

Mapping QTLs for mineral accumulation and shoot dry biomass under different Zn nutritional conditions in Chinese cabbage (*Brassica rapa* L. ssp. *pekinensis*)

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Abstract Chinese cabbage (*Brassica rapa* L. ssp. *pekinensis*) is one of the most important vegetables in China. Genetic dissection of leaf mineral accumulation and tolerance to Zn stress is important for the improvement of the nutritional quality of Chinese cabbage by breeding. A mapping population with 183 doubled haploid (DH) lines was used to study the genetics of mineral accumulation and the growth response to Zn. The genetic map was constructed based on 203 AFLPs, 58 SSRs, 22 SRAPs and four ESTPs. The concentration of 11 minerals was

determined in leaves for 142 DH lines grown in an open field. In addition shoot dry biomass (SDB) under normal, deficient and excessive Zn nutritional conditions were investigated in hydroponics experiments. Ten QTLs, each explaining 11.1–17.1% of the Na, Mg, P, Al, Fe, Mn, Zn and Sr concentration variance, were identified by multiple-QTL model (MQM) mapping. One common QTL was found affecting SDB under normal, deficient and excessive Zn nutritional conditions. An additional QTL was detected for SDB under Zn excess stress only. These

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results offer insights into the genetic basis of leaf mineral accumulation and plant growth under Zn stress conditions in Chinese cabbage.

Keywords *Brassica rapa* L. ssp. *pekinensis* · Quantitative trait loci (QTL) · Minerals · Zinc response

Abbreviations

AFLP	amplified fragment length polymorphism
DH	doubled haploid
ESTP	expressed sequence tagged polymorphism
MQM	multiple-QTL model
QTL	quantitative trait locus
SDB	shoot dry biomass
SRAP	sequence related amplified polymorphism
SSR	simple sequence repeat

Introduction

Chinese cabbage (*Brassica rapa* L. ssp. *pekinensis*) is one of the most important vegetable crops in China with, among vegetable crops, the largest cultivation area and per capita consumption. It is a main source of mineral nutrients for the Chinese population (Ma et al. 2007), and therefore elevating the amount of beneficial minerals in Chinese cabbage can contribute to the reduction of micronutrient malnutrition in China. The improvement of mineral content by plant breeding, also referred to as biofortification (Bouis 2002), is a recent development that requires knowledge about the genetic variation for this trait and genetic markers linked to relevant genes (reviewed in Ghandilyan et al. 2006). However, genetic studies on mineral content in crops have been limited mainly to seeds, such as for common bean (Beebe et al. 2000; Islam et al. 2002), rice (Gregorio et al. 2000), wheat (Monasterio and Graham 2000), maize (Banziger and Long 2000) and also the model species *Arabidopsis thaliana* (Vreugdenhil et al. 2004). QTLs were also identified for mineral concentrations in leaves for P (Bentsink et al. 2003), Cs (Payne et al. 2004) and K (Harada and Leigh 2006) in *A. thaliana* and for Zn in *Thlaspi caerulescens* (Assunção et al. 2006; Deniau et al. 2006). These studies have demonstrated the presence of allelic variation affecting mineral accumulation, although the identity of the underlying genes remains unknown. However, very little is known about the

genetics of mineral concentration in leafy vegetables, such as *B. rapa* vegetables, until recently QTLs were identified for shoot Ca and Mg concentrations in *Brassica oleracea* (Broadley et al. 2008).

Zn is an essential micronutrient as it plays both functional and structural roles in enzyme reactions (Vallee and Auld 1990). However, both Zn deficiency and Zn toxicity occur worldwide, including in China, depending on Zn content and availability in the soils (Nan and Zhao 2000; Hacisalihoglu and Kochian 2003; Liu et al. 2005). In addition to reduced yield due to Zn deficiency or toxicity, the transfer of excess Zn from soils to plants is an important contribution to human health exposure to Zn and more importantly to Cd that goes along with it (Friberg and Nordberg 1986). A potential solution is to breed crop cultivars that are more tolerant to high Zn exposure and thus grow relatively well with an acceptable level of Zn accumulation in the leaves.

In *Brassica rapa* vegetables, an eight, six and 2.5-fold genotypic variation was observed for shoot Zn, Fe and Mn concentration, respectively, and a two-fold variation for relative shoot or root dry biomass production under Zn deficient or toxic conditions (Wu et al. 2007). A two-fold genotypic variation was reported for Ca, Mg, K, Fe, Zn concentration among *B. oleracea* vegetables (Kopsell et al. 2004; Broadley et al. 2008) and for shoot dry biomass ratio-based Zn efficiency for *B. napus* and *B. juncea* (Grewal et al. 1997). The observed natural variation for these traits in *Brassica* crops suggests the possibility to analyze these traits genetically.

Mineral accumulation in above ground organs is a complex trait controlled by a series of processes, including mobilization from the soil, uptake by the roots, translocation and redistribution within the plant, and import and deposition in the organs (Clemens 2001). This is also the case for plant growth under stressful nutrition conditions because different aspects such as availability, uptake and utilization of nutrient elements can be affected and as a consequence growth, development, and yield of the crop will alter (Gupta 2005). Quantitative trait locus (QTL) mapping allows the identification of individual chromosomal regions containing genetic factors that contribute to variation in a complex trait (Alonso-Blanco and Koornneef 2000). Molecular mapping of *B. rapa* was initiated by Song et al. (1991) using RFLP markers and a segregating F₂ population. Subsequently several additional maps were constructed based on different molecular markers and F₂, recombinant inbred lines (RILs) or DH populations

(Chyi et al. 1992; Teutonico and Osborn 1994; Kole et al. 1997; Suwabe et al. 2006; Kim et al. 2006; Choi et al. 2007). The recent development of *Brassica* simple sequence repeat (SSR) markers facilitates the anchoring of linkage groups to one common reference map and allows the comparison of map positions in studies involving different populations (Suwabe et al. 2006; Choi et al. 2007). Furthermore the syntenic relationship with the related genus *Arabidopsis* is now well established (reviewed by Schranz et al. 2006) and allows the comparison of map positions between *Brassica* and *Arabidopsis*. For *B. rapa* QTL analyses have been described for a wide variety of morphological and physiological traits such as seed colour, pubescence, flowering time (Teutonico and Osborn 1994; Song et al. 1995; Nozaki et al. 1997; Lou et al. 2007), oleic acid concentration (Tanhuanpaa et al. 1996), linolenic acid content (Tanhuanpaa and Schulman 2002), clubroot resistance (Suwabe et al. 2006) and black rot resistance (Sogengas et al. 2007). As microspore culture can be applied efficiently in *Brassica* species, doubled haploid (DH) populations are presently the preferred mapping populations for QTL studies because of their immortality and homozygosity which allows replicated experiments.

In the present paper we describe the construction of a molecular linkage map containing 203 amplified fragment length polymorphism (AFLP) markers, 58 simple sequence repeat (SSR) markers, 22 sequence related amplified polymorphism (SRAP) markers and four expressed sequence tagged polymorphism (ESTP) markers using 183 DH lines derived from a cross between two Chinese cabbage DH lines. Plants from this population were analyzed for the concentration of 11 elements in leaves, including four essential micro-elements (Fe, Zn, Mn and Cu), five macro-elements (Na, Ca, K, P and Mg), and also for a non-essential element (Sr) and a toxic element (Al). QTL analysis was performed for mineral accumulation and for shoot dry biomass (SDB) yield under normal, deficient and excessive Zn conditions.

Materials and methods

Mapping population and DNA isolation

The BrIVFhn mapping population of 183 DH lines was derived from a cross between two DH Chinese

cabbage lines. Y177 originated from a winter type Japanese cultivar ‘Jianchun’ and Y195 is derived from a summer type Chinese cultivar ‘Xiayang’. The two cultivars show differences in bolting time, seed colour, trichome density and flower colour (Supplementary Table 1, Supplementary Fig. 1). DNA from the parents and the DH plants was isolated from the mature leaves as described by Wang et al. (2005).

AFLP analysis

AFLP markers were analyzed following Vos et al. (1995), using fluorescently labelled *EcoRI* and *MseI* primers with three selective nucleotides as described by Zhao et al. (2005). AFLPs were scored on the basis of the presence or absence of the band. AFLP markers were named using a code for each *EcoRI* and *MseI* primer followed by the band number in ascending molecular-weight order (Table 1).

PCR based markers

A total of 205 SSR markers derived from *B. rapa*, *B. nigra*, *B. oleracea* and *B. napus* (Lowe et al. 2004; Ge et al. 2005; Suwabe et al. 2002, 2004, 2006; Kim et al. 2006; Choi et al. 2007; T. Osborn, personal communication; G. King personal, communication; G. Bonnema, personal communication) were screened for their polymorphism between the two parental lines. The polymorphic SSR markers with clear bands were used for mapping. Seven Sequence Related Amplified Polymorphism (SRAP) primer combinations (me1/em1, me1/em6, me4/em1, me4/em6, BMe6/BEm12, BMe10/CoEm9, CoMe10/K6), distinguishing the parents, were used to test the DH lines following Li and Quiros (2001). The markers originated from *B. oleracea* (Li and Quiros 2001), *B. napus* (Riaz et al. 2001), buffalo grass (Budak et al. 2004) and cotton (Lin et al. 2003). SRAP markers were scored on the basis of the presence or absence of the band and were named using the primer combination followed by a number in descending molecular-weight order. Seven Expressed sequence tagged polymorphism (ESTP) markers (E018, E039, E060, E101, E120, E129, E138) derived from *B. rapa* cDNA sequences were used to test the population following Choi et al. (2007). PCRs were performed in 20 µl 1 X PCR buffer, containing 20 ng DNA template, 1 U *Taq* DNA polymerase, 0.25 mM of each dNTP, and 250

Table 1 AFLP primer combinations used for *B. rapa* ssp. *pekinensis* linkage map construction

Primer combination	3' Selective nucleotides		No. of polymorphic bands
	<i>EcoRI</i>	<i>MseI</i>	
E32M61	AAC	CTG	6
E33M32	AAG	AAC	12
E33M48	AAG	CAC	8
E33M49	AAG	CAG	10
E33M50	AAG	CAT	6
E33M51	AAG	CCA	10
E33M53	AAG	CCG	3
E33M54	AAG	CCT	4
E33M56	AAG	CGA	2
E33M60	AAG	CTC	8
E34M35	AAT	AAT	15
E35M52	ACA	CCC	7
E35M56	ACA	CCT	2
E36M31	ACC	AAA	8
E36M32	ACC	AAC	12
E36M35	ACC	ACA	6
E36M36	ACC	ACC	4
E36M47	ACC	CAA	5
E36M50	ACC	CAT	13
E36M53	ACC	CCA	2
E36M59	ACC	CTA	5
E37M32	ACG	AAC	12
E37M49	ACG	CAG	4
E37M56	ACG	CGA	2
E38M35	ACT	ACA	9
E38M50	ACT	CAT	10
E38M51	ACT	CCA	11
E38M56	ACT	CGA	3
E40M38	AGC	ACT	6
E40M51	AGC	CCA	6
E41M51	AGG	CCA	9
E41M56	AGG	CGA	6

nM of each primer. Thermocycling was started at 94°C for 5 min and followed by 35 cycles of 94°C for 1 min, 55°C for 1 min, 72°C for 1 min and a final extension at 72°C for 10 min before holding at 12°C. The PCR products were separated on 6% denaturing polyacrylamide gels and visualized by silver staining.

Segregation analysis and map construction

The segregation of each marker and linkage analysis were performed using JoinMap version 3.0 (<http://www.kyazma.nl/>). The linkage groups (LGs) were determined using a LOD threshold ≥ 4.0 and a maximum recombination fraction of 0.4. The Kosambi

mapping function (Kosambi 1944) was used to convert recombination frequencies into map distances.

Phenotypic analyses

The leaf mineral concentration was analyzed for the parents and 142 DH lines grown in an open field of the Henan Academy of Agricultural Sciences in Zhengzhou, China in the autumn of 2004. Seeds were first sown in pots (10 cm) with fertilized potting soil. Thirty days after sowing, plants were transplanted to an open field in a randomized two-block design. Soil analysis was carried out according to the method of Agro Services International (ASI) of Orange City,

Florida, USA (Table 2). Basal fertilizer (750 kg diammonium phosphate per hectare) was applied to plots at the time of transplanting; 375 kg N ha⁻¹ in the form of urea was applied at late rosette stage.

The second fully grown head leaf was harvested from two plants per DH line as two replica, each from a different block, after plants had grown for 30 days in the open field. Harvested leaves were washed with deionized water and lyophilized for 3 days. Samples were ground by mortar and pestle before being ash-digested at 300°C for 1 h and 500°C for 6 h. The digests were dissolved in 1.5 ml 8 M HNO₃ and doubled deionized H₂O to a final volume of 25 ml. Concentrations of 11 minerals, including Na, Ca, K, P, Mg, Fe, Zn, Mn, Cu, Al and Sr, were determined by inductively coupled plasma—atomic emission spectrometry (ICP-AES; Shimadzu 1000 II, Japan).

To investigate the effect of Zn nutritional stress on shoot dry biomass (SDB) the parental lines and 140 randomly selected DH lines were cultivated hydroponically in a greenhouse without climate control in March (experiment 1: Zn deficiency experiment) and April 2006 (experiment 2: Zn toxicity experiment) in Beijing. The environmental conditions were 15–25°C/8–12°C in March and 20–30°C/10–15°C in April (day/night temperature), 540–720 μmol m⁻² s⁻¹ light intensity and 50–60% relative humidity. Half strength Hoagland's nutrition solution (pH 5.5), buffered with 2 mM MES (2-morpholinoethanesulphonic acid), was used for plant cultivation. Each experiment included a treatment with normal Zn supply (2 μM ZnSO₄) and a Zn stress treatment, which was either omitting the addition of ZnSO₄ to the medium, to create Zn deficiency, or supplying 100 μM ZnSO₄ to create Zn toxicity. In both experiments three replicates were used for both control and Zn stress treatment. Seeds were germinated on agar in 1.5-ml Eppendorf tubes of which the bottom tips had been being cut off and put directly in hydroponic culture trays. Each tray

contained 71 DH lines with one plant per line. The trays were shuffled twice a week to reduce a possible effect of location in the greenhouse. After 21 days of growth, whole shoot (aboveground tissue) of each individual was harvested separately and oven-dried at 65°C for 3 days for SDB analysis.

Statistical analysis and QTL mapping

For each DH line, the mean values of 11 mineral concentrations were log₁₀-transformed to improve normality of the distribution. Transformed mineral data were used for QTL analysis. For QTL analysis of SDB under normal Zn supply, data from the two experiments were first standardized, and the mean values of the two experiments were used for QTL analysis. Detection of QTL for SDB under deficient and excessive Zn conditions was conducted with untransformed data. The software package MAPQTL version 5.0 was used to identify and locate QTL on the linkage map by using interval mapping (IM) and multiple-QTL model (MQM) mapping methods as described in the reference manual (<http://www.kyazma.nl>). A sequential procedure was used for QTL detection. The first step involves finding putative QTLs using IM analysis (Van Ooijen 1992). The significance thresholds accepting the presence of QTLs were determined by permutation tests (1,000 replications; Churchill and Doerge 1994) for each trait at a significance level of α=0.05. For different traits, the genome wide significance threshold varied between 2.9 and 3.1. Therefore QTLs with a LOD score ≥3.0 were considered to be significant. Markers at these QTLs were taken as cofactors (significant at P<0.02) to include the effect of the genetic background in MQM analysis (<http://www.kyazma.nl>). A mapping step size of 1 cM was used for both IM and MQM analyses. The QTL positions were estimated as the position with maximum LOD score on a linkage group. Two-LOD support intervals were

Table 2 Soil extractable mineral concentrations for the open field trial plot

pH	O.M. (%)	Element	N	K	Ca	Mg	P	Cu	Fe	Mn	Zn
7.97±0.1	0.11±0.01	Conc. (mg l ⁻¹)	24.3±10	58.7±10.2	1568±185	209±33.5	75.3±12.2	2.0±0.1	34.3±4.0	12.3±2.8	3.2±0.4

Data are presented as means±SD, n=6. K, Cu, Fe, Mn, Zn and P were extracted by extracting solution containing 0.25 mol l⁻¹ NaHCO₃, 0.01 mol l⁻¹ EDTA, 0.01 mol l⁻¹ NH₄F, 0.005% Superfloc 127, while Ca and Mg were extracted by 1 mol l⁻¹ KCl solution containing 0.0025% Superfloc 127. The extracting solution used for O.M. containing 0.2 mol l⁻¹ NaOH, 0.01 mol l⁻¹ EDTA, 2% methanol and 0.005% Superfloc 127.

Conc concentration, O.M. organic matter

established as 95% confidence intervals (Van Ooijen 1992) using MQM mapping implemented with MAPQTL 5.0. For the correlation coefficient (r) test between mineral concentrations for 11 elements, a Bonferroni correction to adjust the 0.05 or 0.01 threshold of significance was applied. Broad sense heritability was estimated for each trait by using the following equation: $h_b^2 = VG/(VG + VE)$; where VG is the variance between DH lines and VE is the variance within DH lines.

The interaction between the QTLs identified for the same trait was tested by analysis of variance (ANOVA) using the corresponding two markers as fixed factors and the trait as dependent variable at a significance level $P \leq 0.05$. For the interaction between genotype and Zn treatment, ANOVA was performed using corresponding markers and Zn treatment as fixed factors and the SDBs as dependent variables. The calculations were carried out using the general linear model module of the SPSS version 12.0.1 statistical package (SPSS Inc., Chicago, IL, USA).

Results

Construction of the BrIVFhn genetic linkage map

Out of the 211 AFLP primer combinations tested on the parental lines, 32 combinations, which each generated over eight polymorphic markers, were selected for genotyping the BrIVFhn mapping population (Table 1). After removing ambiguous markers, in total 202 polymorphic bands were used for mapping. Among these, 110 bands were provided by parent Y177 and 102 by parent Y195. A total of 205 SSR primers were screened for polymorphisms between the parental lines of which 61 displayed a polymorphism and out of these 53 SSRs that could be easily scored using silver stained gels were chosen for genotyping the mapping population. Three of these SSR assays (MAF, OI11B05 and KBRH048011) detected more than one segregating locus. In addition, seven recently reported ESTP markers were screened of which four showed polymorphism between the parental lines. To generate SRAP markers seven primer combinations were used, which amplified two to six polymorphic bands per primer combination in the parents.

In total 287 markers (203 AFLPs, 58 SSRs, 22 SRAPs and four ESTPs) were assigned to 10 linkage

groups (LGs) with a LOD score ≥ 7.0 (Fig. 1). The total length of the map is 1,090 cM, which represents on average one marker for every 3.8 cM. By using the 38 SSRs and four ESTPs with known map positions on the common *B. rapa* reference map (Lowe et al. 2004; Kim et al. 2006; Suwabe et al. 2006; Choi et al. 2007), the 10 linkage groups were assigned to their corresponding chromosomes R01–R10 (Fig. 1). The individual LGs ranged from 76 cM for R01 and R05 to 145 cM for R09. The number of markers on 10 LGs ranged from 21 (R10) to 41 (R03). A map-interval with markers separated more than 20 cM was only observed on R08. The distribution of markers and the marker density will make this map a useful framework for quantitative trait loci identification.

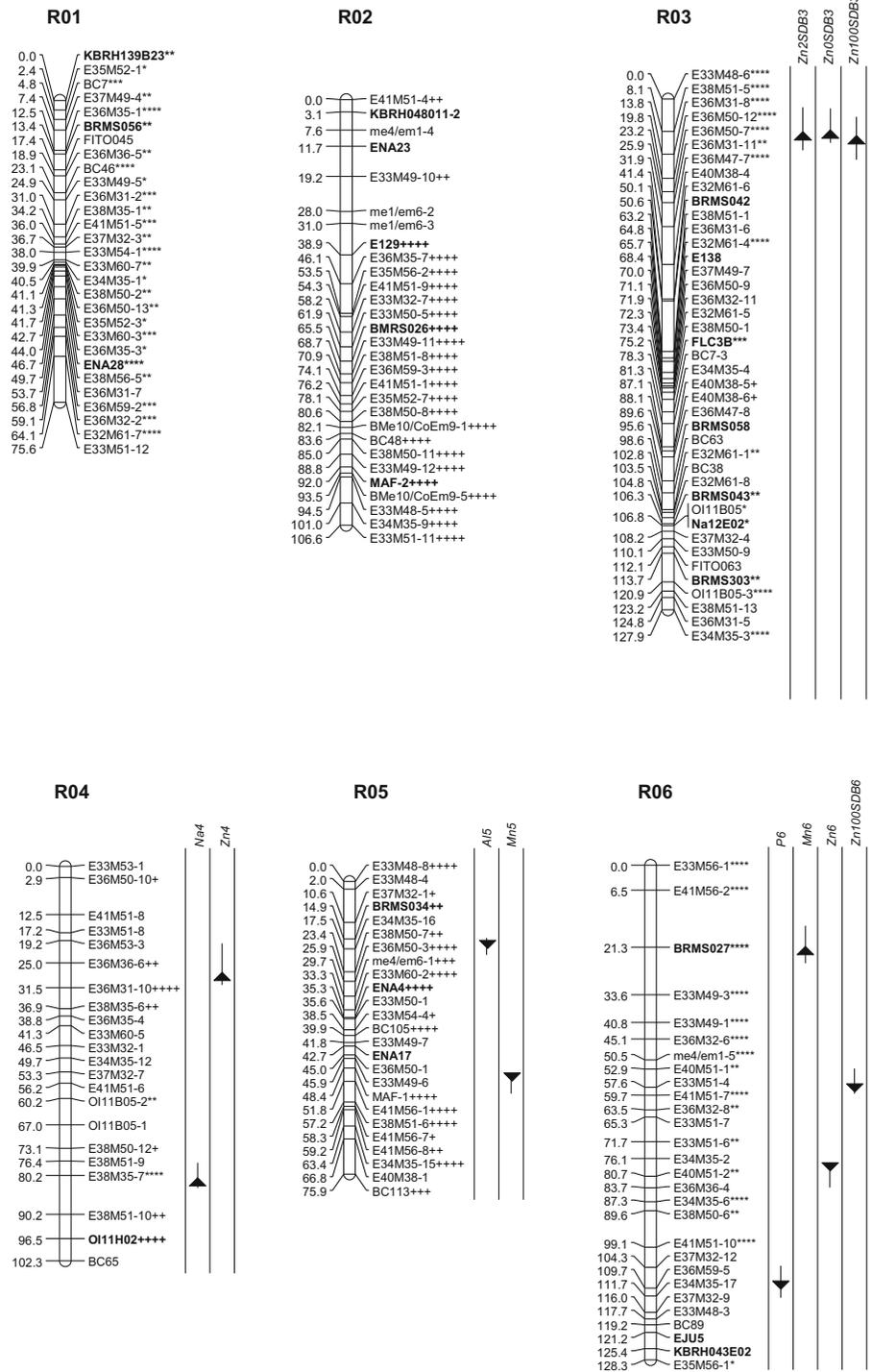
Based on a χ^2 test for goodness-of-fit to the expected 1:1 Mendelian segregation ratio, skewed segregation was detected for 202 loci (70.3%) at $P < 0.05$, including 146 AFLP loci, 38 SSR loci, 15 SRAP loci and three ESTP loci. For 154 of the 202 skewed markers the Y195 allele was in excess. Markers that showed skewness were clustered in LGs R01, R07 and R08 and in specific regions on LGs R03, R09 and R10 for markers with an excess of Y195 alleles, and for LGs R02, and R05 this was the case for Y177 alleles (Fig. 1).

Trait analysis

Broad sense heritability of the shoot mineral concentrations in the field experiment was high, ranging from 0.56 for Mg to 0.74 for Mn, respectively (Table 3). Transgression beyond the parental values was observed for nearly all tested elements, including K, Zn and P concentrations for which parental values hardly differed (Table 3; Fig. 2). Differences between DH lines ranged from two-fold for P to 6.5-fold for Fe. In most cases the extent of transgressive variation was towards the lower rather than the higher values. Correlations between mineral concentrations are shown in Table 4. The highest significant correlations were found between Ca, Mg and Sr concentrations, which were 0.84 (Ca/Mg), 0.86 (Ca/Sr) and 0.84 (Mg/Sr) respectively (Table 4). In this population, the Mn concentration was significantly correlated with all other elements, except for K. In all cases significant correlations were positive except for the correlation between Na and K.

The heritability of SDB under different Zn nutritional conditions was lower compared with that of

Fig. 1 Genetic linkage map based on a population of 183 DH lines of *B. rapa* ssp. *pekinensis*, and positions of QTLs associated with the concentration of leaf minerals and shoot dry biomass (*SDB*). Marker loci are listed to the right and recombination distances (cM) to the left of each linkage group. Skewed marker loci are indicated with a plus sign (skewed to Y177) or asterisk (skewed to Y195), the number of asterisk or plus sign indicates the level of significance with one symbol indicating significance at $P \leq 0.05$; two at $P \leq 0.01$; three at $P \leq 0.005$; and four at $P \leq 0.001$. Locations of QTLs are indicated by bars to the right of the linkage groups. The length of a bar indicates the 2-LOD confidence interval. The direction of the arrowhead indicates the relative phenotypic effect of the Y177 allele with upward for positive and downward for negative effects



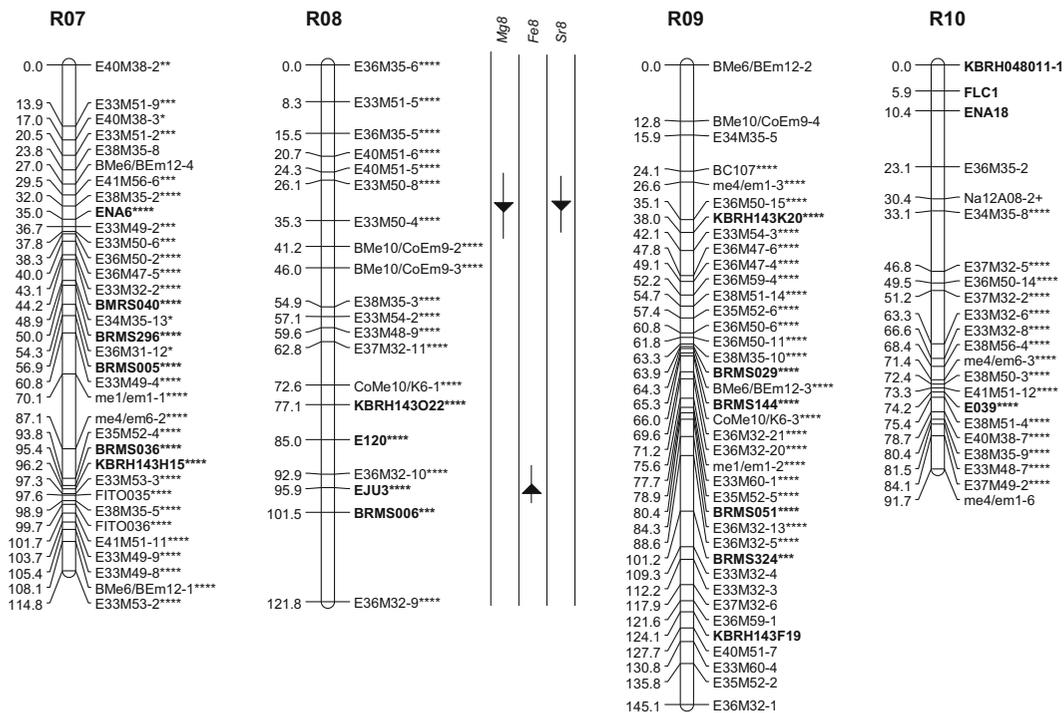


Fig. 1 (continued)

leaf mineral concentrations, with heritability from 0.30 to 0.60. The distribution of the SDB is broad and there is transgression towards lower as well as higher SDB (Table 3). Parental line Y177 has a considerably higher SDB than Y195 under all three tested Zn conditions. On average we found a difference in the SDB of plants growing under normal Zn between experiment 1 and experiment 2, with less biomass for plants in experiment 1, which was carried out earlier in spring. Nevertheless, the correlation of the SDB of DH lines under 2 μ M Zn in two experiments was significant ($r=0.43$, $P\leq 0.01$).

Detection of QTLs

To identify the genetic loci involved in establishing leaf mineral concentration in Chinese cabbage grown in the field and shoot dry biomass production under Zn nutritional stress in hydroponics culture, QTL mapping was performed for the tested 11 minerals and SDB under different Zn nutritional conditions. Ten QTLs were mapped on four LGs for Na, Mg, P, Fe, Mn, Zn, Al and Sr concentrations. No QTL was detected for K, Ca and Cu concentrations in this

population (Fig. 1; Table 5). The most significant of the identified QTLs, localizing on R04, explained 17.1% of the Zn concentration variance. Another QTL for Zn was detected on R06, explaining 13.6% of the phenotypic variance. These two QTLs together explained 21.2% of the Zn concentration variance. When tested by ANOVA, no significant interaction was found between the two QTLs. Two QTLs were detected for Mn concentration, one localized on R05 with explained variance of 13.5% and another one localized on R06 with explained variance of 15.7%. The two QTLs together explained 23.5% of the total Mn concentration variance. For the concentrations of Na, Mg, P, Fe, Al and Sr, only one QTL was found for each element. QTLs for Mg and Sr co-localized on R08 and explained 11.9% of Mg and 12.5% of Sr phenotypic variance. Another QTL on R08 explained 10.5% of the Fe concentration variance. A QTL for Na concentration was found on R04, accounting for 16.2% of the phenotypic variance. For P concentration, a QTL was mapped on R06, explaining 11.1% of the phenotypic variance. The QTL associated with Al concentration is located on R05 with an effect of 11.2% of the phenotypic variance.

Table 3 Leaf mineral concentrations and shoot dry biomass (SDB) of the parents (Y177 and Y195) and the DH population (range, mean) grown in an open field or, for determination of SDB, hydroponically under 0, 2 and 100 μM Zn supply

Trait	Parents		Range	Mean	Heritability
	Y177	Y195			
Macro mineral concentration (mg g^{-1} DW)					
K	35.8	34.4	25.3–58.2	36.7	0.70
Na	8.6	14.1	2.4–15.7	7.5	0.70
Ca	17.5	30.3	10.1–26.0	15.2	0.60
Mg	3.4	5.1	2.1–5.3	3.2	0.56
P	8.8	8.3	5.0–9.6	7.2	0.64
Micro mineral concentration ($\mu\text{g g}^{-1}$ DW)					
Al	77.0	171.5	38.9–175.0	88.3	0.60
Cu	6.0	7.75	3.8–7.9	5.6	0.68
Mn	27.2	30.2	13.9–34.6	20.0	0.74
Fe	89.0	183.1	46.5–224.2	109.2	0.69
Zn	51.4	53.8	31.1–72.3	48.2	0.69
Sr	81.8	145.4	44.2–150.2	78.1	0.63
SDB (mg per plant)					
SDB0 ^a	130	70	20.0–165.0	79.1	0.46
SDB2-1 ^a	120	85	25.0–176.7	84.1	0.30
SDB2-2 ^b	189	84	37.5–254.0	135.9	0.60
SDB100 ^b	107	59	21.0–144.3	86.0	0.59

For each trait the heritability is indicated.

^aData in experiment 1

^bData in experiment 2

QTLs related to SDB were identified on R03 and R06. The locus on R03 affects SDB under all three Zn treatments, explaining 14.8%, 15.3% and 12.3%, of the phenotypic variance for SDB under Zn deficiency, normal and excess conditions, respectively. When tested by ANOVA, no significant interactions between genotype and Zn conditions were found for the SDB phenotypic variance at this locus. This suggests that this QTL affects SDB regardless of the Zn supply. The additional QTL on R06 exclusively affected SDB under Zn excess, accounting for 12.6% of the phenotypic variance. No significant interactions between genotype and Zn conditions were found, neither were there significant interactions between the two SDB QTLs.

Discussion

Construction of a *B. rapa* linkage map

This study reports on the construction of a genetic linkage map for a segregating population of 183 *B.*

rapa DH lines and the first analysis of genetic control of leaf mineral accumulation in *B. rapa* and shoot dry biomass under different Zn nutrition conditions.

The present linkage map includes 203 AFLP markers, 58 SSR markers, 22 SRAP markers and four ESTP marker grouped on 10 LGs, each anchored to the corresponding chromosome of the *B. rapa* reference map (Lowe et al. 2004; Kim et al. 2006; Suwabe et al. 2006; Choi et al. 2007) based on common SSR-markers. Our linkage map covers a total genetic distance of 1,090 cM, which compares well with the 1,005.5 and 1,182 cM of two recently published SSR-based maps (Suwabe et al. 2006; Choi et al. 2007) and the 1,287 cM of a sequence-tag-based map (Kim et al. 2006). The number and distribution of the markers over the genome was also comparable to these maps.

A high percentage of skewed markers (70.4%) were observed in our study. This is higher than that of the genetic linkage map based on a DH population of Chinese cabbage (38.3%; Choi et al. 2007), but it is comparable with a proportion of 64% AFLP and RFLP markers deviating from a 1:1 ratio as observed

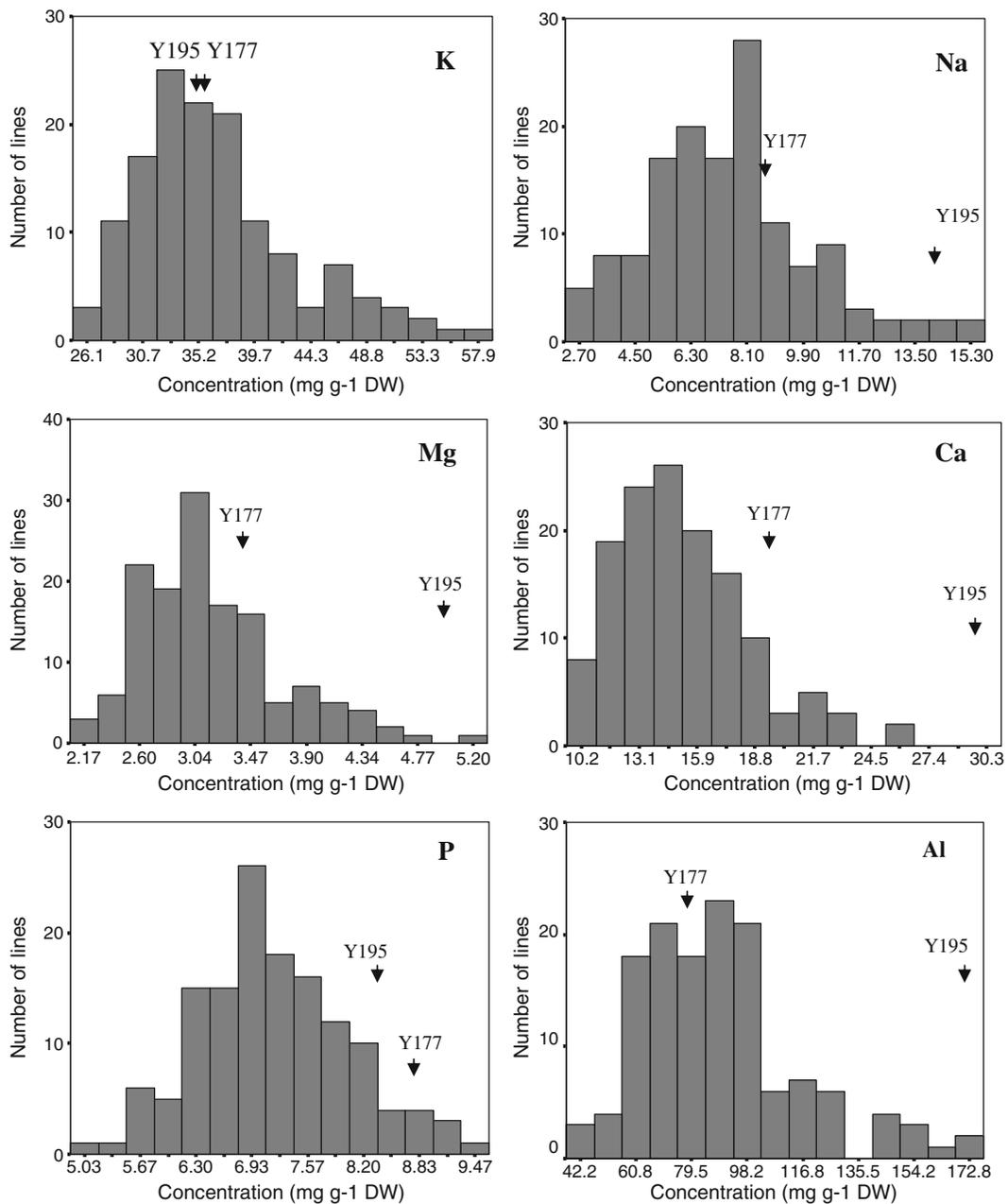


Fig. 2 Frequency distributions of non-normalized data for the concentration of eight minerals in leaves for which QTLs are identified in the DH population of *B. rapa* ssp. *pekinensis*. The

parental values are the mean value of two replicates, indicated with an *arrow* for each parent. The *horizontal bars* represent the range of the parental values

by Voorrips et al. (1997) in a *B. oleracea* DH mapping population. Skewed segregation ratios have often been observed in populations of doubled haploid plants for many other plant species (Graner et al. 1991), and are most likely due to selection during microspore culture (Foisset et al. 1996). Segregation of skewed markers may generate errors

in linkage analysis, resulting in map stretching or a false order of markers on linkage groups. However, simply deleting the markers which show skewed segregation from the map would result in losing all of the skewed segments for trait QTL analysis and thus we decided to include markers with skewed segregation in the map.

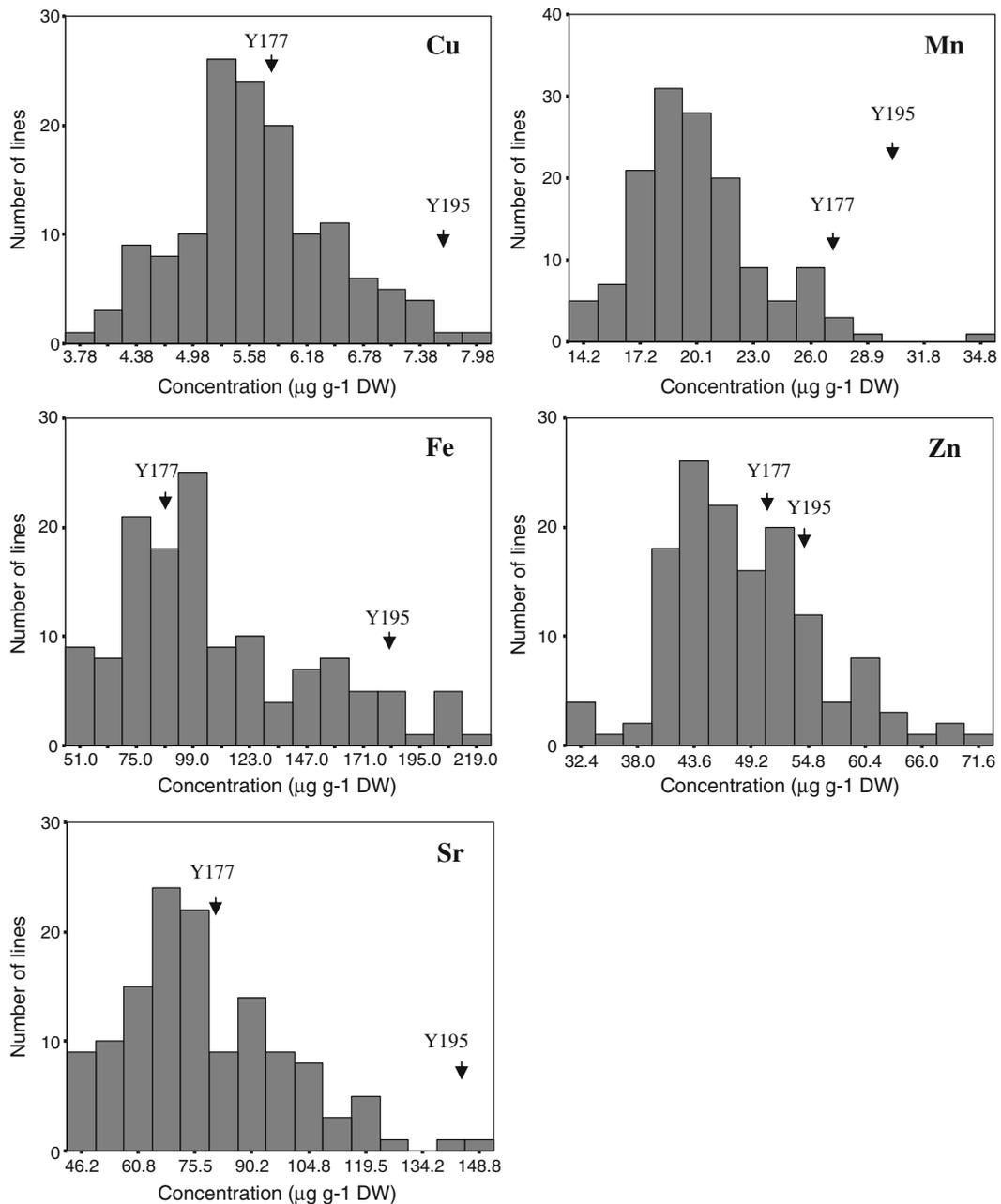


Fig. 2 (continued)

QTLs for mineral accumulation

We analyzed leaf mineral concentration in the mapping population and found transgression for all 11 elements. Nevertheless, no QTL was detected for three of these elements including K, which showed transgressive variation to both sides of the parental values. The failure to detect QTLs associated with

transgressed traits was previously reported for RIL populations of tomato (Saliba-Colombani et al. 2001) and tobacco (Julio et al. 2006). Transgressive segregation in a population is normally due to the presence of complementary QTL alleles in the two parental lines. However if there are several QTLs and the QTL effects are relatively small they may not be detected.

Table 4 Pearson correlation coefficients of the mineral concentrations in the second head leaf in a *B. rapa* ssp. *pekinensis* DH population

Mineral	Pearson correlation									
	Na	Ca	Mg	P	Al	Cu	Mn	Fe	Zn	Sr
K	-0.30**	0.02	0.14	0.41**	0.33**	0.33**	0.20	0.36**	0.37**	-0.12
Na		0.38**	0.38**	0.05	0.11	0.17	0.33**	0.13	0.03	0.43**
Ca			0.84**	-0.07	0.56**	0.17	0.63**	0.46**	-0.02	0.86**
Mg				-0.04	0.61**	0.17	0.62**	0.57**	0.02	0.84**
P					-0.05	0.57**	0.31**	0.04	0.64**	-0.18
Al						0.03	0.49**	0.68**	0.02	0.48**
Cu							0.34**	0.22	0.70**	-0.02
Mn								0.44**	0.25*	0.47**
Fe									0.23	0.41**
Zn										-0.20

* $P < 0.05$, ** $P < 0.01$

The trait analysis showed significant positive correlations among some of the tested minerals, including toxic element Al and non-essential element Sr. This was also the case for seed mineral content in common bean (Beebe et al. 2000) and Arabidopsis (Vreugdenhil et al. 2004). The correlation between

different minerals implicated pleiotropy for genes controlling accumulation of these minerals or close linkage of genes. Therefore consideration must be taken when designing a breeding program for improvement of mineral concentrations, especially to avoid accumulation of toxic minerals. However, we

Table 5 Overview of quantitative trait loci (QTL) affecting leaf mineral concentrations and shoot dry biomass (SDB) under different Zn nutrition conditions of a *B. rapa* ssp. *pekinensis* DH population

QTL ^a	LOD ^b	Position ^c (cM)	Marker ^d	2-LOD (cM) ^e	Exp (%) ^f	Add ^g
Na4	4.2	81	E38M35-7	77–82	16.2	0.15
Mg8	3.4	32	E33M50-4	25–38	12.0	-0.07
P6	3.5	110	E36M59-5	104–111	11.1	-0.04
Al5	3.4	18	E34M35-16	16–20	11.2	-0.10
Mn5	4.7	52	E41M56-1	50–54	13.5	-0.06
Mn6	5.2	21	BRMS027	15–25	15.7	0.07
Fe8	3.1	95	EJU3	92–100	10.5	0.12
Zn4	5.4	28	E36M36-6	20–31	17.1	0.06
Zn6	3.9	79	E40M51-2	77–82	13.6	-0.06
Sr8	3.4	33	E33M50-4	26–37	12.5	-0.10
Zn2SDB3	3.9	8	E38M51-5	4–12	15.3	11.40
Zn0SDB3	4.2	8	E38M51-5	5–10	14.6	10.30
Zn100SDB3	3.0	11	E38M51-5	6–15	12.3	9.10
Zn100SDB6	3.1	59	E33M51-4	53–59	12.7	-9.00

The additive effect for mineral concentrations is given on a \log_{10} scale and the effect for SDB is on the original scale.

^a QTL names are indicated as element followed by chromosome number.

^b Maximum LOD score

^c Map position of the QTL is expressed in cM from the first markers of the linkage group.

^d The corresponding marker is the cofactor used in MQM mapping.

^e Two-LOD support interval of the QTL

^f Explained variance

^g Additive effect of the Y177 allele

did not find as many co-localized QTLs as we found significant correlations between concentrations of different minerals. This could be due to a large number of QTLs involved in mineral accumulation but each with minor effect and therefore is difficult to be detected. Co-localization of QTLs involved in different minerals was reported previously for *Arabidopsis* seed mineral content with most of the co-localizations of K/Ca and K/Ca/Mn QTLs (Vreugdenhil et al. 2004) and recently for *Brassica oleracea* shoot Ca/Mg concentrations (Broadley et al. 2008). Although we found strong correlation between Mg, Ca and Sr concentrations, we only found QTLs for Mg and Sr, but these were the only mineral QTLs that co-localized in the studied population. Although it has been reported that there is no selectivity or interactions between Ca and Sr for transport to the shoots (reviewed by White 2001), the correlation between the concentration of these minerals and the co-location of QTLs indicates that the accumulation of Ca, Mg and Sr in shoots might share a common mechanism. This was also supported by a suggestive QTL in the same region of QTLs on R08 for Ca, with a peak of LOD=2.42. R08 shares high level of collinearity with the chromosome C08 of *B. oleracea* (Parkin et al. 2005), on which Broadley et al. (2008) identified co-localized QTLs for shoot Mg and Ca concentrations. This provides clue that homologous genes in *B. rapa* and *B. oleracea* are likely being involved in Mg and Ca accumulation. However, as no common anchoring marker was used in these two studies, further clarification is needed to prove the alignment of these QTLs for Mg accumulation in the two species.

While the heritabilities for most mineral concentrations were high, only one or two QTLs were detected for individual minerals, with a relatively low percentage of explained variance per locus varying from 10.5–17.1%. This means that many QTLs remained undetected, probably because of small effects of each QTL. This underlines the complex genetic control of leaf mineral accumulation in this population. If this is common in *B. rapa* it will be very difficult to set up an efficient breeding program for improved leaf mineral concentrations. For this population, the parents were not very different in terms of leaf mineral concentrations. Previous work from our group on Fe, Mn and Zn leaf concentrations (Wu et al. 2007) showed that accessions with larger

differences can be found in *B. rapa* germplasm. Choosing such accessions as parents of a segregating population could simplify the genetic complexity and allow identification of more QTLs.

QTL for SDB

We carried out two independent experiments for plant SDB analysis under Zn deficiency and Zn excess conditions. Relative shoot growth based on dry biomass was previously identified as a suitable parameter for tolerance evaluation of *Brassica* crops to Zn stress (Grewal et al. 1997; Wu et al. 2007). Therefore in the present study we investigated SDB under deficient and excess Zn supply. Considering that SDB normally represents a complex trait including production-related characteristics as well as adaptive characteristics that must be changed in plants in order to adapt to (a)biotic stress (Ronnberg-Wastljung and Gullberg 1999), we also analyzed SDB under normal Zn supply to be able to distinguish general SDB QTLs from stress-specific SDB QTLs. Only a few QTLs were detected for SDB, with low explained variance ($\leq 15\%$). This is consistent with previous reports that the analyses of plant responses to environmental stress generally reveal only few major QTL (Koyama et al. 2001), again indicating that a large number of genes contribute to the overall phenotype.

A QTL for SDB was mapped on R03 under different Zn nutrition conditions. It did not show a significant interaction between the genotype of this locus and the Zn treatment, thus this locus controls plant SDB in general. Also for the *Zn100SDB6* QTL on R6, which was identified for excessive Zn only, the interaction between genotype and Zn condition was not significant. As the LOD score of this locus, with a low explained variance, was just above the threshold for QTL determination, such minor QTL could have escaped QTL detection under normal and Zn deficient conditions. Inefficient detection of SDB QTLs under Zn stress conditions, as in the present study, is most likely due to the multigenic inheritance of the trait, which has also been reported for QTL mapping of rice tolerance to Zn deficiency (Wissuwa et al. 2006). More detailed physiological studies that distinguish between sub-processes are likely to improve genetic dissection on tolerance to Zn stress. Also the much higher effect of Zn condition (20.9%)

on SDB than that of genotype of this locus (4.5%), as revealed by ANOVA, indicates that genetic improvement on Zn stress tolerance will need integration of a large number of genes.

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