The genetic architecture of zinc and iron content in maize grains as revealed by QTL mapping and meta-analysis

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Micronutrient malnutrition, especially zinc (Zn) and iron (Fe) deficiency in diets, has aroused worldwide attention. Biofortification of food crops has been considered as a promising approach for alleviating this deficiency. Quantitative trait locus (QTL) analysis was performed to dissect the genetic mechanism of Zn and Fe content in maize grains using a total of 218 F₂;₃ families derived from a cross between inbred lines 178 and P53. Meta-analysis was used to integrate genetic maps and detect Meta-QTL (MQTL) across several independent QTL analyses for traits related to Zn or Fe content. Five significant QTLs and 10 MQTLs were detected. Two informative genomic regions, bins 2.07 and 2.08, showed a great importance for Zn and Fe content QTLs. The correlation between Zn and Fe level in maize grains was proposed by MQTLs as 8 of the 10 involved both traits. The results of this study suggest that QTL mapping and meta-analysis is an effective approach to understand the genetic basis of Zn and Fe accumulation in maize grains.

Key Words: biofortification, grain Zn and Fe, Zea mays L., inbred lines 178 and P53, QTL analysis, meta-analysis.

Introduction

A diversified diet with adequate micronutrients is prerequisite for human health. These nutrient elements include vitamins and trace minerals, of which zinc (Zn), iron (Fe) and vitamin A are defined as the most frequently deficient elements in diets by WHO (2002). The micronutrient Zn, required as a cofactor in over 300 enzymes (Coleman 1998), is essential for living organisms. This element participates not only in DNA transcription, protein, nucleic acid, carbohydrate and lipid metabolism (Broadley et al. 2007, Ishimaru et al. 2011, Palmer and Guerinot 2009), but also in the control of gene transcription and the coordination of other biological processes (Rhodes and Klug 1993, Vallee and Sandstead 2006). A deficiency in Zn leads to diarrhoeal diseases, lower respiratory infections and malaria (Maret and Nestel 2006, Nestel 2006, Shewry 2011). However, many people in developing countries do not eat a balanced diet and rely on a staple diet of cereals such as rice (Oryza sativa) and maize (Zea mays L.), which is low in Zn and Fe content (Bouis 2000, Welch and Graham 2002). Therefore, biofortification, a process relies on conventional plant breeding and biotechnology to increase the micronutrient content in crop plants, has emerged as an alternative approach to tackle malnutrition in the developing world (Bouis and Welch 2010, Nestel 2006, Pfeiffer and McClafferty 2007, White and Broadley 2005).

Finding genes which control accumulation of Zn and Fe in grains of major cereals is the precondition for biofortified breeding programs. Previous studies have shown that the Zn and Fe metabolism, involving processes of mobilization, uptake, translocation and accumulation, is a complex process regulated by many genes (Bashir et al. 2012, Kobayashi and Nishizawa 2012, Lee et al. 2012, Palmgren et al. 2008). In addition, other factors such as metal chaperones, chelators and Zn analogues might also contribute to ultimate mineral density in grains. QTL mapping is a powerful and widely-used tool for dissecting the genetic basis of complex trait in plants and animals. Recent QTL analyses have been performed in Arabidopsis thaliana (Waters and Grusak 2008), rice (Garcia-Oliveira et al. 2009, Stangoulis et al. 2007), bean (Blair et al. 2009, Cichy et al. 2009, Gelin et al. 2007), wheat (Genc et al. 2009, Peleg et al. 2009, Tiwari et al. 2009) and even mungbean (Sompong et al. 2012) to identify loci associated with minerals. A few QTL mapping researches have also been conducted on micronutrient content in maize (Lung’aho et al. 2011, Qin et al. 2012, Šimić et al. 2012).
2012, Zhou et al. 2010); however, the results were inconsistent possibly due to the differences in mapping populations, genotypes and environments used in these studies (Austin and Lee 1996, Li et al. 2007). Therefore, it is necessary to carry out more QTL analyses for such traits as Zn and Fe content in order to detect more loci and ultimately identify consistent QTLs for future fine mapping, marker-assisted selection (MAS) and map-based cloning.

To obtain consensus QTLs and gain more information on the genetic architecture of grain Zn and Fe content, it is necessary to make a comprehensive comparison among QTLs reported in independent studies. QTL meta-analysis is an approach to integrate such QTLs and determine true QTLs with more accurate position and smaller confidence interval (CL) (Arcade et al. 2004, Goffinet and Gerber 2000). This method has been used in various integrations of traits and species (Li et al. 2013, Qi et al. 2011, Shi et al. 2009, Swamy et al. 2011). In maize, 313 QTLs for flowering time found in 22 studies were synthesized into 62 consensus QTLs (Chardon et al. 2004). Truntzler et al. (2010) obtained 26 and 42 MQTLs for digestibility and cell wall composition traits from 50 and 150 QTLs, respectively. Also, compiling and comparing 239 QTLs for drought tolerance under water stressed conditions and 160 detected under control conditions, Hao et al. (2010) identified 39 consensus QTLs under water stressed condition and 36 under control condition from 12 populations tested in 22 experiments.

The objectives of this study were 1) to detect associations between QTLs and Zn and Fe content of maize grains based on QTL mapping with 218 F$_{2:3}$ families derived from a cross between two maize inbred lines with different grain Zn content and 2) to identify consistent QTLs from this study and other independent experiments using meta-analysis and ultimately find informative genetic regions for next research steps in maize.

Materials and Methods

Plant material and field trial

Two maize inbred lines 178 and P53, which were significantly different in grain Zn and Fe content according to our previous study (Gao et al. 2008), were crossed to develop a F$_{2:3}$ mapping population in this study. The 218 F$_{2:3}$ families of the population, along with both parents, were grown in field plots with 4 m long and space between rows being 60 cm and density of 60,000 plants ha$^{-1}$ at the Experimental Station of Hebei Agricultural University, Baoding, China, in the spring of 2009, through single seed descent method. The maize plants were managed following local standard cultivation management practices. When the maize matured, the grains were harvested and used for the evaluation of Zn and Fe content.

Micronutrient testing

All harvested grain samples were cleaned, dried, ground with stainless steel grain crusher and oven-dried at 60–70°C for 2 hours to get sample powder. Two samples were collected for each line and the sample (1 g) was weighed and then charred using electric hot plate at 300°C until smoke appeared. Ashed samples were burned at 500°C for 5 hours by muffle furnace (until the samples were black to white or off-white in color), allowed to cool at room temperature and then dissolved in 5 ml of 1:1 hydrochloric acid (Gorsuch 1970). The same process was carried out for the preparation of blank solution. Micronutrient analysis of the samples was conducted by Atomic Absorption Spectrophotometer (Hitachi Z-5300 AA Spectrophotometer, Hitachi, Japan), following the atomic absorption method.

Map construction and QTL mapping

Leaf samples were collected at seedling (with three leaves) stage for isolation of total genomic DNA using a standard CTAB extraction method (Saghai-Maroof et al. 1984). A total of 240 SSR primer pairs selected from MaizeGDB (http://www.maizegdb.org/) based on their uniform coverage of all 10 maize chromosomes were used in this study. For the detection of SSR amplified products, electrophoreses were performed on 6% polyacrylamide gel. Ultimately, 93 markers were screened according to their polymorphism between the parents, 178/P53. Segregation distortion was tested for each marker (the proportion was 5.4%) and a few markers were excluded. The genetic linkage map was built using MAPMAKER/EXP3.0 (Lincoln et al. 1992) with an overall length of 1,042 cM and an average interval of 13.53 cM. Linkage groups were inferred at a log of the odds (LOD) threshold of 3.0 and map distances were obtained with the Kosambi mapping function (Kosambi 1943).

QTL analysis was conducted using composite interval mapping (CIM) by the software WinQTLCart2.5 (Wang et al. 2007) with the following parameters: standard model, 10 cM window size, five significant background markers, 2 cM walkspeed and 1,000 permutations of the phenotypic data at 1% level to determine the significance threshold for QTL detection.

Statistic analysis

The t test was performed to evaluate the difference between two parents in grain Zn and Fe content according to Microsoft Office Excel 2003. Histograms of grain Zn and Fe content in 218 F$_{2:3}$ families and Kolmogorov-Smirnov test for phenotypic data were conducted by SPSS Statistics17. Broad heritability ($h^2_B$) for the two traits was estimated as $h^2_B = \frac{MS_{\text{between families}}}{MS_{\text{between families}} + MS_{\text{within families}}}$ using one-way ANOVA.

QTL meta-analysis

QTL information was collected from four previous QTL studies which reported maize grain Zn and Fe content and the present study (Table 1). BioMercator2.1 software was used to conduct meta-analysis and estimate the number and positions of MQTLs initially presented by several QTLs mapped on the same or similar chromosome regions for the
same or related traits detected under various conditions (Arcade et al. 2004, Goffinet and Gerber 2000). The integrated genetic map was obtained by projecting all the genetic linkage groups corresponding to reports mentioned above onto the reference map, IBM2 2008 neighbors, with homothetic function. A modified Akaike’s information criterion (AIC) was calculated to select the QTL model with varying numbers of MQTLs and the one with the lowest AIC value was chosen as a significant model to indicate the number of MQTLs on each chromosome. The consensus genetic map with the information of initial QTLs and MQTLs was constructed by MapChart 2.1 software (Voorrips 2002).

**Results**

**Performance for grain Zn and Fe content**

Fig. 1 shows the distribution of two grain mineral nutrients content of the F$_{2:3}$ mapping population and the mean values of two parents grown during 2009. Significant differences (p < 0.05) between two parental lines for Zn and Fe were found by t test, with line 178 having higher mean values relative to P53. A considerable variation was observed for levels of the two traits in 218 F$_{2:3}$ families, presenting a continuous segregation and normal distribution with P value greater than 0.05 (0.731 for Zn and 0.377 for Fe) derived from Kolmogorov-Smirnov test. In addition, transgressive segregation was found for both Zn and Fe content in this population. Broad heritability estimates were 0.57 for Zn and 0.36 for Fe content.

**QTL analysis for Zn and Fe content**

A total of 5 significant QTLs controlling grain Zn and Fe content were detected in this F$_{2:3}$ mapping population (Table 2). For Zn content, 4 QTLs were identified on chromosomes 2, 5 and 10, whereas for Fe content, only one QTL was found on chromosome 5. The detected QTLs for Zn content explained 5.85–17.57% of phenotypic variation with LOD scores ranging from 3.01 to 5.58, of which $qZn5$ located on chromosome 5 exhibited the largest proportion of variance. The detected QTL, $qFe5$, for Fe content accounted for 16.89% of phenotypic variation with LOD score of 3.49. What noteworthy, in this case, was the co-localization of

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**Table 1. Summary of recent QTL studies considered for Zn and Fe content in maize grain**

| QTL study    | Population type | Parents | No. of environment | Software and method                                      | Chromosome | No. of QTL | Range of $R^2$ |
|--------------|-----------------|---------|-------------------|----------------------------------------------------------|------------|------------|---------------|
| Lung’aho et al. (2011) | RIL            | B73/Mo17 | 6                | QTL Cartographer v2.5; Composite Interval Mapping | 2, 5, 9   | 3          | 9.3–12        |
| Šimić et al. (2012) | F$_{4}$          | B84/Os6-2 | 3                | PLABQTL; Composite Interval Mapping               | 4          | 2, 6, 8   | 7.8           |
| Qin et al. (2012) | F$_{2:3}$(Mus)  | Mu6/SDM  | 2                | QTL IciMapping v3.0; Forward Regression Analysis | 1, 2, 6, 7, 9, 10 | 2, 9        | 6.3–21.3   |
| Zhou et al. (2010) | F$_{2:3}$(Mos)  | Mo17/SDM | 2                | QTL Mapper 1.6; Mixed Linear Model                | 2, 3, 4, 6, 7, 10 | 1, 2, 4, 6, 7, 10 | 9.4–48.8   |
| Present study  | F$_{2:3}$       | 178/P53  | 1                | QTL Cartographer v2.5; Composite Interval Mapping | 2, 5, 10  | 5          | 5.9–17.6    |

*Coefficient of determination: percentage of phenotypic variance explained by the QTL.

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**Fig. 1.** Histograms of maize grain Zn (A) and Fe (B) content in 218 F$_{2:3}$ families derived from a cross between two maize inbred lines P53 and 178 grown during 2009. Mean values of the parental lines are shown by arrows.
qZn5 and qFe5 on chromosome 5 in the marker interval umc1429–umc1060. Both additive and dominant effects were detected for all the QTLs on which the alleles from inbred line 178 had positive additive effect, except for qZn10 which had negative effect. The genes that control Zn content were mainly based on additive effect with dominant degrees less than 0.2, while the QTL detected for Fe content was partial dominant with dominant degree of 0.204.

**QTL meta-analysis for Zn and Fe content**

In total, 54 QTLs responsible for Zn or Fe level in maize grains were collected from 5 researches, including this study. Based on the definition of meta-analysis, chromosome regions with only one QTL located were excluded from the analysis and then 28 QTLs were used for meta-analysis. After integration, 10 MQTLs mapped on six chromosomes at CI of 95% were found (Table 3). There were 3 MQTLs on chromosome 2; 2 on chromosome 4 and 9 and one on chromosome 3, 5 and 10 (Fig. 2). The mean phenotypic variation of the QTLs synthesized for corresponding MQTLs ranging from 8.75% to 28.30%. Seven out of 10 MQTLs had phenotypic variation more than 10%, and each MQTL consisted of 2 to 5 initial QTLs. Compared with original QTLs, CIs of all the MQTLs were narrowed, varying from 9.53 cM at bin 2.07 to 212.71 cM at bin 3.04–3.06. All the MQTLs except for MQTL1 and MQTL6 involved both Zn and Fe content related traits. All the QTLs found through QTL mapping in this study were integrated into MQTLs revealed through meta-analysis, of which MQTL2 included qZn2-1 and qZn2-2; MQTL7 included qZn5 and qFe5 and MQTL10 included qZn10.

**Discussion**

**Feasibility of QTL mapping for mineral concentration in maize grains**

Biofortification, especially Zn and Fe enhancement in crop food through genetic improvement, has been receiving much attention in recent years. Previous studies with maize have focused on germplasm assessment related to the genetic potential for increasing the density of Zn and Fe in grains (Bänziger 2000, Oikeh et al. 2003, Šimić et al. 2009). Until very recently, QTL analysis has been applied to biofortification traits in maize, and proved to be a powerful tool in the clarifying the genetic basis and molecular-physiological mechanisms conferring high level of grain micronutrient
QTLs for zinc and iron content in maize grains

(Lung’aho et al. 2011, Qin et al. 2012, Šimić et al. 2012, Zhou et al. 2010). In the F_2:3 mapping population applied in this study, we discovered QTLs for grain Zn and Fe content. Although our results were supported by one environmental experiment, all the five QTLs identified under this genetic background were included in MQTLs revealed through meta-analysis. The results demonstrated that QTL mapping of mineral content in maize grain is feasible and may be a practical approach to proceed with biofortification at the genetic and ultimately molecular level.

**Consensus QTLs and informative genomic regions**

Comparison of QTL results from different studies is difficult and tedious because results of QTL mapping may be influenced by such factors as genetic effects (genotypes, populations and generations), environments, mapping methods and even markers and population sizes (Boer et al. 2007, Brondani et al. 2002, Li et al. 2007, Li et al. 2011, Mihaljevic et al. 2004, Su et al. 2010). Meta-analysis proposed by Goffinet and Gerber (2000) could effectively integrate QTLs detected across different studies and true QTLs with more accurate positions and reduced CIs could be

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**Fig. 2.** MQTLs revealed by meta-analysis of reported Zn and Fe content QTLs. Vertical lines on the right of chromosomes indicate the confidence interval, and figures behind the name of initial QTLs and MQTLs connected by a dash indicate the variance.
provided. In the present study, meta-analysis was used to combine the QTL information related to maize grain Zn or Fe level derived from five independent researches, and total QTL number was decreased by 64% accompanied by reduced rate of CI varied from 29% to 83%. This illustrates the integration power of QTL meta-analysis.

Criteria for choosing MQTLs for MAS has been put forward by Löffler et al. (2009), that is (1) a small CI of the MQTL, (2) a high number of initial QTLs comprised in a MQTL and (3) a high mean effect of the MQTL corresponding to high mean explained phenotypic variances of initial QTLs. Meeting those criteria, 6 active regions for grain moisture content and 2 active regions for ear rot were highlighted by Xiang et al. (2012) through QTL meta-analysis. In this study, the genomic region of MQTL2 located on chromosome 2 (bin 2.07) in the marker interval umc1042–bng1 might be a hot spot of important QTLs controlling grain Zn and Fe amount in maize, because MQTL2 was comprised of 4 initial QTLs with CI less than 10 cM and 12.85% of mean phenotypic variation. In addition, MQTL3 detected on chromosome 2 (bin 2.08) might be interesting to researchers as it consisted of the maximum number of initial QTLs with the mean phenotypic variation up to 15.37%, although it had a relatively broader CI. Since the two chromosome regions of MQTL2 and MQTL3 are informative, they may be worth for further research, such as fine mapping, MAS and map-based cloning. Construction of secondary mapping population, including introgression lines, is our work in progress.

Co-localization for mineral concentration

A co-localization of two QTLs, qFe5 and qZn5 located in the marker interval umc1429–umc1060 on chromosome 5, was discovered in this study. This is consistent with a previous study by Šimić et al. (2012) who reported a co-localization of three QTLs on chromosome 3 for Fe/P, Zn/P and Mg/P ratios and overlapping chromosome regions of 4 QTLs on chromosome 6 for Fe/P ratio, P, Fe and Mg concentration. Co-localizing QTLs for Fe and Zn concentration in bean grains on linkage group B11 were also identified (Blair et al. 2009), coinciding with the same marker or with closely linked markers in the intervals AN034D–V104D and K126G–Bng1.

Meta-analysis is not only a powerful tool for QTL integration, but also an effective approach to reveal the genetic correlations among traits. Eight of the 10 MQTLs found in the present study involved both Zn and Fe content or their related traits, demonstrating a huge tendency of co-localization of the two traits. Co-integration of QTLs responsible for several grain yield components was observed by Li et al. (2011), which were consistent with significant correlations among grain yield and its component traits. Also, 47 out of 55 QTLs related to seed yield co-localized with QTLs for other yield traits (Shi et al. 2009).

The phenomenon of co-localization of nutrient element QTLs may be due to tight linkage of distinct genes or pleiotropism, physiological association of micronutrient accumulation, suggesting the relationship at the molecular level among these traits. MQTLs derived from both Zn and Fe content QTLs would be helpful to crop improvement with enhanced density of grain Zn and Fe because the tightly conjoined traits could be selected simultaneously through MAS.

In conclusion, the results of this study indicate that two candidate chromosome regions, bin 2.07 and 2.08, had obvious associations with QTLs for Zn and Fe content in maize grains. Developing secondary mapping populations with regard to MQTLs is advisable and helpful to a thorough understanding of the genetic mechanism of micronutrient level in grains. In addition, the correlation of Zn content with Fe content at the molecular level revealed by MQTLs comprising both two traits would facilitate the simultaneous improvement of them through MAS.

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