Among the Arabidopsis thaliana accessions Columbia (Col), Landsberg erecta (Ler), and Wassilewskija (Ws), Ler and Ws showed higher copper (Cu) tolerance than Col, while accumulating more Cu. Thus, Cu tolerance did not appear to be related to metal exclusion. Rather, the higher Cu tolerance of Ler and Ws may reflect less Cu-induced nutrient deficiency as they maintained higher sulfur, iron (Fe), and manganese levels than Col under Cu stress. Reverse transcription–polymerase chain reaction was used to compare the leaf transcript levels of nine genes involved in Cu metabolism, oxidative stress resistance or sulfate transport (Sultr 4;1). Excess Cu led to an overall decrease of the transcript levels of plastocyanin and two plastidic Cu transporters, PAA1 and PAA2. The Fe superoxide dismutase (SOD) gene FSD1 was also downregulated in the three accessions, while the cytosolic Cu/Zinc SOD (CSD1) was upregulated compared with the control conditions. These results may be related to differences in Fe and Cu accumulation. The transcript abundance of the Cu chaperones ATX1, CCS and CpCCP showed differential regulation among the three accessions in response to Cu supply. The vacuolar Sultr 4;1 was upregulated by Cu, most likely due to the lower ability to accumulate S. Total non-protein thiol levels were not correlated with Cu tolerance.

Introduction

Copper (Cu) is an essential trace element required by plants for growth and development. It plays important roles as a cofactor in several metabolic processes, including photosynthetic and mitochondrial electron transport, oxidative stress responses, and hormone perception (Himelblau and Amasini 2000). However, the intracellular Cu level must be tightly regulated, as it is toxic for most plants when present in excess. The toxicity induced by this element is visible in non-tolerant plants as reduction of plant growth, likely due to the effect of excess Cu on the accumulation of other essential elements (Tsang et al. 1996), inhibition of root elongation (Murphy and Taiz 1995a), modification of protein and lipid composition of the root plasma membrane (Quartacci et al. 2001), reduction of the thylakoid membrane structure of chloroplasts (Pätsikäi et al. 2002), and alteration of cellular transport and content of several metabolites (Wintz and Vulpe 2002). Cu-induced damage to membranes is mainly due to the formation of

Abbreviations – Col, Columbia; Cu, copper; Fe, iron; Ler, Landsberg erecta; Mg, magnesium; Mn, manganese; Mo, molybdenum; MS, Murashige and Skoog; NPTs, non-protein thiols, PC, plastocyanin, ROS, reactive oxygen species; RT–PCR, reverse transcription–polymerase chain reaction; S, sulfur; Sultr 4;1, sulfate transporter; SOD, superoxide dismutase; UBQ2, ubiquitin; Ws, Wassilewskija; Zn, zinc.
reactive oxygen species (ROS), such as oxygen (O$_2$) and hydroxyl (OH) free radicals, occurring above all in the chloroplast (Levine 1999).

Plant mechanisms controlling metal toxicity include regulation of Cu uptake, intracellular chelation, efflux from cells, sequestration in subcellular compartments, and detoxification of ROS (Wintz and Vulpe 2002). To prevent damage induced by ROS, plants have evolved several detoxification mechanisms including the synthesis of enzymatic [e.g. superoxide dismutases (SODs)] and non-enzymatic (e.g. L-ascorbic acid and glutathione) antioxidant molecules (Kurepa et al. 1997). SODs play a key role in plant cell defense because they catalyze the reaction that converts O$_2^-$ to H$_2$O$_2$ (Bowler et al. 1994, Kliebenstein et al. 1998). In plant cells, three SOD types have been identified, each encoded by a small gene family. They differ with respect to their metal cofactors: there are manganese (Mn)-, iron (Fe)-, and Cu/Zinc (Zn)SODs. Furthermore, the SODs differ in the apoprotein primary sequences (Kliebenstein et al. 1998) and in subcellular location. Typically, MnSODs are active in mitochondria, FeSODs are plastidic, and Cu/ZnSODs are found in both cytosol (CSD1) and plastids (CSD2) (Abarca et al. 2001, Kliebenstein et al. 1998). SODs have been shown to be differentially regulated in response to a number of environmental stimuli such as light, ozone fumigation, and metal stress (Kliebenstein et al. 1998, Kurepa et al. 1997, Tsang et al. 1996). Moreover, the availability of the metal cofactor in the growth medium could lead to the preferential expression of either chloroplast Cu/ZnSOD or FeSOD (Kurepa et al. 1997).

Other than SODs, some sulfur (S)-containing compounds such as glutathione, metallothionein-like proteins and phytochelatins (Jonak et al. 2004) play a role in plant heavy-metal detoxification as they may facilitate the sequestration of the metal in intracellular compartments. The enhanced consumption of cysteine, required for the synthesis of these S-rich metal-chelating compounds has been found to promote the expression of S assimilation and transporter genes (Domínguez-Solís et al. 2001, Nocito et al. 2002).

Despite the importance of preventing both Cu deficiency and toxicity in plants, still much remains to be elucidated about the mechanisms controlling Cu homeostasis and trafficking inside the cell. Members of the CopT family of Cu transporters mediate entry of Cu into the cytosol (Sancenon et al. 2003). Once entered into the cells, Cu must be delivered to various organelles because it is required as cofactor by enzymes in different locations. Soluble Cu-binding proteins known as Cu chaperones bind and transfer the metal to the target enzymes (O’Halloran and Culotta 2000). For instance, the CCS chaperone found in Arabidopsis thaliana chloroplasts mediates the insertion of Cu to the CuSOD plastidic isoform (Abdel-Ghany et al. 2005b). Plant chaperones isolated up to now are mostly homologues of yeast chaperones, suggesting that Cu chaperones have been largely conserved during evolution (Wintz and Vulpe 2002).

As for Cu transport across intracellular membranes, RAN1 is a P-type ATPase that transports Cu to a late secretory compartment, for delivery to ethylene receptors (Hirayama et al. 1999). Furthermore, two P-type ATPases, PAA1 and PAA2, were shown to be required for Cu delivery in chloroplasts of A. thaliana (Abdel-Ghany et al. 2005a, Shikanai et al. 2003). PAA1 and PAA2 are thought to act sequentially in Cu transport over the envelope and the thylakoid membrane, respectively.

To better understand plant Cu tolerance mechanisms, three accessions of A. thaliana differing in Cu tolerance and accumulation were compared with respect to the transcript levels of genes involved in Cu delivery and oxidative stress responses, such as Cu chaperones active either in the cytosol (ATX1) or in the chloroplast stroma (CpCCP and CCS), the PAA1 and PAA2 Cu transporters, and oxidative stress-related genes coding for FSD1 and cytosolic isoform of Cu/Zn SODs (CSD1). To assess the extent of stress due to excess Cu, expression analysis was carried out also on the gene for plastocyanin (PC), a blue Cu protein that functions as electron transporter in photosynthesis. Furthermore, because excess Cu may influence S metabolism, the transcript levels of the tonoplast sulfate transporter (Sultr 4;1) that functions in sulfate efflux in A. thaliana (Kataoka et al. 2004) was also evaluated.

**Materials and methods**

**Plant material: tolerance and accumulation**

Arabidopsis seeds of Columbia (Col), Landsberg erecta (Ler), and Wassilewskija (Ws) were surface-sterilized by rinsing in 96% (v/v) ethanol for 30 s, then in 0.65% (v/v) sodium hypochlorite for 20 min while shaking, and next in sterile distilled water for 5 x 10 min. Seeds of each accession were sown on agar medium containing half-strength Murashige and Skoog (MS) salts and vitamins (M5524; Sigma, St Louis, MO), including 10 g l$^{-1}$ sucrose and 4 g l$^{-1}$ agar, and supplemented with 2.5 mg l$^{-1}$ Cu (40 μM CuSO$_4$), 3.0 mg l$^{-1}$ Cu (48 μM CuSO$_4$) or no supplemental Cu (control). The seedlings were grown on vertically placed plates in a growth chamber with a day/night period of 16/8 h, an air temperature of 25°C and under a photon flux density of 40 μmol m$^{-2}$ s$^{-1}$. On each plate, all three accessions were grown side by side (n = 10 per accession), and three replicate experiments were performed.

To estimate the tolerance of the three accessions to excess Cu, 14-day-old individual seedlings were carefully
harvested, rinsed with distilled water and their root length was measured. Metal tolerance was expressed as relative root length (also known as tolerance index) calculated as root length observed in the presence of the metal divided by root length under the control condition, thus correcting for any possible differences between experiments.

For elemental analysis, Arabidopsis seedlings growing for 3 weeks in control medium or with 2.5 mg l⁻¹ Cu (40 µM CuSO₄) were harvested and subsequently washed in distilled water to remove any Cu bound to the outside of the roots, separated into shoot and root, and dried at 65°C for 48 h. The samples (n = 3, each consisting of at least 10 seedlings) were digested with nitric acid according to the method of Zarcinas (1987) and the total elemental concentration [Cu, Fe, magnesium (Mg), Mn, molybdenum (Mo), sulphur (US), and Zn] in the digests was measured by inductively coupled plasma atomic emission spectroscopy (ICP-AES, Thermo Jarrell Ash, Franklin, MA) according to the method of Fassel (1978), using appropriate standards and quality controls. The values obtained were expressed in mg kg⁻¹ DW.

Expression analysis via semi-quantitative reverse transcription–polymerase chain reaction

Total RNA for reverse transcription–polymerase chain reaction (RT–PCR) was extracted from leaves of Cu-treated and control plant samples and stored at −80°C. Frozen plant tissue (0.2 g) was ground in liquid nitrogen. RNA was isolated using Trizol reagent (Invitrogen, Carlsbad, CA), pelleted by centrifugation, washed in 1 ml of 75% (v/v) ethanol, and re-suspended in 20 µl of RNase-free water. The RNA amount and purity were initially determined via spectrophotometer (Beckman DU 530 UV/VIS; Life Science, Fullerton, CA) comparing the concentration values obtained at 260 and 280 nm. Subsequently, electrophoresis analysis carried out in a 1% (w/v) agarose gel containing 4% formaldehyde confirmed that the RNA was intact. Total RNA (40 µg) was treated with 2.6 U of DNase I (Fermentas, Hanover, MD) placing the samples in a heating block at 37°C for 30 min. To inactivate the DNase, ethylenediaminetetraacetic acid was then added to the samples to a final concentration of 2 mM, followed by incubation for 10 min at 65°C. The RNA was then precipitated by adding 1/10 volume of 3 M LiCl₂. The pellets obtained were washed in 1 ml of 75% (v/v) ethanol and re-suspended in 20 µl of sterile RNase-free water and the RNA amount was estimated as described previously.

After DNase treatment, 2.5 µg RNA was used to synthesize first-strand cDNA by means of 20 U Ŕl⁻¹ of moloney murine leukemia virus (MMLV) reverse transcriptase (Fermentas) and oligo (dT) as primers, in 20-µl reactions. The reaction conditions were 37°C for 60 min, 70°C for 5 min, and 4°C for 5 min.

RT–PCR experiments with specific primers were performed to evaluate the expression level of Cu transporters, Cu chaperones, Sultr 4;1 and oxidative stress-related genes in leaves of seedlings grown with excess Cu (2.5 mg l⁻¹ Cu; i.e. 40 µM CuSO₄) or under the control conditions. For all PCR reactions, 0.5 µl of the cDNA obtained was used in 25-µl reactions, using 3 U Ŕl⁻¹ of Taq-polymerase. Different numbers of cycles ranging from 22 to 30 were tested to determine the optimal number of cycles for each gene where increasing numbers of PCR cycles resulted in a higher amount of PCR product, indicating that the reactions were not in the stationary phase and reaction components were not limiting. PCR reactions were carried out using the following protocol: 50 s denaturation at 94°C, 45-s annealing at 55°C, 90 s extension at 72°C; a 3-min denaturation at 94°C (one cycle) at the beginning of the reaction and a 5-min extension at 72°C at the end were performed for all reactions. A. thaliana ubiquitin (UBQ2) (At2g36170) was used as a constitutive internal standard in order to normalize the obtained gene expression results. In Table 1, the number of amplification cycles and the PCR product size (bp) Forward primer (5’ → 3’) Reverse primer (5’ → 3’)

<table>
<thead>
<tr>
<th>Gene</th>
<th>No. PCR cycles</th>
<th>PCR product size (bp)</th>
<th>Forward primer (5’ → 3’)</th>
<th>Reverse primer (5’ → 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>UBQ2</td>
<td>22</td>
<td>228</td>
<td>CCAAGATCCAGGACAAAGAAGGA</td>
<td>TGGAGACGACGATAAACAACCTGC</td>
</tr>
<tr>
<td>PC</td>
<td>23</td>
<td>650</td>
<td>CGATCGAACCACGAAAGTACAG</td>
<td>GAAGACCTCTTAATGAGTGC</td>
</tr>
<tr>
<td>FSD1</td>
<td>23</td>
<td>500</td>
<td>CAACTCTGGAAAATTACG</td>
<td>TCAAAGCCTGGTATTACGC</td>
</tr>
<tr>
<td>CSD1</td>
<td>26</td>
<td>500</td>
<td>GAGTTGAGAGGTTGAACGC</td>
<td>GAGTGGCAGATTTGGAACGC</td>
</tr>
<tr>
<td>PAA1</td>
<td>25</td>
<td>900</td>
<td>ACACCGCAGAGCCTACCC</td>
<td>ACTGGGACAATGGGACAGGG</td>
</tr>
<tr>
<td>PAA2</td>
<td>25</td>
<td>840</td>
<td>ATTTAAGCTGGGATGCGCCTC</td>
<td>CCGCTCTTCTCGAGGCTCCGC</td>
</tr>
<tr>
<td>Sultr 4;1</td>
<td>24</td>
<td>246</td>
<td>TGACACCTCTCAATTAGAAGCCTG</td>
<td>AATTGTTTGAAGGGCCTGATTTC</td>
</tr>
<tr>
<td>ATX1</td>
<td>24</td>
<td>323</td>
<td>TACCGGGCCTTGAAGGCCTTAAGCTGAAGGCC</td>
<td>ATCCGGGAGCCTTAAGCTGAAGGCCCTACCTTC</td>
</tr>
<tr>
<td>CCS</td>
<td>26</td>
<td>770</td>
<td>TACCGGGCCTTGAAGGCCTTAAGCTGAAGGCC</td>
<td>ATCCGGGAGCCTTAAGCTGAAGGCCCTACCTTC</td>
</tr>
<tr>
<td>CpCCP</td>
<td>23</td>
<td>798</td>
<td>GCAGTCTGGAGAGCTAGATTTGATTTTCCTG</td>
<td>CAGTGCAAGCTTAAGGAGGCTGAATAAT</td>
</tr>
</tbody>
</table>
size are presented for each gene, as well as the primer sequences. RT–PCR analysis was performed using the Eppendorf model Mastercycler gradient (Eppendorf, Westbury, NY) and PCR products were electrophoresed in a 1% agarose gel. The signals were next quantified through the IMAGEJ program. Furthermore, to confirm the expression analysis results, PCR reactions were carried out on cDNAs obtained from two different RNA extractions performed on seedlings of two independent experiments and repeated at least four times for each cDNA.

Non-protein thiol measurements

The three accessions Ler, Col, and Ws were grown for 3 weeks on half-strength MS medium containing 10 g l\(^{-1}\) sucrose and 4 g l\(^{-1}\) agarose (Sigma) with or without CuSO\(_4\) (2.5 mg l\(^{-1}\) Cu). The plants were harvested, washed, separated into roots and shoots, and stored at \(-80^\circ C\). For non-protein thiol (NPT) analysis, three replicates of 100 mg of roots and shoots were ground in liquid nitrogen, and extracted and analyzed as described by Zhu et al. (1999), using Ellman’s reagent.

Statistical analysis

The software program JMP-IN (SAS Institute, Cary, NC) was employed for statistical analysis of metal tolerance and accumulation data. ANOVA was performed followed by pairwise post-hoc analyses to determine as to which of the means differed significantly (\(\alpha = 0.05\)). Statistically significant differences (\(P < 0.05\)) are reported in the text and shown in the figures.

Results

Three accessions of *A. thaliana*, Col, Ler, and Ws, were grown at different Cu concentrations and the root length of individual seedlings was measured as a parameter for metal tolerance (Murphy and Taiz 1995a). In plants treated with excess Cu, the metal was supplied as CuSO\(_4\) at 2.5 mg l\(^{-1}\) Cu (40 \(\mu\)M) or 3 mg l\(^{-1}\) Cu (48 \(\mu\)M). The root length of plants that were grown on control medium did not differ among the three accessions (data not shown). Given the strong toxicity effect of 3 mg l\(^{-1}\) Cu, the accessions were next compared with respect to Cu accumulation, using only seedlings grown with 2.5 mg l\(^{-1}\) Cu or on medium without added Cu (Fig. 2). Under the control conditions, Col showed lower Cu levels than Ws both in root and shoot and accumulated less Cu than Ler in the shoot. When supplied with excess Cu, the highest root Cu level was found in Ws, while no differences in Cu accumulation were observed between Ler and Col. The shoot Cu level was similar in Ler and Ws seedlings and was higher than that measured in the Cu-sensitive Col.

To estimate whether excess Cu differentially altered the accumulation of essential elements in the three accessions, the concentrations of Fe, Mg, Mo, Mn, S, and Zn were also measured. Only the effects of Cu treatment on Fe, S, and Mn levels varied among the three accessions in some respects (Fig. 3).

The three accessions grown under the control conditions did not show differences in S content in shoot, whereas Col was found to contain less S than Ler and Ws in roots (Fig. 3A). After Cu treatment, S accumulation in shoot was enhanced in Ler seedlings with respect to the control values, while in the other two accessions it was negatively affected, especially in Col, which had significantly lower shoot S levels than the other two accessions. Root S content decreased in all three accessions under Cu treatment, but the ability of Ler to accumulate S was the least reduced and Col the most.

As far as Fe accumulation in shoot was concerned, no significant differences were detected among the three accessions grown under the control conditions (Fig. 3B).

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As far as Fe accumulation in shoot was concerned, no significant differences were detected among the three accessions grown under the control conditions (Fig. 3B).
When treated with excess Cu, the ability of Col and Ler to accumulate Fe was reduced, while Ws maintained an Fe concentration as high as the control plants. Col control seedlings showed a lower root Fe content compared with the other two accessions, whereas Ler contained the highest Fe level. Excess Cu reduced the plants’ ability to accumulate Fe in roots in Col and Ws, while Ler maintained a high level of iron.

Exposure to toxic Cu concentration did not negatively affect Mn accumulation in the shoot of the three accessions with respect to the corresponding control values, and Col always showed the lowest Mn content (Fig. 3C). On the contrary, Cu treatment enhanced the ability to accumulate Mn at the root level in the three accessions relative to the control conditions, and this effect was more pronounced in Col and Ws than in Ler.

To obtain more insight into the mechanisms underlying the observed differences in Cu tolerance and accumulation, a semi-quantitative RT–PCR analysis was performed on leaves, where Cu is mostly assimilated, comparing transcript levels of nine genes involved in Cu metabolism, oxidative stress resistance, or Sultr 4;1 (Fig. 4). The constitutively expressed UBQ2 was used as a control, and the expression of each gene was calculated relative to UBQ2 expression. For many of the genes tested, the expression in the leaves of Col, Ler, and Ws varied among the three accessions. The transcript level for the blue Cu protein PC (At2g28660) was higher in Col and Ws under control conditions than in Ler. Excess Cu led to a reduction of PC mRNA level in all three accessions, but there was less reduction in Ws than in the other two accessions.

As far as the transcript level of oxidative stress-related genes was concerned, SODs were found to be differently regulated by excess Cu in the three accessions. The transcript level of FSD1 (At4g25100) was lower in Col than in Ler and Ws, both under control conditions and after Cu treatment. In each of the three Cu-treated accessions, the abundance of FSD1 decreased in comparison with the corresponding control values, and Ler presented the highest transcript level. Under the control conditions, Ler accumulated less of the CSD1 (At1g08830) transcript in its shoot than the other two accessions. While in Col no change in CSD1 mRNA level was observed after Cu treatment, in the Ler and Ws accessions the transcript level of this gene increased in response to Cu.

The analysis of the expression of genes encoding for Cu chaperones revealed different patterns of regulation by Cu stress in the accessions. The Ws control seedlings presented the lowest transcript level of ATX1 (At1g66240), while Col had the highest levels. Following exposure to toxic Cu concentration, the accumulation of transcripts was strongly reduced in Col and to a lesser extent in Ler, whereas it was found to increase in Ws. In the control seedlings, the transcript level of CCS (At1g12520) was similar in all accessions. CCS mRNA accumulation was slightly enhanced in Col and Ws by excess Cu, whereas a reduction in CCS transcript level was apparent in Ler. Both under control conditions and with excess Cu, the transcript abundance of CpCCP (At2g28660) was highest in Col and lowest in Ler. The addition of Cu did not appear to affect the CpCCP transcript level of Ws, but reduced its accumulation slightly in Col and quite strongly in Ler.

The P-type ATPases PAA1 (At4g33520) and PAA2 (At5g21930), required for Cu delivery in the chloroplast, showed similar patterns of expression in Cu-treated...
seedlings. Under both the control and excess Cu treatments, Ler presented the lowest amount of PAA1 transcript. Excess Cu resulted in a reduction of the mRNA level in Ler and, especially, Col; the PAA1 transcript level was much less affected in Ws. Similarly, the accumulation of PAA2 transcript was lowest in the Ler control seedlings, and Cu treatment led to a decrease of transcript accumulation, most evident in Col.

The analysis of the transcript level of Sultr 4;1 (At5g13550) did not reveal differences among the three accessions under control conditions. However, excess Cu induced the accumulation of the Sultr 4;1 transcript in Col and Ws while causing a decrease in Ler.

Total NPT levels were also compared in the three accessions, because some NPT compounds have been associated with Cu tolerance. There were no significant differences between the accessions with respect to NPT levels, in shoot or roots, neither when grown under control conditions nor when supplied with 2.5 mg l⁻¹ Cu (Table 2). Furthermore, no differences in NPT levels were

---

**Fig. 3.** Accumulation of (A) S, (B) Fe, and (C) Mn in the shoot and root of the three *A. thaliana* accessions, Col, Ler, and Ws, grown on 0.5 strength MS medium (control) or on the same medium supplied with 2.5 mg l⁻¹ Cu (+Cu). Data shown represent the mean ± SE. Different letters above bars indicate significant differences (*P* < 0.05) among the three accessions.

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Physiol. Plant. 129, 2007 347
observed in any of the three accessions after Cu exposure in comparison with the control conditions.

Discussion

In this study, it was found that there was significant variation among the three Arabidopsis accessions Col, Ler, and Ws with respect to Cu tolerance and accumulation. The Ws and Ler accessions were found to be more Cu tolerant than Col, while they accumulated more Cu than Col. Previous studies reported that an external Cu concentration higher than 20 μM (approximately 1.3 mg l⁻¹ Cu) was needed to cause visible phytotoxic symptoms in Arabidopsis plants grown on agar medium (Murphy and Taiz 1995a). Indeed, in our study, 2.5 mg l⁻¹ Cu (40 μM Cu) negatively affected the root growth of the three accessions and a drastic reduction was observed when seedlings were supplied with 3 mg l⁻¹ Cu.

Judged from root growth inhibition, the Ws and Ler accessions were significantly more tolerant to excess Cu than Col. Cu exclusion does not appear to be the mechanism involved in Cu tolerance in these accessions, as Ws seedlings accumulated significantly more Cu in its roots than the other two accessions, and Ws and Ler both showed more Cu in shoot than Col. Rather, the observed differences in Cu tolerance may be related to S, Fe, and Mn accumulation, which was differently affected in the three accessions under Cu stress; Mg, Mo, and Zn content did not vary (data not shown). S concentration was lower in the Col accession than in Ws and Ler, both in shoot and roots. Excess Cu did not affect S content in shoots of Ler and only slightly in Ler roots. Because S is involved in heavy-metal detoxification mechanisms (Dominguez-Solis et al. 2001), this result may explain the observed Cu tolerance in Ler. Furthermore, Fe accumulation in the Col accession was strongly reduced by Cu, whereas in the other two accessions the Fe level remained relatively high in comparison with the control conditions. Mn accumulation was enhanced by Cu in the roots of all three accessions. However, the shoot Mn concentration in Col was lower than in Ws and Ler.

Thus, the higher Cu tolerance of the Ws and Ler accessions compared with Col may reflect less nutrient deficiency. Based on reported critical deficiency levels for these elements (Marschner 1995) both S and Fe may have been limiting plant growth in Col plants, but Mn likely was not. Similar to the results presented here, previous studies also showed that the uptake of high Cu amounts affected the accumulation of other essential elements like Fe and, as a consequence, the cation balance of the cell was dramatically compromised (Palma et al. 1987, Welch 1995).

The gene expression analysis indicated that there were differences between the accessions with respect to Cu-regulation of genes involved in Cu metabolism, oxidative stress resistance, and Sultr 4;1. The decrease in PC transcript levels observed here in response to Cu toxicity are in agreement with decreased PC protein levels reported in earlier studies (Abdel-Ghany et al. 2005a), concomitant with a decrease in chlorophyll content. This effect was reduced in plants with a defect in PAA1, a chloroplast envelope Cu transporter, suggesting that thylakoids are a target for Cu toxicity. Indeed, in this study there appears to be a correlation between the PC transcript level and those of the P-type ATPases, PAA1 and PAA2.

![Fig. 4. Expression analysis of genes involved in Cu homeostasis, oxidative stress or S transport, using semi-quantitative RT-PCR. The RT-PCR products presented were obtained using the amplification cycles reported in Table 1. The RT-PCR signals were quantified by employing the ImageJ program and normalized with the constitutive internal standard UBQ2. Numbers shown under the RT-PCR products represent the means of four replicates (±SE).](image)

### Table 2. NPT levels in roots and shoots of the three accessions Col, Ler, and Ws grown with or without excess Cu. Data represent the mean ± SE.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>2.5 mg l⁻¹ Cu</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Col</td>
<td>Ler</td>
</tr>
<tr>
<td>Shoot</td>
<td></td>
<td></td>
</tr>
<tr>
<td>446 ± 78</td>
<td>448 ± 24</td>
<td>486 ± 61</td>
</tr>
<tr>
<td>Root</td>
<td></td>
<td></td>
</tr>
<tr>
<td>372 ± 11</td>
<td>372 ± 9</td>
<td>340 ± 12</td>
</tr>
</tbody>
</table>

Physiol. Plant. 129, 2007
PAA2, required for Cu delivery in chloroplasts. The Ws accession showed a higher PC transcript level than Col and Ler in agreement with the expression pattern of PAA1 and PAA2. In addition, the mRNA levels of PAA1 and PAA2 were low in the Cu-treated accessions in comparison with the control conditions, as observed for the PC gene. The downregulation of these two Cu transporters may be induced by high Cu concentration, and this hypothesis could be extended to the genes encoding the putative Cu chaperones ATX1 and CpCCP. In both cases, transcript levels decreased in Col and Ler, whereas in Ws they appeared to increase.

Cu in excess also differentially regulated the expression of genes encoding SODs in the three accessions. The differences observed are probably related to the nutrient levels in the seedlings, because it is known that Cu and Fe availability in the growth medium, as well as species-specific mechanisms controlling the final cell concentration of these metals, could lead to preferential expression of either Cu/ZnSOD or FeSOD (Kurepa et al. 1997). Indeed, the level of FSD1 transcript was negatively affected by Cu treatment in the three accessions, perhaps due to the lower tissue Fe content compared with the control conditions. This is in agreement with the results obtained from northern blotting (Abdel-Ghany et al. 2005a). The Ws and Ler accessions, being able to accumulate more Fe than Col, also maintained a higher FSD1 transcript level. On the contrary, CSD1 expression was induced by Cu treatment in Ws and Ler relative to the corresponding control plants, and the amount of transcript was higher than in Col, perhaps since more Cu was accumulated, again in agreement with results from northern blotting (Abdel-Ghany et al. 2005a).

Kataoka et al. (2004) reported that the mRNA of the vacuolar Sultr 4;1 was accumulated under S limitation. In this study, an upregulation of the gene encoding Sultr 4;1 was observed in the Col and Ws accessions after exposure to toxic Cu concentration. This may be related to a decreased ability to accumulate S, compared with the control seedlings. This result is in agreement with previous studies, where the induction of high-affinity Sultr 4;1 was observed under heavy metal stress (Nocito et al. 2002).

No effect on the expression of the Sultr 4;1 was detected in Ler, likely because it could maintain a higher S level than the other accessions. There was no correlation between Cu tolerance and total NPT levels, suggesting that these compounds are not rate limiting for Cu tolerance in these accessions. Murphy and Taiz (1995b) also investigated correlations between NPT levels and Cu tolerance in Arabidopsis. There was no significant correlation, although there was a trend for NPT levels to be higher in Cu-tolerant accessions. They found a significant correlation, however, between Cu tolerance and the transcript level of the metallothionein MT2. Incidentally, in Murphy and Taiz (1995b) the Ler accession was found to be somewhat less tolerant to Cu (as CuCl₂) than Col; Ws was the most tolerant of the three accessions. Cu tolerance was assayed slightly differently than in this study: the seeds were initially germinated on control medium, followed by transfer of the seedlings to medium with or without excess Cu. This may, in part, explain the observed differences; also, the accessions may be genetically different from the ones used in this study.

In conclusion, there was significant variation among the three Arabidopsis accessions in Cu tolerance and accumulation. The Ws and Ler accessions were found to be more Cu tolerant than Col, while they accumulated more Cu than Col. Therefore, exclusion does not appear to be the main tolerance mechanism. The Ws and Ler accessions may be more Cu tolerant than the Cu-sensitive Col because they experienced less nutrient deficiency under Cu stress. Expression analysis of Cu-related genes in the three accessions showed some significant differences in transcript levels as influenced by Cu, shedding some light on the potential mechanisms involved in the observed differences in Cu tolerance and accumulation.

Still, a more elaborate transcriptome analysis will be needed to obtain more insight into plant Cu tolerance mechanisms in Arabidopsis.

References


