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Changes in thiol content in roots of wheat cultivars exposed to copper stress

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Abstract

Wheat (*Triticum aestivum* L.) cultivars GK Tiszatáj, Yubileinaya, GK Öthalom and a landrace Kobomugi were grown for 18 d in hydroponic cultures containing 0 (control), 0.1, 1.0 or 10.0 μM Cu^{2+}. On a dry mass basis, cvs. Tiszatáj and Kobomugi accumulated slightly more Cu^{2+} in the root tissues than did cvs. Yubileinaya and Öthalom, but their controls also contained higher amounts of Cu^{2+}. As a result of perturbation in the plasma membrane functions the K^+ content of roots was reduced at 10 μM Cu^{2+} in all cultivars, whereas the K^+/Na^+ ratio decreased significantly only in the roots of cv. Öthalom. In the sensitive cultivar, Öthalom, the dry mass of the roots decreased while the cysteine content, which is a limiting factor for glutathione synthesis, did not satisfactorily increase with increasing tissue Cu^{2+} content. This suggests that in cv. Öthalom the membrane damage of the root cells at 10 μM Cu^{2+} concentration may affect the sulphur availability or metabolism. Concentrations of glutathione and hydroxymethyl-glutathione, a tripeptide which may play a similar biochemical role to glutathione, were also lower in the sensitive cultivar. In the absence of glutathione the root tissues failed to cope with the oxidative stress caused by the excessive amount of Cu^{2+}. A significant accumulation of iron in the roots of the sensitive cultivar at 10 μM Cu^{2+} supply enhanced the oxidative damage.

Additional key words: glutathione, γ-glutamylcysteine, hydroxymethyl-glutathione, phytochelatins, iron accumulation.

Introduction

The tolerance of plants to an excessive amount of heavy metal ions in the soil or nutrient solution may involve contributions of genetically independent physiological mechanisms. Three main types of tolerance exist in plants: the exclusion of metal ions from the cytoplasm by a limited uptake, biochemical detoxification by complex or chelate formation, and compartmentation of the heavy metal ions to the vacuole or other plant parts (Berry 1986). Cu^{2+} modifies the membrane integrity and interferes with the transport processes of plasmalemma (Demidchik *et al.* 1997). Cu^{2+} effectively inhibits the plasma membrane ATPase; this seems to be related to its effect on Mg-ATP binding to the enzyme (Serrano 1990). Copper toxicity is associated, at least in part, with the initiation of oxidative stress in the tissues (Stohs and Bagchi 1995, Weckx and Clijsters 1996).

Plants have several non-enzymatic and enzymatic mechanisms whereby they can scavenge reactive oxygen species. Glutathione (GSH), an intermediate of the Halliwell-Asada ascorbate-glutathione cycle, is one of the most effective antioxidants of plant tissues. Enhancement of the cycle contributes to the defence against oxidative stress both in photosynthetic (Asada and Badger 1984, Foyer *et al.* 1989) and in non-green tissues (Dalton *et al.* 1986, Gupta *et al.* 1999). GSH is also a precursor of a group of heavy metal-binding peptides, the phytochelatins (PCs). These compounds are synthesized by a constitutively expressed PC synthase which is activated in the presence of metal ions (Chen *et al.* 1997; Wojcik and Tukendorf 1999). The genes for GSH synthesis and

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Abbreviations: GSH - glutathione; γ-EC - γ-glutamylcysteine; hmGSH - hydroxymethyl-glutathione; PC - phytochelatin.

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metabolism (Xiang and Oliver 1998) are induced at supraoptimal Cu^{2+} concentrations.

The rate of GSH synthesis is determined by the cysteine concentration (Chen and Goldsbrough 1994) and the activity of γ-glutamylcysteine (γ-EC) synthetase, which catalyses the ATP-dependent synthesis of the GSH precursor, γ-EC (Kocsy *et al.* 1996). At a marginally limiting S supply the reduced sulphur was preferentially allocated into PCs in wheat roots exposed to 100 μM Cd^{2+} (McMahon and Anderson 1998). As a result of the intensive PC synthesis induced by Cu^{2+}, the GSH pool may temporarily decrease (Xiang and Oliver 1998). Hydroxymethyl-glutathione (hmGSH) and homoglutathione, the analogues of GSH, may also function as precursors for PC synthesis (Rauser 1990, Klapheck *et al.* 1994).

By screening for genes participating in metal tolerance, a wheat cDNA, *TaPCS1* has recently been identified which mediates a GSH-dependent Cd^{2+}-tolerance (Clemens *et al.* 1999). The *TaPCS1*-expressing yeast cells accumulated more Cd^{2+} than the controls, and inhibition of GSH synthesis resulted in hypersensitivity to Cd^{2+} and Cu^{2+}.

The Cu^{2+} tolerance of *Silene vulgaris* depended on the rate of PC synthesis after copper exposure. Non-tolerant plants accumulated more Cu^{2+}, and their PC production was much greater than that of tolerant plants, which resulted in oxidative stress after a severe reduction in the GSH content (De Vos *et al.* 1992), but there is controversy in the literature as to whether tolerant or non-tolerant species accumulate larger amounts of heavy metals (Meharg 1993).

Thus, maintenance of a high GSH content under heavy metal stress, especially in the roots is of particular interest. In this paper, we investigate the effects of Cu^{2+} stress on the concentrations of the intermediates of GSH biosynthesis in accordance with copper accumulation in the roots of different wheat cultivars. Transition metal ions, including Cu^{+} and Fe^{2+}, in the presence of H_2O_2 catalyze the formation of $OH^{\bullet}$ radicals chemically and initiate an oxidative chain reaction. Moreover, Fe^{3+} may enhance the autooxidation of ascorbate (Buettner 1988), and in this way increase the oxidative stress of tissues. We were also interested in how secondary processes, such as iron accumulation affect the copper sensitivity of wheat cultivars.

Materials and methods

Seeds of wheat (*Triticum aestivum* L.) cultivars GK Tiszatáj, GK Öthalom and Yubileinaya and a landrace Kobomugi were germinated for 24 h at 26 °C in a dark thermostat. The seedlings were then grown hydroponically (at pH 5.8) in a *Conviron* growth chamber (*Controlled Environments*, Winnipeg, Canada) for 18 d with a day/night temperatures of 22/19 °C, at 75 % relative humidity, under a 14-h photoperiod, at a photon flux density of 180 μmol m^{-2} s^{-1}. The nutrient solution contained [mM]: 0.50 $CaCl_2$; 0.50 $MgSO_4$; 0.50 Na_2HPO_4; 1.0 KH_2PO_4; 1.0 KNO_3 and 0.010 Fe(III)EDTA. The concentrations of microelements [μM]: 1.0 $MnSO_4$; 0.50 $ZnSO_4$; 0.10 $(NH_4)_6Mo_7O_{24}$, 10.0 H_3BO_4; 0.10 $AlCl_3$ and 0; 0.10; 1.0 or 10.0 $CuCl_2$.

For the measurement of metal contents, 100 mg of dry plant material was pulverized and samples were digested for 8 h at 200 °C in 5 cm^3 concentrated HNO_3 (*Merck*) supplemented with 4 cm^3 of 35 % H_2O_2 and then were analysed with a *Hitachi Z-8200* (Tokyo, Japan) atomic absorption spectrometer.

The thiol contents of the samples were determined by the method of Kranner and Grill (1996) as modified by Kocsy *et al.* (2000). 400 mg of plant material was ground in liquid N_2, and homogenized in 5 cm^3 0.1 M HCl containing 1 mM Na_2EDTA. The samples were centrifuged for 20 min at 15 000 *g*. The supernatant was neutralized with 0.2 M 2-[cyclohexylamino]-ethanesulphonic acid (pH 9.3) and disulphides in the extract were reduced with 3 mM dithiothreitol. Derivatization was performed with 15 mM monobromo-bimane and the reaction was stopped with 0.25 % methanesulphonic acid. The derivatized samples were analysed by reverse-phase HPLC (*Waters*, Milford, USA) equipped with fluorescence detector.

Statistical analysis was carried out with a *ProStat* software (*Poly Software International*, Salt Lake City, USA 1996), and the *t*-test was used to test the differences between the means of the results for the copper-free controls and the treated samples. A probability level of ≤ 0.05 was regarded as statistically significant.

Results

Copper exerts a beneficial effect on the growth of seedlings only in a relatively narrow concentration range. Higher concentrations may inhibit the elongation and dry matter accumulation of the roots. The Cu^{2+} demands of wheat cultivars are different and they also display different sensitivities to an excess of copper. Those cultivars (cv. Tiszatáj, Yubileinaya and Kobomugi) which did not exhibit a decrease in root dry mass under copper stress, accumulated a similar amount of or slightly more Cu^{2+} in the root tissues than did cv. Öthalom, which proved to be sensitive. In the 0 - 1.0 µM Cu^{2+} concentration range, the iron content of the tissues reached a very steady level in the roots, which was characteristic for each cultivar. Under copper stress the Fe absorption increased and the accumulation of Fe in the roots was exceptionally high as compared with the control in the sensitive cultivar Öthalom (Table 1).

The 10 µM Cu^{2+} supply reduced K^+ contents in all cultivars and, with the exception of cv. Öthalom, the Na^+ content of roots also decreased. The K^+/Na^+ ratio, which

Table 1. Effects of Cu^{2+} supply on the length, dry mass, Cu and Fe content of roots of different wheat cultivars at the age of 18 d. Means ± SE of three independent experiments. According to Student's *t*-test, means denoted by *; ** and *** are significantly different from the appropriate control at $P \leq 0.05$; 0.01 and 0.001 levels, respectively (ns: not significantly different).

Cultivars	Cu^{2+} [µM]	Root length [cm]	Dry mass [mg $root^{-1}$]	Cu content [µmol g^{-1} (d.m.)]	Fe content [µmol g^{-1} (d.m.)]
Tiszatáj	0	20.55 ± 0.3	9.2 ± 0.8	0.28 ± 0.07	14.16 ± 1.09
	0.1	21.26 ± 1.5 ns	9.8 ± 2.8 ns	0.17 ± 0.08 ***	12.27 ± 1.31 ns
	1.0	20.43 ± 1.1 ns	8.6 ± 2.1 ns	4.36 ± 0.46 ***	13.08 ± 2.14 ns
	10.0	8.71 ± 0.4 ***	10.3 ± 3.2 ns	16.83 ± 1.42***	24.53 ± 4.19 *
Kobomugi	0	13.98 ± 0.8	6.4 ± 0.9	0.20 ± 0.02	13.76 ± 4.88
	0.1	14.44 ± 1.4 ns	6.4 ± 1.7 ns	0.38 ± 0.05 **	13.69 ± 1.20 ns
	1.0	18.08 ± 0.5 **	9.1 ± 1.7 ns	4.81 ± 0.07 ***	14.55 ± 2.01 ns
	10.0	4.37 ± 0.3 ***	8.0 ± 1.4 ns	19.00 ± 0.48***	17.85 ± 2.51 ns
Yubileinaya	0	20.60 ± 1.1	15.1 ± 1.8	0.08 ± 0.03	7.28 ± 0.83
	0.1	22.79 ± 1.0 ns	11.0 ± 2.6 ns	0.12 ± 0.01 **	8.18 ± 2.06 ns
	1.0	19.52 ± 0.6 ns	10.5 ± 0.4 *	3.58 ± 0.64 **	9.36 ± 0.79 *
	10.0	14.70 ± 0.2***	13.6 ± 1.4 ns	13.34 ± 3.23***	17.05 ± 2.96 **
Öthalom	0	19.49 ± 0.5	21.0 ± 1.1	0.06 ± 0.02	4.98 ± 0.45
	0.1	21.39 ± 2.6 ns	17.8 ± 3.3 ns	0.08 ± 0.02 ns	8.69 ± 2.86 ns
	1.0	19.86 ± 1.1 ns	18.6 ± 4.1 ns	2.29 ± 0.19 ***	6.28 ± 1.70 ns
	10.0	12.01 ± 1.0***	16.6 ± 0.1 **	15.37 ± 1.40***	20.47 ± 2.33***

Table 2. Effects of Cu^{2+} supply on the K^+, Na^+ and Ca^{2+} contents and K^+/Na^+ ratio in the roots of different wheat cultivars at the age of 18 d. Means ± SE of three independent experiments. According to Student's *t*-test, means denoted by *; ** and *** are significantly different from the appropriate control at $P \leq 0.05$; 0.01 and 0.001 levels, respectively (ns: not significantly different).

Cultivars	Cu^{2+} [µM]	K^+ content [µmol g^{-1}(d.m.)]	Na^+ content [µmol g^{-1}(d.m.)]	K^+/Na^+	Ca^{2+} content [µmol g^{-1}(d.m.)]
Tiszatáj	0	1850.72 ± 106.9	188.20 ± 33.2	9.84	133.32 ± 13.4
	0.1	1848.16 ± 84.8 ns	144.38 ± 2.4 ns	12.80	132.19 ± 9.5 ns
	1.0	1859.24 ± 29.8 ns	157.78 ± 2.4 ns	11.78	141.55 ± 13.3 ns
	10.0	1526.78 ± 65.3 *	159.20 ± 2.2 ns	9.59	170.14 ± 20.5 ns
Kobomugi	0	1672.74 ± 102.6	356.22 ± 13.2	4.70	165.19 ± 11.7
	0.1	1634.11 ± 40.9 ns	249.95 ± 19.1 **	6.54	139.71 ± 36.1 ns
	1.0	1654.23 ± 21.8 ns	230.65 ± 22.3 **	7.17	143.21 ± 8.0 ns
	10.0	1211.34 ± 30.9 **	231.93 ± 13.8 ***	5.22	130.32 ± 20.4 ns
Yubileinaya	0	1473.50 ± 98.1	167.74 ± 18.6	8.78	95.06 ± 17.3
	0.1	1609.47 ± 32.1 ns	173.00 ± 15.4 ns	9.30	110.82 ± 10.4 ns
	1.0	1557.89 ± 56.5 ns	182.68 ± 4.0 ns	8.53	97.89 ± 10.5 ns
	10.0	1038.31 ± 137.5 *	101.47 ± 15.6 *	10.23	108.99 ± 22.6 ns
Öthalom	0	1306.84 ± 72.0	144.82 ± 1.6	9.02	86.24 ± 14.9
	0.1	1306.84 ± 72.0 ns	173.93 ± 4.3 ***	7.51	79.93 ± 16.7 ns
	1.0	1332.41 ± 17.9 ns	175.44 ± 18.7 *	7.59	94.47 ± 32.2 ns
	10.0	1132.08 ± 37.1 *	179.50 ± 2.0 ***	6.31	100.42 ± 12.7 ns

Table 3. Effects of 0 - 10 μM Cu^{2+} supply on the cysteine, γ-glutamylcysteine (γ-EC) and hydroxymethyl-glutathione (hmGSH) contents in roots of 18-d-old wheat cultivars. Means ± SE, *n* = 3. According to Student's t-test, means denoted by *; ** and *** are significantly different from the appropriate control at $P \leq 0.05$; 0.01 and 0.001 levels, respectively (ns: not significantly different).

Cultivars	Cu^{2+} [μM]	Cysteine [nmol g^{-1}(f.m.)]	γ-EC [nmol g^{-1}(f.m.)]	hmGSH [nmol g^{-1}(f.m.)]
Tiszatáj	0	0.63 ± 0.11	0.04 ± 0.01	1.93 ± 1.65
	0.1	1.30 ± 0.63 ns	1.38 ± 0.781	9.61 ± 5.37 ns
	1.0	2.34 ± 1.41 ns	1.64 ± 0.51 *	8.93 ± 8.65 ns
	10.0	9.14 ± 1.04 **	7.91 ± 1.06 **	35.68 ± 4.10 **
Kobomugi	0	0.69 ± 0.27	0.06 ± 0.01	0.12 ± 0.04
	0.1	0.75 ± 0.07 ns	0.04 ± 0.01 ns	0.15 ± 0.06 ns
	1.0	1.88 ± 0.80 ns	0.74 ± 0.13 **	7.34 ± 1.23 **
	10.0	8.47 ± 1.26 **	4.09 ± 0.71 **	27.35 ± 5.53 **
Yubileinaya	0	0.57 ± 0.03	0.05 ± 0.01	0.15 ± 0.02
	0.1	0.49 ± 0.01 ns	0.02 ± 0.01 ns	0.12 ± 0.10 ns
	1.0	1.02 ± 0.12 *	0.11 ± 0.06 ns	0.65 ± 0.40 ns
	10.0	12.30 ± 2.46 **	6.72 ± 1.34 **	27.78 ± 7.53 *
Öthalom	0	0.57 ± 0.08	0.05 ± 0.02	0.63 ± 0.10
	0.1	0.57 ± 0.06 ns	1.59 ± 0.51 *	0.20 ± 0.16 ns
	1.0	1.00 ± 0.14 ns	0.55 ± 0.23 ns	1.02 ± 0.35 ns
	10.0	2.70 ± 0.70 *	0.85 ± 0.38 ns	3.86 ± 1.65 ns

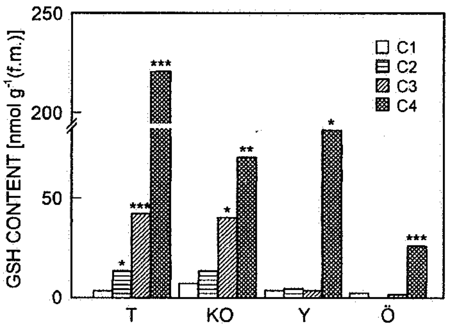

Fig. 1. Effect of Cu^{2+} supply on the glutathione (GSH) content in the roots of 18-d-old wheat plants: T - cv. Tiszatáj, KO - cv. Kobomugi, Y - cv. Yubileinaya, Ö - cv. Öthalom. Concentrations of Cu^{2+} in the nutrient solution were: C1 - control (0 μM), C2 - 0.1 μM, C3 - 1.0 μM, or C4 - 10.0 μM. According to Student's *t*-test, means denoted by *; ** and *** are significantly different from the appropriate control at $P \leq 0.05$; 0.01 and 0.001 levels, respectively.

is an indicator of the selectivity of the K^+/Na^+ uptake or of the activity of Na^+ extrusion from the root tissues, decreased considerably at 10 μM Cu^{2+} in the case of cv. Öthalom as compared to the control. Copper stress did not have a significant effect on the Ca^{2+} content of the roots in these cultivars (Table 2).

The acclimation of plants to copper stress is highly dependent on the capacity of tissues to synthesize GSH. 10 μM Cu^{2+} treatment increased the cysteine content of the roots in three cultivars, but the root tissues of cv. Öthalom contained only a small amount of cysteine. This resulted in a reduced level of γ-EC (Table 3). The concentrations of GSH (Fig. 1), and of a GSH analogue tripeptide, hmGSH, also a presumed precursor of PC synthesis (Rauser 1990), were likewise significantly lower in the roots of cv. Öthalom at 10 μM Cu^{2+} supply (Table 3).

Discussion

The acclimation of plants to toxic concentrations of heavy or transition metals is related to the ability of the roots to cope with the oxidative stress and also to produce PCs, which bind and in this way inactivate the metal ions. A fast and effective induction of PC synthesis upon heavy metal exposure is a prerequisite for the prevention of the oxidative damage to the tissues, but this may also result in a rapid decline in the pool of GSH and its precursors. In this case, the success for acclimation is highly dependent on the ability of plants to regain the GSH pool.

Inhibition of root growth is an early symptom of Cu^{2+} toxicity but the sensitivity of species/varieties to external Cu^{2+} concentrations can be different (Fargašová 2001, Jiang *et al.* 2001). The root elongation of wheat genotypes was effectively reduced at 10 μM Cu^{2+} concentration. The root tissues of cv. Tiszatáj and Kobomugi contained slightly more Cu^{2+} than did the roots of Yubileinaya and Öthalom. Tiszatáj and Kobomugi accumulated more Cu^{2+} in their shoots than did the other two cultivars (Tari *et al.* 1998). On the basis of the dry matter reduction cv. Öthalom proved to be the most sensitive of the four cultivars to copper stress.

Cu^{2+} may exert a strong inhibitory effect on the accumulation of other nutrient elements, including K^+, Na^+, Ca^{2+} or Fe^{2+}/Fe^{3+} (Siedlecka 1995). The K^+ content of the roots decreased significantly as a result of copper stress in all cultivars. The loss of plasma membrane integrity in consequence of the oxidative damage to the membrane constituents or the inhibition of plasmalemma ATPase resulted in a reduced uptake or leakage of K^+ from the cytoplasm (Meharg 1993). The K^+/Na^+ ratio was reduced as compared with the copper-free control only in cv. Öthalom. K^+ may enter the cytoplasm in wheat root cells via a high-affinity K^+ transporter (HKT1) or a potassium channel, which are dependent on the membrane potential, whereas Na^+ influx in the intact roots seems to be voltage-insensitive (Yeo 1998). The function of the membrane potential-dependent plasmalemma antiporter that exchanges cytoplasmic Na^+ for apolastic H^+ could be disturbed as a result of membrane damage under copper stress. These processes may lead to changes in K^+/Na^+ ratio in the roots tissues.

In a short-term experiment the iron content increased at supraoptimal Cu^{2+} concentrations in *Halimione portulacoides* L. plants (Reboredo 1994). Under Cu^{2+} stress, we also found significantly higher Fe contents in the roots. The increased Fe content of the tissues at 10 μM Cu^{2+} may enhance the oxidative damage caused by Cu^{2+}, because both Fe^{2+} or Fe-EDTA as a catalyst generate $OH^{\bullet}$ radicals chemically via the Fenton-Haber-Weiss reaction (Chance *et al.* 1997), which, in the absence of free radical scavenging substances, leads to the oxidative cleavage of macromolecules. In the absence of GSH, copper stress could be enhanced by Fe-induced oxidative stress in cv. Öthalom. In the other cultivars, the active oxygen species generated by similar or even higher Fe concentrations might be scavenged by reduced GSH.

In cv. Öthalom, the pool of GSH and its precursors was highly exhausted at toxic Cu^{2+} concentration because the tissues were unable to adapt to the excessive cysteine demand, which suggests that Cu^{2+} stress may affect sulphate uptake, assimilation and also the cysteine metabolism in this cultivar. The other genotypes maintained or even increased the dry matter production of the roots or of the whole plants at 10 μM Cu^{2+} (unpublished results). In these cultivars (cvs Tiszatáj, Kobomugi and Yubileinaya), 10 μM Cu^{2+} treatment increased the cysteine, γ-EC, hmGSH and GSH concentrations as compared with the controls and these plants maintained relatively high concentrations of GSH after an 18-day exposure. This enabled these genotypes to use the GSH pool for other detoxification mechanisms.

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