Changes in the composition of cell wall proteins in barley roots during germination and growth in aluminium presence

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ABSTRACT

Root growth inhibition and loss of cell viability in barley root cells were induced by Al during germination of barley caryopses on filter paper moistened with 2mM $AlCl_3$. The inhibition of root growth as well as loss of cell viability started on the third day of germination. This time was also needed for induction of Al-induced changes in the composition of cell wall proteins. The accumulation of three salt-extractable polypeptides with relative molecular mass 14, 27, and 29 kDa started 72 h after the beginning of germination of barley caryopses on Al containing filter paper. However, the inhibition of developmentally regulated deposition of three (18, 23 and 28 kDa) salt-extractable CW polypeptides was observed at the same time. The pattern of detergent-extractable CW proteins was not influenced by Al regardless of the duration of Al treatment.

Keywords: spring barley (*Hordeum vulgare* L.); aluminium stress; cell viability; cell wall proteins; root growth; SDS-PAGE

Below pH 5 several plant species are sensitive to a micromolar concentration of soluble Al forms. This fact supports the prevailing opinion that important losses of crop productivity in acid soils are caused by Al toxicity. Many hypotheses have been proposed for the mechanism of Al toxicity, including inhibition of cell division, disjunction of cell wall (CW), inhibition of ion fluxes, disruption of plasma membrane integrity, failure of Ca homeostasis, inhibition of signal transduction pathway and changes in the cytoskeleton structure (for a review see Kochian 1995, Matsumoto 2000, Rout et al. 2001).

The first target site of Al action is probably the apoplast. The distribution of absorbed Al is different in the plant species but a major part of absorbed Al (30–90%) was found in the apoplast (Rengel 1996). Al rapidly interferes with the CW structure where Al strongly binds to the negative charges of pectic matrix of CW affecting its permeability and extensibility. Schmohl and Horst (2000) unequivocally proved that NaCl adapted cells with high pectin content bind more Al and are more sensitive to Al than control cells containing a lower amount of pectin in their cell wall. Blamey et al. (1993) showed that *in vitro* Al reduced water permeability of artificial Ca-pectate membrane. Al-CW interaction affects the uptake of cations and anions probably through the modification of ion homeostasis in the apoplast (Nichol et al. 1993). Al also modifies the metabolism of CW components especially by increasing the production of hemicellulose and ferulic acids as it was shown in wheat roots (Van et al. 1994, Tabuchi and Matsumoto 2001). However, the influence of Al on CW protein composition and metabolism is poorly documented. Al stress significantly increased the level of covalently bound but not salt-extractable CW proteins in pea roots (Kenjebaeva et al. 2001). In contrast, our previous study of barley roots revealed Al-induced accumulation of salt extractable CW polypeptides with low molecular mass in barley roots (Tamás et al. 2001).

The aim of the present study was to characterise the impact of aluminium on protein composition of CW in barley roots during germination and early growth using eight different extraction systems. Al-induced root growth inhibition and viability of root cells were also monitored during an experiment lasting for 96 h.

MATERIAL AND METHODS

Caryopses of barley (*Hordeum vulgare* L.) cultivar Alfor were surface sterilised with 12% H_2O_2 for 10 min and rinsed three times for 10 min with distilled water. After incubation in distilled water for 4 h at 25°C in darkness the caryopses were germinated on filter paper (Whatman No. 1) moistened with 0.2mM $CaCl_2$ solution, pH 4.5 (control), or with 0.2mM $CaCl_2$ containing 2mM $AlCl_3$ solution, pH 4.5 (Al-treated). 48, 72 or 96 h after the placement of barley caryopses on filter paper root tips (10 mm) were harvested and used immediately or stored at –70°C until analysed. Each experiment was repeated at least three times with 60 replications. The experimental data were statistically evaluated using Statgraphics for Windows v. 2.1 package of statistical program.

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Determination of cell viability

The loss of cell viability was evaluated using Evans blue staining method (Baker and Mock 1994). Freshly harvested roots were stained in 0.25% (v/v) aqueous solution of Evans blue for 15 min. After washing in distilled water for 30 min root tips (5 mm) were excised and soaked in N,N-dimethylformamide for 24 h at 4°C. The optical density of released Evans blue was measured spectrophotometrically at 600 nm.

Protein extraction and analysis

Root samples were ground to a fine powder in a cold mortar in liquid nitrogen and the resulting powder was re-homogenised in extraction buffer (40mM succinic acid/NaOH buffer, pH 4.0) with homogeniser (DIAX 900 Heidolph GmbH, Keilheim, Germany). After filtration the homogenate was centrifuged at 1500 *g* for 10 min and the resulting pellet (CW fraction) was washed with 0.05% Na-deoxycholate for 2 h at RT and re-centrifuged at 1500 *g* for 10 min to eliminate cytoplasmic contaminants. Proteins from purified CW were eluted and re-centrifuged step by step with eight different extraction solutions differing in ion strength and detergent concentration:

I. 10mM Tris-maleate buffer, pH 7.3 containing 0.15M NaCl and 1mM EDTA for 1 h at 4°C
II. 10mM Tris-maleate buffer, pH 7.3 containing 1M NaCl and 1mM EDTA for 1 h at 4°C
III. 10mM Tris-maleate buffer, pH 7.3 containing 3.5M LiCl and 1mM EDTA for 1 h at 4°C
IV. 10mM Tris-maleate buffer, pH 7.3 containing 1M $CaCl_2$ for 1 h at 4°C
V. 10mM Tris-HCl buffer, pH 7.0 containing 0.5% EDTA for 1 h at 4°C
VI. 10mM Tris-HCl buffer, pH 8.0 containing 0.1% Nonidet P-40 for 1 h at RT
VII. 10mM Tris-HCl buffer, pH 8.0 containing 1% SDS for 1 h at RT
VIII. 10mM Tris-HCl buffer, pH 7.0 containing 1% SDS and 5% ME for 1 h at 50°C

Proteins from fractions I, II, III, IV and V were divided into two subfractions (BRP – boiling resistant proteins and BSP – boiling sensitive proteins) by 10 min boiling and centrifugation at 12 000 *g* for 10 min. Proteins from BRP-subfractions and from VI, VII and VIII fractions were recovered by precipitation in ice cold acetone. Proteins were solubilised, separated under denaturing conditions on 12–18% gradient polyacrylamide slab gels using the discontinuous buffer system (Laemmli 1970), and silver stained (Heukesloven and Dernick 1985). Protein concentrations were determined according to Lowry et al. (1951) with BSA as the standard. The apparent molecular masses of polypeptides were calculated based on the mobilities of protein standards obtained from Bio Rad (Bio Rad Lab., USA) with gel documentation system (UVP 5000, UK). Each experiment was performed at least three times and the gels from a representative replication are shown.

RESULTS AND DISCUSSION

To mimic natural conditions of plants growing in Al toxic soils we performed a set of experiments with germination and subsequent early growth of barley seedlings on filter paper moistened with aluminium containing $CaCl_2$ solution. Due to the high binding capacity of cellulose filter paper to Al we used a millimolar concentration of Al to attain a similar range of root growth inhibition as we got in hydroponics using micromolar concentration of Al. Easier handling of seedlings as well as diminishing the risk of loss of cell viability and cell wall proteins caused by intensive aeration of root solution in hydroponics were some of the reasons for the realisation of this kind of experiment on filter paper.

In general, germination is a process that is less sensitive to Al than elongation of root cells. As demonstrated by Delima and Copeland (1990), the inhibition of growth of emerging roots in germinated wheat caryopses required the presence of Al concentration higher than 1mM. In our experiments on filter paper the presence of 2mM Al concentration had no effect on germination of barley caryopses (data not shown) and did not inhibit

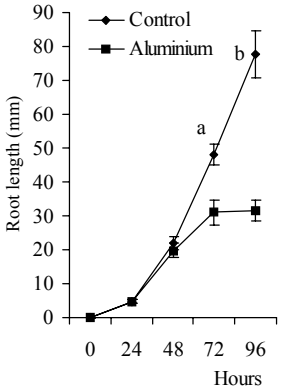

Figure 1. Root growth inhibition of barley germinated on filter paper moistened with 2mM $AlCl_3$ for 24, 48, 72 and 96 h, respectively; mean values ± *SD* (n = 5), a (p < 0.05), b (p < 0.001)

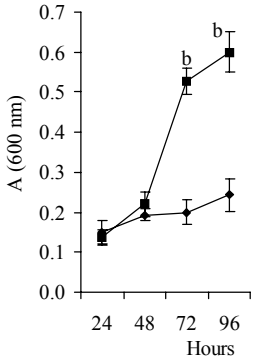

Figure 2. Uptake of Evans blue by barley root tips (0.5 cm) germinated on filter paper moistened with 2mM $AlCl_3$ for 24, 48, 72 and 96 h, respectively; mean values ± *SD* (n = 5), b (p < 0.001)

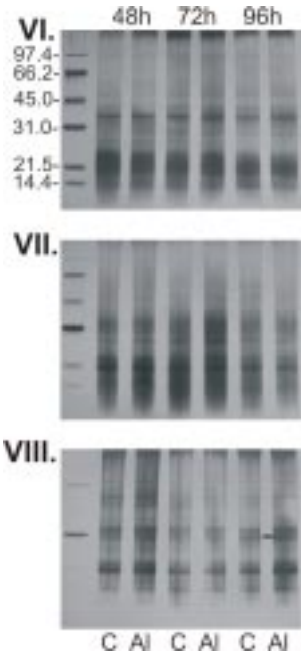

Figure 3. SDS-PAGE of detergent-extractable CW proteins (fraction VI – 0.1% Nonidet P-40, VII – 1% SDS, VIII – 1% SDS and 5% ME) isolated from barley root tips (1 cm) grown on filter paper moistened with 2mM $AlCl_3$ for 48, 72 and 96 h, respectively; C – control; Al – Al-treated plants

root growth within 48 h from the start of germination (Figure 1). However, significant inhibition of root growth was observed during the third day of germination and root growth was completely stopped on the fourth day.

The root growth inhibition was strongly correlated with the loss of root cell viability. The uptake of Evans blue was similar within 48 h after the beginning of germination independently of the presence (Al-treated) or absence (control) of Al (Figure 2). Rapid Al-induced uptake of Evans blue was detected on the third day and a slight increase continued on the fourth day of experiment. These results agree with well-documented Al-induced cell death as a consequence of oxidative stress that is one of the key components of Al toxicity syndrome (Pan et al. 2002, Yamamoto et al. 2002).

Root growth inhibition and significant loss of cell viability were induced on the third day of germination of barley caryopses in the presence of 2mM Al. Therefore we compared the impact of Al on CW proteins before (48 h) and after (72 and 96 h) the occurrence of these Al toxicity syndromes. Figure 3 shows that Al had no effect on the pattern of detergent extractable CW proteins (fractions VI, VII and VIII) in barley roots. During the 48 h period of germination only one polypeptide with molecular mass of about 28 kDa showed an Al-induced change in fraction IV (Figure 4, fraction IV, BSP). This BSP is accumulated at a lower amount in Al-treated roots in comparison with control plants. The lower accumulation of this polypeptide was visible to the same extent also 72 and 96 h after exposure of caryopses to Al, when the loss of cell viability and root growth inhibition were evident. This phenomenon might probably be associated with decreased extractability caused by Al-induced cross-binding of this polypeptide with CW during extraction. On the other hand, Al-induced cross-linking of CW proteins *in vivo* may be the component of Al toxicity mechanism because of increasing CW rigidity. The main Al-induced CW protein was found in the first low salt extractable fraction (0.15M NaCl). The accumulation of 27 kDa BRP (Figure 4, fraction I, BRP) occurred 72 h after the beginning of germination suggesting its function in Al toxicity. Its accumulation was higher in 96 h than in 72 h. Al-induced accumulation of 27 kDa polypeptide was also reported in our previous paper where we analysed roots of older barley plants (Tamás et al. 2001). The accumulation of the second boiling resistant 14 kDa polypeptide

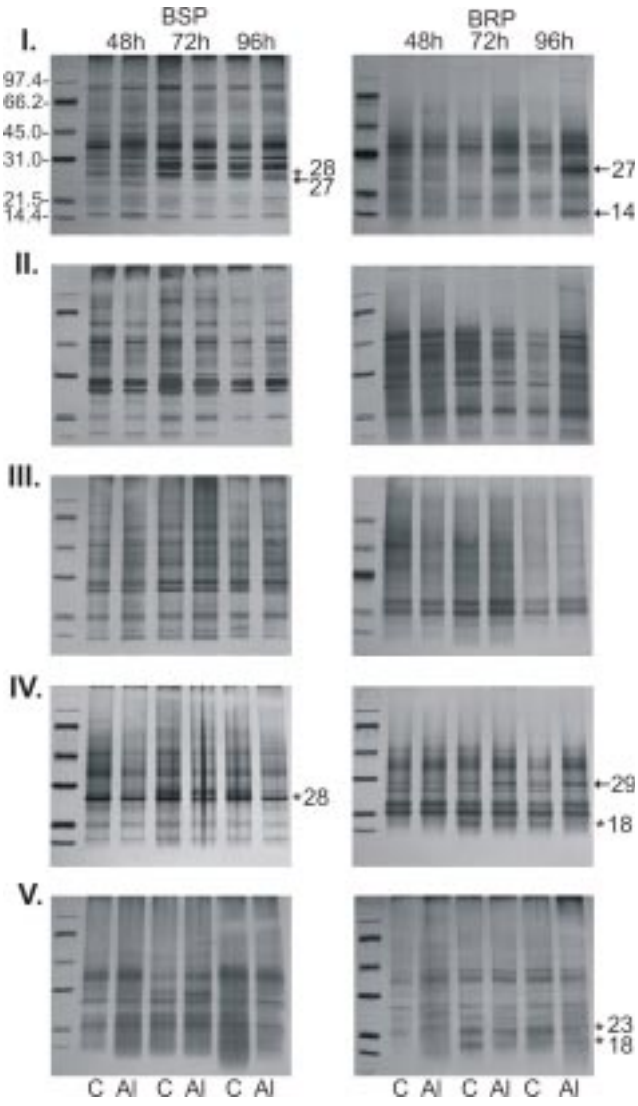

Figure 4. SDS-PAGE of salt-extractable CW proteins (fraction I – 0.15M NaCl, II – 1M NaCl, III – 3.5M LiCl, IV – 1M $CaCl_2$, V – 0.5% EDTA) isolated from barley root tips (1cm) grown on filter paper moistened with 2mM Al for 48, 72 and 96 h; C – control; Al – Al-treated plants; arrowheads indicate induced and asterisks reduced polypeptides

(Figure 4, fraction I, BRP) was evident only after 96 h when root growth completely failed. This fact is consistent with an opinion that this protein has probably a function in the Al toxicity induced destructive mechanism but is not involved directly in the Al toxicity response. Similarly but on a lower level than 27 kDa polypeptide in the first fraction we detected the accumulation of BRP (29 kDa) in the fourth (Ca-extractable) fraction after 72 and 96 h of germination (Figure 4, fraction I, BRP). Basu et al. (1999) reported Al-induced secretion of several polypeptides and one of these exuded proteins co-segregated with Al resistance in wheat. The function of Al-induced CW proteins remains unknown. Some secreted acidic polypeptides bind strongly Al and can act similarly like organic acid chelators in the Al exclusion mechanism (Putterill and Gardner 1988). On the other hand, rapid binding of Al to CW proteins can be involved in Al-induced root growth inhibition. Kenjebaeva et al. (2001) showed that extensin and salt-extractable proteins of pea root tips exhibited a marked Al-binding capacity *in vivo*. In a low salt extractable fraction Al induced accumulation of BSP with the same molecular mass (27 kDa) as in BRP fraction (Figure 4, fraction I, BSP). This is probably due to the insufficient separation methods of BSP and BRP. However, the main difference in this fraction was the Al-induced inhibition of accumulation of 28 kDa polypeptide both in 72 and 96 h after the beginning of germination. This polypeptide was not detected after 48 h of germination either in control or in Al-treated plants. Its accumulation was evident 72 h after the beginning of germination when compared to 48 h in control plants; however, it was fully inhibited in Al-treated roots. A similar pattern was observed in the accumulation of 23 and 18 kDa polypeptides in IV and V fractions (Figure 4, fraction IV and V, BSP). In contrast to 28 kDa in the first fraction these proteins were boiling resistant. This Al-induced inhibition of developmentally regulated deposition of some proteins in CW probably plays a crucial role in the Al-induced toxicity mechanism. One of the mechanisms that could be involved in this inhibition is the dysfunction of protein deposition in CW as a consequence of Al-induced change in ion homeostasis of CW and Al-induced cross-linking of CW materials. On the other hand, the transport of CW material synthesised in the symplast to the apoplast is strongly inhibited in barley roots by Al (Ikeda and Tadano 1993), which could also change CW composition and correct protein deposition in CW.

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Abbreviations

BRP – boiling resistant proteins
BSP – boiling sensitive proteins
CW – cell wall
ME – 2-mercaptoethanol
RT – room temperature
SDS – sodium dodecyl sulphate

REFERENCES

Baker C.J., Mock N.M. (1994): An improved method for monitoring cell death in cell suspension and leaf disc assays using Evans blue. Plant Cell Tiss. Org. Cult., *39*: 7–12.

Basu U., Good A.G., Aung T., Slaski J.J., Basu A., Briggs K.G., Taylor G.J. (1999): A 23-kDa polypeptide co-segregates with aluminium resistance in *Triticum aestivum*. Physiol. Plantarum, *106*: 53–61

Blamey F.P.C., Asher C.J., Edwards D.G., Kerven G.L. (1993): *In vitro* evidence of aluminium effects on solution movement through root cell wall. J. Plant Nutr., *16*: 555–562.

Delima M.L., Copeland L. (1990): The effect of aluminium on the germination of wheat seeds. J. Plant Nutr., *13*: 1489–1497.

Heukesloven J., Dernick R. (1985): Simplified method for silver staining of proteins in polyacrylamide gels and the mechanism of silver staining. Electrophoresis, *6*: 103–112.

Ikeda H., Tadano T. (1993): Ultrastructural changes of the root tip cells in barley induced by a comparatively low concentration of aluminium. Soil Sci. Plant Nutr., *39*: 109–117.

Kenjebaeva S., Yamamoto Y., Matsumoto H. (2001): The impact of aluminium of the distribution of cell wall glycoproteins of pea root tip and their Al-binding capacity. Soil Sci. Plant Nutr., *47*: 629–636.

Kochian L.V. (1995): Cellular mechanisms of aluminium toxicity and resistance in plants. Ann. Rev. Plant Physiol. Plant Mol. Biol., *46*: 237–260.

Laemmli U.K. (1970): Cleavage of structural proteins during the assembly of the head of the bacteriophage T4. Nature, *277*: 680–685.

Lowry O.H., Rosenbrough N.J., Farr A.L., Randall R.J. (1951): Protein measurement with the Folin phenol reagent. J. Biol. Chem., *193*: 265–275.

Matsumoto H. (2000): Cell biology of aluminium toxicity and tolerance in higher plants. Int. Rev. Cytol., *200*: 1–46.

Nichol B.E., Oliveira L.A., Glass A.D.M., Siddiqi M.Y. (1993): The effects of aluminium on the influx of calcium, potassium, ammonium, nitrate, and phosphate in an aluminium-sensitive cultivar of barley (*Hordeum vulgare* L.). Plant Physiol., *101*: 1263–1266.

Pan J., Zhu M., Chen H., Han H. (2002): Inhibition of cell growth caused by aluminium toxicity results from aluminium-induced cell death in barley suspension cells. J. Plant Nutr., *25*: 1063–1073.

Putterill J., Gardner R. (1988): Proteins with the potential to protect plants from Al toxicity. Biochim. Biophys. Acta, *964*: 137–145.

Rengel Z. (1996): Uptake of aluminium by plant cells. New Phytol., *134*: 389–406.

Rout G.R., Samantaray S., Das P. (2001): Aluminium toxicity in plants: a review. Agronomie, *21*: 3–21.

Schmohl N., Horst W.J. (2000): Cell wall pectin content modulates aluminium sensitivity of *Zea mays* (L.) cells grown in suspension culture. Plant Cell Environ., *23*: 735–742.

Tabuchi A, Matsumoto H. (2001): Changes in cell-wall properties of wheat (*Triticum aestivum*) roots during aluminium-induced growth inhibition. Physiol. Plant., *112*: 353–358.

Tamás L., Huttová J., Hajasová L., Mistrík I. (2001): The effect of aluminium on polypeptide pattern of cell wall proteins isolated from the roots of Al-sensitive and Al-resistant barley cultivars. Acta Physiol. Plant., *23*: 161–168.

Yamamoto Y., Kobayashi Y., Devi S.D., Rikiishi S., Matsumoto H. (2002): Aluminium toxicity is associated with mitochondrial dysfunction and the production of reactive oxygen species in plant cells. Plant Physiol., *128*: 63–72.

Van H.L., Kuraishi S., Sakurai N. (1994): Aluminium-induced rapid root inhibition and changes in cell wall components of squash seedlings. Plant Physiol., *106*: 971–976.

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ABSTRAKT

Změny ve složení bílkovin buněčné stěny kořenů ječmene během klíčení a růstu za přítomnosti hliníku

Inhibice růstu kořene a ztráta viability buněk kořene ječmene byla pozorována v průběhu klíčení a růstu ječmene na filtračním papíru nasyceném 2mM konc. $AlCl_3$. Inhibice růstu i ztráta viability kořenových buněk se projevila třetího dne od počátku klíčení. Stejná doba byla potřebná na indukci změn v bílkovinném složení buněčných stěn kořenů. Přítomnost hliníku indukovala akumulaci bílkovin extrahovaných zvýšenou koncentrací solí, zejména polypeptidů s molekulovou hmotností 14, 27 a 29 kDa, za současné inhibice syntézy 18, 23 a 28 kDa polypeptidů. Přítomnost hliníku neměla žádný vliv na složení bílkovin extrahovaných z buněčné stěny pomocí detergentů.

Klíčová slova: jarní ječmen (*Hordeum vulgare* L.); hliníkový stres; viabilita buněk; bílkoviny buněčné stěny; růst kořenů; SDS-PAGE

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