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Genotypic differences and alterations of protein patterns of tomato explants under copper stress

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Abstract

In vitro response of six tomato genotypes to different copper concentrations was studied. Cu was toxic to tomato explants at a relatively high concentration (100 μM), which reduced callus growth and shoot regeneration. Peto-86 followed by UC-97-3 were more tolerant to copper than the other genotypes. Cu (100 μM) induced the synthesis of eight new proteins (70.86 - 14.78 kD) in Peto-86 and six in Western Improve (46.43 - 14.78 kD) and UC-97-3 (77.69 - 14.78 kD). Cu-stress reduced the expression of some enzymatic bands of alcohol dehydrogenase and esterase, meanwhile, one peroxidase band at the locus Prx-1 was newly expressed under Cu-treatment.

Additional key words: alcohol dehydrogenase, esterase, *Lycopersicon esculentum*, peroxidase, tissue culture.

Introduction

The recent increased awareness of environmental pollution by toxic heavy metals initiated extensive studies on the genetical control and evolution of plant species tolerant to these stresses. Plant cell and tissue culture techniques can provide an alternative method for genetic studies, screening and selecting plants which are tolerant to mineral stress. The environment and nutrient conditions can be controlled, uniformly and precisely. The relatively undifferentiated nature of cultured cells reduces the complications of differences in morphology and stages of development (Stavarek and Rains 1984).

Heavy metals such as Cu, Fe, Co, Mn and Zn are required by biological systems as components of proteins and enzymes. In excess, these micronutrients are toxic and plants have developed both a strategy of avoiding uptake of these metal ions and an ability to synthesize proteins and peptides that can tightly bind and sequester these metals. Treatment of plants with copper induced the synthesis of new sets of

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polypeptides with low molecular mass (Lolkema *et al.* 1984, Steffens 1990, Tamás *et al.* 1997). These metal-inducible proteins were described by Grill *et al.* (1987) and Gekeler *et al.* (1989) as phytochelatins. The metal inducibility of these proteins was demonstrated in tomato (Scheller *et al.* 1987, Steffens 1990), *Datura innoxia* (Jackson *et al.* 1985), *Agrostis gigantea* (Rauser and Curvetto 1983, Rauser 1984) and in barley (Tamás *et al.* 1997). These studies suggested that metal-binding proteins/peptides (phytochelatins) are involved in the mechanism of heavy metal tolerance.

In this investigation *in vitro* response of six tomato genotypes to copper stress was studied. The changes in protein and isozyme patterns were analyzed using polyacrylamide gel electrophoresis in order to provide an additional information on the molecular and genetic basis of copper tolerance in tomato.

Materials and methods

Plants and cultivation: Six tomato (*Lycopersicon esculentum* Mill.) genotypes Roma VF, UC-97-3, Media-A, Western Improve, Person Improve and Peto-86 were grown *in vitro* on 1/4 Murashige and Skoog (1962; MS) medium free of growth regulators.

Cotyledon and hypocotyl explants from 10-d-old seedlings were cultured in 100 cm^3 flasks containing 10.0 cm^3 of MS-medium supplemented with 2.0 mg dm^{-3} benzylaminopurine, 2.0 mg dm^{-3} indole-3-acetic acid and 0.8 % agar. The cultures were incubated under 16-h photoperiod of irradiance 376.8 μmol m^{-2} s^{-1} and temperature of 25 °C.

Copper was supplied as $CuSO_4.5\,H_2O$ (Rauser and Curvetto 1983). The cotyledons and hypocotyl segments from each genotype were cultured on media containing 25, 50 and 100 μM Cu. Explants cultured on MS medium supplemented with normal concentration of Cu (0.1 μM) served as the control treatment. The experiment was designated in five replicates, in which 60 explants were cultured in each treatment. After 4 weeks of incubation, the percentage of explant produced shoots, number of shoots per explant, and callus fresh and dry masses were determined. The relative tolerance index was determined according to Reddy and Vaidyanath (1986).

Electrophoresis: Control and treated calli of tolerant (Peto-86), moderate (UC-97-3) and sensitive (Western Improve) genotypes were used to study the changes in protein and isozyme patterns under Cu-stress. Soluble proteins were extracted by crushing 1.0 g of calli in 1.0 cm^3 extraction buffer at pH 7.8 (0.1 M Tris-HCl pH 7.5, 2 mM EDTA, 2 % glutathione). The mixture was centrifuged for 10 min at 13 530 *g*. For peroxidase, esterase and alcohol dehydrogenase analysis, samples were electrophorasing on 7.5 % acrylamide gels under non-denaturing conditions. The activities of the enzymes were determined according to Vallejos (1983). Protein analysis and electrophoresis was carried out according to the method of Laemmli (1970) with 12 % acrylamide and 1 % sodium dodecyl sulphate (m/v) under denaturing conditions. The gels were stained with *Commassie Brillant Blue R*. Then,

gels were destained by repeated immersion in a mixture of methanol/acetic acid/water (1:1:8, by volume). Data were obtained by scanning densitometer *GS 300* (*Hoeffer Scientific Instruments*, USA) of protein profiles. The molecular mass of the individual protein bands were determined by comparison with protein markers which consisted of 77, 67, 48.1, 30, 20 and 14.4 kD, using *GS 365* electrophoresis data system program version *3.01* (*Microsoft Windows* @ version).

Results and discussion

With increasing copper concentration, the percentage of explant produced shoots, number of shoots per explant and callus fresh and dry masses were reduced in all six

Table 1. Effect of different concentrations (0.1, 25, 50, and 100 μM) of Cu on the percentage of explants produced shoots, number of shoots per explant, fresh and dry masses for the six tomato genotypes. Means ± SE, $n = 5$.

Genotypes	Cu [μM]	Explants produced shoots [%]	Number of shoots [explant^{-1}]	Fresh mass [g]	Dry mass [mg]
Roma VF	0.1	96.66 ± 3.34	11.60 ± 2.44	4.86 ± 0.27	337.7 ± 20.70
	25.0	69.96 ± 8.16	4.40 ± 0.58	3.45 ± 0.38	260.0 ± 16.77
	50.0	52.66 ± 6.18	2.95 ± 0.20	3.13 ± 0.21	160.7 ± 12.36
	100.0	22.79 ± 6.34	1.81 ± 0.25	2.19 ± 0.08	98.3 ± 14.24
UC-97-3	0.1	76.68 ± 6.67	7.35 ± 1.57	4.30 ± 0.23	289.3 ± 22.05
	25.0	70.00 ± 6.23	4.28 ± 0.71	3.46 ± 0.09	248.3 ± 21.67
	50.0	40.66 ± 6.25	2.59 ± 0.55	2.80 ± 0.35	193.3 ± 23.83
	100.0	23.99 ± 6.23	1.72 ± 0.22	2.32 ± 0.33	133.3 ± 18.48
Media-A	0.1	63.32 ± 9.72	8.80 ± 1.02	4.47 ± 0.38	306.7 ± 4.96
	25.0	53.34 ± 6.25	6.13 ± 1.51	4.16 ± 0.42	236.7 ± 12.02
	50.0	31.05 ± 6.46	2.62 ± 0.36	2.71 ± 0.44	181.7 ± 19.22
	100.0	10.67 ± 0.02	0.53 ± 0.12	1.86 ± 0.15	106.0 ± 9.71
Person Improve	0.1	86.64 ± 3.34	9.17 ± 0.46	3.69 ± 0.39	263.3 ± 19.30
	25.0	73.34 ± 9.48	4.16 ± 0.57	3.20 ± 0.33	223.3 ± 6.67
	50.0	47.33 ± 9.25	2.39 ± 0.30	2.40 ± 0.24	153.3 ± 12.02
	100.0	23.99 ± 6.23	1.11 ± 0.18	1.83 ± 0.27	128.3 ± 14.81
Western Improve	0.1	100.00 ± 0.00	9.17 ± 0.21	4.56 ± 0.06	306.7 ± 2.90
	25.0	70.00 ± 6.23	3.97 ± 0.43	3.68 ± 0.80	153.3 ± 11.55
	50.0	50.00 ± 6.66	1.85 ± 0.44	1.90 ± 0.41	110.0 ± 16.67
	100.0	8.99 ± 3.26	0.28 ± 0.09	1.27 ± 0.20	53.3 ± 8.82
Peto-86	0.1	96.66 ± 3.34	9.90 ± 0.52	5.17 ± 0.26	340.0 ± 15.27
	25.0	93.32 ± 4.09	6.57 ± 0.62	4.54 ± 0.21	310.0 ± 16.46
	50.0	80.66 ± 6.23	4.98 ± 0.42	3.38 ± 0.58	270.0 ± 10.00
	100.0	50.66 ± 8.50	2.45 ± 0.33	2.75 ± 0.22	204.3 ± 17.29
LSD 0.05		17.36	2.02	0.82	45.39
0.01		23.06	2.69	1.09	60.22

tomato genotypes (Table 1). The differences between the various concentrations of copper were highly significant. Doncheva *et al.* (1996) found that even minimal concentrations of copper above the values necessary for plant growth and development may be toxic. Barker (1972) found that callus fresh mass of cauliflower, lettuce, potato and carrot was lowered with the increase of Cu ions in the culture medium.

Peto-86 followed by UC-97-3 were more tolerant to Cu-stress than the other cultivars. Meanwhile, Western Improve was less tolerant than the other cultivars (Fig. 1). The genotypic differences in tolerance to copper were highly significant for all characters studied. Genotypic differences in the tolerance to Cu-stress were also found by Manyowa (1989) in both wheat and rye genotypes.

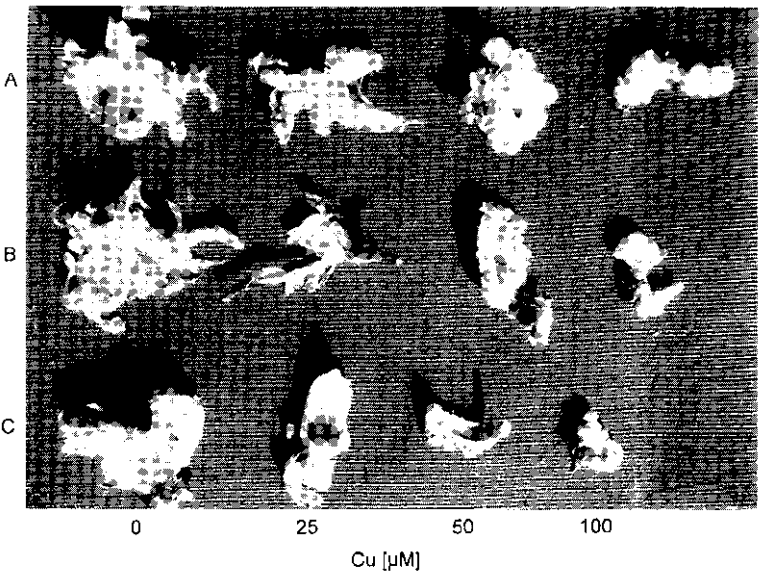

Fig. 1. Callus cultures of tomato genotypes Peto-86 (*A*), UC-97-3 (*B*) and Western Improve (*C*) after four weeks of growth at 0 (control), 25, 50, and 100 μM copper.

Since copper toxicity does occur in cultured tissues, it can be concluded that copper toxicity is not exclusively a whole-plant phenomenon, but appears to be a rather cellular phenomenon which can be studied in tissue culture. Wu and Antonovics (1978) concluded that tolerance to copper is determined genetically and is acting at the cellular level.

Densitometer scans of protein profiles revealed that Cu-stress induced the synthesis of new sets of proteins, as compared to the control treatment (Table 2). The cultures of Peto-86 revealed 8 types of newly induced proteins (70.86 - 14.78 kD). However, six types of protein were newly expressed in Western Improve (46.43 - 14.78 kD) and UC-97-3 (77.69 - 14.78 kD) under Cu-stress.

The results revealed that most of the newly synthesized proteins were of low molecular masses and differ from one genotype to another. Similar observations have

been reported by Steffens *et al.* (1986) and Scheller *et al.* (1987) in tomato, and Reese and Wagner (1987) in cultured tobacco cells.

Table 2. Molecular mass (MM) of induced (I) and reduced (R) proteins in callus cultures of three tomato cultivars grown under 100 μM Cu as compared to the control treatment. Data were obtained by computer program analysis (*Hoeffer*) from densitometer scans of protein profile.

No.	MM [kD]	Western Improve	Peto-86	UC-97-3	No.	MM [kD]	Western Improve	Peto-86	UC-97-3
1	78.15		R		13	31.14			R
2	77.69			I	14	30.24		I	
3	70.86		I		15	28.77		I	
4	58.99			R	16	26.61	R		
5	49.44		R		17	26.54		I	
6	48.43		I		18	25.78		I	
7	46.43	I			19	24.19		I	
8	42.83		R		20	21.10	I		
9	44.62	I		I	21	20.13	I		I
10	41.27			I	22	16.48	I		
11	40.75	R			23	14.78	I	I	I
12	38.18			I					

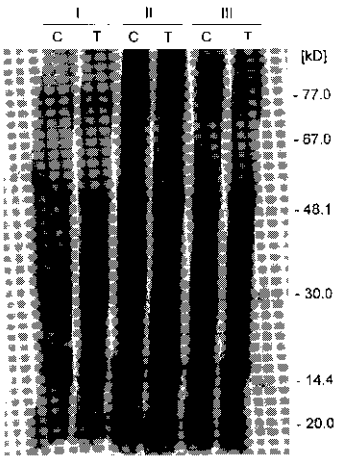

Fig. 2. Polyacrylamide gel electrophoresis of soluble protein fractions from control (*C*) and Cu-treated (100 μM; 4 weeks; *T*) calli of tomato genotypes Western Improve (*I*), Peto-86 (*II*), and UC-97-3 (*III*).

It is interesting to note that the protein band at molecular mass of 14.78 kD was commonly induced in the three genotypes. Furthermore, the two bands 44.62 and 20.13 kD were induced in both copper-sensitive (Western Improve) and moderate (UC-97-3) genotypes. Since these proteins were newly synthesized under Cu-stress, it appears to have a role in the mechanism of copper tolerance which allow to make biochemical and structural adjustments that enable the plant to cope with stress conditions (Sachs and Ho 1986, Scheller *et al.* 1987, Gekeler *et al.* 1989, Tamás *et al.* 1997). Tomsett and Thurman (1988) demonstrated that the metal-resistant gene(s) is directing the synthesis of metal-binding proteins/peptides in plants.

On the other hand, Cu-stress induced a reduction of different protein sets (Table 2 and Fig. 2). Two types of reduced proteins were observed in Western Improve (40.75 and 26.61 kD) and UC-97-3 (58.99 and 31.14 kD). However, three types of proteins (78.15, 49.44 and 42.83 kD) were reduced under Cu-stress in Peto-86 (Table 2).

Generally, the analysis of protein pattern in tomato revealed that Cu-treatment caused changes in gene expression which resulted in changes in protein synthesis. Similar alterations in protein synthesis have also been reported in tomato by Steffens *et al.* (1986) and Scheller *et al.* (1987).

In alcohol dehydrogenase (Adh) zymogram (Fig. 3*A*), two isozyme loci (Adh-1 and Adh-2) were detected in the three genotypes. No quantitative differences in patterns of Adh were observed between the tested genotypes as well as between control and treated calli. This indicated that the tested genotypes possessed similar alleles (allozymes) for alcohol dehydrogenase and these alleles were not affected quantitatively by Cu-treatment. Meanwhile, calli treated with 100 μM Cu exhibited less intensive bands than its controls at the second locus of Adh (Adh-2). These results indicated that the activity of Adh-2 alleles was decreased under Cu-stress.

Four loci (Est-1, Est-2, Est-3 and Est-4) were detected in the esterase zymogram of the genotypes Peto-86, UC-97-3 and Western Improve (Fig. 3*B*). In both control and Cu-treated calli, the three genotypes exhibited similar banding patterns of esterase at the loci Est-2, Est-3 and Est-4. This indicated that these genotypes possessed similar alleles for esterase at these loci. Meanwhile, the two bands 1 and 3 in the Est-1 locus were suppressed under Cu-stress in the genotypes Peto-86 and Western improve. However, UC-97-3 possessed similar banding pattern of Est-1 in both control and treated calli.

The results revealed that Cu treatment reduced the expression of some enzymatic bands of alcohol dehydrogenase and esterase. Similar observations were, also, reported by Walker and Webl (1981) and Stobart *et al.* (1985).

In peroxidase (Prx) zymogram, four loci (Prx-1, Prx-2, Prx-3 and Prx-4) were observed in Peto-86, UC-97-3 and Western Improve (Fig. 4). In both control and Cu-treated, the three genotypes exhibited similar banding patterns of peroxidase at the three loci Prx-2, Prx-3 and Prx-4. This indicated that similar allozymes at these loci were expressed under control and Cu-treatment in the tested genotypes. Meanwhile, allozymes of Prx-1 locus were newly expressed under Cu-stress in the three genotypes (Figs. 4). These results may lead to the suggestion that the genetic

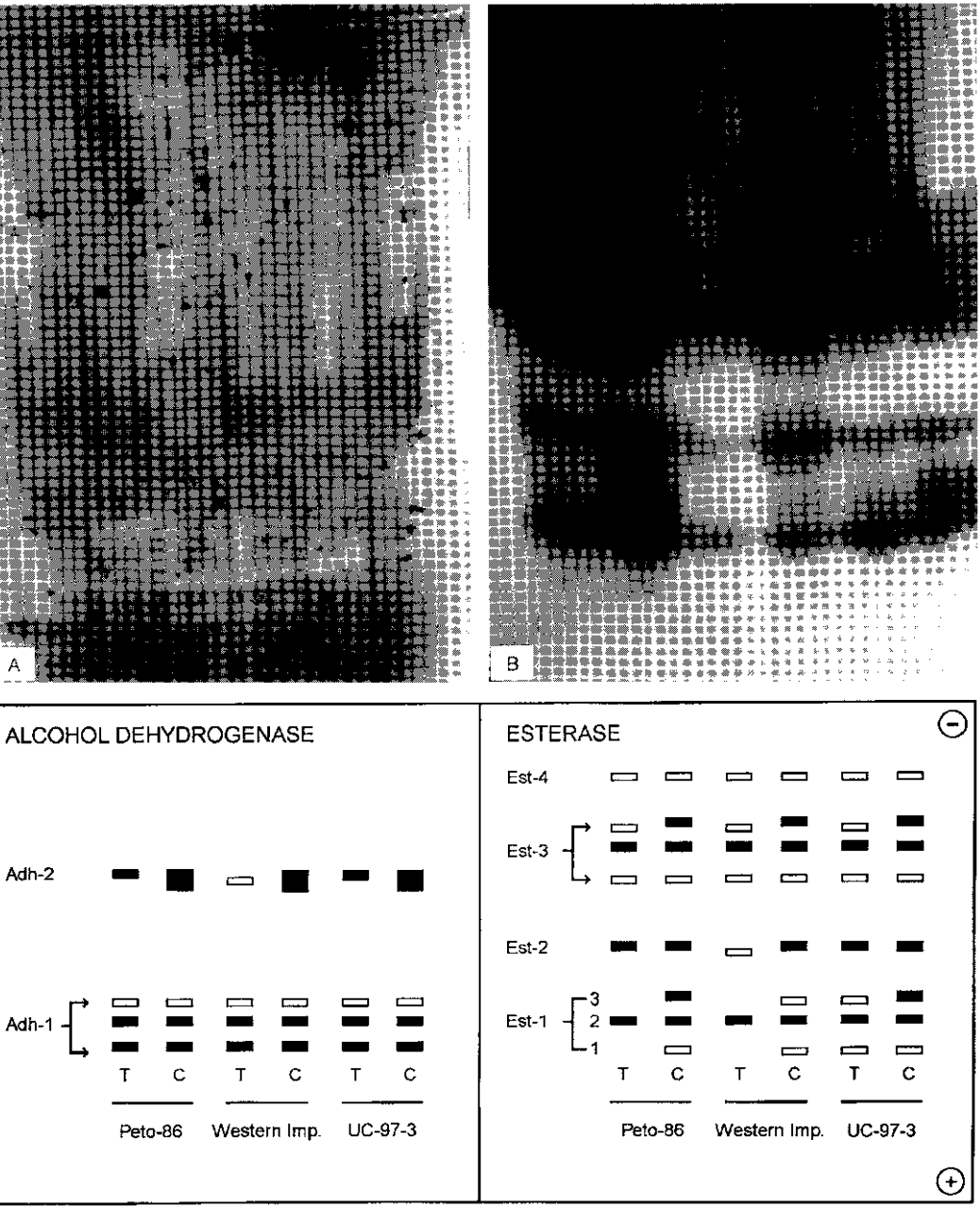

Fig. 3. Alcohol dehydrogenase (*A*) and esterase (*B*) isozyme patterns in callus cultures of three tomato genotypes (Peto-86, Western Improve, and UC-97-3) grown in the presence of 0.1 (*C*) and 100 μM Cu (*T*) for 4 weeks.

programme in tomato was altered by copper treatment to induce the production of these enzymatic bands for specific metabolic pathways involved in copper tolerance.

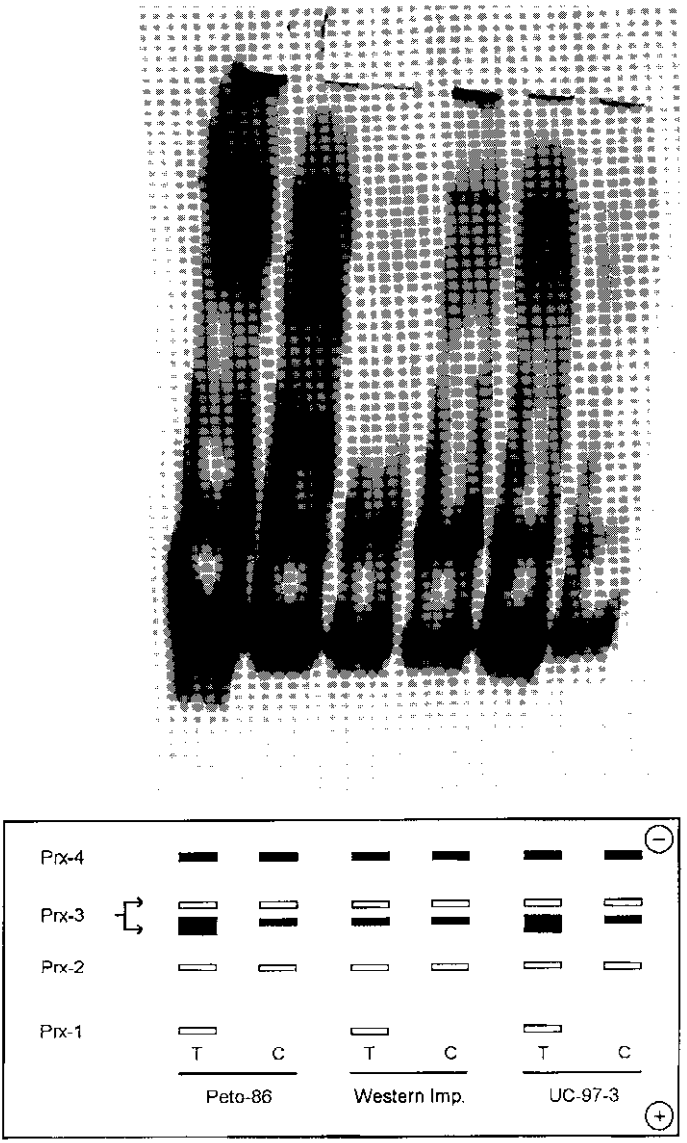

Fig. 4. Peroxidase isozyme patterns in callus cultures of three tomato genotypes (Peto-86, Western Improve, and UC-97-3) grown in the presence of 0.1 (*C*) and 100 μM Cu (*T*) for 4 weeks.

Without knowing the detailed biochemical and physiological basis of Cu tolerance, assumptions regarding the properties of polypeptides involved in Cu tolerance are difficult to make. It is likely that proteins are involved at some level

of the tolerance mechanism unless one invokes a hypothesis in which RNA or DNA itself is the basis of tolerance. Identification of polypeptides involved in Cu tolerance would open up avenues for molecular analysis to identify the genes responsible for the synthesis of metal-binding proteins/peptides in plant. The most direct method will be to determine the amino acid sequence of presumed plant metal-binding proteins/peptides and to synthesize an oligonucleotide predicted from that sequence which can be used as a probe to search for native DNA sequences by hybridization (Tomsett and Thurman 1988). For phytochelatins, the search for genes is more complex because they have γ-carboxamide bonds in their structure which clearly indicate that they can not be gene encoded (Robinson *et al.* 1988). The elucidation of the regulation of phytochelatin synthesis must be rely on characterization of the enzymes involved and the genes which encoded them. The identification of the genes encoding both metal-binding proteins and enzymes involved in phytochelatin synthesis is an obvious first step towards the elucidation of the molecular mechanism of heavy metal tolerance in plants.

The present investigation revealed that Cu-stress caused changes in gene expression which resulted in changes in protein and isozyme synthesis. In addition, the mechanism(s) of copper tolerance in tomato may have its genetic basis at the cellular level, as well as at the whole plant level. This would open the possibility of breeding Cu-tolerant tomato at the cellular level.

References

Barker, W.G.: Toxicity levels of mercury, lead, copper and zinc in tissue culture systems of cauliflower, lettuce, potato and carrot. - Can. J. Bot. **50**: 973-976, 1972.

Doncheva, S., Nikolov, B., Ogneva, V.: Effect of copper excess on the morphology of the nucleus in maize root meristem cells. - Physiol. Plant. **96**: 118-122, 1996.

Gekeler, W., Grill, E., Winnacker, E.L., Zenk, M.H.: Survey of the plant kingdom for the ability to bind heavy metals through phytochelatins. - Z. Naturforsch. **44**: 361-369, 1989.

Grill, E., Winnacker, E., Zenk, M.H.: Phytochelatins, a class of heavy-metal-binding peptides from plants, are functionally analogous to metallothioneins. - Proc. nat. Acad. Sci. USA **84**: 439-443, 1987.

Jackson, P.J., Naranjo, C.M., McClure, P.R., Roth, E.J.: The molecular response of cadmium resistant *Datura innoxia* cells to heavy metal stress. - In: Key, J.L., Kosuge, T. (ed.): Cellular and Molecular Biology of Plant Stress. Pp. 145-160. Alan R. Liss, New York 1985.

Laemmli, U.K.: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. - Nature **227**: 680-685, 1970.

Lolkema, P.C., Donker, M.H., Schonten, A.J., Ernst, W.H.O.: The possible role of metallothioneins in copper tolerance of *Silene cucubalus*. - Planta **162**: 174-179, 1984.

Manyowa, N.M.: The genetics of aluminum, excess boron, copper and manganese stress tolerance in the tribe *Triticeae* and its implications for wheat improvement. - Ph.D. Thesis, Cambridge University, Cambridge 1989.

Murashige, T., Skoog, F.: A revised medium for rapid growth and bioassay with tobacco tissue cultures. - Physiol. Plant. **15**: 473-479, 1962.

Rauser, W.E.: Copper-binding protein and copper tolerance in *Agrostis gigantea*. - Plant Sci. Lett. **33**: 239-247, 1984.

Rauser, W.E., Curvetto, N.R.: Metallothionein occurs in roots of *Agrostis* tolerant to excess copper. - Nature **287**: 563-566, 1983.

Reddy, P.J., Vaidyanath, K.: *In vitro* characterization of salt stress effects and the selection of salt tolerant plants in rice (*Oryza sativa* L.). - Theor. appl. Genet. **71**: 757-760, 1986.

Reese, R.N., Wagner, G.J.: Properties of tobacco (*Nicotiana tabacum*) cadmium-binding peptide(s). - Biochem. J. **241**: 641-647, 1987.

Robinson, N.J., Barton, K., Naranjo, C.M., Sillerud, L.O., Trewhella, J., Watt, K., Jackson, P.J.: Characterization of metal-binding proteins from cadmium resistant plant cells. - Experientia **52** (Suppl.): 323-327, 1988.

Sachs, M.M., Ho, D.T.: Alternation of gene expression during environmental stress in plants. - Annu. Rev. Plant Physiol. **37**: 363-367, 1986.

Scheller, H.V., Huang, B., Hatch, E., Goldsbrough, P.B.: Phytochelatin synthesis and glutathione levels in response to heavy metals in tomato cells. - Plant Physiol. **85**: 1031-1035, 1987.

Stavarek, S., Rains, D.: The development of tolerance to mineral stress. - Hort. Sci. **19**: 13-18, 1984.

Steffens, J.C.: The heavy metal-binding peptides of plants. - Annu. Rev. Plant Physiol. Plant mol. Biol. **41**: 553-575, 1990.

Steffens, J.C., Hunt, D.F., Williams, B.G.: Accumulation of non-protein metal-binding $(\gamma$-glutamyl-cysteinyl$)_n$-glycine in selected cadmium-resistant tomato cells. - J. biol. Chem. **261**: 13879-13882, 1986.

Stobart, A.K., Griffiths, W.T., Ameen-Bukhari, I., Sherwood, R.P.: The effect of Cd^{2+} on the biosynthesis of chlorophyll in leaves of barley. - Physiol. Plant. 63: 293-298, 1985.

Tamás, L., Huttová, J., Žigová, Z.: Accumulation of stress-proteins in intercellular spaces of barley leaves induced by biotic and abiotic factors. - Biol. Plant. **39**: 387-394, 1997.

Tomsett, A.B., Thurman, D.A.: Molecular biology of metal tolerance of plants. - Plant Cell Environ. **11**: 383-394, 1988.

Vallejos, C.: Enzyme activity staining. - In: Tankesley, S.D., Orton, T.J. (ed.): Isozymes in Plant Genetics and Breeding. Pp. 469-516. Elsevier, Amsterdam 1983.

Walker, C.D., Webl, J.: Copper in plants. Forms and behaviors. - In: Loneragan, J.F., Robson, A.D., Graham, R.D. (ed.): Soils and Plants. Pp. 189-212. Academic Press, London 1981.

Wu, L., Antonovics, J.: Zinc and copper tolerance of *Agrosis stolonifera* L. in tissue culture. - Amer. J. Bot. **65**: 268-271, 1978.