BMB
reports

Yeast copper-dependent transcription factor *ACE1* enhanced copper stress tolerance in *Arabidopsis*

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Copper is essential but toxic in excess for aerobic organisms. Yeast transcription factor ACE1 functions as a sensor for copper and an inducer for the transcription of *CUP1*. In addition, *ACE1* can activate the transcription of superoxide dismutase gene (*sod1*) in response to copper. In this study, we introduced the yeast *ACE1* into Arabidopsis and analyzed its function in plant. Under high copper stress, the transgenic plants over-expressing *ACE1* showed higher survival rate than the wild-type. We also found that over-expression of *ACE1* in *Arabidopsis* increased the activities of SOD and POD, which were beneficial to the cell in copper buffering. Excess copper would suppress the expression of chlorophyll biosynthetic genes in *Arabidopsis*, RT-PCR analysis revealed that over-expression of *ACE1* decrease the suppression. Together, our results indicate that *ACE1* may play an important role in response to copper stress in *Arabidopsis*. [BMB reports 2009; 42(11): 752-757]

INTRODUCTION

Copper (Cu) is a vital micronutrient for normal plant growth and development. It is a cofactor for many physiological processes including photosynthesis, mitochondrial respiration, superoxide scavenging, ethylene sensing, and lignification (1). However, excess Cu causes phytotoxicity by inhibiting key cellular processes, including photosynthesis and electron transport, lipid peroxidation, and disruption of protein functions due to Cu-binding to sulphydryl groups, and finally causes the inhibition of plant growth or even death (2-5). Excess Cu also induces the formation of reactive oxygen species (ROS) (6, 7), which has been directly correlated with the damage to the membrane proteins and lipids (8-10). The cells are normally protected from the damage of ROS by operation of intricate anti-oxidative systems comprising of enzymatic [superoxide dismutase (SOD), catalase (CAT), peroxidase (POD) and enzymes of Halliwell-Asada pathway] and non-enzymatic (ascorbate, glutathione and phenolic compounds) systems (11). Additionally, in order to prevent toxicity of heavy metals, plants can synthesize different kinds of metal-binding peptides including phytochelatins and metallothioneins. Consequently, heavy metals can be detoxified by chelation and sequestration in the vacuole (12, 13), and various membrane transport systems play an important role in metal ion homeostasis and tolerance (14).

ACE1 is a transcriptional activator in copper-replete cells. It is a member of a group of fungal transcription factors that contain a copper-fist cysteine-rich DNA-binding domain located in the N-terminal region (15). In *Saccharomyces cerevisiae*, *ACE1* can activate the transcription of the metallothionein encoding genes *cup1* (16-19) and *crs5* (20), and the superoxide dismutase gene *sod1* (21, 22) in response to copper. Recently, Canessa *et al*. (23) found that pc-*ACE1* from *Phanerochaete chrysosporium* activates the transcription of the *mco1* (multicopper oxidases) gene.

So far, the function of *ACE1* in plants has not been reported. Here, we showed direct evidence for the copper stress tolerance of *ACE1* in *Arabidopsis thaliana*. Our results indicated that *ACE1* may play an important role to copper stress tolerance in the transgenic plants as in *S. cerevisiae*.

RESULTS

Transgenic *Arabidopsis* plants with higher expression of *ACE1*

To analyze the potential function of the *ACE1* in plants, the entire coding region of *ACE1* was introduced into *Arabidopsis* cells under the control of the double CaMV 35S (D35S) promoter (Fig. 1A). Eight independent lines of transgenic plants with *ACE1* (T_1 generation) were obtained by selecting for growth on medium containing hygromycin (40 μg/ml), and transgenic plants of the T_2 generation were confirmed by PCR. Among them, three lines (named A1, A4, A5) having high expression of *ACE1* were choose for further experiments. In the next T_2 generation, the three over-expressing lines showed

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Received 27 May 2009 Accepted 13 July 2009

Keywords: *ACE1*, *Arabidopsis*, Copper tolerance, Transgenic plants

strong ACE1 expression (Fig. 1B).

Copper tolerance in seedling growth

Petri dishes were used to copper-tolerance assay on transgenic *Arabidopsis* seedlings. In this assay, Arabidopsis seeds were grown on MS medium containing Cu^{2+} (0 μM, 10 μM, 15 μM) in Petri dish. The root length was measured at 10 d post-germination (dpg). As shown in Fig. 2A and 2B, the root length of *ACE1* transgenic plants were dramatically longer than that of the wild-type.

Fig. 1. (A) Schematic diagram of the vector used in this study. The vector contains the double 35S (DCaMV35S) promoter and the tobacco mosaic virus (TMV) sequence fused to the *ACE1* gene. For steady transmission of the *ACE1* gene, two scaffold attachment regions (SAR) were fused upstream of the DCaMV35S promoter and downstream of the NosS-Terminator (NosS-T). (B) Confirmation of *ACE1* genomic integration in transgenic lines by RT-PCR. Wild type (lane1, WT) and transgenic lines (lane2-4) of *A.thaliana* were used as PCR templates. Specific primer pairs for PCR of *ACE1* were used.

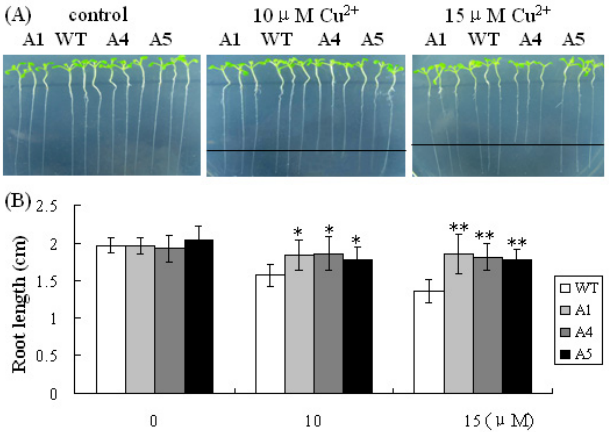

Fig. 2. Copper tolerance in petri dish. Sterilized Arabidopsis seeds were grown on MS medium containing Cu^{2+} (0 μM, 10 μM, 15 μM) in Petri dish, then they were incubated in the darkness at 4°C for 2d and finally transferred maintaining vertically to grow in a controlled environment chamber at 22°C. (A) Comparative image of wild type and transgenic plants 10 dpg on MS medium containing Cu^{2+} (0 μM, 10 μM, 15 μM) in Petri dish. (B) Root length of the transgenic *A.thaliana* and wild-type plants 10 dpg treated with $CuSO_4$ (described as Fig. 2A). Data are the mean ± SD of three replicates. n = 50 seedlings per line for each experiment. The values with significant differences according to t-tests are indicated by asterisks (*P ≤ 0.05; **P ≤ 0.01).

Copper stress tolerance of transgenic plants

To analyze copper stress tolerance, 3-week-old T_2 seedlings grown in pots at 22°C were transferred to water and given $CuSO_4$ (0 μM, 50 μM, 100 μM). The photograph was taken at 10th days after they were watered $CuSO_4$ (0 μM, 50 μM, 100 μM) solution. The leaves of wild-type plants mostly withered and turned yellow, while that of the three over-expressions were dark green (Fig. 3A). Most of the wild-type plants exposed to 100 μM Cu^{2+} died after 10d, whereas ≥70% of the transgenic plants survived (Fig. 3B). It showed that the transgenic plants over-expressing *ACE1* have more tolerance to copper stress than the wild-type plants.

Chlorophyll content

Excess Cu inhibited the photosynthesis and electron transport (2). To analyze the influence of copper stress to photosynthesis, we detected the effects of copper stress on the total

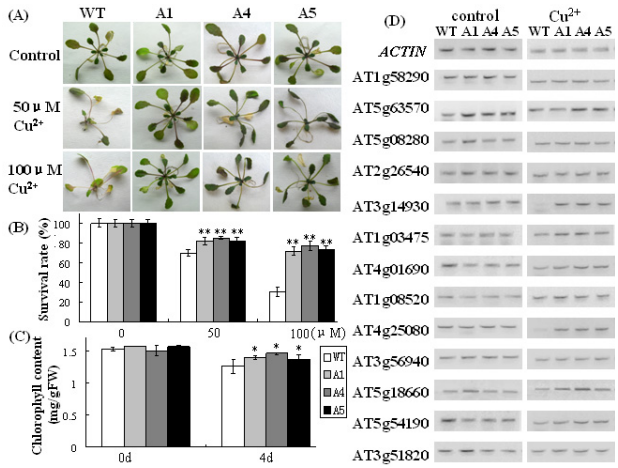

Fig. 3. Copper stress tolerance and chlorophyll biosynthesis pathway. (A) 3-week-old seedlings were transferred carefully from pots into water containing Cu^{2+} [0 mM (control), 50 mM, 100 mM]. The photograph was taken at 10th days after they were watered $CuSO_4$. (B) Survival rates of the transgenic A.thaliana and wild-type plants at three-week-old stage treated with $CuSO_4$ (described as Fig. 3A). Data are the mean ± SD of three replicates. n = 50 plants per line for each experiment. The values with significant differences according to *t*-tests are indicated by asterisks (*P ≤ 0.05; **P ≤ 0.01). (C) 3-week-old seedlings in pots were given Cu^{2+} solution (20 mM) for 4d, and measured the chlorophyll contents of leaves (0d represented the control). Data are the mean ± SD of three replicates. The values with significant differences according to *t*-tests are indicated by asterisks (*P ≤ 0.05; **P ≤ 0.01). (D) Expression pattern of genes coding for chlorophyll biosynthesis enzymes in *Arabidopsis* using RT-PCR. Three- week-old plants were given $CuSO_4$ (20 mM) for 3d and harvested to freeze in liquid nitrogen. A representative experiment is shown. Genes coding for enzymes involved in the chlorophyll pathways were identified by searching the *Arabidopsis* annotation in The *Arabidopsis* Information Resource (http://www.arabidopsis.org).

chlorophyll content. 3-week-old seedlings were given Cu^{2+} solution (20 mM) for 4d, and measured the content in leaves. The content of total chlorophyll in wild type and transgenic plants were decreased after stress. However, the decrease in the transgenic plants was significantly lower than that in the wild-type (Fig. 3C).

Chlorophyll biosynthetic enzymes assay

RT-PCR was used to analyze the chlorophyll biosynthetic enzymes. The genes of uroporphyrinogen decarboxylase (AT3g14930) and methyl transferase (AT4g25080) were not expressed in wild type after copper stress (Fig. 3D), while the expression of other genes were not changed much in transgenic plants after stress treatment.

Malondialdehyde (MDA)

Excess Cu can cause lipid peroxidation (3). The level of lipid peroxidation for plants was measured on the basis of the accumulation of malondialdehyde (MDA), a major product of lipid peroxidation (24). As shown in Fig. 4A, significant differences in the MDA level were detected between wild-type and transgenic plants when they were exposed to copper stress. It demonstrated that less accumulation of lipid peroxidation product was observed in transgenic plants than wild-type.

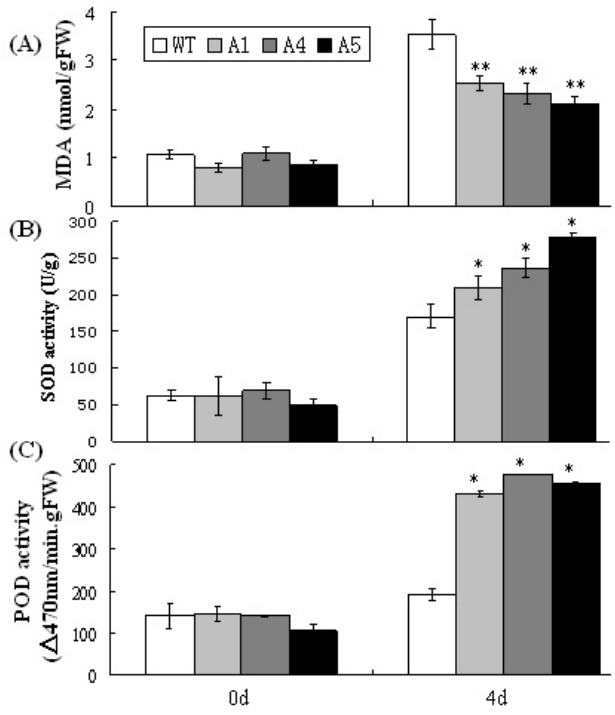

Fig. 4. (A-C) Effects of copper stress on the physiological properties in leaves of wild type and transgenic plants. Data are the mean ± SD of three replicates. The values with significant differences according to *t*-tests are indicated by asterisks (*P ≤ 0.05; **P ≤ 0.01).

Superoxide dismutase (SOD) and peroxidase (POD) activity

Excess Cu can cause the change in the activity of some ROS scavenging enzymes (3). The activities of SOD and POD in wild-type and transgenic plants were increased after copper stress (Fig. 4B and 4C). However, the increase in transgenic plants was significantly higher than that in wild-type.

DISCUSSION

Previous studies have shown that *ACE1* can control Cu resistance and sensitivity in yeast (16, 25-27). Our study showed that *ACE1* can also enhance the copper tolerance in Arabidopsis.

Heavy metal can affect root development. Roots are usually shortened and thickened or poorly developed under heavy metal stress (28). After treated with different copper concentration (0 μM, 10 μM, 15 μM) in Petri dish, the root development was obviously more retarded in wild-type seedling (Fig. 2). It demonstrated transgenic plants have higher copper tolerance than wild-type.

Photosynthesis is also sensitive to excessive Cu, the pigment and protein components of photosynthetic membranes are the targets (29). When high copper stress was imposed on plants, the leaves turned yellow and the chlorophyll content decreased (Fig. 3A, 3C). Chlorophyll synthesis diminution after heavy metal stress may be the consequence of inhibition of enzymes for Chlorophyll synthesis (30). Our result suggested that the uroporphyrinogen III decarboxylase gene (AT3g14930) and magnesium-protoporphyrin IX methyltransferase gene (AT4g 25080) involved in the chlorophyll biosynthesis were suppressed and not expressed in wild-type after copper stress treatment (Fig. 3D). Uroporphyrinogen III decarboxylase catalyzes the stepwise decarboxylation of the four acetate residues of uroporphyrinogen III to form coproporphyrinogen III (31). Although it has been extensively studied in animals, where deficiency causes porphyria disease, the plant enzyme has been relatively little studied. Pontier *et al*. demonstrated that the magnesium-protoporphyrin IX methyltransferase (*CHLM*) gene was essential for the formation of chlorophyll and subsequently for the formation of photosystems I and II and cytochrome b6f complexes using CHLM knock-out mutant in *Arabidopsis* (32). It may explain the reason why the content of chlorophyll in wild type decreased more than transgenic plants after copper stress. The expression of other genes involved chlorophyll biosynthesis pathway seemed not changed much in both control and transgenic plants under stress. Subtle differences in transcript accumulation were difficult to discern by semi-quantitative RT-PCR.

The accumulation of MDA is often used as an indicator of lipid peroxidation (33). Membrane lipid peroxidation occurs from a malfunction of the scavenging system, which can lead to the damage of main cellular components (34). High copper

stress increased MDA content both in wild-type and transgenic plants (Fig. 4A), similar to what has been found in other species (35, 36). Less MDA accumulation in transgenic plants suggested that lipid peroxidation developed less in transgenic plants on high copper stress.

Cu-induced damage is mainly due to the generation of ROS which usually damages the cellular structure, such as membranes, nucleic, chloroplast pigments (37-39). Gralla *et al*. (21) demonstrated that *ACE1* activated copper, zinc superoxide dismutase gene in yeast which encoded copper, zinc superoxide dismutase (EC1.15.1.1), a copper-containing enzyme that functions as preventing oxygen toxicity. Further, higher ROS concentration increase the activities of POD which scavenge the H_2O_2 resulting from SOD activity (40). In our study, we found that the activities of SOD and POD were increased after copper stress treatment (Fig. 4B and 4C). Moreover, the increasing activities of both enzymes were significantly higher in transgenic plants than in wild-type plants. This might contribute towards the decreases in the damage of cellular components caused by ROS. These made the transgenic plants having higher tolerance and survival rate than wild-type when they were exposed to high copper stress (Fig. 3A and 3B).

Together, our results suggested that over-expression of *ACE1* in *Arabidopsis* enhances the copper tolerance due to the accumulation of defense factors, such as SOD and POD. Further investigation is needed to elucidate the function and regulatory mechanism of *ACE1* in plant-stress responses.

MATERIALS AND METHODS

Isolation and transformation of a cDNA encoding the putative *ACE1*

The full cDNA was cloned by PCR from the yeast *Saccharomyces cerevisiae* cDNA library. Reactions were performed using special primers (the forward primer was based on the ATG start codon: 5′-GGATCCATGGTCGTAATTAACGGGGTCAAA TATG-3′ and the reverse primer was based on the TAA stop codon: 5′-GAGCTCTTATTGTGAATGTGAGTTATG-3′).The products were cloned into TA clone vector Simple pMD-18 (Takara Co., Ltd, Dalian, China) and the integrity of the construct was verified by sequencing (the Chinese National Human Genome Center, shanghai, China). The full-length *ACE1* cDNA was digested with *Bam* HI and *Sac* I, and cloned into the binary vector pYG7221 under the control of an enhanced double CaMV 35S promoter and the tobacco mosaic virus TMVΩ sequence to strengthen their translation efficiency (41). The constructs were introduced into *Agrobacterium tumefaciens* GV3101 by electroporation. *A. thaliana* was transformed by the floral dip method (42).

Plant material, growth condition, and treatments

Plants (*Arabidopsis thaliana* ecotype Colubia) were grown on Murashige and Skoog medium (43) for 15d, and transferred into the pots filled with a 9 : 3 : 1 mixture of vermiculite/peat moss/perlite in a controlled environmental chamber at 22°C kept on a 16/8 h day/night cycle.

For copper stress, 3-week-old seedlings were transferred carefully from pots into water containing Cu^{2+} (0 μM, 50 μM, 100 μM).

For the assay of chlorophyll biosynthetic enzymes, 3-week-old seedlings in pots were given Cu^{2+} solution (20 mM) for 3d and harvested to freeze in liquid nitrogen.

For the measurement of the physiological properties (chlorophyll, MDA, SOD, POD), 3-week-old seedlings in pots were given Cu^{2+} solution (20 mM) for 4d, and measured the contents using leaves (0d represented the control).

Petri dish copper tolerance assay

Sterilized Arabidopsis seeds were grown on MS medium containing Cu^{2+} (0 μM, 10 μM, 15 μM) in Petri dish, then they were incubated in the darkness at 4°C for 2d and finally transferred maintaining vertically to grow in a controlled environment chamber at 22°C kept on a 16/8 h day/night cycle. The root length was measured in 10 dpg.

Reverse transcription-PCR analysis

Total RNA was extracted with the multisource total RNA miniprep kit (Axygen Scientific, Inc., Union City, CA, USA) according to the manufacturer's instruction and was digested with DNase I (Takara Co., Ltd, Dalian, China) to remove genomic DNA. The first strand of cDNA was synthesized using 5 μg of total RNA as a template with the Reverse Transcription System (Promega, Madison, WI, USA) in a 20 μl reaction volume. In order to improve the reliability of RT-PCR, the *A. thaliana* actin gene (AtAc2, accession number: NM112764) was synthesized by two primers (AtAc2Z1: 5′-GCACCCTGTTCTTCTTAC CGAG-3′ and AtAc2F1: 5′-AGTAAGGTCACGTCCAGCAAGG-3′) and used as an internal standard. The PCR reaction was carried out in 27 cycles of 30s at 94°C, 30s at 56°C, 30s at 72°C, and a final extension at 72°C for 5 min. A 200-bp fragment of the *ACE1* gene was amplified using two specific primers according to the sequence of the *ACE1* gene and similar PCR reaction conditions as for AtAc2. The PCR products were separated on 2% agarose gel. The DNA intensity ratio of the *ACE1* gene to *AtAc2* was analyzed with a Shine Tech Gel Analyser (Shanghai Shine Science of Technology Co., Ltd., China) to evaluate the expression pattern of the *ACE1* gene. The same results were obtained for three independent experiments. Therefore, only the result from one experiment is presented.

Chlorophyll biosynthetic enzyme assay

3-week-old seedlings were given Cu^{2+} solution (20 mM) for 3d and harvested to freeze in liquid nitrogen. RNA extract and reverse transcription were same to above. The PCR reaction was carried out in 30 cycles of 30s at 94°C, 30s at 54-58°C (the anneal temperature was different according to different primers), 30s at 72°C, and a final extension at 72°C for 5 min.

Chlorophyll and malondialdehyde (MDA) content

Chlorophyll was extracted from individual leaves with 95% ethanol and its content determined as described previously by Lichtenthaler (44).

The level of lipid peroxidation in leaf tissue was determined in terms of malondialdehyde (MDA) content by thiobarbituric acid (TBA) reaction as described by Heath and Packer (45). Leaf tissue (500 mg) was homogenized in 5.0 ml of 0.1% (w/v) trichloroacetic acid (TCA). The homogenate was centrifuged at 10,000 × g for 5 min. Of this solution, 2.0 ml was boiled for 30 min at 95°C in a water bath with 2.0 ml of 0.5% TBA (prepared in 20% TCA) and then cooled quickly on ice bath. The resulting mixture was centrifuged at 10,000 × g for 15 min and absorbance of the supernatant was measured at 532 nm. The measurements were corrected for nonspecific absorbance by subtracting the absorbance at 600 nm.

Enzyme extraction

Fresh leaves tissue (0.1 g) was homogenized in 1.6 ml of chilled 50 mM phosphate buffer (pH 7.8) using chilled pestle and mortar kept in ice bath. The homogenate was filtered with twofold muslin cloth and centrifuged at 20,000 × g for 10 min in a refrigerated centrifuge at 2°C. The supernatant was stored at 2°C and used for enzyme assays within 4 h.

Enzyme assays

SOD (EC 1.15.1.1) activity was assayed by using the photochemical NBT method. The assay was performed in terms of SOD's ability to inhibit reduction of nitroblue tetrazolium (NBT) to form formazan by superoxide by the method described previously (46). The enzyme extract 50 µl in 3 ml contained 50 mM phosphate buffer, pH 7.8, 13 mM L-methionine, 75 µM NBT, 10 µM EDTA-Na_2, and 2.0 µM riboflavin. The photoreduction of NBT (formation of purple formazan) was measured at 560 nm. One unit of SOD was defined as that being present in the volume of extract that caused inhibition of the photo-redution of NBT by 50%.

POD (EC 1.11.1.7) activity was measured using the method of MacAdam *et al*. (47) with modification. The POD reaction solution contained 50 ml phosphate buffer (50 mM), pH 7.8, 20 mM guaiacol, 40 mM H_2O_2, and 50 µl enzyme extract. Changes in absorbance of the reaction solution at 470 nm were determined every 30 s. One unit POD activity was defined as an absorbance change of 0.01 units per min.

Acknowledgements

This research was supported by Shanghai and National Natural Science Foundation (30670179, 08ZR1417200); 863 Program (2006AA10Z117, 2006AA06Z358, 2008AA10Z401); Shanghai Project for ISTC (08540706500); The Key Project Fund of the Shanghai Municipal Committee of Agriculture (No. 2008-7-5) and Shanghai Rising-Star Program (08QH14021).

REFERENCES

1. Maksymiec, W. (1997) Effect of copper on cellular processes in higher plants. *Photosynthetica*. **34**, 321-342.
2. Mocquot, B., Vangronsveld, J., Clijsters, H. and Mench, M. (1996) Copper toxicity in young maize (Zea mays L.) plants: effects on growth, mineral and chlorophyll contents, and enzyme activities. *Plant. Soil*. **182**, 287-300.
3. Weckx, J. E. J. and Clijsters, H. M. M. (1996) Oxidative damage and defense mechanisms in primary leaves of Phaseolus vulgaris as result of root assimilation of toxic amounts of copper. *Physiol. Plant*. **96**, 506-512.
4. Chen, L. M., Lin, C. C. and Kao, C. H. (2000) Copper toxicity in rice seedlings: changes in antioxidative enzyme activies, H_2O_2 level, and cell wall peroxidase activity in roots. *Bot. Bull. Acad. Sin*. **41**, 99-103.
5. Sandmann, G. and Böger, P. (1980) Copper-mediated Lipid Peroxidation Processes in Photosynthetic Membranes. *Plant. Physiol*. **66**, 797-800.
6. Cuyers, A., Vangronsveld, J. and Clijsters, H. (2000) Biphasic effect of copper on the ascorbate-glutathione pathway in primary leaves of Phaseolus vulgaris seedlings during the early stages of metal assimilation. *Physiol. Plant*. **110**, 512-517.
7. Groppa, M. D., Tomaro, M. L. and Benavides, M. P. (2001) Polyamines as protectors against cadmium or copper-induced oxidative damage in sunflower leaf discs. *Plant. Sci*. **161**, 481-488.
8. De Vos, C. H. R., Schat, H., De Waal, M. A. M., Vooijs, R. and Ernst, W. H. O. (1991) Increased resistance to copper-induced damage of the root cell plasmalemma in copper tolerant Silene cucubalus. *Physiol. Plant*. **82**, 523-528.
9. Murphy, A. and Taiz, L. (1997) Correlation between potassium efflux and copper sensitivity in ten Arabidopsis ecotypes. *New Phytol*. **136**, 211-222.
10. Drążkiewicz, M., Skórzyńska-Polit, E. and Krupa, Z. (2004) Copper-induced oxidative stress and antioxidant defence in Arabidopsis thaliana. *BioMetals*. **17**, 379-387.
11. Foyer, C. H., Lopez-Delgado, H., Dat, J. F. and Scott, I. M. (1997) Hydrogen peroxide and glutathione-associated mechanisms of acclamatory stress tolerance and signaling. *Physiol. Plant*. **100**, 241-254.
12. Clemens, S. (2001) Molecular mechanisms of plant metal tolerance and homeostasis. *Planta*. **212**, 475-486.
13. Cobbett, C. and Goldsbrough, P. (2002) Phytochelatins and metallothioneins: roles in heavy metal detoxification and homeostasis. *Annu. Rev. Plant. Biol*. **53**, 159-182.
14. Hall, J. L. and Williams, L. E. (2003) Transition metal transporters in plants. *J. Exp. Bot*. **54**, 2601-2613.
15. Hu, S., Fürst, P. and Hamer, D. (1990) The DNA and Cu binding functions of ACE1 are interdigitated within a single domain. *New Biol*. **2**, 544-555.
16. Thiele, D. J. (1988) *ACE1* regulates expression of the Saccharomyces cerevisiae metallothionein gene. *Mol. Cell. Biol*. **8**, 2745-2752.
17. Culotta, V. C., Hsu, T., Hu, S., Furst, P. and Hamer, D. (1989) Copper and *ACE1* regulatory protein reversibly induce yeast metallothionein gene transcription in a mouse extract. *Proc. Natl. Acad. Sci. U.S.A*. **86**, 8377-8381.

18. Evans, C. F., Engelke, D. R. and Thiele, D. J. (1990) *ACE1* transcription factor produced in E. coli binds multiple regions within yeast metallothionein upstream activation sequences. *Mol. Cell. Biol.* **10**, 426-429.
19. Thorvaldsen, J. L., Sewell, A. K., McGowen, C. L. and Winge, D. R. (1993) Regulation of metallothionein genes by the ACE1 and AMT1 transcription factors. *J. Biol. Chem.* **268**, 12512-12518.
20. Culotta, V. C., Howard, W. R. and Liu, X. F. (1994) CRS5 encodes a metallothionein-like protein in Saccharomyces cerevisiae. *J. Biol. Chem.* **269**, 25295-25302.
21. Gralla, E. B., Thiele, D. J., Silar, P. and Valentine, J. S. (1991) *ACE1*, a copper-dependent transcription factor, activates expression of the yeast copper zinc superoxide dismutase gene. *Proc. Natl. Acad. Sci. U.S.A.* **88**, 8558-8562.
22. Carri, M. T., Galiazzo, F., Ciriolo, M. R. and Rotilio, G. (1991) Evidence for co-regulation of Cu, Zn superoxide dismutase and metallothionein gene expression in yeast through transcriptional control by copper via the *ACE1* factor. *FEBS. Lett.* **278**, 263-266.
23. Canessa, P., Alvarez, J. M., Polanco, R., Bull, P. and Vicuna, R. (2008) The copper-dependent *ACE1* transcription factor activates the transcription of the mco1 gene from the basidiomycete Phanerochaete chrysosporium. *Microbiology*. **154**, 491-499.
24. Esterbauer, H., Schauer, R. J. and Zollner, H. (1991) Chemistry and biochemistry of 4-hydroxynonenal, malonaldehyde and related aldehydes. *Free. Radical. Bio. Med.* **11**, 81-128.
25. Fogel, S. and Welch, J. W. (1982) Tandem gene amplification mediates copper resistance in yeast. *Proc. Natl. Acad. Sci. U.S.A.* **79**, 5342-5346.
26. Fogel, S., Welch, J. W., Cathala, G. and Karin, M. (1983) Gene amplification in yeast. Development of copper resistance is mediated by an increase in CUPI gene copy number. *Curr. Genet.* **7**, 1-9.
27. Welch, J. W., Fogel, S., Cathala, G. and Karin, M., (1983) Industrial yeasts display tandem gene iteration at the CUPI region. *Mol. Cell. Biol.* **3**, 1353-1361.
28. Casella, S., Frassinetti, S., Lupi, F. and Squartini, A. (1988) Effect of cadmium, chromium and copper on symbiotic and free-living Rhizobium leguminosarum biovar trifolii. *FEMS. Microbiol. Lett.* **49**, 343-347.
29. Pätsikkä, E., Kairavuo, M., Šeršen, F., Aro, E. M. and Tyystjärvi, E. (2002) Excess copper predisposes photosystem II to photoinhibition *in vivo* by outcompeting iron and causing decrease in leaf chlorophyll. *Plant. Physiol.* **129**, 1359- 1367.
30. Böddi, B., Oravecz, A. R. and Lehoczki, E. (1995) Effect of cadmium on organization and photoreduction of protochlorophyllide in dark-grown leaves and etioplast inner membrane preparations of wheat. *Photosynthetica*. **31**, 411-420.
31. Beale, S. I. (1999) Enzymes of chlorophyll biosynthesis. *Photosynth. Res.* **60**, 43-73.
32. Pontier, D., Albrieux, C., Joyard, J., Lagrange, T. and Block, M. A. (2007) Knock-out of the magnesium protoporphyrin IX methyltransferase gene in Arabidopsis: effects on chloroplast development and on chloroplast-to-nucleus signaling. *J. Biol. Chem.* **282**, 2297-2304.
33. Smirnoff, N. (1993) The role of active oxygen in the response of plants to water deficit and dessication. *New Phytol.* **125**, 27-58.
34. Monk, L. S., Fagerstedt, K. V. and Crawford, R. M. M. (1989) Oxygen toxicity and superoxide dismutase as an antioxidant in physiological stress. *Plant. Physiol.* **76**, 456-459.
35. Chaoui, A., Mazhoudi, S., Ghorbal, M. N. and Ferjani, E. E. (1997) Cadmium and zinc induction of lipid peroxidation and effects on antioxidant enzymes activities in bean (Phaseolus vulgaris L.). *Plant. Sci.* **127**, 139-147.
36. Zhu, B., Xiong, A. S., Peng, R. H., Xu, J., Zhou, J., Xu, J. T., Jin, X. F., Zhang, Y., Hou, X. L. and Yao, Q. H. (2008) Heat stress protection in Aspen sp1 transgenic Arabidopsis thaliana. *BMB Rep.* **41,** 382-387.
37. Fang, W. and Kao, C. H. (2000) Enhanced peroxidase activity in rice leaves in responses to excess iron, copper and zinc. *Plant. Sci.* **158**, 71-76.
38. Gupta, M., Cuypers, A., Vangronsveld, J. and Clijsters, H. (1999) Copper affects the enzymes of ascorbate-glutathione cycle and its related metabolites in the roots of Phaseolus vulgaris. *Physiol. Plant.* **106**, 262-267.
39. Teisseire, H. and Guy, V. (2000) Copper-induced changes in antioxidant enzymes activities in fronds of duckweed (*Lemna minor*). *Plant. Sci.* **153**, 65-72.
40. Becana, M., Moran, J. F. and Iturbe-Ormaetxe, I. (1998) Iron- dependent oxygen free radical generation in plants subjected to environmental stress: toxicity and antioxidant protection. *Plant Soil*. **201**, 137-147.
41. Gallie, D. R., Sleat, D. E., Watts, J. W., Turner, P. C. and Wilson, T. M. A. (1987) The 5'-leader sequence of tobacco mosaic virus RNA enhances the expression of foreign gene transcripts *in vitro* and *in vivo*. *Nucleic. Acids. Res.* **15**, 3257-3273.
42. Zhang, X. and Henriques, R. and Lin, S. S. (2006) Agrobacterium-mediated transformation of Arabidopsis thaliana using the floral dip method. *Nat. Protoc.* **1**, 641-646.
43. Murashige, T. and Skoog, F. (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant.* **15**, 473-497.
44. Lichtenthaler, H. K. (1987) ChlorophyIls and carotenoids: pigments of photosynthetic biomembranes. *Meth. Enzymol.* **148**, 350-382.
45. Heath, R. L. and Packer, L. (1968) Photoperoxidation in isolated chloroplast, I. Kinetics and stoichiometry of fatty acid peroxidation. *Arch. Biochem. Biophys.* **125**, 180-198.
46. Beyer, W. F. and Fridovich, Y. (1987) Assaying for superoxide dismutase activity: some large consequences of minor changes in conditions. *Analytical biochemistry*. **161**, 559- 566.
47. MacAdam, J. W., Nelson, C. J. and Sharp, R. E. (1992) Peroxidase activity in the leaf elongation zone of tall fescue, Plant. *Physiol.* **99**, 872-878.