Quantitative trait loci (QTL) of seed Zn accumulation in barley population Clipper x Sahara

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Abstract

Aims: There is little information regarding the chromosomal location of genes conferring Zn efficiency in barley.

Methods: With the aim of developing markers for Zn efficiency, a population of 150 lines derived from a cross between Clipper (low-Zn-accumulator) and Sahara 3771 (high-Zn-accumulator) was screened in the field and glasshouse.

Results: Two regions located on 2HS and 2HL were found to be associated with seed Zn concentration and content of field-grown plants. The first region was flanked by Xbcd175 and Xpsr108; the second region was flanked by *vrs*1 and XksuF15 markers. These two regions accounted for 45% of the total variation in seed Zn concentration and 59% of the total variation in seed Zn content. In glasshouse experiment, these two regions (2HS and 2HL) were also associated with seed Zn concentration and content; and explained 37% and 55% of the total variation in seed Zn concentration and content, respectively.

Conclusions: The identification of these QTLs provides an important starting point for transferring and pyramiding genes that may contribute to the improvement of barley productivity and nutritional quality in Zn-deficient environments.

Keywords: QTL, seed Zn accumulation, barley (*Hordeum vulgare*)

Introduction

Low availability of Zn in the agricultural soils is one of the most widespread abiotic stresses recorded in approximately 50% of the world soils used for cereal production (Graham and Welch 1996). Among micronutrients, Zn deficiency appears to be the most critical deficiency in crop production. Wheat and barley grown in conditions of low plant-available Zn showed reduced growth and yield (McDonald et al. 2001; Sadeghzadeh et al. 2009) as well as a decreased amount of Zn in seed (Cakmak et al. 1999; Erdal et al. 2002;

Sadeghzadeh 2008). Zinc plays multiple key roles in different metabolic and physiological processes; its malnutrition afflicts over 3 billion people in the world, particularly in developing countries (Alloway 2004; Hotz and Brown 2004; Welch and Graham 2004; WHO 2002).

Zinc deficiency causes large reduction in crop nutritional quality (Kochian and Garvin 1999). Increasing the amount of Zn in food crops can contribute to improving Zn nutrition of people. Furthermore, micronutrient-dense seeds result in greater seedling vigour, bigger root system (Wissuwa et al. 2006) and higher crop yield when the seeds are sowed to micronutrient-poor soils (Welch 1999).

In recent years, there has been a growing research interest in the utilization of barley in a wide range of human food applications (Bhatty 1999; Bilgi and Celik 2004; Erkan et al. 2006; Koksel et al. 1999). Barley flour is unique among cereals in having a higher amount of healthy β-glucan, which is reported to be effective in lowering cholesterol levels (Kahlon and Chow 1997; McIntosh et al. 1991), regulating blood glucose level and insulin response in diabetics (Cavallero et al. 2002) and even reducing the cancer risk (Jacobs et al. 1998).

During the breeding process over the past few decades, enhanced nutrient content in seed has only been a breeding objective occasionally. Nevertheless, the efficiency of nutrient uptake and seed nutrient content have been successfully improved (for example, see Bouis 2000). To provide the macronutrient and micronutrient needs of estimated 8 billion people by 2025, it is likely that both conventional crop technology and biotechnology will be needed. However, progress in enhancing the grain nutrient content so far has mainly relied on the conventional plant breeding approaches and agronomic biofortification (applying micronutrient-containing fertilizers; see (Cakmak 2008; Rengel et al. 1999)) that have had limited success.

Improving mineral content of seeds through plant biotechnology has perhaps the greatest potential to benefit human health and increase crop yields (Cakmak et al. 2002; Ghandilyan et al. 2006; Poletti et al. 2004; Welch and Graham 2004; Zimmerman and Hurrel 2002). Using genetic engineering, however, requires a comprehensive exploration of potential genetic resources and an in-depth understanding of micronutrient accumulation mechanisms. In contrast, DNA marker approach based on a genetic tag for the desired trait does not usually require a detailed understanding of relevant plant physiological and biochemical processes.

The implementation of molecular markers in breeding programs is likely to be the most beneficial approach in developing micronutrient-dense grains in staple food crops. Molecular markers are genetic tools that are not affected by environmental influences governing plant growth and nutrient availability, which pose difficulties in the conventional plant breeding. Pot culture bioassays can be used easily in developing molecular makers; however, demonstrating the relevance of these genes under field conditions could be a major challenge (Schachtman and Barker 1999).

Determining quantitative trait locus/loci (QTL) is a powerful genetic approach to study complex traits, such as Zn accumulation, and to reveal genetic differences within species. The QTL analysis (i) identifies chromosomal regions of the important loci without any prior knowledge about the genes involved, and (ii) unravels their possible genetic effects leading to micronutrient-dense seeds (Ghandilyan et al. 2006), which can be employed as a starting point for the detection and cloning of such genes (Paran and Zamir 2003). QTL analysis, in case of mineral accumulation in seed, might target the genes encoding transporters and chelators

as well as regulatory factors such as membrane receptors, protein kinases and transcription factors (Vreugdenhil et al. 2004).

The QTL analysis for grain quality traits of cereals has been pursued by several research groups (Aluko et al. 2004; Li et al. 2004; Septiningsih et al. 2003). To identify the genetic loci involved in establishing seed Zn content in Arabidopsis, Vreugdenhil et al. (2004) found that the four QTLs on chromosomes 1, 2, 3 and 5 are involved in seed Zn content, explaining up to 42% of the variation. In an F2 cross of *Arabidopsis halleri* with *A. petraea*, three QTLs were identified on chromosomes 4, 6 and 7 that are involved in determining the amount of Zn in seeds (Filatov et al. 2007). In common bean, one QTL on linkage group IV was associated with seed Zn content, explaining 15% of the phenotypic variance for this trait (Guzman-Maldonado et al. 2003). In a doubled-haploid barley population (derived from a cross between Clipper and Sahara), Lonergan et al. (2001) identified a region on the long arm of chromosome 4H as being associated with both shoot Zn concentration and content. In a related study, it has been found that one QTL on the short arm of chromosome 2H is also involved with increased seed Zn content in barley (Lonergan 2001). Recently, Sadeghzadeh et al. (2010) developed a PCR-based molecular marker (SZnR1), located on the short arm of chromosome 2H, involved in controlling both seed Zn concentration and content.

At present, the knowledge of the genes controlling specific steps of Zn storage in seed of barley is still rudimentary. Increased knowledge about genetic loci controlling seed Zn accumulation is a necessary step to improve seed Zn reserves. This understanding is expected to improve crop nutritional value and yield under Zn-deficient conditions. The objective of the present study was to identify chromosomal regions affecting seed Zn accumulation; this information can be used for marker-assisted selection in barley breeding programs.

Materials and Methods

Field experiment

The plant material used for the map construction and phenotyping [150 doubled-haploid (DH) lines] was derived from a cross between the Australian cultivar two-rowed Clipper (low-Zn-accumulator) and Algerian landrace six-rowed Sahara 3771 (high-Zn-accumulator). The population was produced by the *Hordeum bulbosum* method (Finnie et al. 1989).

Two independent experiments were conducted in the field and glasshouse. Doubled-haploid lines, together with the parents, were sown under irrigated condition in a completely randomised design with two replications (each including six plants) at the University of Western Australia Field Station, Shenton Park (31.9°S, 114.9°E) in the autumn. Field soil properties in the experimental site were: pH_{water} 6.8, organic matter 8.5 g/kg, P 43 mg/kg soil, and DTPA (diethylenetriamine pentaacetic acid)-extractable Zn 1.0 mg/kg soil.

The field management was done as per the standard practice. Soil was fertilized with 320 kg/ha Super Potash (P, 50 kg/ha; K, 95 kg/ha; S, 62 kg/ha) before sowing, and 50 kg/ha urea was applied every two weeks from week 6 to week 18 after sowing. At maturity, all ears were harvested by hand in each replicate, air-dried to preserved seed viability, threshed and counted to obtain 1000-kernel weight. Fifteen seeds from each individual DH lines and the parents were oven-dried at 70°C for 72 hours. After weighing, the seed samples were ashed at 550°C for 14 hours, and solubilized in 10 mL of 30% (v/v) hydrochloric acid (HCl) for 30

minutes at 50°C. Seed concentration of Zn was determined by inductively coupled plasma-mass spectrometry (ICP-MS). The accuracy of mineral determinations was checked by using certified values of Zn in the reference samples. Seed Zn content was calculated by multiplying seed dry weight with seed Zn concentration.

Glasshouse experiment

The same set of 150 DH lines and the parents used in the field experiment was studied in the glasshouse experiment. Seeds were hand sorted to a uniform size, surface sterilized and pre-germinated on filter paper in Petri dishes. Zn-deficient Lancelin soil (1.5 kg) was placed into plastic-bag-lined milk cartons (70 × 70 × 200 mm, 1-L volume). The soil used for glasshouse experiment had a sandy texture, pH_{water} 6.1, organic matter 12 g/kg, P 3.3 mg/kg soil, and DTPA-extractable Zn 0.1 mg/kg soil. Basal nutrients (in mg/kg of dry soil) 91 KH_2PO_4, 145 K_2SO_4, 147 $CaCl_2.2H_2O$, 21 $MgSO_4.7H_2O$, 2 $CuSO_4.5H_2O$, 15 $MnSO_4.H_2O$, 0.7 H_3BO_3, 0.2 $Na_2MoO_4.2H_2O$ and 93 NH_4NO_3 together with 0.8 mg Zn/kg soil as $ZnSO_4.7H_2O$ were applied to the soil. Nine pre-germinated seeds of each genotype were sown per pot in a completely randomised block design with three replications. Seedlings were thinned for uniformity to seven plants per pot at the two-leaf stage. Pots were rotated within a block daily to minimize the effect of microenvironments. Plants were grown in a glasshouse and watered with deionized water daily by weight, keeping water content at 90% of the field capacity, where moisture content of Lancelin soil at field capacity was 10% (w/w).

Two harvests were performed; four plants per pot were harvested at the five-leaf stage (60 days after sowing) and the remaining three plants at maturity. Harvested plants were washed under running deionised water and then dipped into three changes of double-deionised water. Plant material digestion, chemical analyses and measured traits were similar to those described for the field experiment in this paper.

A genetic linkage map of Clipper × Sahara population was used to identify chromosomal regions associated with Zn accumulation in barley grown in field and glasshouse conditions. Analysis of the Clipper × Sahara population in producing the genetic map has been detailed in Karakousis et al. (2003), with map containing 302 RFLP, SSR (simple sequence repeats) and morphological markers spanning all seven chromosomes. QTL analyses were performed using the software package QTLNetwork (Yang et al. 2007). The data were analysed for QTL with additive effects and epistatic interactions. One thousand permutation tests were carried out to control the genome-wide type I error rate at 0.05. A minimum separation of 10 cM ('filtration window') was used to define individual adjacent QTL. QTL effects were estimated by Bayesian method of mixed linear model via Gibbs sampling. The analysis calculated a *P* value for each of the estimates of QTL effects. A threshold of $P \leq 0.05$ was used to declare significant QTL effects. In addition, the correlation analysis (using Pearson's coefficient; two-tailed test) was performed to determine if there was a relationship between all the measured traits in the DH lines using SPSS software (Version 10; SPSS Inc. Chicago, IL, USA).

Results

The DH population and their parents varied considerably in both mean values and ranges for seed Zn in the field and glasshouse conditions (Figure 1). The distribution of the progeny mean values was normal for all

measured traits. Seed Zn concentration in DHs showed a range of 22-61 mg/kg dry matter in the field study, and 16-48 mg/kg in the glasshouse experiment. The large range among DH progeny sometimes mirrored significant differences in the parental means for the measured traits (e.g. seed Zn concentration in the field and glasshouse experiments), whereas in other cases parental lines were similar for the measured traits. The frequency distribution of DHs for seed Zn concentration in the field study was slightly skewed towards the higher values, contrasting with glasshouse experiment that displayed a more normal distribution (Figure 1).

Seed Zn content of DHs ranged from 0.7 to 2.9 µg/seed in plants grown in the field, and 0.6 to 2.2 µg/seed in plants grown in the glasshouse. The distribution of seed Zn content was normal in both growing conditions. Distribution of the traits for the DH population indicated the presence of transgressive lines, which are higher or lower than the parents (Figure 1). A simple correlation analysis for the measured parameters in a pair-wise fashion showed significant and positive correlation between seed Zn concentration (r=0.5) and content (r=0.67) of plants from field and glasshouse experiments.

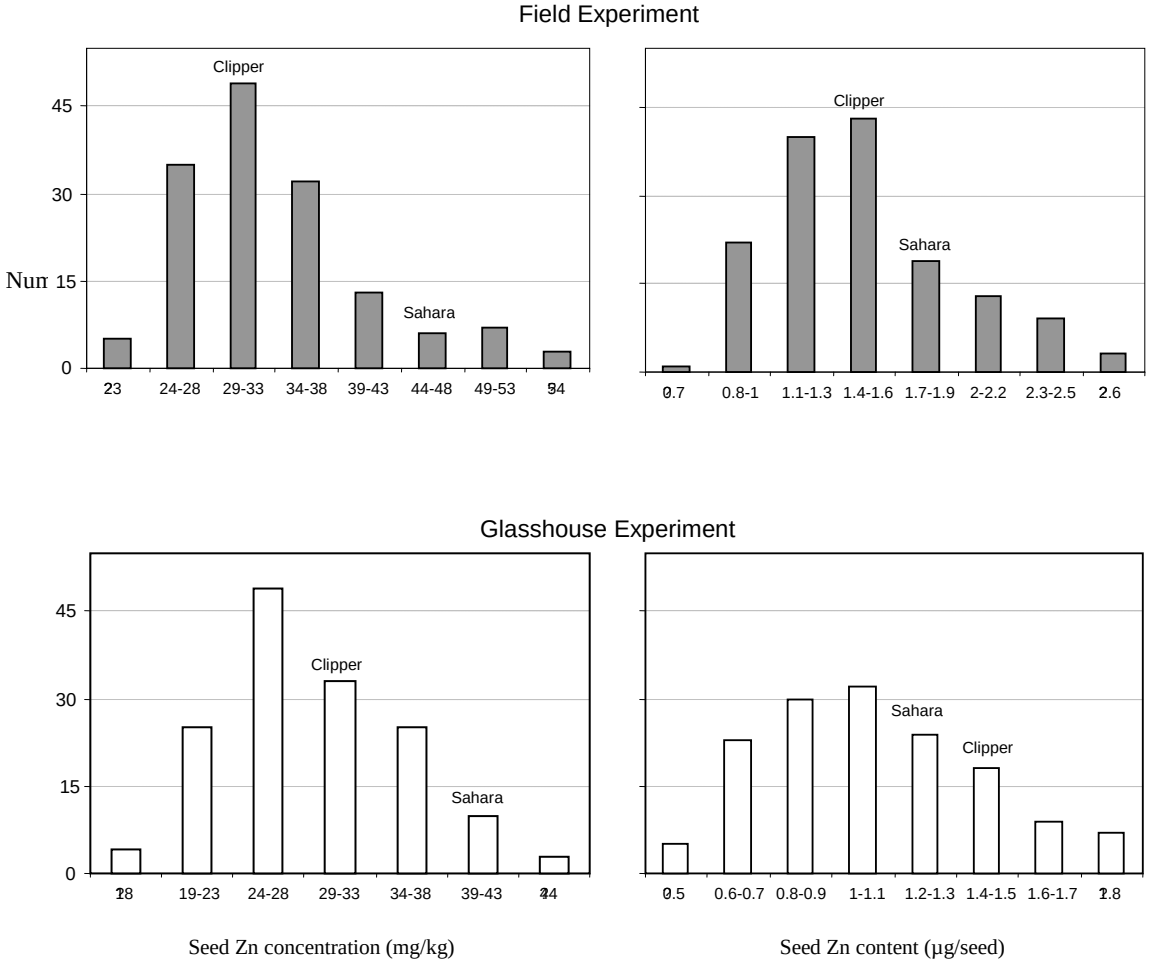

Figure 1. Frequency distribution of seed Zn concentration and content in the 150 doubled-haploid barley lines derived from a Clipper x Sahara cross grown in the field and glasshouse.

A comprehensive molecular map of DH population with 302 markers enabled the identification of QTLs for Zn accumulation characteristics in the seed (Table 1). The density and coverage (3.8 cM/locus) of this map were satisfactory for QTL analysis. Table 2 shows the positions of QTLs associated with Zn accumulation in seeds. In the field experiment, two regions were found to be associated with seed Zn concentration on chromosome

2H in the mapping population. These two regions accounted for 45% of the total variation in seed Zn concentration. The first of these regions on short arm of chromosome 2H was flanked by the RFLP markers Xbcd175 and Xpsr108, which accounted for 18% variation in seed Zn concentration. The second region on the long arm of chromosome 2H accounted for 27% variation in the concentration and was flanked by the morphological marker six/two rowed *vrs1* and RFLP marker XksuF15.

Interestingly, two locations on chromosome 2H associated with seed Zn concentration in the field experiment were also found to be associated with Zn concentration of seed in the glasshouse experiment (Table 1). Two additional regions were also identified to be associated with seed Zn concentration in the glasshouse experiment. These locations were on the long arm of chromosome 3H, flanked by RFLP marker Xwg178 and SSR marker HVM60, and the short arm of 4H flanked by RFLP marker Xcdo358 and SSR marker awbma29. These four QTLs could explain 50% of the total variation in seed Zn concentration.

Table 1. Estimated additive genetic effects and chromosomal regions of the barley genome associated with seed Zn concentration and content in the 150 doubled-haploid lines derived from a Clipper x Sahara cross grown in field and glasshouse conditions.

Trait		Chromo-some	Region	QTL position (cM)	A genetic effect[a]	Explained variance (%)	F value	*P* value
Field Experiment	**Seed Zn concentration** (mg/kg dry matter)	2HS	bcd175 - psr108	22.7	-3.3 ***	18	43	0.000000
		2HL	*vrs1* - ksuF15	103.4	3.9 ***	27	39	0.000000
	Seed Zn content (µg/seed)	2HS	bcd175 - psr108	23.7	-0.2 ***	11	30	0.000000
		2HL	*vrs1* - ksuF15	102.4	0.3 ***	48	122	0.000000
Glasshouse Experiment	**Seed Zn concentration** (mg/kg dry matter)	2HS	bcd175 - psr108	20.7	-2.4 ***	11	21	0.000000
		2HL	*vrs1* - ksuF15	102.4	3.6 ***	26	34	0.000000
		3HL	wg178 - HVM60	68	-1.8 ***	7	20	0.000032
		4HS	cdo358 - awbma29	51	-1.7 ***	6	15	0.000030
	Seed Zn content (µg/seed)	2HS	bcd175 - psr108	21.7	-0.12 ***	11	21	0.000000
		2HL	*vrs1* -ksuF15	102.4	0.25 ***	44	100	0.000000

a : Additive effect estimated as one-half the difference in homozygotes carrying either parental allele; a positive additive effect indicates the first parent is contributing a positive allele and the second parent a negative allele.

*** : Indicates marker effect is statistically different from zero at P<0.001

Seed Zn content of plants grown in the field and glasshouse was significantly associated with two QTLs located on the short and long arm of chromosome 2H. They could explain 59% of the total variation in seed Zn content in field experiment and 55% in the glasshouse trial. These two regions were also associated with seed Zn concentration of plants grown in the field and glasshouse. The presence of Sahara allele on chromosome 2HS and Clipper allele on 2HL conferred 11% and 44% increase in seed Zn content in plants from glasshouse study, respectively.

Discussion

Concentration of mineral nutrients in plant tissues depends on genetic and environmental factors (Ernst and Nelissen 2000; Lickfett et al. 1999; Vreugdenhil et al. 2004). A wide range of values was observed in the doubled-haploid lines (DH) for all traits measured in this study (Figure 1). The frequency distribution of seed Zn concentrations and content showed transgression in both directions. This suggests that both parents carry genes with alleles involved with either increased or decreased Zn concentration or content. This indicates that improved Zn accumulation over the parental average could be achievable by selection of a progeny containing a combination of parental alleles if an easy way of identification of the beneficial alleles could be found for this quantitative trait. Further, the range and distribution of DHs for Zn concentration and content indicated polygenic variation in the DH population. Polygenic control for the amount of Zn in the seeds has been reported in different plant species (Filatov et al. 2007; Guzman-Maldonado et al. 2003; Lonergan 2001; Vreugdenhil et al. 2004). In wheat, several candidate genes for seed Zn accumulation have been identified on chromosomes 6A and 6B (Cakmak et al. 2000).

Genotype by environment interaction is an important factor in determining the adaptation of genotypes to the physical environment, and has received considerable attention in plant breeding programs. The correlation for seed Zn concentration and content between field and glasshouse trials presented here suggested that these traits were not greatly influenced by environmental conditions in this study. This will simplify detecting the stable QTLs for seed Zn concentration and content. The existence of correlation for Zn accumulation between field and glasshouse conditions is one possible explanation for detecting similar loci on 2H affecting Zn accumulation under both conditions (Table 1).

Large phenotypic variation for Zn accumulation enabled mapping of genomic regions associated with seed Zn concentration and content. In the study presented here, a number of the Zn-accumulation QTLs found in the Clipper x Sahara population were repeatable across environmental conditions. Two QTLs were identified in the marker intervals Xbcd175-Xpsr108 on the long arm of 2H and *vrs1-ksuF15* on the short arm of 2H controlling both seed Zn concentration and content in the field trials. These loci were also suggested by the interval mapping data of the glasshouse experiment. Similarly to the results of this study, marker Xbcd175 has been previously reported for seed Zn concentration and content in the Clipper x Sahara population (Lonergan 2001). Recently, in another study, Lonergan et al. (2009) reported that two-rowed locus (*vrs1*) is the main contributor to seed Zn content but not concentration in the same Clipper × Sahara population. The consistent identification of the QTLs mapped around Xbcd175 and *vrs1* confirms the robustness of phenotyping data of seed Zn concentration and content used in the mapping of these traits. Further, the QTL mapped to the same chromosomal location across different experimental condition provides validation and increases the value of this QTL for use in the marker-assisted selection.

Two additional QTLs, with a minor role in controlling seed Zn concentration, were detected on 3HL and 4HS chromosomes in the glasshouse experiment. Detection of the role of different chromosomes (4 loci: 2HS, 2HL, 3HL and 4HS) contributing directly to Zn accumulation in barley confirmed the significant underlying genetic complexity of this quantitative trait. The polygenic character of seed Zn concentration was reported

earlier in barley (Lonergan 2001; Lonergan et al. 2009), Arabidopsis (Filatov et al. 2007; Vreugdenhil et al. 2004) and bean (Beebe et al. 2000; Cichy et al. 2005). The beneficial allele of 2HS and the possible minor QTL on 3HL and 4HS (Table 1) were contributed by Sahara, whilst the beneficial 2HL allele originated from two-rowed Clipper that has bigger seed size. The 2HL locus is quite closely linked to *vrs1*, which has a confounding effect on seed Zn content because of its contribution to seed size (Hori et al. 2003).

In the present study, seed Zn content in field and glasshouse experiments was largely influenced by *vrs1* locus on the long arm of chromosome 2H, which controls two- and six-rowed spikes in barley (Komatsuda et al. 1999; Tanno et al. 2002). This region have also been found to be associated with 1000-kernel weight (TKW) in the population of barley recombinant inbred lines (RIL) derived from the cross of Russia6 and HES4 (Hori et al. 2003). The two-rowed type has a larger seed, and thus presumably higher Zn content, than the six-rowed type. However, the same locus also controls Zn concentration in seed in our study. Thus, the co-location of seed Zn content, Zn concentration and TKW traits may be due to a pleiotropic effect or simply be based on the presence of two or more different and closely linked genes that either directly or indirectly influence seed Zn content. More probably, one gene has the pleiotropic effect on both traits because *vrs1* locus was found to control two- and six-rowed spikes in barley (Komatsuda et al. 1999; Tanno et al. 2002). It should be borne in mind that in the QTL studies, differentiation between pleiotropy and linkage is not always possible (Barua et al. 1993; Thomas et al. 1995). The study of populations derived from different generations, in which a variety of recombination rounds take place, makes it possible to discriminate these traits, as the probability of breaking a linkage increases with an increase in the number of crossing-overs.

A locus on chromosome 4HS appears to be particularly important. Lonergan (2001) found that this locus controls leaf zinc concentration in a DH population from the cross of Clipper and Sahara. Increased Zn accumulation might be due to enhanced soil uptake by release of Zn chelators (Cakmak et al. 1996; Rengel et al. 1998), and increased maximum uptake rate (Rengel and Graham 1996). Furthermore, this locus controls the synthesis of mugineic acid from 2'-deoxymugineic acid (Mori and Nishizawa 1989). It is also closely linked to a homeologous region of rye that confers part of the Zn efficiency trait (Graham 1984). The synthesis of mugineic acid phytometallophores, with enhanced expression in Zn-deficient barley roots, is encoded by five genes (Suzuki et al. 2006). The 4H locus probably regulates the abundance or release of these gene products, or is a variant allele of a rate-limiting step in mugineic acid biosynthesis.

In short, the present study identified genomic locations associated with genotypic variation in seed Zn concentration and content. Identified QTLs affecting seed Zn concentration and content were repeatable in the field and glasshouse conditions, suggesting their robustness across environments as well as their value in marker-assisted selection. The use of molecular markers associated with the QTL on the short arm of chromosome 2H could be effective in the improvement of seed Zn amount, which is important in alleviation of human Zn deficiency and for improved crop yield on Zn-deficient soils. However, the assessment of any additional genetic variation in seed Zn accumulation not represented in this DH population will be important for obtaining an inclusive view of breeding potential for these traits in barley and to extend this research to other main crops.

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