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Scientific paper

CADMIUM AND ZINC INDUCED SIMILAR CHANGES IN PROTEIN AND GLYCOPROTEIN PATTERNS IN TOBACCO (*NICOTIANA TABACUM* L.) SEEDLINGS AND PLANTS

Petra PEHAREC ŠTEFANIĆ[1], Sandra ŠIKIĆ[2], Petra CVJETKO[1], and Biljana BALEN[1]

Department of Molecular Biology, Faculty of Science, University of Zagreb, Zagreb, Croatia[1], Department of Ecology, Institute of Public Health, Zagreb, Croatia[2]

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The effects of 10 μmol L^{-1} and 15 μmol L^{-1} cadmium (Cd), a nonessential toxic element and 25 μmol L^{-1} and 50 μmol L^{-1} zinc (Zn), an essential micronutrient, on proteins and glycoproteins of *Nicotiana tabacum* L. seedlings and plants were investigated after exposure to each metal alone or to their combinations. Changes in only few polypeptides related to heavy metal treatments were observed in tobacco seedlings and leaves of adult plants, while the greatest change in total soluble protein pattern was observed in plant roots. Differences between control and treated tobacco tissues were more pronounced in the glycoprotein pattern, which was analysed by application of different lectins. The majority of the detected glycoproteins in leaves and roots of adult plants can be considered as a result of enhanced glycosylation due to heavy metal stress. The difference in glycoproteins between Cd and Zn application on tobacco seedlings and adult plants could not be determined since enhanced glycosylation was noticed after treatment with either metal alone or in combination. Therefore, it can be concluded that both metals induced N- and O-glycosylation as a result of changed environmental conditions.

KEY WORDS: *heavy metals, lectins, protein pattern*

Agricultural plants face a variety of abiotic and biotic stresses, which are major causes limiting crop production. Among abiotic stressors, heavy metal contamination represents a global environmental problem endangering humans, animals, and plants. Cadmium (Cd) is very toxic and without any metabolic significance (1). It is especially dangerous due to its long biological half-life. When released into the environment, Cd becomes accessible to plants, where its toxic effects involve a decrease in chlorophyll and carotenoid content, as well as changes in photosynthetic activity (2), growth inhibition and root damage (1), reduction of water and nutrient uptake (3), lipid peroxidation (4) and protein degradation (1, 5). Toxicological properties of Cd originate from its chemical similarity to zinc (Zn), an essential micronutrient of great chemical and metabolic significance in plant systems (6). Zn plays a fundamental role in several cellular functions such as protein metabolism, gene expression, chromatin structure, photosynthetic carbon metabolism, and indole acetic acid metabolism (7) and is involved in the catalytic function of many enzymes and structural stability of various cell proteins (8). Cd frequently accompanies Zn minerals in the environment (9), which is why they have often been investigated together for their mutual effect on different plants. Up to this moment, mostly antagonistic interaction was suggested; it has been

found that Zn can lessen physiological damage caused by Cd (10), suppress Cd uptake (6), and decrease Cd-induced oxidative stress (11).

It has been documented that exposure to heavy metals can cause changes in the expression of cellular plant proteins (12, 13). Some heavy metals, such as manganese (Mn) and Zn, stimulate protein synthesis (14), while other metabolites, such as Cd, interfere with this process (15). Sobkowiak and Deckert (16) reported that Cd induced changes in the protein pattern in soybean cell suspension culture. Moreover, dramatic changes in the protein pattern of barley seedlings exposed to Cd were revealed by SDS-PAGE (17), while a proteomic approach has been adopted for the analysis of protein profile alternations during the germination of rice following exposure to Cd (18).

Posttranslational modifications such as glycosylation are important for altering the properties and functions of proteins. The oligosaccharide chain can be either N- or O-linked. N-glycosylation occurs in the endoplasmic reticulum (ER) and the primary oligosaccharide chain is further processed when it exits the ER and passes through the Golgi apparatus (GA). The latter cell compartment is also a site of protein O-glycosylation. Glycosylation can change basic biological functions of a protein including immunogenicity, specific activity, and the ligand-receptor interaction. Only few studies have been conducted on plants to reveal whether environmental conditions act upon protein glycosylation. The analysis of N-linked glycans of soluble endogenous glycoproteins from the leaves of tobacco demonstrated that developmental processes and different growth conditions could influence glycosylation (19, 20). It was found that N-glycosylation of extracellular (21-23) as well as cellular proteins (22, 24-27) was affected by *in vitro* culture conditions. However, modest information about the abiotic stress related to glycoprotein patterns is available so far. It has been revealed that the mutation in *STT3* gene, which encodes important subunits of the enzyme oligosaccharyltransferase involved in protein N-glycosylation, increases the sensibility to salt and osmotic stress (28). Moreover, Kang et al. (29) found that salt tolerance of *Arabidopsis thaliana* requires of N-glycosylated proteins to mature in the GA. In the basal parts of rice leaf sheaths, cold stress altered the glycosylation profile of calreticulin, a key protein that regulates the quality control of other proteins (30). Carpena et al. (31) suggested that glycoprotein accumulation might be considered as a useful indicator of Cd-induced stress in white lupin nodules. In a recent publication Zhang et al. (32) suggest that protein N-glycoslyation might have a key role in plant development and abiotic stress response. The role of O-glycosylation in plant cells grown in a stressful environment is even less studied than N-glycosylation. The report by Johnson et al. (33) indicates that arabinogalactans, a group of plant specific O-glycoslyated proteins, are likely to be important during plant development and in response to abiotic stress.

Tobacco, *Nicotiana tabacum* L., became a model plant in many studies due to its relative tolerance to environmental stress and wide distribution (34). All of the first achievements in plant genetic engineering are mostly based on the work with tobacco. This plant has been employed in studies on the production of useful recombinant proteins and antibodies, which have an application in medicine and industry (35). Moreover, tobacco has been found to be an extremely versatile system for all aspects of abiotic stress research, including the studies of physiological mechanisms, which lead to adverse environmental conditions (36, 37).

The aim of the present work was to investigate the effects of Cd and Zn, applied separately or in combinations, on protein and glycoprotein patterns of soluble cellular proteins in seedlings and adult plants of tobacco.

MATERIALS AND METHODS

Chemicals and instrumentation

$CdCl_2$, $ZnCl_2$, $AgNO_3$, glycine, sucrose, ascorbic acid, methanol, HCl and NaCl were obtained from Kemika (Zagreb, Croatia), while polyvinylpyrrolidone (PVP), bovine serum albumin (BSA), Tris, Phytagel, Tween® 20, EDTA, Con A, 4-chloro-1-naphthol and peroxidase were purchased from Sigma (Steinheim, Germany). Distilled and deionized water from Milli-Q water systems (Millipore, Bedford, MA, USA) was used to prepare the nutrient media and extraction buffers. UV-VIS spectrophotometer ATI/Unicam UV4-100, (Cambridge, UK) was used to determine the protein concentration in extracts. Pure nitrocellulose membrane – 0.45 mm as well as electrophoresis system for protein separation and electroblotting (Mini-PROTEAN 3 Cell and Mini Trans-Blot Cell)

were purchased from Bio-Rad (Bio-Rad Laboratories GmbH München, Germany). Protein Molecular Weight Marker was obtained from Fermentas (St. Leon-Rot, Germany). Ponceau-S stain and digoxigenin-labeled lectins GNA, DSA, PNA MAA, and SNA (their descriptions are given in Table 1) (DIG Glycan Differentiation Kit) were obtained from Roche Applied Science (Mannheim, Germany). Gels and membranes were scanned using HP Scanjet 2400 scanner (Hewlett-Packard Company, USA).

Plant material and heavy metal treatments

Seeds of *Nicotiana tabacum* L. cv Burley were surface sterilised with 50 % NaOCl, washed with distilled H_2O several times and subsequently germinated in sterilised nutrient medium. The medium was prepared according to Murashige and Skoog (38) with addition of the 500 mg L^{-1} MES [2-(N-morpholino)ethanesulfonic acid], 1.5 g L^{-1} sucrose, and 2.2 g L^{-1} Phytagel (pH 5.6) (39) at 24 °C with 16 h : 8 h light/dark cycle and light intensity of 90 μE m^{-2} s^{-1}. In these conditions seedlings were grown for 90 days until adult plants were obtained.

For heavy metal treatment of seedlings, tobacco seeds were germinated for 30 days on solid MS nutrient medium with the addition of Cd, Zn or their combinations. Cd treatments in concentrations of 10 μmol L^{-1} and 15 μmol L^{-1} were prepared by adding a stock solution of $CdCl_2$ to the nutrient medium. Zn was added as $ZnCl_2$ to the nutrient medium in the amounts suitable to achieve concentrations of 25 μmol L^{-1} and 50 μmol L^{-1}. Tobacco seedlings were also exposed to the combinations of metals (10 μmol L^{-1} or 15 μmol L^{-1} Cd with 25 μmol L^{-1} and 50 μmol L^{-1} Zn). For protein extraction, whole tobacco seedlings were used.

For exposure to heavy metals, adult plants obtained on the solid MS medium were transferred to the liquid medium of the same composition but with the addition of either metal alone (10 μmol L^{-1} or 15 μmol L^{-1} Cd and 25 μmol L^{-1} or 50 μmol L^{-1} Zn) or their combinations (10 μmol L^{-1} or 15 μmol L^{-1} Cd with 25 μmol L^{-1} and 50 μmol L^{-1} Zn) and then treated for 7 days. For protein and glycoprotein pattern analyses, leaves and roots were analysed separately.

Protein extracts, SDS-PAGE and electroblotting

Total soluble proteins from tobacco seedlings were extracted by grinding 0.4 g of fresh tissues in 1.5 mL of 0.1 mol L^{-1} Tris/HCl buffer, pH 8.0 at 4 °C containing 17.1 % sucrose, 0.1 % ascorbic acid, 0.1 % cystein (40), and (10 to 15) mg of PVP. For the extraction of total soluble proteins from leaves and roots of adult plants, 0.4 g of fresh tissue was homogenized in 1.0 mL cold (0 to 4) °C extraction buffer (pH 7.5) containing 30 mmol L^{-1} Tris/HCl, 5 mmol L^{-1} EDTA, 20 mmol L^{-1} NaCl, and (10 to 15) mg of PVP. Homogenates were centrifuged at 20,000 x*g* and 4 °C for 15 min. Supernatants were centrifuged again at 20,000 x*g* and 4 °C for 60 min. The supernatant was collected and protein content was determined according to Bradford (41) using BSA as a standard. Obtained supernatants were used for the analysis of total soluble proteins and glycoproteins.

Total soluble proteins were analysed by SDS-PAGE in 8 % to 18 % T (2.67 % C) gradient gels, with the buffer system of Laemmli (42). For the SDS-PAGE, the same amount of protein (10 μg) per sample was loaded. Proteins migrated through stacking and separating gels at 100 V and 200 V, respectively. Protein bands were visualised by silver staining (43) and gels were scanned as an 8 bit grey scale Tiff-images.

Table 1 *Major N- and O-linked carbohydrate binding specificities of different plant lectins applied.*

Lectin	Taxonomic lectin source	Specificity toward carbohydrate moieties	Type of glycans
Con A	*Canavalia ensiformis*	*α*-D-Glu *α*-D-Man	core N-glycan structure (44)
GNA	*Galanthus nivalis*	*α*-D-Man	high-mannose N-glycans (44)
PNA	*Arachis hypogaea*	*β*-Gal-(1,3)-GalNAc	O-glycosidically linked carbohydrates (45)
DSA	*Datura stramonium*	Gal-(1,4)-GlcNAc	complex and hybrid N-glycans (46)
MAA	*Maackia amurensis*	NeuAc-α(2,3)-Gal	sialic acid in complex N- and O-glycans (47)
SNA	*Sambucus nigra*	NeuAc- α (2,6)-Gal	sialic acid in complex N- and O-glycans (47)

For lectin assays, proteins were separated by SDS-PAGE in 12 % T (2.67 % C) polyacrylamide gels (42). The same amount of protein (15 µg) per sample was loaded. Proteins migrated through stacking and separating gels at 100 V and 200 V, respectively. Subsequently, proteins were electroblotted to the nitrocellulose membrane in a mini trans-blot cell at 60 V for 60 min. The transfer buffer was 20 mmol L^{-1} Tris-HCl, 150 mmol L^{-1} glycine and 10 % methanol. The membrane was stained with Ponceau-S stain to confirm the complete transfer of proteins. The stain was washed off with distilled water. The unoccupied sites of the membrane were blocked by incubating the membrane with 0.1 % Tween® 20 in TBS buffer, pH 7.5 at 4 °C overnight. Glycoproteins with D-manose in their glycan component were detected on nitrocellulose membrane by the reaction with Con A. Bands were visualised by peroxidase reaction using 4-chloro-1-naphthol as a substrate (44). The glycan part of proteins was further characterised according to binding of digoxigenin-labelled lectins GNA(45), DSA (46), PNA (46), MAA (47), and SNA (47) (Table1). The staining procedure was performed following the manufacturer's instructions. Membranes were scanned as an 8 bit grey scale Tiff-images.

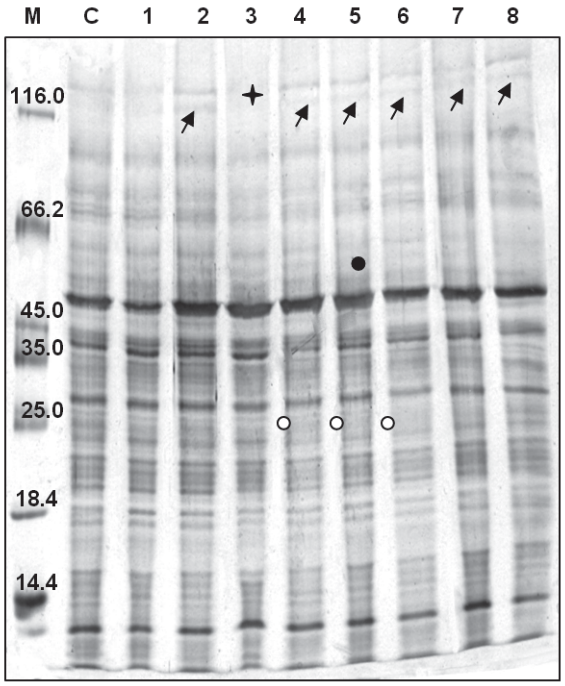

Figure 1 *Total soluble cell proteins of tobacco seedlings separated by SDS-PAGE in gradient 8 % to 18 % gel and silver stained. M – protein molecular weight marker, C – control; (1 to 10) µmol L^{-1} Cd; (2 to 15) µmol L^{-1} Cd; (3 to 25) µmol L^{-1} Zn; (4 to 50) µmol L^{-1} Zn; (5 to 10) µmol L^{-1} Cd and 25 µmol L^{-1} Zn; (6 to 10) µmol L^{-1} Cd and 50 µmol L^{-1} Zn; (7 to 15) µmol L^{-1} Cd and 25 µmol L^{-1} Zn; (8 to 15) µmol L^{-1} Cd and 50 µmol L^{-1} Zn.*

RESULTS

Total soluble cell proteins

We observed changes in only few polypeptides related to heavy metal treatments in tobacco seedlings (Figure 1): protein band of 116 kDa (arrows) was present in all samples, although with weaker intensity in the control and treatments with lower concentration of both metals, 10 µmol L^{-1} Cd and 25 µmol L^{-1} Zn. Moreover, seedlings exposed separately to 10 µmol L^{-1} Cd and 25 µmol L^{-1} Zn exhibited weak expression of 118 kDa protein (star) in comparison with other treatments, while the 56 kDa (black circle) was missing after the combined treatments with 10 µmol L^{-1} Cd and 25 µmol L^{-1} Zn as well as with 15 µmol L^{-1} Cd and 50 µmol L^{-1} Zn. 25 kDa protein (white circles) was missing after the treatment with 50 µmol L^{-1} Zn as well as with combinations of 10 µmol L^{-1} Cd and both concentrations of Zn.

In tobacco leaves, a protein of 120 kDa (arrows) was expressed after exposure of adult plants to 10 µmol L^{-1} Cd, both Zn concentrations, and combined treatments with both Cd concentrations and 50 µmol L^{-1} Zn (Figure 2A). The 60 kDa polypeptide (star) was specific for combined treatments of 15 µmol L^{-1} Cd with 25 and 50 µmol L^{-1} Zn. In comparison to control, all leaves from treated plants exhibited a stronger expression of 45 kDa, 50 kDa and 55 kDa proteins (black circles).

The greatest change in total soluble protein pattern was observed in roots of adult plants (Figure 2B). The 20 kDa and 25 kDa proteins had a stronger expression in control in comparison with treated roots (stars). After the exposure to 50 µmol L^{-1} Zn, protein bands of 50 kDa, 70 kDa and 116 kDa (black circles) were observed in roots of adult plants with the strongest staining intensity. The 45 kDa proteins (black squares) were expressed after the treatment with 15 µmol L^{-1} Cd, 25 µmol L^{-1} Zn as well as in combined treatment with 15 µmol L^{-1} Cd and 50 µmol L^{-1} Zn, although they were present as very faint bands in combined treatments with 10 µmol L^{-1} Cd and 50 µmol L^{-1} Zn as well as 15 µmol L^{-1} Cd and 25 µmol L^{-1} Zn. Roots treated with 15 µmol L^{-1} Cd in combination with both

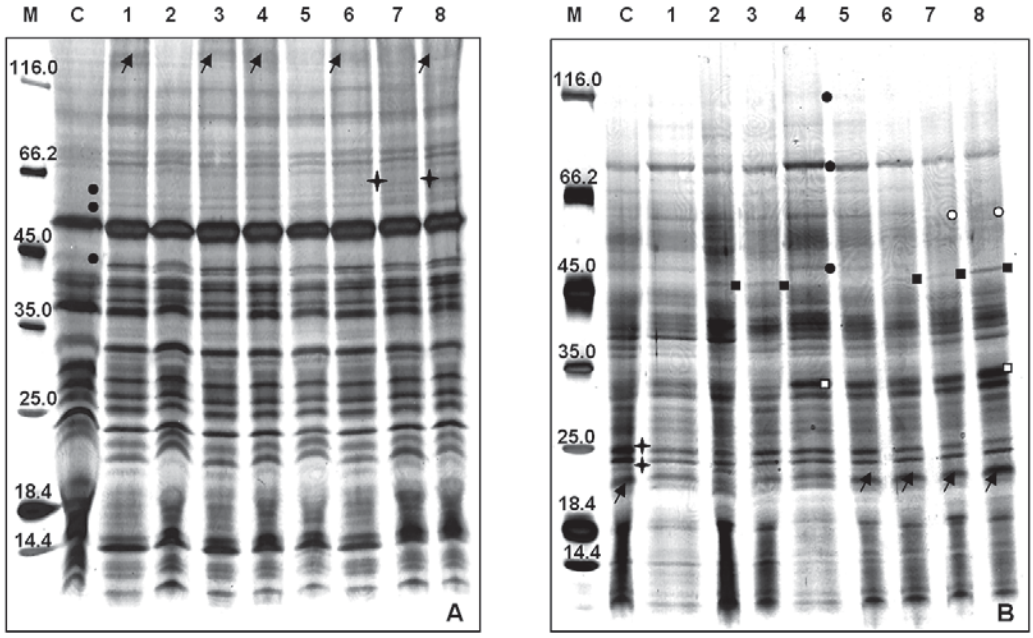

Figure 2 *Total soluble cell proteins of A) leaves and B) roots of adult tobacco plants separated by SDS-PAGE in gradient 8 % to 18 % gel and silver stained. For lane labels see Figure 1.*

Zn concentrations were devoid of the 60 kDa protein (white circles). Protein band of 30 kDa (white squares) had a stronger expression after the treatment with 50 µmol L^{-1} Zn alone and in combination with 15 µmol L^{-1} Cd in comparison with other treatments, while the 20 kDa polypeptide (arrows) was more pronounced in control roots and those exposed to combinations of metals compared to each metal alone.

Glycoproteins

In control and in treated seedlings, the 45 kDa glycoprotein (black frame) was detected, although in the treatment with 15 µmol L^{-1} Cd and 50 µmol L^{-1} Zn was present as a very faint band (Figure 3A). The 50 kDa glycoprotein (grey frame) was revealed in all samples, except in combined treatment with 15 µmol L^{-1} Cd and 50 µmol L^{-1} Zn. Control leaves and those treated with 10 µmol L^{-1} Cd and 50 µmol L^{-1} Zn alone and with combination of 10 µmol L^{-1} Cd and 50 µmol L^{-1} Zn, as well as 15 µmol L^{-1} Cd with 25 µmol L^{-1} Zn were characterised by the 35 kDa band (dashed frame). GNA revealed glycoproteins of 40 kDa (white circle) and 43 kDa (black circle) of the strongest staining intensity (Figure 3B), which were present in control and all treatments, except in combined treatment with 15 µmol L^{-1} Cd and 50 µmol L^{-1} Zn. Glycoproteins of 46 kDa (grey arrow) and 50 kDa (black arrow) were detected as very faint bands and were more pronounced in treatments with either metal alone than in combined treatments. The 20 kDa glycoprotein (star) was detected in control seedlings as well as in those treated with 10 µmol L^{-1} Cd, 50 µmol L^{-1} Zn, 15 µmol L^{-1} Cd and 25 µmol L^{-1} Zn, as well as 10 µmol L^{-1} Cd and 50 µmol L^{-1} Zn. Exposure to 50 µmol L^{-1} Zn resulted in the appearance of 15 kDa, 16 kDa and 17 kDa bands (asterisks), which were also detected in treatments with 10 µmol L^{-1} Cd and 50 µmol L^{-1} Zn as well as with 15 µmol L^{-1} Cd and 25 µmol L^{-1} Zn, but with weaker expression. In control and in treated seedlings, PNA revealed the 43 kDa glycoprotein (black arrow), whose expression was the strongest after treatment with 50 µmol L^{-1} Zn (Figure 3C). Combined treatment with 15 µmol L^{-1} Cd and 50 µmol L^{-1} Zn was devoid of the 38 kDa band (grey arrow), whose expression was the strongest in the treatment with 50 µmol L^{-1} Cd. Control seedlings and those exposed to 50 µmol L^{-1} Zn and combinations of 10 µmol L^{-1} Cd and 50 µmol L^{-1} Zn as well as 15 µmol L^{-1} Cd and 25 µmol L^{-1} Zn, were characterised by the presence of the 18 kDa glycoprotein (black circle). In the above mentioned treatments 17 kDa band was detected (star), while the exposure to 50 µmol L^{-1} Zn and to a combination of 15 µmol L^{-1} Cd and 25 µmol L^{-1} Zn resulted in the appearance of the 16 kDa glycoprotein (asterisk). DSA revealed only

two glycoproteins (Figure 3D). The 43 kDa one (black arrow) was missing from the treatments with 15 μmol L^{-1} Cd, 25 μmol L^{-1} Zn as well as from combined treatment with 15 μmol L^{-1} Cd and 50 μmol L^{-1} Zn. Exposure to 50 μmol L^{-1} Zn alone and to combinations of 10 μmol L^{-1} Cd and 50 μmol L^{-1} Zn as well as 15 μmol L^{-1} Cd and 25 μmol L^{-1} Zn resulted in the appearance of the 38 kDa glycoprotein (grey arrow). Nine glycosylated proteins were detected in seedlings with MAA (Figure 3E). The 43 kDa (black arrow) and 45 kDa (grey arrow) glycoproteins were present in control and all treatments as very strong bands, while the 66 kDa (black circle) and 68 kDa (white circle) ones were visible as proteins with weak staining intensity. Seedlings exposed to 10 μmol L^{-1} Cd and 50 μmol L^{-1} Zn as well as 15 μmol L^{-1} Cd and 25 μmol L^{-1} Zn were characterised by the presence of the 55 kDa glycoprotein (star), which was also detected as a very faint band in control seedlings and in those treated with single 10 μmol L^{-1} Cd and 50 μmol L^{-1} Zn. The 22 kDa band (asterisk) was present in treatments with 50 μmol L^{-1} Zn as well as with 15 μmol L^{-1} Cd and 25 μmol L^{-1} Zn, while the 20 kDa one (white star) was detected in control and in treatments with 10 μmol L^{-1} Cd, 50 μmol L^{-1} Zn, 10 μmol L^{-1} Cd and 50 μmol L^{-1} Zn as well as 15 μmol L^{-1} Cd and 25 μmol L^{-1} Zn. Combined treatment with 15 μmol L^{-1} Cd and 25 μmol L^{-1} Zn revealed the 16 kDa (white square) and 17 kDa (black square) glycoproteins. SNA revealed the 35 kDa glycoprotein (black arrow) in control and all treatments except for the combined treatment with 15 μmol L^{-1} Cd and 50 μmol L^{-1} Zn (Figure 3F). Glycosylated protein of 32 kDa (grey arrow) was missing from the

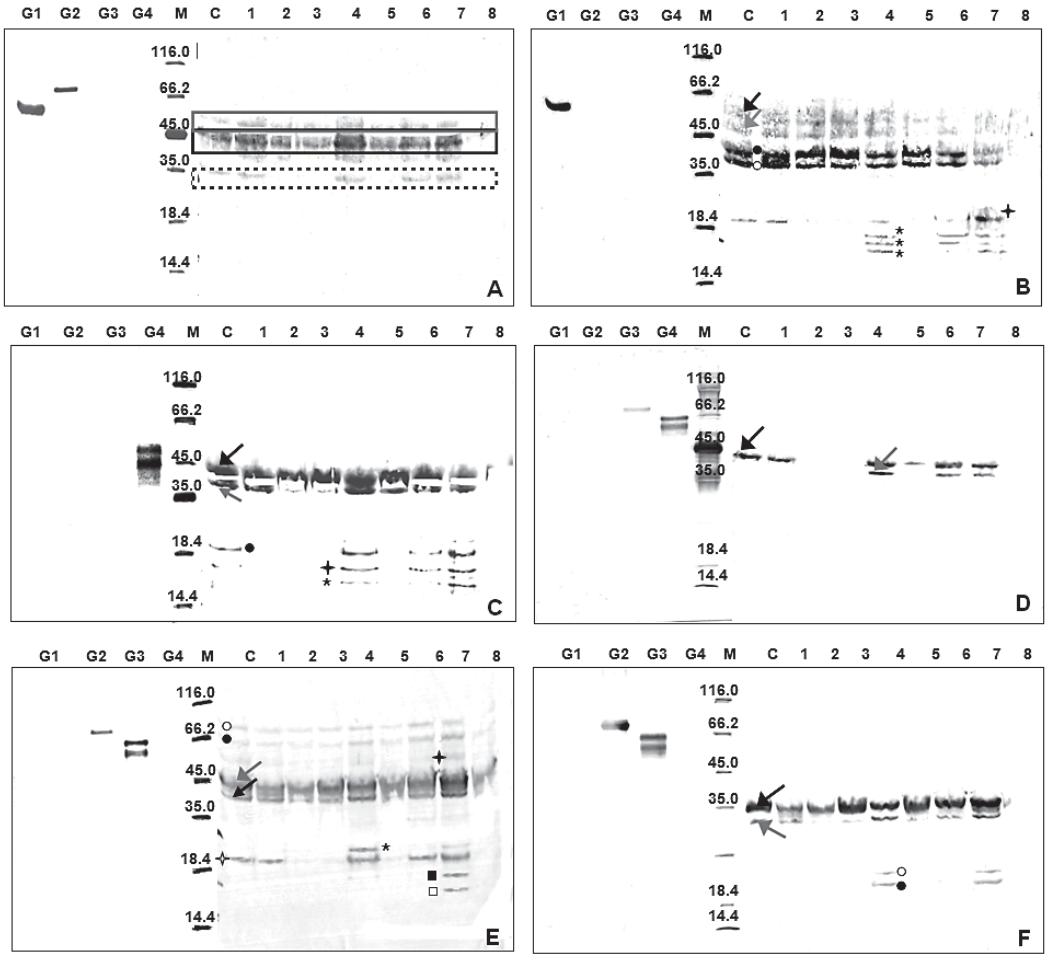

Figure 3 *Glycoprotein pattern of tobacco seedlings detected by lectins A) Con A, B) GNA, C) PNA, D) DSA, E) MAA, and F) SNA. G1 – control glycoprotein carboxypeptidase Y (positive control for Con A and GNA), G2 – control glycoprotein transferrin (positive control for MAA and SNA), G3 – fetuin (positive control for DSA, MAA and SNA), G4 – asialofetuin (positive control for DSA and PNA). For lane labels see Figure 1*

treatment with 15 µmol L^{-1} Cd as well as with 15 µmol L^{-1} Cd and 50 µmol L^{-1} Zn. Very faint bands of 22 kDa and 23 kDa (black and white circle) were present only after exposure to 50 µmol L^{-1} Zn and combination of 15 µmol L^{-1} Cd and 25 µmol L^{-1} Zn.

Three glycosylated proteins were detected by Con A (Figure 4A), according to which all leaves of tobacco adult plants were characterised by the presence of 60 kDa glycoprotein (grey frame), which was the most pronounced one. This glycoprotein band showed a stronger expression in all treatments in comparison to control. Control leaves and those treated with 15 µmol L^{-1} Cd were devoid of the 43 kDa (dashed frame) and 68 kDa glycoprotein (black frame) of weaker staining intensities. The pattern of glycosylated proteins detected with GNA (Figure 4B) was very similar to that obtained with Con A; 43 kDa (grey arrow) and 60 kDa glycoproteins (black arrow) were present in leaves of all treated plants, while they were missing in control plants. Moreover, GNA revealed two additional bands of very weak expression; 15 kDa glycoprotein (black circle) was present in the treatment with 10 µmol L^{-1} Cd and 50 µmol L^{-1} Zn, while the 20 kDa (white circle) one was detected after exposure to 25 µmol L^{-1} Zn and combined treatment with 10 µmol L^{-1} Cd and 50 µmol L^{-1} Zn. PNA revealed the 40 kDa protein (black arrow) of very strong expression in all treated samples except in control leaves (Figure 4C), while very faint band of 68 kDa (grey arrow) was detected in leaves of plants exposed to single 10 µmol L^{-1} Cd and 50 µmol L^{-1} Zn. Exposure to 10 µmol L^{-1} Cd as well as to both concentrations of Zn alone resulted in very low expression of 35 kDa glycoprotein (black circle). The 27 kDa band (white circle) was missing from the control and treatments with 15 µmol L^{-1} Cd alone and

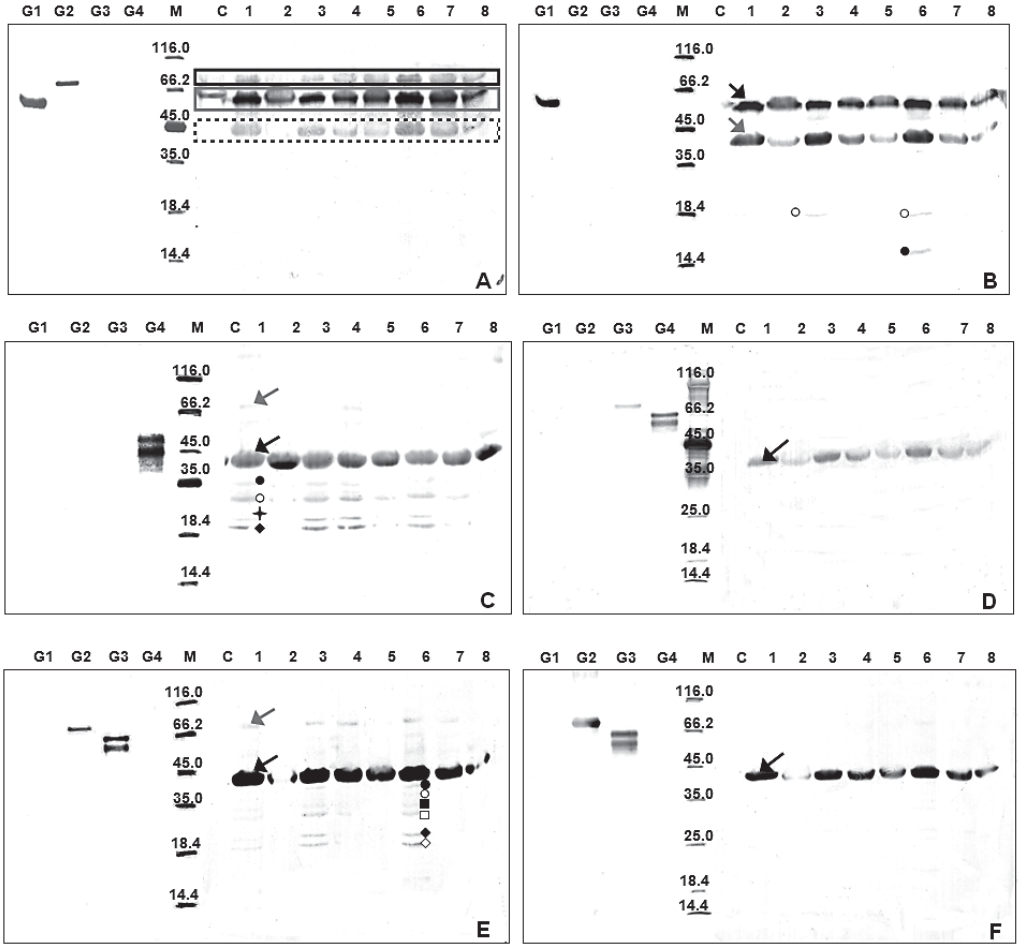

Figure 4 *Glycoprotein pattern of leaves of adult tobacco plants detected by lectins A) Con A, B) GNA, C) PNA, D) DSA, E) MAA, and F) SNA. G1 – control glycoprotein carboxypeptidase Y (positive control for Con A and GNA), G2 – control glycoprotein transferrin (positive control for MAA and SNA), G3 – fetuin (positive control for DSA, MAA and SNA), G4 – asialofetuin (positive control for DSA and PNA). For lane labels see Figure 1*

combination of 15µmol L^{-1} Cd and 50 µmol L^{-1} Zn, while the 20 kDa (diamond) and 22 kDa (star) ones were observed in treatments with single 10 µmol L^{-1} Cd, 25 µmol L^{-1}, and 50 µmol L^{-1} Zn as well as in combined treatments of 10 µmol L^{-1} Cd with 25 µmol L^{-1}, and 50 µmol L^{-1} Zn. Leaves from adult plants exposed to all investigated treatments revealed with DSA only one glycoprotein of 40 kDa (black arrow), whose expression was the strongest in the treatments with single 10 µmol L^{-1} Cd and 25 µmol L^{-1} Zn as well as in combined treatment with 10 µmol L^{-1} Cd and 50 µmol L^{-1} Zn (Figure 4D). Control leaves were devoid of any glycoproteins after the MAA was applied (Figure 4E). In total, eight bands were detected with this lectin in leaves of treated plants, among which the 43 kDa one (black arrow) was present in all the treatments with the strongest staining intensity. The 70 kDa protein (grey arrow) of the weaker expression was detected in all treatments except in the 15 µmol L^{-1} Cd treatment and in combined treatments with 10 µmol L^{-1} Cd and 25 µmol L^{-1} Zn as well as 15 µmol L^{-1} Cd and 50 µmol L^{-1} Zn. Very faint glycoproteins of 20 kDa (white diamond), 22 kDa (black diamond), 32 kDa (white square), and 36 kDa (black square) were detected after exposure to single 10 µmol L^{-1} Cd and 25 µmol L^{-1} Zn as well as to combined treatment with 10 µmol L^{-1} Cd and 50 µmol L^{-1} Zn, while the 40 kDa one (black circle) was present only in treatment with 25 µmol L^{-1} Zn alone as well as with combination of 10 µmol L^{-1} Cd and 50 µmol L^{-1} Zn. Combined treatment of 10 µmol L^{-1} Cd and 50 µmol L^{-1} Zn was characterised by the presence of 38 kDa glycosylated protein (white circle). After incubation with SNA,

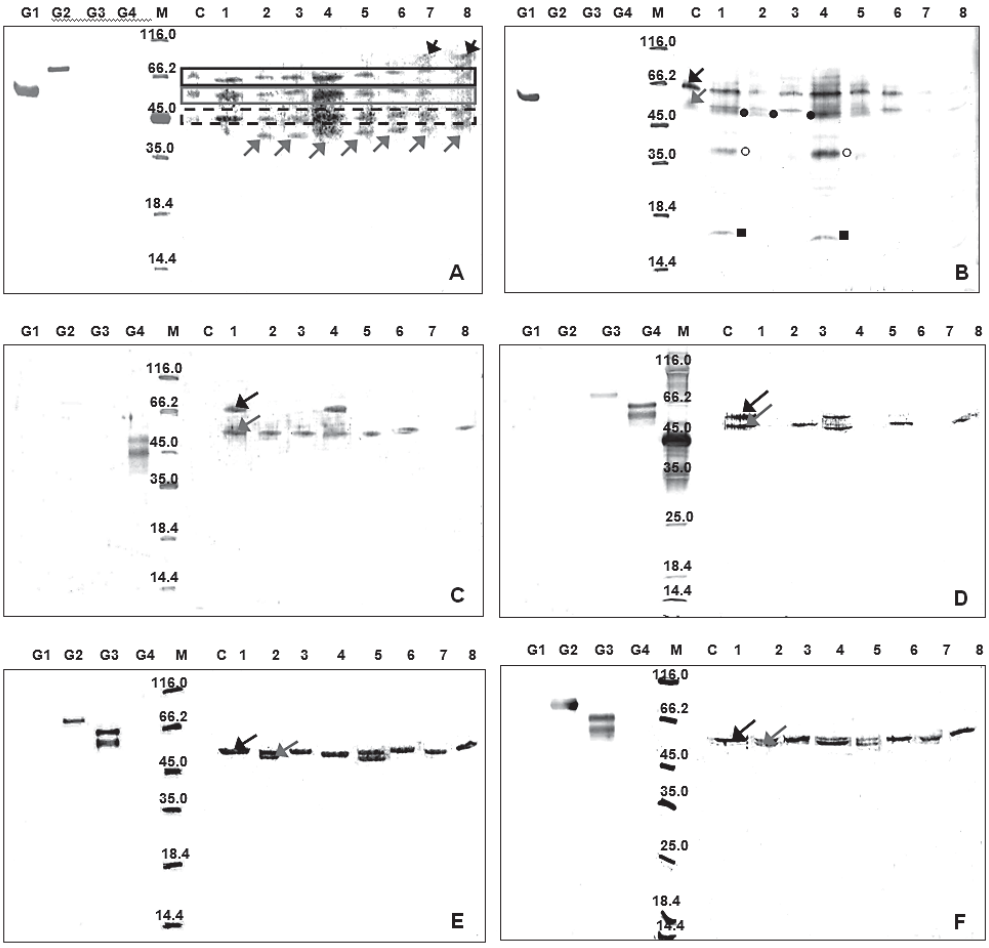

Figure 5 *Glycoprotein pattern of roots of adult tobacco plants detected by lectins A) Con A, B) GNA, C) PNA, D) DSA, E) MAA, and F) SNA. G1 – control glycoprotein carboxypeptidase Y (positive control for Con A and GNA), G2 – control glycoprotein transferrin (positive control for MAA and SNA), G3 – fetuin (positive control for DSA, MAA and SNA), G4 – asialofetuin (positive control for DSA and PNA). For lane labels see Figure 1*

leaves from the treated plants revealed only one glycoprotein of 43 kDa (black arrow), the expression of which was much weaker after exposure to 15 μmol L^{-1} Cd in comparison with other treatments (Figure 4F).

Roots of adult plants revealed in total five glycoprotein bands with Con A (Figure 5A). The 45 kDa (dashed frame), 60 kDa (grey frame), and 66 kDa (black frame) glycoproteins were detected in control and all the treatments, although their expression was the strongest after exposure to 50 μmol L^{-1} Zn. Combined treatments with 15 μmol L^{-1} Cd and 25 μmol L^{-1} Zn as well as 15 μmol L^{-1} Cd and 50 μmol L^{-1} Zn were characterised by the presence of 70 kDa band (black arrow). The 43 kDa glycoprotein (grey arrows) was detected in roots of adult plants exposed to single 15 μmol L^{-1} Cd and to Zn at both concentrations as well as to all combined treatments, with the strongest staining intensity after exposure of single 50 μmol L^{-1} Zn. Five glycoprotein bands were detected in roots of adult plants with GNA (Figure 5B). The 50 kDa (grey arrow) and 60 kDa (black arrow) bands were present in control and all treatments except in the combined exposure to 15 μmol L^{-1} Cd with 25 μmol L^{-1} or 50 μmol L^{-1} Zn. The treatments with single Cd at both concentrations as well as with 50 μmol L^{-1} Zn were characterised by the presence of the 48 kDa band (black circles). The 16 kDa (black squares) and 40 kDa (white circles) glycoproteins appeared in roots after exposure to single 10 μmol L^{-1} Cd and 50 μmol L^{-1} Zn. Only roots of adult plants exposed to metal treatments revealed bands with PNA; the 66 kDa band (black arrow) was detected in treatments with single 10 μmol L^{-1} Cd and 50 μmol L^{-1} Zn, while the 50 kDa one (grey arrow) was present in all treatments, except for the combination of 15 μmol L^{-1} Cd and 25 μmol L^{-1} Zn (Figure 5C). DSA also reacted with root proteins of adult plants (Figure 5D). The 60 kDa glycoprotein (black arrow) was detected after exposure to single 10 μmol L^{-1} Cd and 50 μmol L^{-1} Zn, with stronger staining intensity in Cd treatment. The 10 μmol L^{-1} Cd treatment gave strong expression of 50 kDa band (grey arrow), while the same glycoprotein was also detected in treatments with single Zn at both concentrations and in combined treatments with 10 μmol L^{-1} Cd and 50 μmol L^{-1} Zn as well as 15 μmol L^{-1} Cd and 50 μmol L^{-1} Zn. MAA (Figure 5E) and SNA (Figure 5F) lectins revealed two glycoprotein bands in roots of treated plants. All treatments were characterized by the presence of the 55 kDa band (black arrow) with both lectins. The 53 kDa band (grey arrow) appeared in treatments with 15 μmol L^{-1} Cd and combinations of 10 μmol L^{-1} Cd and 25 μmol L^{-1} Zn with MAA, while with SNA the same band also appeared in the treatment with single 50 μmol L^{-1} Zn.

DISCUSSION

Proteins, as direct gene products, reflect characteristic gene expression. Numerous researches revealing the full genome sequence of model plants, such as Arabidopsis and rice, have been published recently (48-50). However, gene sequence does not give complete information about gene function, developmental and regulatory biology or biochemical kinetics (51). Proteins are macromolecules directly responsible for most biological processes in a living cell, while protein function is directly influenced by posttranslational modifications, which cannot be followed by genome studies. Therefore, it is necessary to conduct proteomic studies which elucidate protein presence and role under certain environmental conditions (52). It is well known that heavy metal stress can activate a range of potential cellular mechanisms in plants, some of which being the mobilization of specific molecules such as phytochelatins and stress proteins that play a very significant role in Cd detoxification and tolerance in plants (53, 54). In a study performed on *Datura innoxia*, a Cd-induced expression of numerous proteins in a molecular mass range from 10 kDa to 70 kDa was reported (55), while Cd-binding proteins have been isolated from Cd-exposed pea plants (56, 57). In our study, changes in protein expression were observed in tobacco seedlings as well as in leaves and roots of adult plants exposed to both cadmium and zinc. An enhanced expression of 116 kDa protein was noticed in seedlings grown on media supplemented with both Cd and/or Zn, except on the medium containing lower Cd and Zn concentration. Moreover, in leaves of adult plants, a 120 kDa protein appeared after treatment with 10 μmol L^{-1} Cd, both concentrations of Zn, as well as in combined treatments of 10 μmol L^{-1} and 15 μmol L^{-1} Cd with 50 μmol L^{-1} Zn. In general, no specific effect of cadmium on protein profiles could be observed based on the SDS-PAGE results obtained in this study. Namely, both metals induced similar changes in protein patterns in tobacco seedlings and plants. In a study of Cd and Zn effect on leaves of *Arabis paniculata*, most identified

proteins suggested that Zn and Cd share similar pathway to certain extent (58). However, our investigation was restricted to cellular proteins which can be easily extracted due to their high solubility in aqueous buffers at neutral pH. Several publications report that heavy metals affect the rigidity of the cell wall and the cell membrane as well as the adsorption of certain secreted proteins to the cell wall (59-61). Therefore, in search of a more reliable molecular marker of heavy metal stress, some additional analyses should be performed. First of all, in order to identify the complete set of cadmium- and/or zinc-induced proteins the SDS-PAGE analyses of expression patterns should be extended to membrane and extracellular proteins. Moreover, 2D-PAGE electrophoresis should be applied, since this technique has a higher resolving power for separating complex mixtures of heterogenous polypeptides than SDS-PAGE. Finally, employing 2D-PAGE in combination with mass spectrometry analyses will allow some of the heavy metal stress related proteins to be identified and their localization, modifications, interactions, activities, and ultimately their function to be determined.

Jomová and Morovič (13) found enhanced protein signals in the pattern of cellular stress protein expression in root tips of *Lupinus luteus* after exposure to Cd and suggested that increased proteosynthetic activity indicated the possible participation of these proteins in the cell defence reactions. It has been documented that heavy metals, as other abiotic stressors, can induce formation of reactive oxygen species (ROS) (62). The excess ROS produced under abiotic stress leads to oxidative damages of lipids, proteins, and nucleic acids (63). When exposed to such unfavourable environmental conditions, plants activate an arsenal of defence mechanisms, both passive and active. The active defence responses require *de novo* synthesis of proteins, some of these being antioxidative enzymes such as the superoxide dismutase, ascorbate peroxidase, gluthatione reductase, and class III plant peroxidases (64). Since elevated activities of these particular enzymes as well as an increased number of their isoforms were found in tobacco tissues exposed to Cd and/or Zn (Šikić et al. unpublished results), it is possible that at least some of the detected proteins belong to this group of defenders of plant cells. A study conducted with Cd on whole rice seeds, including the endosperm during germination, showed that similar proteins increased in response to Cd as with Cu, so it appears that these proteins are involved in a general metal or stress response (18).

Except at the proteome level, abiotic stress can induce changes in posttranslational modifications, among which glycosylation is the most frequent one. Many proteins destined for secretion or expression at the surface of plant cells are glycoproteins. N- as well as O-glycosylation are essential protein modifications required for many different aspects of their structure and function, including their targeting to the appropriate destinations, their stability, solubility and antigenicity, as well as their capacity to be recognized by receptors. In several studies it was reported that protein glycosylation could have an important role in plant response to different abiotic stresses (28, 29, 33). However, the influence of heavy metals on protein glycosylation has been poorly studied so far and only one extensive investigation could be found (31). In our study, characterization of glycosylated proteins was performed by lectin-based glycan profiling, the essence of which is to extract core information (like N-glycosylated or O-glycosylated, high-mannose type or complex type, core-fucosylated or not, fully or partially sialylated and other information) about glycan structures by means of lectin affinity technology (65). The use of lectins in glycan profiling provides considerable advantages, such as discrimination between the isomers on the basis of biological rather than physicochemical principles (66). Lectin-based glycan profiling has already been successfully applied in several plant glycoprotein studies (23, 26, 27). In the present research, differences between control and treated tobacco tissues were more pronounced in glycoprotein compared to soluble protein pattern, which suggests that the oligosaccharide structures could be important determinants for heavy metal stress.

Analysis of N-glycosylated proteins from tobacco seedlings, leaves, and roots revealed common glycoproteins, which implies an enhanced protein glycosylation in all tobacco tissues after exposure to heavy metal stress. Namely, numerous papers indicate that N-glycans can protect protein from proteolytic degradation and are responsible for thermal stability, solubility, and biological activity of the protein (32, 67). Komatsu et al. (30) reported that stress induced by a low temperature changed reactivity of 12 out of 22 glycoproteins detected with Con A in rice. Moreover, in tobacco roots, additional glycoproteins of 43 kDa and 70 kDa were detected with Con A, which is in agreement with the fact that plant roots

are the main site of heavy metals uptake and thus serve as the first barrier in defence mechanism. Interestingly, when GNA lectin was applied, all treated tobacco tissues revealed the bands, which according to their molecular weight (40 kDa in seedlings as well as 43 kDa in leaves and roots) corresponded to signals detected with horseradish peroxidase antibody (Šikić et al. unpublished results). Since most of the plant peroxidases are glycoproteins, this result suggests enhanced expression and glycosylation of this enzyme which has a very important role in plant defence against ROS (64). However, to be completely certain about this, more elaborated techniques for characterising protein and glycan part of glycoproteins further by their isoelectric point and molecular weight as well as by mass spectrometry should be applied. This would give significant information about the expressed glycoslyated proteins that are apparently subdued to changes in glycosylation during the exposure to heavy metal treatments. Lectin GNA revealed N-glycosylated proteins of high-mannose type of lower molecular weight (up to 28 kDa) in seedlings, leaves and roots of tobacco exposed to heavy metals, which proves enhanced protein glycosylation that occurs in ER. Zhang et al. (32) reported that N-glycosylation has a key role in response to abiotic stress and its absence leads to accumulation of damaged proteins in ER.

Lectin DSA, which mostly binds to Gal-(1,4)-GlcNAc, detected glycoproteins with complex and/or hybrid N-glycans in tobacco seedlings and adult plants. In treated leaves, only the glycoprotein of 40 kDa was observed, while glycoslyated proteins of 50 kDa and 60 kDa were detected in root tissue after exposure to certain treatments. This result confirms enhanced modification of N-glycan component, which occurs in GA, where complex and hybrid N-glycans are formed (68, 69). Correct N-glycosylation is important for normal growth and morphology of plant cells (70). Moreover, recent investigations, conducted on the model plant *Arabidopsis thaliana*, suggest that plant cell capability to form complex N-glycan is very important in response to abiotic stress (28, 32). Kang et al. (29) reported that *A. thaliana* resistance to salt stress requires maturation of N-glycosylated proteins in GA. Moreover, von Schaewen et al. (71) reported that *A. thaliana* mutants defective in complex N-glycans show enhanced salt sensitivity, establishing that complex N-glycans protect plants from salt and osmotic stress.

Information about plant O-glycans are scarce. In plants, O-glycosylation has been described mainly for the hydroxyl groups of Hyp, Ser, and Thr residues. Plant and mammalian O-glycans are usually considered structurally different. The main O-glycosylated proteins in plants, extensins and arabinogalactan proteins (AGPs), belong to a large group of glycoproteins known as Hyp-rich glycoproteins (HRGPs). HRGPs are involved in many aspects of plant growth and development (72), and many effects of O-linked glycosylation on the biological activity of these proteins have been described (73). In our study, PNA, which specifically recognizes the β-Gal-(1,3)-GalNAc sequence present in O-glycans, revealed several glycoproteins that also reacted with GNA. This result suggests that these proteins might possess both N- and O-glycosylation sites, although PNA and GNA may alternatively recognize different glycoproteins of similar molecular size, which are not resolved in SDS-PAGE (26). In seedlings treated with 50 μmol L^{-1} Zn and combined treatments 10 μmol L^{-1} Cd and 50 μmol L^{-1} Zn as well as 15 μmol L^{-1} Cd and 25 μmol L^{-1} Zn PNA detected glycoproteins of 16 kDa and 17 kDa, which were not present in control tissues. In tobacco leaves and roots no band was detected in control tissues with PNA, while the majority of treatments responded with at least one band. Interestingly, the greatest number of PNA-reacting proteins was found after exposure to 10 μmol L^{-1} Cd as well as to 50 μmol L^{-1} Zn in tobacco leaves. These findings indicate that heavy metal stress can also induce O-glycosylation, even though it is difficult to say which type of plant O-glycoproteins these bands belong to. To get this answer, some more powerful techniques such as HPAEC-PAD and mass spectrometry should be applied.

MAA and SNA detected sialic acids in glycoproteins of tobacco tissues. Sialylated glycoconjugates have already been found in suspension-cultured cells of *Arabidopsis thaliana* (47), in cellular and extracellular proteins of sugar beet tissue lines (23) as well as in soluble cellular glycoproteins of *Mammillaria gracilis* (24), which suggests that a genetic and enzymatic basis for sialylation exits in plants. In our study, the 43 kDa glycoprotein from all tobacco seedlings reacted with MAA, but also with GNA and PNA. These findings indicate that the 43 kDa is a multiglycosylated protein which has sialic acid (NeuAc) α(2,3)-linked to galactose (Gal). This suggests that this could be a stress-related protein. Balen et al. (27) reported enhanced sialylation of proteins in plant tissues

obtained in *in vitro* culture, which indicates that sialylation could be induced by stressful environmental conditions. Arillo et al. (74) pointed out that the sialic acid content can be used as an index for environmental stress. Moreover, it was found that increased ROS generation in human (75, 76) and animal cells (77) can be correlated with the overproduction of sialoglycoproteins.

Considering all the glycoprotein results, the greater number of glycosylated proteins was detected in tobacco seedlings compared to both leaves and roots of adult plants. In this study, control seedlings reacted with all the applied lectins, while the majority of detected bands were common for all samples. This result is in accordance with the findings that glycosylation has an important role during plant growth and development (19, 20, 22-25). On the other hand, in leaves and roots of adult plants, only Con A among the applied lectins reacted with control samples. Therefore, the majority of detected glycoproteins in these tobacco tissues can be considered as a result of enhanced glycosylation due to heavy metal stress. Furthermore, the difference in glycosylation pattern between Cd and Zn application on tobacco seedlings and adult plants could not be determined since enhanced glycosylation was noticed after treatment with either metal alone or in combination. Therefore, it can be concluded that both metals induced N- and O-glycosylation as a result of changed environmental conditions, although more sophisticated analytical techniques should be employed to acquire more information.

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Sažetak

KADMIJ I CINK INDUCIRAJU SLIČNE PROMJENE U UZORKU PROTEINA I GLIKOPROTEINA U KLIJANCIMA I ODRASLIM BILJKAMA DUHANA (*NICOTIANA TABACUM* L.)

Ispitivali smo učinke 10 μmol L^{-1} i 15 μmol L^{-1} kadmija (Cd), neesencijalnoga toksičnog elementa i 25 μmol L^{-1} i 50 μmol L^{-1} cinka (Zn), esencijalnog mikronutrijenta, na proteine i glikoproteine u klijancima i odraslim biljkama *Nicotiana tabacum* L. nakon izlaganja svakomu metalu posebno ili njihovim kombinacijama. Promjene kod nekoliko polipeptida koje su uočene u klijancima i listovima odraslih biljaka nisu bile brojne, dok su one najvažnije zabilježene u uzorku ukupnih topljivih proteina u korijenu biljke. Razlike između kontrole i tretiranog tkiva duhana bile su izraženije kod glikoproteina koji su analizirani primjenom različitih lektina. Većina glikoproteina uočenih u listovima i korijenu odraslih biljaka može se smatrati rezultatom povećane glikozilacije zbog stresa koji uzrokuju teški metali. Nije bilo moguće utvrditi razliku u glikoproteinima između tretiranja klijanaca i odraslih biljaka duhana kadmijem i cinkom jer je povećana glikozilacija utvrđena i nakon tretmana svakim metalom posebno i nakon tretmana njihovim kombinacijama. Stoga se može zaključiti da su, kao rezultat promijenjenih uvjeta u okolišu, oba metala potaknula N- i O-glikozilaciju.

KLJUČNE RIJEČI: *glikozilacija, lektini, teški metali, uzorak proteina*

CORRESPONDING AUTHOR:

Biljana Balen, PhD
Department of Molecular Biology,
Faculty of Science, University of Zagreb
Horvatovac 102a, Zagreb, Croatia
E-mail: *bbalen@zg.biol.pmf.hr*