QTL Mapping of Genome Regions Controlling Manganese Uptake in Lentil Seed

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ABSTRACT This study evaluated Mn concentration in the seeds of 120 RILs of lentil developed from the cross “CDC Redberry” × “ILL7502”. Micronutrient analysis using atomic absorption spectrometry indicated mean seed manganese (Mn) concentrations ranging from 8.5 to 26.8 mg/kg, based on replicated field trials grown at three locations in Turkey in 2012 and 2013. A linkage map of lentil was constructed and consisted of seven linkage groups with 5,385 DNA markers. The total map length was 973.1 cM, with an average distance between markers of 0.18 cM. A total of 6 QTL for Mn concentration were identified using composite interval mapping (CIM). All QTL were statistically significant and explained 15.3–24.1% of the phenotypic variation, with LOD scores ranging from 3.00 to 4.42. The high-density genetic map reported in this study will increase fundamental knowledge of the genome structure of lentil, and will be the basis for the development of micronutrient-enriched lentil genotypes to support biofortification efforts.

KEYWORDS
DArT
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Genome Report

Manganese (Mn) is an essential micronutrient with a recommended daily intake of 0.7 to 22.0 mg for adults (Santos et al. 2007). Micronutrient analysis using atomic absorption spectrometry indicated mean seed manganese (Mn) concentrations ranging from 8.5 to 26.8 mg/kg, based on replicated field trials grown at three locations in Turkey in 2012 and 2013. A linkage map of lentil was constructed and consisted of seven linkage groups with 5,385 DNA markers. The total map length was 973.1 cM, with an average distance between markers of 0.18 cM. A total of 6 QTL for Mn concentration were identified using composite interval mapping (CIM). All QTL were statistically significant and explained 15.3–24.1% of the phenotypic variation, with LOD scores ranging from 3.00 to 4.42. The high-density genetic map reported in this study will increase fundamental knowledge of the genome structure of lentil, and will be the basis for the development of micronutrient-enriched lentil genotypes to support biofortification efforts.
et al. 2016), wheat (Monasterio and Graham 2000; Garvin et al. 2006; Gupta et al. 2015; Prasad et al. 2015), barley (Ma et al. 2004; Rodrigo et al. 2013), and lentil (Ates et al. 2016; Aldemir et al. 2017). Identifying the quantitative trait loci (QTL) that control the concentration of Mn in lentils would aid in the development of biofortified cultivars through the use of closely linked molecular markers, which would allow breeders to screen and select for micronutrient dense genotypes. QTL controlling Mn uptake and identified using QTL analysis have been published for cabbage (Wu et al. 2008), Lotus japonicus (Klein and Grusak 2009), clover (Sankaran et al. 2009), canola (Ding et al. 2010), and Arabidopsis (Willems et al. 2010). To date, however, no QTL controlling the concentration of Mn in lentil seeds have been identified. The objectives of this study were to (i) determine the Mn concentration of lentil seeds from recombinant inbred lines (RILs), (ii) calculate genetic variation of Mn concentration among RILs, locations, and years, and (iii) identify QTL controlling Mn concentration in lentil seeds.

MATERIALS AND METHODS

Soil analysis

Soil samples were collected from experimental fields in three locations in Turkey (Izmir, Adana, and Sanliurfa) to determine the physical and chemical properties of each soil. Soil pH analysis (Black 1965), total soluble salt analysis (Richards 1954), texture analysis (Black 1965), CaCO₃ analysis (Schlichting and Blume 1966), and macro- and micro-nutrient analysis (Bingham 1949; Pratt 1965; Lindsay and Norvell 1978) were carried out at the Department of Plant and Soil Science at Ege University in Izmir, Turkey.

Plant materials

A population of 120 RILs was developed from the cross "CDC Redberry" (P1) × "ILL7502" (P2) and designated LR-8. P1 was developed from a cross made in 1997 between CDC breeding lines 1049F3 / 819-5R. Line 1049F3 was derived from the cross 567-16 / 545-8. Line 819-5R was derived from the cross 86-360 / (458-258G / (458-122 / C8L27- Rc // Precocx) F2) F1 (Vandenbergh et al. 2006). P2 is a lentil cultivar released in Bangladesh (Sarker et al. 1999). The LR-8 population was generated by advancing F₂ plants from the simple cross to the F2 generation, and the RILs developed by single seed descent from the F2 to the F2 generation. The RILs were produced at the University of Saskatchewan, Canada where resources for genetic and genomic studies of lentil have been developed since 2001.

Micronutrient analysis and heritability

The RILs were grown in 2 years (2012 and 2013) in three different locations in Turkey—Ege University Izmir (27°09' E, 38°25' N), Cukurova University in Adana (35°18' E, 37°01' N), and Harray University in Sanliurfa (38°46' E, 37°08' N)—and placed with three replications in a randomized complete block design (RCBD) with three factors (year, location, genotype) for micronutrient analysis. An atomic absorption spectrometer (AAS) (Varian, SpectraAA 220/ FS, California, USA) was used to estimate Mn concentrations in all seed samples. Samples were prepared for analysis as per a previous study (Kacar 1972). Seed samples (2 g) were first washed with tap water and then with pure water to remove surface contaminants. The washed seeds were dried in a hot air oven at 65°. The dried samples were ground using an analytic mill (IKa, A11, Staufen, Germany) and then each 2-g ground sample placed into a 150-mL flask to which 24 mL of 4:1 nitric: perchloric acid were added to decompose the samples. All procedures were performed with three replications. Spectrophotometric readings for total Mn concentrations were converted to mg/kg concentration in seed (Kacar 1972; Kacar and Inal 2008). To confirm the accuracy of the AAS, standard Mn solutions (1.0, 2.0, 3.0, 4.0, 5.0 ppm) were analyzed to form a calibration curve ($r^2 = 0.9999$). Heritability (H) based on lentil population means was calculated with the formula $H = \frac{MS_{among~families} - MS_{fixed~family}}{MS_{among~families}}$ where MS is the mean square (Courtney et al. 2008).

Variance analysis

Analysis of variance (ANOVA) was used to determine variation in Mn concentrations of the LR-8 RIL population grown in different years and locations using TOTEMSTAT software (Acikg oz et al. 2004). Genotypes were accepted as fixed while year and location were random. Variation of year (Y) × location (L), Y × genotype (G), L × G, and Y × L × G interactions were calculated and significance was accepted at the $P \leq 0.01$ and $\leq 0.05$ levels.

DNA isolation

Young leaves from 4- to 6-week-old seedling of all lentil genotypes grown at the Izmir location were harvested and placed in labeled aluminum foil containers and then immediately placed in liquid nitrogen. Frozen leaf samples were kept in a deep freezer (-86°C) until analysis. Genomic DNA from 120 RILs and the parents were extracted from frozen leaf tissue using the Fermentas DNA Isolation Kit (Thermo Scientific, Hanover, MD, USA). Purity of the DNA was confirmed on 1% agarose gel and the purified DNA then quantified with a Qubit2.0 Fluorometer (Life Technologies, US).

DArT analysis

DArT analyses were carried out following Aldemir et al. (2017). The raw data for SNP discovery are presented as supplemental file 1 (File S1).

Linkage mapping and QTL analysis

JoinMap4.0 software described by Van Ooijen and Voorrips (2004) was used for linkage mapping analysis. A maximum recombination frequency of 0.50 and the kosambi function were used as options in linkage mapping. Distorted markers were eliminated. MapQTL version 6.0 (Van Ooijen 2009) was used for QTL analysis. The effects and positions of QTL were determined following composite interval mapping (CIM). The significant threshold was calculated based on 1000 permutations at the $P \leq 0.01$ and $\leq 0.05$ levels (Van Ooijen and Voorrips 2004), and QTL that passed the threshold significance are reported. The proportion of observed phenotypic variation explained due to a particular QTL was estimated by the coefficient of determination ($R^2$) using maximum likelihood for CIM.

Data Availability

File S1 contains SNP data. File S2 contains Mn phenotyping data.

RESULTS

Soil properties

The physico-chemical properties of soil samples from Izmir, Adana, and Sanliurfa locations are presented in Table 1. Soil samples from all locations were slightly alkaline, non-saline, and calcareous. Soil from Adana had a loamy clay texture and soils from the other two locations had a loamy texture. The bioavailability of Mn by plants from soil is the degree to which an extractable solid-phase quantity is correlated with measured tissue concentration, which is called available Mn (Lindsay and Norvell, 1978). Available Mn contents were low for all three soils.
Mn concentration in seeds of the LR-8 population

Mn concentrations in P1 and P2 of the LR-8 lentil population are shown in Table 2. The overall mean Mn concentration in seeds of the parents was 9.6 mg/kg for P2 and 27.6 mg/kg for P1. Mn concentrations in seeds of the RILs of the LR-8 population varied from 8.5 to 26.8 mg/kg with a mean of 17.6 mg/kg. The highest concentration of Mn was detected in RIL LR8-113. Heritability for Mn concentrations was detected as 0.76 and 0.74 for 2012 and 2013, respectively (Table 2). This means that Mn accumulation in the seed is affected by genetics rather than the environment. The frequency distribution of Mn concentrations in seeds of the LR-8 population as a mean across three locations (Izmir, Adana, and Sanliurfa) and 2 years (2012 and 2013), respectively (Table 2). Variance analysis showed that Mn concentration in seeds among RILs was significant at the P ≤ 0.01 level. It was also statistically significant among locations (Table 3). The effects of Y, X, Y × G, L × G, and Y × L × G interactions were statistically significant. Genotypes accumulated Mn in seed at different levels according to year and location.

Table 2 Minimum, maximum, and mean Mn concentration in seeds of the LR-8 lentil population grown at Izmir, Adana, and Sanliurfa in 2012 and 2013

| Location   | Izmir | Adana | Sanliurfa |
|------------|-------|-------|-----------|
| Year       | 2012  | 2013  | 2012  | 2013  | 2012  | 2013  | Mean     |
| P1         | 25.5  | 29.3  | 24.0  | 32.1  | 27.5  | 27.1  | 27.6     |
| P2         | 7.7   | 8.8   | 9.5   | 10.6  | 8.0   | 12.7  | 9.6      |
| Minimum    | 7.7   | 10.8  | 7.5   | 9.2   | 8.0   | 9.0   | 8.5      |
| Maximum    | 27.8  | 31.9  | 27.5  | 26.3  | 28.5  | 25.1  | 26.8     |
| Mean       | 17.8  | 20.4  | 17.5  | 17.3  | 17.8  | 17.3  | 17.6     |
| Mn heritability | 2012 | 2013 | 0.76  | 0.74  | 0.76  | 0.74  | 0.76     |
| Std dev    | 4.2   | 4.0   | 4.2   | 4.0   | 4.2   | 4.0   | 4.2      |

DISCUSSION

Micronutrient malnutrition affects more than one-half of the total human population, with children and women at the highest risk (Ahmed et al. 2016). Biofortification aims to increase the total amount of minerals in the edible parts of crops by increasing the concentration of compounds, such as Mn, thus promoting their uptake by humans (Graham and Welch 1996). The biofortification strategy for alleviating this form of malnutrition is to increase the consistent daily intake of food staples by all family members, especially children and women, and to target the bridge between human nutrition and agriculture (Graham et al. 1999).

Mn accumulation in seed was determined to be quantitatively inherited in lentil. Supporting our results, previous QTL studies show...
that Mn concentration of seeds is quantitatively inherited in cabbage (Wu et al. 2008), Lotus japonicus (Klein and Grusak 2009), clover (Sankaran et al. 2009), canola (Ding et al. 2010), and Arabidopsis (Willems et al. 2010). Therefore, this study is also important with respect to understanding the genetic nature of Mn accumulation in seed. To date, no studies have identified QTL for Mn concentration in lentil. Identification of the QTL associated with high Mn concentration in lentil seeds could help select lines containing high Mn concentration in lentil breeding programs. This type of knowledge can be used to develop genetic strategies for molecular breeding to help increase the micronutrient content of edible parts of the lentil plant. Increased consumption of lentil with elevated levels of micronutrients could help to overcome micronutrient deficiency (Cichy et al. 2009), and the large variation in Mn concentration among the RILs could be the basis for developing such a strategy (Beebe et al. 2000).

**Mn variation**

Mean Mn concentrations of seeds of RILs in the LR-8 population grown at three locations in 2 years varied from 8.5 to 26.8 mg/kg and represented a ~threefold variation (Table 2). Previous reports indicate Mn concentrations range from 11.5 to 16.2 mg/kg for lentil landraces and from 11.5 to 15.4 mg/kg for lentil cultivars (Karakoş et al. 2012). Mn concentrations reported by Karakoş et al. (2012) are lower than those from the current study, which could be due to the different genotypes they used as well as different soil chemical properties of their experimental field. Per capita global lentil consumption is being increased rapidly and lentil fortification is a simple and promising approach to help decreased Mn deficiency (Podder et al. 2018). The data show that the Mn concentrations we observed could provide a significant amount of the required daily Mn from lentil in a given meal. For example, daily cooked lentil dal (50g/day) contains approximately 1 mg Mn (Mn concentrations in the current research found as a mean of 17.6 mg/kg, Table 2) which falls into recommended daily allowance (RDA) indicated by Santos et al. (2004) (0.7 to 22.0 mg for adults). In previous studies, Mn concentration was detected as a mean of 14 mg/kg in common bean seeds (Pinheiro et al. 2010). The Mn concentration was ranged between 9.2 -14.6 mg/kg in pea, between 4.4 and 12.6 mg/kg in buckwheat (Beitane and Krumin-Zemture 2017) and 16.8 mg/kg in seeds of chickpea (Kahraman et al. 2017). Mn value detected in the current study was higher as compared to other legumes.

The ANOVA for Mn concentration shows that location, year, and genotype interactions are statistically significant (Table 3). Interactions among genotypes, locations, and years are likely due to different environmental conditions affecting the availability of Mn in the pool of soil micronutrients available for plant uptake (Sankaran et al. 2009).

**Linkage mapping**

DArT analysis allowed the construction of high-density linkage maps with a very large number of SNPs. In the current study, the DArT method generated 10,552 SNPs. Using this DArT approach on the parental RIL populations, a total of 5,385 SNPs were mapped (Table 4). The amount of data used for mapping purposes was similar to previous DArT analysis studies (Poland et al. 2012; Li et al. 2014; Han et al. 2016).

In this study, the linkage map of lentil consisted of seven linkage groups with 5,385 SNP markers. The total map length (973.1 cM) in the current study is shorter than for many previous lentil mapping studies, e.g., 1,073 cM (Eujayl et al. 1997), 1,868 cM (Tullu et al. 2008), 1,396.3 cM (Tanyolac et al. 2010), 3,843 cM (Gupta et al. 2012), and 834.7 cM (Sharpe et al. 2013). Recently, the lentil genome was mapped with 1,784 markers (including SNP and SSR) covering a genome size of 4,060.6 cM using genotype by sequencing (GBS) in RILs developed from “PI 320937” × “Eston” parents (Ates et al. 2016). On the other hand, a total map length of 784.1 cM, which is close to our map length, was detected using a few markers [100 Random Amplified Polymorphic DNA (RAPDs), 11 Inter Simple Sequence Repeats (ISSRs) and 3 Resistance Gene Analogs (RGAs)] (Rubeena et al. 2003). Another study constructed a genetic linkage map using 6 SSRs and 537 contigs covering a genome size of 834.7 cM (Sharpe et al. 2013). Overall, the genetic map in this study is more robust compared to previous QTL mapping studies in lentil (Eujayl et al. 1997; Tullu et al. 2008; Tanyolac et al. 2010; Gupta et al. 2012; Sharpe et al. 2013).

The seven major LGs constructed in the current study correspond to the seven haploid chromosome number of lentil (Sharpe et al. 2013; Ates et al. 2016). Differences in the estimated distances of both parental maps may reflect differences in the recombination frequencies of both parents. Putative causes for the difference between the two estimated parental genome maps include marker distribution along the chromosome that varies between parents, and male and female gametes that probably display different recombination frequencies (Khadari et al. 2010).

**QTL analysis of Mn**

This study is the first to map QTL for Mn concentration in lentil seeds and uses a larger number of SNPs than previous studies mapping the lentil genome. PI (CDC Redberry) is adapted to the northern temperate

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**Table 3 ANOVA for Mn concentrations in seeds of LR-8 lentil RILs grown at three different locations for two years**

| Source of Variation | df | Mean Square | F | F prob. 5% | F prob. 1% |
|---------------------|----|-------------|---|------------|------------|
| Block               | 2  | 4.1         | 2.4 ns | 3.1        | 4.8        |
| Year (Y)            | 1  | 55.7        | 31.6 * | 3.9        | 6.9        |
| Location (L)        | 2  | 14.1        | 8.0 * | 3.1        | 4.8        |
| Genotype (G)        | 119| 306.0       | 173.4 *| 1.4        | 1.5        |
| Y X L               | 2  | 18.5        | 10.5 *| 3.1        | 4.8        |
| Y X G               | 119| 3.1         | 1.8 * | 1.4        | 1.5        |
| L X G               | 238| 4.0         | 2.3 * | 1.3        | 1.4        |
| Y X L X G           | 238| 3.8         | 2.2 * | 1.3        | 1.4        |
| Error               | 1246| 1.765      |      |            |            |
| General             | 1871|            |      |            |            |

Coefficient of variation (CV)= 7.71%

df: degree of freedom. ns: not significant.

*: Significant at P ≤ 0.01.

**Table 4 Characteristics of the linkage groups of the LR-8 lentil population**

| Linkage group | Length (cM) | Number of SNP markers | Number of SNP markers (%) | Average distance between markers (cM) |
|---------------|-------------|-----------------------|---------------------------|--------------------------------------|
| LG1           | 151.8       | 1,102                 | 20.5                      | 0.13                                 |
| LG2           | 175.2       | 676                   | 12.5                      | 0.25                                 |
| LG3           | 167.7       | 940                   | 17.4                      | 0.17                                 |
| LG4           | 169.2       | 835                   | 15.5                      | 0.20                                 |
| LG5           | 102.5       | 849                   | 15.8                      | 0.12                                 |
| LG6           | 117.9       | 439                   | 8.1                       | 0.26                                 |
| LG7           | 88.8        | 544                   | 10.1                      | 0.16                                 |
| Total         | 973.1       | 5,385                 |                           | 0.18                                 |

Average: 0.18
Figure 2 Genetic linkage map for lentil derived from the cross P1 x P2. Left bar of the LGs is cM and the right bar is marker names. QTL for Mn marked with red.
zone, and was the parent that had the highest seed Mn concentration. A total of 6 QTL for Mn concentration in seeds of the LR-8 population were identified using CIM. These were distributed across three linkage groups in the LR-8 lentil population (Table 5).

In a study of QTL associated with Mn concentration in Lotus japonicus (Klein and Grusak 2009) two QTL explaining 35.2% of the phenotypic variation were identified on chromosomes 1 and 2. Ten QTL for Mn concentration were distributed across 8 chromosomes, with LOD scores ranging between 3.34-6.55, explaining between 9.06 and 16.43% of the phenotypic variation in Brassica napus (Ding et al. 2010). In other similar studies, six QTL for Mn concentration were found in one of two wheat populations (Pu et al., 2013), two QTL were mapped in soybean (Ramamurthy et al., 2014), and four QTL associated with Mn concentration were identified in rice (Yu et al., 2015). Here, the number of QTL detected was high; micronutrient accumulation in seeds continues to be a complex process controlled by poly-genes (Grusak and DellaPenna 1999).

For Mn in lentil seeds, six QTL were statistically significant, and the phenotypic variation ranged from 16.1 to 24.1% with LOD scores of 3.02-4.42. QTL analysis of nutrient element accumulation in seeds of other crops shows that the value for explaining phenotypic variation typically ranges between 9.06 and 35.2% (Ding et al., 2010; Klein and Grusak 2009; Yu et al., 2015). The value we found falls within the same range, and our estimates of phenotypic variation of seed Mn concentration in lentil were similar to those for canola (Ding et al., 2010) and wheat (Pu et al., 2013).

Conclusions

The LR-8 lentil population studied here demonstrated large phenotypic variation in terms of Mn concentrations in seeds. Mn concentrations in lentil seeds were observed to be quantitatively inherited. DArT analysis allowed the construction of high-density linkage maps with a large number of SNPs. The QTL that were stable across 2 years and three locations were unaffected by environmental conditions, and therefore could be used in marker-assisted selection in lentil breeding programs. We believe that this work is the first to map QTL for Mn concentrations in lentil seeds. The discovery of QTL for seed Mn concentration could have significant implications for biofortification breeding strategies. The QTL analysis might help to resolve some of the complexity with respect to Mn accumulation in lentil grain. RIL LR8-113, which contained the highest Mn concentration, could be used as a parent in breeding programs. The results of this study can be applied to the development of lentil genotypes with higher Mn concentrations. The high-density maps could increase fundamental knowledge of the genome structure of lentil, help in future construction of physical maps, and serve as a basis for map-based cloning in lentil.

Table 5 Characteristics and locations of QTL regions for Mn concentrations in seeds of the LR-8 lentil population

| QTL region | LG   | Position (cM) | Number of SNPs | % explanation | Additive effect | LOD  | Year/location |
|------------|------|---------------|----------------|---------------|----------------|------|---------------|
| MnQTL1.1   | LG1  | 26.0-26.3     | 72             | 24.1          | +              | 3.25 | 2012 Adana    |
| MnQTL1.2   | LG1  | 37.0-37.7     | 87             | 16.1          | —              | 3.02 | 2012 Adana    |
| MnQTL3.1   | LG3  | 27.0-27.6     | 24             | 18.0          | —              | 3.70 | 2013 Sanliurfa |
| MnQTL3.2   | LG3  | 56.6-57.7     | 10             | 22.4          | —              | 4.38 | 2012 Izmir; 2013 Izmir, Sanliurfa |
| MnQTL3.3   | LG3  | 114.6-124.1   | 103            | 21.6          | —              | 4.22 | 2012 Izmir, Sanliurfa |
| MnQTL7.1   | LG7  | 2.3-7.7       | 14             | 16.1          | +              | 4.42 | 2013 Adana, Izmir; Sanliurfa |

* Positive (+) values of additive effect mean that the positive allele comes from parent P1, while negative (-) values mean that the positive allele comes from parent P2.

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