Quantitative trait loci analysis of zinc efficiency and grain zinc concentration in wheat using whole genome average interval mapping

Y. Genc · A. P. Verbyla · A. A. Torun · I. Cakmak · K. Willsmore · H. Wallwork · G. K. McDonald

Abstract Zinc (Zn) deficiency is a widespread problem which reduces yield and grain nutritive value in many cereal growing regions of the world. While there is considerable genetic variation in tolerance to Zn deficiency (also known as Zn efficiency), phenotypic selection is difficult and would benefit from the development of molecular markers. A doubled haploid population derived from a cross between the Zn inefficient genotype RAC875-2 and the moderately efficient genotype Cascades was screened in three experiments to identify QTL linked to growth under low Zn and with the concentrations of Zn and iron (Fe) in leaf tissue and in the grain. Two experiments were conducted under controlled conditions while the third examined the response to Zn in the field. QTL were identified using an improved method of analysis, whole genome average interval mapping. Shoot biomass and shoot Zn and Fe concentrations showed significant negative correlations, while there were significant genetic correlations between grain Zn and Fe concentrations. Shoot biomass, tissue and grain Zn concentrations were controlled by a number of genes, many with a minor effect. Depending on the traits and the site, the QTL accounted for 12–81% of the genetic variation. Most of the QTL linked to seedling growth under Zn deficiency and to Zn and Fe concentrations were associated with height genes with greater seedling biomass associated with lower Zn and Fe concentrations. Four QTL for grain Zn concentration and a single QTL for grain Fe concentration were also identified. A cluster of adjacent QTL related to the severity of symptoms of Zn deficiency, shoot Zn concentration and kernel weight was found on chromosome 4A and a cluster of QTL associated with shoot and grain Fe concentrations and kernel

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Y. Genc (✉) · K. Willsmore · H. Wallwork · G. K. McDonald
Molecular Plant Breeding CRC, Waite Campus,
PMB 1,
Glen Osmond, SA 5064, Australia
e-mail: yusuf.genc@adelaide.edu.au

Y. Genc · A. P. Verbyla · G. K. McDonald
School of Agriculture, Food and Wine,
The University of Adelaide,
Adelaide, SA 5064, Australia

A. P. Verbyla
Mathematical and Information Sciences, CSIRO,
Glen Osmond, SA 5064, Australia

A. A. Torun
Department of Soil Science and Plant Nutrition,
Cukurova University,
01330 Adana, Turkey

I. Cakmak
Faculty of Engineering and Natural Sciences,
Sabanci University,
81474 Tuzla, Turkey

K. Willsmore · H. Wallwork
South Australian Research and Development Institute,
Glen Osmond, SA 5064, Australia

weight was found on chromosome 3D. These two regions appear promising areas for further work to develop markers for enhanced growth under low Zn and for Zn and Fe uptake. Although there was no significant difference between the parents, the grain Zn concentration ranged from 29 to 43 mg kg^{-1} within the population and four QTL associated with grain Zn concentration were identified. These were located on chromosomes 3D, 4B, 6B and 7A and they described 92% of the genetic variation. Each QTL had a relatively small effect on grain Zn concentration but combining the four high Zn alleles increased the grain Zn by 23%. While this illustrates the potential for pyramiding genes to improve grain Zn, breeding for increased grain Zn concentration requires identification of individual QTL with large effects, which in turn requires construction and testing of new mapping populations in the future.

Keywords Biofortification · QTL analysis · Zinc deficiency · Whole genome analysis

Introduction

Zinc (Zn) deficiency reduces yield and quality of crops over large areas of the world's agricultural land (Graham et al. 1992; Cakmak et al. 1998). In the past decade there has been a great deal of research to determine genetic variation in tolerance to Zn deficiency (also called Zn efficiency) in wheat (Graham et al. 1992; Cakmak et al. 1998), rice (Gao et al. 2005) and barley (Genc et al. 2002). Although these studies have demonstrated considerable genetic variation and improved our understanding of the physiological mechanisms responsible for tolerance, no varieties of any cereal species have been bred specifically for this trait. The lack of success has been because firstly, screening methods are generally labour-intensive and slow, and in field trials show a large environmental influence, and secondly the Zn efficiency in cereals is a physiologically complex trait (Rengel 1999; Gao et al. 2005; Genc et al. 2006). There has been little work to characterise the genetic basis of Zn efficiency in wheat, but an analysis of the Opata x Synthetic bread wheat mapping population showed continuous variation in Zn efficiency suggesting that it is a quantitative trait and polygenic (Y. Genc unpublished), which is consistent with the few studies in other cereals (barley: Lonergan 2001; rice: Wissuwa et al. 2006).

Recently, a major focus of breeding has been to improve the grain Zn and Fe concentration of staple food crops as a means of overcoming chronic nutrient deficiencies that afflict large numbers of people in developing countries (Graham et al. 1999). Genotypic variation in grain Zn and Fe concentration has been demonstrated (Graham et al. 1999; Rengel et al. 1999; Cakmak et al. 2004), but at present we know very little about their genetic control. Grain Zn concentration in a bread wheat mapping population showed continuous variation suggesting that it is a quantitative trait controlled by several genes (Shi et al. 2007). Nutrient efficiency and grain nutrient loading are considered to be under separate genetic control (Cakmak et al. 2004), although there has been no systematic genetic analysis of the relationship between these traits. While plant nutrient uptake may not be directly related to grain nutrient levels, without an adequate pool of Zn and Fe in the vegetative tissue, grain Zn and Fe concentrations may be low, irrespective of the degree of remobilisation of nutrients and loading into the grain. Evidence of this is seen in the large increases in grain Zn concentrations that can be achieved from foliar applications of Zn prior to grain filling (Cakmak 2008; Peck et al. 2008).

The physiological complexity of plant Zn and Fe nutrition and the slow progress in phenotypic selection for enhanced Zn efficiency and grain nutrient concentrations suggests that marker assisted selection would be a powerful tool to select for enhanced plant nutrient status. There have been only a few studies that have identified QTL linked to Zn nutrition in cereals. One study identified QTL for Zn deficiency tolerance in rice (Wissuwa et al. 2006), where QTL were based on visual observations of Zn deficiency (deficiency-induced plant mortality and leaf bronzing) and dry matter; there were no QTL reported on plant Zn concentration in this study. Lonergan (2001) reported QTL linked to leaf Zn concentration in barley on chromosome 4HS and three QTL linked to grain Zn concentration in barley (on chromosomes 2HS, $2HL_1$ and 5HL). Recently a study in wild emmer wheat [*Triticum turgidum* ssp. *dicoccoides* (Korn) Thell] identified a QTL on chromosome 6B affecting grain Zn, Fe and protein accumulation

(Distelfeld et al. 2007). In a *Triticum monococcum* mapping population with 168 recombinant inbred lines and grown in 4 locations, a major QTL has been identified on chromosome 5 that is associated with high grain contents of Zn, Fe, copper (Cu) and manganese (Mn) (Ozkan et al. 2006).

There has been one recent report on QTL analysis of grain Zn (Shi et al. 2007), but there have been no studies in which QTL analysis of vegetative growth, grain yield and nutrient concentrations has been conducted on the same population in bread wheat. Such an analysis may provide additional insight into the physiological and genetic bases of nutrient uptake and its translocation to the grain.

Using QTL analysis for trait dissection is a common approach to examine complex traits and improvements in the method may increase its power. Composite interval mapping (CIM) and multiple interval mapping (Jansen 1994; Zeng 1994) are the standard methods of QTL analysis. However, recently Verbyla et al. (2007) proposed an improved method of analysis using a mixed model extension of interval mapping. The method, which has been called whole genome average interval mapping, or WGAIM, performs whole genome QTL analysis. The method uses a working model, a stopping rule based on a likelihood ratio test of significance of random QTL sizes, and an outlier detection method to select QTL. In a comprehensive simulation study, Verbyla et al. (2007) showed that the WGAIM approach outperforms CIM by a considerable margin. It is also a much simpler method to use.

Multi-environment analysis of QTL has received some attention (Tinker and Mather 1995; Hackett et al. 2001; Verbyla et al. 2003; Vargas et al. 2006) and this is particularly relevant to studies on nutrient efficiency and nutrient uptake since plants are grown under different levels of nutrient stress and may show significant genotype by environment interactions. This paper describes a QTL analysis of a doubled haploid (DH) population grown under deficient and adequate levels of Zn in a number of growth room and field experiments. QTL linked to seedling growth under low Zn supply and tissue and grain Zn and Fe concentrations were identified using a powerful extension of the work of Verbyla et al. (2007). Much of the previous work on genetic variation has only looked at grain or vegetative traits. This study examined whether there were genetic links between biomass production, shoot Zn and Fe concentrations, and grain Zn and Fe concentrations. The aims of this paper are first to illustrate the value of an improved technique of data analysis to identify QTL associated with Zn nutrition in wheat, and second to present the results of the QTL analysis and its interpretation.

Materials and methods

Plant material The study used a DH population of 90 lines from a cross between wheat genotypes RAC875-2 and Cascades. Previous screening of bread wheat genotypes at a range of soil Zn levels showed this pair had the largest difference in Zn efficiency (Genc et al. 2006). The DH population was increased in University of California soil mix (Barker et al. 1998) in the glasshouse to minimize variation in grain Zn content within the population since high seed Zn content can significantly affect growth under Zn-deficient conditions (Rengel and Graham 1995; Yilmaz et al. 1998; Genc et al. 2000). The seed Zn concentration was the same for the parental genotypes [24 mg kg^{-1} dry weight (DW)], but ranged from 18 to 32 mg kg^{-1} DW in the population. Grain Zn content was 1.4 μg Zn $seed^{-1}$ for both parents, while it varied from 0.9 to 1.7 μg Zn $seed^{-1}$ in the population.

Phenotypic data Three experiments were conducted; two experiments for seedling biomass and shoot nutrient concentration and one experiment for grain traits. Vegetative responses to varied Zn supply were examined in pot experiments in Adelaide (South Australia) and Adana (Turkey) under different experimental conditions. A field trial was conducted in South Australia to examine the grain yield response to applied Zn and variation in grain Zn concentration.

Experiment 1: Adelaide growth room study

Plants were grown in a sandy soil (Mt. Compass sand) which was collected near Mt. Barker, South Australia. The soil was washed three times with deionised water and air-dried. The DTPA-extractable Zn was 0.10 mg kg^{-1} soil. One kg of air-dried soil was placed into plastic bags and calcium carbonate (0.5% *w/w*) was mixed through the soil to raise the pH to 8.0 at which Zn availability is reduced. A pH of

7.5–8.0 is common in Zn-deficient calcareous soils (Cakmak et al. 1996). Basal nutrients consisting of (mg kg^{-1} soil) NH_4NO_3 (350); K_2HPO_4 (90); K_2SO_4 (120); $MgSO_4$, (90); $MnSO_4$, (3.0); $CuSO_4$ (5); H_3BO_3 (0.1); $CoSO_4.7\ H_2O$ (1); $FeSO_4.7\ H_2O$ (1.4); MoO_3 (0.005); $NiSO_4.6\ H_2O$ (0.15), and the two Zn treatments were added to the surface of the soil and allowed to dry. The contents of the plastic bags then were mixed again and placed in lined PVC pots with approximate dimensions of 6.5 × 30 cm (diameter × depth). The two Zn levels used in this experiment were 0.025 and 1.0 mg Zn kg^{-1} soil for deficient and adequate treatments, respectively. These levels gave the best discrimination between the two parents of the mapping population in a previous experiment.

Seeds were surface sterilised and germinated at 20°C. Four pre-germinated seeds of each genotype were transplanted into each pot and thinned to two per pot after the emergence. Plants were grown in a growth cabinet set at 20/15°C day/night temperature, 14 h photoperiod and 300 μmol m^{-2} s^{-1} light intensity at plant hight. Pots were randomised and watered daily to maintain the soil moisture content at 12% *w/w* (field capacity). All the DH lines and the two parents were harvested 32 days after transplanting when the difference in visual scores between the two parents was largest. The severity of deficiency symptoms, plant biomass and nutrient concentrations were measured.

Symptoms of Zn deficiency were recorded only on the deficient Zn treatment using a 1–9 scale [1=healthy green plants, 2=reduction in shoot growth with no foliar symptoms, 3=foliar symptoms (chlorotic areas) appearing on first leaves, 4=chlorotic areas scattered across the first leaves, 5=large chlorotic areas on the first leaves, 6=leaves collapsing in the middle, 7= chlorotic areas developing on second leaves, 8=both first and second leaves turning pale yellow, and 9= dead growing points]. At harvest, shoots were cut at the soil surface, rinsed in double-deionised water and dried at 65°C for 48 h. After shoot dry matter was recorded, shoot samples were chopped finely with stainless steel scissors. 0.6 g of oven-dried and finely chopped shoot samples was digested with 11 ml of nitric acid (HNO_3)/ perchloric acid ($HClO_4$) mixture (10:1 v/v), boiled down to approx. 1 ml of $HClO_4$ and made to 25 ml final volume using de-ionised water. This final solution was then analysed for nutrients on Inductively Coupled Plasma Optical Emission Spectrometer (ICP-OES; ARL 3580 B, Appl. Res Lab. SA, Ecublens, Switzerland) based on the method of Zarcinas et al. (1987).

Experiment 2: Adana glasshouse study

The experiment used a severely Zn-deficient soil from Central Anatolia, Turkey (Cakmak et al. 1996) which had very low Zn availability (DTPA 0.08 mg Zn kg^{-1}soil). Plants were grown either without applied Zn or with 5 mg Zn kg^{-1}soil applied as $ZnSO_4.7\ H_2O$. Each pot received a basal fertilizer application containing 200 mg N kg^{-1} soil (as $Ca(NO_3)_2.4H_2O$), 100 mg P kg^{-1} soil (as KH_2PO_4), 50 mg S kg^{-1} soil (as $CaSO_4.2H_2O$) and 5 mg Fe kg^{-1} soil (as Fe-EDTA). Each pot was filled with 1.650 kg dry soil and eight seeds were sown per pot and after germination (when seedling were 5–6 cm high) seedlings were thinned to five in each pot. The pots were brought to field capacity moisture and then irrigated regularly with deionized water. Plants were grown in a glasshouse with evaporative cooling system. The daily temperature was variable during the course of the experiment ranging between 18°C and 28°C.

Based on the severity of symptoms plants were harvested after 68 days of growth. Shoot biomass and shoot nutrient concentrations were measured as described by Cakmak et al. (1999).

Experiment 3: Field trial

A field trial was conducted at Claypans, South Australia in 2004 on a calcareous soil. The 90 DH lines were grown with the parents and check varieties consisting of commercial varieties and advanced breeding lines at two levels of Zn in a split-plot randomised complete block design trial with three replications. Genotype was the whole plot and the subplot was Zn treatment. Seed for all genotypes used in this trial were obtained from a seed increase nursery at the Charlick Experiment Farm Strathalbyn, South Australia the previous season. The plots were six rows (15 cm spacing) by 5 m with a seeding rate of 180–190 seeds m^{-2}. At sowing, all plots received a basal fertilizer application of 25 kg N ha^{-1} and 28 kg P ha^{-1}. One of each split-plot was sown with granular Zn (zinc oxysulphate, 32%Zn) at 7 kg Zn ha^{-1}. Eight weeks after sowing, a foliar spray of zinc sulphate

(Zincsol®, 16.7% Zn) at 2 L ha^{-1} [(1.1 ml of Zincsol® plus 82 ml of water) $plot^{-1}$] was applied to the split-plot that received Zn at sowing using a small plot sprayer.

The plots were harvested and the grain weight per plot measured. Samples of 20 grains of each line and the parental genotypes were randomly selected from the harvested grain, dried, weighed and processed for nutrient analysis by ICP-OES as described in Experiment 1. Only grain from the Zn treatment were analysed because our preliminary studies found that there was very little (and generally non-significant) variation in grain Zn concentration under severe Zn deficiency in the nil Zn treatment, whereas genetic variation in the Zn concentration was high when Zn was applied. Zn concentration is expressed on a dry weight basis (mg kg^{-1} DW).

DNA extraction and genotyping DNA extraction for the DH lines was achieved using a mini-prep adapted from Rogowsky et al. (1991) with the following variations. Of extraction buffer and phenol–chloroform–isoamyl alcohol (25:24:1), 750 µL was used for DNA extraction. The extraction buffer consisted of 0.1 M Tris–HCl (pH 8), 10 mM EDTA, 0.1 M NaCl and 1% sarkosyl. Deoxyribonucleic acid was precipitated by the addition of 0.1 vol. of 3 M sodium acetate (pH 4.8) and 1 vol. of isopropanol.

A total of 667 restriction fragment length polymorphism (RLFP), amplified fragment length polymorphism (AFLP), single squence repeat (SSR), gene-based and diversity arrays technology (DArT) markers were used to construct a linkage map. RFLP analysis, restriction endonuclease digestion and Southern hybridisation, followed the methods described by Guidet et al. (1991). AFLP analysis was performed according to Vos et al. (1995) using *PstI* and *MseI* restriction enzymes. Samples were electrophoresed on an Applied Biosystems ABI 3700 capillary electrophoresis instrument and analysed using GeneScan 3.5.1 software. SSR markers were amplified using a 'touchdown' PCR protocol (Williams et al. 2002), in 10-µL reaction mixtures containing 20 mM Tris–HCl (pH 8.4), 50 mM KCl, 1.0 mM dNTP, 1.875 mM $MgCl_2$, 15 ng of each primer, 0.5 U of Taq DNA polymerase (Qiagen), and 16 ng of template DNA. SSR PCR products were separated on 1-mm-thick 8% polyacrylamide gels at constant 300V for 3 h. Gels were stained with ethidium bromide and visualised under UV light prior to photography. For the gene-based markers, *Rht1* and *Rht2*, PCR and electrophoresis conditions were used as described by Ellis et al. (2002). DArT (Akbari et al. 2006) analysis used the standard cultivated wheat array, version 2.3, which has approximately 2000 polymorphic markers. Clones with $P>80\%$ and a call rate of at least 80% were initially selected for mapping; with clones with P between 75 and 80% being later incorporated into the map provided the number of introduced crossover events was minimised. Genotyping of each DH line for the non-DArT markers was achieved by visually scoring each marker in accordance with the corresponding parental alleles. The polymorphic DArT markers were converted from a binary code into genotype scores ('A', 'B') according to the scores of the parents.

Map construction The map was constructed using Map Manager QTX [version QTXb20, Manly et al. (2001)] using Kosambi mapping function with a threshold value of $P=0.01$. Genotypic data from the doubled haploid population was initially arranged into groups via the "Make Linkage Groups" function. New markers were integrated into these chromosomes using the "Links report" function, then in conjunction with the "ripple" function and published maps, an order of markers was established, with the aim of minimising double recombinants and chromosome length. Data was rechecked if these factors were compromised.

The original 667 markers were reduced to 470 for QTL analysis. Redundancies in markers occurred because of either zero or tiny recombination fractions between those markers. The genetic distances were initially estimated using MapManager QTX version QTXb20 (Manly et al. 2001) and checked using the Lander and Green (1987) algorithm in the R (R Development Core Team 2006) package qtl (Broman et al. 2006). There was an average of 23 markers per chromosome with an average spacing of 26 cM. However several chromosomes consist of several linkage groups with some of the chromosomal assignment being based on other doubled haploid populations (www.genica.net.au).

Data analysis The data to be analysed in this paper came from three trials. Thus combining information across trials and allowing for possible genotype by

environment (trial) interaction was required. Genotype by environment interaction is discussed by Smith et al. (2005) and the methods they presented provide the foundation for the developments in this paper. There were three sets of data to be analysed (1) Zn scores from plants grown under low Zn in Experiment 1; (2) dry weight and Zn concentration of shoots from both Zn treatments in Experiments 1 and 2 (3) and kernel weight and grain Zn concentration for lines grown in the field trial (Experiment 3). For simplicity, in the description of the analysis of the seedling traits, the low Zn treatment will be referred to as nil Zn although the amount applied to the soil in the Adelaide (0.025 mg Zn kg^{-1}) and Adana (0 mg Zn kg^{-1}) differed. In the analysis, shoot biomass at nil Zn will be used as the indicator of Zn efficiency. Often, the ratio of shoot biomass or grain yield under deficient and adequate levels of Zn is used to estimate Zn efficiency (Graham et al. 1992), however, variation in Zn efficiency is most strongly affected by shoot biomass under Zn deficiency. Rather than use a derived value of relative growth, the ability to grow vigorously under low Zn supply is the primary interest of the study. QTL analysis was done using WGAIM (Verbyla et al. 2007). Some traits were converted to a log-scale because of heterogeneity in the data. A detailed description of the statistical models used for the analysis is given in Appendix.

Results

Responses to zinc, distributions and relationships between traits

For most traits, there was evidence of transgressive segregation (Table 1). Applying Zn significantly increased shoot biomass and tissue Zn concentration in Experiments 1 and 2. The severity of Zn deficiency and the reduction in growth was greater in Experiment 2 (Adana) than in Experiment 1 (Adelaide); this is not surprising given the difference in Zn application in the two experiments. Without additional Zn applied to the soil in Experiments 1 and 2 , plants became severely deficient with mean shoot Zn concentrations being 6–9 mg kg^{-1} DW, well below the critical level of 15–20 mg kg^{-1} DW (Reuter and Robinson 1997). RAC875-2 was more sensitive to Zn deficiency than

Table 1 Range and average values of score of Zn deficiency symptoms (0–9 score), seedling biomass (mg $plant^{-1}$), shoot Zn and Fe concentrations (mg kg^{-1}), grain yield (kg ha^{-1}), kernel weight (mg) and grain Zn and Fe concentrations (mg kg^{-1}) in the three experiments used for QTL analysis

Experiment	Trait	Zinc treatment	Mean	Range	Parental means	
					RAC875-2	Cascades
Exp. 1	Zn score	Nil	4.2	2.5–7.0	6.3	3.1
	Shoot dry matter	Nil	680	471–892	640	829
		Zn	920	630–1322	947	1018
	Shoot Zn conc.	nil	8.6	7.0–12.0	8.19	9.13
		Zn	68.9	49.0–90.5	63.9	68.0
	Shoot Fe conc.	nil	76.8	53.3–112.8	77.5	84.1
		Zn	59.0	40.5–83.4	57.0	63.5
Exp. 2	Shoot dry matter	nil	1270	730–1810	1,070	1,329
		Zn	1990	1130–2640	1,920	2,065
	Shoot Zn conc.	nil	6.6	7.8–9.7	6.43	7.80
		Zn	42.9	30.1–61.2	44.5	42.0
	Shoot Fe conc.	nil	82.1	46.0–151.5	101.6	83.7
		Zn	41.1	29.9–65.5	45.5	43.1
Exp. 3	Grain yield	nil	669	439–964	724	603
		Zn	648	354–942	609	632
	Kernel weight	Zn	30.8	24.8–34.7	33.0	29.3
	Grain Zn conc.	Zn	35.2	29.0–42.7	30.3	33.9
	Grain Fe conc.	Zn	36.6	28.6–46.8	35.8	36.3

Zinc treatments: *nil* No Zn application, *Zn* Zn application

Cascades. It showed more severe symptoms and lower shoot Zn concentrations (Table 1).

Growth and grain yield from the field trial (Experiment 3) were reduced greatly by drought, with a mean grain yield of 657±12.3 kg ha^{-1}. There was no significant response to applied Zn, although there were significant differences in grain yield among the genotypes. The grain Zn concentration of the parents were not significantly different.

Residual correlations between seedling biomass and shoot Zn and Fe concentrations varied in Experiment 1, but were consistently negative in Experiment 2 (Table 2). The residual correlations between shoot Zn and Fe concentrations varied between experiments and Zn treatments. Under Zn deficiency, the score for symptoms of Zn deficiency was not correlated with shoot Zn concentration or shoot biomass (Table 2). The genetic correlations showed more consistent relationships. Shoot Zn and Fe concentrations were negatively correlated with biomass, whereas shoot Zn and Fe concentrations were positively correlated with each other. There was no genetic correlation between Zn score and shoot biomass production or between Zn score and shoot Zn concentration.

In the field experiment (Experiment 3), the residual correlations between grain Zn concentration, grain yield and Fe concentration were generally weak and non-significant apart from a significant correlation between grain Zn and Fe (r=0.562). There were positive genetic correlations between grain Zn and kernel weight (r_g=0.539), between grain Fe and kernel weight (r_g=0.316), and between grain Zn and grain Fe (r_g=0.635).

QTL analysis

Zinc score Four QTL were identified for the Zn deficiency scores on chromosomes 2A, 4A, 7B and 7D (Table 3) with the QTL on chromosome 7B associated with the largest shift in Zn score. The RAC875-2 alleles on chromosomes 2A and 4A contributed to a lower Zn score (i.e., less severe symptoms), while the RAC875-2 allele on chromosome 7B increased Zn score. Fitting the QTL effect reduced the polygenic variance from $\hat{\sigma}^2_g = 1.10$ to $\hat{\sigma}^2_g = 0.73$ so that 34% of the total genetic variance was explained by the QTL identified by the analysis (see Eq. 3, Appendix).

Table 2 Residual and genetic correlation coefficients[a] between shoot biomass (on the log-scale), shoot Zn concentration (on the log-scale), shoot Fe concentration (on the log-scale) and visual scores of Zn deficiency among 90 lines of the RAC875-2 × Cascades doubled haploid population grown at two levels of Zn in experiments at Adelaide, South Australia and Adana, Turkey

Parameter	Zn deficiency score	Zn concentration		Fe concentration	
		Nil	Zn	Nil	Zn
Residual correlations					
Adelaide					
Shoot biomass	−0.113	0.136	−0.840	−0.176	0.403
Zn concentration	0.034			0.418	−0.504
Adana					
Shoot biomass		−0.227	−0.683	−0.185	−0.547
Zn concentration				−0.022	0.460
Genetic correlations					
Adelaide					
Shoot biomass	0.000	−0.721	−0.755	−0.455	−0.512
Zn concentration	−0.046			0.652	0.841
Adana					
Shoot biomass		−0.719	−0.638	−0.412	−0.713
Zn concentration				0.766	0.548

The correlations have been calculated within each treatment in each of the two experiments.

[a] The genetic correlations were found using a model that incorporated the four traits in a single analysis. They are calculated using the estimated genetic variance–covariance **G** matrix from model (1) of Appendix. Residual correlations are found in a similar fashion and are based on the estimated **R** in model (1) of Appendix

Table 3 Putative QTL intervals the size of the effect and its significance for Zn deficiency score and shoot biomass of seedlings grown in a growth room in Adelaide (GR-Adel.) or a glasshouse in Adana, Turkey (GH-Adana) under deficient (Nil) and adequate (Zn) levels of soil Zn

Chrom.	Site	Zn treat	Marker interval	Spanning Distance (cM)	Additive effect[a]	P-value
Zn deficiency score						
2A	GR-Adelaide	Nil	wms614-barc124	8.2	–0.306	0.0033
4A	GR-Adelaide	Nil	P40/M60-217-gwm397	2.4	–0.373	0.0003
7B	GR-Adelaide	Nil	wmc273A-P43/M60-284	6.7	0.449	0
7D	GR-Adelaide	Nil	gwm437-P38/M50-240	2.4	0.380	0.0002
Shoot biomass						
4B	GR-Adelaide	Nil	*Rht1*-gwm6	15.3	0.099	0
4B	GR-Adelaide	Zn	*Rht1*-gwm6	15.3	0.070	0
4B	GH-Adana	Nil	*Rht1*-gwm6	15.3	0.103	0
4B	GH-Adana	Zn	*Rht1*-gwm6	15.3	0.106	0
4D	GR-Adelaide	Nil	*Rht2*-wPt.8321	17.0	–0.084	0
4D	GR-Adelaide	Zn	*Rht2*-wPt.8321	17.0	–0.086	0
4D	GH-Adana	Nil	*Rht2*-wPt.8321	17.0	–0.085	0
4D	GH-Adana	Zn	*Rht2*-wPt.8321	17.0	–0.081	0

[a] Positive and negative values indicate that RAC875-2 and Cascades alleles increased the phenotypic values, respectively. The additive effect for shoot biomass is given on a $\log_e$ scale and the effect for Zn score is on the original scale

Seedling biomass Eight intervals were identified as putative QTL for shoot biomass, with all the QTL being the same in Experiments 1 and 2 (Table 3). All intervals (chromosomes 4B and 4D) were associated with the height genes, *Rht1* and *Rht2* (Ellis et al. 2002), and these had an impact on dry matter regardless of location or treatment. Higher biomass was associated with the RAC875-2 allele on chromosome 4B and with the Cascades allele on chromosome 4D.

The estimated polygenic variance matrix (see Appendix) can be found for both the base model (i.e. prior to QTL analysis) and the final model (i.e. after QTL analysis) with selected QTL for shoot biomass (on the log-scale) (Table 4). Shoot biomass showed a high genetic correlation between the two experiments and between the two Zn treatments. Comparing the genetic variances (the diagonal entries in each matrix) before and after the QTL analysis shows that fitting the QTL decreased the polygenic variances by 65% (Adelaide, nil), 49% (Adelaide, Zn), 51% (Adana, nil) and 55% (Adana, Zn), indicating that the QTL identified in the analysis are accounting for 50% or more of the observed genetic variance. The correlations have in general decreased but the QTL that remain undetected are probably mostly in common, of small size and may be numerous. Epistatic interactions also remain to be investigated.

Shoot Zn concentration Twelve putative QTL intervals were found for shoot Zn concentration (Table 5). A single QTL common to both experiments and treatments was found on chromosome 4D, which is one of the height genes (*Rht2*). Of the significant QTL for Zn concentration, this had the largest effect, with the RAC875-2 allele associated with higher concentrations. The allele increased shoot Zn concentration under nil Zn by 6–7%, equivalent to 0.4–0.7 mg kg^{-1} DW. Under adequate Zn, the allele increased tissue Zn concentration by 6–7%, equivalent to an increase of 3–4 mg kg^{-1} DW. This QTL was also identified in the analysis of seedling biomass (Table 3), where the effect of the RAC875-2 allele was to reduce shoot biomass. The remaining QTL, which were independent of height, had smaller effects of about 3–5%. The QTL on 4A is a part of a sequence of two QTL associated with Zn score and shoot Zn concentration (Tables 3 and 5). The RAC875-2 allele for these two QTL was associated with lower Zn score and Zn concentration.

Polygenic variance matrices before and after QTL selection for log (Zn concentration) indicated there was a high genetic correlation in shoot Zn concentration among the treatments and experiments (Table 4). While the same QTL were observed in the Adelaide and Adana experiments, the QTL accounted for a larger proportion of the genetic variation in the

Table 4 Matrices of polygenic variances and genetic correlations[a] for shoot biomass, shoot Zn and Fe concentrations before and after QTL analysis in Experiments 1 and 2. Variances are shown on the diagonal and the pairwise genetic correlations are shown above the diagonal. The values derived prior to QTL analysis represent the variation in the trait using the baseline models and includes the variation associated with the marker effect and non-marker effects, while values obtained after the QTL analysis is the variation in the population after the variation associated with the QTL has been removed

Parameter	Before QTL analysis				After QTL analysis			
	Adelaide Nil	Adelaide Zn	Adana Nil	Adana Zn	Adelaide Nil	Adelaide Zn	Adana Nil	Adana Zn
Log_e(Shoot biomass)								
Adelaide Nil	0.023	0.85	0.84	0.83	0.008	0.69	0.62	0.61
Adelaide Zn		0.022	0.84	0.84		0.011	0.67	0.70
Adana Nil			0.030	0.87			0.015	0.72
Adana Zn				0.028				0.013
Log_e(Shoot Zn concentration)								
Adelaide Nil	0.007	0.57	0.60	0.44	0.002	0.17	0.13	0.17
Adelaide Zn		0.011	0.74	0.54		0.001	0.59	1.00
Adana Nil			0.013	0.57			0.010	0.77
Adana Zn				0.017				0.011
Log_e(Shoot Fe concentration)								
Adelaide Nil	0.016	0.91	0.62	0.60	0.003	1.00	0.60	0.51
Adelaide Zn		0.008	0.68	0.66		0.005	0.56	0.51
Adana Nil			0.030	0.45			0.028	0.31
Adana Zn				0.014				0.011

The difference between the two values is the genetic variation explained by the markers

[a] The estimated polygenic variances and genetic correlations were found using the estimated genetic variance–covariance matrix **G** from model (1) of Appendix, that is before QTL analysis, and using the estimated genetic variance-covariance matrix **G** from the final model which contains the QTL

Adelaide experiment compared to the Adana experiment: the percentage of polygenic variance explained by the fitted QTL were 77% (Adelaide, nil), 94% (Adelaide, Zn), 24% (Adana, nil) and 34% (Adana, Zn). The stronger effect in the Adelaide experiment resulted in the genetic correlations within the Adelaide experiment being essentially reduced to zero after the QTL were fitted, whereas correlations with the Adana experiment were still high.

Shoot Fe concentration There were ten putative QTL intervals for shoot Fe concentration on chromosomes 2B, 3D, 4B and 4D (Table 5). The common QTL across experiments were the height genes on chromosomes 4B and 4D. The presence of the RAC875-2 allele at QTL on chromosome 4B reduced tissue Fe concentration by 3–7% while on chromosome 4D the allele increased Fe concentration by a similar amount. As with the QTL associated with Zn concentration, the effects of the RAC875-2 allele on tissue Fe concentration showed an opposite trend to its effect on shoot biomass. The remaining two QTL on chromosomes 2B and 3D were found only for the nil treatment in the Adelaide trial and were associated with a change in Fe concentration of 3–5%. The effects of these QTL were independent of the shoot biomass QTL and the QTL on chromosome 2B had relatively large effects, increasing shoot Fe concentration by 5%, or approximately 4 mg kg^{-1} DW.

The QTL for Fe concentration explained 78% (Adelaide, nil), 36% (Adelaide, Zn), 6% (Adana, nil) and 23% (Adana, Zn) of the polygenic variation (Table 4). The impact of the QTL selected on the polygenic correlations is variable.

Kernel weight The analysis identified 17 putative QTL located on nine chromosomes (Table 6). Fitting the QTL reduced the polygenic variance, $\hat{\sigma}^2_g$ from 0.088 to 0.016, thus explaining 82% of the genetic variance. Most of the QTL identified for increased kernel weight were associated with the RAC875-2 allele, while the Cascades allele resulted in single largest increase. Out of 17 QTL, those located on chromosomes 4A, 4B and 6D had the greatest effects on kernel weight, with a shift in kernel weights of

Table 5 Putative QTL intervals the size of the effect and its significance for Zn and Fe concentrations of seedlings grown in a growth room in Adelaide (GR-Adel.) or glasshouse in Adana, Turkey (GH-Adana) under deficient (Nil) and adequate (Zn) levels of soil Zn

Chrom.	Site	Zn treat.	Marker interval	Spanning Distance (cM)	Additive effect[a]	*P*-value
Zn concentration						
2D	GR-Adelaide	Zn	gwm296-P40/M56-118	12.9	0.044	0.0004
4A	GR-Adelaide	Zn	gwm397-gwm269	11.2	0.036	0.003
4B	GR-Adelaide	Nil	wms149-gwm113	7.9	−0.035	0.0001
4D	GR-Adelaide	Nil	*Rht2*-wPt.8321	17.0	0.074	0
4D	GR-Adelaide	Zn	*Rht2*-wPt.8321	17.0	0.060	0
4D	GH-Adana	Nil	*Rht2*-wPt.8321	17.0	0.062	0
4D	GH-Adana	Zn	*Rht2*-wPt.8321	17.0	0.070	0
5B	GH-Adana	Zn	gwm271b-gwm400	18.0	0.067	0
5D	GH-Adana	Zn	gwm190-P32/M57-191	45.0	−0.062	0
5D	GR-Adelaide	Zn	P36/M50-44-wPt.2847	2.3	−0.041	0.0009
6A	GR-Adelaide	Zn	P32/M52-219-barc146	2.4	−0.050	0.0001
6A	GR-Adelaide	Zn	P39/M53-229-wPt.1159	5.9	0.029	0.019
Fe concentration						
2B	GR-Adelaide	Nil	wPt.7092-wmc332	2.4	−0.052	0
3D	GR-Adelaide	Nil	gdm8-gdm136	1.1	0.027	0.0002
4B	GR-Adelaide	Nil	*Rht1*-gwm6	15.3	−0.069	0
4B	GR-Adelaide	Zn	*Rht1*-gwm6	15.3	−0.061	0
4B	GH-Adana	Nil	*Rht1*-gwm6	15.3	−0.032	0.125
4B	GH-Adana	Zn	*Rht1*-gwm6	15.3	−0.059	0.0001
4D	GR-Adelaide	Nil	barc098-P42/M49-70	1.1	−0.055	0
4D	GR-Adelaide	Zn	barc098-P42/M49-70	1.1	0.026	0.0098
4D	GH-Adana	Nil	barc098-P42/M49-70	1.1	−0.041	0.0349
4D	GH-Adana	Zn	barc098-P42/M49-70	1.1	−0.023	0.09

The additive effect is given on a $\log_e$ scale

[a] Positive and negative values indicate that RAC875-2 and Cascades alleles increased the phenotypic values, respectively

0.19–0.79 mg. The Cascades alleles for QTL on 4A and 6D increased kernel weight, while the RAC875-2 allele for QTL on 4B, which was associated with one of the height genes (*Rht1*), increased kernel weight. The QTL on 4D collocated to a QTL for shoot Fe concentration (Tables 5 and 6), with the effect of the RAC875-2 allele to decrease tissue Fe and kernel weight. it should be noted that the QTL on 4A was located on a very wide interval, between two markers that are essentially unlinked. The marker gwm160 was placed on 4A on the basis of other linkage maps and in fact it is this marker that is strongly associated with kernel weight. If the marker is omitted, the QTL on 4A is no longer detected. Thus it is important to use as much information as possible in the analysis, and this includes information from other populations.

Grain Zn concentration Four QTL were found on chromosomes 3D, 4B, 6B and 7A (Table 6), with RAC875-2 and Cascades contributing alleles associated with high grain Zn. Fitting the QTL accounted for 92% of the genetic variance, reducing the estimated polygenic variance from 2.79 to 0.23. The additive effect of the allele from RAC875-2 on grain Zn concentration ranged from a decline of 0.95 mg kg^{-1} DW to an increase of 1.17 mg kg^{-1} DW. The effects of the 16 different combinations of the parental alleles for these QTL are shown in Fig. 1. Average grain Zn increased by 23%, from approximately 31 to 38 mg kg^{-1} DW, by combining the four alleles that contributed to high grain Zn concentration. The QTL for grain Zn concentration on chromosome 4B corresponded to a QTL for shoot Zn concentration identified in Experiment 1 (Table 5). The allele from RAC875-2 increased grain Zn concentration and the Cascades allele increased shoot Zn concentration. There were no coincident QTL for kernel weight and grain Zn concentration.

Table 6 Putative QTL intervals the size of the effect and its significance for kernel weight and grain Zn and Fe concentrations of field-grown plants at Claypans, South Australia

Chrom.	Marker interval	Spanning distance (cM)	Additive effect[a]	*P*-value
Kernel weight				
2A	barc124-P40.M48.4	3.2	0.071	0.0001
2A	gwm71-barc15	1.1	0.083	0
2D	P39/M47-1-wPt.0935	3.2	−0.082	0
3A	P34/M51-9-gwm5	3.3	0.069	0.0001
3A	gwm155-wPt.0544	10.5	−0.054	0.0076
3A	P38/M50-1-P44/M54-6	22.7	0.066	0.0017
3D	Gdm8-gdm136	1.1	0.078	0
4A	wPt.5175-wPt.1179	1.2	−0.054	0.0023
4A	psr119b-abg75A	3.9	0.083	0
4A	bcd129b-gwm160	656.1[b]	−0.788	0.0001
4B	*Rht1*- gwm6	15.3	0.115	0
4D	barc098-P42/M49-7	1.1	−0.063	0.0006
5A	mwg2053b-wPt.3605	5.2	−0.040	0.0264
6D	gwm325-P40/M56-1	21.5	−0.190	0.0001
6D	mwg798A-P38/M50-3	1.0	0.083	0
6D	P42.M48.2-gwm325	4.5	0.077	0.0688
6D	P43/M54-8-P31/M58-3	3.3	0.054	0.0085
Grain Zn concentration				
3D	Gdm136-gwm3	43.5	0.919	0.001
4B	wms149-gwm113	7.9	1.166	0
6B	barc146a-P41/M48-76	14.5	−0.949	0.0001
7A	gwm282-gwm63	6.9	0.690	0.0034
Grain Fe concentration				
3D	gdm8- gdm136	1.1	1.78	0

[a] Positive and negative values indicate that RAC875-2 and Cascades alleles increased the phenotypic values, respectively.

[b] The marker gwm160 is not linked to bcd129b in the RAC875-2 × Cascades population but is linked in other populations. Examination of this QTL showed the marker gwm160 to be clearly associated with kernel weight. Including this marker allowed the association to be detected

Grain Fe concentration A single QTL was found on chromosome 3D (Table 6) which collocated to a QTL for shoot Fe concentration (Table 5) and to a QTL for kernel weight. The RAC875-2 allele increased grain Fe concentration by 1.8 mg kg^{-1} DW, or 5%, and this was associated with an increase in tissue Fe concentration under nil Zn of approximately 2 mg kg^{-1} DW. The RAC875-2 allele contributed to high kernel weight at this QTL. The QTL accounted for 47% of the genetic variance.

Discussion

Whole genome average interval mapping

The analysis illustrated the value of WGAIM for identifying QTL from multi-environment, multi-treatment experimental data sets. The decomposition of a FA model into two parts, one for common QTL effects (but with varying levels of association) and one for individual components or contrasts allows improved understanding of interval trait association over a number of different environments. These models can be fitted using ASReml (Butler et al. 2007; Gilmour et al. 2006). The advantage of using this method of QTL analysis is that it is a more powerful method than CIM and is better able to detect QTL in small populations (Verbyla et al. 2007). This should not be seen as a reason for restricting population size, but it does allow useful information to be obtained from experiments where, for various reasons, population sizes are smaller than desirable. In addition, the approach allows non-genetic effects to be included in the analysis. This means that naïve and simplistic calculation of the phenotype of interest is avoided. The impact of non-genetic sources of variation can be assessed and allowed for in the full QTL analysis.

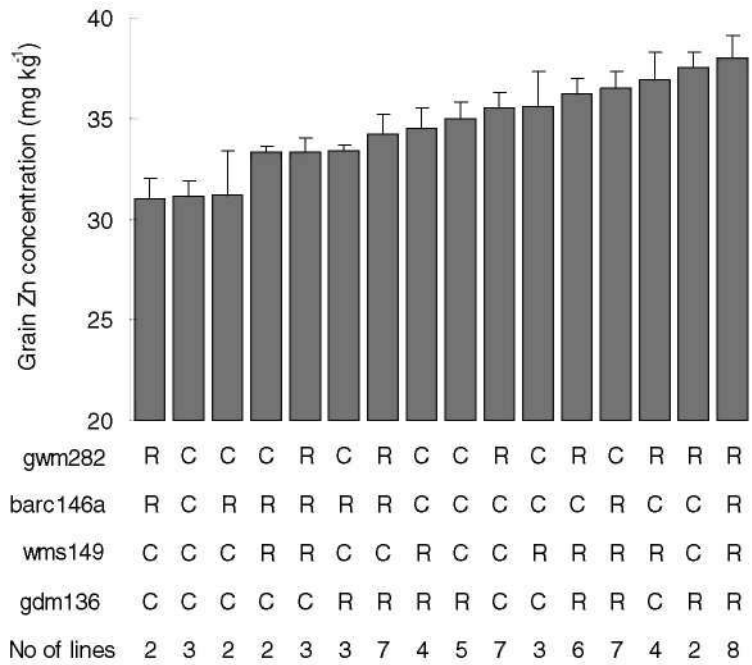

Fig. 1 The effects on grain Zn concentration of the different combinations of the parental alleles at each of the four putative QTL identified for grain Zn concentration (gdm136, wms149, barc146a and gwm282). 22 DH lines which could not be genotyped at one or more of those four putative QTL according to parental scores ('A', 'B') were excluded from the analysis. The data have been ranked from lowest (31 mg kg^{-1}) to highest (38 mg kg^{-1}) grain Zn concentration. *Error bars* are the standard error of the means. *R*=RAC875-2 allele; *C*=Cascades allele

One additional aspect of the analyses conducted is the inclusion of markers that are either weakly or not linked to other markers in a linkage group (or chromosome). There are several examples where markers were included in linkage groups on the basis of other populations. The inclusion of weakly linked or not linked markers in the present study resulted in finding QTL which otherwise would not have been detected. WGAIM down-weights wide intervals and the fact that the QTL were found indicates their impact is substantial. This is another good feature of the method.

Shoot biomass production

Vegetative growth under both deficient and adequate levels of Zn was greatly influenced by height genes. This is consistent with previous studies on early vigour in wheat which showed the presence of the *Rht1* and *Rht2* dwarfing genes reduced seedling biomass (Coleman et al. 2001; Rebetzke et al. 2001). There were consistent negative genetic correlations between shoot biomass and Zn and Fe concentrations at both levels of Zn nutrition in the two experiments (Table 2). The QTL analysis indicated that part of this effect was caused by the presence of the height genes, because the alleles conferring greater height and biomass also were associated with lower Zn concentrations. Given the strong influence of the height genes on Zn and Fe concentrations, it is perhaps not surprising that there was a consistent positive genetic correlation between Zn and Fe concentrations in the shoot (Table 2). However, it does illustrate that where a population is segregating for height, interpretation of tissue nutrient concentrations needs to take account of differences in vigour of the seedlings that result from the effects of the these height genes. It also emphasises the importance of selection of parents which show similar levels of vigour in developing DH populations for future studies in Zn efficiency.

There was evidence of a cluster of genes associated with response to Zn deficiency by the seedling. Two adjacent QTL associated with Zn score under Zn deficiency and shoot Zn concentration were identified on chromosome 4A: a low Zn score was associated with high Zn concentration. Reviewing the genetics of micronutrient uptake, Graham and Stangoulis (2003) noted that traits linked to Zn, Mn and Cu efficiency in a number of cereals were found on the same homeologous regions of chromosome 4 and this, in turn was associated with a region of the chromosome that controls phytosiderophore production. The association of Zn concentration and expression of Zn deficiency symptoms on a similar region of chromosome 4 is consistent with the presence of genes controlling uptake from the soil.

The alleles from the less efficient genotype (RAC875-2) on chromosome 4A were associated with enhanced Zn nutrition. This apparent inconsistency with the observed phenotypic difference between the parents is because most of the Zn-related traits are associated with multiple QTL, often of small effect individually. Each parent contributed alleles associated with both increased and reduced sensitivity to Zn deficiency, and in the case of the cluster of QTL on chromosome 4A, it is the contribution from RAC875-2 that is enhancing growth under Zn deficiency. That RAC875-2 shows greater sensitivity to Zn deficiency suggests that the positive influence of the QTL is insufficient to overcome the effects of the other QTL linked to greater sensitivity to Zn deficiency. Nevertheless, if the importance of these QTL is verified, this region on chromosome 4A may be useful to target to improve growth under low available Zn.

Apart from chromosome 4, QTL on chromosome 7 may also contribute to enhanced Zn nutrition. QTL for Zn deficiency symptoms were identified on chromosomes 7B and 7D. In rye, chromosome 7R (along with 1R) was shown to be responsible for higher tolerance to Zn deficiency (Graham 1984; Cakmak et al. 1997). Further work is required to establish if these QTL are linked.

The QTL analysis also suggested a gene cluster associated with Fe nutrition on chromosome 3D: A QTL associated with tissue Fe concentration on chromosome 3D coincided with a QTL for grain Fe concentration and was adjacent to a QTL for kernel weight. It is possible that all three traits coincide. In this case high shoot Fe concentration was associated with a larger kernel weight and higher grain Fe. Alleles that contributed to high shoot Fe concentration also contributed to high grain Fe concentration and high kernel weight. Again, this region may be useful for further study as it suggests it is linked to Fe uptake by the shoot during vegetative growth and remobilisation to the grain.

Kernel weight, grain Zn and Fe concentrations

The low residual correlations between kernel weight and grain nutrient concentrations in this study are consistent with previous analyses that showed little or no relationship between these traits (McDonald et al. 2008; Distelfeld et al. 2007). However, there was a positive genetic correlation between kernel weight and grain Zn. This suggests the ability to load Zn into the grain may have been linked to the ability to fill grain in this experiment.

The dry seasonal conditions limited grain yield in the field experiment and it is likely that this resulted in high grain Zn grain concentrations. The average value in the experiment (35 mg kg^{-1}) was greater than that commonly measured in bread wheat grown under rainfed conditions in this environment (approximately 20 mg kg^{-1}). However, the 'yield-concentration' effect of the low yields may not have had a large effect on the ability to identify QTL for grain Zn concentration. Grain Zn concentration and grain yield are often negatively correlated when a range of genotypes with differences in grain yield is analysed (McDonald et al.2008). In such cases part of the variation in grain Zn concentration is associated with the variation in grain yield. While there was a significant difference in grain yield among the lines, there were no phenotypic or genetic correlations between grain yield and grain Zn concentration, suggesting the differences in grain Zn concentration were largely independent of grain yield. Therefore, we contend that while the dry conditions may have enhanced grain Zn concentration, the QTL for grain Zn concentration identified in this study are unlikely to have been influenced markedly by differences in yield potential among the lines. The fact that grain Zn QTL identified under low yielding conditions in itself is a useful trait in low rainfall regions where Zn deficiency can occur. However, clearly the value of the QTL to grain Zn needs to be tested under less adverse seasonal conditions.

The parents did not differ significantly in their grain Zn concentration and variation within the population was due to transgressive segregation of genes with relatively small effects. Four QTL for grain Zn concentration were identified and each was associated with an effect of approximately 1 mg Zn kg^{-1} DW. These QTL were on separate chromosomes (3D, 4B, 6B and 7A) which may allow pyramiding of the alleles conferring high grain Zn. Within the population at Claypans, a shift in grain Zn of approximately 7 mg kg^{-1} DW occurred when the four alleles associated with high grain Zn were combined (Fig. 1). This shift in grain Zn concentration may be considered physiologically significant in terms of human nutrition (RD Graham pers. comm.). It also occurred under conditions that would have severely limited Zn uptake by the crops because there

was no effect of Zn treatment on yield. Despite this the change in grain Zn associated with these QTL is of a similar magnitude, both in relative and absolute terms, to that reported for the high protein *Gpc-B1* locus among recombinant substitution lines grown under much more favourable conditions (Fahima et al. 2006; Distelfeld et al. 2007). The results demonstrate in principle, the ability to pyramid genes to enhance the grain Zn concentration, although from a practical viewpoint it is unlikely that plant breeders would select for four QTL to increase grain Zn density. The RAC875-2 x Cascades DH population was not developed specifically for studies on grain nutrient density and the relatively small effects of each of the QTL reflects the small difference between the parents. It is likely that QTL associated with larger effects would be identified if a DH population derived from parents with a bigger difference in grain Zn concentration were used.

The QTL identified on 3D, 4B, 6B and 7A have not been reported previously. However, the QTL on chromosome 6B (Table 6) supports the findings of a previous study with wheat-*dicoccoides* substitution lines that chromosome 6B carried genes determining high grain Zn density (Cakmak et al. 2004). It is yet to be determined if the QTL on 6B in the present study is on the same region as the genes proposed in the latter study.

One QTL for grain Fe was identified. Although significant, the QTL was associated with a shift in grain Fe concentration of about 1.8 mg Fe kg^{-1} DW equivalent to a total difference between the alleles of 3.6 mg Fe kg^{-1} DW. The QTL for grain Fe concentration collocated with a QTL for shoot Fe concentration, with the alleles for high Fe concentration coming from the same parent. Similarly, a QTL that was linked to shoot Zn concentration and grain Zn was found on chromosome 4B. Much of the work on biofortification has focussed on the remobilisation and translocation of nutrients to the grain as the means of improving grain nutrient concentration, but the results of this QTL analysis suggests that an ability to accumulate Fe and Zn in the vegetative tissue is also associated with elevated grain Fe and Zn. Agronomic biofortification achieves this by directly applying nutrients to the foliage, but the QTL analysis illustrates the underlying principle that a way of increasing grain nutrient concentration genetically is to enhance nutrient uptake (Cakmak 2008).

Several loci are involved in tolerance to Zn deficiency and grain Zn and Fe density

Earlier studies in barley (Genc et al. 2003) and rice (Gregorio et al. 2002) suggested that tolerance to Zn deficiency is controlled by a single gene or by major QTL. However, the current results clearly show that Zn efficiency, shoot and grain nutrient concentrations are each controlled by a number of genes, often with relatively small effects. The complexity of the traits associated with enhanced Zn nutrition means that marker aided selection has the potential to improve selection for Zn efficiency and grain Zn concentration if robust QTL can be identified. The present study also identified a number of chromosome regions that may be useful to improve Zn and Fe concentrations in the shoot and grain of wheat. The consistency of some of these QTL across experiments together with our understanding of the physiological basis on Zn and Fe nutrition suggest they might be useful candidates for further marker development.

Putative genetic linkages between QTL for Zn deficiency symptoms and Cu/ZnSOD gene

The most significant QTL for Zn deficiency symptoms in this study was located on the long arm of 7B. The expression of Zn deficiency symptoms on the leaves of cereals has been found to be related to the activity of the enzyme copper (Cu)/Zn superoxide dismutase (Cu/ZnSOD) (Cakmak et al. 1997, 1998; Hacisalihoglu et al. 2003). High levels of Cu/ZnSOD help protect leaf tissue against oxidative damage (Cakmak 2000) and thereby contribute to Zn efficiency (Cakmak et al. 1995; Hacisalihoglu et al. 2003). Wu et al. (1999) located the chloroplast Cu/ZnSOD genes on the long arms of the group 7 chromosomes, but the exact location of the Cu/ZnSOD gene is not known. The linkage between this gene and QTL identified in our study warrants further investigation.

Conclusions

An improved method of QTL analysis enabled a number of significant QTL with large effects to be identified from a relatively small population. Plant height, determined by the presence of the *Rht1* and *Rht2* dwarfing genes, was an important influence on

the growth, shoot Zn concentration and grain Zn concentration, irrespective of the level of available soil Zn. These effects have not been described previously. The ability to accumulate Fe in the vegetative tissue appears to influence the concentration of Fe in the grain. The regions of adjacent QTL on chromosomes 4A and 3D that were associated with improved growth and Zn and Fe concentrations appear promising for the future development of molecular markers to improve Zn and Fe nutrition in wheat. While the presence of several QTL for grain Zn concentration indicates that pyramiding genes for can significantly increase grain Zn density, breeding for increased grain Zn concentration will require identification of individual QTL with large effects.

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Appendix

Statistical models: baseline models without QTL

The mixed model forms the basis of the analysis and is given by

$$\mathbf{y} = \mathbf{X}\boldsymbol{\tau} + \mathbf{Z}\mathbf{u} + \mathbf{Z}_l\mathbf{g} + \mathbf{e} \tag{1}$$

where $\mathbf{X}$, $\mathbf{Z}$ and $\mathbf{Z}_l$, and are known design matrices for the fixed effects, random effects and genetic effects respectively, τ is the vector of fixed effect parameters, $\mathbf{u}$ is a vector of random effects and $\mathbf{e}$ is a vector of residual random effects. These latter two effects are assumed independent, mean zero with covariance matrices $\mathbf{G}_u$ and $\mathbf{R}$ and respectively. The form of $\mathbf{G}_u$ and $\mathbf{R}$ will depend on the application.

We are specifically interested in the genetic line effects $\mathbf{g}$. Two classes of analysis are required, firstly for the Zn scores and grain Zn and Fe concentrations, and secondly for the seedling traits. The two situations involve different models for $\mathbf{g}$.

Zinc score Since only the nil Zinc plants were scored, $\mathbf{g}$ represents a single set of genotypic line effects under the nil treatment, and it is assumed

$$\mathbf{g} \sim N\left(\mathbf{0}, \sigma_g^2 \mathbf{I}_{n_g}\right) \tag{2}$$

where σ_g^2 is the genetic line variance and n_g is the number of lines. The base model fitted was

$$\text{ZincScore} = \text{Type} + \mathbf{Block} + \mathbf{Variety} + \mathbf{Error}$$

where the bold terms are random effects. The term Type is a factor of three levels (DH, RAC875-2, Cascades) and hence distinguishes the DH lines from the parents. The **Variety** term represents the polygenic line effects.

It was assumed that the **Error** term was such that $e \sim N(0, \sigma^2 \mathbf{I}_n)$, so that $\mathbf{R}=\sigma^2\mathbf{I}_n$. Thus is the genetic variance associated with the doubled haploid lines, while σ^2 is the residual or error variance.

Plant traits Shoot dry matter, shoot Zn and Fe concentration were measured under both the Nil and the Zinc treatments, and in Experiments 1 and 2. The resulting four combinations will be considered a (structured) set of four environments. Thus we assume

$$\mathbf{g} \sim N\left(\mathbf{0}, \sigma^2\left(\mathbf{G} \otimes \mathbf{I}_{n_g}\right)\right) \tag{3}$$

where $\mathbf{G}$ is a 4×4 variance-covariance matrix that provides the genetic variances across lines for the trait grown under the two treatments in the two experiments (hence 4) and the genetic covariances of the trait between pairs of environments; $\mathbf{G}$ may be an unstructured (and hence fully parameterized) variance–covariance matrix, or it may take on another form. In the analysis of multi-environment trials, this matrix is taken to be a factor analytic (FA) structure (Smith et al. 2005). For the case of t environments, the FA model is given by

$$\mathbf{g} = \left(\wedge \otimes \mathbf{I}_{n_g}\right)\mathbf{f} + \xi$$

where if there are n_f factors, $\mathbf{f}$ represents a vector of latent unobserved factors of size $n_f n_g \times 1$, $\wedge$ is $t \times n_f$ an matrix of factor loadings for each of the treatments and each factor, and ξ is a residual random vector for genetic variation not explained by the factors. To ensure identifiability, $\mathbf{f} \sim N\left(\mathbf{0}, \mathbf{I}_{n_f n_g}\right)$ and $\xi \sim N\left(\mathbf{0}, \Psi \otimes \mathbf{I}_{n_g}\right)$ where Ψ where is a diagonal

matrix. These FA models provide a parsimonious structure in many applications (see Smith et al. 2005).

Dry matter was multiplied by 100 to improve the scale for both estimation and reporting. However, there was clear variance heterogeneity for this and the other traits and the analysis was conducted on the log-scale. For the three plant traits (seedling biomass, shoot Zn and Fe concentrations), the base model fitted was:

$$\log(\text{trait}) = \text{Type.Env} + \mathbf{at(Site).Block} + \mathbf{Env.Variety} + \mathbf{Error}$$

where Site represents the two experiments (Adelaide and Adana), **Env** is a factor of four levels, being a 2×2 factorial structure of level of Zinc (Nil, Zinc) by experiment (Adelaide, Adana) and **Block** reflects the randomized complete block design. The term **Env. Variety** allows for the interaction of genetic effects with the environment, and the latter is determined by the treatment by experiment combinations. The term **at (Site).Block** allows for separate block effects at each Site.

The **Error** term allowed for differing residual variances for each experiment by treatment combination; thus four residual variances were estimated.

Grain traits Kernel weight, Zn and Fe concentration were measured on grain from the Zn-treated plants in Experiment 3 (the field trial). The model for **g** as given by (2) is again appropriate. The base model fitted for kernel weight was

$$\text{kwt} = \text{Type} + \mathbf{Block} + \mathbf{Column} + \mathbf{Variety} + \mathbf{Error}$$

where **Block** reflects the design of the field trial, and the random **Column** effect allows for between column variation in the field. The **Error** term allowed for spatial variation in the field as indexed by the row and column position of the plots (see Gilmour et al. 2006).

For grain Zn concentration, the base model fitted is given symbolically as

$$\text{zc} = \text{Type} + \mathbf{Block} + \mathbf{Variety} + \mathbf{Error}$$

where terms are very similar to the kernel weight model. For grain Fe concentration, the base model fitted was:

$$\text{fe} = \text{Type} + \text{lin(Column)} + \mathbf{Block} + \mathbf{spl(Column)} + \mathbf{Variety} + \mathbf{Error}$$

where additional terms were required to account for smooth spatial field dependence across rows that was evident in the analysis.

Statistical Models for QTL analysis

Zinc score and grain traits The approach used for QTL analysis is based on Verbyla et al. (2007) who provide an approach that uses the full linkage map simultaneously in a staged analysis. A working model is used in which all intervals are assumed to possibly contain a QTL. The vector of the sizes of QTL effects for all intervals is denoted by **a** and is assumed to be a random effect with mean zero and variance (σ_a^2). If this variance is significant, an outlier detection technique is used to sequentially select putative QTL. At each selection step, the putative QTL interval is moved to the fixed effects part of the model and selection ceases when the between interval variation (σ_a^2) is no longer statistically significant.

The initial mixed model that is used for the QTL analysis is given by

$$\mathbf{y} = \mathbf{X}\boldsymbol{\tau} + \mathbf{Zu} + \mathbf{M}_E\mathbf{a} + Z_I\mathbf{g} + \mathbf{e}$$

where the matrix $\mathbf{M}_E$ consists of pseudo-markers that relate to intervals on the linkage map (see Verbyla et al. 2007 for details). An important summary of the contribution of the selected QTL is the percentage of genetic variance explained by the QTL. An overall measure is given by

$$\%\,var = \frac{\hat{\sigma}^2_{g,b} - \hat{\sigma}^2_{g,q}}{\hat{\sigma}^2_{g,b}} \times 100$$

where $\hat{\sigma}^2_{g,b}$ is the estimated polygenic genetic variance for the baseline model (that is, without marker effects) and $\hat{\sigma}^2_{g,q}$ is the equivalent estimated polygenic genetic variance after finding the QTL. This quantity is reported in the results for each analysis.

Plant traits A common approach for QTL analysis was used for plant traits and is based on the whole genome approach of Verbyla et al. (2007) in the case of multi-environment and multi-trait data (based on work in preparation by Verbyla and Cullis). A FA model (Smith et al. 2005) is used to allow detection of QTL that affect the trait in multiple environments or under multiple treatments or both. The initial mixed model for QTL analysis in this case is given by

$$\mathbf{y} = \mathbf{X}\tau + \mathbf{Zu} + (\wedge_a \otimes \mathbf{M}_E)\mathbf{f}_a + (\mathbf{I} \otimes \mathbf{M}_E)\xi_a + \mathbf{Z}_{l\,\mathbf{g}} + \mathbf{e}$$

where the term$(\wedge_a \otimes \mathbf{M}_E)\mathbf{f}_a$ allows for a common QTL effect across the 4 environments but with differential association in terms of size of effect, and$(\mathbf{I} \otimes \mathbf{M}_E)\xi_a$ allows for environment specific QTL effects. The selection of QTL proceeds by choosing common effects until that term is no longer statistically significant. Subsequently, environment specific QTL are selected using the second term. Once this term is no longer statistically significant, the selection process is concluded.

Note that there are four polygenic "traits", and hence four polygenic variances. Thus the calculations given in equation (3) can be conducted for each of the polygenic variances as given by the diagonal elements of **G**. Not only can the percentage of genetic variance be given for the combination of location and treatment, the variances and the correlations between the four combinations can be reported both before QTL and after QTL analysis thereby allowing the assessment of the impact of the QTL selected. Thus in the analysis of the plant traits the estimated **G** before and after QTL analysis is presented and discussed.

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