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Mapping of QTLs affecting copper tolerance and the Cu, Fe, Mn and Zn contents in the shoots of wheat seedlings

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Abstract

Quantitative trait loci (QTLs) for Cu-tolerance were determined in wheat grown in control and Cu-treated soil in greenhouse. In addition, loci having an influence on the shoot Cu-, Fe-, Mn- and Zn-contents under non-stressed and Cu-stressed environments were mapped. One major QTL for Cu-tolerance was found on chromosome 5DL, while slighter effects were determined on the chromosomes 1AL, 2DS, 4AL, 5BL and 7DS. QTLs affecting the shoot Mn- and Zn-contents were found on the chromosomes 3BL and 3AL, respectively. The centromeric region on the chromosome 3B plays a role in the regulation of the shoot Fe-contents in the stressed plants. Under Cu-stress QTL affecting shoot Cu-content was found on chromosome 1BL, while on the chromosome 5AL a QTL influencing the Cu-accumulation ability of wheat from Cu-polluted soil was determined.

Additional key words: micronutrients, phytoremediation, shoot heavy metal content, *Triticum aestivum* L.

Introduction

Copper (Cu) is an essential plant micronutrient, which can be phytotoxic if present in soils in high concentrations (Lanaras *et al.* 1993). The toxic effect of the excess copper is often studied (recently, *e.g.*, Ouzounidou and Ilias 2005, Agrawal and Sarma 2006), however, there is a little information about the genetic background of the copper tolerance in the economically important plants. The cereals are sensitive to the high soil copper concentration: the phytotoxic effect could result in reduced fertility and in yield loss of field-cultivated wheat (Moustakas *et al.* 1997). Selection of more resistant genotypes could provide gene sources for breeding Cu-tolerant wheat, and tolerant cultivars, which can accumulate high concentration of Cu in their shoots, could be directly used for the extraction of the metals from the polluted soils. Based on the studies of wheat substitution series 14 of the 21 wheat chromosomes were signed as having an effect on Cu-tolerance (Manyowa and Miller 1991, Bálint *et al.* 2003, Ganeva *et al.* 2003), suggesting the complex and polygenic nature of this character in wheat. The reported numerous chromosomes affecting the Cu-tolerance make it difficult to determine the major or common QTLs, however, using a mapping population originated from a wide cross could be helpful to find them, because in these populations a wider range of allelic differences exist compared to populations originated from crosses between common wheat cultivars.

Beside the toxicity of heavy metals, the micronutrient malnutrition is also a serious problem worldwide (Underwood and Smitasiri 1999). Common wheat often contains very low amounts of essential Fe, Zn, Cu and Mn (Salunkhe and Desphande 1991). When there are higher Cu-, Fe-, Mn- and Zn-contents in the shoot tissues, also higher mineral contents in the grains can be expected (Welch 1986). Therefore, mapping of QTLs determining higher shoot micronutrient contents could be used for the marker assisted breeding of wheat cultivars with a better nutritional value. The aim of our study was to determine QTLs for Cu-tolerance and for the shoot Cu-, Fe-, Mn- and Zn-contents under control and Cu-stressed conditions.

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Abbreviations: ITMI - International *Triticeae* Mapping Initiative; LOD - logarithmic odds; LR - likelihood ratio; MT - metallothionein; QTL - quantitative trait locus; RIL - recombinant inbred line; SMA - single marker *ANOVA*.

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Materials and methods

Plants: Wheat (*Triticum aestivum* L.) recombinant inbred lines (RILs) from the ITMI (International *Triticeae* Mapping Initiative) mapping population developed by a single seed descent F_8 from the cross of the synthetic hexaploid wheat (W7984) with the spring wheat cultivar Opata 85 was examined. Synthetic was generated via a wide cross of the diploid *Aegilops tauschii* (DD genome) with the tetraploid wheat Altar 84 (AABB genome). The population contains 114 RILs, which were mapped with 332 RFLP markers, on average 15.8 marker for each chromosome (Nelson *et al.* 1995a,b,c, Marino *et al.* 1996).

Screening for copper tolerance: A subset of 53 RILs was randomly selected and screened together with the two parents for copper tolerance. The plants were tested in a soil pot experiment in greenhouse, using control [contents of Cu, Fe, Mn, and Zn were 1.8, 2.1, 16.3, and 5.3 mg kg^{-1}(air-dry soil mass), respectively] and Cu-enriched soil with 1000 mg(Cu) kg^{-1}(dry soil mass). $CuSO_4 \cdot 5\,H_2O$ was pulverized and then mixed uniformly to the soil. The plants were tested in 3 independent replications, in each replication 4 - 6 plants were analysed. Two weeks after germination the shoot dry masses (d.m.) were determined and the ratios of shoot dry masses under Cu-stress and non-stress conditions were used as a tolerance index (Macnair 1993).

Mineral content determination: The two parents and 42 lines from the 53 RILs having the lowest variability for shoot dry mass between replications were used for the investigations. The determination of Cu-, Fe-, Mn- and Zn-contents was carried out on the whole dried plant shoots using atomic absorption spectrophotometer (*Spectr AA 10 Plus*, *Varian*, Mulgrave, Australia). The mean values were calculated from data of the 3 replications; in each replication a homogenized mixture of 4 - 6 plants was measured. The ratio of the shoot Cu-contents of Cu-treated and non-treated plants was used to characterize the Cu-accumulation ability of the plants.

Statistical analysis: The significance of differences between mean values was determined by one-way analysis of variance (*ANOVA*). Correlations between investigated parameters were established by calculating simple pair-wise correlation coefficients. Distribution of the phenotypical trait data were tested using *Statistica 6.0* software (*StatSoft Inc.* 2001) with the χ^2-test.

QTL mapping: QTL analysis was initially performed using the software *QTL Cafe* (http://www.biosciences.bham.ac.uk/labs/kearsey/applet.html) with the mean value of the phenotypic data using single marker *ANOVA* method. The QTLs determined from the mean phenotypic values were accepted as significant, when from the 3 replications at least two were found to be significant and in nearly the same position. When putative QTLs were found, these locations were further confirmed using simple interval mapping with regression method (Haley and Knott 1992), and for significance a threshold 9.2 of the likelihood ratio (LR) test was accepted, which correspond to a LOD value of 2.0 (Haley and Knott 1992). The locations of the QTLs were further confirmed applying the software *MapQTL 5* (Van Ooijen 2003) using the simple interval mapping with maximum likelihood method (Lander and Botstein 1989). For Cu-tolerance, in addition, the single marker *ANOVA* method was applied, using the significance threshold at $P \le 0.05$.

Results

Phenotypic analysis: The shoot dry masses of the Cu-treated RILs were significantly reduced compared to the control lines for both the RILs and parents. Significant differences were also detected between the shoot dry masses of the RILs grown in the two different environments (Table 1). Both parents have nearly the same tolerance against Cu, however, for the RILs we could find lines where the tolerance was significantly higher and lower compared to the parents (Table 1). The continuous distribution of the trait indicated that Cu-tolerance should be polygenic in nature and quantitatively inherited. As the consequence of the Cu-treatment the shoot Cu-contents were increased in the RILs and in both of the parents. However, in the Cu-treated lines the contents of the other investigated metals (Fe, Mn and Zn) were decreased to various extent (Table 1). The mean Cu-content of the RILs was 1.75 times higher as in the control plants. The total plant shoot Cu-contents (Cu-concentration × shoot d.m.) were higher after Cu-treatment compared to the shoots of the control plants. The mean, maximum and minimum Cu-contents in the control plants were 0.60, 0.86 and 0.45 µg $plant^{-1}$, but in the Cu-treated plants 0.89, 1.31 and 0.59 µg $plant^{-1}$, respectively. On average the Cu-content in the shoots of the Cu-treated plants is 1.48 times higher as in the shoots of non-stressed plants. Significant negative correlation was found between Cu-tolerance and the Cu-accumulation ability of the plants ($r = -0.31$, $P \le 0.05$). Interestingly, the shoot Cu-contents of the control plants correlated positively with the contents of the other metals in control environment (Cu - Fe: $r = 0.67$, $P \le 0.001$, Cu-Mn: $r = 0.53$, $P \le 0.001$, Cu - Zn: $r = 0.35$, $P \le 0.05$).

Interval mapping analysis: One QTL determining shoot d.m. of the control plants was found on the chromosome 5BL (Table 2, Fig. 1). Analyzing the dry masses of the

Table 1. Normality of the distribution of the phenotypic traits, phenotypic means, ranges of variation (minimum/maximum) and differences among the phenotypic data of the recombinant inbred lines (RILs) and the two parental genotypes. *, ** and *** - significant at $P \leq 0.05$, 0.01 and 0.001, respectively. ns - not significant. [1] Comparison of the distribution of the phenotypic data of the RILs with the normal distribution. F-values are calculated using the χ^2-test. [2] F-values are calculated using *ANOVA*-method.

Investigated traits		F-values[1]	Phenotype of the parents			Phenotype of the RILs			
			Opata 85	W7948	differences among parents[2]	number of tested RILs	mean value	range	differences among RILs[2]
Shoot d.m.	control	15.771**	0.0358	0.0433	81.331***	53	0.0438	0.0374 - 0.0536	2.478***
[g]	Cu-treated	10.918*	0.0302	0.0350	4.706ns	53	0.0374	0.0300 - 0.0451	2.866***
Cu-tolerance index		2.552ns	0.844	0.809	1.365ns	53	0.860	0.6670 - 1.0170	4.652***
Cu content	control	0.784ns	15.9	12.4	14.982**	42	13.8	9.9 - 17.0	2.922***
[mg kg^{-1}(d.m.)]	Cu-treated	1.040ns	27.2	22.1	7.486*	42	24.2	16.6 - 31.7	4.074***
Fe content	control	7.251*	79.3	69.3	4.089ns	42	82.4	54.2 - 113.1	4.919***
[mg kg^{-1}(d.m.)]	Cu-treated	3.984ns	42.9	49.2	4.474ns	42	48.6	33.0 - 65.5	2.113**
Mn content	control	7.631ns	177.2	106.9	32.862***	42	160.5	88.2 - 220.0	6.509***
[mg kg^{-1}(d.m.)]	Cu-treated	3.979ns	117.6	68.0	15.330**	42	102.6	62.4 - 167.9	5.472***
Zn content	control	3.094ns	84.9	67.4	10.180**	42	81.4	52.5 - 122.9	6.783***
[mg kg^{-1}(d.m.)]	Cu-treated	7.781ns	60.3	55.5	0.850ns	42	64.1	39.4 - 113.7	8.714***
Cu-accumulation		2.303ns	1.71	1.79	0.942ns	42	1.75	1.22 - 2.23	6.776***

Table 2. Results of the QTL mapping for Cu-tolerance traits and for shoot dry mass [g] and shoot Cu, Fe, Mn and Zn contents [mg kg^{-1}(d.m.)] under non-stress and Cu-stressed conditions. [1]F-value for the interval mapping with regression method (Haley and Knott 1992) using the software QTL Cafe. [2]LR-value for the interval mapping with regression method (Haley and Knott 1992) using the software QTL Cafe. [3]LOD-value for the interval mapping with maximum likelihood method (Lander and Botstein 1989) using the software MapQTL 5 (Van Ooijen 2003). [4]Phenotypic variance explained by the individual QTL for the interval mapping using the software MapQTL 5 (Van Ooijen 2003). [5]Positive value: effect contributed by W7948; negative value: effect contributed by Opata 85. [6]SMA: F-value for single marker *ANOVA* analysis and the corresponding probability levels (*, **, *** - $P \leq 0.05$, 0.01 and 0.001) for the mean phenotypic values by the RILs.

Investigated traits		Chromosome/ nearest marker	Int [1]F-value	LR[2]	LOD[3]	% Var[4]	Additive effect[5]	SMA[6]
Shoot d.m.	control	5B/*Xabg473b*	11.736**	10.976	2.23	17.7	0.002	13.217***
	Cu-treated	1A/*Xksue11b*	12.572**	11.678	2.69	20.9	-0.002	11.093**
	Cu-treated	5D/*Xcdo57b*	18.906**	16.712	3.64	27.1	0.002	18.967***
Cu-tolerance index	Cu-treated	5D/*Xcdo412b*	14.674**	13.402	3.19	28.9	0.037	13.614***
Cu content	Cu-treated	1B/*Xksui27a*	17.851**	15.497	3.01	31.7	-2.200	14.924***
Fe content	Cu-treated	3B/*Xfba091a*	12.719**	11.596	2.57	32.0	-3.200	11.348**
Mn content	control	3B/*Xpsr903a*	12.346**	11.298	1.91	19.0	-12.500	12.927**
	Cu-treated	3B/*Xpsr903a*	13.672**	12.349	2.43	23.6	-10.700	16.375***
Zn content	control	3A/*Xmwg30*	10.632**	9.899	2.00	28.5	7.600	5.572*
	Cu-treated	7A/*Xcdo545b*	11.716**	10.789	1.91	23.2	7.400	9.464**
Cu-accumulation	Cu-treated	5A/*Xbcd1355*	11.043**	10.239	2.13	25.0	0.120	9.834**

treated plants 2 QTLs were detected on the chromosomes 1AL and 5DL, respectively. One QTL having a LR value higher than 9.2 was found for Cu-tolerance (Table 2), which located on the long arm of the chromosome 5D nearly in the same position as the QTL controlling the shoot d.m. under Cu-stress (Table 2, Fig. 1). Because the relatively low number of the investigated lines and high number of used markers the "Type I" error (false positive QTL) could be increased. In order to check the reliability of our results, we removed randomly 50 % of the markers, and performed again the interval mapping for Cu-tolerance on the chromosome 5D (using the software *MapQTL5*), and repeated it 5 times. In all cases we got a QTL for Cu-tolerance with the LOD values varying between 2.11 - 3.21, while the phenotypic variance caused by the individual QTLs varied between 30.6 and 49.3 %, showing the goodness of our results. For the shoot Cu-contents in the treated plants one QTL was detected on the chromosome 1BL (Table 2, Fig. 1). On chromosome 3B we localized 3 QTLs: one of them is for shoot Fe-contents in the treated plants, and 2 of them for shoot Mn-contents under non-stressed and Cu-treated environments. The latter 2 QTLs were in the same position. For the shoot Zn-concentration in the non-stressed plants a QTL on chromosome 3A, and in the Cu-treated plants on chromosome 7A were determined. The contributions of the individual QTLs to the phenotypic variances of the shoot metal contents were varying between 19.0 - 32.0 % (Table 2). For the Cu-accumulation ability a QTL on the chromosome 5AL close to the centromere was found (Table 2, Fig. 1).

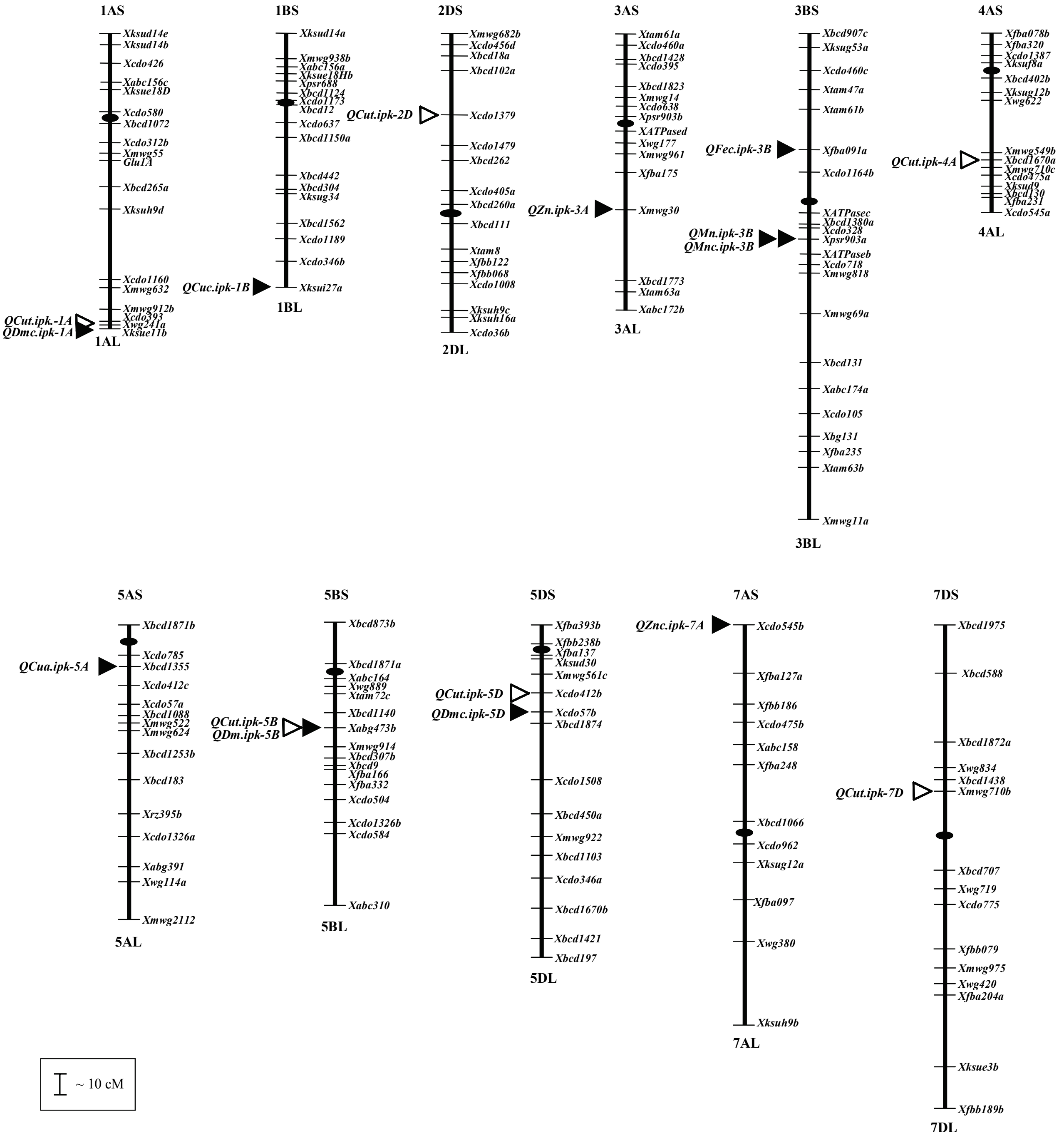

Fig. 1. Map positions of the QTLs expressed in the RILs of the cross W7984 × Opata 85. The markers are signed on the right side of the chromosomes. *On the left*, the QTLs detected by interval mapping analysis (*closed arrows*) and single marker *ANOVA* (*open arrows*) are indicated. Abbreviations for the QTLs: *QDw*: shoot dry mass in the control plants, *QDwc*: shoot dry mass in the Cu-treated plants, *QCut*: Cu-tolerance, *QCuc*: shoot Cu-contents in the Cu-treated plants, *QFec*: shoot Fe-contents in the Cu-treated plants, *QMn*: shoot Mn-contents in the control plants, *QMnc*: shoot Mn-contents in the Cu-treated plants, *QZn*: shoot Zn-contents in the control plants, *QZnc*: shoot Zn-contents in the Cu-treated plants *QCua*: shoot Cu-accumulation ability (relative shoot Cu-contents). The centromere position is indicated by *ellipses*.

Single marker *ANOVA* analysis for Cu-tolerance: Six markers were identified having an effect on Cu-tolerance when $P \leq 0.05$ was used as significance threshold (Table 3, Fig. 1). The greatest effect was found on the chromosome 5D in the same position, where the QTL for Cu-tolerance was detected using interval mapping analysis. The tolerance was contributed by W7948; however, tolerance with lower significance was also contributed by Opata 85.

Discussion

Testing chromosome substitution lines the polygenic character of the Cu-tolerance of wheat was reported earlier: significant effects were found on the chromosomes 3D, 5A, 5B, 5D, 6B and 7D (Bálint *et al.* 2003), 1A, 1D, 3A, 3B, 3D, 4A, 4D and 7A (Ganeva *et al.* 2003) and on the chromosomes 5A, 4D, 7A, 7B, 7D (Manyowa and Miller 1991). The role of the different chromosomes in the different experiments suggests not only the polygenic character, but the possibility of different expressions of the genes against distinct toxic contents of copper in different populations. In this study regions affecting Cu-tolerance were found on the chromosomes 1A, 4A, 5B, 2D, 5D and 7D (Tables 2, 3, Fig. 1). The role of chromosomes 1A, 4A (Ganeva *et al.* 2003), 5B, 5D (Bálint *et al.* 2003) and 7D (Manyowa and Miller 1991, Bálint *et al.* 2003) in the Cu-tolerance was reported earlier. Some of these influences are probably not specific for Cu-tolerance, but could play a role in the control of general adaptation to the environment, or in the control of plant development. This hypothesis is supported by the findings that in the region affected Cu-tolerance on the chromosomes 2DS and 4AL major QTLs affecting flowering time, grain mass per ear, plant height, ear emergence time, ear length, grain number and plant height were mapped earlier in the same population (Börner *et al.* 2002).

Table 3. Results of single marker *ANOVA* analysis for Cu-tolerance. *, ** and *** - significant at $P \leq 0.05$, 0.01 and 0.001, respectively.

Marker	Chromosome	F-value	Tolerance contributed by
Xcdo412b	5D	13.614***	W7948
Xwg241a	1A	9.330**	Opata 85
Xmwg710b	7D	6.866*	W7948
Xbcd1670a	4A	6.589*	Opata 85
Xabg473b	5B	4.792*	Opata 85
Xcdo1379	2D	4.033*	Opata 85

The contents of Cu, Fe, Mn and Zn in the shoots are affected by different QTLs, which suggest the strongly metal-specific uptake and/or translocation of these elements. In the control plants there was a significant positive correlation among the shoot metal contents, therefore, if a genotype had a higher shoot Cu-concentration, higher Fe-, Mn- and Zn-contents could be also expected. This phenomenon could be useful for the breeding of wheat having higher shoot metal contents. The centromeric region on the chromosome 3B plays a role in the regulation of the shoot Fe-contents in the stressed plants and Mn-contents in both the non-stressed and stressed plants. QTL determining shoot Cu-concentration was found only under Cu-stressed environment in the telomeric region of chromosome 1B, whereas a QTL affecting the Cu-accumulation ability was determined on chromosome 5AL. The role of the homoeologous group 5 chromosomes in the regulation of the shoot Cu-concentration was reported earlier: in rye chromosome 5R was described to have an effect on the shoot Cu-concentration analyzing wheat-rye addition lines (Schlegel *et al.* 1997).

Based on the negative correlation between Cu-tolerance and shoot Cu-accumulation ability, the key mechanism for enhanced Cu-tolerance by the tolerant lines could be the restriction of uptake of Cu in the roots, or the reduced translocation from root to shoot. This mechanism is comparable with the tolerance mechanism against boron toxicity in wheat reported earlier by Jefferies *et al.* (2000). The base mechanism for the exclusion could be the chelating of the free metals in the rhizosphere through chelators excreted by the plants. The citrate and malate exudations from wheat roots induced by the excess of Cu were also documented (Nian *et al.* 2002), however, their role in the tolerance is not clear yet. The role of metal binding substances, such as metallothioneins (MTs) or phytochelatines (PCs) has also remained a mystery: *e.g.* the amount of MT-like transcripts could be decreased or increased after the exposures of the metals, depending on species and metals (Hall 2002). The genes encoding the MT-proteins in wheat are positioned on the long arm of chromosomes 1A, 1B and 1D (Gale, personal communication, cit. in: Kawashima *et al.* 1991), however, we found only a small effect on the long arm of chromosome 1A. To clarify the detailed mechanism of the tolerance further investigations are needed.

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