

Identification of QTL affecting seed mineral concentrations and content in the model legume *Medicago truncatula*

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Abstract Increasing the amount of bioavailable micronutrients such as iron and zinc in plant foods for human consumption is an international goal, intended especially for developing countries where micronutrient deficiencies are an ongoing health risk. Legume seeds have the potential to provide the essential nutrients required by humans, but concentrations of several minerals are low when compared to other foods. In order to increase seed mineral concentrations, it is important to understand the genes and processes involved in mineral distribution within the plant. The main objectives of this study were to use a *Medicago truncatula* recombinant inbred population (Jemalong-6 × DZA 315.16) to determine loci governing seed mineral concentrations, seed mineral content, and average seed weight, and to use these loci to propose candidate genes whose expression might contribute to these traits. Ninety-three lines in 2004 and 169 lines in 2006 were grown for seed harvest and subsequent analysis of seed Ca, Cu, Fe, K, Mg, Mn, P, and Zn concentrations and content. Quantitative trait loci

(QTL) cartographer was used to identify QTL using composite interval mapping (CIM). CIM identified 46 QTL for seed mineral concentration, 26 for seed mineral content, and 3 for average seed weight. At least one QTL was detected for each mineral trait, and colocation of QTL for several minerals was found in both years. Results comparing seed weight with seed mineral concentration and content QTL demonstrate that seed size can be an important determinant of seed mineral concentration. The identification, in this model legume, of transgressive segregation for nearly all the minerals suggests that allelic recombination of relevant mineral-related genes in agronomic legumes could be a successful strategy to increase seed mineral concentrations above current levels.

Introduction

More than 3 billion people globally are affected by micronutrient malnutrition resulting in overall poor health and higher rates of mortality (Cakmak 2002; Pfeiffer and McClafferty 2007). Cereals and legumes are important nutrient sources of protein and dietary minerals for humans, especially in developing countries where plant foods comprise a significant portion of the diet. However, the concentrations of certain minerals, especially iron and zinc, are low when compared to animal food products (Wang et al. 2003). Plants require 14 essential minerals for their normal growth and development, of which 12 of these also are essential nutrients required by humans (Grusak and DellaPenna 1999). However, the concentrations of these dietary minerals in most plant foods are not sufficient to meet the daily dietary requirements of humans when these foods are consumed in typical amounts. Hence there has been an interest in improving the mineral concentration of various seed crops.

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Biofortification is a new approach which involves both conventional breeding and modern biotechnology to increase the bioavailable nutrients such as iron and zinc in staple food crops (Pfeiffer and McClafferty 2007). However, breeding for elevated micronutrient content requires the enhancement of several processes. This is because in plants, mineral movement involves long distance systems as well as membrane-localized transport proteins that are necessary to move water and nutrients to different parts of the plant. The amount of mineral nutrients in sink tissues, such as the seeds, depends on a number of processes; these include mobilization and uptake from the soil into the roots, xylem translocation to the transpiring shoots, storage in leaves, and finally, mobilization and redistribution into the seeds via the phloem (Marschner 1995). To increase the amount of mineral nutrient in seeds and fruit crops, understanding the processes and genes involved in transport and accumulation of these minerals is important.

One of the major problems in trying to improve the nutritional quality of seeds is the lack of understanding of the different pathways and the rate limiting steps involved in transporting minerals to the seeds. Recent studies have identified and characterized a number of gene families that encode proteins involved in metal transport processes in plants (Fox and Guerinot 1998; Lopéz-Millán et al. 2004; Maser et al. 2001, Ramesh et al. 2003). There have been several studies identifying the genes involved in uptake of different minerals from the rhizosphere and very few for translocation to different vegetative tissues and ultimately accumulation in seeds (Grotz et al. 1998; Eren and Arguello 2004; Green and Rogers 2004; Hussain et al. 2004; Verret et al. 2004; Pittman 2005; Andrés-Colás et al. 2006; Grotz and Guerinot 2006; Colangelo and Guerinot 2006; Durrett et al. 2007). While studies with specific transporters help us to understand their function, whole-plant studies also are needed to determine which transporters are most relevant to seed mineral delivery. Moreover, there have been few studies on phloem loading and regulation of minerals and very limited knowledge of phloem-expressed genes involved in mineral loading and mobilization in different sink tissues (Hocking and Pate 1977; Grusak 1994; Marentes and Grusak 1998; Grusak 2000; Zhang et al. 2002) Furthermore, each of these processes is probably controlled by a number of genes, many of which are yet to be identified.

Until recently, breeders have focused on increasing yield and disease resistance in various crops. A wide range of genetic variation in micronutrient concentrations is already available in a number of crops such as wheat and rice. However, studies trying to understand processes involved in moving the minerals to seeds using quantitative trait loci (QTL) analysis have been limited to common bean, rice, wheat and *Arabidopsis* (Gregorio et al. 2000; Ortiz-Monasterio and Graham 2000; Guzman-Maldonado et al. 2003;

Vreugdenhil et al. 2004; Waters and Grusak 2008b). In this work, we have used recombinant inbred lines (RIL) of the model legume, *Medicago truncatula* to identify loci affecting seed mineral concentration, and mineral content per seed. QTL were mapped for average seed weight in an attempt to assess the potential colocalization of this trait with seed mineral concentration and content loci, because a few studies have indicated that the difference in seed mineral concentration between different individuals might simply represent a dilution effect due to seed size (Cakmak et al. 2000; Imtiaz et al. 2003). QTL have been presented for two growth cycles to verify the reproducibility of these QTL. We have also identified potential candidate genes (genes with known or predicted function that could influence seed mineral concentration) in the identified loci, using available *M. truncatula* sequence information.

Materials and methods

Plant material

A set of 175 F6 plants of a *M. truncatula* RIL population named LR4 was obtained by crossing Jemalong-6 × DZA 315.16. Jemalong-6 was derived from the Australian cultivar Jemalong by selfing twice and DZA315.16 was derived from an Algerian ecotype which was also selfed twice (Julier et al. 2007). All the RILs were planted in synthetic soil (two parts Metro-Mix 360; Sungro Horticulture, Bellevue, Washington, USA and one part premium grade Sunshine Strong Lite vermiculite, Bellevue, Washington, USA) and grown in a greenhouse at the USDA-ARS Children's Nutrition Research Center, Houston, TX. Plants were maintained at controlled light and temperature conditions (natural lighting plus supplemental lighting using metal halide lamps with a 15-h day and 9-h night photoperiod, $22 \pm 3^\circ\text{C}$ day/ $20 \pm 3^\circ\text{C}$ night). RILs were grown in 2004 and again in 2006. In 2004, 93 lines were planted in September, and harvested in January 2005 while in 2006 169 lines were planted in January 2006 and harvested in July 2006. Even though the lines were grown under the same greenhouse temperature conditions during both years, there was variation in light intensity due to the different times at which the study was conducted. In 2004, when the lines were planted in September, the average mid-day light intensity of photosynthetically active radiation was $\sim 700 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ and this gradually decreased by January to $\sim 280 \mu\text{mol photons m}^{-2} \text{s}^{-1}$. On the contrary, in 2006 light intensity was $\sim 280 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ in January during planting, while at the time of harvest in July the light intensity was $\sim 900 \mu\text{mol photons m}^{-2} \text{s}^{-1}$. Plants were watered three times daily (automatic drip irrigation system) with nutrient solution containing 1 mM KNO_3 , 0.4 mM

Ca(NO₃)₂, 0.15 mM KH₂PO₄, 0.1 mM MgSO₄, 25 μM CaCl₂, 25 μM H₃BO₃, 2 μM MnSO₄, 2 μM ZnSO₄, 0.5 μM CuSO₄, 0.5 μM H₂MoO₄, 0.1 μM NiSO₄, and 1 μM Fe(III)EDDHA (as Sprint 138; Becker-Underwood, Ames, IA, USA). Three plants of each RIL (together in a single pot) were grown to reproductive maturity. All the pods from the three plants were combined and seeds were separated from the pods for mineral analysis.

Tissue analysis

Pods from each line were collected and seeds were obtained by opening a subset of the pods collected to obtain a total seed mass of ~2 g. All seed samples were dried at 60°C to constant mass. 0.25 g of dried tissue sample was digested using 4 mL of concentrated nitric acid and 2 mL of perchloric acid at temperatures up to 200°C and then taken to dryness. The digests were then resuspended using 2% nitric acid. The acids used were trace metal grade (Fisher Scientific, Pittsburgh, Pennsylvania, USA) and the water was deionized via a MilliQ system (Millipore, Billerica, MA, USA). Samples were analyzed for eight different minerals, Ca, Cu, Fe, Zn, Mg, Mn, K, and P using inductively coupled plasma-optical emission spectroscopy (CIROS ICP Model FCE12; Spectro, Kleve, Germany).

Seed mineral concentration, mineral content per seed, and average seed weights were calculated for all the lines. Average seed weight was obtained by dividing the seed weight of 50 seeds by the number of seeds (50). Mineral content per seed was calculated by multiplying each sample's concentration by the average seed weight.

Marker development for the Jemalong-6 × DZA315.16 RIL population

A framework genetic map of the LR4 population with 374 codominant microsatellite markers was already available (T. Huguet et al., unpublished). In addition, 85 previously mapped SSR markers were selected from the A17 × A20 genetic linkage map (Mun et al. 2006; <http://www.medicago.org/genome/map.php>) to be tested and mapped in the LR4 RIL population, in order to populate chromosomes 2 and 7 with additional markers. These chromosomes were chosen because Fe and Zn QTL had been detected during a pilot run of the quantitative data.

DNA was extracted from 25 mg fresh leaf tissue using DNAzol direct method (Molecular Research Center, OH). For each PCR reaction, 20 nanograms of DNA was used. The PCR reactions were run under standard conditions for all the 90 primers used for screening using *Taq* DNA polymerase with 10X PCR reaction buffer (Clontech, CA, USA) or *Ex Taq* polymerase (Takara Bio USA, WI, USA), 50 μM dNTPs, 0.2 μM of the forward and reverse primer in a 10 μl

reaction. Amplification was initiated with a denaturation step for 10 min at 95°C, followed by 40 cycles each of 30 s denaturation at 95°C, annealing between 45 and 65°C depending on the primer pair (optimum annealing temperatures were used for the respective primer pairs), elongation for 30 s at 72°C, and a final extension step of 6 min at 72°C. All the primers were initially screened using *Taq* polymerase. *Ex Taq* polymerase was used for the primers that amplified non-specific bands with the *Taq* polymerase. Amplified PCR products were separated on 2% agarose gels stained with ethidium bromide. Polymorphism was determined between the parents based on the size of the bands. Markers showing polymorphism between the parental lines (22 out of the 85 tested) were used to screen the entire LR4 RIL population. The PCR products were resolved as described above and all the lines were genotyped as homozygous maternal (Jemalong-6) "A", homozygous paternal (DZA 315.16) "B", heterozygous "H", and missing data "*". Previously mapped markers and the new markers were used to perform the linkage analysis. Linkage analysis was performed using JoinMap version 4.0 (Van Ooijen 2006). Lines with >50% of markers missing were excluded from the analysis. Marker order along each linkage group was obtained using the sequential method in Joinmap. Marker order and distances between markers were calculated using the Kosambi map function.

QTL analysis

A total of 367 of the 396 markers available from the linkage map were used for the QTL analysis. Twenty overlapping markers and nine unlinked markers were excluded for the QTL analysis. WINDOWS QTL CARTOGRAPHER 2.5 (<http://statgen.ncsu.edu/qtlcart/WQTLCart.htm>) (Wang et al. 2006) was used to identify and locate QTLs linked to molecular markers using the composite interval mapping (CIM) method. The genome wide LOD score threshold for QTL detection was determined using the permutation test (1,000 repetitions) at a *P* value of 0.05 for normally distributed data. The LOD score threshold was set between 2.7 and 2.9 for all traits to declare the presence of a QTL. The estimated additive effect and the percentage of variance explained by all the QTL affecting a trait were obtained by the software in the CIM model. Linear regression analysis and correlation coefficients were calculated using SPSS for Windows (Version 10.0, SPSS Inc.). MapChart (Version 2.2) was used to produce the genetic linkage map with all 367 markers used for QTL analysis.

Gene searches

The Medicago genome sequencing site (<http://www.medicago.org/genome>) provides access to physical and genetic

maps, molecular markers, bacterial artificial chromosome (BAC) sequences, genome browsers and annotation. For the mineral-related loci determined in this study, database searches were used to identify BACs that fell within the borders of each locus (i.e., the region defined by LOD scores \geq the LOD significance threshold). The chromosomal location of each BAC was determined on the genetic map provided on the website (<http://www.medicago.org/genome/map.php>). All annotated BACs within the loci of interest were searched (10th June 2008) for known mineral-related genes.

Results

Genetic linkage map

Based on the screening for polymorphism between the parents, 22 new markers were chosen for mapping from the A17 genetic linkage map. As many as 396 codominant microsatellite (SSR) markers including the 22 new markers were used to construct a genetic linkage map for the RIL population which was assigned to 8 linkage groups (Supplementary Fig. 1). The total map length was 756.26 cM with an average distance of 2.25 cM between markers and the largest distance between markers was 31 cM located in LG3. The genetic size of the 8 linkage groups varied between 25 and 102 cM.

Variation and correlation of mineral concentrations among the RIL population

The frequency distributions of all the mineral traits showed continuous phenotypic variation and transgressive segregation (lines with lower values than the lowest parent or higher values than the highest parent) in both directions suggesting multiple gene action. The range for some minerals differed markedly between years. Concentrations and content per seed were higher in 2006 than 2004 for most minerals except Fe and Zn, which had similar values for both years. Seed weight was also similar for both years in the RIL population (Figs. 1, 2). Between the parents, DZA had similar or higher concentrations for Ca, Cu, Fe, Mn, and Zn when compared to Jemalong. DZA had a lower average seed weight (3.15 mg) relative to Jemalong (3.72 mg). Parental values for these traits are presented in Supplementary Table 1. Analysis of seeds collected from replicate plantings of the parents (grown on different carts within the greenhouse) in 2004 indicated that within-parent variability across all traits was low, averaging 2.8%.

Significant positive and negative correlations were observed for seed concentration between several minerals, while only significant positive correlations were observed

between different minerals for content per seed (Tables 1, 2). Some of the minerals were highly correlated while many of them had weak positive correlations. Significant positive correlations were observed between the different macronutrients.

Significant positive correlations were observed between K and P for concentration for both years. Significant positive correlations were observed between the micronutrients, Cu, Fe, Mn, and Zn. Negative correlations were observed for mineral concentrations between K and Mn, and K and Fe and between Cu and Mg. Significant positive and negative correlations were observed between average seed weight and most mineral concentrations (Ca, Cu, Fe, K, Mn, P, and Zn) except for Mg (Table 1).

For content, significant positive correlations were observed between K and P and Ca and P. Correlations between the micronutrients were observed for Mg, Mn, and K for both years. Significant positive correlations were also observed between Cu, Fe, Mn, and Zn.

Identification of seed mineral and seed weight QTL

Quantitative trait loci analysis was performed for all the seed mineral traits in the RIL population using a set of 367 SSR markers. CIM identified a total of 46 significant QTL for mineral concentrations and 24 for mineral content per seed and 3 for seed weight. The percent explained variance for the QTL varied from 5.7 to 42.2%. Colocations of different concentration, content, and seed weight QTL were observed in both years. Tables 3 and 4 and Supplementary Fig. 2 summarize the QTL detected for all minerals.

Four QTL were observed for seed Ca concentration in 2004 with a total explained variance of 49.5% and one in 2006 with an explained variance of 8.9%. Three QTL in 2004 had Jemalong as the contributing parental allele. Three Ca content QTL were observed with a total explained variance of 45.6% in 2004 and two of the QTL (LG1 and 4) had Jemalong as the contributing allele for the trait.

Two Cu concentration QTL on LG4 and 7 with a total explained variance of 37.6% and three on LG1 and 2 with an explained variance of 24.1% were observed in 2004 and 2006, respectively. Three QTL for Cu content were identified in 2004 on LG1, 4, and 6 with an explained variance of 44.9% and Jemalong being the contributing parent while two QTL on LG2 and 3 with an explained variance of 12.5% were identified in 2006. Jemalong was the contributing parent for QTL on LG3 while DZA was the contributing parent for the QTL on LG2.

One Fe QTL with an explained variance of 35.5% was identified on LG7 in 2004. Two QTL were identified in 2006, one on LG7 which overlapped with the QTL from 2004 and the other QTL was located on LG8 with a total explained variance of 21.6%. The contributing parental

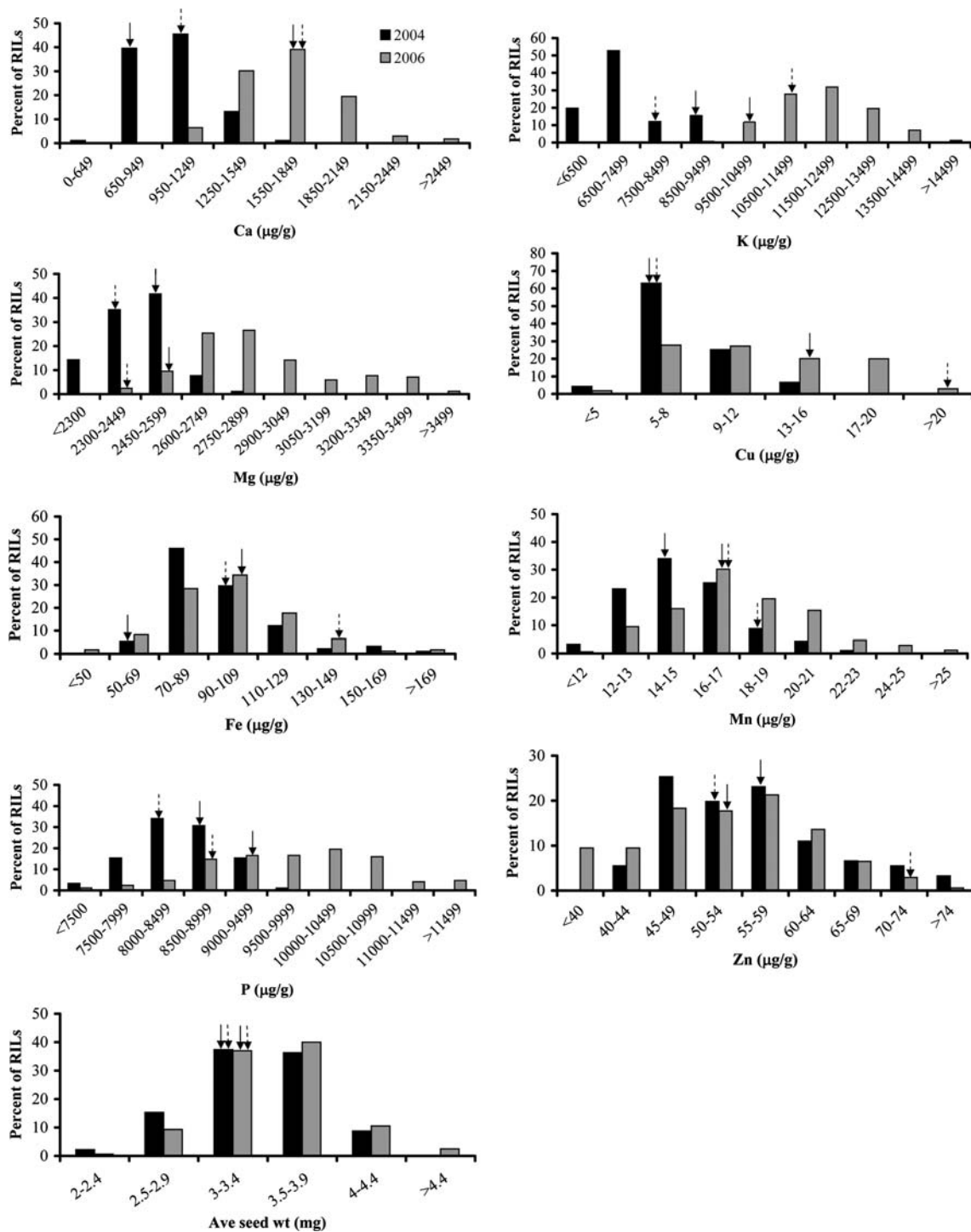


Fig. 1 Frequency distributions of seed mineral concentrations and average seed weight in the Jemalong × DZA RIL population measured during 2004 and 2006. *Arrows* indicate the level of the parental lines:

allele for all three QTL was DZA. No QTL were identified for Fe content in the seeds.

Three K concentration QTL on LG3, 5, and 7 with an explained variance of 33.7% and one QTL on LG8 with a phenotypic variance of 8.9% were identified in 2004 and 2006, respectively. Two QTL for K content with an

solid arrows indicate the Jemalong parent and *dashed arrows* indicate the DZA parent

explained variance of 52.9% were identified in 2004 and none in 2006. All of the QTL except the concentration QTL on LG3 had Jemalong as the contributing parent.

Five QTL for seed Mg concentration explaining 55.7% of the phenotypic variance on LG1, 2, 3, and 8 were identified in 2004, while four QTL were identified in 2006

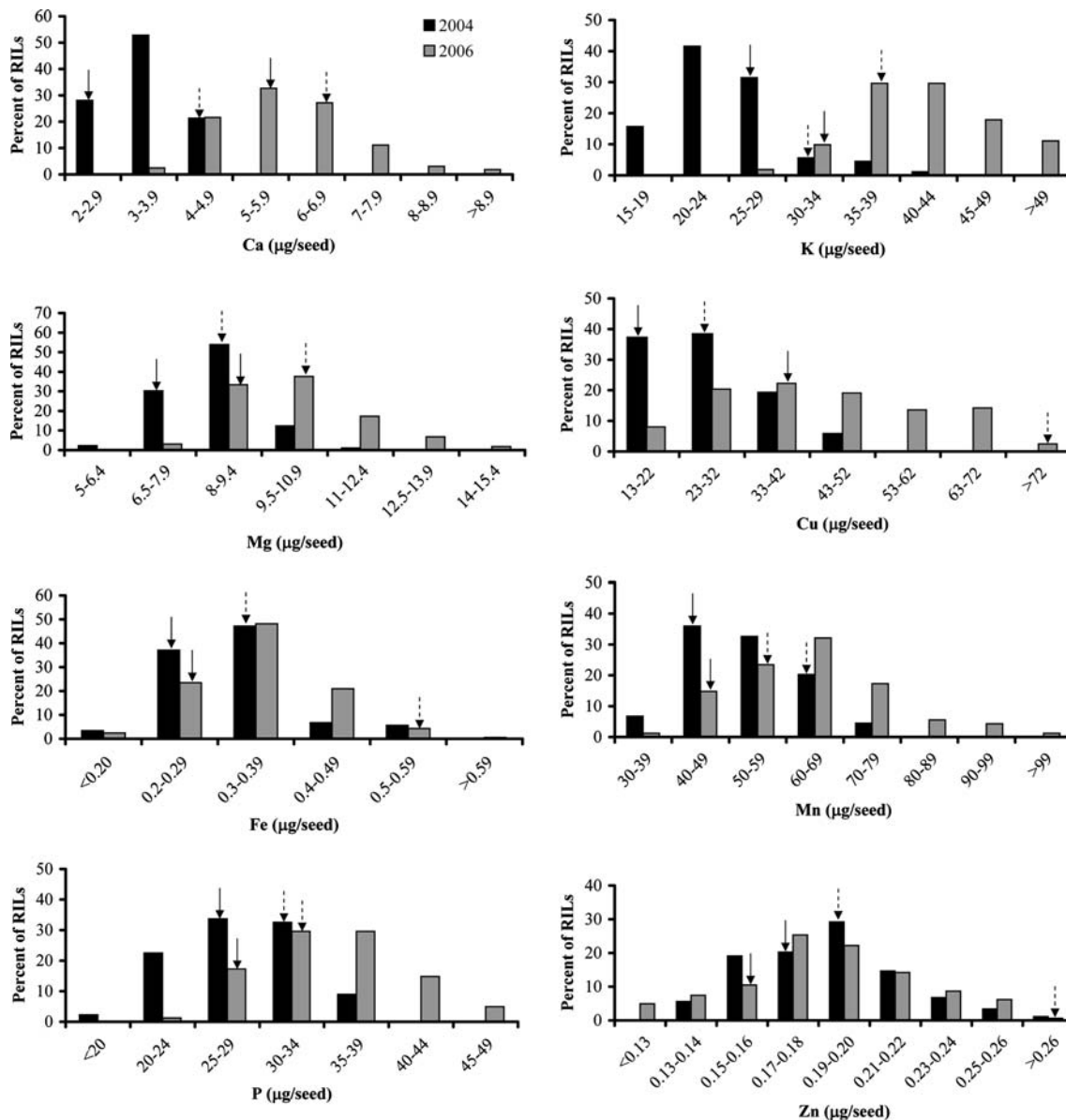


Fig. 2 Frequency distributions of seed mineral content in the Jemalong \times DZA RIL population measured during 2004 and 2006. *Arrows* indicate the level of the parental lines: *solid arrows* indicate the Jemalong parent and *dashed arrows* indicate the DZA parent

located on LG2 and 6 with a total explained variance of 39.3%. One QTL for Mg content was identified on LG7 with a total explained variance of 32.7%. All the QTL except the one on LG3 and one on LG2 had Jemalong as the contributing parent.

Four QTL for Mn concentration were identified on LG1, 2, and 7 explaining 46.3% of the phenotype in 2004 while two on LG2 and 6 explaining 15.2% of the variance were identified in 2006. Four QTL explaining 35.4% of the phenotypic variance were identified on LG2, 3, 5, and 7 for Mn content. QTL on LG1 and 7 for concentration and LG7 for content had Jemalong as the contributing parental allele.

Four QTL for P concentration with a total explained variance of 46.7% on LG1, 7, and 8 and one on LG7 explaining 9.2% of the variance were identified in 2004 and 2006, respectively. Four QTL for P content explaining 75.5% of the explained variance and one on LG7 explaining 9.4% of the variance were identified for P content in 2004 and 2006, respectively. All QTL except the P QTL on LG3 and 5 for content had Jemalong as the contributing parental allele.

Four QTL for Zn concentration with an explained variance of 47.8% and one with an explained variance of 7.2% were identified on LG2, 4, 7, and 8 in 2004 and 2006, respectively. Two QTL for Zn content on LG4 and 5

Table 1 Correlation coefficients between different mineral concentrations and average seed weight in the seeds of the Jemalong-6 × DZA315.16 RIL population

	Ca	Cu	Fe	K	Mg	Mn	P	Zn	Seed weight
Ca		0.34	ns	ns	ns	0.23	ns	0.21	ns
Cu	0.34		0.51	ns	−0.17	0.32	ns	0.45	−0.3
Fe	ns	0.48		−0.21	ns	0.39	−0.17	0.59	−0.25
K	ns	ns	ns		0.54	−0.23	0.52	ns	−0.18
Mg	ns	ns	ns	ns		0.26	0.57	0.2	ns
Mn	0.49	0.48	0.25	ns	ns		ns	0.48	ns
P	ns	ns	ns	0.41	0.39	ns		ns	ns
Zn	0.46	0.61	0.35	ns	ns	0.5	ns		−0.25
Seed weight	−0.46	−0.29	ns	0.25	ns	ns	0.47	−0.37	

Values below the empty diagonal are for 2004 and above the empty diagonal are for 2006

ns Not significant

Significance $P < 0.05$

Table 2 Correlation coefficients between different mineral contents in seeds of the Jemalong-6 × DZA315.16 RIL population

	Ca	Cu	Fe	K	Mg	Mn	P	Zn
Ca		0.32	0.21	0.27	0.42	0.48	0.36	0.37
Cu	0.27		0.44	ns	ns	0.31	ns	0.38
Fe	0.23	0.44		ns	0.3	0.48	ns	0.57
K	ns	ns	0.36		0.81	0.33	0.84	0.27
Mg	0.27	ns	0.41	0.75		0.6	0.84	0.44
Mn	0.5	0.48	0.49	0.53	0.65		0.48	0.62
P	0.26	ns	0.37	0.83	0.94	0.65		0.34
Zn	0.37	0.52	0.46	0.32	0.36	0.61	0.39	

Values below the empty diagonal are for 2004 and above the empty diagonal are for 2006

ns Not significant

Significance $P < 0.05$

explaining 33% of the phenotypic trait and two on LG1 and 3 explaining 16.3% of the variance were observed in 2004 and 2006, respectively. Except for the concentration QTL on LG7 in 2006 and content QTL on LG1, the rest had Jemalong as the contributing parental allele.

Quantitative trait loci for some minerals repeated during both years for seed mineral concentration and content. Common loci were detected for seed mineral concentration and mineral content on LG1, 3, 4, 5, 7, and 8. QTL that colocalized mostly had the same parental allele except on LG3, 7, and 8 which had both the parental alleles for different traits in the same loci. The trait enhancing allele for most QTL was from the Jemalong parent.

Seed weight QTL were identified on LG5, 6, and 7 with a total explained variance of 38.6% in 2005 and 17.8% in 2006. Only QTL on LG7 repeated during both years. Seed weight QTL on LG5 and 7 colocalized with some seed mineral concentration and seed content QTL.

Identification of putative mineral-related genes at loci of interest

By taking advantage of the available sequence information and existing knowledge of mineral-related proteins, we have identified potential candidate genes (genes with known or predicted function that could influence seed mineral concentration) in the loci of interest for further investigation. Sequence search revealed the presence of several orthologs of mineral-related gene families on all the linkage groups (Table 5): cation diffusion facilitator (CDF) gene family; cation exchangers (CAX and CHX); ferric chelate reductase (FRO) gene family; HMA, a heavy metal transporting subfamily of the P-type ATPases; mitochondrial RNA splicing2 (MRS2) gene family; the nicotianamine synthase (NAS) gene family, required for the synthesis of nicotianamine; the oligopeptide transporter (OPT) family; KUP/HAK/KT gene family of potassium transporters; and the yellow stripe-like (YSL) gene family.

Discussion

A number of groups are interested in increasing seed mineral concentrations of plant foods such as rice, wheat, beans, and maize (Pfeiffer and McClafferty 2007). One of our interests is to increase the mineral concentration in legume seeds. In this study, we have identified QTL affecting mineral concentration and content in a RIL population of the model legume, *M. truncatula* in an effort to understand ways to increase the mineral density of important staple crops. For this, a genetic linkage map for the Jemalong-6 × DZA315.16 was constructed using 396 codominant microsatellite markers of which 387 were assigned to 8 linkage groups. The total map length was 756.26 cM and

Table 3 Location and description of QTL affecting seed mineral concentrations and average seed weight in the Jemalong-6 × DZA315.16 RIL population

Trait	Chromosome	Closest marker	Peak (range)	LOD	Explained variance (%) ^a	Effect ^b
2004						
Ca	1	mtic487	115.8 (114–116)	3.0	6.3	J
	1	mtic233	119.5 (116–120)	3.7	9.5	J
	4	mtic244	69.68 (56–79)	8.6	25.8	J
	7	MtB289	85.05 (84–86)	2.9	7.8	D
Cu	4	mtic510	10.69 (2–22)	5.4	16.5	J
	7	mtic1091	72.45 (66–82)	6.6	21.1	D
Fe	7	MTE125	68.71 (59–76)	10.2	35.5	D
K	3	mtic672	58.46 (57–60)	3.2	11.0	D
	5	MTE32	17.07 (14–19)	3.5	12.3	J
	7	mtic246	64.22 (63–66)	3.0	10.4	J
Mg	1	mtic738	110.6 (107–111)	4.0	11.2	J
	1	mtic641	124.1 (123–127)	3.6	9.9	J
	2	mtic1088	24.09 (18–25)	3.0	8.3	J
	3	mtic501	64.91 (58–67)	3.8	10.7	D
	8	mtic681	1.92 (0–11)	5.2	15.6	J
Mn	1	mtic1075	82 (80–84)	3.0	11.3	J
	1	mtic641	124.1 (122–124)	3.4	12.6	J
	2	mtic671	86.71 (82–86)	3.3	10.6	D
	7	mtic1100	58.73 (57–61)	3.7	11.8	J
P	1	mtic637	131.3 (120–131.3)	5.7	11.4	J
	7	mtic432	55.07 (53–58)	5.4	13.3	J
	7	MTE125	68.71 (67–69)	7.6	16.3	J
	8	mtic681	1.92 (0–8)	3.0	5.7	J
Zn	2	mtic589	56.06 (53–60)	3.8	9.8	J
	4	mtic510	10.69 (0–21)	5.5	14.1	J
	7	MtB296	90.44 (88–92)	6.5	16.8	J
	8	mtic113	0.5 (0–6)	3.1	7.1	J
Seed weight	5	Mtic575	94.2 (87–100)	3.6	11	J
	6	MTE40	13.7 (4–15)	3.2	13	J
	7	MTE124	68 (59–71)	4.8	14.7	J
2006						
Ca	8	mtic481	75.14 (72–79)	4.0	8.9	D
Cu	1	mtic1075	82 (80–82)	2.9	6.2	J
	2	h2_32e10d	63.39 (60–65)	3.5	8.1	D
	2	h2_128a17a	72.12 (70–78)	4.5	9.7	D
Fe	7	mtic1181	66.7 (63–67)	3.3	7.3	D
	8	mtic681	3.92 (0–7)	3.9	14.3	D
K	8	MTE58	80.36 (77–81)	3.5	8.9	J
Mg	2	MTE12	18.8 (12–25)	4.0	8.5	J
	6	mtic1109	30.29 (21–30)	5.1	11.5	J
	6	MTE67	48.03 (48–52)	4.5	9.7	D
Mn	6	MTE41	65.83 (61–78)	3.9	9.6	J
	2	mtic149	79.71 (70–82)	3.7	8.6	D
	6	MTE43	91.31 (85–91)	3.1	6.7	J
P	7	h2_8g20b	71.81 (64–72)	4.2	9.2	J
Zn	7	005E07	81.33 (79–83)	3.4	7.2	D
Seed weight	7	Mtic470	65.6 (55–73)	8.3	17.8	J

J indicates that the Jemalong allele at the marker increases the level of minerals, *D* indicates that the DZA allele at the marker increases the level of minerals

^a The amount of phenotypic variation explained by each identified QTL

^b Allelic effect

Table 4 Location and description of QTL affecting seed mineral content in the Jemalong-6 × DZA315.16 RIL population

Trait	Chromosome	Closest marker	Peak (range)	LOD	Explained variance (%) ^a	Effect ^b
2004						
Ca	1	mtic487	115.8 (113–118)	3.2	8.6	J
	4	MTE28	85.4 (75–89)	8.91	27.2	J
	5	mtic617	14.3 (11–15)	3.7	9.8	D
Cu	1	mtic641	124.1 (122–124.1)	3.3	10.6	J
	4	mtic510	10.7 (7–21)	4.3	14.2	J
	6	Mte42	81.5 (78–87)	5.3	20.1	J
K	3	MTE84	47.1 (43–47)	5.4	16.7	J
	7	MTE92	67.6 (65–68)	10.8	36.2	J
Mg	7	MTE92	67.6 (60–81)	11.7	32.7	J
Mn	2	mtic149	79.7 (78–81)	3.4	8.7	D
	3	mtic651	60 (57–61)	3.9	10.4	D
	5	mtic670	65.5 (62–69)	3.5	8.6	D
	7	mtic432	53.1 (52–58)	3.0	7.7	J
P	3	mtic538	51.6 (47–54)	5.0	11.5	D
	5	mtic238	59.6 (55–62)	3.2	6.6	D
	5	mtic60	96.5 (90–100)	6.6	15.2	J
	7	mtic470	65.7 (53–76)	15.1	42.2	J
Zn	4	mtic668	12.4 (1–21)	4.4	16	J
	5	mtic60	97.9 (94–100)	4.9	17	J
2006						
Cu	2	mtic652	14.7 (13–18)	2.5	6.0	D
	3	mtic359	74.7 (73–75)	2.7	6.5	J
P	7	h2_8g20b	71.8 (59–82)	3.9	9.4	J
Zn	1	Mtic726	116.6 (114–118)	2.7	7.0	D
	3	mtic675	62.6 (60–72)	3.6	9.3	J

J indicates that the Jemalong allele at the marker increases the level of minerals, *D* indicates that the DZA allele at the marker increases the level of minerals

^a The amount of phenotypic variation explained by each identified QTL

^b Allelic effect

ranged from 25 to 102 cM spanning across different linkage groups. This map is very reliable for quantitative analysis due to excellent distribution of markers (the average distance between 2 markers is 2.25 cM), small number of unlinked markers and above all, a good number of markers (387) being distributed across the eight linkage groups. This map also compares well with other published *M. truncatula* maps used for other mapping studies (Thoquet et al. 2002; Julier et al. 2007).

Genetic variation in the population is essential for achieving significant improvements in mineral content. Such variation can be utilized to identify QTL associated with mineral concentration in seeds and in turn can help in identifying rate limiting steps and different genes involved in mineral movement to different tissues. Significant genetic diversity in mineral concentrations has been reported for a number of crop species such as wheat, rice, maize, cassava and beans (Beebe et al. 2000; Chavez et al. 2000; Ortiz-Monasterio and Graham 2000; Gregorio et al. 2000; Ozkan et al. 2007). Transgressive segregation was observed for all the minerals (concentration and content) and for seed weight, suggesting that these are quantitatively

inherited traits and they are controlled by multiple genes. A large genetic variation was also observed for most minerals in this population even though the two parental lines chosen were not very different for most traits (except for Fe, whose parental concentration differences were noted in a preliminary study and were the basis for choosing this population). For most minerals, concentration and content were higher in 2006 when compared to 2004. The variations between the 2 years (two different environments) were large for some minerals possibly due to different environmental conditions, emphasizing the importance of environmental factors on quantitative traits (Figs. 1, 2).

Different traits in a population can be positively or negatively correlated due to different factors such as linkage, pleiotropy or environmental effects (Aastveit and Aastveit 1993; Paterson et al. 1988). Correlation between different mineral traits in the RIL population might indicate cosegregation of genes for traits. Several minerals were positively correlated with each other, possibly pointing to common pathways, or to common transporters controlling the uptake and movement of these minerals in different tissues. Significant correlations were found between different macro and

Table 5 Putative mineral-related gene orthologs identified in different linkage groups of *Medicago truncatula*

Linkage group	Mineral QTL	BAC accession	Gene orthologs	Species
1	Ca, Cu, Mg, Mn, P, Zn	AC149805	<i>YSL3</i>	<i>Arabidopsis thaliana</i>
2	Cu, Mg, Mn, Zn	AC123898	<i>OPT2</i> ; <i>MRS2</i>	<i>A. thaliana</i>
2	Cu, Mg, Mn, Zn	AC135102	<i>FRO6</i>	<i>A. thaliana</i>
2	Cu, Mg, Mn, Zn	AC152185	<i>NAS3</i>	<i>A. thaliana</i>
3	Cu, K, Mg, Mn, P, Zn	AC147002	<i>MTPC3</i>	<i>A. thaliana</i>
3	Cu, K, Mg, Mn, P, Zn	AC149303	<i>CHX</i>	<i>A. thaliana</i>
4	Ca, Cu, Zn	AC144564	<i>OPT2</i>	<i>A. thaliana</i>
4	Ca, Cu, Zn	AC153000	<i>MRS2</i>	<i>A. thaliana</i>
4	Ca, Cu, Zn	AC140029	<i>MRS2</i>	<i>A. thaliana</i>
4	Ca, Cu, Zn	AC151817	<i>MRS2</i>	<i>A. thaliana</i>
5	Ca, K, Mn, P, Zn	AC144459	<i>KUP7</i>	<i>A. thaliana</i>
6	Cu, Mg, Mn	AC146910	<i>HMA</i>	<i>A. thaliana</i>
7	Ca, Cu, Fe, K, Mg, Mn, P, Zn	AC137986	<i>CAX7</i>	<i>A. thaliana</i>
7	Ca, Cu, Fe, K, Mg, Mn, P, Zn	AC159089	<i>HMA</i>	<i>A. thaliana</i>
7	Ca, Cu, Fe, K, Mg, Mn, P, Zn	AC148343	<i>CHX</i>	<i>A. thaliana</i>
7	Ca, Cu, Fe, K, Mg, Mn, P, Zn	AC135465	<i>HMA</i>	<i>A. thaliana</i>
8	K, Mg, P, Zn	AC136841	<i>HMA</i>	<i>A. thaliana</i>
8	K, Mg, P, Zn	AC144721	<i>HMA</i>	<i>A. thaliana</i>
8	K, Mg, P, Zn	AC140032	<i>HMA</i>	<i>A. thaliana</i>
8	K, Mg, P, Zn	AC146708	K ⁺ transporter	<i>Medicago truncatula</i>
8	K, Mg, P, Zn	AC152057	<i>HAK5</i>	<i>A. thaliana</i>
8	K, Mg, P, Zn	AC135229	<i>HMA5</i>	<i>A. thaliana</i>
8	K, Mg, P, Zn	AC135313	<i>HMA2</i>	<i>A. thaliana</i>

micronutrients (Tables 1, 2). For example, significant positive correlations were observed between Cu, Fe, Mn, and Zn concentrations (Table 1). Similar results for Fe and Zn have been reported by Peterson et al. (1986), Graham et al. (1999), Cakmak et al. (2004) and Ozkan et al. (2007) for wheat and for bean by Beebe et al. (2000). Correlations among micronutrients indicate that improvement in one micronutrient may simultaneously improve the concentration of other micronutrients (Ozkan et al. 2007). Significant positive and negative correlations were also observed between mineral concentrations and average seed weight (Table 1). Similar to the study by Moraghan and Grafton (2001) comparing seed weight and mineral concentration, a significant negative correlation was observed between Ca concentration and seed weight and a positive correlation between P concentration and seed weight. Negative correlations were also observed between Cu and Zn concentration and seed weight in 2005. The rest of the significant correlations were positive suggesting that seed weight might have a “dilution effect” on concentration, thus demonstrating the importance of reporting mineral content in the context of improving the mineral density in seeds.

Several QTL for different minerals were identified for concentration, content and average seed weight (Tables 3, 4; Supplementary Fig. 2). Most traits had more than one

QTL localized in different regions of the genome contributing to a small percentage of the phenotypic variation. This suggests that the transport and accumulation of minerals in seeds is a complex trait involving several genes. Several mineral QTL colocalized with each other for both concentration and content and several of these also colocalized with seed weight QTL. In particular, most of the micronutrients analyzed colocalized with each other. Most striking was the colocalization of Cu, Fe, Mn, and Zn on linkage group 7 and Cu, Mg, Mn, P, and Zn on linkage group 1. These loci might point to a common transporter such as a ZIP gene family member, which is capable of transporting both Mn and Zn in addition to Fe, or to a synthetic protein for nicotianamine, a metal chelator involved in Fe, Zn, Cu, and Mn homeostasis (Curie and Briat 2003; Delhaize et al. 2003; Higuchi et al. 1999; Guerinot 2000).

The regulation of metal homeostasis is complex, requiring transport proteins located in different membranes as well as long distance transport systems to move the nutrients throughout the plant. These short and long distance systems are tightly controlled by several metal specific and non-metal specific genes. Putative genes related to mineral transport or homeostasis that were located in the vicinity of identified QTL have been listed in Table 5.

For the macronutrients, our search has revealed the presence of mitochondrial RNA splicing2 (*MRS2*) gene family members. *MRS2* is involved in the movement of Mg^{2+} in plants (Gardner 2003). Putative *MRS2* genes were located on LG2 and 4. Various cation exchangers, CHX (Cation/H⁺ exchanger), CAX (Ca^{2+} cation antiporter), and KUP and HAK of the KUP/HAK/KT family of potassium transporters were also identified on LG3, 5, 7, and 8 (Maser et al. 2001; Gierth et al. 2005).

Proteins from several gene families involved in the transport of micronutrients were identified. Heavy metal transporting P_{1B} -ATPases (HMAs) are responsible for the transport of a variety of cations across cell membranes. In *Arabidopsis*, eight members of the type1B ATPases (HMA1–HMA8) have been identified. HMA1, HMA2, HMA3, and HMA4 are involved in the transport of divalent cations. HMA2, HMA3, and HMA4 have been reported to be important in the root to shoot translocation of Zn while HMA5, HMA7, and HMA8 are monovalent Cu transporters (Hussain et al. 2004; Andrés-Colás et al. 2006). Several putative *HMA* genes were located on LG6, 7, and 8 within Cu, Fe, and Zn QTL.

Nicotianamine is a metal chelator that is required for the homeostasis of Fe and other micronutrients. Nicotianamine and nicotianamine synthase (NAS) play key roles in iron nutrition, and the regulation of the *NAS* gene is required for both Fe acquisition and distribution (Colangelo and Gueriot 2006). An *NAS* gene whose product is similar to AtNAS3 was identified on LG3. The *YSL* gene family encodes metal-nicotianamine transporters (DiDonato et al. 2004). A putative *YSL* gene was identified on LG1 within Cu and Zn QTL; this gene's product showed homology to AtYSL3. AtYSL1 and AtYSL3 have been shown to be required for the delivery of metal micronutrients to and from vascular tissues (Waters et al. 2006). *ysl1ysl3* double mutants in *Arabidopsis* have been shown to over accumulate Cu and Zn in leaves (Waters and Grusak 2008a).

A putative member of the ferric reductase oxygenase (*FRO*) gene family, whose product shows homology to AtFRO6, was identified on LG2. AtFRO6 is expressed in shoots and flowers and is required to maintain Fe homeostasis in shoot tissues (Wu et al. 2005). A homolog of *AtMTPc3*, a gene belonging to the CDF family with a role in Zn transport (Broadley et al. 2007), was identified on LG3 within a Zn QTL.

An *OPT* gene encoding a protein similar to AtOPT2 was identified on LG2. OPTs (oligopeptide transporters) are involved in the transport of peptides and nicotianamine-metal chelates (Vasconcelos et al. 2008). Several OPTs have been identified in *Arabidopsis* and rice (Koh et al. 2002; Vasconcelos et al. 2008); analysis of their expression patterns has revealed distinct tissue-specificity, including expression both in roots (site of mineral absorption) and

source tissues such as leaves (sites of mineral remobilization and/or transit).

The presence of these putative mineral-related genes on linkage groups with several mineral QTL suggests that these genes are good candidates explaining the transport and accumulation of different minerals in seeds, and are worthy of further investigation.

A number of factors such as genetic variation in individuals, environmental variation, population size, number of markers and experimental error can influence the detection of QTL in a segregating population (Collard et al. 2005). One of the goals of this study was to determine the effect of seed size on seed mineral concentration. The relationship between seed concentration and seed size is of importance when trying to improve the nutritional content of legume seeds because the variation in concentration may simply represent a dilution effect due to size or weight variation. Reporting mineral levels based on content in seeds can partially remove the masking effect of seed size (Pfeiffer and McClafferty 2007). In our study, we determined the mineral content per seed in order to assess the phenotypic correlation between seed weight and mineral concentration. Both positive and negative correlations between seed weight and different mineral concentrations were observed. In our study, for example, K and P concentration QTL on linkage group 7 overlapped with K and P seed content QTL and Jemalong was the contributing parental allele for both these QTL. This overlap of concentration and content QTL might suggest that variation in concentration is probably not influenced by seed weight but probably due to other transport processes. On the other hand, the occurrence of minerals that had concentration QTL overlapping with seed weight QTL could suggest that seed concentration might be indirectly affected by variations in seed size. Thus, our results suggest that seed size is an important determinant in seed mineral concentration and further stresses the need to focus both on seed mineral content and concentration when trying to improve the nutritional quality of seeds.

Environmental factors have a large effect on QTL detection. In our study, we replicated the experiments over 2 years but during different times of the year, which resulted in different environmental conditions. During 2004, the lines were planted in fall and in 2006 the lines were planted in spring. Due to the difference in timing, there were variations in light intensity in the greenhouse. During fall there was a decrease in light intensity when the plants were in the seed set stage while the reverse was true in 2006. This difference in light intensity could be one of the reasons for the QTL differences observed between the 2 years. Another reason could be that different population sizes were used in 2004 and 2006. Even though some of the QTL in 2004 accounted for a relatively large amount of variance (>10%) they were not stable across different envi-

ronments and were lost in 2006. The observed inconsistency in our study could be due to the difference in population size between the 2 years or altered gene expression in response to environmental pressure (Beavis 1994). For example, QTL for Zn on LG4 with explained variances of 13 and 14% were lost in 2006. Inconsistent QTL across different environments is not uncommon (Paterson et al. 1991). This inconsistency between different environments also indicates that mineral transport and redistribution from the roots to seeds requires multiple steps and is truly quantitative.

In summary, several QTL have been identified for both mineral concentration and mineral content, with some of these being identified in both years, thereby indicating that these QTL are robust and can be targeted for fine mapping genes of interest. In fact, a number of mineral-related genes were found to reside within these QTL; they will serve as primary candidates for further studies aimed at developing biofortified crops. Several significant QTL were found in this study, even with the use of a RIL population (LR4) whose parents were not highly variable for all the traits of interest. This suggests that existing RIL populations in agronomic species, including those populations developed for non-mineral traits (e.g., yield, disease resistance, abiotic stresses), could also be exploited for the identification of seed mineral QTL.

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