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Induction of heavy-metal binding phytochelatins by inoculation of cell cultures in standard media

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ABSTRACT

A large increase in phytochelatin (PC) synthesis occurred when cell cultures of different plant species were transferred from spent medium to fresh standard media. Phytochelatin accumulation correlated with the initial concentration of zinc ions in the nutrient solution. After reaching stationary growth phase, phytochelatins had almost disappeared from the cells which indicates a high turnover of these molecules under normal conditions. No significant formation of the heavy-metal complexing phytochelatins was observed if the microelement ions zinc and copper were omitted from the nutrient solutions for plant cell cultures. Both the induction and degradation phenomena of these peptides indicate that phytochelatins are involved in metal ion homeostasis in plants.

INTRODUCTION

Inoculation of plant suspension cells from logarithmic or stationary growth phases into fresh cell culture media causes considerable changes in the metabolism of the cultured cells. For instance, the activation of protein synthesis (Verma and Marcus, 1974), induction of nitrate and nitrite reductases (Filner, 1966; Chroboczek-Kelker and Filner, 1971), specific increase of enzymes of the phenylpropanoid pathway (Hahlbrock and Wellmann, 1973) and degradation of starvation related proteins (Walter and Hahlbrock, 1985) have been reported.

One of the general features which occur during the cell transfer process from spent to fresh medium is the exposure of the cells to a fresh supply of micronutrients such as Zn^{2+} and Cu^{2+}. These heavy metals are essential for plant metabolism and are contained in various amounts in every cell culture medium. We have shown previously that addition of sublethal quantities of many metals such as Cd^{2+}, Pb^{2+}, Zn^{2+}, Hg^{2+}, Cu^{2+}, Ni^{2+}, etc. to cell suspension cultures resulted in the synthesis of a new class of heavy metal-binding peptides with the general structure $[\gamma\text{-Glu-Cys}]_n\text{-Gly}$ (n = 2-11), the PC's, or the homo-PC's $[\gamma\text{-Glu-Cys}]_n$ -ß-Ala (n = 2-8) (Grill et al., 1985, 1986). The occurrence of these peptides in metal-exposed cell cultures has been independently verified by Steffens et al. (1986) and Scheller et al. (1987). The function of PC's was assumed to be detoxification and homeostasis of heavy metal ions in plants (Grill et al., 1987). Metal ions such as Cu^{2+} and Zn^{2+} can interfere with sulfhydryl groups of proteins, therefore, these reactive metal ions should be physiologically inactivated in the cell to allow undisturbed metabolism in plants. This could be achieved by the sequestration of the metals with specific organic molecules within the cell. These molecules must play a dual role, firstly they have to complex and thus store the metals and secondly they have to transfer the essential metal to newly synthesized apoenzymes which require Cu or Zn ions for catalytic activity. As part of our current studies on metal metabolism in plants we report in this communication that the homeostasis of essential heavy metal ions seems to be fulfilled by formation and degradation of PC's. The synthesis of these heavy metal-binding peptides is induced by the low concentrations of zinc and copper ions present in standard media for plant cell culture.

MATERIAL and METHODS

Plant material Cell cultures were provided by our cell culture laboratory. These strains had been kept for at least the past 10 years in culture in the media indicated in Table 1. The cells were grown (300 ml Erlenmeyer flasks, 75 ml medium) on a gyratory shaker (100 rpm) at 23°C in continuous light (650 lux), and inoculation into fresh medium was performed at late stationary phase using approximately a 20% inoculum (fwt).

Media The media used were as compiled in Street (1973). In the experiments indicated, cells were grown in the same media but omitting both Cu and Zn salts.

Assay for phytochelatins Suspension cells were separated from media by suction filtration. Aliquots (0.4 g fwt) were suspended in a solution (0.4 ml) containing 1 N NaOH and 1 mg of $NaBH_4$ per ml. After sonication and centrifugation (5 min, 11 000 x g) of the sample, the supernatant (0.5 ml) was acidified with 3.6 N HCl (100 µl). Precipitating material was sedimented again, and the cleared extract was separated by HPLC (Nucleosil C-18, flow rate 2.0 ml/min) at pH 2.7 (0.05% phosphoric acid in 0 to 20% acetonitrile-H_2O). Detection of the sulfhydryl-containing PC's was performed by post-column derivatization with 75 µM 5,5'-dithio-bis-(2-nitrobenzoic acid), Ellman's reagent, in 50 mM potassium phosphate buffer (pH 7.6) at a flow rate of 2 ml/min and detection at 410 nm. Zn and Cu ions were detected by atomic absorption (Perkin-Elmer flame mode and graphite furnace technique, respectively).

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RESULTS and DISCUSSION

The first indication that PC's were involved in heavy metal ion homeostasis was the observation that plant suspension cells grown in standard medium lacking zinc and copper ions contained no detectable or very low levels of PC's. However, inoculation of these cells into the unmodified medium providing 'physiological' concentrations of essential heavy metal ions led to an immediate rise in PC concentration which reached its maximum in mid log to late log phase. Subsequently the level of metal-binding peptides declined. This is exemplified by Fig. 1.

Rauvolfia serpentina cells were grown for one growth cycle in a Cu- and Zn-free medium to deplete PC's. Transfer of the cells into the complete nutrient solution of Linsmaier and Skoog resulted in an at least 100-fold increase in the total amount of the metal-chelating peptides on the 6th day after inoculation. This PC induction was not observed in cells cultivated in the absence of zinc and copper ions. Omission of the heavy metal ions for one or two growth cycles did not influence cell mass production on a dry weight basis. During stationary phase a strong decrease of the total amount of the metal-chelating peptides in the cells was observed. Since no PC's could be identified in the culture medium, this finding clearly indicates that PC's are degraded, probably to their amino acid constituents.

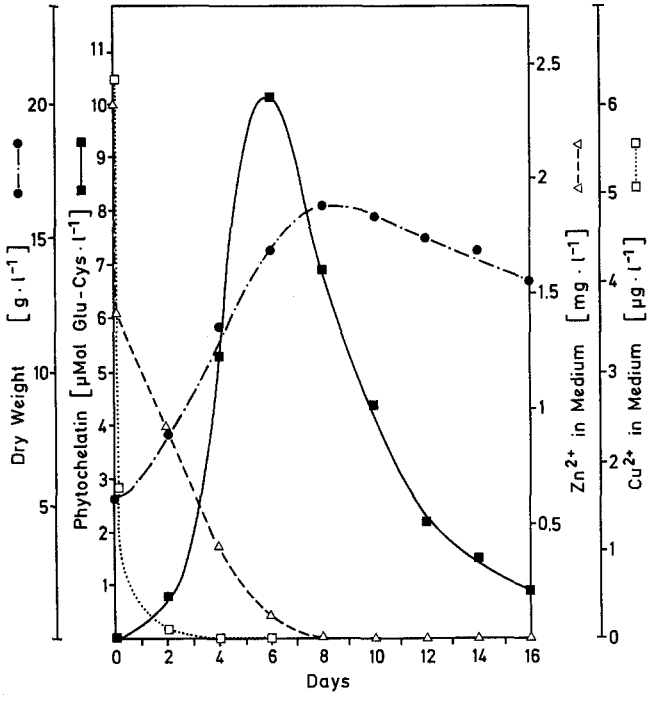

Fig. 1 Time course for growth (as dry weight (●)) and total phytochelatin synthesis (■) of Rauvolfia serpentina cells upon dilution of cells into fresh complete Linsmaier-Skoog medium (80 g fwt x l^{-1}). The disappearance of zinc (Δ -Δ) and copper (□ ··· □) ions from the medium was monitored by atomic absorption spectroscopy. The cells were previously grown in modified Linsmaier-Skoog medium for one growth cycle in the absence of copper and zinc ions to deplete endogenous phytochelatins. Cells transferred into medium without these heavy metal ions revealed no reduced growth rate and showed a phytochelatin level below 0.3 µmol Glu-Cys units per liter of culture throughout the cultivation period.

The loss of Zn and Cu ions from the medium showed a biphasic course. After inoculation of Linsmaier and Skoog medium with cells there was a rapid disappearance of both metals from the medium. About 40% of Zn and 70% of the Cu ions present in the medium were most likely adsorbed to the cell walls within half an hour after transfer of the cells. This phase was followed by a slower, probably metabolic phase in which the copper and the zinc ions were depleted from the medium within 2 days and 6 - 8 days, respectively. The fresh medium contained 0.1 µM copper ions and 37 µM zinc ions. Concomitant with the drop in heavy metal ions in the medium, an increase in PC's in the cells occurred. The highest intracellular PC concentration in the culture was observed at the time when the Zn ion had almost completely disappeared from the medium. This induction of the formation of heavy-metal chelating peptides in the R. serpentina cell suspension culture was not restricted to a specific medium. As shown in Fig. 2,

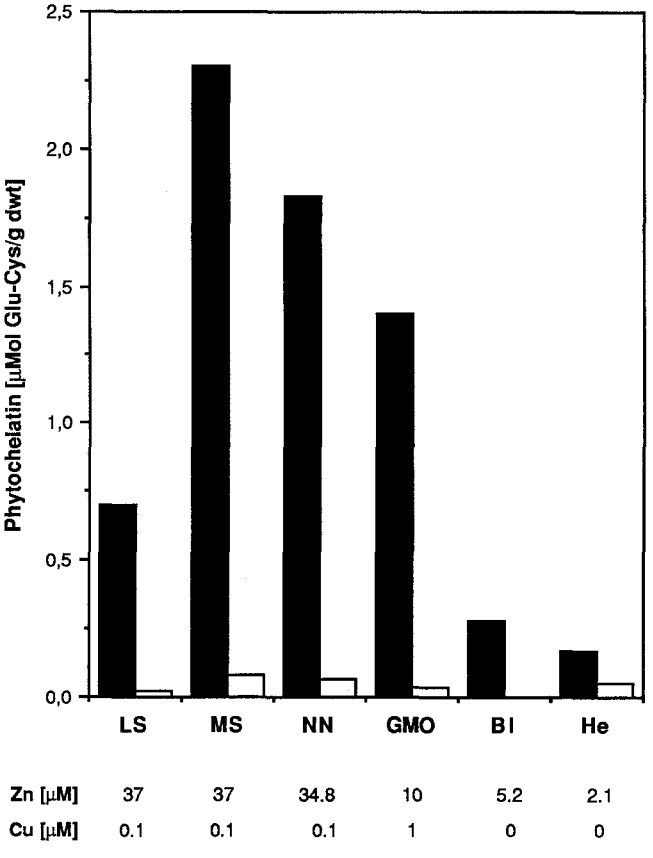

Fig. 2 Induction of phytochelatins after transfer of R. serpentina cells into various plant cell culture media. Cells were depleted in phytochelatins (<0.05 µmol/g dwt) by cultivation in heavy metal-free Linsmaier-Skoog medium for one week. Phytochelatin level was analyzed 6 days after inoculation into fresh complete (shaded bars) and zinc- and copper-free (open bars) medium. Growth in all cases was 14 g dwt x l^{-1}. Standard media employed: LS = Linsmaier and Skoog, MS = Murashige and Skoog, NN = Nitsch and Nitsch, GMO = Gamborg, Miller and Ojiama, Bl = Blaydes, He = Heller. Heavy metal concentrations in these media are indicated below the histogram.

the induction of heavy metal-sequestering peptides was observed in cells cultivated in all frequently used media for plant cell cultures. The zinc and copper ions present in the media were responsible for PC induction since omission of these ions resulted in negligible PC levels. Cobalt ions, which are also present in the nutrient solution, are not able to induce PC's(Grill et al., 1987). Thus PC formation correlated with the concentration of the heavy metals zinc and copper in the various media. The highest peptide induction was observed in Murashige-Skoog medium which contains 37 µM heavy metals, the smallest induction in Heller's medium with 2.1 µM $ZnSO_4$ and no Cu ions. Invariably, however, Murashige-Skoog medium gave a higher peptide yield than Linsmaier-Skoog medium which is surprising since both media contain the same levels of zinc and copper ions and may reflect a different uptake or turnover of the metal ions. The dependence of PC formation on the concentration of zinc ions in the medium is clearly shown in Fig. 3. Supplementation of heavy metal-free Linsmaier and Skoog medium with varying amounts of zinc ions leads to a linear relationship between PC accumulation and zinc ion concentration in the medium.

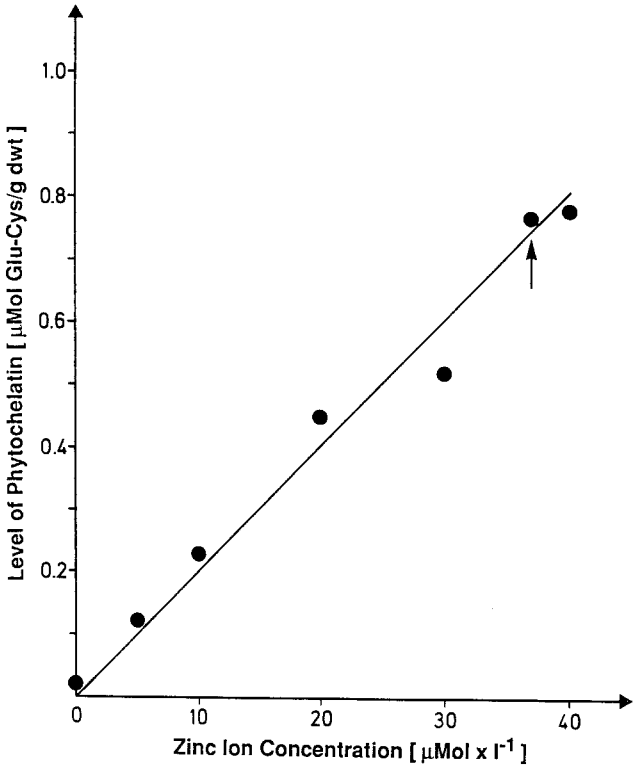

Fig. 3 Dependence of phytochelatin induction on the concentration of zinc ions in the medium. Rauvolfia serpentina cells cultivated for one growth cycle in medium lacking zinc and copper ions were transferred to copper-free Linsmaier and Skoog medium supplemented with varying concentrations of zinc ions. After 5 days of cultivation the phytochelatin level was determined. Arrow indicates the normal concentration of Zn^{2+} in the Linsmaier and Skoog medium.

Formation of heavy metal-binding peptides after transfer of cells into fresh medium is not confined to Rauvolfia serpentina. Exposure of several plant species to the low concentrations of zinc and copper ions present in Linsmaier and Skoog or Murashige and Skoog medium, indicated a general phenomenon of PC induction by 'physiological' concentration of zinc and copper ions (Table 1). Cells in the stationary growth phase were transferredinto fresh culture medium. The PC content of the inoculum was without exception very low (<50 nmol per gram dry weight). During the logarithmic growth phase (day 6) the PC level of the cultures increased by a factor of 10 to 30, and then decreased at the stationary phase (day 12) generally 4 to 10-fold. Analysis of the spent medium gave no indication that PC's, either in the metal-complexed or in the metal-free form, were excreted by the cells. This transient rise and decline in PC concentration corresponded to the metabolic metal uptake and, possibly, to the release of micronutrients. It will be of interest to investigate the fate of the heavy metal ions in the stationary phase as to whether these ions are transferred from the PC complex to metal requiring proteins or are released into vacuoles or cell walls for storage.

Days of cultivation	0	6	12	0	6	12
Culture	Phytochelatin content (µMol Glu-Cys within phytochelatin x l^{-1})			Cell mass (g dwt x l^{-1})		
Agrostis tenuis	0.42	4.30	0.30	10.8	20.5	18.8
Beta vulgaris	0.22	5.28	1.78	4.8	9.9	12.4
Lupinus vulgaris	0.03	0.90	0.23	2.4	7.1	16.6
Minuartia verna	0.17	1.34	0.79	5.7	14.6	18.8
Rauvolfia serpentina	0.08	6.43	0.78	3.4	7.4	14.7
Rosa canina	0.61	6.20	1.17	5.9	16.7	15.6
Silene cucubalus	0.10	2.20	0.98	1.5	3.6	12.5

Table 1 Phytochelatin concentration and growth (as dwt) of different plant species inoculated from late stationary phase (in complete medium) into fresh media. Analysis was performed 0 (inoculum), 6 and 12 days after inoculation into fresh medium. B. vulgaris cells were grown in Murashige-Skoog medium, all others in Linsmaier-Skoog medium.

In view of these findings, we can assume that zinc and copper ions sequestration by phytochelatin peptides invariably occurs if cells are transferred into fresh plant culture media. This is a general phenomenon which has escaped attention up to now. Phytochelatins under these conditions are transient metabolites and show considerable turnover. They could fulfill the requirements of metal homeostasis by complexing the cellular

surplus of heavy metal ions which are rapidly taken up from the culture media. In this form the metal ions are stored and prevented from interfering with metal-sensitive functional groups of enzymes and structural proteins. In the course of the growth process, the metals are probably released from the phytochelatin complex by transfer to the apoforms of metalloproteins and also to a final storage site within the cells. Thus the metal-free phytochelatin peptides are subsequently degraded. Phytochelatins therefore play not only a role in the detoxification of metal ions such as Cd^{2+}, Hg^{2+}, Pb^{2+}, Ag^{+}, but are part of normal reactions involved in the homeostasis of essential metals, the microelements Zn, Cu and probably Ni (Brown et al., 1987).

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