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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 × 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 × 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 × 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 × 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

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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 80^0C. 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 125^0C 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 µl. Reaction conditions consist of initial denaturation for 5 min at 94°C (to minimize primer - dimer formation and to activate the *Taq* polymerase), subsequently 10 cycles of denaturation for 15 seconds at 94°C, annealing at 61°C to 52°C for 20 seconds, the annealing temperature for each cycle is reduced by 1°C and extension at 72°C for 30 seconds followed by 40 cycles of denaturation at 94°C for 10 seconds, annealing at 54°C for 30 seconds and extension at 72°C for 30 seconds followed by final extension at 72°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 × 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 × 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

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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 × ICGV 93468 were validated on alternate $F_{2:3}$ population derived from the cross ICGV 06040 × 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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References

Berhanu, D.B., Naveen, G.K., Rakhi, S. and Shashidhar, H.E. 2013. Genetic evaluation of recombinant lines of rice (*Oryza sativa* L.) for grain zinc concentrations, yield related traits and identification of associated SSR markers. *Pakistan J Biol Sci*., **16**: 1714 - 1721.

Cuc, L.M., Mace, E.S., Crouch, J.H., Quang, V.D., Long, T.D. and Varshney R.K. 2008. Isolation and characterization of novel microsatellite markers and their application for diversity assessment in cultivated groundnut (*Arachis hypogaea* L). *BMC Pl Biol*., **55**: 1-11.

Davis, J., Berzonsky, W.A. and Leach, G.D. 2006. A comparison of marker-assisted and phenotypic selection for high grain protein content in spring wheat. *Euphytica*, **152**: 117-134.

Ender, M., Terpstra, K. and Kelly, J.D. 2008. Marker-assisted selection for white mold resistance in common bean. *Mol Breed*., **21**: 149-157.

Ferguson, M.E., Burow, M.D., Schulze, S.R., Bramel, P.J., Paterson, A.H., Kresovich, S. and Mitchell, S. 2004. Microsatellite identification and characterization in peanut (*A. hypogaea* L.). *TheorApp Genet*., **108**: 1064-1070.

Gande, N.K., Pavan, J.K., Soman, R., Rajeswari, A., Ashwathanarayana, A., Bekele, B.D. and Shashidhar, H.E. 2014. Identification of putative candidate gene markers for grain iron and zinc content using recombinant inbred lines (RIL) population of IRRI38 × Jeerigesanna. *African J Biotech*., **13**: 657-663.

Guo, B., Zou, M. and Wagner, A. 2012. Pervasive indels and their evolutionary dynamics after the fish-specific genome duplication. *Mol. Biol. Evol*., **29**: 3005-3022.

He, G., Meng, R., Newman, M., Gao, G., Pittman, R.N. and Prakash, Ch.S. 2003. Microsatellites as DNA markers in cultivated peanut (*Arachis hypogaea* L.). *BMC Pl. Biol*., **3**: 1-6.

Mace, E.S., Buhariwalla, H.K. and Crouch, J.H. 2003. A high-throughput DNA extraction protocol for tropical molecular breeding programs. *Pl. Mol. Biol. Reporter*, **21**: 459-460

Miklas, P.N. 2007. Marker assisted backcrossing QTL for partial resistance to *Sclerotinia* white mold in dry bean. *Crop Sci*., **47**: 935 - 942.

Moretzsohn, M.C., Leoi, L., Proite, K., Guimarães, P.M., Leal-Bertioli, S.C.M., Gimenes, M.A., Martins, W.S., Valls, J.F.M., Grattapaglia, D. and Bertioli, D.J. 2005. A microsatelite-based, gene-rich linkage map for the AA genome of Arachis (Fabaceae). *Theor. App. Genet*., **111**: 1060- 1071.

Nagy, E., Guo, Y., Khanal, S., Taylor, C., Knapp, S., Oziasakins, P., Stalker, H.T. and Nielsen, N. 2010. Developing a high-density molecular map of teh A-genome species *A. duranensis.* American Peanut Research and Education Society Proceedings (APRES). **42**: 25.

Nestel, P, Bouis, H.E., Meenakshi, J.V. and Pfeiffer, W. 2006. Biofortification of staple food crops. *J. Nutri*., **136**: 1064-1067.

Srivastava, P.P., Kar, P.K., Awasthi, A.K. and Urs, S.R. 2007. Identification and association of ISSR markers for thermal stress in polyoltine silkworm (*Bombyx mori*). *Russian J. Genet.,* **43**: 858-864.

United Nations System Standing Committee on Nutrition (UNSSCN). 2004. 5th Report on the World Nutrition Situation Nutrition for Improved Development Outcomes. SCN, Geneva.

Upadhyaya, H.D., Naresh, D., Singh, S. and Dwivedi, S.L. 2012. Variability and stability for kernel iron and zinc content in the ICRISAT mini-core collection of peanut. *Crop Sci*., **52**: 2628-2637.

Wang, X.J., Su, L., Quan, X.Q., Shan, L., Zhang, H.T. and Bi, Y.P. 2006. Peanut (*Arachis hypogaea* L.) EST sequencing, gene cloning an Agrobacteria-mediated transformation. *In*: International groundnut conference on groundnut Aflatoxin

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and genomics. (5th -9th November, 2006, Guangzhou, China). 59-60.

Wheal M.S., Fowles T.O. and Palmer, L.T. 2011. A cost-effective acid digestion method using closed polypropylene tubes for inductively coupled plasma optical emission spectrometry (ICP-OES) analysis of plant essential elements. *Analytical Methods*, 3: 2854-2863.

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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	
2	GM1991	GAAAATGATGCCGAGAAATGT	GGGGAGAGATGCAGAAAGAGA	B06	92.89	122	
3	GM1742	GCCTTGTTGCAATCATCACA	ACCTCCAACAGGAACATTGC	B10	38.69	270	
4	GM2536	AGCCTCCACCTTCTCCTATTG	GATGCAGTGGAGGGATAACAA	A06	115.72	336	
5	GM1577	GCGGTGTTGAAGTTGAAGAAG	TAACGCATTAACCACACACCA	A05	53.75	278	Nagy *et al.* (2010)
6	GM2032	GCCGATGATGTACGTTTCTTC	GAGACGGCATGTCAAAAGAAT	B10	24.30	149	
7	GM2638	ATGCTCTCAGTTCTTGCCTGA	CAGACATAACAGTCAGTTTCACC	A04	86.55	107	
8	GM2746	TCAACCTCAAGGGTGATTGTC	ACACAAACCCGCTCACTCTAA	B08	60.30	120	
9	GM2120	TCCACTGCCACCTCTATCATC	TCCACCCACATAGACAGAAGC	B09	90.39	139	
10	TC3B05	GGAGAAAACGCATTGGAACT	TTTGTCCCGTTGGGAATAGT	A08	23.09	248-270	
11	TC9F10	ATCACAATCACAGCTCCAACAA	GGCAAGTCTAATCTCCTTTCCA	A08	73.94	286-320	
12	TC7E04	GAAGGACCCCATCTATTCAAA	TCCGATTTCTCTCTCTCTCTCTC	A03	127.20	300	
13	TC1E05	GAAGGATAAGCAATCGTCCA	GGATGGGATTGAACATTTGG	A08	60.27	215-260	Moretzsohn *et al.* (2005)
14	TC1B02	AACATGCATGCAAATGGAAA	GCCAAAGTCACTTGTTTGCTT	B02	55.56	220-270	
15	TC4G02	GATCCAACTGTGAATTGGGC	CACACCAGCAACAAGGAATC	B03	88.70	130-166	
16	TC4F12	GATCTTTCCGCCATTTTCTC	GGTGAATGACAGATGCTCCA	A02	34.51	230	
17	GM2053	ACAAGGAAAACCCATCCAATC	ACGTGATGGATTCTTGTGGAG	B03	74.42	405	
18	GM2301	GTAACCACAGCTGGCATGAAC	TCTTCAAGAACCCACCAACAC	B03	113.75	137	Guo *et al.* (2012)
19	GM2079	GGCCAAGGAGAAGAAGAAAGA	GAAGGAGTAGTGGTGCTGCTG	B03	115.71	418	
20	IPAHM689	GATGACAATAGCGACGAGCA	GTAAGCCTGCAGCAACAACA	A06	52.22	240	Cuc *et al.* (2008)

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Table 2. Contd.,

S. No.	Marker	Sequence		Linkage Group	Position (cM)	Size (bp)	References
		Forward	Forward				
21	IPAHM245	CCCAAGGACCTAGTGACCAA	GGACCCTTAGCACATTCCAA	A06	55.16	290	
22	IPAHM103	GCATTCACCACCATAGTCCA	TCCTCTGACTTTCCTCCATCA	A03	133.84	160	Cuc *et al.* (2008)
23	IPAHM524	GCCATGGATAAGAACCTGAAA	CAGTAAGCTGAGCTGGCAGA	B02	46.11	300	
24	PM36	ACTCGCCATAGCCAACAAAC	CATTCCCACAACTCCCACAT	A05	54.89	190-240	He *et al*. (2003)
25	S109	AAGGGAGCACAATCATA	GAGCACGAGTTCATACAC	A04	55.62	370-430	Wang *et al.* (2006)
26	SEQ2B09	GCAACATGCTCTGAATTTTGAC	TGTGCAACCCAATTCAATAACTT	B09	82.55	259	
27	SEQ5D01	TGGCCAAAACAACTGATTGA	TCCCAACTTTTCCGTTCTTG	A01	65.76	264	
28	SEQ17E03	TTTCCTTTCAACCCTTCGTG	AATGAGACCAGCCAAAATGC	A09	85.93	193	
29	SEQ19G07	ATTCAATTCCTCTCTCCCCC	TCAATCAATCAATCGCAGGA	A03	106.08	149	Ferguson et al. (2004)
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	TTGGTGATGGTGTTGGAGAA	TTAAACCAGGCCAAAAGTGG	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, R^2= Phenotypic variation (%) explained

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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