THE ACTIVITY OF CELL-WALL MODIFYING β-1,3- GLUCANASES IN SOYBEAN GROWN IN PRESENCE OF HEAVY METALS

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Abstract: Cell walls represent the first barrier that can prevent the entrance of toxic heavy metals into plants. The composition and the flexibility of the cell wall are regulated by different enzymes. The ß-1,3-glucanases control the degradation of the polysaccharide callose as a flexible regulation mechanism of cell wall permeability and/or its ability to bind metals under stress conditions. The profile and activity of ß-1,3-glucanases in the presence of heavy metals, however, has rarely been studied. Here we studied these enzymes in four soybean varieties (*Glycine max*) grown in the presence of cadmium ions. These analyses revealed three acidic and one basic enzyme isoforms in each soybean variety, but only two of the acidic isoforms in the variety Moravians were substantially responsive to the presence of Cd^{2+}. Since the responses of certain glucanases were detected mainly in the varieties sensitive to metal and accumulating high amounts of metals, we assume their role in the defense rather than strategic metal sequestration.

Key words: metal stress, glucanhydrolase, cadmium, plant defense, PR proteins

1. Introduction

Increasing industrialization and urbanization result in emission of pollutants in the environment including toxic heavy metals. Among the different heavy metals contaminating the environment, cadmium raises great concern, as it is ecotoxic and as such can heavily impact ecosystems. Moreover, once taken up by plants it can enter the food chain endangering animals and humans.

The cell wall is the first structure that comes in contact with heavy metals in soil. Considerable amount of literature demonstrates the roles of the cell wall in heavy

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metal response in sequestering heavy metals. The cell wall composition confers the ability to bind heavy metals via different functional groups (e.g., carboxyl, hydroxyl) deriving from the different polysaccharides, thus represents a both barrier and target of heavy metals. The binding and accumulation of Cd in the cell wall prevents it from penetrating inside the cell (extracellular compartmentalization) where it can cause damage. On the other hand Cd pollution might have severe repercussions on the development of secondary cell walls, by reducing their rigidity and robustness (POPPER *et al.,* 2011).

The composition and the flexibility of the cell wall are regulated by different enzymes, of which many have been shown as sensitive to metals. These include proteins involved in the oxidation and peroxidation of cell wall components (CHAOUI *et al.,* 2004) and pathogenesis-related defense proteins of the glucanase family. The latter are responsible for degradation of the polysaccharide callose as one of the earliest sign of metal intoxication (PIRŠELOVÁ *et al.*, 2011). Results indicate that the callose accumulation is not related to decreased enzymatic activity of glucanases and reinforce the hypothesis that accumulation of callose is primarily dependent on a higher rate of synthesis and/or deposition (PIRŠELOVÁ *et al.*, 2012; 2013). The callose-decomposing glucanases have been rarely studied in the context of heavy metal stress. To our knowledge there is available a single study on the activity of these enzymes after exposure to metal toxicity in the roots of maize and soybeans (PIRŠELOVÁ *et al.,* 2011). In this work we studied the activity of glucanases in a set of soybean cultivars and evaluated their potential role in metal detoxification.

2. Materials and Methods

2.1. Plant material

Soybean seeds (Glycine max L.), cultivars Moravians, Kent, Gallec, Cardiff were obtained from Matex, s.r.o (Veľké Kapušany). Seeds were sterilized with 0.5 % sodium hypochlorite for 5 minutes and germinated on wet filter paper in Petri dishes. Roots (6 – 8 mm) were replaced into Petri dishes with experimental solutions of 50 mg/dm^3 Cd^{2+} in a form of $CdCl_2$. Control plant material was incubated on a distilled water. The seedlings were incubated in dark at 23 °C for 48 hours. Fresh weight (FW) of seedling's roots was measured at the end of each treatment on analytical scale (Explorer Pro EP 114 CM). Index of tolerance (IT, %) was calculated as a ratio of the weight of roots of experimental group to weight of roots of control group x 100 %. All biological determinations were performed in triplicate.

2.2. Protein isolation

Protein extract was isolated by extraction buffer containing 20 % (v/v) glycerol, 1.5 % (w/v) polyvynilpyrrolidon, 10 % (v/v) 1 M Tris-HCl (pH 8.0), 0.02 % (v/v) β-mercaptoethanol and 1 % (w/v) 100 mM phenylmetylsulfonylfluorid (Sigma).

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Concentration of proteins in the extract was determined according to Bradford (1976) spectrophotometrically at wave lenghth 525 nm (ULTROSPEC 1 000 UV/VisibleSpectropotometer, Shimadzu, Tokyo, Japan).

2.3. Spectrophotometry detection of glucanase activity

Overall glucanase activity in protein extracts was determined spectropfotometrically according to MILLER (1959). Briefly, samples were mixed with 1 % (w/v) 3,5-dinitrosalicylic acid (DNS) and 30 % (w/v) potassium sodium tartrate, and subsequently boiled for 5 minutes. After cooling the absorbance of samples was measured spectrophotometricaly at 540 nm (UV-1800 UV / VIS (Shimadzu).

2.4. In-gel detection of protein fractions with glucanase activity

Gel-electrophoresis separation of proteins was performed according to LAEMMLI (1970) on the Mini-PROTEAN Tetra Cell Module (Bio-Rad). The 12.5 % (w/v) polyacrylamide gels contained 1 % (w/w) laminarin (Sigma) as a substrate. Electrophoresis was running under constant power of 18 mA in the stacking gel and 24 mA in the separation gel, for 4 h. After electrophoresis, proteins were re-natured by shaking the gels in 50 mM sodium acetate buffer (pH 5.0), 1 % (v/v) Triton X-100 over night. For subsequent enzyme detection, the gels were transferred into fixing solution (7 % (v/v) acetic acid, 20 % (v/v) metanol) for 5 minutes. The fixed gels were boiled 3 – 10 minutes in a solution of 1 M NaOH and 0.1 % (v/w) triphenyl tetrazolium chlorid (Sigma) until red strips appeared indicating to fractions with glucanase activity. The gels were scanned by Bio Rad – GS-800 Calibrated Densitometer and stored in a 7 % (v/v) acetic acid. After detection of glucanases, the gels were stained for detection of total proteins with Coomassie Brilliant Blue. Proteins were loaded on the gels without heat treatment. Molecular weights of proteins were estimated by co-electrophoresis of a protein ladder (Mark 12 Unstained Standard, Invitrogen) ranging from 2.5 to 200 kDa.

2.5. Statistical analysis

Each experiment was performed in triplicate. After verification of normal distribution and variance homogeneity the data was analysed by *t* test.

3. Results and Discussion

Soybean is an important staple food especially in Asia, while its high nutrition value is well known. Since soybean is considered as sensitive to metal toxicity and variable (genotype dependent) accumulation has been demonstrated (SOCHA *et al.,* 2015), we conducted a research aiming to study the soybean responses to metals

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focusing on the activity of cell-wall modifying enzymes ß-1,3-glucanases. Seeds of 4 soybean varieties, namely Cardiff, Gallec, Kent a Moravians, were germinated and exposed to solution with cadmium ions that has previously been shown as toxic but sub-lethal to soybean (SOCHA *et al.,* 2015). Indeed, a strong negative impact on root growth and development was observed for each variety ($P \leq 0.01$) (Fig. 1).

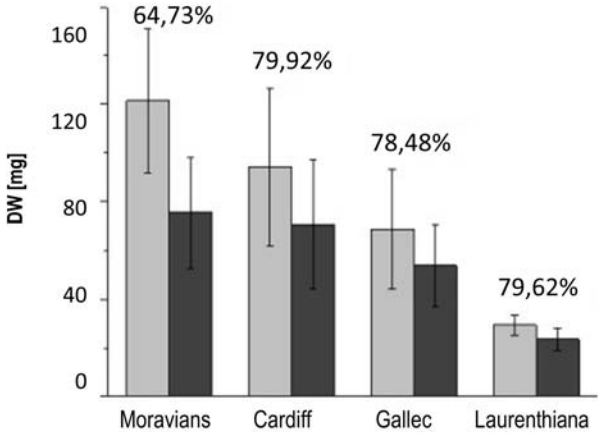

Fig. 1. Data on root biomass of soybean varieties grown in presence (dark columns) and absence (light columns) of cadmium ions. Shown are the means ± SD (n = 4). Above columns the tolerance index values are indicated for each variety.

The effect of heavy metals on root biomass is considered a good indicator for sensitivity / tolerance to metal stress (PIRŠELOVÁ *et al.,* 2011), hence the measured values of tolerance index assigned the variety Moravians as the most sensitive and the variety Laurentiana the most sensitive to cadmium (Fig. 1). These data are consistent with the amount of cadmium accumulated in roots: the metal uptake was the largest in the sensitive variety Moravians, and relatively lower in the tolerant variety Laurentian (Fig. 2).

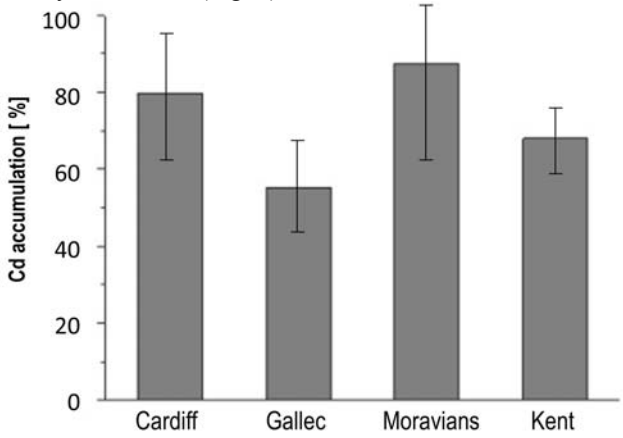

Fig. 2. Accumulation of Cd by roots of soybean genotypes. The data indicate average values from three experiments ± SD (n = 4).

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The enzymes of ß-1,3-glucanases belong to the repertoire of plant´s defense under different stresses, including metal toxicity. Their total activities in plants, however, depend on many factors, including age, developmental stage and genotype (DOBROVICZKÁ *et al.,* 2013). Total activity of glucanases in the roots of tested soybean varieties was variable (Fig. 3).

Genotypic variations in the levels of enzyme have previously been confirmed for e.g. the chitinases - another type of defense proteins (METWALLY *et al.,* 2005). In each variety we measured significant changes in enzyme activity ($P \leq 0.05$); while in the varieties Cardiff and Laurentian the enzymes were induced, in Moravians and Gallec we detected a significant reduction of their activity (Fig. 3). Previously PIRŠELOVÁ *et al.* (2012) have described the induction of the overall activity of the glucanases of soybean (cv. Korada) under the action of 500 mg/dm^3 Pb^{2+}, and 300 mg/dm^3 Cd^{2+}. On the other hand, inhibition of the activity was observed for 100 mg/dm^3 As^{3+}. In contrast in maize roots no significant changes were observed (PIRŠELOVÁ *et al.,* 2012).

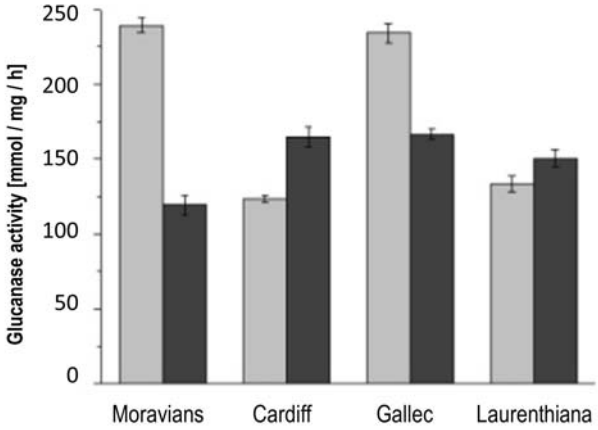

Fig. 3. Total activity of glucanase in roots of soybean cultivars under Cd stress. Shown are the means ± SD (n = 4).

Since the total enzyme activity comprises an entire set of isoforms in tissues (MÉSZÁROS *et al.,* 2013), we studied the soybean glucanases in in more detail after separation in polyacrylamide gels. In the enzyme profile of each variety we recorded the presence of at least 6 isoforms with glucanolytic activity of sizes ~ 140, 100, 70, 50, 40 and 25 kDa (FIG. 4a). Previously PIRŠELOVÁ *et al.* (2011) have detected in the variety Korada only 5 isoforms of smaller sizes (65, 35, 32, 25 and 18 kDa). Such differences in protein size between the two studies may be due to the variety or technical reasons (different conditions of separation and isolation of proteins, the sensitivity of the detection system, etc.). Polymorphism in the profile of defense proteins has rarely been described (MÉSZÁROS *et al.,* 2013).

None of the detected enzyme fractions (separated based on size exerted responsivity to cadmium (data not shown). In contrast, PIRŠELOVÁ *et al.* (2011) have observed a significant induction of two isoforms (32 and 35 kDa) at much

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higher metal doses. We further analyzed the individual glucanases separately for acidic / neutral and alkaline / neutral isoforms. These analyses revealed three acidic and one basic isoforms in each soybean variety (Fig. 4b,c). Two of the acidic isoforms in the variety Moravians were substantially responsive to the presence of Cd^{2+} ($P < 0.05$); the acid isoform A was significantly induced, while the isoform C was inhibited by the metal (Fig. 4d,e). However, no such response was observable for any of the acidic isoforms in the other varieties (Fig. 4d). Previously a single acidic glucanase has been described in soybean (PIRŠELOVÁ *et al.,* 2011) that was induced by high Cd concentration. In view of the differences in experimental conditions of the two studies, however, the equality of these isoforms remains uncertain. Concerning the alkaline proteins we detected a single basic isoform with glucanase activity (FIG. 4c), which was significantly induced by the presence of Cd^{2+} ($P < 0.05$) in the varieties Moravians and Cardiff (Fig. 4d). In earlier studies no basic isoform has been detected so far in soybean (PIRŠELOVÁ *et al.,* 2011).

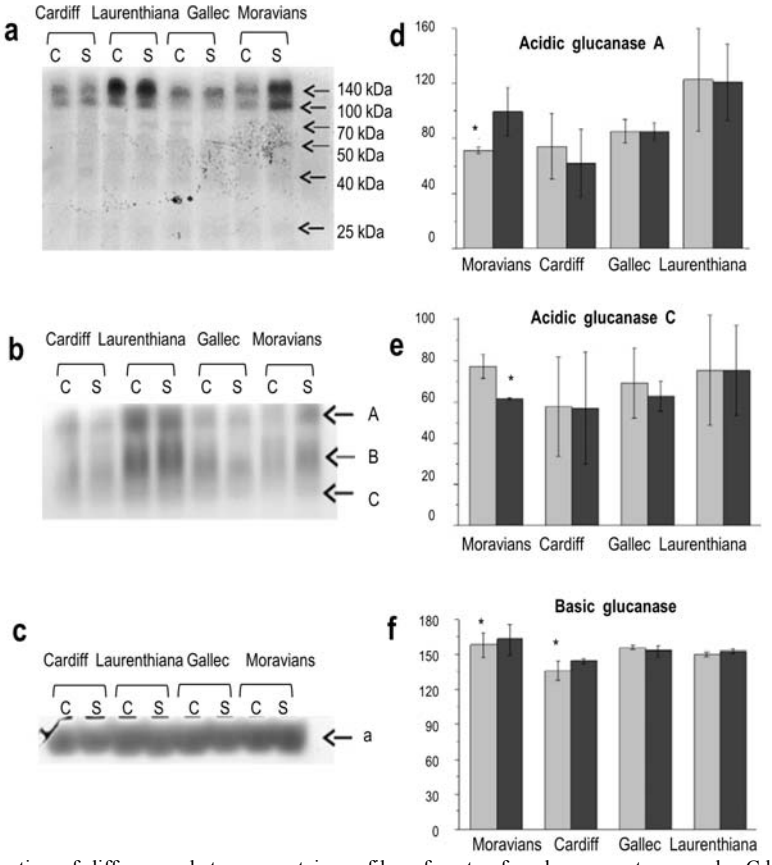

Fig. 4. Detection of differences between protein profiles of roots of soybean genotypes under Cd stress. Proteins were separated in laminarine-containing gels on SDS–PAGE (a) and native gels (b, c). Band intensities of isoforms of acidic glucanases of soybean roots of 4 genotypes under Cd stress (d, e, f) – shown are the means ± SD ($n = 4$).

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The increase but also the reduction of the activity of the individual isoforms suggests that glucanases likely play a different role in this type of stress. Since the responses of certain glucanases were detected mainly in the varieties sensitive to metal and accumulating high amounts of Cd^{2+} (Fig. 1, 2, 4 c,d), we assume their role in the defense (e.g. by limiting cell wall permeability), rather than strategic metal sequestration. Conversely, the minor changes of corresponding activities in the other cultivars might indicate to low importance of glucanases in this type of stress. Metal tolerant varieties might utilize other components of the defense/tolerance e.g. the composition and thickness of the cell walls (YOKOYAMA NISHITANI, 2004) or effective mechanisms for removal of oxidative stress (TOUNEKTI *et al.,* 2011).

In the varieties Moravians and Cardiff the identified glucanase isoforms might modulate the callose content in cell walls and affect their permeability and/or ability to bind metals (CORRALES *et al.,* 2008). The glucanase enzymes in general regulate the callose turnover on the different types of stress (including toxic metals), or might contribute to a more specific defense against various types of metals (BÉKÉSIOVÁ *et al.,* 2008; MÉSZÁROS *et al*., 2013; this study). For example, deposition of callose as a local and very flexible process was observed in roots cells exposed to various types of toxic metals (Cd, Pb and As) in soybean and corn (PIRŠELOVÁ *et al.,* 2012).

4. Conclusions

In conclusion, we identified glucanase isoforms in selected varieties of soybean that respond to presence of cadmium in growth media. Studying the variety of cell wall responses to metals will allow us to identify specific mechanisms used to tolerate large doses of such pollutants. Those mechanisms (including general biochemical responses or sequestering in the vacuole/cell wall) contribute to the chemical variety of cell walls and provide several possibilities for the cell walls to interfere with the toxic activity of metals. In this context, it is important to identify the natural targets of Cd at the cell wall level and to figure out which of these targets can be modified to generate plants that either cannot absorb Cd or can sequester Cd in the cell wall thereby reducing or avoiding detrimental effects in terms of growth or productivity.

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