

# Characterization of natural variation for zinc, iron and manganese accumulation and zinc exposure response in *Brassica rapa* L.

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**Abstract** *Brassica rapa* L. is an important vegetable crop in eastern Asia. The objective of this study was to investigate the genetic variation in leaf Zn, Fe and Mn accumulation, Zn toxicity tolerance and Zn efficiency in *B. rapa*. In total 188 accessions were screened for their Zn-related characteristics in hydroponic culture. In experiment 1, mineral assays on 111 accessions grown under sufficient Zn supply ( $2 \mu\text{M ZnSO}_4$ ) revealed a variation range of  $23.2\text{--}155.9 \mu\text{g g}^{-1}$  dry weight (d. wt.) for Zn,  $60.3\text{--}350.1 \mu\text{g g}^{-1}$  d. wt. for Fe and  $20.9\text{--}53.3 \mu\text{g g}^{-1}$  d. wt. for the Mn concentration in shoot. The investigation of tolerance to excessive Zn ( $800 \mu\text{M ZnSO}_4$ ) on 158 accessions, by using visual toxicity symptom parameters (TSPs), identified different levels of tolerance in *B. rapa*. In experiment 2, a selected sub-set of accessions from experiment 1 was characterized

in more detail for their mineral accumulation and tolerance to excessive Zn supply ( $100 \mu\text{M}$  and  $300 \mu\text{M ZnSO}_4$ ). In this experiment Zn tolerance (ZT) determined by relative root or shoot dry biomass varied about 2-fold. The same six accessions were also examined for Zn efficiency, determined as relative growth under  $0 \mu\text{M ZnSO}_4$  compared to  $2 \mu\text{M ZnSO}_4$ . Zn efficiency varied 1.8-fold based on shoot dry biomass and 2.6-fold variation based on root dry biomass. Zn accumulation was strongly correlated with Mn and Fe accumulation both under sufficient and deficient Zn supply. In conclusion, there is substantial variation for Zn accumulation, Zn toxicity tolerance and Zn efficiency in *Brassica rapa* L., which would allow selective breeding for these traits.

**Keywords** *Brassica rapa* L. · Mineral accumulation · Zn excess tolerance · Zn efficiency

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## Introduction

Zinc (Zn) is an essential micronutrient required by all organisms for its role in many physiological processes as a structural or catalytic component of proteins. Unfortunately Zn deficiency is a widespread problem by affecting humans in case of Zn shortage in food. About 20% of rural children are at risk of inadequate Zn intake in China

(Ma et al. *in press*). Zn deficiency is also affecting crops in case of poor Zn availability in soil. In China Zn deficiency is prevalent on calcareous soil in North China and calcareous alluvial soils of the Middle and Lower Yangtse River valley (Liu 1994). Breeding and growing of crops with high Zn content and Zn efficiency are promising and sustainable approaches to solve the Zn deficiency problems in humans and soil (Cakmak et al. 1996).

Knowledge on genetic variation of Zn accumulation and Zn efficiency is the prerequisite for breeding of Zn content/efficiency-improved crop cultivars. Previous studies on genetic variation of micronutrients were mainly limited to staple food crops, including wheat (Graham et al. 1997), rice (Graham et al. 1999), bean (Beebe et al. 2000) and maize (Banziger and Long 2000). Little is known about micronutrient content in leaves, which is the main edible organ of leafy vegetables (Kopsell et al. 2004). The ability of a genotype to grow and yield well in soils that are too deficient in Zn for a standard cultivar to grow and yield well, is defined as Zn efficiency (Graham et al. 1992). Progress has been made in screening Zn efficient genotypes and understanding the physiological and biochemical mechanisms of Zn efficiency (Reviewed in Hacisalihoglu and Kochian 2003). However, knowledge on Zn efficiency in vegetables is limited (Hacisalihoglu et al. 2004).

*Brassica rapa* L. comprises several cultivar types producing edible roots, stems, leaves, buds or flowers as vegetables (Gomez-Campo and Prakash 1999). Some of these are the most important vegetables in eastern Asia, especially in China, Korea and Japan, both in terms of production and per capita consumption (Opena et al. 1988). As vegetables are one of the main micronutrient sources of the population in China (Ma et al. *in press*), we studied *B. rapa* vegetables to collect more information on the extent of genotypic variation for Zn accumulation and Zn efficiency and their potential for genetic improvement of these traits.

While a shortage of Zn is a problem for plant growth, an excess of Zn is even more detrimental. Zn heavy metal pollution is prevalent in China's industrialized areas (Liu et al. 2005; Nan and

Zhao 2000). *B. rapa* is not known to be a metal hyperaccumulator and showed a significant decrease in biomass with increased root and shoot Zn concentration upon exposure to toxic Zn levels (Ebbs and Kochian 1997; Coolong and Randle 2003; He et al. 2004). However, in general only one accession was tested in each case. We therefore intended to determine the natural variation for excess Zn tolerance (ZT) among *B. rapa* germplasm.

The objective of this study is to characterize the genotypic variation for Zn accumulation and Zn response in *B. rapa* upon exposure to different Zn concentrations. Understanding the range of genotypic variation in Zn accumulation and response to Zn nutritional stress will provide a genetic basis for micronutrient and Zn stress tolerance breeding of *B. rapa* vegetables and for further genetic studies on Zn accumulation and tolerance to Zn nutritional stress.

## Materials and methods

### Plant material

To determine the genetic variation of Zn accumulation and response to Zn stress, a total of 188 *Brassica rapa* accessions belonging to nine cultivar groups (Table 1) were screened. About 184 accessions were obtained from the Institute of Vegetables and Flowers of the Chinese Academy of Agricultural Sciences (IVF-CAAS); two were obtained from the Dutch Crop Genetic Resources Centre (CGN) in Wageningen, and the other two were obtained from Dr. T. Osborn (University of Wisconsin, Madison, USA). About 111 accessions were used for shoot (aboveground tissue) mineral analysis and 158 lines were screened for their tolerance to Zn excess stress. On the basis of their performance in this large scale screening experiment (experiment 1), 15 accessions were selected for a detailed accumulation and tolerance testing (experiment 2) as described below. In experiment 2, six additional accessions were added, which are the parents of additional doubled haploid (DH) populations that are under development.

**Table 1** Overview of *B. rapa* accessions, according to cultivar group used in the described experiments

Cultivar group	Total	No. of accessions					
		ZA		ZT		ZE	
		Exp 1	Exp 2	Exp 1	Exp 2		
Chinese cabbage (sp. <i>pekinensis</i> )	69	45	7	46	1	1	
Pak Choi (sp. <i>Chinensis</i> )	64	39	6	61	2	2	
Caixin (sp. <i>parachinensis</i> )	23	15	1	23	1	1	
Turnip (sp. <i>rapa</i> )	11	6	1	10			
Wutacai (sp. <i>narinosa</i> )	8	4	1	8	1	1	
ZiCaitai (sp. <i>chinensis</i> var. <i>purpurea</i> )	5	2	1	5			
Mizuna (sp. <i>nipposinica</i> )	6	4	2	5	1	1	
Oil seed (Yellow Sarson) (sp. <i>tricoloris</i> )	1	1	1				
Rapid cycling	1	1	1				
Total	188	117	21	158	6	6	

ZA: Zn accumulation experiment; ZT: Zn tolerance experiment; ZE: Zn efficiency experiment; Exp 1: experiment 1; Exp 2: experiment 2

### Plant culture

For experiment 1, three plants for each accession were grown in a greenhouse without climate control in Beijing, China, from mid March till May. The environmental conditions were 20–30°C/10–15°C (day/night temperature), 30,000–40,000 Lux light intensity and 50–60% relative humidity. Seeds were germinated in vermiculite and watered every 3 days with half-strength Hoagland's nutrient solution after germination. After 14 days (mineral accumulation experiment) or 7 days (Zn tolerance experiment) seedlings were transferred to hydroponic culture trays each containing three individuals from 24 accessions in 20 l half-strength Hoagland's nutrient solution. The solution was buffered with 2 mM MES (2-morpholinoethanesulphonic acid) at pH 5.5. A concentration of 2  $\mu\text{M}$   $\text{ZnSO}_4$  was used as sufficient Zn supply. Nutrient solutions were replaced once a week until harvesting. After 7 days at sufficient Zn, plants for the ZT experiment were transferred to

excess Zn nutrient solution containing 800  $\mu\text{M}$   $\text{ZnSO}_4$ , and were exposed for 14 days. The solution was refreshed after 1 week.

For experiment 2, plants were grown in a climate-controlled growth cabinet set at 75% humidity and 22/16°C (16 h/8 h) day/night temperature regime. Seeds were germinated in fertilized potting soil watered with tap water. Seedlings were transferred to hydroponic solution after 14 days for the mineral accumulation experiment or after 7 days for the ZT and Zn efficiency experiments. For the mineral accumulation experiment plants were grown for 14 days in medium with sufficient Zn (2  $\mu\text{M}$   $\text{ZnSO}_4$ ). For the ZT experiment plants were first grown for 7 days in medium with sufficient Zn (2  $\mu\text{M}$   $\text{ZnSO}_4$ ) before exposure to excess Zn for 14 days. Instead of the very high concentration of 800  $\mu\text{M}$   $\text{ZnSO}_4$ , plants were transferred to 100 and 300  $\mu\text{M}$   $\text{ZnSO}_4$  as excess Zn concentrations and to 2  $\mu\text{M}$   $\text{ZnSO}_4$  as the sufficient Zn control. For the Zn efficiency experiment, one-week-old germinated seedlings were transferred directly to a nutrient solution without  $\text{ZnSO}_4$  or with 2  $\mu\text{M}$   $\text{ZnSO}_4$  as control and grown for 15 days before assessment. For each line three pots were used with one plant per pot for the mineral accumulation experiment and three plants per pot for the Zn efficiency and ZT experiments. In all these experiments the nutrient solutions were refreshed twice a week.

### Mineral determination

In experiment 1, shoots were harvested from plants with a similar size after 27–36 days of growth. For each accession shoots from 2 to 3 individual plants were combined in one sample. Harvested shoots were washed with de-ionized water and lyophilized. Samples were ground by mortar and pestle before wet-digestion in concentrated  $\text{HNO}_3:\text{HClO}_4$  (87:13, V/V) subsequently at 60°C for 3 h, 100°C for 1 h, 120°C for 1 h and 195°C for 2.5 h. The digests were diluted with 5 ml 20% HCl and deionized  $\text{H}_2\text{O}$  to a final volume of 20 ml before analysis by inductively coupled plasma-atomic emission spectrometer (ICP-AES) (Leeman-DRE DR6009, USA) at the IVF-CAAS in Beijing.

In experiment 2, shoots and roots were harvested separately per plant. After oven-drying at 65°C for 3 days, shoot and root dry biomass were measured. Shoot samples were ground by mortar and pestle before wet-digestion in concentrated HCl:HNO<sub>3</sub> (1:4, V/V) at 140°C for 7 h. Mineral assays were performed by using a flame Atomic Absorption Spectrometer (AAS) (model 1100, Perkin–Elmer) at the Vrije Universiteit, Amsterdam. Seed mineral content of the 21 accessions used for experiment 2 was determined in samples of about 100 mg ground seeds. Seed mineral determination assays were as described for shoots.

### Zn efficiency and tolerance

Shoots and roots were harvested separately and dried at 65°C for 3 days to determine their dry biomass. Zn efficiency (ZE) was calculated for shoots and roots based on relative biomass production using the following calculation:  $ZE (\%) = [\text{dry biomass at } 0 \mu\text{M Zn} / \text{dry biomass at } 2 \mu\text{M Zn}] * 100\%$ .

In the first ZT experiment, a ranked set of five Toxicity Symptom Parameters (TSPs) representing different levels of deterioration of the leaves was used to score plant response after exposure to 800  $\mu\text{M ZnSO}_4$ : 1 = slight chlorosis of leaves, plant is still growing; 2 = chlorosis of leaves; 3 = severe chlorosis of leaves, leaves started withering; 4 = most of the leaves seared; 5 = plant has died. Accessions with mean TSP values below 2 were classified as Zn tolerant and above 4 as Zn sensitive.

For the second ZT experiment, ZT was calculated in two different ways. One calculation was made based on dry root or shoot biomass:  $ZT_{\text{bio-mass}} (\%) = [\text{dry biomass at toxic level Zn} / \text{dry biomass at } 2 \mu\text{M Zn}] * 100\%$ . Another calculation was based on root elongation during exposure. For this analysis, roots were dyed with active charcoal before transferring plants to excess Zn medium. Root elongation during exposure (non-stained part of the root) was measured according to Schat and Ten Bookum (1992):  $ZT_{\text{root}} (\%) = [\text{root elongation at toxic level Zn} / \text{root elongation at } 2 \mu\text{M Zn}] * 100\%$ .

### Statistics

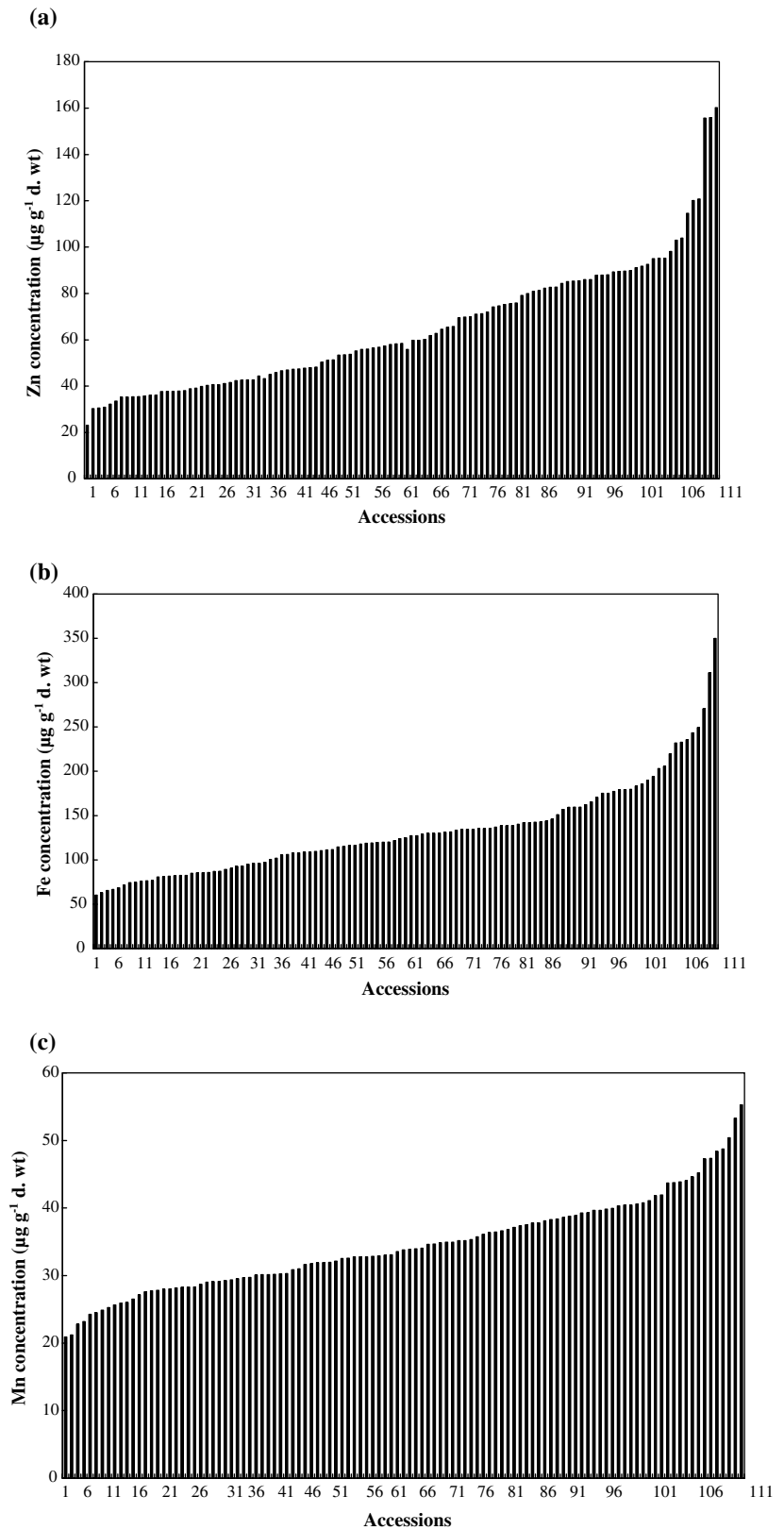
Statistical analyses of metal concentration and root length were conducted using one-way ANOVA followed by the Student-Neuman-Keuls posthoc analysis (SigmaStat, SPSS Science, Chicago, IL, USA). The variation within the mean is presented as the standard error. Significance of correlation was determined using simple linear regression. We considered differences significant at  $P \leq 0.05$ .

## Results

### Zn, Fe and Mn accumulation

When examining the shoot Zn concentration for 111 accessions, belonging to seven cultivar groups of *B. rapa*, grown in hydroponic culture with sufficient Zn supply for about 4 weeks, large variations were found between accessions, ranging from 23.2 to 155.9  $\mu\text{g Zn g}^{-1}$  d. wt. (Fig. 1a). Accessions with a Zn concentration lower than 50  $\mu\text{g g}^{-1}$  d. wt., between 50 and 100  $\mu\text{g g}^{-1}$  d. wt. and above 100  $\mu\text{g g}^{-1}$  d. wt. accounted for respectively 37%, 56% and 7% of the total. The same samples were used to determine the Fe and Mn concentrations. The Fe concentration varied from 60.3 to 350.1  $\mu\text{g g}^{-1}$  d. wt. (Fig. 1b). Accessions with a Fe concentration lower than 100  $\mu\text{g g}^{-1}$  d. wt., between 100 and 200  $\mu\text{g g}^{-1}$  d. wt. and above 200  $\mu\text{g g}^{-1}$  d. wt. accounted respectively for 28%, 71% and 1% of the total. The Mn concentration ranged from 20.9 to 53.3  $\mu\text{g g}^{-1}$  d. wt. (Fig. 1c). The proportions of accessions with a Mn concentration lower than 30  $\mu\text{g g}^{-1}$  d. wt., between 30 and 50  $\mu\text{g g}^{-1}$  d. wt. and above 50  $\mu\text{g g}^{-1}$  d. wt. were respectively 28%, 69% and 3% of the total. There was no significant difference in average Zn or Fe concentration between the different cultivar groups, however the average Mn concentrations in Wutacai and Mizuna accessions were significantly higher compared to those of the other cultivar groups (Table 2). Zn concentration varied most in Wutacai with a variation coefficient as high as 60%.

**Fig. 1** Genotypic variation of shoot Zn (a), Fe (b) and Mn (c) concentrations of 111 *B. rapa* accessions grown in half-strength Hoagland's nutrient solution containing 2  $\mu\text{M}$  Zn for 27–36 days. Data are based on a mixed sample of 2–3 plants per accession. Accessions are ordered according to increasing mineral content



**Table 2** Average Zn, Fe and Mn concentrations ( $\mu\text{g g}^{-1}$  d. wt.) in shoots of a number of *B. rapa* accessions (No. acc.) according to cultivar groups

Cultivar group	No. acc.	Zn	Fe	Mn
Chinese cabbage (sp. <i>pekinensis</i> )	44	60 $\pm$ 30	142 $\pm$ 50	32 $\pm$ 7b
Pak Choi (sp. <i>chinensis</i> )	37	69 $\pm$ 24	118 $\pm$ 46	34 $\pm$ 5b
Caixin (sp. <i>parachinensis</i> )	15	60 $\pm$ 21	125 $\pm$ 72	37 $\pm$ 5b
Wutacai (sp. <i>narinosa</i> )	4	83 $\pm$ 50	153 $\pm$ 48	45 $\pm$ 7a
Turnip (sp. <i>rapa</i> )	5	44 $\pm$ 10	145 $\pm$ 48	39 $\pm$ 6b
Mizuna (sp. <i>nipposinica</i> )	3	75 $\pm$ 10	142 $\pm$ 37	44 $\pm$ 5a
ZiCaitai (sp. <i>chinensis</i> var. <i>purpurea</i> )	2	80 $\pm$ 15	185 $\pm$ 67	34 $\pm$ 0b

All plants were grown in half-strength Hoagland's nutrient solution containing 2  $\mu\text{M}$   $\text{ZnSO}_4$ . Data are presented as means  $\pm$  SE. Only for Mn significant differences ( $P \leq 0.001$ ) were found between the cultivar groups as indicated by different letters. Significance was determined by ANOVA followed by Student Neuman-Keuls posthoc analysis

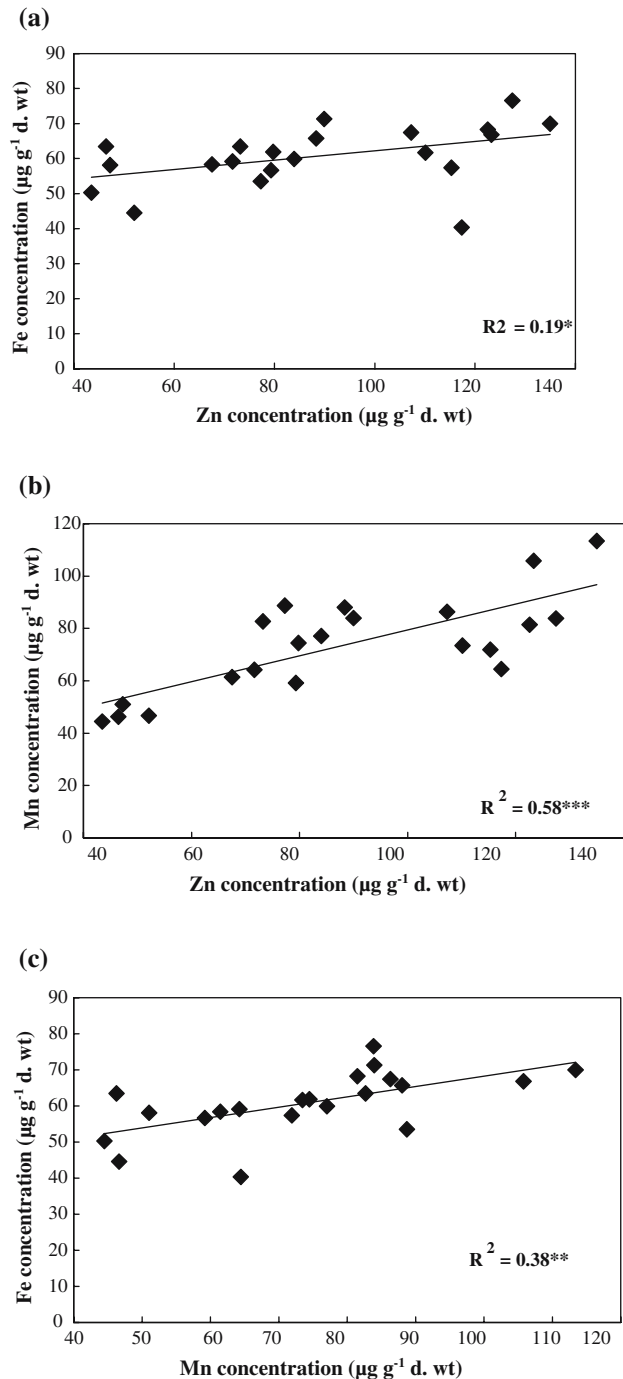
Five accessions with low Zn concentration (on average 40  $\mu\text{g g}^{-1}$  d. wt.), four accessions with moderate Zn concentration (on average 67  $\mu\text{g g}^{-1}$  d. wt.) and six accessions with high Zn concentration (on average 135  $\mu\text{g g}^{-1}$  d. wt.) were selected for further confirmation in a subsequent experiment, with plants grown under climate-controlled conditions (Experiment 2). Six additional accessions, which are the parents of recently developed DH populations, were also included. In experiment 2, the range of Zn concentration was slightly less (43.5–135.0  $\mu\text{g g}^{-1}$  d. wt.) than in experiment 1 (Table 3). The mean Zn concentration was comparable in both experiments. In general, the Fe concentration was lower in experiment 2 compared to experiment 1 (ranging from 40.4 to 70.6  $\mu\text{g g}^{-1}$  d. wt.), whereas it was the reverse for the Mn concentration (ranging from 44.5 to 113.4  $\mu\text{g g}^{-1}$  d. wt.). The data from the two experiments were not significantly correlated ( $R^2 = 0.10$  for Zn, 0.06 for Fe and 0.24 for Mn). When comparing the 21 accessions, the Fe concentrations were not significantly different, but there were significant differences for the Zn and Mn concentrations (Table 3). The Zn, Fe and Mn

concentrations of these accessions were positively correlated (Fig. 2). The correlation between Zn and Mn ( $R^2 = 0.58$ ,  $P \leq 0.001$ ) was much higher than that of Zn and Fe ( $R^2 = 0.19$ ,  $P \leq 0.05$ ), however, when excluding the data of outlier accession L144 from the data set, the correlation between Zn and Fe concentrations was significant at  $P \leq 0.005$  ( $R^2 = 0.47$ ). Omitting this accession from the correlation analysis did not affect the significance level for the correlation between Zn and Mn concentrations ( $R^2 = 0.67$ ,  $P \leq 0.001$ ) or Fe and Mn concentrations ( $R^2 = 0.43$ ,  $P \leq 0.005$ ). In addition to shoot mineral concentration, the seed weight, seed mineral content and plant biomass were determined for these accessions (Table 4). When comparing the shoot mineral concentration to the dry shoot or root biomass (Table 4), shoot Fe concentration was positively correlated with dry shoot biomass ( $R^2 = 0.32$ ,  $P \leq 0.05$ ) and root biomass ( $R^2 = 0.34$ ,  $P \leq 0.05$ ), while neither Zn concentration nor Mn concentration was correlated with biomass. There was no significant correlation between shoot concentration and content per seed for Zn and Fe (Table 4), however, a significant correlation was found for seed Mn content and shoot Mn concentration ( $R^2 = 0.25$ ,  $P \leq 0.05$ ).

### Zn tolerance

Although *B. rapa* is not known to be particularly tolerant to excess Zn exposure, we assessed the initial set of 158 accessions for their tolerance to 800  $\mu\text{M}$   $\text{ZnSO}_4$  exposure for 14 days (Experiment 1). Tolerance was determined using TSPs, and tolerant, average and sensitive accessions accounted for respectively 8%, 45% and 46% of the total (Fig. 3). Exposure to 800  $\mu\text{M}$   $\text{ZnSO}_4$  is rarely encountered by plants in the field. To determine if comparable results could be obtained when exposing plants to less extreme Zn concentrations, two tolerant accessions (L56w and L86w), three average accessions (L58w, L64w and L203w) and one sensitive accession (L66w) were used in experiment 2. In this experiment, plants were exposed to 2  $\mu\text{M}$   $\text{ZnSO}_4$  as normal Zn supply and 100 and 300  $\mu\text{M}$   $\text{ZnSO}_4$  as excess Zn supply. Zn tolerance was determined in terms of dry biomass production rather than with TSPs

**Fig. 2** Correlations between shoot Zn, Fe and Mn concentrations of 21 selected accessions of 21 selected accessions grown in half-strength Hoagland's nutrient solution with 2  $\mu\text{M}$   $\text{ZnSO}_4$  for 14 days. **(a)** Correlation between shoot Zn and Fe concentrations. **(b)** Correlation between shoot Zn and Mn concentrations. **(c)** Correlation between shoot Fe and Mn concentrations. Correlation between shoot Zn and Fe concentrations was significant at  $P \leq 0.005$  ( $R^2 = 0.47$ ) when excluding the low Fe outlier L144. \*, \*\* and \*\*\* are statistically significant at  $P \leq 0.05$ ,  $P \leq 0.005$  and  $P \leq 0.001$  levels respectively. Significance was determined by simple linear regression;  $R^2$  = squared linear regression coefficient



(Table 5). As expected, biomass was reduced when plants were grown at high Zn concentrations and the growth inhibition increased along with the increase in Zn concentration (Table 5). ZT based on shoot dry biomass varied almost 2-fold among accessions at both 100  $\mu\text{M}$  and

300  $\mu\text{M}$   $\text{ZnSO}_4$ . A comparable range was found for ZT based on root dry biomass. In line with the initial selection based on TSP values, the two tolerant accessions L56w and L86w maintained a high relative shoot growth at 300  $\mu\text{M}$  Zn (94%), whereas the sensitive accession L66w showed

**Table 3** Comparison of Zn, Fe and Mn concentrations ( $\mu\text{g g}^{-1}$  d. wt.) in shoots of 21 selected *B. rapa* accessions (Acc.) for experiments 1 and 2

Cultivar group	Acc	Zn		Fe		Mn	
		Exp 1	Exp 2	Exp 1	Exp 2	Exp 1	Exp 2
Chinese Cabbage	L107w	104	127 ± 1 ab	244	77 ± 6 a	40	84 ± 10 ae
	L113w	160	107 ± 7 ae	127	68 ± 6 a	38	86 ± 5 ad
	L64w	57	80 ± 6 ae	90	62 ± 3 a	29	75 ± 2 ce
	L127w	120	79 ± 16 ae	203	57 ± 1 a	28	59 ± 6 ce
	L140w	156	72 ± 3 be	87	59 ± 4 a	40	64 ± 5 ce
	L23w	48	52 ± 3 de	134	45 ± 3 a	37	47 ± 0 de
	L146	n.d.	46 ± 3 e	n.d.	64 ± 13 a	n.d.	46 ± 7 de
Pak Choi	L196w	156	135 ± 12 a	151	70 ± 5 a	47	113 ± 2 a
	L86w	64	123 ± 10 ac	168	67 ± 2 a	50	106 ± 4 ab
	L66w	48	123 ± 4 ac	160	68 ± 5 a	38	82 ± 3 ae
	L175	n.d.	115 ± 21 ac	n.d.	57 ± 4 a	n.d.	72 ± 2 be
	L67w	115	110 ± 13 ad	157	62 ± 7 a	34	73 ± 7 be
	L78w	23	90 ± 1 ae	98	71 ± 0 a	23	84 ± 7 ae
Mizuna	L203w	42	68 ± 1 ce	160	58 ± 4 a	39	61 ± 2 ce
	L19	n.d.	44 ± 6 e	n.d.	50 ± 6 a	n.d.	45 ± 4 e
Wutacai	L56w	41	88 ± 2 ae	110	66 ± 3 a	38	88 ± 3 ac
Caixin	L58w	71	73 ± 4 be	117	64 ± 2 a	34	83 ± 7 ae
Zi Caitai	L62w	76	84 ± 5 ae	90	60 ± 6 a	37	77 ± 5 ae
Yellow Sarson	L143	n.d.	77 ± 7 ae	n.d.	54 ± 5 a	n.d.	89 ± 10 ac
Rapid cycling	L144	n.d.	117 ± 6 ac	n.d.	40 ± 2 a	n.d.	64 ± 10 ce
Turnip	L115	n.d.	47 ± 6 e	n.d.	58 ± 5 a	n.d.	51 ± 3 ce
Correl		0.10		0.06		0.24	
Mean		85	89	102	61	37	74

The accessions are arranged according to cultivar groups and in declining order of Zn concentration in experiment 2. Data in experiment 1 are values of mixed samples of 2–3 individual plants; data in experiment 2 are presented as means ± SE,  $n = 3$ . All plants were grown in half-strength Hoagland's nutrient solution containing  $2 \mu\text{M ZnSO}_4$ . Different letters indicate significant differences at  $P \leq 0.001$ . Significance was determined by ANOVA followed by Student Neuman-Keuls posthoc analysis. The squared correlation coefficients (Correl) were determined between data in the two experiments for each mineral by linear regression

only 50% relative shoot growth at  $300 \mu\text{M Zn}$  (Table 5). The range of relative growth was different between roots and shoots (Table 5). The  $300 \mu\text{M Zn}$  treatment induced a drastic decrease in root biomass by 65–82%, while this induced only a moderate reduction in shoot biomass of between 6 and 50%.

When determining ZT in terms of root elongation, it became clear that the effect on root biomass was not reflected by an effect on root length (Table 6), as the relative growth calculated for the increase of maximum root length is not consistent with the previously determined ZT based on root dry biomass. Root elongation of accessions L56w and L86w, that showed the highest relative root dry biomass with respectively 35% and 26% of the control, was strongly inhibited at  $300 \mu\text{M Zn}$  to respectively 11% and 9% of the control. Root

elongation of all six accessions was inhibited at  $100 \mu\text{M Zn}$  and this inhibition was enhanced at  $300 \mu\text{M Zn}$ . The difference in root elongation between accessions decreased with increased Zn concentration. When plants were grown at  $300 \mu\text{M Zn}$  there was no significant difference in the mean root elongation among the six accessions.

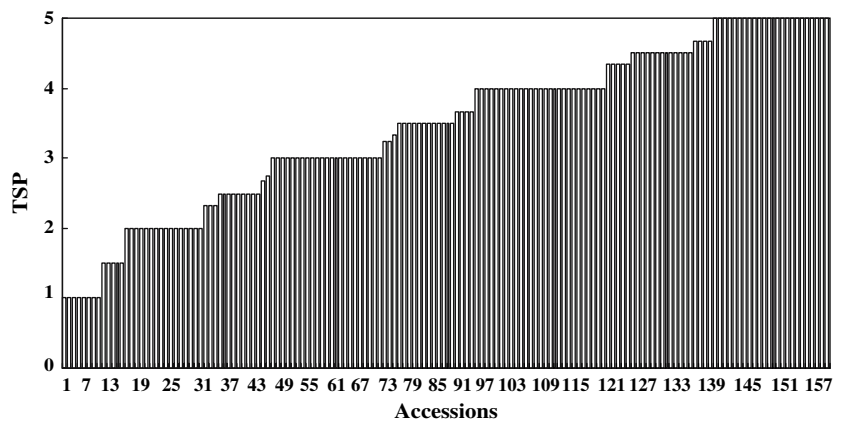
#### Zn efficiency

In experiment 2, the six accessions (L56w, L58w, L64w, L66w, L86w, L203w) used in the ZT experiment were also examined for their Zn efficiency (ZE), i.e. the ability to grow under low Zn supply. Plants began to show typical symptoms of Zn deficiency, such as interveinal chlorosis, a purple stem and reduced growth, already after growing for 1 week in Zn deficient medium. After 2 weeks, the



**Table 4** Seed weight (mg per 100 seeds), seed mineral content (ng seed<sup>-1</sup>) and dry biomass (mg plant<sup>-1</sup>) after 28 days of growth under sufficient Zn supply of the 21 *B. rapa* accessions used in experiment 2

Cultivar group	Acc.	Seed weight	Zn	Fe	Mn	Dry shoot biomass	Dry root biomass
Chinese Cabbage	L107w	428	199	289	153	880 ± 65	55 ± 6
	L113w	154	107	145	67	380 ± 68	32 ± 4
	L64w	267	238	309	91	517 ± 42	46 ± 6
	L127w	248	139	153	87	645 ± 47	36 ± 2
	L140w	233	124	214	87	345 ± 44	23 ± 4
	L23w	218	179	227	74	175 ± 54	9 ± 4
Pak Choi	L146	246	245	237	112	754 ± 101	48 ± 4
	L196w	266	176	278	124	474 ± 98	34 ± 7
	L86w	318	222	314	140	422 ± 29	36 ± 4
	L66w	306	189	246	138	601 ± 10	43 ± 1
	L175	199	134	209	83	321 ± 41	22 ± 4
	L67w	179	143	229	102	361 ± 14	21 ± 5
Mizuna	L78w	287	242	391	173	449 ± 38	34 ± 6
	L203w	297	221	332	117	413 ± 117	22 ± 6
Wutacai	L19	123	73	222	74	606 ± 97	40 ± 9
	L56w	203	137	202	96	182 ± 21	11 ± 1
Caixin	L58w	189	85	201	80	465 ± 56	35 ± 6
Zi Caitai	L62w	202	128	173	92	356 ± 70	29 ± 1
Yellow Sarson	L143	448	261	469	187	342 ± 41	40 ± 6
Rapid Cycling	L144	124	94	88	101	57 ± 17	5 ± 1
Turnip	L115	183	141	206	74	434 ± 3	28 ± 2

**Fig. 3** Excess Zn stress response of 158 *B. rapa* accessions grown in half-strength Hoagland's nutrient solution with 800 μM Zn for 14 days. TSP (Toxic Symptom Parameter): 1 = slight chlorosis, still growing; 2 = chlorosis; 3 = severe chlorosis, leaves started withering; 4 = most of the leaves seared; 5 = dead. Values are means of two or three plants per accession

difference in phenotype compared to untreated plants was easily distinguished by eye with the exception of accessions L64w and particularly L56w, which appeared comparatively healthy (Fig. 4).

Both root and shoot dry matter production was reduced in all accessions due to Zn deficiency (Table 7), but this reduction was much less in L64w for both shoot (69.8%) and root (98.0%). L64w is therefore considered to be the most zinc efficient accession of the six accessions tested. ZE varied more for shoot biomass (2.6-fold) than for root biomass (1.8-fold). In addition to ZE we also

examined the relative root:shoot biomass ratio (RSR) for the six accessions. Zn deficiency enhanced the RSR in most accessions and thus has a stronger effect on shoot biomass production than on root biomass production. However, since the most Zn efficient accession L64w has a very similar RSR as the least Zn efficient accession L86w, there does not seem to be a very strong correlation between ZE and RSR. When comparing ZE based on shoot biomass, root biomass or RSR with seed weight and mineral content per seed (Table 4), no significant correlation was found (data not shown).

**Table 5** Average dry shoot and root biomass (mg plant<sup>-1</sup>) and Zn tolerance (ZT) of shoots and roots of six accessions (Acc.) grown for 14 days in half-strength Hoagland's nutri-ent solution containing 2  $\mu$ M, 100  $\mu$ M or 300  $\mu$ M ZnSO<sub>4</sub>. ZT (in%) is calculated as relative biomass compared to 2  $\mu$ M ZnSO<sub>4</sub>

Acc.	2 $\mu$ M		100 $\mu$ M			300 $\mu$ M				
	Shoot	Root	Shoot	ZT	Root	ZT	Shoot	ZT	Root	ZT
L56w	247 $\pm$ 49	20 $\pm$ 5	257 $\pm$ 28	104	15 $\pm$ 3	74	231 $\pm$ 20	94	7 $\pm$ 1	35
L86w	441 $\pm$ 58	71 $\pm$ 10	354 $\pm$ 40	80	46 $\pm$ 5	65	417 $\pm$ 6	94	18 $\pm$ 1	26
L58w	476 $\pm$ 62	62 $\pm$ 12	341 $\pm$ 29	72	37 $\pm$ 5	59	312 $\pm$ 32	66	13 $\pm$ 2	21
L64w	688 $\pm$ 77	73 $\pm$ 6	491 $\pm$ 67	71	48 $\pm$ 7	66	429 $\pm$ 61	62	14 $\pm$ 3	19
L203w	660 $\pm$ 44	109 $\pm$ 11	440 $\pm$ 95	67	37 $\pm$ 11	34	391 $\pm$ 50	59	20 $\pm$ 2	18
L66w	516 $\pm$ 38	58 $\pm$ 6	274 $\pm$ 34	53	24 $\pm$ 3	41	256 $\pm$ 44	50	11 $\pm$ 2	19

Data are presented as means  $\pm$  SE,  $n = 9$ **Table 6** Comparison of root elongation (RE; in mm) and relative root growth (RG; in%) of six accessions (Acc.) grown in half-strength Hoagland's nutrient solution with 100  $\mu$ M or 300  $\mu$ M Zn supply for 14 days

Acc.	2 $\mu$ M	100 $\mu$ M		300 $\mu$ M	
	RE	RE	RG	RE	RG
L56w	17 $\pm$ 3 c	13 $\pm$ 3 b	77	2 $\pm$ 1 a	11
L86w	33 $\pm$ 6 ab	17 $\pm$ 3 b	52	3 $\pm$ 3 a	9
L58w	16 $\pm$ 3 c	13 $\pm$ 4 b	82	5 $\pm$ 2 a	31
L64w	49 $\pm$ 8 a	35 $\pm$ 4 a	72	4 $\pm$ 1 a	8
L203w	45 $\pm$ 9 a	19 $\pm$ 5 b	42	11 $\pm$ 4 a	24
L66w	29 $\pm$ 6 ab	12 $\pm$ 5 b	41	7 $\pm$ 4 a	24

RG is expressed as percentage of the growth at 2  $\mu$ M Zn. Data are presented as means  $\pm$  SE,  $n = 9$ . Values followed by different letters are significantly different at  $P \leq 0.05$ . Significance is determined by ANOVA followed by Student Neuman-Keuls posthoc analysis

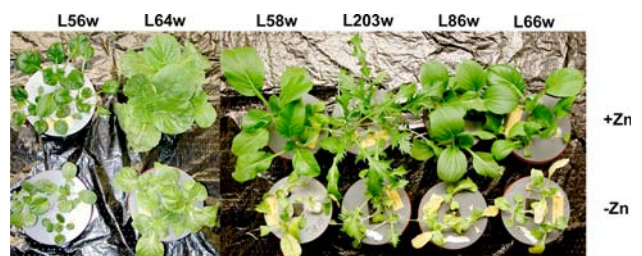
Shoot Zn concentration did not differ among the six accessions after plants were grown under Zn deficient condition for 15 days with a reduction of 76–84% (Table 8). There was no significant correlation between ZE and the shoot Zn concentration at 0  $\mu$ M or 2  $\mu$ M Zn (data not shown). In general, both shoot Fe and Mn concentrations were increased under Zn deficient condition when compared to Zn sufficient condition (Table 8). Four out of six accessions showed

a significant increase in shoot Mn concentration, while two of these accessions also increased significantly in Fe concentration. There was no correlation between ZE and shoot Mn or Fe concentration of plants grown at 0 or 2  $\mu$ M Zn (data not shown).

## Discussion

In total 117 *B. rapa* accessions were screened for Zn, Mn and Fe accumulation characteristics as a general survey for genotypic variation among *B. rapa* vegetables. This survey showed that there is considerable genotypic variation for shoot Zn, Mn and Fe concentration in *B. rapa*. This variation is not limited to one or a few cultivar groups and there is also no clear correlation between mineral concentration and cultivar group. When the selected accessions were re-examined at a second location, different results were obtained for some of these accessions and in general the correlation between locations was lower than expected. This does not reflect errors in sampling or measuring mineral concentrations, but largely illustrates the difficulty associated with studying a trait that is easily affected by genotype  $\times$  environment

**Fig. 4** Visible phenotypic response of *B. rapa* accessions to Zn deficiency. Plants were grown in half-strength Hoagland's nutrient solution for 15 days with (2  $\mu$ M Zn; +Zn) or without ZnSO<sub>4</sub> supply (0  $\mu$ M Zn; -Zn)



**Table 7** Average dry biomass (in mg plant<sup>-1</sup>), Zn efficiency (ZE; in%) of roots and shoots and the relative root:shoot biomass ratio (RSR) of six accessions (Acc.)

Acc.	Root			Shoot			Root:Shoot		
	0 $\mu$ M	2 $\mu$ M	ZE	0 $\mu$ M	2 $\mu$ M	ZE	0 $\mu$ M	2 $\mu$ M	RSR
L64w	44 $\pm$ 1	45 $\pm$ 6	98.0	379 $\pm$ 26	543 $\pm$ 32	69.8	0.113	0.083	1.4
L66w	15 $\pm$ 2	25 $\pm$ 4	60.0	140 $\pm$ 12	290 $\pm$ 31	48.5	0.107	0.086	1.2
L56w	19 $\pm$ 1	29 $\pm$ 3	66.0	160 $\pm$ 11	352 $\pm$ 11	45.4	0.094	0.082	1.2
L203w	20 $\pm$ 1	52 $\pm$ 4	38.7	276 $\pm$ 30	647 $\pm$ 49	42.9	0.072	0.080	0.9
L58w	23 $\pm$ 4	53 $\pm$ 6	43.4	175 $\pm$ 40	434 $\pm$ 42	42.7	0.131	0.122	1.1
L86w	24 $\pm$ 3	48 $\pm$ 2	50.6	197 $\pm$ 23	529 $\pm$ 6	37.2	0.122	0.091	1.3

ZE is determined as the biomass at 0  $\mu$ M Zn compared to 2  $\mu$ M Zn (in%). RSR is determined as the root:shoot ratio (Root:Shoot) at 0  $\mu$ M Zn compared to 2  $\mu$ M Zn (in%). Data are presented as means  $\pm$  SE,  $n = 3$

**Table 8** Shoot Zn, Fe and Mn concentrations ( $\mu$ g g<sup>-1</sup> d. wt.) of plants grown for 15 days in half-strength Hoagland's nutrient solution without (0  $\mu$ M) or with (2  $\mu$ M) Zn supply

Acc	Zn		Fe		Mn	
	0 $\mu$ M	2 $\mu$ M	0 $\mu$ M	2 $\mu$ M	0 $\mu$ M	2 $\mu$ M
L64w	6 $\pm$ 0 *	33 $\pm$ 1	90 $\pm$ 17	45 $\pm$ 2	51 $\pm$ 2	53 $\pm$ 2
L66w	8 $\pm$ 1 *	41 $\pm$ 2	297 $\pm$ 135	43 $\pm$ 1	164 $\pm$ 30*	43 $\pm$ 1
L56w	7 $\pm$ 1 *	43 $\pm$ 3	131 $\pm$ 29 *	45 $\pm$ 2	128 $\pm$ 18*	65 $\pm$ 1
L203w	6 $\pm$ 1 *	27 $\pm$ 1	59 $\pm$ 8	45 $\pm$ 2	65 $\pm$ 5 *	46 $\pm$ 3
L58w	7 $\pm$ 1 *	36 $\pm$ 2	99 $\pm$ 54	52 $\pm$ 1	88 $\pm$ 16	66 $\pm$ 1
L86w	6 $\pm$ 0 *	32 $\pm$ 1	84 $\pm$ 6*	50 $\pm$ 4	123 $\pm$ 9 *	50 $\pm$ 4

Data are presented as means  $\pm$  SE,  $n = 3$

\* significantly different from 2  $\mu$ M Zn supply ( $P \leq 0.05$ ). Significance is determined by one-way ANOVA

interactions, which is often the case for mineral accumulation. Genetic variation within accessions is another factor that may have caused differences in mineral concentration. Although the accessions had been propagated for some generations in the resource collection they originated from, the occurrence of self-incompatibility, which is common in *B. rapa*, is expected to maintain some genetic variation within each accession.

Previous studies revealed variation ranges of 10–60  $\mu$ g g<sup>-1</sup> for Zn and 10–90  $\mu$ g g<sup>-1</sup> for Fe in seeds (Beebe et al. 2000; Graham et al. 1999; Graham et al. 1997; Banziger and Long 2000). Kopsell et al. (2004) reported that leaf Zn concentration based on fresh weight ranged from 29.1 to 71.9 mg g<sup>-1</sup> and Fe concentration ranged from 53.1 to 114.2 mg g<sup>-1</sup> in *B. oleracea* vegetables. In the present study a wider variation range (7-fold) and higher highest concentration were found for Zn and Fe in *B. rapa* shoots. Of course

grown for 15 days in half-strength Hoagland's nutrient solution without (0  $\mu$ M) or with (2  $\mu$ M) Zn supply

this may reflect a physiological difference in the accumulation process between shoot and seed. Shoot accumulation largely depends on xylem transport, whereas seed accumulation requires additional phloem transport. Also different from screening plants in hydroponic culture, as was done in our study, the investigations on above staple crops were all carried out in soil, which might cause lower mineral availability. Seed weight and mineral content influence plant growth at the early vegetative stage (Rengel and Graham 1995a) and therefore affect shoot mineral accumulation. In the present study the correlation of Mn concentration in shoot and Mn content in seed supports the previous conclusion, however, there was no correlation between shoot concentration and seed content for Zn and Fe. Thus, the variation for Zn and Fe concentration as observed in this study suggests that there is sufficient genetic variation to dissect the genetic mechanism controlling shoot Zn and Fe accumulation by quantitative trait locus (QTL) analysis and/or to improve Zn and Fe content in *B. rapa* vegetables by breeding.

In addition to growth under sufficient Zn supply we also studied the response to Zn excess and deficiency in *B. rapa*. Both relative shoot and root growth have been suggested as good indices of tolerance to excess Zn in different species (Bert et al. 2000; Escarre et al. 2000; Meerts and Van Isacker 1997; Schat and Ten Bookum 1992; Yang et al. 2004). The comparable range in variation of ZT we observed, which was based on dry shoot and root biomass, suggests that the same holds for *B. rapa*. However, ZT determined by maximum root

length did not correlate with the ZT determined by dry biomass, although both root elongation and biomass increase were inhibited in a concentration-dependent manner when exposed to toxic Zn concentrations. Our results thus support the suggestion by Ebbs and Kochian (1997) that the toxic effect of excess Zn on the root development in *Brassica* ssp. has more effect on lateral root elongation than on lateral root density.

To examine the response to deficient Zn exposure, relative shoot growth was reported as a suitable index to determine Zn efficiency (Cakmak et al. 1999; Grewal et al. 1997; Hacisalihoglu et al. 2004; Rengel and Römheld, 2000), although also the relative root:shoot biomass ratio (RSR) has been suggested to be adequate (Rengel and Graham, 1995b). In the present study, both the relative shoot and relative root biomass index were effective in distinguishing differences in Zn efficiency. Accession L64w clearly stood out as the least affected by low Zn supply when compared to the other accessions. The RSR generally increases under Zn deficiency as an initial response to Zn deficiency (Grewal et al. 1997; Khan et al. 1998; Loneragan et al. 1987). Higher RSRs correlating with Zn efficiency have also been reported for *B. napus* and *B. juncea* (Grewal et al. 1997). A comparable result was obtained in the present study, with the exception that, based on biomass production, the relatively Zn inefficient accession L86w had a similarly high RSR as the Zn efficient accession L64w. Considering this, the RSR does not seem to be the optimal Zn efficiency index for *B. rapa*.

While variation for both ZT and Zn efficiency was observed among the six accessions, there was no significant correlation between these traits. The absence of correlation between Zn efficiency and seed weight, seed mineral content or shoot mineral concentration also indicates that Zn efficiency is genetically independent from these traits. When grown under Zn deficiency, the limited Zn supply resulted in an almost uniform shoot Zn concentration for all accessions that was below  $10 \mu\text{g g}^{-1}$  d. wt. Since a leaf Zn concentration below  $10\text{--}15 \mu\text{g g}^{-1}$  d. wt. is considered to be the critical Zn deficiency level for normal plant growth (Marschner 1995), this explains the negative effect of the Zn deficiency treatment on growth of the *B. rapa* accessions.

In addition to an effect on Zn concentration, we found that when plants were grown under Zn deficient conditions, both Fe and especially Mn concentration in shoots increased, comparable to what has been found in wheat (Rengel and Graham 1996) or *Arabidopsis* (van de Mortel et al. 2006). Most of the known metal transporters belong to large gene families covering a broad range of metal specificities. Several of the Zn transporters can also transport Fe or Mn (Connolly et al. 2002; Mills et al. 2003; Vert et al. 2001). Decreased shoot Fe and Mn concentration was found for *Brassica* plants grown in high-level Zn conditions (Ebbs and Kochian 1997). This is also in line with the correlations we observed between shoot Zn, Fe and Mn concentrations when plants were grown under sufficient Zn supply (Fig. 2). In both experiments (sufficient and deficient Zn supply), the strongest correlation was found between Zn and Mn, suggesting that Zn and Mn accumulation share more common elements than Fe and Mn or Fe and Zn accumulation.

Another observation was that the difference in Zn efficiency based on biomass was not fully reflected in the visual appearance of the accessions. Accessions L64w and L56w seemed to suffer little from deficient Zn supply in terms of plant size and degree of leaf senescence or yellowing of the leaves (Fig. 4). However, when scored for biomass production, L56w did not perform better than the other accessions with a comparable ZE value. A similar difference in visible appearance and biomass production was previously found for wheat (Genc and McDonald 2004). This suggests that the plant response to low zinc can act at different levels and the effect on biomass production is not always easily visible by eye. It also shows that visual selection of Zn efficient plant genotypes by breeders may be misleading with respect to yield.

Based on the screening of a large set of *B. rapa* accessions, we conclude there is substantial genotypic variation for Zn, Fe and Mn accumulation and for tolerance to excessive or deficiency inducing levels of Zn. Relative shoot and root growth calculated on dry biomass yield are suitable indices both for the evaluation of excess ZT and for Zn efficiency. There is a close relationship

between Zn, Mn and Fe accumulation. Our results underline that breeding for improved Zn content, whether or not in combination with enhanced fertilization with Zn, is likely to substantially increase the Zn content of *B. rapa* vegetables and thus offer a desirable Zn supplementation to a vegetarian human diet.

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