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ORIGINAL PAPER

# QTL for seed iron and zinc concentration and content in a Mesoamerican common bean (*Phaseolus vulgaris* L.) population

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**Abstract** Iron and zinc deficiencies are human health problems found throughout the world and biofortification is a plant breeding-based strategy to improve the staple crops that could address these dietary constraints. Common bean is an important legume crop with two major genepools that has been the focus of genetic improvement for seed micronutrient levels. The objective of this study was to evaluate the inheritance of seed iron and zinc concentrations and contents in an intra-genepool Mesoamerican × Mesoamerican recombinant inbred line population grown over three sites in Colombia and to identify quantitative trait loci (QTL) for each mineral. The population had 110 lines and was derived from a high-seed iron and zinc climbing bean genotype (G14519) crossed with a low-mineral Carioca-type, prostrate bush bean genotype (G4825). The genetic map for QTL analysis was created from SSR and RAPD markers covering all 11 chromosomes of the common bean genome. A set of across-site, overlapping iron and zinc QTL was discovered on linkage group b06 suggesting a possibly pleiotropic locus and common physiology for mineral uptake or loading. Other QTL for mineral concentration or content were found on linkage groups b02, b03, b04, b07, b08 and b11 and together with the b06 cluster were mostly novel compared to loci found in previous studies of the Andean genepool or inter-genepool crosses. The discovery of an important new locus for seed iron and zinc concentrations may facilitate crop improvement and biofortification using the high-mineral genotype especially within the Mesoamerican genepool.

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## Introduction

Iron and zinc deficiencies are among the most common nutritional deficiencies in human beings and both iron and zinc are important in people's growth and development due to their essential role as co-factors in critical proteins, such as hemoglobin, cytochromes and transcription factors (Welch and Graham 1999). Several options for solving the problem of iron and zinc deficiency are to increase the supply of these minerals in the diet, either through supplementation, fortification or biofortification (Welch 2002; Bouis 2003).

Biofortification consists in improving the concentration of essential minerals in the edible portions of commonly consumed crops, such as common bean or *Phaseolus vulgaris* (Graham et al. 2001; Welch and Graham 2004). This process has been initiated for seed iron and zinc in various

classes of common beans and has concentrated on identifying sources of high minerals and using them in a plant-breeding strategy (Beebe et al. 2000). Essential to this effort has been the analysis of the inheritance of seed mineral concentration and crosses within and across genepools to transfer the genes for high iron and zinc (Blair et al. 2009a; Cichy et al. 2009).

Common bean varieties can be classified into two major genepools within the species based on their overall seed size and origin in terms of domestication and center of diversity (Singh et al. 1991). One domesticated genepool, the Mesoamerican, is from Central America and Mexico and has small to medium-sized seed while the other, the Andean, is from South America and generally has large seed. The differences between the genepools are also reflected in their protein (isozymes and phaseolin) and molecular marker profiles (Blair et al. 2009b). Common bean varieties can also be distinguished by their growth habit with variability ranging from determinate or indeterminate bush beans to climbing beans. Common beans are self pollinating and have a small genome and simple diploid inheritance, which makes them excellent subjects for genetic analysis.

Nutritional quality in common beans has been found to be high, with large amounts of minerals and vitamins provided by the seed in conjunction with a background of high percentage protein, 'slow digestion' complex carbohydrates and low oil content (Broughton et al. 2003). Iron and zinc concentration of common bean and other legume seeds are higher than in the cereals and are generally retained through harvest and processing unlike for milled grains (Wang et al. 2003; Beebe et al. 2000).

Iron and zinc are taken up by the plant's roots from the soil and transferred through vascular transport and partitioning mechanisms to the seeds with all of these processes influenced by transporters and storage reserves (Frossard et al. 2000; Grusak 2002). Common bean is a strategy I plant and therefore uses rhizosphere acidification, with iron reductase for iron reduction and an iron transporter for cross-membrane root uptake of the mineral (Marschner and Römheld 1994; Briat and Lobreaux 1997). Once iron and zinc are taken up into the plant root's epidermal cells, various metal transporters are involved in movement throughout the plant (Grotz and Guerinot 2006). The minerals are then used for vegetative growth, where iron homeostasis is mediated by ferritin, an iron storage protein (Briat and Lobreaux 1997). During reproductive phases minerals are remobilized to seeds (Frossard et al. 2000).

The inheritance of nutrition traits appears to be mostly quantitative and only somewhat influenced by the environment, but varies depending on the source genotype (Guzman-Maldonado et al. 2003; Cichy et al. 2005, 2009; Blair et al. 2009a). Andean and Mesoamerican genotypes generally differ in seed mineral concentration with Mesoamerican beans having lower concentrations of iron than Andean beans, but higher zinc levels (Islam et al. 2002). Therefore, biofortification for increased iron without sacrificing zinc is challenging in Mesoamerican beans and hence the genetic analysis of the seed mineral trait in this genepool is a priority. In addition, most breeding programs for beans work within a genepool along commercial class lines and therefore an understanding of the inheritance of nutritional traits in each genepool and differences between genepools for these traits is a necessary first step for improving common beans for micronutrient quality.

The objective of this study was to determine which QTL control seed iron and zinc accumulation in the Mesoamerican genepool specifically through the analysis of a Mesoamerican × Mesoamerican cross. Previous studies had only analyzed inter-genepool or Andean crosses, hence the goal of this study was to determine which QTL are important in the Mesoamerican genepool and especially when combined with the Carioca commercial class, which is the predominant bean grown in Brazil, is the largest producer of common beans in the world. Specific objectives were to identify which QTL control both mineral concentration and per seed mineral content in the population.

## Materials and methods

### Plant materials

A population of 110 recombinant inbred lines (RILs) in the F10 generation was developed from the cross G14519 × G4825 at the International Center for Tropical Agriculture (CIAT). The parents were identified by Islam et al. (2004) as highly contrasting genotypes for seed iron and zinc concentration based on a survey of the CIAT core collection. Both parents are from the Mesoamerican genepool and the cross was of an intra-genepool type as classified by Blair et al. (2006a) based on a molecular marker survey. G14519 also known as 'Hickman pole bean' is a high seed iron and zinc landrace from the United States with medium brown-colored seed with type IVa climbing bean growth habit. G4825 also known as 'Carioca' is a type III bush bean landrace variety from Brazil which has low concentrations for both minerals and small seed that is cream colored with brown stripes. The population was created by advancing F1 plants to the F2 generation and then the population was advanced through single seed descent from the F2 to the F8 generation. The individual F8 selections were planted for seed increase to the F10 generation which was used for the experiments.

## Experimental sites

The population was analyzed over three locations in Colombia: (1) Darién, Valle which is at 1,400 m above sea level (masl) elevation and has an average yearly temperature of 20°C, annual rainfall of 1,500 mm and an Udand soil type with pH of 5.6; (2) Palmira, Valle which is at 1,000 masl elevation and has an average yearly temperature of 24°C, annual rainfall of 905 mm and a Haplustoll soil type with pH 7.8; and (3) Popayán, Cauca which is at 1,730 masl elevation and has an average yearly temperature of 18°C, annual rainfall of 2,124 mm and a Dystrudepts soil type with pH of 6.1. Soil iron and zinc concentrations for the sites were determined using three soil samples from the top 15 cm collected in different parts of the fields used in each site that were dried, sifted and mixed thoroughly before extraction with HCl (0.05 M) and $H_2SO_4$ (0.0125 M) according to the methods in Page (1982). Analysis of the extracted soil samples was then performed with atomic absorption spectroscopy and values were estimated by comparison to known standards for each mineral.

Although the Palmira site had no major soil fertility problems, the sites in Darién and Popayán had low phosphorus and were fertilized with 60 kg of P $ha^{-1}$ as superphosphate. For the experiments in Popayán and Darién, the trials consisted in randomized complete block designs with 3 and 2 replications, respectively, while the experiment in Palmira was a single replicate of the same lines in the other two sites. All the experiments were planted with trellis supports since the population is predominantly made up of climbing bean genotypes. Pests and pathogens were controlled in all locations, and plots were hand harvested to avoid contamination by metal machinery.

## Mineral analysis

A sub-sample of 10 seeds were picked at random from the bulk seed harvest of each plot and were washed with sterile water and then dried in an oven for 2 days at 45°C before grinding to a fine flour with zirconium grinding balls in a modified paint shaker using Teflon chambers. These chambers and balls were chosen to be free of iron so as to avoid mineral contamination. The seed was randomly selected from the full plot rather than pods from one plant to ensure as much sampling of the plot as possible.

One principal method of mineral analysis was then implemented for all three experiments, namely atomic absorption spectroscopy (AAS). Sample preparation for AAS involved separating 0.5 g of the bean flour, digesting with nitric/perchloric acid (5 mL of a 2:1 mixture of 65% nitric acid ($HNO_3$) and 70% perchloric acid ($HClO_4$)) for 2 h followed by a heat treatment for 2 h and re-suspension in 25 mL of de-ionized water. The resulting samples were analyzed in a UNICAM969 mass spectrometer with acetylene flame as described in Blair et al. (2009a).

A second method, namely inductive coupling plasma (ICP) was used to confirm mineral analysis for the Popayán harvested seed. Sample preparation for ICP also involved digesting the bean flour with nitric/perchloric acid, but followed this with analysis on an ARL 3580 ICP with optical emission spectroscopy as described in Blair et al. (2009a). The AAS technique was carried out in the CIAT analytical services laboratory while the ICP technique was carried out at the Waite laboratory of the University of Adelaide. In addition to analyzing mineral concentrations, the 100 seed weight of grain from each RIL was determined so as to evaluate seed content of each mineral.

## Genetic mapping

DNA was extracted for each of the RILs with the method of Mahuku (2004) followed by a chloroform:octanol (24:1) cleanup, isopropanol precipitation and ethanol (70%) wash. DNA extraction was based on young trifoliate leaf tissue that was ground in liquid nitrogen. The resulting purified DNA was re-suspended in TE buffer, quantified in a Hoefer DyNA Quant 2000 fluorometer and used for all marker analysis as described below at a concentration of 5 ng/ul. A total of 573 simple sequence repeat (SSR) markers were surveyed for polymorphism on the parents of the populations, these included genomic and genic markers described in Blair et al. (2006a), the AT-rich microsatellites from Blair et al. (2008), the cDNA-derived microsatellites from Blair et al. (2009b) and the small insert derived microsatellites from Buso et al. (2006) and from Blair et al. (2009c).

In addition, 10 randomly amplified polymorphic DNA (RAPD) primers from Islam et al. (2002) were analyzed on the RILs. Amplification and band detection for these DNA-based markers were as described in these previous studies. Polymorphisms and segregation patterns were scored for each of the markers and bands and a genetic map was constructed using Mapmaker 3.0 (Lander et al. 1987) and a minimum LOD of 3.0. The Kosambi function was used to convert recombination fractions into genetic distances in centiMorgans (cM). Linkage groups were oriented and named according to Blair et al. (2003) based on the comparative map position of microsatellite loci.

## QTL analysis

QTL were detected with composite interval mapping (CIM) analysis using QTL Cartographer v. 2.5 (Wang et al. 2007) for mineral concentration, content and seed weight data and with single point analysis (SPA) using qGENE (Nelson 1997) for mineral content and seed weight and in all cases using the newly constructed genetic map as described

above. The following parameters were used for CIM analysis: 10 cM window size, 1 cM walkspeed, 5 significant background markers, analysis by forward and backward multiple linear regression for each chromosomal position with a global significance level of 5% and probability thresholds of 0.05 for the partial $F$ test for both marker inclusion or exclusion. In the CIM analysis, determination coefficients were calculated for each interval separately ($R^2$) and for each interval given the background markers ($TR^2$) to determine the phenotypic variance explained by a single QTL (either alone or in conjunction with all other significant intervals). In the case of the CIM analysis, likelihood ratio (LR) thresholds for the QTL for each trait were determined by the generation of 1,000 permutations of the data for that trait and regions surpassing that threshold were considered to be significant (Churchill and Doerge 1994). In the case of the SPA analysis, probability thresholds of $P < 0.05$ and $P < 0.01$ were used for significant marker associations in the regression analysis.

## Results

### Population distributions and parental differences

Population distributions of RILs for seed iron and zinc concentrations measured in µg/g or parts per million (ppm) were continuous in all the trial sites and for each of the analytical methods showing a wide range of values for all locations and quantitative inheritance for both mineral traits (Fig. 1). The parents were highly contrasting for both minerals with G14519 averaging 80 and 34 ppm, respectively, for seed iron and zinc AAS values over all sites and G4825 averaging 46 ppm iron and 25 ppm zinc over all sites. Transgressive segregation was not very evident as the parents of the populations were mostly at the extremes of the distribution. This was especially the case for iron concentration; however, for zinc concentration the low-mineral parent tended to be middle of the range, while the high-mineral parent was at the edge of the distribution in Darién and Palmira. Normal distributions were observed to be significant for most of the combinations of mineral concentration, location and method; however, some skewing toward the low-mineral parent G4825 was observed for iron and zinc especially in Darién. Among the sites, the RILs grown in Palmira had a higher average in iron concentration than Darién and Popayán as measured with AAS while zinc was lower both in Palmira and in Darién and higher in Popayán with this method (Table 1). For the alternative methods of analysis, the average iron and zinc concentrations in Popayán for ICP were higher than for AAS at the same site, although the total ranges in values between the highest and lowest iron and zinc genotypes were similar.

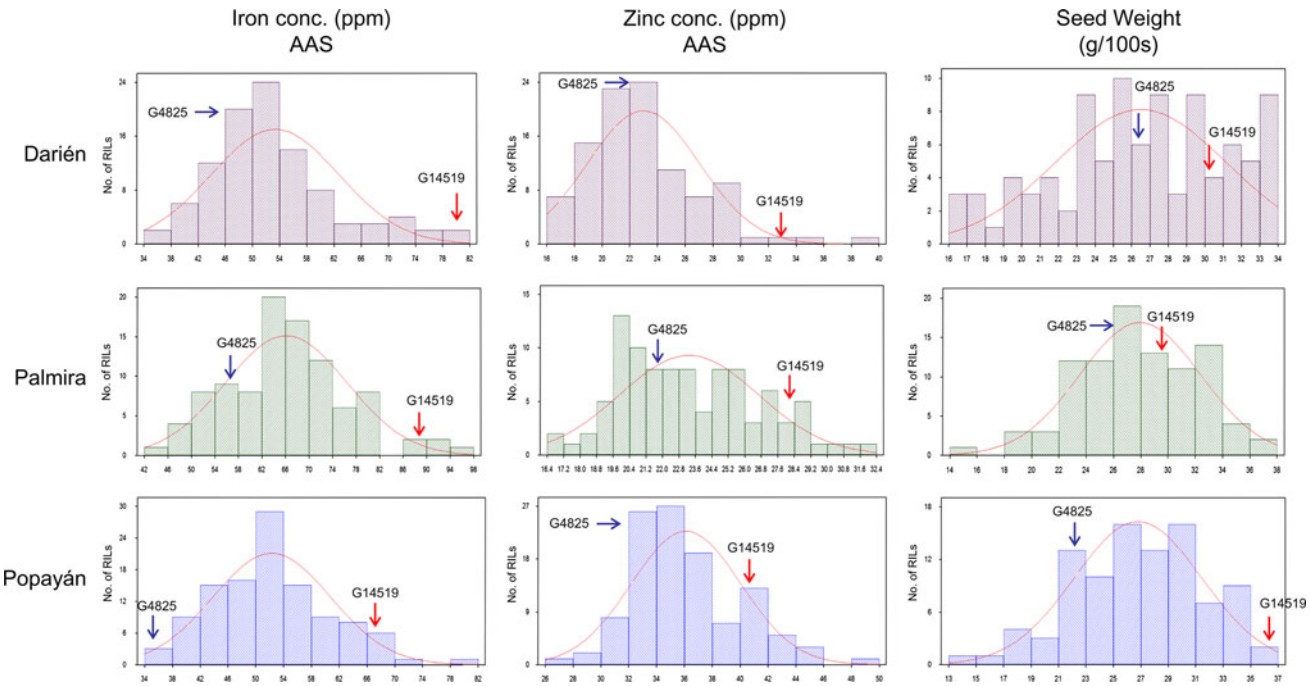


**Fig. 1** Population distributions for seed iron and zinc concentrations measured in parts per million (ppm or µg/g) with atomic absorption spectroscopy (AAS) and seed weight measured in grams for 100 seed in the G14519 × G4825 recombinant inbred lines grown over three locations (Darién, Palmira and Popayán). Parental mineral values are indicated by *arrows*

**Table 1** Descriptive statistics for seed iron and zinc concentrations (in μg/g) and content (in μg/seed) in the G14519 × G4825 recombinant inbred line population

| Trait | Location | Population Mean | Population SD | Population Min | Population Max | Skew | Kurtosis | Normality test (*W*)[a] |
|---|---|---|---|---|---|---|---|---|
| Iron concentration: AAS | Popayán | 52.3 | 8.5 | 35.7 | 81.2 | 0.6 | 0.5 | 0.973* |
| Iron concentration: ICP | Popayán | 59.4 | 7.2 | 44.0 | 77.0 | 0.3 | −0.2 | 0.987 ns |
| Iron concentration: AAS | Darién | 53.4 | 9.0 | 35.4 | 80.9 | 0.8 | 0.7 | 0.948** |
| Iron concentration: AAS | Palmira | 66.0 | 10.2 | 43.4 | 97.1 | 0.4 | 0.3 | 0.984 ns |
| Zinc concentration: AAS | Popayán | 36.0 | 4.0 | 27.3 | 49.5 | 0.7 | 0.4 | 0.961* |
| Zinc concentration: ICP | Popayán | 38.8 | 4.1 | 30.0 | 49.0 | 0.2 | −0.4 | 0.984 ns |
| Zinc concentration: AAS | Darién | 22.9 | 3.9 | 16.8 | 39.5 | 1.2 | 2.5 | 0.924** |
| Zinc concentration: AAS | Palmira | 23.3 | 3.4 | 16.6 | 32.2 | 0.5 | −0.4 | 0.970* |
| Iron content: AAS | Popayán | 13.9 | 3.4 | 8.3 | 28.6 | 1.1 | 2.6 | 0.937*** |
| Iron content: ICP | Popayán | 15.6 | 3.4 | 8.9 | 26.1 | 0.6 | 0.2 | 0.971* |
| Iron content: AAS | Darién | 11.8 | 2.3 | 7.0 | 17.5 | 0.1 | −0.3 | 0.987 ns |
| Iron content: AAS | Palmira | 18.3 | 3.7 | 10.7 | 32.2 | 0.7 | 1.5 | 0.962* |
| Zinc content: AAS | Popayán | 9.6 | 2.1 | 5.2 | 17.1 | 0.5 | 0.8 | 0.981 ns |
| Zinc content: ICP | Popayán | 10.3 | 2.1 | 5.8 | 18.2 | 0.5 | 0.9 | 0.978 ns |
| Zinc content: AAS | Darién | 14.4 | 5.2 | 4.0 | 26.6 | 0.3 | −0.6 | 0.976 ns |
| Zinc content: AAS | Palmira | 6.5 | 1.3 | 3.4 | 10.1 | 0.3 | 0.4 | 0.986 ns |

Population mean, minimums and maximums as well as skewness and kurtosis and results from normality test results (*W*) are presented for each combination of mineral, location and analytical technique

*AAS* atomic absorption spectroscopy, *ICP* inductively coupled plasma–optical emission spectroscopy

[a] Level of significance corresponding to $P < 0.05$ (*) and $P < 0.01$ (**)

## Correlations between methods and sites

Despite the differences in average mineral concentrations, the correlations between AAS and ICP for the set of RILs were very high and significant ($r = 0.750$ and 0.724 for iron and zinc, respectively, $P < 0.001$). Correlations between the three sites for mineral concentrations in RILs using AAS (Table 2) were intermediate to high ranging from 0.474 to 0.562 for iron and 0.420 to 0.526 for zinc; all being highly significant ($P < 0.001$). Meanwhile, the correlations between iron and zinc in RILs were high ranging between $r = 0.483$, 0.636 and 0.683 for the AAS method at the three sites of Palmira, Popayán and Darien, respectively and up to $r = 0.736$ for the RILs with the ICP method for Popayán, with all of these being highly significant ($P < 0.001$). Correlations of ICP in Popayán and AAS in the other two sites were also significant ranging from $r = 0.400$ ($P < 0.01$) to 0.590 ($P < 0.001$).

**Table 2** Correlations between locations and mineral detection methods for seed iron and seed zinc concentrations and between minerals (in μg/g) within each location in the G14519 × G4825 recombinant inbred line population

| Location | Iron versus Iron | | | Zinc versus Zinc | | | Iron versus zinc |
|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 1 | 2 | 3 | |
| 1. Darien: AAS | 1.000 | – | – | 1.000 | – | – | 0.683 |
| 2. Palmira: AAS | 0.562 | 1.000 | – | 0.526 | 1.000 | – | 0.483 |
| 3. Popayan: AAS | 0.485 | 0.474 | 1.000 | 0.420 | 0.494 | 1.000 | 0.636 |

## Genetic map construction

The final genetic map for the G14519 × G4825 population was constructed with a total of 68 SSR loci and 46 RAPD markers (Table 3). The total polymorphism was 11.9% for SSR markers based on the screening of 573 primer pairs while the RAPD markers generated an average of 4.6 segregating bands per reaction. The total number of markers included in the map was 114 with a total map coverage of 11 linkage groups which is equivalent to the haploid chromosome number in common beans (Blair et al. 2003). The map had a total genetic distance of 915.4 cM, an average length between markers of 8.0 cM and an average length per linkage group of 83 cM. Meanwhile, the length of the individual linkage groups was higher than the average for b02, b04, b06, b08 and b10 and lower than average for b03, b09 and b11. This was due to the heavier saturation of the former six linkage groups and lower saturation of the latter linkage groups. As a result the average distance between markers for each linkage group varied from 4.3 cM (for b09) to 19.4 cM (for b11), but most linkage groups had an average distance between markers of 5 to 9 cM.

Among the mapped SSR markers, 19 were from the BM and BMd series evaluated by Blair et al. (2006a),

**Table 3** Number of SSR and RAPD markers integrated into linkage groups (LG) of the new genetic map for the G14519 × G4825 population

| LG | SSR | RAPD | Total | Distance (cM) | Average distance | Segregation distortion |
|---|---|---|---|---|---|---|
| B01 | 2 | 3 | 5 | 69.5 | 13.9 | NA |
| B02 | 9 | 3 | 12 | 100.4 | 8.4 | NA |
| B03 | 2 | 1 | 3 | 15.6 | 5.2 | NA |
| B04 | 11 | 4 | 15 | 120.9 | 8.1 | NA |
| B05 | 7 | 10 | 17 | 98.5 | 5.8 | NA |
| B06 | 10 | 6 | 16 | 136.9 | 8.6 | G4825 |
| B07 | 7 | 1 | 8 | 74.1 | 9.3 | G14519 |
| B08 | 8 | 8 | 16 | 123.7 | 7.7 | NA |
| B09 | 5 | 1 | 6 | 25.9 | 4.3 | G14519 |
| B10 | 5 | 9 | 14 | 111.2 | 7.9 | NA |
| B11 | 2 | 0 | 2 | 38.7 | 19.4 | G14519 |
| Total | 68 | 46 | 114 | 915.4 | 8.0 | NA |

4 were from the PV series developed by Buso et al. (2006), 26 were AT-rich markers from Blair et al. (2008) and 19 remaining markers were from more recently developed BM and BMc series markers (Blair et al. 2009b, c). The polymorphism rate for each type of SSR marker varied between 30% for AT-rich markers to below 5% for the cDNA-based markers, such as the BMc and BMd markers.

Two other observations on the genetic map were that SSR markers were more common on linkage groups b02, b04 and b06, while RAPD markers were more common on b05, b08 and b10; and finally, segregation distortion based on a Chi-square test for a 1:1 ratio ($P < 0.05$) was observed for sections of linkage groups b06 (toward G4825), b07, b09 and b11 (toward G14519). The final genetic map was deemed well-saturated for QTL analysis using both CIM and SPA which are described below and shown along with marker distribution in Fig. 2a, b.

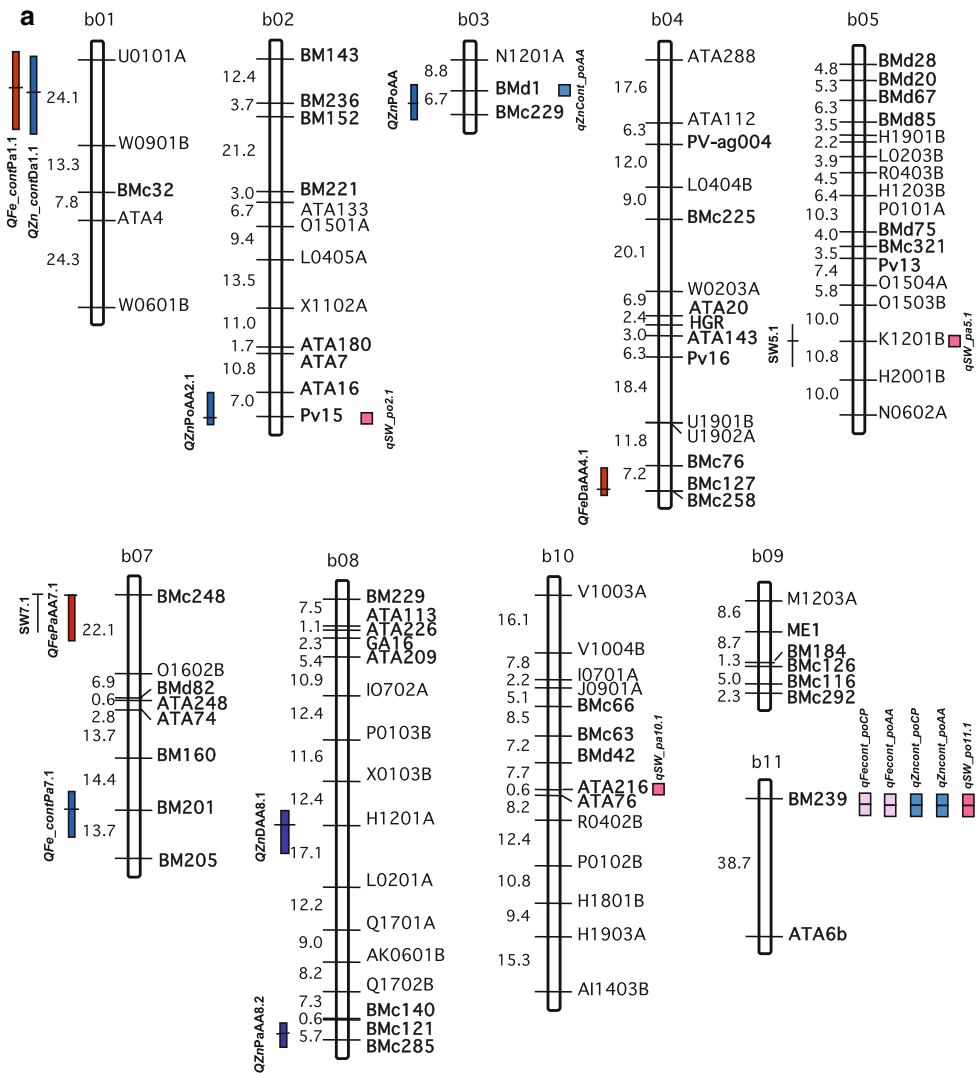


**Fig. 2** Genetic map for the G14519 × G4825 recombinant inbred line mapping population showing linkage groups b01 through b11 with QTL for the concentration and content of seed iron and zinc (**a**) or highlighting the cluster of QTL on linkage group b06 (**b**). *Vertical lines* to the left of the linkage groups for each QTL represent the region in which the marker–phenotype associations are above the LOD threshold for composite interval mapping (CIM) analysis with iron QTL indicated in *red* and zinc QTL indicated in *blue*. *Horizontal marks* on the lines indicate the LOD peak for the QTL. *Small boxes* to the right of the linkage groups and in different shading represent markers that were significant in single point analysis (SPA) for the analysis of iron and zinc content or seed weight at probability levels of $P < 0.05$ (one box) and $P < 0.01$ (two boxes). QTL names for CIM analysis are as listed in Table 4 while those from SPA analysis are listed in Table 5. Mapped microsatellite markers are indicated in *bold*

QTL identification in the three trials

A total of 13 QTL were identified for seed iron and zinc concentrations through CIM analysis (Table 4, QFe and QZn, respectively) with 6 of these located near the same set of 3 markers, BM137, BM158 and R0405B on linkage group b06. Of the QTL in this cluster, three were for iron based on phenotypic data obtained with both AAS and ICP detection methods and seed from Darién, Palmira and Popayán. Meanwhile, three QTL for zinc were also found near the same position on linkage group b06, with these based on both the AAS and ICP methods. In all the QTL for this part of linkage group b06, the positive allele for higher mineral concentration was from the high-mineral parent, G14519. The QTL near BM158 could be considered to be a single major gene; or alternatively a tight cluster of genes controlling the concentrations of both minerals.

Four other QTL for mineral concentrations were found on linkage group b04 and b07 for iron and on linkage groups b02, b03 and b08 for zinc. All of these QTL except for the QTL on b04 and b08 were contributed by the G4825 allele rather than from G14519 allele. A final QTL for zinc which was also derived from G4825 was found associated with V1001B on a different part of b06 than the previously described QTL near BM137, BM158 and R0405B. Coefficients of determination ($R^2$ values) for all five of these QTL ranged from 9.6% for the iron QTL on b07 to 17.8% for the zinc QTL on linkage group b08. Therefore, these QTL represented more minor QTL or genes that were independent of the major QTL on linkage group b06 and tended to be specific to only one site.

Meanwhile, the overlapping QTL on linkage group b06 had higher determination coefficients than the other QTL on the other linkage groups. For example, the QTL on linkage group b06 explained up to 21.3% of phenotypic variation for iron and 38.4% for zinc. This was not surprising since the QTL on linkage group b06 were consistent across sites. Some slight differences were noted for the determination coefficients between sites for the QTL on linkage group b06; namely the $R^2$ values for this locus were higher for iron concentration in Popayán and Palmira than in Darién and for zinc concentration in Darién and Popayán compared to Palmira. It was also notable that the $R^2$ values were somewhat higher for QTL from ICP than from AAS for the zinc data from Popayán although $R^2$ values were comparable for ICP and AAS for the iron data there. Coefficients of determination for background markers (TR2) for the iron and zinc QTL on b06 ranged from 33.1 to 50.4% suggesting that other minor QTL that did not surpass the permutation threshold contributed to the inheritance of the seed mineral traits. However, it was notable that the QTL for iron and zinc concentrations on linkage group b06 produced an increase of up to 4.78 and 8.77 ppm of each mineral, respectively, while the QTL for iron and zinc on other linkage groups produced lower increments only.

In terms of seed weight (SW), two QTL were identified with CIM analysis, one near the marker K1201B on linkage group b05 and another near the BMc248 marker on linkage group b07. The latter marker was also the one associated with iron concentration QTL on linkage group b07. The low number of QTL for seed weight may be due to the small variation for this trait in the population since the range in seed size between largest and smallest seeded RILs was only 15.5 to 36.5 g per 100 seed which is in the range of small to medium-sized seed found in the Mesoamerican genepool. It was notable that the parents were similar in seed weight at 22 and 23 g per 100 seed for G14519 and G4825, respectively, and therefore transgressive segregation had occurred for the trait. This may have resulted from differences in seed dimensions, since G14519 seed is wide but flat, while G4825 seed is narrow but plump. The lack of more associations between mineral QTL and seed size QTL was a reflection of the low correlations between seed weight and iron which were non-significant ranging from $r = -0.120$ to $-0.200$ or the correlations of seed weight with zinc which were also non-significant ranging from $r = -0.050$ to $-0.310$.

Given these low correlations we decided to analyze seed mineral content separately from seed mineral concentration and with both CIM and SPA analyses since content would be a correction for concentration based on seed size. Seed mineral content was calculated from the weight per seed in grams and the μg/g concentration of each mineral and a total

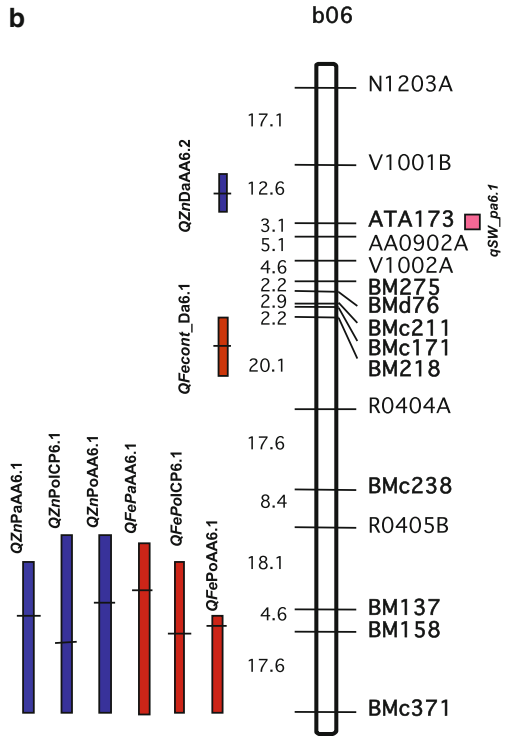


**Fig. 2** continued

**Table 4** Quantitative trait loci (QTL) for iron and zinc concentrations (parts per million, ppm) or contents (μg/seed) and seed size with associated markers and linkage groups (LG) identified by composite interval mapping in the G14519 × G4825 population

| Trait | Location | Method[a] | QTL[b] | LG | Marker | LOD[c] | $R^2$ | TR$^2$ | Additivity | Source |
|---|---|---|---|---|---|---|---|---|---|---|
| Iron concentration (ppm) | Darién | AAS | QFeDaAA4.1 | 4 | BMc127 | 2.74 | 10.82 | 33.06 | 3.00 | G14519 |
| | Palmira | AAS | QFePaAA6.1 | 6 | R0405B | 4.71 | 21.27 | 34.66 | 4.78 | G14519 |
| | Palmira | AAS | QFePaAA7.1 | 7 | BMc248 | 2.99 | 9.57 | 41.74 | 3.18 | G4825 |
| | Popayán | AAS | QFePoAA6.1 | 6 | BM158 | 5.10 | 19.80 | 34.30 | 3.83 | G14519 |
| | Popayán | ICP | QFePoICP6.1 | 6 | BM158 | 5.42 | 19.26 | 36.05 | 3.26 | G14519 |
| Zinc concentration (ppm) | Darién | AA | QZnDaAA6.2 | 6 | V1001B | 5.24 | 38.42 | 65.10 | 8.77 | G4825 |
| | Darién | AAS | QZnDaAA8.1 | 8 | H1201A | 4.44 | 17.83 | 26.68 | 3.30 | G14519 |
| | Palmira | AAS | QZnPaAA6.1 | 6 | BM158 | 4.07 | 14.39 | 36.96 | 1.29 | G14519 |
| | Palmira | AAS | QZnPaAA8.2 | 8 | H1201A | 2.85 | 10.05 | 37.02 | 1.18 | G4825 |
| | Popayán | AAS | QZnPoAA2.1 | 2 | PV15 | 3.92 | 11.94 | 43.11 | 1.41 | G4825 |
| | Popayán | AAS | QZnPoAA3.1 | 3 | BMd1 | 2.92 | 10.52 | 46.24 | 1.32 | G4825 |
| | Popayán | AAS | QZnPoAA6.1 | 6 | BM158 | 5.34 | 17.36 | 43.98 | 1.72 | G14519 |
| | Popayán | ICP | QZnPoICP6.1 | 6 | BM158 | 4.92 | 29.91 | 50.36 | 2.27 | G14519 |
| Seed weight (g/100 s) | Darién | 100SW | SW5.1 | 5 | K1201B | 2.76 | 10.32 | 26.47 | 1.94 | G14519 |
| | Darién | 100SW | SW7.1 | 7 | BMc248 | 2.53 | 10.58 | 28.52 | 1.82 | G14519 |
| Iron content (mg/seed) | Darién | AAS | QFe_contDaAA6.1 | 6 | BM218 | 7.54 | 55.17 | 56.20 | 0.53 | G4825 |
| | Palmira | AAS | QFe_contPaAA1.1 | 1 | W0901B | 3.56 | 20.13 | 41.73 | 0.28 | G14519 |
| | Palmira | AAS | QFe_contPaAA7.1 | 7 | BM201 | 2.80 | 11.14 | 30.76 | 0.25 | G4825 |
| Zinc content (mg/seed) | Darién | AAS | QZn_contDaAA1.1 | 1 | W0901B | 2.73 | 14.16 | 30.61 | 0.08 | G14519 |

[a] Methods refer to atomic absorption spectroscopy (AAS or AA) and inductively coupled plasma–optical emission spectroscopy (ICP)

[b] QTL name based on method and association with iron (Fe) or zinc (Zn) as well as location of experiment in Darién (Da), Palmira (Pa) or Popayán (Po) and QTL order on linkage group

[c] QTL surpassing empirical LOD thresholds based on 1,000 permutations recommended by Churchill and Doerge (1994)

**Table 5** Markers significantly associated with iron and zinc content (in μg/seed) and seed size identified with single point regression analysis in the G14519 × G4825 population

| QTL[a] | Chromosome | Marker | LOD | $R^2$ | Significance | Source |
|---|---|---|---|---|---|---|
| qSW_po2.1 | 2 | ATA16 | 0.96 | 40.31 | 0.047* | G14519 |
| qSW_po11.1 | 11 | BM239 | 2.14 | 13.80 | 0.002** | G14519 |
| qSW-pa5.1 | 5 | K1201B | 0.96 | 4.52 | 0.038* | G14519 |
| qSW-pa6.1 | 6 | ATA173 | 0.89 | 5.68 | 0.046* | G14519 |
| qSW-pa10.1 | 10 | ATA216 | 1.17 | 5.41 | 0.022* | G14519 |
| qFeCont_poICP | 11 | BM239 | 2.07 | 11.90 | 0.002** | G14519 |
| qFeCont_poAA | 11 | BM239 | 1.86 | 9.96 | 0.004** | G14519 |
| qZnCont_poAA | 3 | BMd1 | 0.97 | 6.54 | 0.037* | G14519 |
| qZnCont_poAA | 11 | BM239 | 1.80 | 12.23 | 0.005** | G14519 |
| qZnCont_poICP | 11 | BM239 | 1.80 | 10.90 | 0.005** | G14519 |

[a] QTL name based on method either atomic absorption spectroscopy (AA) or inductively coupled plasma–optical emission spectroscopy (ICP) and association with iron (Fe) or zinc (Zn) as well as location of experiment in Darién (Da), Palmira (Pa) or Popayán (Po) and QTL order on linkage group

[b] Level of significance corresponding to $P < 0.05$ (*) and $P < 0.01$ (**)

of three CIM-based QTL were found for iron content (QFe_cont) on linkage groups b01, b06 and b07 and one CIM-based QTL for zinc content (QZn_cont) on linkage group b01. The QTL for iron and zinc content on linkage group b01 both overlapped at the W01901B marker while the QTL for iron content on linkage group b06 was located between the QTL for zinc concentration associated with V1001B and the QTL for iron and zinc concentrations near the markers BM137, BM158 and R0405B. The SPA analysis found that QTL for seed weight (qSW) or iron and zinc contents (qFe_cont, qZn_cont) overlapped with QTL for iron or zinc concentration on linkage groups b02, b03 and b06 and that an additional set of iron and zinc contents QTL might be located on the poorly saturated linkage group b11 (Table 5).

## Discussion

The results from this research were interesting and novel for several reasons. First a stable, cross-location QTL was found for seed mineral concentration that was associated with both iron and zinc levels. Previous QTL have either not been analyzed for stability across locations only across years (Cichy et al. 2005; 2009) or tended to be somewhat variable across locations (Blair et al. 2009a). The consistency of the QTL effects of the locus on linkage group b06 may be due to the high-mineral concentration found in the G14519 source which was selected from among 380 genotypes in the Mesoamerican genepool for having the highest levels of iron and zinc concentrations (Islam et al. 2004). Indeed the positive allele for each of the QTL found was from this high-mineral parent.

In terms of QTL × environment stability, the b06 locus may not be greatly influenced by climatic conditions or soil types that vary between the three locations, although these environmental influences did influence the overall range in seed mineral concentrations as seen in the distributions of seed mineral concentration for each site. For example in the neutral pH, high fertility soils of Palmira, soil iron levels are higher (3.5 ppm) than in the acidic pH, low-fertility soils of Darién (1.7 ppm) and Popayán (2.5 ppm) and this was reflected in the average seed iron and zinc concentrations at these locations. Meanwhile, Darién produced lower seed iron and zinc values than Popayán. The high correlations between locations showed the robustness of the sampling/evaluation method which was based on the amount of seed that fit in our iron and zinc-free grinding system of Teflon chambers and zirconium balls, an improvement over the method in Blair et al. (2009a).

The high correlations across locations for seed mineral concentrations for the population also helped to explain the consistency in QTL detection and suggested that seed mineral concentration is a stably heritable trait. Overlap in the QTL for seed iron and zinc, meanwhile, might be explained by the high correlations between these mineral concentrations in all locations, especially Darién and Popayán. The lower correlation between minerals in Palmira might have been due to the different soil characteristics of this site. Blair et al. (2009a) also found high correlation coefficients among iron and zinc concentrations in Darién and Popayán, but did not test the Palmira site. The correlation between iron and zinc concentrations may indicate a physiological relationship between uptake or transport and seed accumulation of both minerals and has been observed before by Beebe et al. (2000) in other genotypes.

Among the methods used to analyze mineral concentrations, we found that AAS and ICP gave similar and highly correlated results as was shown before (Blair et al. 2009a). Therefore, we found it useful to use the lower cost AAS method across all the sites, where the Mesoamerican population was grown and only used ICP to confirm results in Popayán. ICP analysis was recommended by previous studies (Beebe et al. 2000; Guzman-Maldonado et al. 2003; Welch et al. 2000), but is more costly and Cichy et al. (2009) used AAS exclusively without ICP confirmation for an evaluation of Andean genotypes. Both AAS and ICP results produced very reliable data that were consistent with previous results on the concentrations of iron and zinc of 55 and 35 ppm, respectively, as averages for the species according to Islam et al. (2002).

Another interesting result of this study was the creation of an inter-genepool genetic map for the discovery of nutritional trait QTL within the Mesoamerican genepool. The genetic map had fewer markers than Andean × Mesoamerican intra-genepool genetic maps (Blair et al. 2003), but was saturated enough to carry out QTL analysis since markers were spaced at less than 10 cM average distance. Genetic map coverage in terms of marker number varied per linkage group, with b02, b04, b05, b06, b08 and b10 having the best coverage overall and b03, b09 and b11 having the least coverage. This was partly due to the distribution of SSR markers with many residing on b02 and b04.

Saturation of b02 and b04 by SSR markers has been observed before and there may be some preference for microsatellites to be located on these linkage groups (Blair et al. 2003, 2008). RAPD markers were preferentially located on linkage groups b05, b06, b08 and b10, but the reason for this is not known, although some association of RAPD bands with retrotransposons in bean heterochromatin has been postulated (Blair et al. 2006b). The major advantage of the microsatellites over the RAPD markers was that they allowed the anchoring of the genetic map to known linkage group and chromosomal positions as defined by Blair et al. (2003, 2008). The RAPD markers meanwhile had the advantage of being fairly polymorphic while microsatellites required the screening of a large number of primer pairs due to their low polymorphism within the Mesoamerican genepool as observed previously (Blair et al. 2006a; Gelin et al. 2007). In this regard, the ideal microsatellite markers were the higher polymorphism ATA-based markers from Blair et al. (2008) compared to the CA/GA and gene-based markers evaluated by other authors (Blair et al. 2006a, b, c; Buso et al. 2006).

In terms of QTL discovered in this study, we were originally surprised to find fewer QTL for iron and zinc concentrations than in the inter-genepool population analyzed by Blair et al. (2009a). This may have been an effect of analyzing an intra-genepool population as Cichy et al. (2009) found only a moderate number of QTL in an Andean × Andean cross and Gelin et al. (2007) found only one QTL for zinc and none for iron in a narrow cross of two navy beans from the Mesoamerican genepool. The difference

between inter and intra-genepool crosses may be that in the former population type there are many genes involved in the inheritance of any given trait and more transgressive segregation while in the latter population type fewer underlying factors control inheritance of the trait. This may be one reason why Cichy et al. (2005) postulated one gene controlling seed zinc concentration for Mesoamerican beans. It is interesting that in this study as well as in Blair et al. (2009a) and Cichy et al. (2009) the QTL for iron and zinc concentrations or content overlap with each other at several loci.

In this sense, the important QTL we found on linkage group b06 is likely to be a novel locus for mineral concentration for several reasons. First, it consisted of a cluster of iron and zinc QTL around the loci BM137, BM158 and R0405B appearing therefore to be closely linked group of genes or perhaps a pleiotropic locus controlling both iron and zinc concentrations while most QTL discovered to date have been for each mineral separately (Guzman-Maldonado et al. 2003; Blair et al. 2009a). Second, the QTL is located in a different location than other potentially pleiotropic QTL for iron and zinc concentrations, such as those on the upper part of b06 (Cichy et al. 2009) or on linkage groups b07 and b11 (Blair et al. 2009a). Finally, the other QTL found on linkage group b06 in the studies of Blair et al. (2009a) and Cichy et al. (2009) have been in different positions except for one QTL located near Bng46 for zinc which is in the region of the markers BM137 and BM158 according to Blair et al. (2003). BM137 was not associated with iron or zinc concentrations in the study by Cichy et al. (2009).

Both the b06 and other QTL for iron and zinc concentrations on linkage groups b02, b03 and b08 located in this population are likely to be different from those in Gelin et al. (2007) who predicted a gene for seed zinc concentration in navy beans to be located on linkage group b09 based on the same cross used by Cichy et al. (2005). In that study there was an association of zinc concentration with two microsatellite loci (BM154 and BM184) which are linked at 17 cM according to Blair et al. (2003). These results seem to confirm the non-allelism of this putative gene with the QTL found in the present study. It is not possible to determine the allelism of the QTL in this study with those of Guzman-Maldonado et al. (2003) since those QTL were found with an un-anchored, AFLP-based genetic map. In addition to the QTL for iron or zinc concentrations, the mineral content of QTL found on linkage groups b01, b06 and b07 is novel since content has not been measured in most previous studies in common bean.

Mineral content has been highlighted as a trait in studies of smaller seeded model legumes (Klein and Grusak 2009; Sankaran et al. 2009) and may have an important agronomic function in providing nutrients for early seedling establishment (Bouis 2003). We found that in a food legume like common bean, mineral content per seed is much higher than that in the model legumes reaching on average 11.8, 18.3 and 13.9 μg/seed iron and 14.4, 6.5 and 9.6 μg/seed zinc for the RILs evaluated by AAS in Darién, Palmira and Popayán, respectively (Table 1). This would be explained by the much larger average seed size even of Mesoamerican RILs (266 to 280 mg/seed) across the three site in this study compared to Medicago (approximately 4 mg/seed) for example (Sankaran et al. 2009). The alignment of the iron and zinc contents QTL on linkage groups b07 and b11 with the important QTL for iron and zinc concentrations on these chromosomes found by Blair et al. (2009a) or the QTL on linkage group b01 and b11 with ones found by Cichy et al. (2009) will be pursued through further fine mapping, especially with markers near the phaseolin locus for linkage group b07.

In conclusion, the inheritance of iron and zinc accumulation in the Mesoamerican population was shown to be quantitative, but with much of the variation explained by a single locus that was common to both minerals. The independent location of this QTL from those of previous studies suggests that inheritance within the Mesoamerican genepool is simpler than that in inter-genepool crosses (Blair et al. 2009a) and different than that in the Andean genepool (Cichy et al. 2009). The existence of co-localizing QTL and correlation in iron and zinc concentrations, suggested that both minerals are of similar inheritance as observed before (Blair et al. 2009a; Cichy et al. 2009). Correlation in seed iron and zinc concentrations and overlap of the corresponding QTL have also been observed in other plant species (Peleg et al. 2009; Sankaran et al. 2009). Meanwhile, the association of QTL for mineral concentration and seed size found in some other small-seeded legumes like *Lotus* (Klein and Grusak 2009) may not hold for the larger seed common bean (Blair et al. 2009a). However, the overlapping QTL on linkage group b07 for both iron concentration and seed size indicate that dilution or concentration effects of larger seed size on mineral accumulation may be important at least for some segregants in the intra-genepool population.

In terms of common bean breeding, the co-localization of QTL for seed iron and zinc and the major locus on linkage group b06 could be useful for marker-assisted selection and would allow the rapid improvement of various commercial classes for micronutrient concentration through backcrossing for example. The G14519 source of high seed mineral concentration has been used already for the improvement of red mottled Andean beans (Blair et al. 2010) through a single backcross. Meanwhile, the results of this study are also important for the genetic improvement of Carioca type beans since the genetic background of the cross included G8425 which is a typical cream and brown

striped genotype of this commercial class. It is notable that Carioca beans are the most important bean type found in Brazil which in turn is the largest producer of beans in the world. Therefore, the overall results are promising for the biofortification process in common beans and show that micronutrient concentration can be improved in the Mesoamerican genepool.

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