115	Nagy <i>et al.</i> (2010)
2	GM1991	GAAAATGATGCCGAGAAATGT	GGGGAGAGATGCAGAAAGAGA	B06	92.89	122	
3	GM1742	GCCTTGTGCAATCATCACA	ACCTCCAACAGGAACATTGC	B10	38.69	270	
4	GM2536	AGCCTCCACCTTCTCCTATTG	GATGCAGTGGAGGGATAACAA	A06	115.72	336	
5	GM1577	GCGGTGTTGAAGTTGAAGAAG	TAACGCATTAAACCACACACCA	A05	53.75	278	
6	GM2032	GCCGATGATGTACGTTTCTTC	GAGACGGCATGTCAAAAGAAT	B10	24.30	149	
7	GM2638	ATGCTCTCAGTTCTTGCTGA	CAGACATAACAGTCAGTTTCACC	A04	86.55	107	
8	GM2746	TCAACCTCAAGGGTGATTGTC	ACACAAACCCGCTCACTCTAA	B08	60.30	120	Moretzsohn <i>et al.</i> (2005)
9	GM2120	TCCACTGCCACCTCTATCATC	TCCACCCACATAGACAGAAGC	B09	90.39	139	
10	TC3B05	GGAGAAAACGCATTGGAAC	TTTGTCCCGTTGGGAATAGT	A08	23.09	248-270	
11	TC9F10	ATCACAATCACAGCTCCAACAA	GGCAAGTCTAATCTCCTTTCCA	A08	73.94	286-320	
12	TC7E04	GAAGGACCCCATCTATTCAAA	TCCGATTTCTCTCTCTCTCTC	A03	127.20	300	
13	TC1E05	GAAGGATAAGCAATCGTCCA	GGATGGGATTGAACATTTGG	A08	60.27	215-260	
14	TC1B02	AACATGCATGCAAATGGAAA	GCCAAAGTCACCTTGTGCTT	B02	55.56	220-270	
15	TC4G02	GATCCAACGTGAATTGGGC	CACACCAGCAACAAGGAATC	B03	88.70	130-166	Guo <i>et al.</i> (2012)
16	TC4F12	GATCTTTCCGCCATTTCTC	GGTGAATGACAGATGCTCCA	A02	34.51	230	
17	GM2053	ACAAGGAAAACCCATCCAATC	ACGTGATGGATTCTTGTGGAG	B03	74.42	405	
18	GM2301	GTAACCACAGCTGGCATGAAC	TCTTCAAGAACCCACCAACAC	B03	113.75	137	
19	GM2079	GGCCAAGGAGAAGAAGAAAGA	GAAGGAGTAGTGGTGCTGCTG	B03	115.71	418	Cuc <i>et al.</i> (2008)
20	IPAHM689	GATGACAATAGCGACGAGCA	GTAAGCCTGCAGCAACAACA	A06	52.22	240	

Table 2. Contd.,

S. No.	Marker	Sequence		Linkage Group	Position (cM)	Size (bp)	References
		Forward	Forward				
21	IPAHM245	CCCAAGGACCTAGTGACCAA	GGACCCTTAGCACATTCCAA	A06	55.16	290	Cuc <i>et al.</i> (2008)
22	IPAHM103	GCATTACCACCATAGTCCA	TCCTCTGACTTTCCTCCATCA	A03	133.84	160	
23	IPAHM524	GCCATGGATAAGAACCTGAAA	CAGTAAGCTGAGCTGGCAGA	B02	46.11	300	
24	PM36	ACTCGCCATAGCCAACAAAC	CATTCCCACAACCTCCCACAT	A05	54.89	190-240	He <i>et al.</i> (2003)
25	S109	AAGGGAGCACAATCATA	GAGCACGAGTTCATACAC	A04	55.62	370-430	Wang <i>et al.</i> (2006)
26	SEQ2B09	GCAACATGCTCTGAATTTTGAC	TGTGCAACCCAATTCAATAACTT	B09	82.55	259	Ferguson <i>et al.</i> (2004)
27	SEQ5D01	TGGCCAAAACAACCTGATTGA	TCCCAACTTTTCCGTTCTTG	A01	65.76	264	
28	SEQ17E03	TTTCCTTTCAACCCTTCGTG	AATGAGACCAGCCAAAATGC	A09	85.93	193	
29	SEQ19G07	ATTCAATTCTCTCTCCCCC	TCAATCAATCAATCGCAGGA	A03	106.08	149	
30	SEQ1B09	CGTTCTTTGCCGTTGATTCT	AGCACGCTCGTTCTCTCATT	A02	38.49	282	
31	SEQ3A08	ATACGTGACTTGGGCCAGAC	AGTGAAAAATACACCCAACGAA	A08	53.56	152	
32	SEQ9G05	CAAATTGTGCAGCCAAGAGA	CATATGCCCAGGAAGAGGAA	B05	32.05	273	
33	SEQ19B01	TTGGTGATGGTGTGGAGAA	TAAACCAGGCCAAAAGTGG	A09	54.44	198	

Table 3. Markers found associated with kernel iron and zinc concentration in F_{2:3} mapping population of cross ICGV 06099 × ICGV 93468 in groundnut by Single Marker Analysis (SMA)

For kernel iron concentration		
Marker	Probability	R ² (%)
IPAHM245	0.009 **	2.19
SEQ1B09	0.049 *	0.23
SEQ9G05	0.002 **	6.24
For kernel zinc concentration		
Marker	Probability	R ² (%)
GM2638	0.038 *	1.75
IPAHM245	0.012 *	2.25
SEQ9G05	0.001 **	6.01

Where, R²= Phenotypic variation (%) explained

Table 4. Kernel iron and zinc concentrations of entries in the cross ICGV 06040 × ICGV 87141 which showed similar scoring as that of entries of genotyping population using three SSR markers each associated with kernel iron and zinc concentrations of the cross ICGV 06099 × ICGV 93468

Sample No.	Kernel iron concentration (mg/kg)	Kernel zinc concentration (mg/kg)
1	25.42	43.29
2	24.16	43.39
3	25.48	41.18
4	21.74	40.38
5	23.69	36.82
6	23.54	38.55
7	21.65	35.38
8	21.94	43.58
9	19.39	34.97
10	22.58	38.87
11	23.59	42.62
12	27.22	43.83
13	24.18	43.40
14	21.12	39.44
15	20.80	36.69
16	19.89	39.65
17	20.22	35.06
18	26.12	39.60
19	25.60	40.44
20	23.04	43.59
21	20.33	41.43
22	19.59	41.08
23	24.31	45.90
24	19.38	36.30
25	21.27	41.32
26	23.92	40.13
27	19.03	34.49
28	20.69	37.55
29	23.00	42.36
30	20.13	40.95
31	21.83	40.17
32	21.96	43.08
33	20.03	43.12
34	12.84	26.51