

Genes differentially expressed in soybean lines sensitive and tolerant to aluminium stress

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Abbreviations

°C	Grad Celsius
μ	Micro-
35S	35S promoter from Caliumflower Mosaic Virus
A	Adenine
ABA	Abscisic acid
Acc No	Accession number
Al	Aluminium
Al ³⁺	Aluminium ion
bp	Base pairs
C	Cytosine
CaMV	Cauliflower mosaic virus
cDNA	DNA complementary to mRNA
Ci	Curie
CO ₂	Carbon dioxide
cv	Cultivar
D	Daltons
d	Desoxy-
DNA	Deoxyribonucleic acid
e.g.	<i>Exempli gratia</i> – for example
EDTA	Ethylendiaminetetraacetate
g	Gram
G	Guanine
Gfr. wt.	Grams of fresh weight
h	Hours
kg	Kilogram
kbp	Kilo base pairs (thousand of base pairs)
l	Liter
m	Milli/meter
M	Molar
min	Minute
mM	Millimolar concentration
mRNA	Messenger RNA
n	Nano-
nM	Nanomolar concentration
nt	Nucleotide
μg	Microgram
μM	Micromolar concentration
p	Pico-
PCR	Polymerase Chain Reaction
RNA	Ribonucleic acid
RT	Room temperature
T	Thymin
t	Time
u	Unit
UV	Ultra violet (light)
wt	Wild type

1. Introduction

1.1 Uptake, distribution and accumulation of Al in plants

Aluminium is the most abundant metal in the Earth's crust. Bound as oxides and complex aluminosilicates, it normally exists in insoluble non-toxic form. Surface water concentrations of aluminium ion are minimal due to insolubility of aluminium hydroxide complexes at neutral pH. The acidification of soils and, as a consequence, soil waters, dramatically releases Al^{3+} . The most complete analysis of aluminium chemistry in solutions with different pH was presented by MAC DONALD and MARTIN (1988). The mole fraction of free Al^{3+} , referred as total aluminium, ranges from $<10^{-6}$ at pH 7.0 to 1.0 at pH 4. In solutions with pH more acidic than 5.0, Al ion exists as the octahedral hexahydrate, $Al(H_2O)_6^{3+}$, often abbreviated as Al^{3+} . As a solution becomes less acidic, $Al(H_2O)_6^{3+}$ undergoes successive deprotonations to yield $Al(OH)_2^+$ and $Al(OH)^{2+}$. Neutral solutions give $Al(OH)_3$ precipitate, which redissolves in basic solutions, due to formation of tetrahedral $Al(OH)_4^-$. At pH 7.0 virtually all the soluble aluminium ion occurs as $Al(OH)_4^-$. Living organisms have developed at pH 7.0 and cannot cope with high concentration of toxic soluble aluminium form (FOY, 1984). Toxicity of soil acidification is therefore mainly determined by biological effects of aluminium ion (FOY, 1984). Despite its ubiquity, aluminium does not exert any known function in plant metabolism and belongs to unessential metals. Since acid soils comprise up to 40% of worlds' arable land, aluminium toxicity could be considered as one of the major limiting factors of crop productivity in the world (FOY et al., 1978).

According to aluminium content in different plant organs the plants relatively tolerant to aluminium stress could be divided into three groups (FOY et al., 1978). The first group consists of species, in which aluminium content in upper part of a plant is not different, however the roots of tolerant varieties contain substantially less of the metal in comparison to the sensitive ones. To this type could be attributed triticale, barley, soybean, haricot and others. Aluminium content in the upper part of the second plant type is quite lower in tolerant varieties compared to the sensitive one, however, in the roots the metal content is much higher. To this group belong rice, azalea, rye, alfalfa and others. The third type contains the plants with symplastic tolerance mechanisms accumulating aluminium in the upper part, for example tea can accumulate 30 000 mg/kg dry weight. For wheat, rape and oats this amount is quite a bit lower, 50mg/kg dry weight. Tea, buckwheat, mangroves, some tropical coniferous trees and some grasses are in this group along with some others (FOY et al., 1978).

Aluminium (Al) penetration into roots occurs very rapidly during plant exposure to toxic Al^{3+} . Pattern of Al uptake into plant roots was studied by the most sensitive technique in this field, secondary ion mass spectrometry (SIMS). With use of SIMS it was shown that Al accumulated in the cells of *Zea mays* root tips (0.3-0.8 mm from the apex) after already 30 min exposure to 38 μM Al^{3+} activity (LAZOF et al., 1994). Such short period of uptake is in agreement with the time frame of the first plant morphological changes detected, e.g. inhibition of root growth. Aluminium was accumulated in the cells, in the cytoplasm and vacuolar vesicles but not in the cell wall (LAZOF et al., 1994; LAZOF et al., 1997). Initially Al accumulation in the cells of the root periphery was 140 nmol per g fr. wt. Later on, during 4h exposure, Al entered the cells in more differentiated regions of the maize roots and the rate of accumulation was higher than in root tip. In the primary root bearing lateral roots between 2 and 4 cm length, the rate of Al absorption after 4h of exposure was about 3 times higher than that found in the root tip. (LAZOF et al., 1997).

It was shown by ARSHAMBAULT et al. (1996) that kinetics of aluminium uptake into wheat roots was biphasic with a rapid, non-linear phase in the first 30min, and a linear phase for the remainder of the uptake period of 3h. In these experiments accumulation of Al in the cell walls contributed only 8% of total non-exchangeable pool, when concentration of Al^{3+} in solution was less than 200 μM (moderate Al-stress).

MARIENFELD et al. (2000) as well as earlier Delhaize et al. (1993) reported, however, in the contrast to the previous results, about much higher apoplastic vs symplastic Al concentrations. This was documented by ^{26}Al labelling and uptake assays in *Chara* cells (RENGEL and REID, 1997). More than 99% of absorbed Al was localized in the cell walls and cytoplasmic concentrations were calculated to be in nM range. MARIENFELD et al. (2000) discussed that the data from Lazof et al. (1994, 1997) may be influenced by the extended washing of roots by citrate prior to aluminium penetration measurements. Citrate strongly complexes and immobilises Al and possibly could influence the membrane permeability.

MANGABEIRA et al. (1999) studied pattern of Al accumulation in *Vicia faba* seedlings during relatively long exposure times (24-96hours). Aluminium accumulated in the cells of root surface and in cortical parenchyma cells of the roots. Cortex cells were disintegrated, had damaged plasma membranes and large deposits of Al-complexes in the vacuoles. No accumulation was observed in the vascular cylinder, as well as no detectable Al has been found in stems or leaves of the seedlings studied. MARIENFELD et al. (2000) detected that the radial mobility of Al was much lower in the roots of *Vicia faba* than in *Zea mays*. Aluminium reached the stele already after 60 min of the treatment start, while it was confined to the

rhizodermis and outer cortex in *Vicia faba* indicating stronger binding of Al in the cell wall of dicots.

It has been directly demonstrated (RYAN et al., 1992) that only the terminal 2-3 mm of maize root including the meristem and root cap needed to be exposed to Al in order to cause inhibition of root growth. Later DE LA FUENTE et al. (1997) found that Al penetrates root hairs and affects root hair development. Now the terminal 2-3 mm of roots and root hairs are widely recognized as primary sites of aluminium toxicity and studied most intensively (DE LA FUENTE et al., 1997; KOLLMEIER et al., 2000).

1.2. *Mechanisms of aluminium toxicity in plants*

Aluminium primarily inhibits root growth already in the first 30 minutes of exposure to toxic concentrations, as it was mentioned above (see 1.1). Both cell division and cell elongation of the root tips are sensitive to Al, whereas the mature parts of the root remain unaffected (RYAN et al., 1993; KOCHIAN, 1995). The short time of action suggests aluminium-mediated inhibition of root cell elongation, rather than division, since cell cycle in roots takes 24 hours (KOCHIAN, 1995). Al treated roots become thick and brown, inefficient in absorbing water and nutrients (RENGEL, 1992). Another effect measurable in the first hours of exposure to toxic aluminium is induction of callose formation in root cells (ZHANG et al., 1994; HORST, 1995). In experiments on *Zea mays* root tips and protoplasts callose formation correlated with inhibition of root elongation, so that species tolerant to aluminium showed less Al-induced callose accumulation in comparison to the sensitive ones (HORST et al., 1997). SIVAGURU et al. (2000) showed that Al induced root growth inhibition is closely associated with blockage of cell-to-cell coupling caused by Al induced callose deposition at plasmodesmata responsible for inhibition of symplastic nutrition transport.

After longer exposure to toxic aluminium (24 h to several days), a lot of symptoms appear in roots and shoots. For example, inhibition of cell division in plant roots (CLARKSON, 1995), suppression of mitochondrial activity (DE LIMA and COPELAND, 1994), disturbance of cation and especially K^+ -transport across plasma membrane (HUANG et al, 1992; GASSMANN and SCHRÖDER, 1994), disruption in water and nutrient uptake (BALIGAR, 1993). Still the question whether any of these effects is the primary cause of Al toxicity, remains open.

Another important question has to be clarified in understanding of Al toxicity mechanisms, is it necessary for aluminium to penetrate into the cell to cause toxic syndrome (symplastic toxicity) or it is enough to remain outside the cell blocking water and nutrient exchange (apoplastic toxicity)?

Investigations of root apical ion transport showed that only Ca^{2+} and possibly Mg^{2+} influx were inhibited rapidly enough to be involved in Al toxicity (HUANG et al., 1992, KOCHIAN, 1995). Inhibition of root apical Ca^{2+} influx correlated well with suppression of root growth. It was also shown that micromolar activities of Al^{3+} effectively blocked Ca^{2+} channel in plasmalemma of root apex. In both Al-sensitive and Al-resistant cultivars, however, inhibition of Ca^{2+} influx was differential, so that it was affected less in Al-resistant plants (HUANG et al., 1996). The difference of responses was suggested to be the result of Al-triggered organic acid release, resulting in Al exclusion from the root apex (RYAN et al., 1994). In another study a growth solution containing 1.5mM Al^{3+} activity and moderate Ca^{2+} activity (200mM) stopped the growth of wheat roots without inhibiting apical Ca^{2+} influx. Therefore, the inhibition of root growth by low levels of Al^{3+} does not appear to be the result of inhibited Ca^{2+} influx (RYAN et al., 1994).

Another effect of aluminium stress on cell membranes is dramatic decrease of membrane lipid fluidity (VIERSTRA and HAUG, 1978) as well as increase of plasma membrane lipid peroxidation (CAKMAK and HORST, 1991; ONO et al., 1995). In soybean cell suspension culture under conditions of 15 μ M Al activity the cells showed increase in lipid peroxidation within 4h. This effect could be suppressed by lipophilic antioxidants (RATH and BARZ, 2000). Cell peroxidase isozyme activity in general is also increased under Al stress (CAKMAK and HORST, 1991), which is common for many stresses, for example oxidative burst. Aluminium can bind to plasma membrane outer surface where it stimulates Fe^{2+} -induced peroxidation of membrane lipids (OTEIRA, 1994). That is a free radical chain reaction, which can lead to a loss of membrane integrity and affect functions of membrane proteins (HALLIWELL and GUTTERIDGE, 1989). Enhanced lipid peroxidation is not confined to Al toxicity, but can result from many stress factors. After set of investigations (HORST et al., 1992; YAMAMOTO et al., 2000; ZHENG et al., 2000) it was concluded that lipid peroxidation was a consequence rather than a cause of Al toxicity.

Free cytoplasmic Al^{3+} activity is largely dismissed because in cytoplasm pH value is approximately 7.5 and most of monomeric Al would be in non-toxic form. Taking into account abundance of potential ligands, free cytoplasmic Al^{3+} is estimated to be at picomolar or nanomolar range (KOCHIAN, 1995). However, nanomolar or sub-nanomolar cytoplasmic Al^{3+} activities could be toxic when aluminium interacts with enzymes regulated by Mg^{2+} and Ca^{2+} . Due to size similarities between Mg^{2+} and Al^{3+} ions, displacement of Mg^{2+} by Al^{3+} is much more likely than Al^{3+} - Ca^{2+} interactions. For example, Al^{3+} -ATP complex binds to the enzyme hexokinase 1000 times more strongly than does Mg^{2+} -ATP complex. This is the

proposed mechanism of enzyme inhibition by Al^{3+} (MARTIN, 1988). Another effect of aluminium toxicity in the cell is dramatic decrease of RNA and protein biosynthesis (KOCHIAN, 1995).

The morphological changes induced by aluminium ion in both roots and root hairs, which include a cessation of elongation and swelling of the root or root hair tip, points to aluminium-induced alteration in the cytoskeleton (KOCHIAN, 1995). Al caused modifications of actin network in soybean cells (GRABSKI et al., 1995) and altered polymerisation of actin microfilaments in the distal part of the transition zone (SIVAGURU et al., 1999). Additionally, Al caused rigidification and alteration of cytoskeleton orientation in root elongation zone of maize (BLANCAFTOR et al., 1998; SEJU and LEE, 1998; SIVAGURU et al., 1999).

Interactions between Al^{3+} and components of phosphoinositide signalling pathway were relatively well characterized in animal cells (BERRIDGE, 1987) and are starting to be understood in plants (COTE et al., 1993). When the results from number of laboratories are summarised, it can be concluded that Al^{3+} either binds a GTP-binding protein (possibly at the Mg^{2+} binding site) or interacts directly with the phospholipase C (KOCHIAN, 1995). SIEGEL and HAUG (1983) proposed the role of Al-induced calmoduline modification in Al toxicity as well. MA et al. (2000) suggested the involvement of extracellular calmoduline in short-term effects of aluminium on pollen germination and pollen tube elongation.

Main sites and ways of aluminium toxicity are not clear yet. Recent studies brought some light into the issue, however, the picture of this process is still to be understood.

1.3 Plant resistance to aluminium stress

High variability in plant natural tolerance to aluminium stress allows suggesting that several plant species evolved certain mechanisms to survive under stress conditions. The molecular basis of such tolerance is still poorly understood. Possible strategies of plant resistance to the stress could be divided into two groups (DE LA FUENTE-MARTINEZ, 1999). Both resistance strategies are intensively discussed with respect to their role and importance in Al tolerance in plants.

1. Neutralisation of toxic Al within the cell (symplastic mechanisms).
2. Al exclusion from entry into root apex and root hairs (apoplastic mechanisms).

1.3.1. Symplastic mechanisms of plant resistance

Although little is known about components of symplastic mechanisms of resistance so far, it was demonstrated that some plant species accumulated Al in the roots, such as some

cultivars of azalea, cranberry, rice, triticale, and rye (FOY et al., 1978). Large amounts of Al could also be accumulated in the aerial parts of the plants – tea, some Hawaiian grasses, pine trees, mangrove and others – mainly in the apoplasm or vacuoles of leaf cells (DE LA FUENTE-MARTINEZ and HERRERA-ESTRELLA, 1999).

Substantial increase of Al resistance in both aluminium tolerant and sensitive wheat cultivars was reported by ANIOL et al. (1984) after preincubation of the seedlings with sub lethal levels of Al for 48 hours. This work is the most widely cited one about induction of plant resistance by aluminium stress. RINCON and GONZALES (1991) repeated almost the same experiments, but the results for Al-induction of the tolerance were less convincing. It was demonstrated in other works, however, that root elongation under conditions of moderate Al-concentrations was initially inhibited but partially recovered afterwards (CUMMING et al., 1991; HORST et al., 1983; PCTON et al., 1991). These findings present evidence for plant capability to evolve tolerance against toxic aluminium concentrations.

Recent investigations of aluminium resistance in buckwheat (MA et al., 1998) showed that Al was accumulated in leaf sap in complex with oxalic acid. ^{13}C nuclear magnetic resonance study revealed only one signal, indicating that Al was bound to carboxylic group of oxalic acid. The same signal was achieved with magnetic resonance experiment on intact roots. The purified cell sap was not toxic to root elongation in corn as a test plant. All these results demonstrated that aluminium tolerance in roots and leaves of buckwheat was achieved by formation of a nonphytotoxic Al-oxalate complex with aluminium to oxalate ratio of 1:3.

It was shown that Al stress induces synthesis of some proteins in the root apex (BASU et al., 1984) despite that protein biosynthesis is decreased in general during the stress (KOCHIAN, 1995). The synthesis of two microsomal proteins was induced in root apex of Al-resistant wheat cultivar by 24-96 h Al exposures. Such proteins were not found in the Al-sensitive cultivar. Induction of 51-kD protein synthesis after 24h exposure to $75\mu\text{M Al}^{3+}$ was reported in the work of TAYLOR and co-workers (1997). This protein was accumulated in the root tips of Al-tolerant wheat cultivar PT741 and its association with the tonoplast was demonstrated as well. Until now, however, structures and functions of these proteins are not clear. Transient activation of 48 kD protein kinase and irreversible repression of 42 kD protein kinase were observed in Al resistant wheat cultivar having enhanced malate efflux after very short Al-elicitation (OSAWA and MATSUMOTO, 2001).

Two 51 kD proteins were discovered to be induced by aluminium in resistant wheat cultivar cv PT741 (HAMILTON et al., 2001). The protein sequences were homologous to B subunit of

H⁺-ATPase and also α and β -subunits of mitochondrial ATP-synthase. Both enzymes were elicited by aluminium in dose-dependent manner only in resistant PT741, but not in the other ones. It is suggested that the proteins are responding specifically to Al stress with ATP required for its activity supplied by ATP synthase to maintain energy balance within the cell.

The ability to maintain undisturbed flux through the pentose phosphate pathway seems to play an important role in the mechanism of aluminium tolerance. This pathway provides intermediates such as pentoses, erythrose-4-phosphate and NADPH. These intermediates are involved in the synthesis of amino acids, nucleic acids and coenzymes, which, in their turn could be involved in the process of plant resistance. Rapid increase of activity for the two enzymes of pentose phosphate pathway was suggested to participate in aluminium resistance of wheat (SLASKI et al., 1996). The activity of glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase increased after already 6 hours of exposure to 100 μ M Al³⁺ but only in root tips (terminal 5mm of the roots) of Al-tolerant cultivars. No differences in both enzymes' activity were detected in root tips of sensitive cultivars.

Small metal binding peptides, i.e. phytochelatins play very important role in plant defence against heavy metal stresses (GRILL et al., 1985; RAUSER, 1990; FENIK et al., 1995). In this, like in many other ways, aluminium toxicity stays somewhat apart from heavy metal toxicity phenomenon, however, detailed connection between these stress reactions is very much unclear. One from Al-induced genes, wali1 (found in wheat, SNOWDEN and GARDNER, 1993), showed some homology with metallothionein-like proteins, however, other indications on the role of metallothioneins have not been reported so far.

1.3.2. Apoplastic mechanisms of plant resistance

There are several hypotheses that explain plant resistance by apoplastic mechanism involving Al exclusion from the root apex. Such hypotheses were suggested due to the differences in Al-accumulation between sensitive and tolerant plant genotypes. In nearly isogenic wheat lines, differing in Al-resistance, Al-sensitive genotypes accumulated 3- to 8-fold more Al in the root apex, the critical site for Al-toxicity (DELHAIZE et al., 1993; RINCON and GONZALES, 1992; TICE et al., 1992). No differences were registered in Al content of the tissues from the root upper part between resistant and sensitive genotypes (RINCON and GONZALES, 1992).

Some physiological mechanisms were proposed to explain Al exclusion from entry into plant roots, such as the ones based on alteration in rhizosphere pH, low cation exchange capacity of the cell wall, Al³⁺-efflux across the plasma membrane (KOCHIAN, 1995) but most of the

hypotheses are highly speculative. Relatively well-documented ones are release of organic acids acting as Al-binding ligands (MIYASAKA et al., 1991; PELLET et al., 1995; MA et al., 1997; MA et al., 1998; ZHENG et al., 1998; LI et al., 2000; YANG et al., 2000) and Al-induced alteration of rhizosphere pH (TAYLOR, 1991).

The potential role of organic acid exudation in aluminium resistance was initially proposed by MIYASAKA et al. (1991). In that work increased organic acid exudation was reported in Al-tolerant snap bean cultivar under stress conditions. The root systems of the tolerant plants released 70 times as much citrate in the presence of Al as the plants of Al-sensitive cultivar grown under the same conditions. The most complete analysis of the possible role for organic acids as Al-resistance mediators comes from the work by DELHAIZE and co-workers (1993). Using near-isogenic wheat lines differing at the Al-tolerance locus (Alt-1), it was found that malate exudation from the root apex in tolerant wheat lines upon Al treatment is 10-fold higher in comparison to the sensitive varieties. The amount of malic acid excreted was dependent on Al concentration and Al-tolerance trait cosegregated with high rates of malate excretion, so that it was proposed that Alt1 locus in wheat encoded the component of aluminium tolerance mechanism based on Al-induced malate efflux from the root apex.

In resistant to Al stress soybean cultivar Suzunari citric acid exudation was induced by exposition to 15µM Al already after 6h. The exudation of citric acid was 2-fold higher from the roots of resistant cultivar than of the sensitive one, Shishio, after 6 hours of stress and 2.7-fold higher after 24 hours. Fourteen days of phosphate deficiency did not elicit citrate exudation, while application of Al to Pdeficient plants rapidly induced citrate excretion in both cultivars, confirming the specificity of the response to Al.

Al-activated anion channel was found in plasmalemma of the protoplasts isolated from roots of the Al-tolerant wheat genotype ET8, containing Alt1 gene (RYAN et al., 1997). In the recent work by PINEROS and KOCHIAN (2001) Al activated channels were studied in the protoplasts isolated from the terminal 5 mm of the root apex of aluminium resistant maize hybrid. The study performed using patch-clamp technique showed that acidification of extracellular environment stimulated inward K^+ currents while inhibiting outward K^+ currents. Addition of extracellular Al^{3+} inhibited the remaining K^+ outward currents, blocked inward K^+ ones and caused activation of an inward Cl^- current (anion efflux). Further studies revealed the existence of Al-dependent anion channels, which were highly selective for anions over cations. Such channels were activated by Al^{3+} in isolated membrane patches indicating that the machinery required for Al^{3+} activation and probably the whole root response is localized

into root cell plasma membrane. Al^{3+} -activated anion channel may also be permeable to organic acids mediating Al-tolerance response, for example citric acid.

The investigations of ZHANG et al. (2001) provide the evidence that the difference between Al induced malate efflux from roots of tolerant and sensitive wheat cultivars lies in differing ability of Al^{3+} to activate malate permeable channels and cation channels for sustained malate release.

The type of organic acid actually involved in the process of plant resistance is dependent on the plant specie. Malic acid was released upon aluminium induction in wheat (*Triticum aestivum*, BASU et al, 1994; PELLET et al., 1996), oxalic acid in taro (*Colosalia esculenta* L., MA et al., 1998a) and buckwheat (*Fagopyrum esculentum* Moench. cv Jianxi, ZHENG et al., 1998) and citric acid in maize (*Zea mays*, PELLET et al., 1995), *Cassia tora* L. (MA et al., 1997) and soybean (*Glycine max* cv Suzunari, YANG et al., 2000).

The method of modified vibrating microelectrode was used to show that wheat alr-104 mutant had a 2-fold increase in net H^+ -influx in the root tip, which led to increase in pH of 0.15 points (DEGENHARDT et al., 1998). This mutant did not show an increased exudation of organic acids and its increased resistance disappeared after addition of the buffer preventing the change of pH. It has been concluded that the resistance has been mediated by alkalization of the rhizosphere. Changes of rhizosphere pH were also found along root tips of aluminium-tolerant wheat cultivars, however, with no evidence of such changes in aluminium resistance mechanisms (MIYASAKA et al., 1989; PELLET, 1996).

Aluminium also binds in apoplastic spaces to the cell wall pectin molecules or negatively charged components of the cell wall on the root epidermal and cortical cells (DELHAIZE et al., 1993; MARIENFELD et al., 2000; SCHMOHL and HORST, 2000; SCHMOHL et al., 2000). The free carboxylic groups of demethylated pectin molecules bind toxic aluminium ion complex. In the work of SCHMOHL et al. (2000) pectin methylesterase (PME) treatment of *Zea mays* suspension cultured cells modulated diminished aluminium resistance. In addition, transgenic potato plants overexpressing PME proved to be more sensitive to aluminium than both aluminium tolerant and sensitive cultivars. From the presented results could be concluded that pectin matrix in the apoplast of root apical cells may play an important role in mediating stress signals to cytoskeleton of the cells. High aluminium accumulation in the root apoplast appears to be characteristic for Al sensitivity (RINCON and GONZALES, 1992; SCHMOHL and HORST, 2000).

1.4. General and molecular genetics of Al tolerance; genes induced by aluminium stress

The genetics of Al resistance has been studied in several crop plants with particular emphasis on wheat. In experiments on crossings of 25 aneuploid lines of Chinese Spring wheat cultivar (ANIOL A., 1990) it was shown that Al tolerance is controlled by at least three different factors. These factors were located on chromosomes 5As, 2DI and 4DI. In some cases, tolerance to Al could be controlled by major dominant and several minor dominant genes (e.g. Alt1 locus; DELHAIZE et al., 1993). Major genes for tolerance to Al in rye located on 3R, 6RS and the other minor genes on 4R (ANIOL and GUSTAFSON, 1984). Aluminium tolerance in maize is a dominant trait and is controlled at a single locus by a multiple allelic series (RHUE, 1978), which was shown by screening of F₂ hybrids and backcross populations for aluminium tolerance. After series of experiments genetic control of aluminium tolerance in general is considered to be a dominant, multigenic trait.

Along with field and greenhouse crossings and analyses, studies on the genes involved in aluminium stress have been performed since 1993, when the first five genes up-regulated by aluminium in plants were found by SNOWDEN and GARDNER (1993). These genes, wali1-wali5, were isolated from a cDNA library constructed with use of RNA of root tips from Al-stressed wheat. Al-induction of the gene expression was followed in time frame from 0.5 to 96 hours. Wali2 was the earliest gene to be induced already after 0.5 h of exposure to 10µM Al³⁺. The induction of wali 1 and wali 3 started from 24 hours of exposure and reached maximum at about 48 hours. Wali4 and wali5 were expressed at 24-96 and 48-96 hours, respectively. Among all the studied genes wali1 and wali3 demonstrated the highest expression level. Wali4 as well as wali5 were expressed in quite small amount.

In order to evaluate possible role of isolated genes in aluminium resistance, database search for homologies at the level of encoded proteins was performed (SNOWDEN and GARDNER, 1993). Wali1 (7.4 kD) had homology with metallothionein-like proteins; wali2, encoding 37.5 kD protein was without any significant homology in the database. Wali4 had homology to phenylalanine ammonialyase. Wali3 and wali5 showed 62% of nucleotide identity to each other, however no significant homology of predicted amino acid sequence to any known protein in the database.

Another two genes up regulated by aluminium, wali6 and wali7, were found in the same cDNA library, (RICHARDS et al., 1994). Their expression started after 1 day of exposure to inhibitory Al concentration with peak at day 2 for wali6, while the expression level gradually increased for wali7 with maximum at day 4. Wali6 encoded a small protein that was related to the previously isolated wali3 and wali5 genes (see above). All three polypeptides had a

hydrophobic leader sequence and showed homology to Bowman-Birk proteinase inhibitors (IKENAKA and NORIOKA, 1986). The reading frame encoded by the nucleotide sequence of wali7 is incomplete. However, comparison to a "stem-specific" gene from *Nicotiana tabacum* (SEURINCK et al., 1990) suggests that it may lack only the first nucleotide in ATG initiation codon.

Isolation of 4 cDNA clones, corresponding to the genes induced not only by Al stress (50 to 200 μ M of AlCl₃), but also by acid stress and phosphate (Pi) starvation in suspension cultured tobacco cells was reported by EZAKI and co-workers (1995). The two of them, pAL139 and pAL111, showed the most profound difference in expression level between plants subjected to acid stress and Pi starvation alone or together with Al-stress particularly after 10 hours of treatment. pAL141 showed the same induction during Al-stress as by Pi starvation and pAL142 demonstrated only slight difference between putative two stresses. All cDNA fragments showed high homology with the members of the group of auxin down-regulated genes with glutathione-S-transferase activity (*par* genes, TAKAHASHI et al., 1989). Later the same group isolated another gene, GDP-dissociation inhibitor, pAL546 (EZAKI et al., 1997).

The complete Sali 5-4a and Sali3-2 cDNA sequences from Al-tolerant cultivar of *Glycine max* (Peking) were isolated by RHAGLAND and SOLIMAN (1997). They were up regulated by elevated levels of soluble Al (75 μ M Al³⁺) in the terminal 5mm to 1cm of the roots after 2 days of treatment. Sali5-4a was nearly identical (99%) in nucleotide sequence and identical (100%) in predicted amino acid sequence to auxin down-regulated gene ADR6 from *Glycine max* (DATTA et al., 1993). Sali3-2 was 88% and 81% homologous in nucleotide sequence and amino acid sequence, respectively, to the same gene.

Several genes were discovered to be up regulated by aluminium in *Arabidopsis thaliana* (RICHARDS et al., 1998). The corresponding mRNAs were induced early in Al treatment by 50 μ M Al³⁺. Transcripts of pEARLI8 and pEARLI1 were induced already within first 15 minutes after treatment start, the transcripts of aldolase, pEARLI2 and pEARLI4 were induced a bit later (30min to 4h) and for longer period. Another group, "late induced genes" included glutathione-S-transferase, peroxidase, pEARLI5 and blue copper-binding protein. Delhaize et al. (1999) found that a newly cloned phosphatidylserine synthase gene transcript level is enhanced in response to 8h aluminium stress by 10 μ M Al³⁺ in wheat roots.

All the genes found to be up regulated by Al stress are summarised in Table1 (see above) together with the two ones published only in the Internet by MENOSSI et al. (1997), accession number AF031083 and GAMAS (1998), accession number Y18226. In general, the genes

represented in the Table1 are either the genes of general plant stress response, e.g. wali4 coding for phenylalanine ammonialyase or the genes of unknown or not identified functions, e.g. Wali2.

Table1. Known genes up regulated by Al stress.

No	gene name	plant	function or homology and reference
1	Wali1 ¹	Wheat	Metallothionein-like proteins from several plants (SNOWDEN and GARDNER, 1993).
2	Wali2	Wheat	No sufficient homology, unknown function (SNOWDEN and GARDNER, 1993).
3	Wali3, Wali5, Wali6	Wheat	51 to 54% amino acid similarity to Bowman-Birk Ser proteinase inhibitors from several plant species (SNOWDEN and GARDNER, 1993; RICHARDS et al., 1994).
4	Wali4	Wheat	Clone encoding part of phenylalanine ammonialyase (SNOWDEN and GARDNER, 1993).
5	Wali7	Wheat	No sufficient homology, unknown function (RICHARDS et al., 1994).
6	pAL139, pAL141, pAL142	Tobacco	Strong similarity with glutathione S-transferase gene family from many plant species (EZAKI et al., 1995).
7	pAL111	Tobacco	Identical to auxin-regulated <i>parA</i> gene (EZAKI et al., 1995; TAKAHASHI ET AL, 1989).
8	Zmal1	Maize	Gene coding for protein with expression restricted to root tip (MENOSSI et al., 1999).
9	Mtn29	Barrel medic	Gene induced during nodule development (GAMAS et al., 1996).
10	Sali 5-4 Sali3-2	Soybean	High homology to auxin down regulated gene ARD6 from soybean (DATTA ET AL, 1993; RHAGLAND and SOLIMAN, 1997).
11	pEARLI1	<i>Arabidopsis</i>	Unknown function, (RICHARDS et al., 1998).
12	pEARLI2	<i>Arabidopsis</i>	Unknown function, no homology (RICHARDS et al., 1998).
13	pEARLI4	<i>Arabidopsis</i>	20% identity to extensin (RICHARDS et al., 1998).
14	pEARLI5	<i>Arabidopsis</i>	Reticulate oxidoreductase (RICHARDS et al., 1998).
15	pEARLI8	<i>Arabidopsis</i>	98% identity with EST clone h36573 with unknown function (RICHARDS et al., 1998).
16	BCB	<i>Arabidopsis</i>	Blue-copper-binding protein (RICHARDS et al., 1998).
17	Peroxidase	<i>Arabidopsis</i>	Peroxidase (INTAPRUK et al., 1994; RICHARDS et al., 1998).
18	GST	<i>Arabidopsis</i>	Glutathione-S-transferase (RICHARDS et al., 1998).
19	Aldolase	<i>Arabidopsis</i>	Homologous to EST clone t43001 (RICHARDS et al., 1998).
20	TaPSS1	Wheat	Phosphatidylserine synthase (DELHAIZE et al., 1999).

1.5. Acquisition of Al-tolerance by over expression of transgenes in yeast and plants

Up to now two attempts have been published of functional analyses of genes involved in aluminium stress and study Al-responsiveness of transgenic organisms.

DE LA FUENTE et al. (1997) performed ectopic expression of the gene for citrate synthase from *Pseudomonas aeruginosa* under control of constitutive 35S promoter from cauliflower mosaic

virus (CaMV). *Nicotiana tabacum* L. var. xanthi was chosen as a test plant as it is convenient for plant transformation and sensitive to aluminium toxicity. Seedlings from transgenic plants showed about 10-fold increase of citrate content in the root tissues and up to 4-fold increase in citrate exudation in comparison to wild type. They possessed some degree of increased tolerance to Al. Transgenic tobacco was able to germinate under conditions of high (300 μ M AlCl_3) aluminium concentration but no root system development was observed. Under control conditions, however, root formation took place. Lower aluminium activities (50 to 75 μ M) allowed germination with slightly affected root growth but severely impaired root hair development.

LOPEZ-BUCIO et al. (2000b) found that the plants overproducing citrate yielded better root growth as compared to the wild type under conditions of aluminium stress. The same plants also gave more biomass and had better developed root system under Pi-starvation conditions (LOPEZ-BUCIO et al., 2000a)

However, DELHAIZE et al. (2001) characterized the same transgenic plants and after a set of tests concluded that cytoplasmic expression of bacterial citrate synthase gene in plants is unlikely to be a robust and reproducible way to mediate aluminium tolerance.

EZAKI et al. (1999) expressed several plant genes in *Saccharomyces cerevisiae* to test if it confers Al-resistance. Al-sensitivity tests showed that expression of two of 12 studied plant genes, the *Arabidopsis* gene for blue copper binding protein (*AtBCB*) and the tobacco one for the GDP dissociation inhibitor (*NtGDI1*) resulted in enhanced Al tolerance. Determinations of total content and localization of Al ions in the corresponding transformants suggested that the *BCB* gene product functions in restricting Al uptake while the product of the *NtGDI1* gene may promote release of Al ion after uptake. All the other genes (see Table 1) identified in different groups as Al-induced (DATTA ET AL., 1993; SNOWDEN and GARDNER, 1993; RICHARDS et al., 1994; EZAKI et al., 1995; GAMAS et al., 1996; RHAGLAND and SOLIMAN, 1997; RICHARDS et al., 1998; MENOSSI et al., 1999) did not induce yeast cells' tolerance to Al. The authors speculated that their role could be in protecting the plants during the stress, not conferring Al tolerance.

In order to verify if the genes mediating aluminium resistance in yeast will play the same role in plants, *Arabidopsis thaliana* plants were transformed with several genes up regulated under aluminium stress conditions (EZAKI et al., 2000). An *Arabidopsis* blue-copper-binding-protein gene (*AtBCB*), a tobacco glutathione S-transferase gene (*parB*), and a tobacco GDP dissociation inhibitor gene (*NtGDI1*) conferred a degree of resistance to Al showing

significantly higher root growth of transgenic plants under conditions of 200 μ M AlCl₃ in comparison to the wild type. The increase of the root growth was about 1.5 times higher than in the controls. When 300 μ M AlCl₃ was used, the difference between control and transgenic plants was not observed.

Expression of phosphatidylserine synthase gene from wheat conferred increased resistance to aluminium toxicity in yeast but high level of transgene expression in *Arabidopsis* and tobacco led to appearance of necrotic lesions and was not associated with increased Al tolerance in transgenic plants (DELHAIZE et. al., 1999).

Until now the expression of Al up-regulated genes in plants did not lead to really enhanced aluminium tolerance of transgenic plants or contributed to the functional analysis of the genes involved in the tolerance mechanisms.

2. Scientific Goals of the Work

Despite isolation of some genes and proteins, molecular biology of plant response to aluminium toxicity is not clarified so far. The goal of this work was to study the genes involved in molecular processes of plant resistance to aluminium toxicity in soybean.

Two main directions of the investigations have been chosen. The first one involved testing candidate genes, which have been chosen during literature search for genes up regulated during aluminium stress in plants. Another direction was search for cDNA fragments corresponding to the genes induced upon the stress by the method of DD RT-PCR. Marathon PCR has been applied as the method for cDNA fragment prolongation and further verification. In order to obtain the full protein coding sequence of putative genes, cDNA library construction from the root tips and suspension cultured cells from tolerant soybean under stress conditions was necessary with further library screening using the fragments obtained after Marathon PCR.

As a basic selection principle for the genes studied in all the methods applied comparison of aluminium tolerant and sensitive cultivars under stress conditions as well as comparison with non-stressed control was used. The genes showing transcript enhancement as compared to both non-stressed controls and sensitive aluminium stressed samples were favoured through all gene search process.

In order to study the role of corresponding genes in the tolerance mechanisms, overexpression of the best candidate protein coding cDNA fragments have been done in yeast and in thale cress (*Arabidopsis thaliana*) plants with subsequent characterization of transgenic plant responsiveness to aluminium, Al penetration into stressed roots and distribution of Al-elicited callose accumulation.

This study was a part of a BMBF-funded project within the framework of German-Indonesian cooperation.

3. Materials and Methods

3.1 Materials

3.1.1. Chemicals and Enzymes

AGS GmbH, Heidelberg, Germany	Qualex Gold Agarose.
Amersham Pharmacia Biotech, Uppsala, Sweden	α - ³² P-dCTP(3000Ci/mM), α - ³² P-dATP(3000Ci/mM), α - ³³ P-dCTP, Bind-Silane, Repel-Silane, Restriction Endonucleases: BamHI, EcoRI, EcoRV, HindIII, NcoI, NotI, KpnI, PstI, SacI, SalI, Scal, SmaI, XbaI, T4 DNA Ligase, Shrimp Alkaline Phosphatase.
Boehringer Mannheim, Mannheim, Germany	Bovine Serum Albumine.
Clontech Laboratories, Inc., Palo Alto, California, USA	Advantage Polymerase Mix.
Difco, Detroit, USA	Bacto-Agar, Bacto-Trypton, Bacto-Yeast Extract, BeTek-Agar.
Duchefa, Netherlands	Murashige Scoog Medium, modified 4, Myo-Inosit, Nicotine acid, Pyridoxin x HCl, Thiamine x HCl.
Karl Roth GmbH, Germany	Ampicillin, Kanamycin, Ca(NO ₃) ₂ x4H ₂ O, MgSO ₄ x7H ₂ O, KNO ₃ , KH ₂ PO ₄ , Na ₂ HPO ₄ , NaCl.
Life Technologies GmbH, New York, USA	Super Script Reverse Transcriptase.
MBI Fermentas, Vilnius, Lithuania	Desoxynucleotidetriphosphates, Klenow Enzyme, ATP.
Merck Eurolab GmbH, Hanover, Germany	Formamide, Sodium Dodecylsulfate, AlCl ₃ , H ₃ BO ₃ , Acrylamide, Ammonium Persulfate, TEMED, β -Mercaptoethanol, Urea, H ₃ PO ₄ , KOH, NaOH.
Perkin Elmer Applied Biosystems, Foster City, California, USA	AmpliTaq Polymerase, Stoffel Fragment.
Serva Electrophoresis GmbH A Novex Company, Heidelberg, Germany	CuSO ₄ x5H ₂ O, (NH ₄) ₆ Mo ₇ O ₂₄ x4H ₂ O, MnCl ₂ x4H ₂ O, ZnSO ₄ x7H ₂ O, CaCl ₂ x 2H ₂ O, FeSO ₄ x 7H ₂ O, Na ₂ EDTA x 2H ₂ O, NH ₄ NO ₃ .
Sigma-Aldrich Chemie GmbH, Deisendorf, Germany	Ethidium Bromide, IPTG, X-Gal, Tetracycline, Rifampicin, Carbenicillin.
TaKaRa Biomedical, Japan	PyroBest polymerase

3.1.2. Kits and Ladders

Amersham Pharmacia Biotech, Uppsala, Sweden	Megaprime DNA Labelling Kit, FlexiPrep Kit.
Clontech Laboratories, Inc., Palo Alto, California, USA	Marathon cDNA Amplification Kit.
DYNAL, Oslo, Norway	Dynabeads mRNA Purification Kit.
Eurogentech, Seraing, Belgium	Mass Ladder
Macherey-Nagel, Düren, Germany	NucleoSpin Extract Kit
MBI Fermentas, Vilnius, Lithuania	1kb Ladder
Millipore GmbH, Eschborn, Germany	Dyalysis Filters, Type HA, 0,45µm.
QIAGEN GmbH, Hilden, Germany	QIAGEN Plasmid Midi Kit, QIAquick Gel Extraction Kit, QIAquick Nucleotide Removal Kit.
Stratagene, La Jolla, California, USA	PCR-Script Cloning Kit, ZAP Express cDNA Synthesis Kit and Zap Express cDNA Gigapack III Gold Cloning Kit.

3.1.3. Oligonucleotides for different PCR applications

63end Express2	5'-GATTTTGGATGGTTTCATACTTCAAAGGGCGGCCGCATA-3'
63stExpress	5'-AATCCATGGCGCCGCCTCCCAAGCCAGTGCC-3'
AP1	5'-CCATCCTAATACGACTCACTATAGGGC-3'
AP2	5'-ACTCACTATAGGGCTCGAGCGGC-3'
Coli PEPC End	5'-GCCGGTATTACGCATACCTGCC-3'
Coli PEPC St	5'-ATGAACGAACAATATTCCGC-3'
Mals255	5'-GGAAGCAT(AT)**AGGGC(AT)**ACTGTTC-3'
Mals280	5'-CCATTC(AT)**GTGGG(CT)**TTGAA(CT)**TG-3'
TA22L	5'-GCTGTACAGAAGCACCTGTGCAATTCCGCC-3'
TA22s	5'-GCACCTCTGCAATTCCGCC-3'
TA52L	5'-CCAAAGAAGTTACATCTTGTTAAACATTGG-3'
TA52s	5'-CATCTTGTTAAACATTGGC-3'
T ₁₂ NA	5'-TTTTTTTTTTTTTN*A-3'.
T ₁₂ NC	5'-TTTTTTTTTTTTTN*C-3'.
T ₁₂ NG	5'-TTTTTTTTTTTTTN*G-3'.
Wali11	5'-GC(AT)**ACATN*N*CAACACGT-3'
Wali12	5'-GAA(CT)***(AT)**(AT)**(AG)****GGTTGCAGG-3'

Wali4v1	5'-TTCTTGTGGAGCACGCCT -3'
Wali4v2	5'-TGCAATCTTTGGCATTGG-3'

* - any nucleotide, A, C, G or T. ** - A or T nucleotides. *** - C or T nucleotides **** - A or G nucleotides

3.1.4. Fluoresceine Labeled Primers for Sequencing

M13 Reverse	5'-CAGGAAACAGCTATGAC-3'
M13 Universal	5'-GTAAAACGACGGCCAGT -3'
131RI	5'-GGCCTTGCGGCGCCGTTTCG-3'
131bbRII	5'-GTTCCGGAAGCACGGTTTGG-3'
131BBR2	5'-CAGCTTCTAGATGAGG-3'
131bbUI	5'-GGTGCTCATCGTGA CTACC-3'
131bbU2	5'-GGATTTGAGGATAAGTCG-3'
58 1b2rF	5'-CCGTTACAAGGAAATTGAGAATGG-3'
58 1b2uF	5'-CTAGTACACACCCAAAAATTC-3'
58 1b2RII	5'-CCAGGACCTCCTTACAGG-3'
58 1b2UII	5'-CCTTATCAATAAAACCATG-3'
58 1b2R2	5'-GCTCCCTCGATGTGCTTC-3'
58 1b2U2	5'-GGAGGTCAAGTGCTAAGTG-3'
63 2a3rF	5'-GGACGAATCATACCGACAAG-3'
63 2a3uF	5'-CCCACGAAATCAGTGTTCTTCC-3'
63 2a3R2	5'-CCATTTGTACTACTCAGG-3'
63 2a3U2	5'-CTGATACACATAAGCGCC-3'

3.1.5. Vectors and Plasmids

Vector	Bacterial marker	Plant marker	Reference
PBI121	Kan ^r	Kan ^r	Stratagene, La Jolla California, USA
PBIN 19	Kan ^r	Kan ^r	Bevan et al. 1984
PBK-CMV	Kan ^r	--	Stratagene, La Jolla California, USA
PCR-Script SK+	Amp ^r	--	Stratagene, La Jolla California, USA
PS5'UT	Amp ^r	Kan ^r	Hirsch et al.
λ-ZAP Express TM .	Kan ^r	--	Alting-Mees et al, 1992

3.1.6. Bacterial and Yeast Strains

E. coli strains

Strain	Genotype	Reference
HB101	F ⁻ leuB6 proA2 recA 13 thi-1 ara-14 lacY1 galK2 xyl-5 mtl-1 rpsL20 supE44 hsdS20	ATCC Bacteria and Bacteriophages, 19 th edition, 1996
XL1 Blue	RecA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac[F' proAB lacI ^q ΔM15 Tn10(Tet ^r)] ^c .	Stratagene, La Jolla California, USA
XL10 Gold	Tet ^r Δ (mcrA) 183 Δ(mcr CB-hsdSMR-mrr)173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac Hte [F' proAB lacI ^q ΔM15 Tn10 (Tet ^r) Amy Cam ^r]	Stratagene, La Jolla California, USA
XL1 Blue MRF'	Δ(mcrA)183, Δ(mcrCB-hsd SMR-mrr)173, endA1, supE44, thi-1, recA1, gyr A96, relA1, lac[F' proAB lacI ^q ΔM15 Tn10(Tet ^r)]	Stratagene, La Jolla California, USA

Agrobacterium tumefaciens strains

Strain	Reference
C58C1 [pGV2260]	BRIDGE and SUMMERFELT, 1990
GV3101	VAN LABEREKE et al., 1974

Saccharomyces cerevisiae strain

Strain	Genotype	Reference
Inv Sc2	MATa his3 [?] leu2 trp-298 ura3-52	EZAKI et al., 2000

3.1.7. Plant Material

We used in our Al stress experiments soybean lines: *Glycine max* cv Tambora and *Glycine max* cv Wilis (tolerant to Al stress); *Glycine max* cv Malabar and *Glycine max* cv Lumut (sensitive to Al stress). The lines *Glycine max* cv Wilis and *Glycine max* cv Lumut were used in cell suspension culture work. As host plants for gene transfer experiments we have chosen *Arabidopsis thaliana* ecotype Columbia (Col-0).

3.1.8. Media

Bacterial Media

E. coli strains were grown in LB(Luria -Bertrani) medium (SAMBROOK et al., 1989) at 37°C. *A. tumefaciens* was grown in CPY medium (0.1% yeast extract, 0.5% trypton, 0.5% saccharose, 2mM MgSO₄, pH 7.2) at 28°C or in YEB medium (0.5% beef extract, 0.1% yeast extract, 0.5% peptone, 0.5% saccharose, 2mM MgSO₄).

λZAP cDNA library was plated on NZY medium (SAMBROOK et al., 1989).

Media for plant transformation

LS medium (LINSMAIER AND SCOOG, 1965); MS Medium (MURASHIGE AND SCOOG, 1962).

CPY medium (HORSCH ET AL., 1985): 0.5% casein peptone, 0.1% yeast extract, 0.5% Saccharose, 2mM MgSO₄.

Modified Hoagland solution: (5mM Ca(NO₃)₂, 1mM MgSO₄, 5mM KNO₃, 1mM KH₂PO₄ with micronutrients: 50µM H₃BO₃, 0.3µM CuSO₄, 0.1µM (NH₄)₆Mo₇O₂₄, 4.5µM MnCl₂, 3.8µM ZnSO₄).

3.2. Methods

3.2.1. Plantlets in water culture: growth conditions and Al treatment

The appropriate soybean seeds were germinated for 3 days in the dark on the paper wet by 1mM CaCl₂ with aeration, then the root tips were removed to enhance growth of secondary roots. After that the plantlets were placed into modified Hoagland solution (5mM Ca(NO₃)₂, 1mM MgSO₄, 5mM KNO₃, 1mM KH₂PO₄ with micronutrients: 50µM H₃BO₃, 0.3µM CuSO₄, 0.1µM (NH₄)₆Mo₇O₂₄, 4.5µM MnCl₂, 3.8µM ZnSO₄) for the next 4 days in the green house with aeration. Subsequent Al stress experiment was performed by titrating solution pH to 4.2 by 37% HCl and applying AlCl₃ in final concentration 300µM for 4, 24 or 48 hours. After Al treatment the plantlets were removed from the green house, roots washed with cold water, root tips (approximately 1-2cm from the end of the roots) were cut on ice, frozen in liquid nitrogen and stored at -70°C till RNA isolation (not more than two weeks).

3.2.2. Plant suspension culture and Al treatment

Suspension cultured cells were obtained from Dr A. Tewes, IPK, Gatersleben. Al treatments were made according to STASS and HORST (1995). Seven-day-old cultured cells were washed twice with a solution containing 2% sucrose and 500µM CaCl₂, pH 4.3. Then they were resuspended in the same solution with a density of 3g cells per 100 mL. Aliquots of 10mL of this cell suspension were immediately pipetted into 250 mL Erlenmeyer flasks, containing 90 mL of the same solution supplemented with AlCl₃ to final concentration of 100µM. The cells

were incubated for 4 hr with horizontal shaking (140 rpm, r=12.5 mm) and collected for further RNA isolation.

3.2.3. RNA isolation

RNA extraction has been performed with guanidine hydrochloride method (LOGEMANN et al., 1987). Plant material has been frozen in liquid nitrogen, grinded to a fine paste. 3ml Z6 Buffer (8M guanidine hydrochloride; 20 mM MES; 20 mM EDTA, pH 8.0; 50 mM β -mercaptoethanol) and 3ml phenol: chloroform: isoamyl alcohol (24:24:1) per g of material have been added and this mixture centrifuged 30 min at 4°C, at maximal speed. 0.7 volume ethanol and 0.2 volumes 1M acetic acid have been added to the upper phase after centrifugation and incubated on ice for 30min. After centrifugation 15 min at maximum speed the pellet has been washed two times by precooled 3M sodium acetate (pH 5.2) and RNA has been pelleted by centrifugation 15 min on maximum speed at 4°C. The pellet has been washed by cold 70% ethanol, dissolved in DEP water and stored at -70°C. Quality and quantity of RNA have been verified by denaturing RNA agarose gel electrophoresis according to SAMBROOK et al. (1989).

3.2.4. RT-PCR

To find the most conservative regions in the gene targeted for RT-PCR, search for homologies has been made with BLAST internet resource on the DNA and protein level. Primers were constructed inside such conservative regions and alignment on DNA level was used to avoid too high primer degeneracy. Ampli Taq DNA polymerase (Perkin Elmer Applied Biosystems) was used for RT-PCR. The conditions of amplification were standard according to producer's manual. Products of PCR were resolved on 1% agarose gel.

3.2.5. Differential Display RT-PCR; reamplification and cloning of chosen cDNA fragments

DD RT-PCR was made in general according to Liang et al. (1993). For 3'-end poly-A nested primers were used T₁₁NA, T₁₁NG and T₁₁NC. Random 11-mers were used as 5'-end ones. PCR conditions were used exactly according to Liang et al.(1993). ³³P- α -dCTP was used in amplification reaction as a radioactive label. All PCR reactions were made twice with the same cDNA sample. 3 μ l aliquotes of amplification products were resolved on 6% acrylamide sequencing gel. After exposing the gel to the autoradiographic film, the bands of interest were positioned with use of the film, cut out and incubated in TE buffer at 37°C 4-5 hours. This mixture was used for the band reamplification. Reamplified bands were cloned into PCR Script SK⁺- cloning vector (Stratagene).

3.2.6. Marathon cDNA amplification

Marathon cDNA amplification was made with use of Clontech Marathon cDNA amplification kit and Advantage Polymerase (Clontech). The primers for Marathon PCR were made with use of sequence information of either Differential Display bands or appropriate sequences found in Erez Nucleotide Sequence Search:

(<http://www.ncbi.nlm.nih.gov/Entrez/nucleotide.html>).

Typical PCR reaction included: 94°C, 3min, 30 cycles of: 94°C, 5sec; 68°C, 4 min; then 68°C, 3 min. Touch down approach was also applied in several cases, which is highly recommended to reduce background. PCR scheme with touch down looked like this: 94°C, 2 min; 10 cycles of: 94°C, 30 sec; 63°C (65°C or 68°C were applied according of primer melting temperature), 30sec; 68°C, 2 min, subsequently 30 cycles of: 94°C, 30 sec; 60°C(63°C or 65°C were applied according of primer melting temperature), 30sec; 68°C, 2 min, then at the end 68°C, 5 min. The products of PCR reaction were resolved on 1% agarose gel.

3.2.7. Cloning and sequencing

Standard methods of restriction analysis, gel-electrophoretic resolution of nucleic acids and proteins were made according to protocols by SAMBROOK et al.(1989). PCR fragment isolations were made by NucleoSpin Extract Kit (Macherey-Nagel). Plasmid DNA isolations were made by FlexiPrep kit (Amersham Pharmacia Biotech). Midi preparations of plasmid DNA were made by Plasmid Midi kit (Qiagen), some purifications were made by QIAquick PCR Purification kit (Qiagen). *E. coli* strains XL-1 Blue, XL-10 Gold and HB101 were transformed with use of heat shock method according to HANAHAN (1988). Cloning of PCR fragments was performed with use of PCR-Script Cloning kit (Stratagene), according to the producer's recommendations. DNA sequencing was done according to SANGER et al. (1977) and CHEN and SEEBURG (1985) with use of automated A.L.F.- sequencing equipment in sequencing laboratory (S. KÖNIG, IPK, Gatersleben). DNAMAN Sequence Analysis Software, Version 4.1 (Lynnon BioSoft) was used for sequence analysis and editing. Homology search for nucleic acid and protein sequences was made by BLAST (Basic Local Alignment Search Tool, ALTSCHUL et al., 1990; <http://www.ncbi.nlm.nih.gov/BLAST/>).

3.2.8. Reverse Northern and Northern Blotting

Reverse Northern Blotting was performed according to Clontech protocol for hybridization with cDNA. cDNA synthesis was done with use of Super Script RNaseH⁻ Reverse Transcriptase (Gibco BRL), according to protocol of producer in total volume of 80 µl. 27µl of reaction mixture were used in random prime labelling reaction performed with use of Megaprime DNA Labelling Kit (Amersham). ³²P-α-dCTP and ³²P-α-dATP were used both for

labelling reaction. Labelled cDNA probe was purified by NucTrap columns (Stratagene). Hybridization was performed in Church buffer (CHURCH and GILBERT, 1984) at 60°C with membranes containing 10 µg of appropriate cloned Differential Display fragments or purified PCR products after Marathon cDNA amplification. Washing procedure included: 4 times, each 20 min, in 2x SSC; 1% SDS at 60°C and 2 times, each 20 min, in 0.1x SSC; 0.5% SDS at 50°C.

Northern blotting was made according to standard protocol of SAMBROOK et al. (1989) with some variations. 100ng to 1µg of DNA probe were labelled either by random prime labelling method with use of Megaprime DNA Labelling Kit (Amersham), or, in case of small Differential Display fragments, by 3'-end labelling procedure with use of Klenow enzyme (MBI Fermentas). Labelled DNA fragments were purified by NucTrap columns (Stratagene). Hybridization was made in Church buffer (CHURCH and GILBERT, 1984) at 60°C. After overnight incubation the membranes were washed by 2x SSC; 0.1% SDS, 2 times 20 minutes each and 2-3 times, 20 minutes as well by 1xSSC; 0.1% SDS. In any case the results of hybridizations were analysed with use of Phosphor Imager BAS 2000, Fujix and Tina 2.09 software (Raytest Isotopenmeßgeräte GmbH).

3.2.9. cDNA library construction and screening

cDNA library was constructed with use of λ-ZAP Express cDNA synthesis kit. 75µg of RNA extracted from aluminium treated (see 2.2.1.) root tips of *Glycine max* cv Tambora and 75µg RNA extracted from aluminium treated suspension cultured cells of *Glycine max* cv Willis were used for cDNA synthesis. Gigapack III Gold Packaging Extract was used for packaging. The library was amplified and screened by purified PCR fragments obtained by Marathon cDNA amplification. Approximately 100 ng of each fragment were used for screening. Hybridizations were made in Church buffer (CHURCH and GILBERT, 1984) at 60°C. The clones obtained in screening were subjected to *in vivo* excision and subsequent sequencing.

3.2.10. Yeast transformation and characterization of Al responsiveness of transgenic yeast

A Yep-type vector, pYES2 was used for the introduction of full protein-coding fragment into yeast strain Inv Sc2. Transformation was performed according to GIETZ et al., 1992. Exponential-phase cells grown in LPM medium at pH 4.0 supplemented with 2% glucose were centrifuged and resuspended in LPM medium with 2% galactose or 2% glucose and grown for 9 hours to initiate the gene expression. Then the part of the cells was diluted 1:10 in LPM medium and placed in diluted and undiluted form in drops onto LPM agar adjusted to pH 4.0 containing aluminium chloride in concentrations of 0, 1, 5, 7.5 and 10mM. The results of the cell growth were analysed after overnight incubation.

3.2.11 Construction of expression plasmids for over expression in plants

The structural genes from the clones 318 and 633 were amplified with use of plasmid DNA as a template by high fidelity PyroBest DNA polymerase (TaKaRa). The primers were designed to obtain corresponding full protein coding sequences in frame for protein synthesis with restriction sites *Sma*I in the beginning and *Not*I in the ends of the sequences. The PCR products were resolved on 1% agarose gel, purified with use of NucleoSpin Extract kit (Macherey-Nagel) and cloned into the plasmid pRTRA7/3 (kind gift from S. Miroshnichenko, IPK, Gatersleben) containing Cauliflower Mosaic Virus 35S promoter, c-myc-tag for detection of foreign protein expression and poly-A region sequence for enhancement of expression in transgenic plants. After recombinant plasmid verification by restriction analysis partial restriction was made with use of *Hind*III restriction enzyme. Vector plasmid pBIN19 was also restricted by *Hind*III and dephosphorylated to block self-ligation according to SAMBROOK et al., 1989. The constructs consisting of the CMV 35S promoter, structural gene (either 318 or 633), c-myc-tag and poly-A region were cloned into pBIN19 in *Hind*III sites. After restriction verification recombinant plasmids were ready for plant transformation.

3.2.12. Plant transformation and characterization of transgenic plants

The plant transformation was performed according to CLOUGH and BENT (1998) using *Arabidopsis thaliana* cv Columbia as a host for transgene expression. F₁ transgenic lines were selected on MS agar containing 30µg/ml kanamycin. Kanamycin resistant seedlings were transferred to soil and grown to maturation. Kanamycin resistant progeny in F₂ were selected in the same way.

The test for aluminium resistance was performed on 4 to 7 day old seedlings. They were transferred to Hoagland solution, pH 5.7 for the control or pH 4.0 as acidic stress control and Hoagland solution, pH 4.0 supplemented with different concentrations of aluminium chloride (in the range of 50 to 300µM).

Morin staining experiment as a test for aluminium penetration into the roots of transgenic and wild type plants was performed according to LARSEN et al. (1996). The seedlings were treated by 200µM aluminium chloride in Hoagland solution, pH 4.0 for 2h, washed in 5mM NH₄OAc, pH 5.0 for 10 min and stained in 100µM morin (Fluka) in 5mM NH₄OAc, pH 5.0 for 1h. After washing in 5mM NH₄OAc, pH 5.0 for 10 min stained roots were visualised under fluorescence microscope (Zeiss, Axioplan II) using fluorescence filter for DAPI-FITC-Rhodamine fluorescence.

Evaluation of stress induced callose biosynthesis has been performed using aniline blue staining (SCHMOHL et al., 2000). The seedlings were subjected to aluminium stress for 48 hours under conditions of 200 μ M aluminium chloride in Hoagland solution, pH4.0. After the treatment roots were cut, immediately washed in tridest for 5 min and stained by aniline blue for 2-3 minutes with subsequent wash in tridest for 5 min. The wild type and transgenic plants were stained and washed in parallel to ensure the uniformity of staining procedure. After staining the roots were visualized under fluorescence microscope using the same filter combination as for morin staining procedure.

4. Results

4.1. Influence of aluminium stress on root morphology and suspension cultured cells

The aim of the work was to study gene activities responsible for stress resistance of soybean under conditions of moderate aluminium stress. We tried to restrict the plant response to this specific stress.

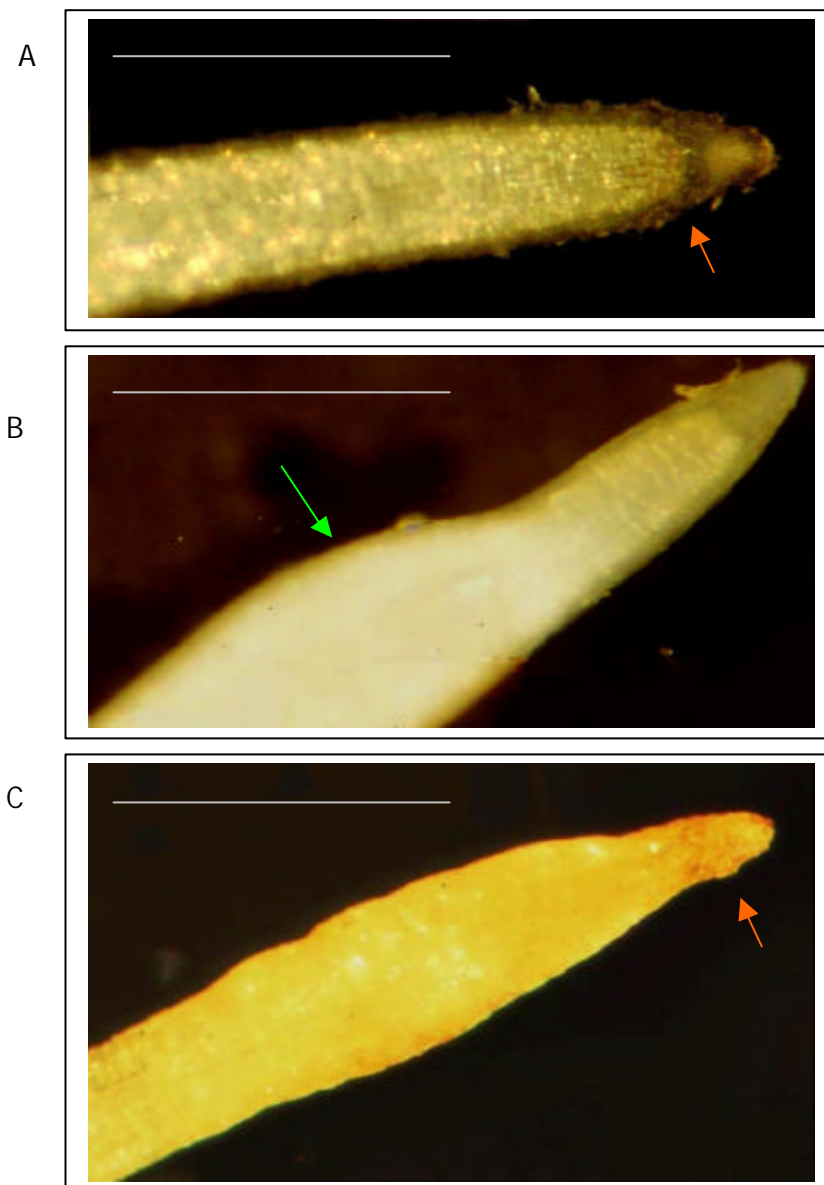


Fig. 1. Influence of aluminium treatment on root tip morphology.

A - control root of soybean cv Tambora (tolerant). B - the root of soybean cv Tambora (tolerant) after the treatment with 300µM AlCl_3 for 48 h. C - the root of soybean cv Tambora (tolerant) after the treatment with 1000µM AlCl_3 for 48 h.

The red arrows show the root meristem undergoing the changes already after the treatment by 300µM AlCl_3 . Morphology of the meristem changes but not so dramatically as in case of the treatment by 1000µM AlCl_3 . After this treatment the form of the root changes dramatically and the root meristem shape clearly visible in control disappears. The green arrow shows root swelling which is typical for the roots after 300µM aluminium treatment. The bars in the upper left corners are 1000µm long.

Therefore the experiments were performed to evaluate root shape changes dependent on AlCl_3 content in the nutrient solution in order to find the lowest stressor concentration causing the first morphological changes of the root tips in comparison to the untreated control. $300\mu\text{M AlCl}_3$ induced the first visible alterations of the root tip shape, disruption of the meristem zone and swelling of the main roots (Fig. 1B). $1000\mu\text{M AlCl}_3$ caused severe damage of the root tip shape, completely destroying the normal structure of the meristem zone clearly visible in the control roots (Fig.1, A and C). The concentration of $300\mu\text{M AlCl}_3$ was chosen for the further aluminium stress experiments on plants in the nutrient solution.

Based on STASS and HORST'S (1995) work on aluminium treatment of *in vitro* cell suspension culture the concentration of $100\mu\text{M}$ has been chosen for the aluminium stress in our *in vitro* cell culture studies. This moderate Al was used because $50\mu\text{M}$ concentration is the lowest one causing enhanced callose formation, one of the most sensitive markers of aluminium toxicity. On the other hand increasing of AlCl_3 concentration up to $500\mu\text{M}$ inhibited K^+ -net efflux (indicator of the ion exchange through the membrane) to the lowest range whereas lipid peroxidation is started indicating already the massive stress in the cells (STASS and HORST, 1995).

To study the gene expression in soybean plants as well as *in vitro* cell suspension culture two different approaches were used. The first one included hybridization experiments with the known genes potentially involved in the mechanisms of aluminium resistance. The second one consisted of a direct study of gene expression pattern using the method of Differential Display.

4.2. Evaluation of the expression of genes potentially involved in Al resistance

The mechanisms of aluminium toxicity and plant resistance are not yet clear. There are a number of potential genes, which could be involved in the processes of plant defence against aluminium stress (see *Introduction*). In order to study gene expression pattern several candidates from the literature have been chosen. The reasons for choosing each individual gene are presented in Table 1.

Some of the candidate genes could be verified directly since they were available as cloned cDNA fragments. Others, however, either were not at our disposal or were from too genetically distant plants so that it was necessary to use the sequence information from such genes in Marathon PCR approach (see *Methods*, chapter 2.2.6) with subsequent evaluation of corresponding gene expression by Reverse Northern hybridization technique (*Methods*, chapter 2.2.8).

Table 2. Northern hybridization experiments, the reasons for using candidate genes.

No	Gene	Reason for the gene choice
1	Calreticulin	Ca-binding protein. Participates in second messenger pathway, one of possible sites of aluminium attack (KOCHIAN, 1995) as well as involved in callose accumulation in plasmodesmata, mediating aluminium blockade of symplastic transport (SIVAGURU et al., 2000)
2	Metallothionein, MT4	Metallothionein-like proteins are induced by aluminium in wheat (SNOWDEN and GARDNER, 1993).
3	Metallothionein, MT1	Metallothionein-like proteins are induced by aluminium in wheat (SNOWDEN and GARDNER, 1993).
4	Pea Metallothionein	Metallothionein-like proteins are induced by aluminium in wheat (SNOWDEN and GARDNER, 1993).
5	Glutathione-S-Transferase	Antioxidation enzyme. Induced by aluminium in tobacco (EZAKI et al., 1996; EZAKI et al., 2000)
6	GTP-binding protein	Induced by aluminium in tobacco (EZAKI et al., 1996)
7	Malate Dehydrogenase	Malic acid is one of the acids used in organic acid exudation mechanism, one of plant strategies to tolerate aluminium stress (KOCHIAN, 1995; LI et al., 2000).
8	Phosphoenolpyruvate (PEP) Carboxylase	The component of organic acid biosynthesis. The organic acids such as malic and citric acids are used as external chelators of aluminium in plants (KOCHIAN, 1995).

4.2.1. Evaluation of gene transcript abundance by Northern hybridization

The study of the gene expression level for the available cDNA fragments involved verification of the gene responsiveness to aluminium stress by Northern hybridization. The RNA for such experiments was extracted from the root tips of Tambora (tolerant to Al stress) and Malabar (susceptible to Al stress) soybean cultivars.

The aluminium treatment was performed for 48 h as described in *Methods*, chapter 2.2.1. The majority of the genes probed showed the same expression level during stress and in non-treated controls, both in case of tolerant and sensitive cultivars (Fig.2). This result indicates that all of them are not involved in aluminium stress response of soybean.

Phosphoenolpyruvate (PEP) carboxylase was up regulated in soybean root tips during aluminium stress for 48 h. Its expression was enhanced in root tips of both tolerant cv Tambora and sensitive cv Malabar. In case of tolerant cultivar Tambora the increase was 2.8-fold in comparison to untreated controls during moderate acidic stress (pH of nutrient solution lowered to 4.0) and 5.4-fold during 300µM $AlCl_3$ treatment for 48h. Sensitive to Al stress cv Malabar reacted somewhat weaker.

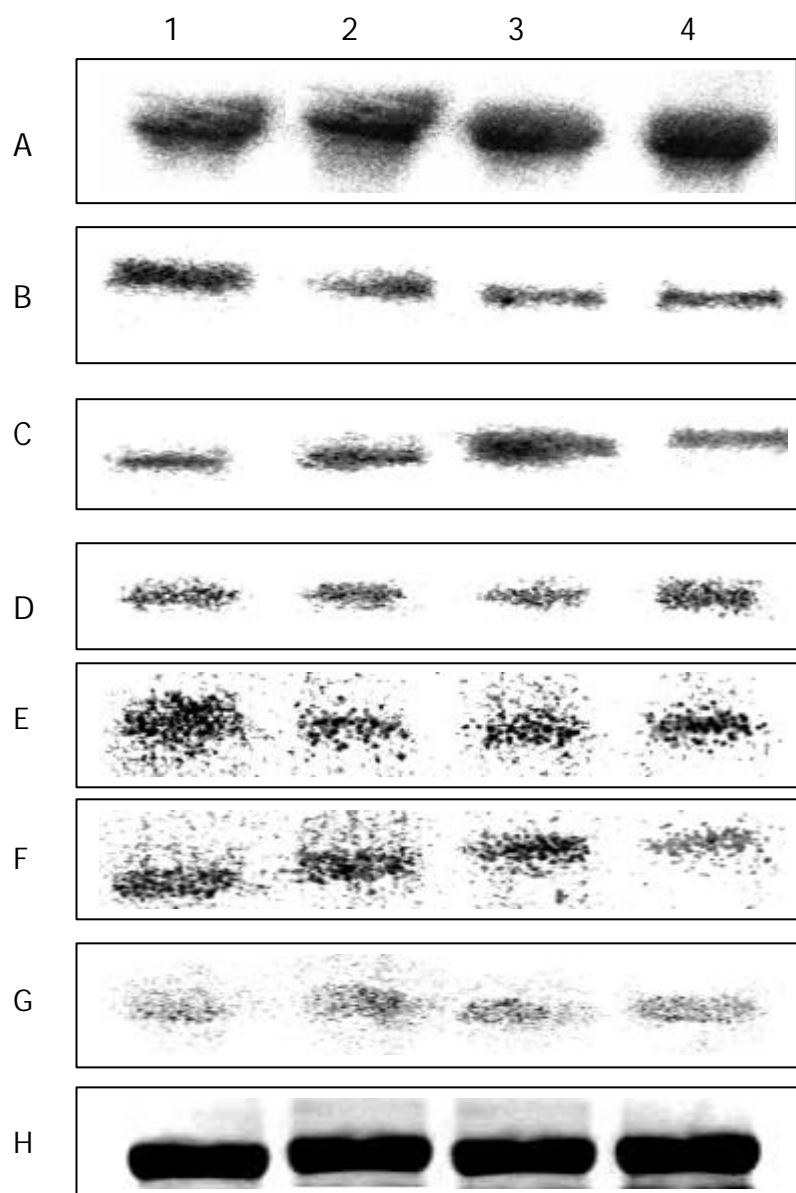


Fig. 2. Evaluation of the transcript abundance for the genes possibly involved in the plant response to aluminium stress.

Northern hybridization with probes: A. Calreticulin, tobacco (*Nicotiana tabacum*). B. Metallothionein MT4, tomato (*Lycopersicum esculentum*). C. Metallothionein MT1, tomato (*Lycopersicum esculentum*). D. Metallothionein, pea (*Pisum sativum*). E. Glutathione-S-transferase, tobacco (*Nicotiana tabacum*). F. GTP-binding protein gene, tobacco (*Nicotiana tabacum*). G. Malate dehydrogenase gene, tomato (*Lycopersicum esculentum*). H. 18s rDNA as loading control. 1. Control: RNA from root tips of Malabar (sensitive), non-treated. 2. RNA from root tips of Malabar (sensitive), treated with 300 μM AlCl₃ for 48h. 3. Control: RNA from root tips of Tambora (tolerant), non-treated. 4. RNA from root tips of Tambora (tolerant), treated with 300 μM AlCl₃ for 48h.

The increase in PEP carboxylase expression under identical stress conditions was 2.6-fold as compared to untreated tolerant control plant, however, the control PEP carboxylase expression in cv Malabar was beyond the level of detection in this experiment indicating that level of enzyme expression is lower in susceptible plants in comparison to tolerant ones (Fig. 3). The increased transcript level of PEPC under Al stress in tolerant soybean cultivars suggests that this enzyme belonging to organic acid metabolism could be involved in Al resistance mechanism of soybean.

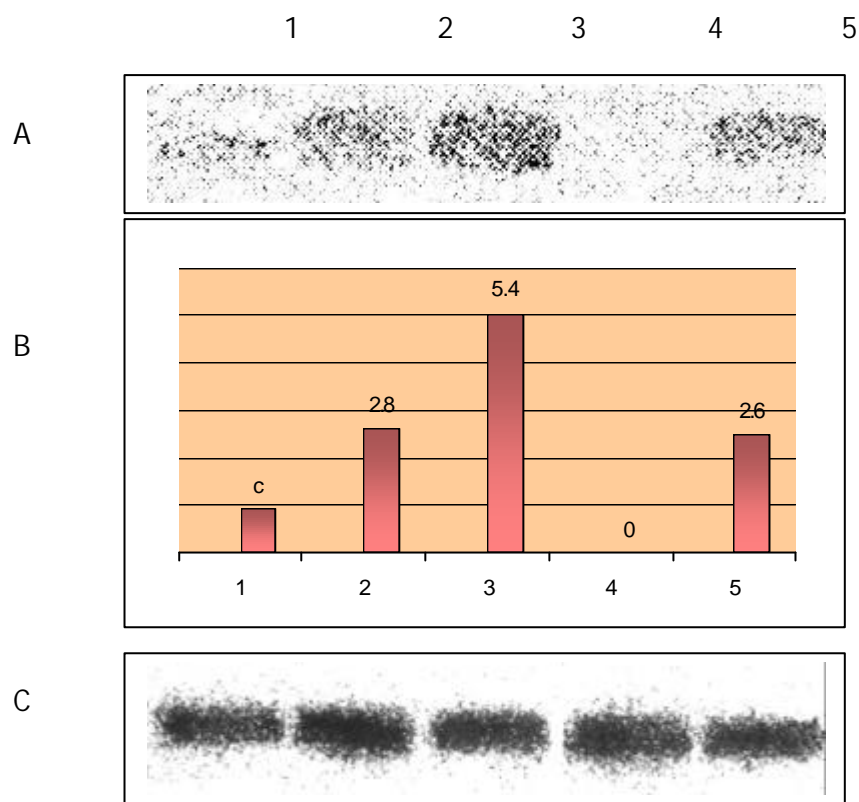


Fig.3. Evaluation of RNA abundance for PEP Carboxylase during aluminium stress.

A. Northern hybridization with PEP Carboxylase cDNA probe from tobacco (*Nicotiana tabacum*). B. Quantification of the relative hybridization signal intensity. C. Hybridization with 18s rDNA probe as loading control.

1. Control: RNA from root tips of soybean cv Tambora (tolerant), non-treated. 2. RNA from root tips of soybean cv Tambora (tolerant), treated only with low pH (4.0) for 48h. 3. RNA from root tips of soybean cv Tambora (tolerant), treated with 300 μ M AlCl₃ for 48h. 4. Control: RNA from root tips of soybean cv Malabar (sensitive), non-treated. 5. RNA from root tips of soybean cv Malabar (sensitive), treated with 300 μ M AlCl₃ for 48h. The numbers on the graph show the expression level enhancement of appropriate sample in comparison to the control marked as „c“.

4.2.2. Evaluation of gene expression by Reverse Northern hybridization

In case of several genes not available directly another method of evaluation was used: Marathon PCR combined with Reverse Northern hybridization. Sequence information of the gene conservative regions was used to create primers for Marathon amplification. Since the aim of the work was to find the genes specifically involved in aluminium stress response of soybean, the most effort was directed to extract the amplified fragments yielded only in the reaction with cDNA from tolerant to Al stress soybean cultivars treated by AlCl₃ but not in controls or susceptible cultivars also stress treated. By this approach the cDNA fragments corresponding to non-stressor genes (PCR fragments were in reactions with all cDNA samples or in controls only) and the genes of general stress response (PCR fragments were in reactions with cDNA samples from susceptible cultivars treated with aluminium stress) were eliminated from the further experiments.

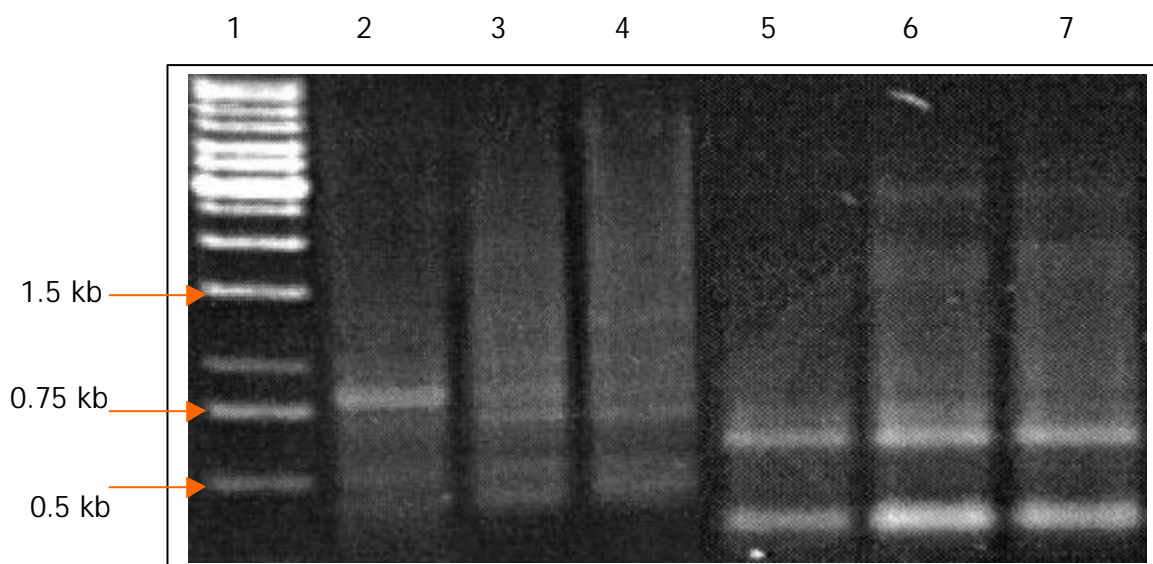


Fig. 4. Marathon amplification for GST and pAL genes.

1. DNA Ladder 2. Marathon amplification with GST primers with use of cDNA from control root tips of soybean cv Tambora (tolerant). 3. Marathon amplification with GST primers with use of cDNA from root tips of soybean cv Tambora (tolerant) treated with 300 μ M AlCl₃ for 48 h. 4. Marathon amplification with GST primers with use of cDNA from root tips of soybean cv Malabar (sensitive) with 300 μ M AlCl₃ for 48 h. 5. Marathon amplification with pAL primers with use of cDNA from control root tips of soybean cv Tambora (tolerant) 6. Marathon amplification with pAL primers with use of cDNA from root tips of soybean cv Tambora (tolerant) treated with 300 μ M AlCl₃ for 48 h. 7. Marathon amplification with pAL primers with use of cDNA from root tips of soybean cv Malabar (susceptible) treated with 300 μ M AlCl₃ for 48 h. The arrows indicate the molecular sizes of the ladder fragments.

Marathon amplification fragments were extracted and applied in Reverse Northern hybridization. Reverse Northern allows verifying many fragments in one experiment, which is extremely important in case of such PCR study with possible output of multiple fragments. This method was applied for several genes, such as glutathione-S-transferase (GST), phenylalanine ammonia-lyase (pAL), and Wali1 (SNOWDEN and GARDNER, 1993).

Marathon PCR results for GST showed that the major PCR fragment was only in the reaction with control cDNA from root tips of soybean cv Tambora (tolerant to Al stress) but not in cDNA samples from root tips of stress treated plants and also not in cDNA sample from root tips of soybean cv Malabar (sensitive to Al stress) also treated by stress (Fig. 4, lanes 2, 3, and 4). This result indicates that GST belongs to the genes that are not involved in the stress response of soybean. That is in agreement with Northern hybridization pattern in experiment with GST gene from tobacco where one can find the same expression level for GST in control and stress treated samples from tolerant and sensitive soybean plants (Fig. 1 E).

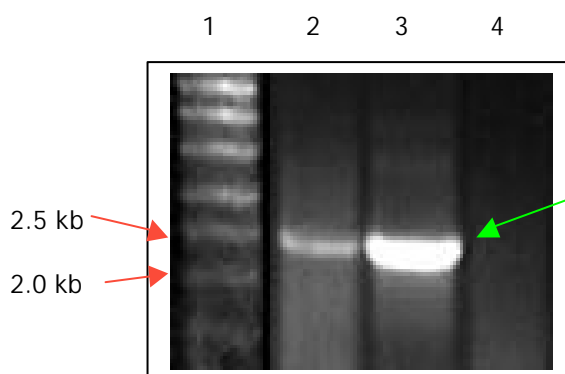


Fig. 5. Marathon amplification for Wali1 gene induced by aluminium in wheat (SNOWDEN and GARDNER, 1993).

1. Smart Ladder 2. Marathon amplification with Wali1 primers with use of cDNA from control root tips of soybean cv Tambora (tolerant). 3. Marathon amplification with Wali1 primers with use of cDNA from root tips of soybean cv Tambora (tolerant) treated with 300 μ M AlCl₃ for 48 h. 4. Marathon amplification with Wali1 primers with use of cDNA from root tips of soybean cv Malabar (susceptible) treated with 300 μ M AlCl₃ for 48 h. The arrows indicate the position of the Smart Ladder bands of 2.0 kb and 2.5 kb. The green arrow shows Marathon PCR fragment extracted and used further in Reverse Northern hybridization (Fig. 6).

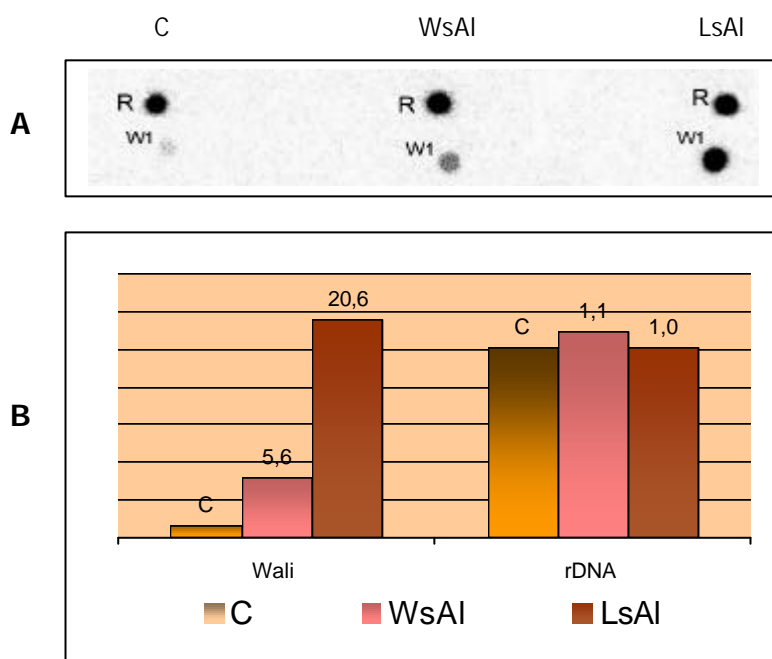


Fig. 6. Evaluation of Wali1 gene expression by Reverse Northern hybridization.

A – Reverse Northern hybridization with Wali1 Marathon PCR fragment (see Fig. 5, green arrow). C – hybridization with control cDNA. WsAl – hybridization with cDNA isolated from the suspension cultured cells of soybean cv Willis (tolerant to Al stress) treated by 100 μ M AlCl₃ for 4h. LsAl – hybridization with cDNA isolated from the suspension cultured cells of soybean cv Willis (susceptible to Al stress) treated by 100 μ M AlCl₃ for 4h, R – 18S rDNA fragment, W1 – Wali1 Marathon PCR fragment.

B – Quantification of the signal intensities made by TINA software. The numbers on the quantification show enhancement of expression in comparison to control values marked as „c”.

The experiment with pAL showed the same PCR fragment pattern for all the three cDNAs (Fig. 4, lanes 5, 6, 7), from control and treated tolerant plants (cv Tambora) and stress treated sensitive plant (cv Malabar). This result presents evidence that pAL expression

remains steady during stress and non-stress conditions and therefore can't belong to the stress genes, either responsible for general or aluminium specific reaction of soybean. Amplification for the Wali1 gene has been performed with use of cDNA from suspension cultured soybean cells, tolerant (cv Wilis) and sensitive (cv Lumut) to the stress conditions.

Marathon PCR with Wali1 primers revealed much higher amplification fragment intensity for cDNA sample from soybean cv Wilis (tolerant) treated by 100 μ M Al chloride for 4 h (see *Methods*, chapter 2.2.2.) in comparison with non treated controls (Fig.5, lanes 2 and 3). No PCR fragments could be seen in reaction with Lumut (susceptible) cDNA from suspension culture also treated under the same stress conditions (Fig.5, lane 4). This amplification results indicated possible influence of Wali 1-like gene on the process of soybean response to aluminium stress.

Marathon PCR fragment was extracted and used in Reverse Northern hybridization. Wali1 gene was expressed about 6 times higher in cv Wilis (tolerant) suspension cultured cells under conditions of aluminium stress for 4h. As much as 20-fold higher expression has been observed in cv Lumut (susceptible) suspension cultured cells under the same stress conditions (Fig. 6). This result shows that Wali1 gene is the one of general stress response, being expressed more in susceptible soybean cultivars where the stress effect is more profound.

During the experiments on aluminium responsiveness evaluation 9 genes were tested with the methods of Northern and Reverse Northern blotting in combination with Marathon cDNA amplification. Two of them showed up regulation during the stress, wali 1 and PEP carboxylase. Higher RNA abundance of PEP carboxylase in tolerant soybean under stress conditions indicated the participation of PEPC in mechanisms of Al resistance, whereas Wali1 gene belongs to the genes of general stress phenomenon.

4.3. Direct evaluation of gene expression under stress conditions using Differential Display and Marathon amplification techniques

Direct evaluation of the gene expression pattern in stressed and non-stressed root tips was made by Differential Display RT-PCR (DD). Expression patterns of tolerant soybean lines, Tambora (root tip experiments) and Wilis (experiments with suspension cultured cells) were compared to the ones obtained with use of susceptible soybean lines Malabar (root tip experiments) and Lumut (experiments with suspension cultured cells). In general, however, DD pattern was differing very little between aluminium treated samples and non-treated

controls (data not shown). That was supporting the choice of relatively low aluminium concentration for the stress treatments. The majority of cDNA fragments showing different expression patterns in controls and stressed samples were down regulated supporting the well-known phenomenon of general gene expression decrease under aluminium stress conditions (KOCHIAN, 1995). However, the stress-related genes up regulated during the stress might act specifically against aluminium toxicity. If some gene is involved in the general stress response, its expression should be enhanced in root tip cells (major site of aluminium toxicity) as well as in suspension-cultured cells of tolerant and susceptible soybean cultivars subjected to the stress conditions. In case of the genes involved in the mechanisms of the stress protection the highest expression level should be expected in tolerant stress treated soybean cultivars, but not in controls or sensitive ones where the specific genes can't be so much up regulated.

Therefore cDNA fragments have been taken, which were up regulated in Al-treated samples from tolerant lines, but not in the controls or in the stress treated samples obtained from susceptible soybean cultivars. By this approach the pool of obtained cDNA fragments has been enriched with the ones corresponding to the genes specifically involved in soybean resistance against aluminium toxicity. Each DD experiment was made twice and only the fragments appearing in both independent PCR sets were used for further experiments.

During DD experiments we found that resolution pattern was highly reproducible and differed only slightly between controls and samples obtained from root tips of tolerant Tambora soybean treated by 300 μ M AlCl₃ for 48 hr. DD resolution pattern from samples treated by 1000 μ M AlCl₃ showed quite many down regulated bands (results not shown), which is in agreement with inhibition of DNA and RNA synthesis by aluminium stress (KOCHIAN, 1995). In the set of DD experiments 12 DD fragments up regulated during aluminium stress were found.

Two of obtained DD fragments, TA52 and TA22, are presented on the Fig. 7 as they appeared on DD pattern. Intensity of cDNA fragment signal on the DD pattern corresponds to the expression level for the appropriate gene in appropriate sample. In that way it could be seen that expression level of the gene corresponding to the TA52 fragment is quite low in both susceptible (obtained with use of cDNA from the root tips of soybean cv Malabar) and tolerant (obtained with use of cDNA from the root tips of soybean cv Tambora) controls (Fig. 7 A, lanes 1 for susceptible control pattern and 6 for the tolerant one). The fragment signal intensity is enhanced already in the sample subjected to Al stress (for the conditions of the stress see *Methods*, chapter 2.2.1) for 4 h (Fig. 7 A, lane 3) as well as in the one after 24

and 48 h of stress (lanes 4 and 5). After 48h stress the expression of the gene corresponding to the TA52 fragment is maximal of all 6 samples used for the study (lane 5) in the case of tolerant cultivar but practically not different from the control in sensitive one.

In case of TA22 cDNA fragment the signal is virtually invisible on control DD patterns (Fig. 7 B, lanes 1 for sensitive control (non-stressed root tips of cv Malabar) and 6 for the tolerant one (non-stressed root tips of cv Tambora)). However, the signal appears on DD pattern of the tolerant cultivar root tips already after 4 h of aluminium treatment (Fig. 7 B, lane 3) and its intensity is the highest after 48h treatment of tolerant cv Tambora (Fig. 7 B, lane 5) but invisible in susceptible cv Malabar (Fig. 7 B, lane 2), as in control samples.

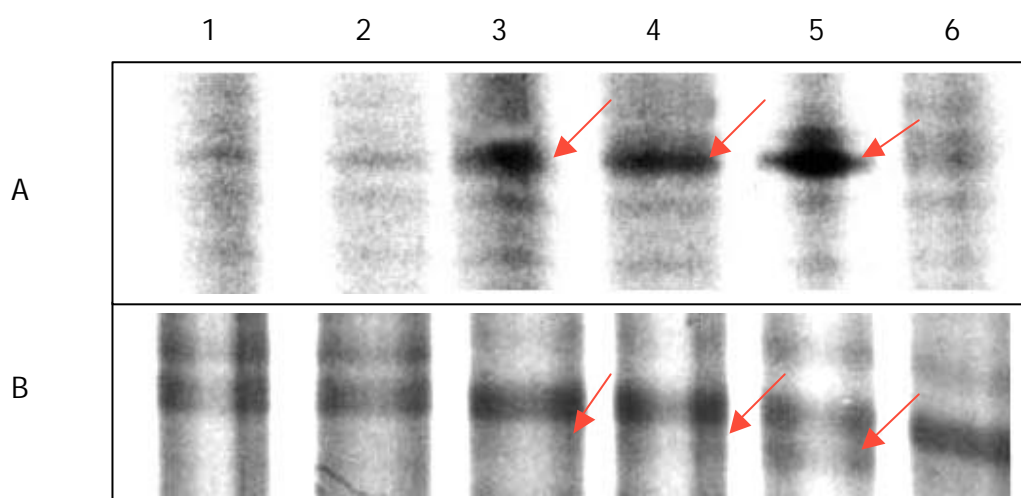


Fig. 7. TA52 and TA22 cDNA fragments on the Differential Display pattern of cDNA from tolerant and susceptible soybean lines.

Lane1: Control soybean cv Malabar (susceptible) root tip RNA. Lane 2: Soybean cv Malabar (susceptible) RNA from root tips treated with $AlCl_3$ for 48 h. Lane 3: Soybean cv Tambora (tolerant) RNA from root tips treated with $AlCl_3$ for 4 h. Lane 4: Soybean cv Tambora (tolerant) RNA from root tips treated with $AlCl_3$ for 24 h. Lane 5: Soybean cv Tambora (tolerant) RNA from root tips treated with $AlCl_3$ for 48 h. Lane 6: Control soybean cv Tambora (tolerant) root tip RNA.

A. TA52 DD fragment. B. TA22 DD fragment. The appropriate cDNA fragments are indicated by arrows.

Such DD patterns indicate that the genes corresponding to the cDNA fragments TA52 and TA22 are involved in the process of specific soybean response to the moderate aluminium stress.

All DD fragments were extracted, reamplified and cloned into Stratagene PCR-Script cloning vector. As it is well known, each DD band can contain several different co-migrating cDNA fragments (LIANG et al., 1993), which is supported by the resolution picture of the DD

fragment reamplification (Fig. 8). Thus after the extraction and reamplification a pool of cloned DD fragments was obtained.

Reverse Northern hybridization was used to eliminate the false positive clones typical for the DD method. This hybridization method gives the possibility to probe a lot of cloned DNA fragments in one experiment to find the ones, which correspond to the genes showing really up regulated expression pattern during aluminium stress. Such experiment was made for all the DD fragments under study and enhanced expression of the genes corresponding to TA22 and TA52 cDNAs has been confirmed by Reverse Northern hybridization (Fig. 9 and 10).

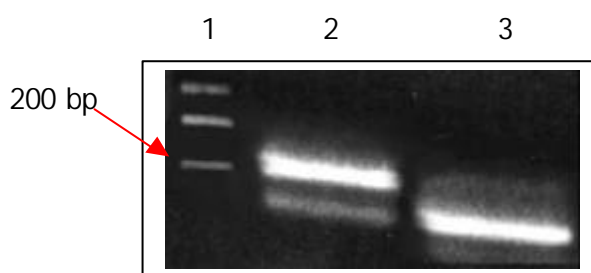


Fig. 8. Reamplification of DD cDNA fragments. 1. Smart Ladder 2. TA22 band reamplification (the major band is of size 212 bp). 3. TA 52 band reamplification (the major band is of size 75 bp).

The expression of the gene corresponding to TA22 fragment (Fig. 9) in root tips of cv Tambora (tolerant to aluminium stress) has been enhanced 7-fold under the same stress conditions. To obtain one more evidence for the up regulation of the genes corresponding to TA52 and TA22 DD fragments Northern hybridization experiment has been performed for the both of them.

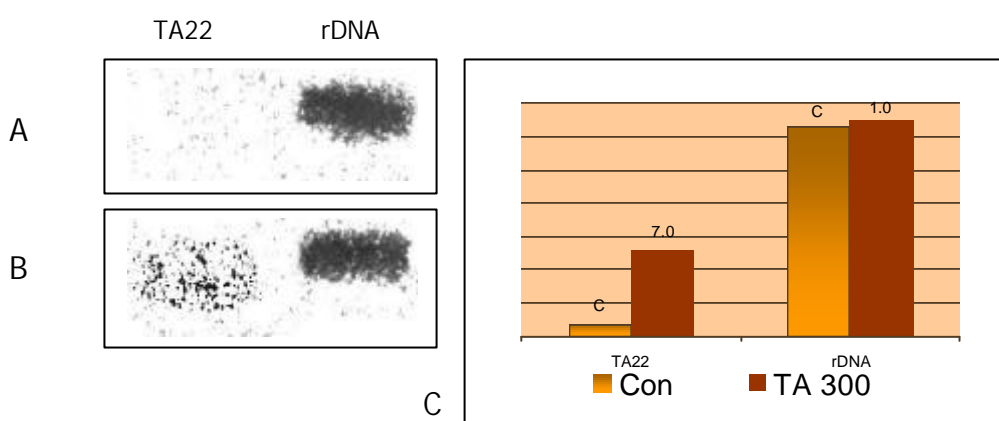


Fig. 9 Evaluation of expression level for the gene corresponding to TA22 DD fragment using Reverse Northern blotting.

A – hybridization with cDNA isolated from control root tips of soybean cv Tambora (tolerant). RNA level on the quantification is indicated by “Con”. B – hybridization with cDNA isolated from root tips of soybean cv Tambora (tolerant) treated by 300µM AlCl₃ for 48h. RNA level on the quantification is indicated by “TA300”. rDNA – 18s rDNA fragment, TA22 –TA22 DD fragment. C – Quantification of the signal intensities made by TINA software. The numbers show enhancement of the gene expression level in comparison to control marked as „c”.

As it is shown on Fig. 10, the expression level of the gene corresponding to TA52 DD fragment was enhanced 7.4-fold in the root tips of cv Tambora (tolerant to aluminium stress) after 48h of treatment by 300 μ M aluminium chloride (the exact conditions of the treatment see in *Methods*, chapter 2.2.1) in comparison to non-treated control.

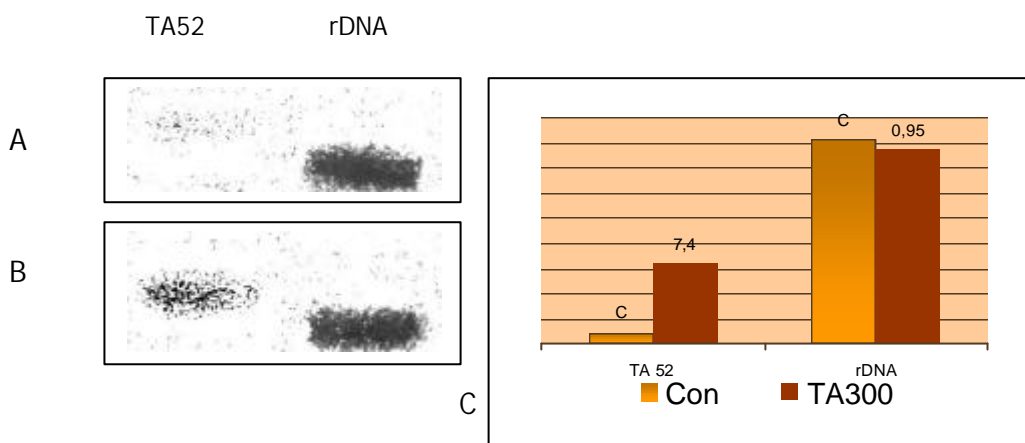


Fig. 10 Evaluation of expression level for the gene corresponding to TA52 DD fragment using Reverse Northern blotting.

A – hybridization with control cDNA from root tips of soybean cv Tambora (tolerant). RNA level on the quantification is indicated by “Con”. B – hybridization with cDNA isolated from root tips of soybean cv Tambora (tolerant) treated by 300 μ M AlCl₃ for 48h. RNA level on the quantification is indicated by “TA300”. rDNA – 18s rDNA fragment (loading control), TA52 –TA52 DD fragment. C – quantification of the signal intensities made by TINA software. The numbers show enhancement of the gene expression level in comparison to control marked as „c”.

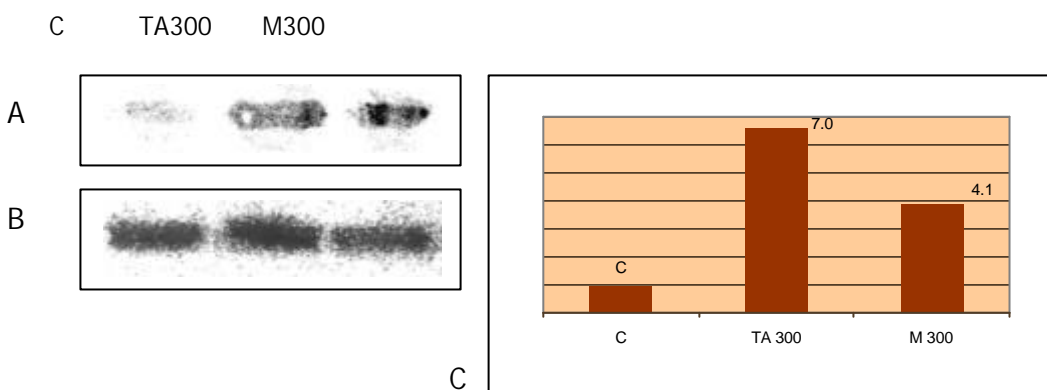


Fig. 11. Evaluation of the expression level for the gene corresponding to TA52 DD cDNA fragment using Northern blotting.

A – Northern hybridization with TA52 DD fragment. B – Northern with 18s rDNA (loading control). C – quantification of signal intensities on Northern hybridization with TA52 DD fragment made by TINA software. „c” - Control RNA from root tips of soybean cv Tambora. „TA300” - RNA from root tips of soybean cv Tambora treated with 300 μ M AlCl₃ for 48 h. „M300” - RNA from root tips of soybean cv Malabar treated with 300 μ M AlCl₃ for 48 h. The numbers on the quantification show the enhancement of the expression level in comparison to the control values marked as „c”.

The gene corresponding to the TA52 fragment showed 7.0-fold enhancement of expression level in Tambora (tolerant) root tips after 48h stress under conditions of 300 μ M Al chloride in the nutrient solution in comparison to the control expression value (Fig. 11 A and C, TA300).

Under the same conditions the putative gene showed only 4.1-fold increase of the expression level in cDNA from the root tips of Malabar (sensitive) which was 1.7-fold less up regulated than in tolerant plants (Fig. 11 A and C, M300). Such result showed the probable involvement of the studied gene in specific plant stress response against aluminium stress in soybean.

Northern hybridization experiments with TA22 DD fragment showed that the expression level of the gene corresponding to this fragment was beyond the level of detection for the method (data not shown).

The method of Differential Display is designed to obtain the pattern of cDNA fragments of the size up to 500 bp from the part of cDNA just near the poly-A tail, normally the untranslated region. This methodical drawback hardly allows DD fragments to be used in cDNA bank screening. That is why obtained DD fragments had to be prolonged. Since all the fragments were originated from 3'-part of cDNA, the probability was high that in the pool of all cDNA molecules from studied tissues several cDNAs corresponding to one DD fragment could be found. Therefore the special method has been chosen for the experiments on DD fragment prolongation, Marathon cDNA amplification (see *Methods*, chapter 2.2.6). This method creates an uncloned cDNA library and the search for the cDNA fragments is performed by amplification with use of the primers specific to some part of them. Another primer is taken to anneal to the special adapters, which does not allow the PCR reaction without the primer specific for the cDNA of interest. Thus with use of this method all cDNAs having the sequence of putative DD fragment in 3'-part could be found and studied for the expression of corresponding genes under stress and non-stress conditions in further experiments.

The cells in the root tip region are highly differentiated structurally and functionally and their stress response cannot be uniform. In order to obtain the uniform stress response, we used RNA from suspension-cultured cells of tolerant and sensitive soybean cultivars on the level of Marathon amplification (for the conditions of the experiments see *Methods*, chapter 2.2.2.). Since all the cells in the long-term suspension culture are cultivated and stress-treated under the same conditions, the sensitivity of the gene search could be reasonably enhanced because in this case some genes expressed in the plant only in specific tissue layers could be detected also.

The sequence information of several clones containing DD fragments was used for the primer construction and the primers were applied in Marathon PCR. Amplification experiments were performed with cDNAs from the root tips of tolerant soybean cv Tambora

and sensitive cv Malabar as well as suspension cultured cells of tolerant soybean cv Willis and sensitive cv Lumut. Root tips were subjected to moderate acidic stress with and without aluminium stress for 48h (see *Methods*, chapter 2.2.1). Suspension cultured cells were stressed by aluminium treatment for 4 h (see *Methods*, chapter 2.2.2).

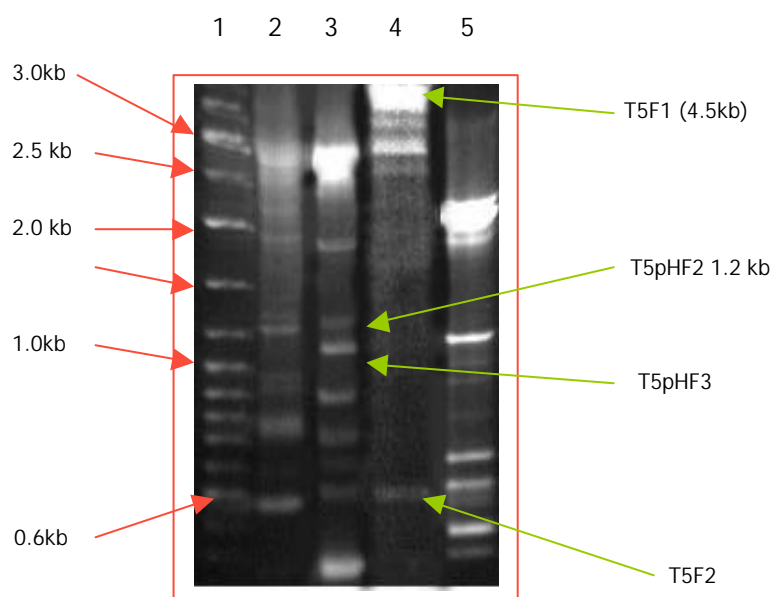


Fig. 12. cDNA fragments obtained by Marathon amplification with use of TA52 specific primers.

Lane 1. DNA Ladder. The red arrows indicate the sizes of ladder fragments in kb. Lane 2. PCR with control cDNA from root tips of soybean cv Tambora (tolerant). Lane 3. PCR with cDNA from root tips of soybean cv Tambora (tolerant) treated by moderate acidic stress (pH of the nutrient solution lowered to 4.0) for 48 h. Lane 4. PCR with cDNA from root tips of soybean cv Tambora (tolerant) treated by 300 μ M AlCl₃ for 48 h. Lane 5. PCR with cDNA from root tips of soybean cv Malabar (susceptible) treated by 300 μ M AlCl₃ for 48 h. The green arrows indicate appropriate cDNA fragments.

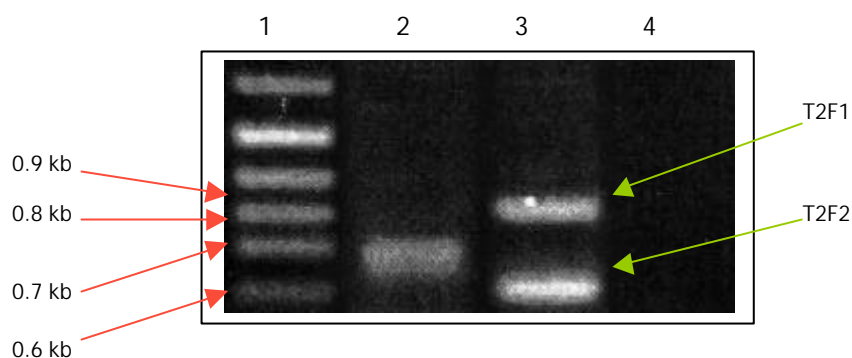


Fig. 13. cDNA fragments obtained by Marathon amplification with use of TA22 specific primers. Lane 1. DNA Ladder. The red arrows indicate the sizes of ladder fragments in kb. Lane 2. PCR with control cDNA from root tips of soybean cv Tambora (tolerant). Lane 3. PCR with cDNA from root tips of soybean cv Tambora (tolerant) treated by 300 μ M AlCl₃ for 48 h. Lane 4. PCR with cDNA from root tips of soybean cv Malabar (susceptible) treated by 300 μ M AlCl₃ for 48 h. The green arrows indicate appropriate cDNA fragments.

In this way Marathon amplification patterns for all the DD fragments studied were compared between controls, low pH and aluminium stressed root tip and suspension cultured cells of sensitive and tolerant soybean cultivars.

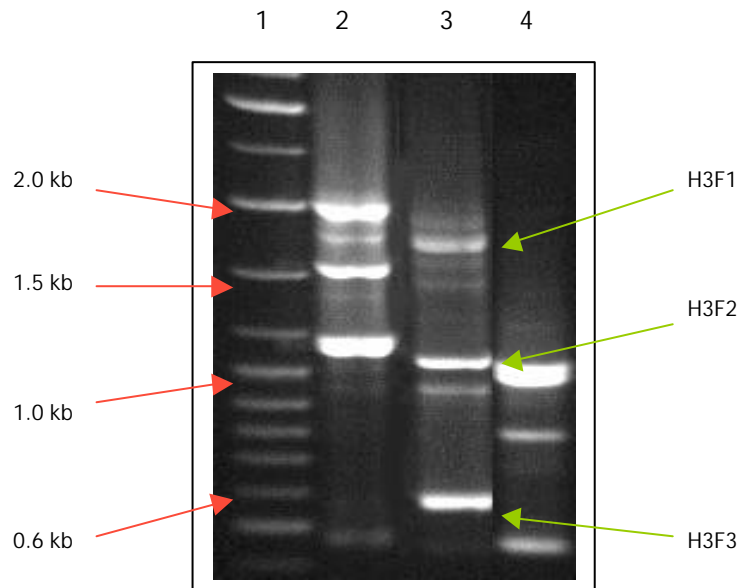


Fig. 14. cDNA fragments obtained by Marathon amplification with use of HT31 specific primers in the experiment with suspension-cultured cells from aluminium stressed cv Tambora. Lane 1. . DNA Ladder. The red arrows indicate the sizes of ladder fragments in kb. Lane 2. PCR with control cDNA from suspension cultured cells of soybean cv Willis (tolerant). Lane 3. PCR with cDNA from suspension cultured cells of soybean cv Willis (tolerant). treated by 100 μ M AlCl₃ for 4 h. Lane 4. PCR with cDNA from suspension cultured cells of soybean cv Lumut (susceptible) treated under the same conditions as the cells of tolerant cv Willis. The green arrows indicate appropriate cDNA fragments.

The clones chosen for this approach were the ones containing the both cDNA fragments verified for their aluminium responsiveness TA52 (Fig. 9 and Fig. 11) and TA22 (Fig. 10), and also the clone containing DD fragment HT31, which could not be verified in the form of DD fragment since the expression level for the appropriate gene was beyond the level of detection in both Northern and Reverse Northern hybridizations. During Marathon experiments only the PCR fragments from the amplification with cDNA from tolerant soybean cultivars treated either by acidic or by aluminium stress were selected, but not the ones existent in the amplification reactions with cDNAs from controls or stress treated susceptible cultivars, as it was explained in more detail above for DD experiment design.

Finally, 9 cDNA fragments have been obtained as the result of PCR with use of DD fragments TA52, TA22 and HT31. The cDNA fragments T5F1, T5F2 were observed only in PCR reaction using TA52 derived primers with cDNA from cv Tambora (tolerant) root tips stressed by Al

for 48h, but not in controls or in the amplification of cDNA from sensitive cv Malabar root tips. T5pHF2 and T5pHF3 appeared only in reaction with cDNA from cv Tambora (tolerant) root tips subjected to moderate acidic stress for 48h, but not in controls or in the amplification of cDNA from aluminium treated sensitive cv Malabar root tips (Fig. 12).

cDNA fragments T2F1, T2F2 were observed only in PCR reaction using TA22 derived primers with cDNA from cv Tambora (tolerant) root tips stressed by Al for 48h, but not in controls or in the amplification of cDNA from sensitive cv Malabar root tips (Fig. 13). In the amplification experiments with cDNA from the samples subjected only to acidic stress or coming from suspension cultured cells no additional cDNA fragments could be isolated (data not shown).

cDNA fragments H3F1, H3F2 and H3F3 were observed only in PCR reaction using HT31 derived primers with cDNA from cv Wilis (tolerant) suspension cultured cells stressed by Al for 4h, but not in controls or in the amplification of cDNA from sensitive cv Lumut (Fig. 14). The amplification patterns for the root tips, control and aluminium stressed as well as for moderate acidic stress were attempted to be verified as well, however, no result was obtained (data not shown). The information about all cDNA fragments obtained after Marathon amplification experiments is summarized in the Table 2.

Table 3. cDNA fragments obtained with use of TA52, TA22 and HT31 sequences in Marathon amplification experiments.

No	Marathon PCR fragment	Primers were obtained with use of DD fragments:	The bands were in Marathon PCR sample from:	cDNA originated from	Fragment size, kb
1	T5pHF2	TA52	Tambora (tol.) pH	Root tips	1.3
2	T5pHF3	TA52	Tambora (tol.) pH	Root tips	1.1
3	T5F1	TA52	Tambora (tol.) Al	Root tips	3.6
4	T5F2	TA52	Tambora (tol.) Al	Root tips	0.7
5	T2F1	TA22	Tambora (tol.) Al	Root tips	0.85
6	T2F2	TA22	Tambora (tol.) Al	Root tips	0.6
7	H3F1	HT31	Wilis (tol.) Al	Suspension cultured cells	1.9
8	H3F2	HT31	Wilis (tol.) Al	Suspension cultured cells	1.05
9	H3F3	HT31	Wilis (tol.) Al	Suspension cultured cells	0.6

Tambora (tol) pH – cDNA isolated from the root tips of tolerant to aluminium stress soybean cultivar Tambora treated by moderate acidic stress (pH of nutrient solution was lowered to 4.0 by HCl). Tambora (tol) Al – cDNA isolated from the root tips of tolerant to aluminium stress soybean cultivar Tambora treated by 300µM AlCl₃ for 48h. Wilis (tol.) Al – cDNA fragments isolated from suspension-cultured cells treated by aluminium stress for 4 hours.

All Marathon PCR fragments were extracted and studied for their Al stress responsiveness by Reverse Northern Hybridization. The hybridization experiment were performed with use of cDNAs from stressed and non-stressed root tips of tolerant to Al stress cv Tambora and

susceptible cv Malabar as well as with stressed and non-stressed suspension cultured cells of stress-tolerant cv Willis and susceptible cv Lumut.

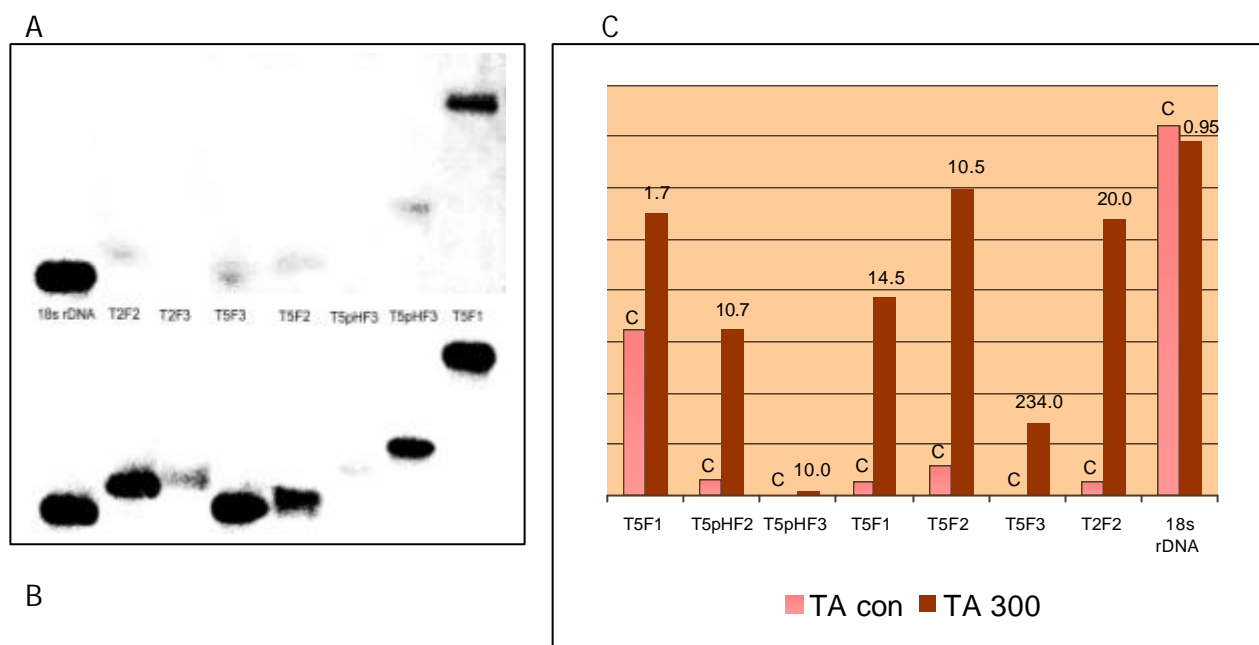
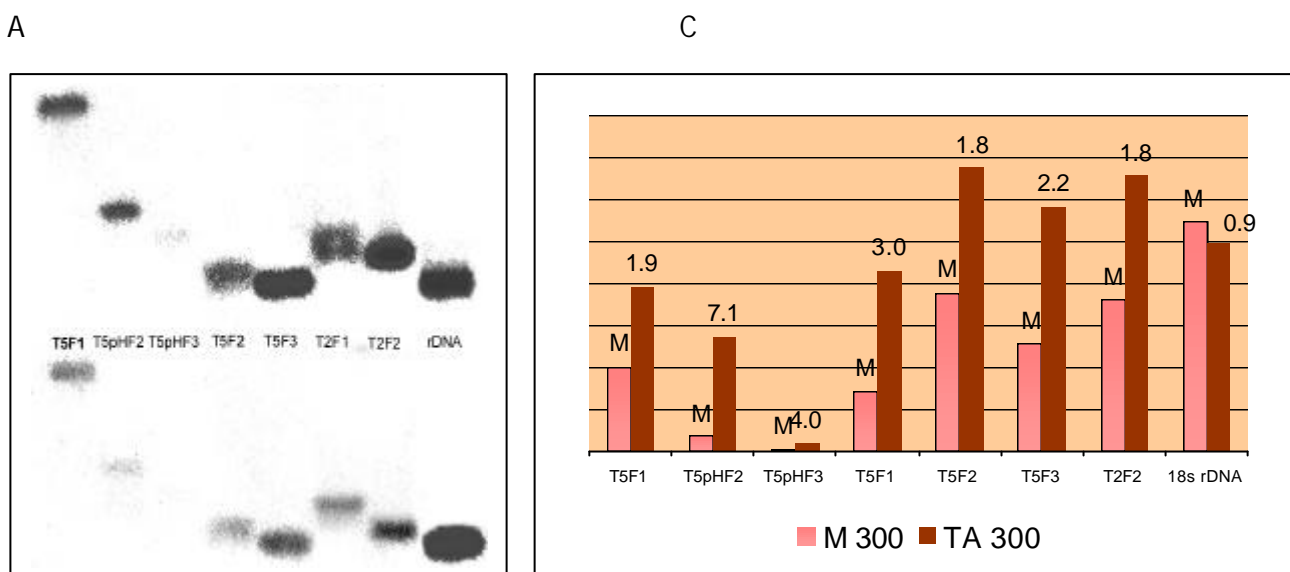


Fig. 15. Analysis of expression level for the genes corresponding to cDNA fragments obtained by Marathon amplification with use of TA52 and TA22 DD fragments. Comparison of gene expression in control and stressed root tips of tolerant cv Tambora.

A – Reverse Northern hybridization with cDNA of control root tips from cv Tambora (TA con). B – Reverse Northern hybridization with cDNA of root tip from cv Tambora stressed by AI for 48h (TA 300). C – Quantification of Reverse Northern signal intensity made by TINA software. The numbers on the quantification picture show the enhancement of expression level (TA 300) as compared to non-treated control (TA con). 18srDNA is marking the fragment corresponding to cloned 18s rDNA as loading control.

The results of the comparison between the levels of expression of the genes corresponding to putative Marathon amplification fragments are presented on Fig. 15. Practically all Marathon cDNA fragments have shown the enhanced level of corresponding gene expression. The highest enhancement showed T5F3 and T2F2 fragments, with 234.0 and 20.0-fold increase of hybridization signal in comparison to the control sample. A bit lower value was obtained for the fragments T5F1, T5F2 (14.5 and 10.5-fold increase) and T5pHF2 along with T5pHF3 (10.7 and 10.0-fold increase). T5F1 fragment showed 1.7-fold increase in the signal intensity, with initially quite high signal level in the control sample.

A very important part of Marathon fragment analysis was the comparison of expression levels for corresponding genes between tolerant and sensitive soybean varieties. The results of this experiment are shown on the Fig. 16.



B
 Fig. 16. Analysis of expression level for the genes corresponding to cDNA fragments obtained by Marathon amplification with use of TA52 and TA22 DD fragments. Comparison of gene expression in stressed root tips of tolerant to Al stress cv Tambora and susceptible cv Malabar.

A – Reverse Northern hybridization with cDNA of root tip from cv Tambora stressed by Al for 48h (TA 300). B – Reverse Northern hybridization with cDNA of root tip from cv Malabar stressed by Al for 48h (M 300). C – Quantification of Reverse Northern signal intensity made by TINA software. The numbers on the quantification picture show the enhancement of expression level (TA 300) as compared to expression level in susceptible cv Malabar (M 300). 18srDNA is marking the fragment corresponding to cloned 18s rDNA as loading control.

Not all the genes corresponding to candidate cDNA fragments with the best transcript level difference as compared with untreated controls, showed also the best transcript level enhancement in comparison to sensitive soybean varieties. The best candidates in the first blotting, namely T5F3 and T2F2 were not so promising in this case, showing only 2.2 and 1.8-fold enhancement for the corresponding gene expression. However the fragments T5pHF2 and T5pHF3 with moderate increase of the gene expression in comparison to the control sample (10.7 and 10.0-fold, Fig. 15) showed the best results in this experiment with 7.1 and 4.0 increase, respectively. The results of the last blotting were taken as critical for the choice of Marathon cDNA fragments for further work.

Northern hybridization experiments were performed for the cDNA fragment H3F2 obtained by Marathon hybridization with the primers made on the basis of HT31 DD fragment. Expression of appropriate gene is higher 3.8 times after 4h aluminium treatment of Willis (tolerant) suspension culture.

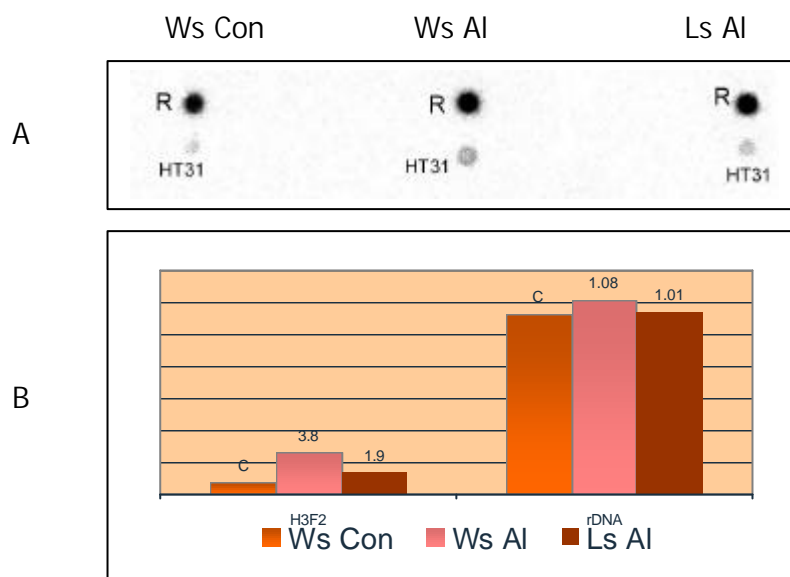


Fig. 17. Analysis of expression level for the gene corresponding to H3F2 obtained by Marathon amplification.

A – Reverse Northern hybridization picture. Ws Con – cDNA from control suspension cells of cv Wilis (tolerant). Ws AI – cDNA from suspension cells of cv Wilis (tolerant) treated by AI for 4h. Ls AI – cDNA from suspension cells of cv Lumut (sensitive) treated by AI for 4h. rDNA – 18s rDNA as loading control. HT31 – HT31 DD fragment as a probe. B – Quantification of Reverse Northern signal intensity made by TINA software. The numbers on the graph show the enhancement of the signal intensities in comparison to the control level marked as “c”.

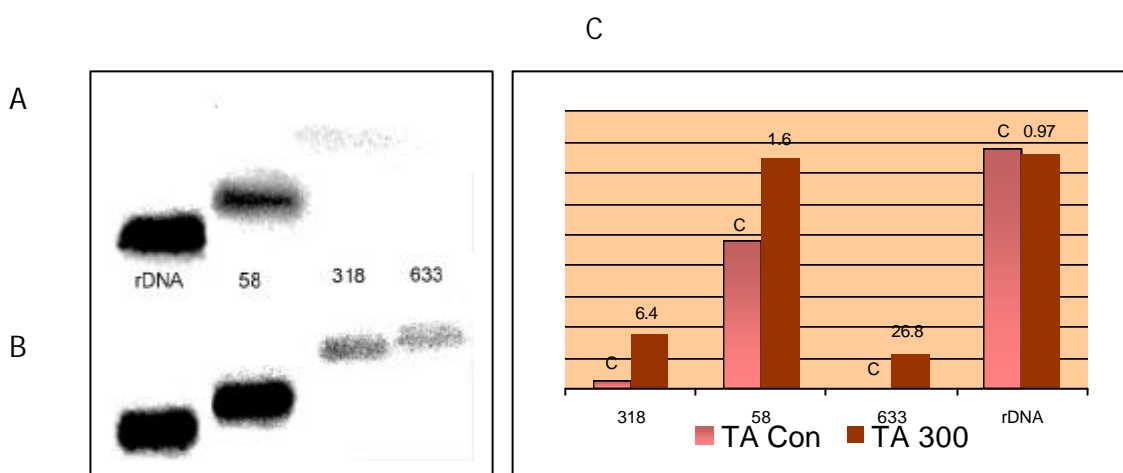


Fig. 18. Evaluation of expression levels for the genes corresponding to full protein coding cDNA fragments obtained after cDNA library screening.

A – Reverse Northern hybridization with control cDNA from root tips (TA Con) of cv Tambora (tolerant). B – hybridization with cDNA from AI-treated root tips (TA 300) of cv Tambora (tolerant). C – Quantification of Reverse Northern signal intensity made by TINA software. rDNA – cloned 18s rDNA fragment as loading control. 58, 318 and 633 – full protein coding cDNA fragments after cDNA bank screening. The numbers on the graph show enhancement of the blotting signal intensities in comparison to the control level indicated as “c”.

The expression level in sensitive soybean cells in suspension culture treated under the same conditions is 2 times lower as in tolerant ones. It could be seen that expression pattern of the gene corresponding to H3F2 fragment is quite different from the genes of the general

response (for example Wali1, see Fig. 6) and indicates that the gene is probably involved in specific response to Al stress (Fig. 17).

In order to obtain the full protein coding sequences of selected and verified cDNA fragments λ -ZAP cDNA library was prepared from mRNA of aluminium treated tolerant soybean lines (Tambora, root tips and Willis, suspension cultured cells, ratio 1 to 1). The cDNA library was screened with extracted Marathon PCR fragments. DNA of the full protein coding clones was used for Reverse Northern hybridization experiment to prove the enhanced expression of appropriate gene under aluminium stress conditions (Fig. 17).

For the three clones clear expression enhancement of corresponding genes has been shown in root tips under stress conditions (Fig. 18). They were named 318 clone, up-regulation of appropriate gene was 6.4-fold under stress conditions as well as clones 58 and 633 with 1.6-fold and 26.8-fold up-regulation under stress conditions, respectively.

4.4 Sequence analysis and characterization of full-length cDNAs.

These full-length cDNAs were completely sequenced. The first one, the clone 318 is coding for protein of 636 amino acids (Fig. 19). This protein has homology with yeast and E. coli ABC transporters, the members of quite diverse superfamily of proteins involved in ATP-dependent transport of quite different substances through tonoplast or plasmalemma. Some of the functions for the proteins from this superfamily can involve compartmentation of metabolites in different organelles or regulation of the transport through cell membranes (DAVIES and COLEMANN, 2000; THEODOULOU, 2000; WILLIAMS et al., 2000).

1 GAAAAACCTAAAGTGGCGGAAG**ATG**ACAGAGGTGGCGAGATCAGTGGTGCACGACGTTTTGGGGCAAAGGGTGGTC
 1 M T E V A R S V V H D V L G Q R V V
 76 GACGTGGACCAGCCAATCGTCGATTACATCGTAAACGTCCTCGCCGACGATGACTTCGATTTGGCCTCGACGGC
 19 D V D Q P I V D Y I V N V L A D D D F D F G L D G
 151 CAAGGCGCCTTCGAAGCCCTCGGCGAGCTCCTCGTCGCCCGGCTGCGTCGACGATTTCTCCACTGCCGCTCC
 44 Q G A F E A L G E L L V A A G C V D D F S H C R S
 225 GTGTGCAGCACGCTGTGCGACAAGTTCGGGAAGCACGGTTTGGTGAAGGAGAAGCCCGTGTTCGAGGCCTTGGC
 69 V C S T L C D K F G K H G L V K E K P A V R G L A
 300 GCGCCGTTTCGAATGAACGAGGGGATGGACGACGTGCAAGCTCCCAAGAAGAAGCCCGAGCCCGTTCGACGGCCCT
 94 A P F R M N E G M D D V Q A P K K K P E P V D G P
 375 CTTCTCTCGGAACGCGACCGGTTAAAGCTGGAGAGGCGAAAACGCAAGGATGAACGCCAGCGAGAGGCACAATAC
 119 L L S E R D R L K L E R R K R K D E R Q R E A Q Y
 450 CAAATGCACTTGGCGGAGATGGAGGCGGCGAGCGGGGATGCCGGTGGTGTGTGTGAGACATGATAACAGCGGG
 144 Q M H L A E M E A A R A G M P V V C V R H D N S G
 525 GGACCAAACGTGAAGGACATTCATATGGAGAATTTCAACATCTCTGTTGGTGGGCGTGATCTCATTGTGGATGGT
 169 G P N V K D I H M E N F N I S V G G R D L I V D G
 600 TGTGTACGCTTTTCATTTGGAAGGCATTATGGTTTGGTGGGAAGAAATGGGACGGGGAAAACCACTTTTCCTTAGC
 194 C V T L S F G R H Y G L V G R N G T G K T T F L R
 675 CACATGGCTATGCATGCCATTGATGGTGTCCGAGGAATTGTCAGATACTGCATGTGGAGCAAGAGGTGACAGGT
 219 H M A M H A I D G V P R N C Q I L H V E Q E V T G
 750 GATGCTACGACTGCTTTGCAGTGTGTCTCAACTCTGACATTGAGAGGACTCAGCTTCTAGATGAAGAAGCTCAG
 244 D A T T A L Q C V L N S D I E R T Q L L D E E A Q
 825 TTAGTTGCCCAACAGAGGGAATTCGAGGACAAAATCGAAAAGGGTGATTTCGAATGGAGTGGTCCGGTAGGGATGAC
 269 L V A Q Q R E F E D K I E K G D S N G V V G R D D
 900 ATTTCAAAAAGGCTTGAGGAAATATATAAGAGGCTTGAACACATTGATGCTGATTCCGGCTGAGGCACGAGCAGCA
 294 I S K R L E E I Y K R L E H I D A D S A E A R A A
 975 TCAATACTAGCAGGTTTGGATTTTCACTCCTGAGATGCAGAAGAAGGCAACAAAAACATTTTCTGGAGGATGGCGG
 319 S I L A G L S F T P E M Q K K A T K T F S G G W R
 1050 ATGCGAATAGCTCTTGCTCGTGCATTGTTTATAGAGCCTGATATATTGCTTCTTGATGAGCCTACGAATCATCTT
 344 M R I A L A R A L F I E P D I L L L D E P T N H L
 1125 GATCTTCATGCTGTTAGAGAATTTTTAAACACGGTGGTACTGATATAATTCACCTACAAAACAAAAGCTGACT
 369 D L H A V R E F L N T V V T D I I H L Q N Q K L T
 1200 ACTTATAAAGGGAATTATGATGCTTTTGGAGAAGACTCGAGAAGAACAAGTTAAAAACCACTTATGGCTTGAATCG
 394 T Y K G N Y D A F E K T R E E Q V K N H L W L E S
 1275 TACTTGGTTAAATGGCCAAAACATTTATCGTTGTTTTCATGCGCAAAAAGCATTAGAGGCAAACGAACGTGCT
 419 Y L V K W P K T F I V V S H A Q K A L E A N E R A
 1350 AGATCTCATATGCAGACCTTCATTGACAAGTTCGGTTATAATGCGAAGAGGGCATCACTTGTTCATCTAGAATC
 444 R S H M Q T F I D K F R Y N A K R A S L V Q S R I
 1425 AAGGCTTGGATCGAATGGGCCATGTGGATGAAATTGTTAATGATCCTGACTACAAATTTGACTTCCCCACTCCA
 469 K A L D R M G H V D E I V N D P D Y K F D F P T P
 1500 GATGATAGACCTGGTGCACCAATAATAAGTTTCAGTGATGCATCATTGGTTATCCTGGGGGCCCACTTCTGTTT
 494 D D R P G A P I I S F S D A S F G Y P G G P I L F
 1575 AAGAATTTGAACTTTGGGATTGATCTCGACAGTCGATTGCAATGGTTGGACCAAATGGTATTGGCAAATCAACT
 519 K N L N F G I D L D S R I A M V G P N G I G K S T

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1650 ATACTTAAGTTAATTGCTGGGGATCTTCAGCCCAGTTCTGGGACTGTCTTCCGATCTGCTAAGGTTTCGTATAGCA
544  I L K L I A G D L Q P S S G T V F R S A K V R I A

1725 GTGTTTCAGTCAGCACCATGTTGATGGACTCGACTTATCCTCAAATCCTCTTCTGTATATGATGCGCTGCTATCCT
569  V F S Q H H V D G L D L S S N P L L Y M M R C Y P

1800 GGAGTTCAGAACAGAAGCTTCGAGCTGCACTTAGGTTCTTTTGGTGTAAACCGGAAATCTTGGCTGCAGCCAAT
594  G V P E Q K L R A A L R F F W C N R K S C A A A N

1875 GTATACTTTGTCAGGTGGTCAGAAAAGCAGGGTTGCCTTTGCAAAGATAACTTTTAAAGAACCCACACATAATATTGCTTGATG
619  V Y F V R W S E K Q G C L C K D N F -

1958 AGCCACCCAATCATCTGGATTGGATGCTGCTGAGCGCTTATTCAAGGTCTTGTGCTGTTCCAAGGAGGCATTCTCATGGTGAGTCACGATGAGCACCTC
2058 ATCTCTGGAAGCGTGGAGGAACATGGGTTGTATCCGAAGGAAGAGTGGCACCATTCATGGCACATTCCAAGATTATAAGAAGATACHCCAATCATCCT
2258 AGAAATGTCAAATTTTGTGGATGTATACTGGAAGACCCCTGGATCACATTTTTTTGCTGTTCCCTCTCAGCTGTCATACAATTTTGCAATTTATCCAAA
2358 CCTATGTATTTCAAGGCAATTTTATGATTTTATGAACACGAGGTTAAATTAAC TATTAATAATTTTGTAAATAACCTTATAATTATAAGATGTATAATTT
2449 ATACTTTCAC TAAAAAAAAAAAAAAAAAAAAA

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Fig. 19. Complete cDNA of the clone 318 and predicted protein sequence.

cDNA sequence consisted of 2488 nucleotides. ORF was coding for the protein of 636 amino acid residues.

The search for homologies has been performed with use of DNA and protein sequence of cDNA clone at <http://www.ncbi.nlm.nih.gov/blast/blast.cgi>. Average identity of predicted protein coded by 318 cDNA to four other closely related proteins is 26.7 per cent (Fig. 20), which is relatively low similarity in general. Some really conserved regions, however, concentrated in the middle of the protein sequence and to C-terminal end. These regions were located between 316-326, 339-398 and 529-572 amino acids and corresponded to ABC transporter signature motif (amino acids 338-358, see below and Fig. 20) and the second ATP-GTP binding motif (amino acids 535-542, see below and Fig. 20).

Protein motif search was performed at

http://www.isrec.isb-sib.ch/software/PFSCAN_form.html

(Profile Scan server at Swiss Institute for Experimental Cancer Research) and at

<http://www.expasy.ch/tools/scnpsit1.html>

(Scan Prosite EXPASy server at Swiss Institute of Bioinformatics). It showed that this protein

sequence contain several probable motifs and posttranslational modification sites, such as:

2 N-glycosylation sites: amino acids 181-184 (NISV), and amino acids 209-212 (NGTG); 2 cAMP- and cGMP-dependent protein kinase phosphorylation sites, amino acids 332-335 (KKAT) and 459-462 (KRAS); 6 Protein kinase C phosphorylation sites, amino acids 121-123 (SER), 211-213 (TGK), 295-297 (SKR), 414-416 (TYK), 562-564 (SAK), 625-627 (SEK);

318 proteinMTEVARSVVDVLDGQRVVDVDPQIVDYIVNVLADDDDFDGLDGQG	45
ABC E. coli	0
ABC Haemophi.	0
ABC Yeast	0
GCN20 Yeast	MASIGSQVRKAASSIDPVTDYAVGYFNHLSGITFDVAVQSKQVDLSTEVQFVSDLLIDAGASKAKVKELS	70
318 protein	AFEALGELLVAAGCVDDFVSHCRSVCSTLCKDFGKHLVKEKPAVRGLAAPPFRMNEGMDVQAPKKKPEPV	115
ABC E. coli	0
ABC Haemophi.	0
ABC YeastM	1
GCN20 Yeast	ESILKQLTTLQKENEAKLELTGDTSKRLLDINVLSKSHNSKSDINVSLSMLGVNGDIEHTGRKMETRVLDL	140
318 protein	DGPLLSERDRCLKLERRKRKDERQREAOYQMHLEAMEAARAGMPVVCVRHDNSGGPNVKDIHMENFNISVG	185
ABC E. coli	0
ABC Haemophi.	0
ABC Yeast	PPVSASKAKRDAKKAEREAKKAAAGKTIKRLGRKKEAAAAEESVDAAREIKMMKLQDDKDGSDRVVVTG	71
GCN20 Yeast	KLAKAEQKIAKVKAKRNNKFKYEASKLINDQKEEDYDSFFLQINPLEFGSSAGKSKDIHIDTFDLYVGD	210
318 protein	GRDLIVDGCVTLVSGFRHYGLVGRNGTGT'FLHMHAMHAIDGVPRIC'QILHVEQV'VTGDATTALQVVLNS	255
ABC E. coliMIVFS SLQIRRGVRLVDNIT'ATINPGQKVLGKNGCGKS'TLLAL'LNKNEISADGGSYTFP	61
ABC Haemophi.MI IFSNLSLKRQGTTELLENASATINPKQKVLGKNGCGKS'SSLFAL'LNKELMPEGGVNYP	61
ABC Yeast	VLSSLETSRDIKLS SVSLLF'GKVL IQDSGLEINYGRR'GLLG'INGCGKS'TFLK'LATREYPIPEIIDY	141
GCN20 Yeast	GQRILSNAQLTSLFGHRYGLVGRNGIGKST'LLRALSRRELIV'KHIVS . ILHVEQ'LRGDDTKALQVVLDA	279
318 protein	DIERTQLLDE'EAQLVAQQE'FEDKIEK'DSNQ'VGRDDISKRLLEEIKRLEHDADSAERAAAS	319
ABC E. coli	GSWQLAWVNO'ETPALPQAALYV IDGDFE'YR'LEA(L'HD)AIE'ERNDGHAIA'ITIG'LDLDAIDAWS'ISRAAS	131
ABC Haemophi.	ANWRVSWVNO'ETPALDISAIDV'IQGDFE'YCFL'QQLE'ERAE'ERNDGNAIAI'IG'LETLDAWTI'SRAAS	131
ABC Yeast	LLDEPAEPSELSALDYVVE'IAQHELKRIE'DLVEK'TL'HDGIE'SELLEPLYERID'DLDPDTFE . . SRAAI	208
GCN20 Yeast	DVWRKQLLSE'FAKINERLKE'MIVLRQEF'E'EDSL'EVKKID'NEREDLDNHLI(I'D)LDL'VDMESDKAE'RAAS	349
318 protein	ILAGLSFT'EFMQKATK'IFSGGWRMRI'LA'ALF'IPD'ILLLDE'PTNHLDL'IAV'WLE'YL'LVVWPT'FFVV	389
ABC E. coli	LLHGLGFSNE'QLERPVS'IFSGGWRMR'LN'AQALIC'R'DLLLE'PTNHLDL'IAV'WLE'WLLK'YQ'TL'LL	201
ABC Haemophi.	LLHGLGFS'CE'ETIQPV'KAFSGGWRMR'LN'AQALIC'P'DLLLE'PTNHLDL'IAV'WLE'WLLV'YQ'TL'LL	201
ABC Yeast	ILIGLGFNKK'TILKTK'DMSGWKMVR'V'LA'ALF'V'FP'ILLLDE'PTNHLDL'IAV'WLE'Y'K'K'FP'D'LL'LL	278
GCN20 Yeast	ILYGLGFS'IEAQQCPT'N'FSGGWRMR'LA'ALF'FC'PD'LLLE'EP'N'LDV/P'ILY'LA'Y'K'K'Y'PT'V'VLT	419
318 protein	VSHARE'FLNTVVT'DIHLNQK'L'ITY'GNV'D'FE'YTR'HE'QVK'Q'QKALEAIE'ERAR'HIQ'Q'FFIDKFFYYNA	458
ABC E. coli	ISHDR'DFLDPIVDK'ILHIECQ'SMIFY'IGNV'SFE'VQR'ATRLA'QQAIV'E'QQ'ERV'HHQS'IIDRFRAAAT	271
ABC Haemophi.	ISHDR'DFLDPIVT'KILH'LENQK'LNEY'GDY'SFE'VQR'ATKLA'Q'TAY'R'QQ'QKI'HIQ'Q'IIDRFKAAAT	271
ABC Yeast	VSHSQD'FLNGVCT'NMI'DMRAQK'L'ITY'GNV'ISY'HTR'SE'LET'QMK'Y'NQ'Q'EEI'GH'IKK'IIASAGYAN	348
GCN20 Yeast	VSHDR'AF'LN'EVAT'DIT'YQHN'ERL'DY'Y'G'QDFDT'F'T'HE'ERRKNAQREYD'IQ'MYVR'KHL'Q'FFIDKYYNA	489
318 protein	KRASLVQSRIKALDRMGHVDEIVNIP'DYF'F'FP'P'DDRPG/P'II'SF'DAS'G'Y'G'G'IP'LFKN . . LNFFGI	525
ABC E. coli	KAKQAQSR'IKML'ERME'LIAPAV'DNP'FRF'SFFA'P'ESLPP'LLMMEKV'AAG'GBDRI'ILDS . . . I'INLLVP	337
ABC Haemophi.	KAKQAQSRMK'AL'ERME'LIAPAV'DNP'FT'F'EFF'P'QSLP'P'LV'II'EQAS'AG'G'BI'CESAVE'ILSK'I'INLLVP	341
ABC Yeast	LVKQAKSR'CK'ILDKME'ADGLVQPV'D'KVF'F'RF'PQVERLIP'IV'LAID'DIS'FHE'SNP'ENLYE'LNFFGV	418
GCN20 Yeast	AKSQEAQSR'IKKLEKLPVLEPPEQDKT'II'F'F'P'ECDKL'P'P'II'QL'D'VS'G'YIE'ENNLL'LDK . . VNLLDV	555
318 protein	DLD'SRIAMV'G'NG'IGKST'ILK'L'JAGD'L'CP'SG'V'IR:SAV'IA'AV'CH'HD'D'LDL'SSPI'LLMMRC'PPGV	595
ABC E. coli	G..SRI'GLL'G'KNG'AGKST'ILK'L'JAGE'L'AP'VSG'IT'GLAK(I'HLG'YF'CH'QJE'LRAD'SPI'Q'LLARL'PPQE	405
ABC Haemophi.	G..SRI'GLL'G'KNG'AGKST'ILK'L'JAGE'L'IAISG'V'QLAK(V'LG'YF'CH'QID'D'LRAD'SALL'WMMQ'KL'P'PQE	409
ABC Yeast	DMD'SRI'ALV'G'NG'VKGST'LLK'I'MIGEL'IP'SG'IV'SR:HTV'ILG'Y'Y'CH:IS'D'D'LLT'SALLE'VVRDKYSNI	488
GCN20 Yeast	QMD'SRI'ALV'G'NG'CGK'T'LLK'I'MEQ'L'FP'L'G'V'SR:NPHL'IG'YF'CH'HD'D'MDLT'SAV'DMMSK'S'PPGK	625
318 protein	PEQKLR'AAALRF'FCWNRKSCAAANVY'V'VRWSEKQGL'CKDNF	636
ABC E. coli	LEQKLR'DYLG'CF'GF'G'DKVIE'ETR'RF'SGG'EK'AR'L'ALIVWQRPNL . . . LLD'EP'INNLD'DLDMR'QLLTAAL	473
ABC Haemophi.	TEQQR'DYLG'S'FAFG'DKVQ'AVK'SFSGG'EK'AR'L'ALIVWQRPNL . . . LLD'EP'INNLD'DLDMR'QLLTAAL	477
ABC Yeast	SQDFQ'WRGQLGRYGLT'GEGQ'IVMQATLSE:G'R'SFV'FALLALEQPNLLLD'EP'INNLD'IPTID'LLAAAI	558
GCN20 Yeast	TDEEY'RRH'LG'S'GIT'G'TLGIQ'KMQLSGGQK'S'VA	660
318 protein	636
ABC E. coli	IEFEGALVVVSHDRHLLRSTTDDLYIVHDKRVEPFIGDLEIY'QQIL'SDVQKQENQTD'EPAPKENANSAQAR	543
ABC Haemophi.	IVDYEGLVVVSHDRHLLRNTVEEFYIVHDKVVEEFIGDLEIY'QKIL'SEQNST'ENKVNSEKVGDNENSVQN	547
ABC Yeast	NEFNGGVVVVSHDFRLLDKIAQDIFV'ENKTATRWIGS'IL(Y'KNIL'AKNVVL	610
GCN20 Yeast	660
318 protein	636
ABC E. coli	KD.QKRREAE'LRQTP'LRKEIARLEKEMEKLNAQLAQAEKLGDS'ELYDQSRKAELTAC'LOQQASAKSG	612
ABC Haemophi.	RKEQKRREAE'LRQQTAP'LRKKITQLEEKMNK'FSS'ELANIENQLAD'ETELYNAENKEKLTALLAQ'QVDVKKKA	617
ABC Yeast	610
GCN20 Yeast	660
318 protein	636
ABC E. coli	LEECEMAWLEAQEQLEQMLLEGQS	636
ABC Haemophi.	LDDVET'EWMTAQEELEMLQA	638
ABC Yeast	610
GCN20 Yeast	660

Fig. 20. Multiple protein alignment for 318 clone (26.7% identity).

--- >50% homology; --- >75% homology; --- 100% homology on alignment.

ABC E. coli – Hypothetical ABC transporter ATP-binding protein from *Escherichia coli*; ABC Haemohi – Hypothetical ABC transporter ATP-binding protein from *Haemophilis elegans* HI0658 (Acc. No P44808); ABC Yeast – probable ATP-dependent transporter YER036c from *Saccharomyces cerevisiae* (Acc. No P40024); GCN20 Yeast – ATP-binding cassette protein from *Saccharomyces cerevisiae* (Acc. No P43535).

7 Casein kinase II phosphorylation sites, amino acids 72-75 (TLCD), 121-124 (SERD), 255-258 (SDIE), 425-428 (TREE), 449-452 (TFID), 492-495 (TPDD), 503-506 (SFSD); 2 Tyrosine kinase phosphorylation sites, amino acids 137-143 (RQREAQY), 296-302 (KRLEEIY); 6 N-myristoylation sites, amino acids 45-50 (GAFEAL), 210-215 (GTGKTT), 227-232 (GVPRNC), 417-422 (GNYDAF), 538-543 (GIGKST), 577-582 (GLDLSS).

To the functional motifs on this protein sequence could be attributed two ATP/GTP-binding site motifs A (P-loop) at amino acids 207-214 (GRNGTGKT) and 535-542 (GPNGIGKS) as well as ABC transporters family signature, amino acids 338-352 (FSGGWRMRLALRAL).

Analysis of hydrophobic regions performed by DNAMAN software (see *Methods*, chapter 2.2.7) showed that there are four probable hydrophobic sites on the protein sequence (Fig. 21).

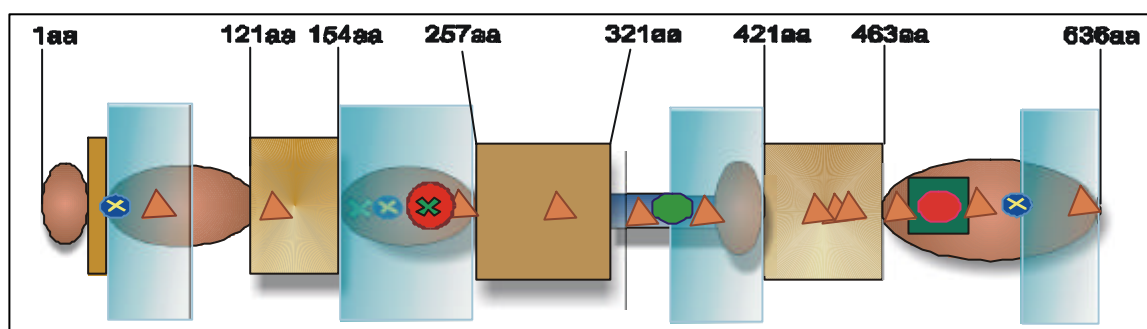
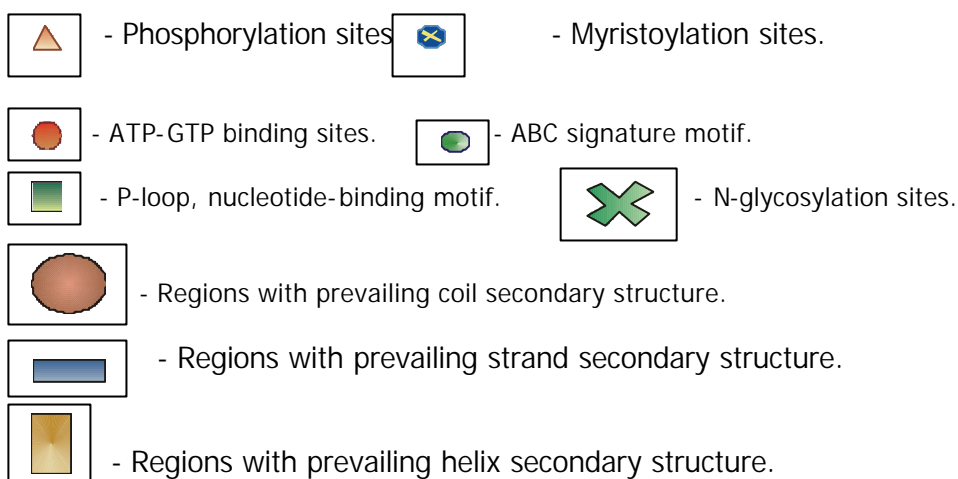


Fig. 21. Hypothetical scheme of the predicted protein structure coded by 318 cDNA.



The semi-transparent zones indicate regions with prevailing hydrophobic amino acids. Numbers on the picture show amino acid position on the protein sequence.

The myristoylation sites and hydrophobic regions indicate possible membrane association or protein-protein interaction domains, however, typical transmembrane domain could not be

found in the protein sequence. The phosphorylation sites indicate possible regions of protein activation-deactivation by protein kinases.

In case of the protein coded by cDNA of the clone 318 one could assume that the main protein features are quite intensive phosphorylation, four hydrophobic regions, two ATP-GTP binding motifs and ABC-signature. The myristoylation sites are located in N- and C-terminal regions as well as in the middle of the protein near to ATP-GTP-binding site overlapping with possible N-glycosylation site.

This information leads to speculation that N-terminal and C-terminal regions of the protein either could be membrane associated or be involved in protein-protein interactions. ABC-signature motif is flanked by phosphorylation sites, which indicates probable sites of functional regulation, as well as in the helix between amino acids 421 and 463, where 3 phosphorylation sites are located.

The same pattern is valid for the region with P-loop, coupled with ATP-GTP-binding motif flanked by phosphorylation sites as in the case of ABC transporter signature. The most conservative regions with local homologies 70-90% overlap with the region of ABC-signature motif (position between 379 – 399 amino acids) and P-loop, ATP-GTP-binding site with flanking phosphorylation sites (amino acids 529 – 573). All the information about this sequence is schematically presented on Fig. 21. Taking together homology search and protein secondary structure prediction as well as analysis of potential functional motif distribution, it could be assumed that the protein coded by 318 cDNA belongs to the group of ABC transporters with two nucleotide binding domains and no transmembrane domains (DAVIES and COLEMANN, 2000; THEODOULOU, 2000), as it was shown for GCN20 enzyme from yeast (DE ALDANA et al., 1995).

The cDNA of the second clone, 58, consisted of 833 base pairs and the only ORF was coding for the protein of 180 amino acids (Fig. 22). Analysis of cDNA and protein sequences has been made with use of the same software as for the clone 318. The search for the homologies showed that this protein is highly homologous to many Translationally Controlled Tumour Proteins (TCTP) from different plants.

TCTP proteins were initially described as tumour growth related proteins in human and mice. The function of them is not clearly understood. However, it is known that TCTPs form very closely related family of abundant Ca-binding proteins.

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1          C C A G T T T C A C C G C A C G A A T A T C T T G T T G T T G G A G C G A T C C G A G A A A A C T C T G T A A A C A T G T T G G T T T A C C A G
1
74 G A C C T C C T T A C A G G T G A T G A G C T T C T C T G A C T C C T T C C G T T A C A A G G A A A T T G A G A A T G G A A T G C T G T G G G A A
5   D L L T G D E L L S D S F R Y K E I E N G M L W E

150 G T T G A G G G A A G T G G G T T G T T A A A G G A G C A G T T G A T G T A G A C A T T G G T G C A A A C C C T T C T G C T G A G G G T G G A G G A
30  V E G K W V V K G A V D V D I G A N P S A E G G G

225 G A A G A T G A G G G A G T T G A T G A T G C A G C T G T T A A G G T T G T T G A C A T T G T T G A C A C A T T C A G A C T T C A G G A G C A A C C C
55  E D E G V D D A A V K V V D I V D T F R L Q E Q P

300 G C A T T C G A T A A G A A G C A G T T T G T T A C C T T C A T G A A G A G G T T T A T C A A G A A T T T G A C T C C C A A G C T C G A T G C A G A G
80  A F D K K Q F V T F M K R F I K N L T P K L D A E

375 C A A C A G G A G T T G T T T A A G A A G C A C A T C G A G G G A G C A A C T A A A T A C C T G C T C T A A G A T T A A A G A T T T C C A A T T T
105 Q Q E L F K K H I E G A T K Y L L S K I K D F Q F

400 T T T G T T G G T G A G A G C A T G G G T G A T G A T G C T T G C T T G G T C T T T G C C T A C T A C A A G G A C G G C G C T G C T G A T C C A A C A
130 F V G E S M G D D A C L V F A Y Y K D G A A D P T

475 T T C C T G T A C T T T T G C A T A T G C C T T A A A G G A G G T C A A G T G C T A A G T G A C T T A A T G T A C C G A G C T A T C T A C T C A T T T T
155 F L Y F A Y A L K E V K C * V T * C T E L S T H F

550 A T C T A G T T T T T T T T A C G T T C T G T C C T A A G T T A C T T G A C T T T G A C C T G G T A T T T T G A T T T T R A T G G T G A G A C G T G T G C T A C T G C T A C A T C A
180 I -

642 T A A T T C C C A T C A A A T T T A A T T A T C G A T G G G T T A G A A T T T C A T T A A T T A T G A T T T T G G T C G T G C T A G A T T C C C G G A A G T T T C T T T T G A A G A A T T T
737 T T G G G T G T G T A C T A G A A A T A T T T T A A T T T G G C T T C A T G G T T T T A T T G A T A A G G C T T T G A A T A C G A C T T G G G A A T T T A A T A A A A A A A A A A A A A A A A
832 A A A

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Fig. 22. Complete cDNA of the clone 58 and predicted protein sequence.

cDNA sequence consisted of 834 nucleotides. ORF was coding for the protein of 180 amino acid residues.

They can be up regulated by darkness in plants as it was shown on *Pharbitis nil* cv. Violet (SAGE-ONO et al., 1998) and also elicited by heavy metal stress in earthworm *Lumbricus rubellus* (STÜRZENBAUM et al., 1998). Identity on the protein level with 7 plant proteins is 87.43% (Fig. 23), which is very high for the proteins. The homology tree shows that the most related protein to the one encoded by cDNA of the clone 58 is TCTP from Hevea (Fig. 24).

The search for different motifs has been performed with use of the same software as for the protein coded by cDNA of the clone 318 (see above). Number of potential modification sites and functional motifs has been found for this sequence. They are: 3 protein kinase C phosphorylation sites, amino acids 17-19 (SFR), 73-75 (TFR) and 99-101 (TPK); 2 casein kinase II phosphorylation sites, amino acids 912 (TGDE) and 135-138 (SMGD), 2 N-myristoylation sites, amino acids 46-51 (GANPSA) and 59-64 (GVDDAA). Two functional motifs have been found also, Translationally controlled tumour protein signatures 1 (amino acids 45-55, IGANPSAEGGG), and 2 (amino acids 130-147, FFVGESMGDDACLVFAYY).

58 prot	MLVYQDLLTGDELLSDSFYRKEIENGMLWEVEGKQVVGAVDVDIGANPSAEG..GGEDE	58
Pea	MLVYQDLLTGDELLSDSYPYKEIENGMLWEVEGKQVVGAVDVNIGANPSAE..GGEDE	57
Tobac	MLVYQDLLSGDELPSSDFSYTELGNGLWEVQGGKQVVGAVDVNIGANPSAEG..ADEDE	58
Hevea	MLVYQDLLTGDELLSDSFYRKEIHNGILWEVEGKQVVGAVDVGANPSAEG..ADEDE	58
Medicag	MLVYQDLLTGDELLSDSYPYKEIENGMLWEVEGKQVVGAVDVNIGANPSAE..GGEDE	57
Rice	MLVYQDLLTGDELLSDSFYRKEIENGILWEVVDGKQVVGAVDVGANPSAEG..GGDDE	58
Potato	MLVYQDLLTGDELLSDSFYRKEIENGMLWEVHGKQVVGAVDVNIGANPSAEG..GGEDE	58
Straw	MLVYQDLLSGDELLSDSFYRKEIENGMLWEVVDGKQVVGAVDVGANPSAEGADADADE	60
Fir	MIVYQDLLSGDELLSDSFYRKEIENGMLWEVEGKQVVGAVDVGANPSAEGGDE...E	57
58 prot	GVDDAVKVVVDIVDTFRLQEQPAFDKKQFVTFMKRFIKNLTPLDQAEQELFKKHIEGAT	118
Pea	GVDDTAVKVVVDIVDTFRLQEQPPFDKKQFLGFVKKYIKLLTPKLEAEKQEIFKKNIEGAT	117
Tobac	GVDDQAIKVVVDIVDTFRLQEQPSFDKKQFVAYMKKYIKNLTPLKLAEQEEVFKNIEGAT	118
Hevea	GVDDQAVKVVVDIVDTFRLQEQPAFDKKQFVTFMKRFIKNLTPLDQAEQELFKKHIEGAT	118
Medicag	GVDDTAVKVVVDIVDTFRLQEQPAFDKKQFLGFVKKYIKLLTPKLAEKQELFKKHIEGAT	117
Rice	GVDDQAVKVVVDIVDTFRLQEQPPFDKKQFVTFMKRYIKNLSAKLDAEKQEEFKKNIEGAT	118
Potato	GVDDQAVKVVVDIVDTFRLQEQPAFDKKQFVTFMKRYIKNLSAKLDAEKQEEFKKNIEGAT	118
Straw	GVDDQAVKVVVDIVDTFRLQEQPAFDKKQFVTFMKRYIKNLSAKLDAEKQEEFKKNIEGAT	120
Fir	GVKIRLVKVVVDIVDTFRLQEQPPFDKKQFLGFVKKYIKNLTATKLEERQAEFKKNVEGAA	117
58 prot	KYLLSKIKDFQFVFGESMGDDACLVAFYKDGAAADPTFLYFAYALKEVK	167
Pea	KYLLGKLDLQFVFGESMHDDGSLVFAFYKDGAAADPTFLYFSAFALKEIK	166
Tobac	KYLLSKLSDLQFVFGESMADDTGMVFAFYKDGATDPTFLYLAHGLKEVK	167
Hevea	KFLLSKLSDLQFVFGESMHDDGSLVFAFYRGGATDPTFLYFAYALKEVK	167
Medicag	KYLLCKLKLQFVFGESMHDDGSLVFAFYKDGAAADPTFLYFAYALKEIK	166
Rice	KYLLGKLDLQFVFGESMHDDGSLVFAFYKDGATDPTFLYFSAFALKEIK	167
Potato	KFLLSKLKLQFVFGESMHDDGSLVFAFYKDGAAADPTFLYFAYALKEIK	167
Straw	KFLLSKLSDLQFVFGESMGDDTSLVFAFYKDGATDPTFLYLAHGLKEVK	169
Fir	KMLVSKLSDLQFVFGESMHDDGSMVFAFYKDGATDPTFLYFADGLKEVK	166

Fig. 23. Multiple alignment for the protein coded by 58 cDNA (86.73% identity).

■ -- >50% homology; ■ -- > 75% homology; ■ -- 100% homology on alignment.

58 prot – protein coded by cDNA of the clone 58; Pea – TCTP from pea (*Pisum sativum*, Acc. No P50906); Tobac – TCTP from tobacco (*Nicotiana tabacum*, Acc. No Q9XHL7); Hevea – TCTP from Hevea (*Hevea brasiliensis*, Acc. No Q9ZSW9); Medicag – TCTP from medicago (*Medicago sativa*, Acc. No P28014); Rice -- TCTP from rice (*Oryza sativa*, Acc. No P35681); Potato – TCTP from potato (*Solanum tuberosum*, Acc. No P43349); Straw – TCTP from strawberry (*Fragaria x ananasa*, Acc. No O03992); Fir – TCTP from fir (*Pseudotsuga menziesii*, Acc. No Q9ZRX0).

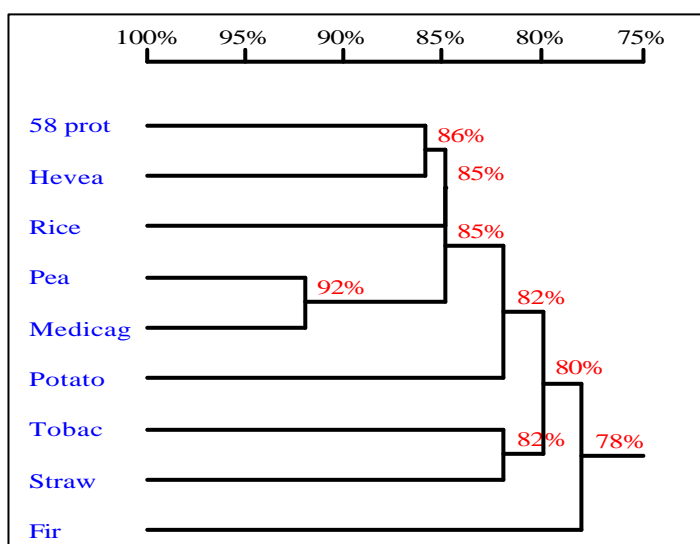


Fig. 24. Homology tree for protein coded by cDNA of 58 clone.

58 Prot – protein coded by cDNA of the clone 58; Hevea – TCTP from Hevea (*Hevea brasiliensis*, Acc. No Q9ZSW9); Rice -- TCTP from rice (*Oryza sativa*, Acc. No P35681); Pea – TCTP from pea (*Pisum sativum*, Acc. No P50906); Medicag – TCTP from medicago (*Medicago sativa*, Acc. No P28014); Potato – TCTP from potato (*Solanum tuberosum*, Acc. No P43349); Tobac – TCTP from tobacco (*Nicotiana tabacum*, Acc. No Q9XHL7); Straw – TCTP from strawberry (*Fragaria x ananasa*, Acc. No O03992); Fir – TCTP from fir (*Pseudotsuga menziesii*, Acc. No Q9ZRX0).

Analysis of probable secondary structure as well as for hydrophobic regions of the protein has been made by DNAMAN software (*Methods*, chapter 2.2.8). All these data are summarised in the model of the protein structure (Fig. 25). This protein contains no clear hydrophobic regions. Potential TCTP signature motifs (see above) overlap with myristoylation (TCTP signature 1) and phosphorylation (TCTP signature 2) sites.

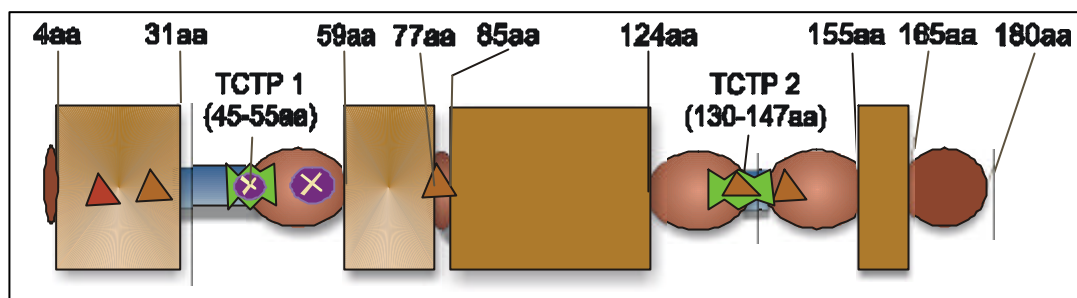
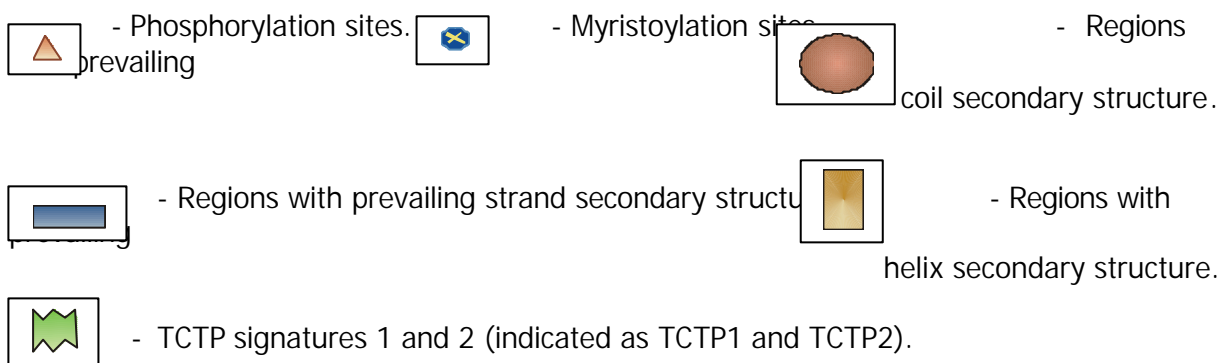


Fig. 25. Hypothetical scheme of the predicted protein structure coded by 58 cDNA.



The semi-transparent zones indicate regions with prevailing hydrophobic amino acids. Numbers on the picture show amino acid position on the protein sequence.

cDNA of the third clone, 633, consisted of 1470 base pairs and was coding for the protein of 337 amino acids (Fig. 26). Analysis of cDNA and corresponding protein sequences has been made with use of the same software as for the clones 318 and 58. The search for the homologies showed that this protein has similarities with Inosine Monophosphate Dehydrogenases (IMPDH) from different organisms. IMPDHs catalyse the rate-limiting step in de novo biosynthesis of guanine nucleotides and have an essential role in providing necessary precursors for DNA and RNA synthesis and in signal transduction pathways mediating cell differentiation (COLLART et al., 1996). Identity on the protein level was 67% average (Fig. 27) with quite high homologies at the C-terminal part of the other IMPDHs.

The protein sequence encoded by the clone 633 has following probable functional sites. 2 protein kinase C phosphorylation sites: amino acids 23-25 (SEK); amino acids 253-255

(TAK); 4 Casein kinase II phosphorylation sites: amino acids 75-78 (motif TRED); 103-106 (SIYD); 153-156 (TTQE); 297-300 (SAHD).

1	AATTCGGCACCAGGCACCTTCGCGCGTCTGGAGCAGATGACGCCTTCGGGGCCTCCCCCTTC
64	CTTCTCGTCACCGACACTGGCACCTCCGCCGGAACTCCTCGGCTACGTCGCGAAGAGTGACTGGACGAATCATACCACAAG
147	GGCTTGAGAGTCGGCGACTAC ATGGCGCCACCTCCCAAGCCAGTGCCATGGAACGCCGACCTAAACAAAATTAATGAA
51	M A P P P K P V P W N A D L N K I N E
221	ATATTTGAGAGTGAGAAAAGTGGTGTCTGGCTTTGGAGAAGGATGGTGAGGTTGTTGATTGGTGGTGAGGGAG
20	I F E S E K S G A V A L E K D G E V V D L V V R E
296	GAGGTGGAGAGGGTGAAGGGTTACCCGAACTGGTGGCGCCGGCGACGGTGGGGCGGACGGGGAGTTTATGGTG
45	E V E R V K G Y P K L V A P A T V G A D G E F M V
371	GGGCGCGGTTGGGACGAGGGAAGATGATAAGGAGAGGTTGGAGCATTGGTGAAGGCTGGGTTGAATGTTGTA
70	G A A V G T R E D D K E R L E H L V K A G L N V V
446	GTGTTGATAGTTCTCAGGGAACTCAATTTATCAGTTAGAGATGGTGAAGTATGTGAAGAGGGTTTACCCTGAG
95	V L D S S Q G N S I Y Q L E M V K Y V K R V Y P E
521	CTTGATGTGATTGGGGGAATGTTGTGACTATGTACCAGGCTGAGAATCTGATTGAGGCTGGGTTGATGGGTTG
120	L D V I G G N V V T M Y Q A E N L I Q A G V D G L
596	AGGTTTGAATGGGGTCTGGGTCCATTTGTACTACTCAGGAGGTTTGTGCTGTGGGGCGTGGTCAGGCAACTGCT
145	R V G M G S G S I C T T Q E V C A V G R G Q A T A
671	GTTTACAAGGTCTCGTCCATTGCTTATAAAAGTGGTGTCTCTGTGATTGCTGATGGTGGCATCTCAAACCTGCT
170	V Y K V S S I A Y K S G V P V I A D G G I S N S G
746	CATATTGTTAAGGCTTTGTCATTGGGAGCGTCAACTGTTATGATGGGAAGCTTCTTAGCTGGTAGTCTTGGAGGCT
195	H I V K A L S L G A S T V M M G S F L A G S L E A
821	CCTGGGCTTATGTGTATCAGAATGGTCAACGTGTGAAAAAGTACAGAGGAATGGGTTCCCTAGAAGCCATGACT
220	P G A Y V Y Q N G Q R V K K Y R G M G S L E A M T
896	AAAGGGAGCGATGCAAGGTACTTGGGTGATACAGCAAAGCTAAAAATTGCTCAGGGGTTGTTGGAGCTGTTAAA
245	K G S D A R Y L G D T A K L K I A Q G V V G A V K
971	GATAAGGGCTCTGTCTTGAATTCATACCATAACCTTGAAGCAGTCAGGCAAGGGTTTCAGGATATCGGTGCC
270	D K G S V L N F I P Y T L Q A V R Q G F Q D I G A
1046	AACTCTTACAGTCTGCTCATGACCTTCTAAGATCCAGGGTGTAAAGACTGGGAGTCCGGAGTGGAGCAGCACAA
295	N S L Q S A H D L L R S R V L R L G G P E W S S T
1121	AGTTGGAAGGTGGAATTCATGGGCTGGTTTCTTATGAAAAGAAATACTTT TGA AGTATGAAACCATCCAAAAATCAT
320	S W K V E F M G L V S Y E K K Y F -
1196	AAGCGGTGCTATAATCCCTTGCTGCAGTTCAGCATAAAAGAGGGGGACACTGTTATTGGGGTTAGTGGCAACTTGTGCTACT
1280	GTGTCTAGCGAATTGAAGTCTATACCAATAGAATATCGTGGGAAATTTTCCCTTGAGTTTCTGAAGGAAGAACACTGATTCGTG
1364	GGTTTTTTTATGTCATTAGATTTGTTTGTCAAATGTGCATAAACTTTCCTAAATATATTTTGCATGTTTCTGTCCAAAA
1448	AAAAAAAAAAAAA

Fig. 26. Complete cDNA of the clone 633 and predicted protein sequence.

cDNA sequence consisted of 1460 nucleotides. ORF was coding for the protein of 337 amino acid residues.

There are three hypothetical functional motifs also, in decreasing probability they are: amino acids 85 - 306 -- IMPDH - IMP dehydrogenase / GMP reductase motif; amino acids 124 - 173 -- IMP dehydrogenase / GMP reductase signature; amino acids 178 - 213 – motif characteristic for the proteins binding FMN and related compounds.

This protein has 12 probable myristoylation sites on it, such as: at amino acids 70-75 (motif GAAVGT); 125-130 (GNVVTM); 143-148 (GLRVGM); 149-154 (GSICTT); 163-168 (GOATAV);

186-191 (GGISNS); 187-192 (GISNSG); 208-213 (GSFLAG); 236-241 (GSLEAM); 261-266 (GVVGAV); 270-275 (GSVLNF); 320-325 (GGIHL).

633 protein	0
SoyMDFTPPIEDGFTA ⁵⁰ AEK ⁵⁰ LFTQ ⁵⁰ GFSY ⁵⁰ TYDDVIFLPHY ⁵⁰ IDFAADAVDLSTR ⁵⁰ LRRL ⁵⁰	54
ArabMSTLEDGFPADKLFAGQYSYTYDDVIFLPHYIDFSTDAVSLSTRLSRRV ⁵⁰	50
Human	MADYLISSGGTGYVPE ⁵⁰ DGLTA ⁵⁰ QQLFASADDLYNDFLILPGFIDFLADEVDLTSALTRKIT ⁵⁰	60
Mouse	MADYLISSGGTSYVPPD ⁵⁰ DGLTA ⁵⁰ QQLFNCGDGLTYNDFLILPGYIDFTADQVDLTSALTKKIT ⁵⁰	60
Hamst	MADYLISSGGTSYVPPD ⁵⁰ DGLTA ⁵⁰ QQLFNCGDGLTYNDFLILPGYIDFTADQVDLTSALTKKIT ⁵⁰	60
633 protein	0
Soy	LAVDFV ⁷⁵ ASPM ⁷⁵ DTV ⁷⁵ ESAMAA ⁷⁵ AMASLGGI ⁷⁵ AVVHSN ⁷⁵ VPAAVQA ⁷⁵ AILRRAKSRRV ⁷⁵ ILSDPAF ⁷⁵	114
Arab	LSIP ⁷⁵ CVS ⁷⁵ SPMD ⁷⁵ TV ⁷⁵ ESHMAA ⁷⁵ AMASLGGIGIVHYNC ⁷⁵ 3IAAQASI ⁷⁵ IRQAKSLKH ⁷⁵ IASDA ⁷⁵ FV	110
Human	LKTP ⁷⁵ LIS ⁷⁵ SPMD ⁷⁵ TV ⁷⁵ EADMAI ⁷⁵ AMALMGGIGF ⁷⁵ IHHNCTPEFQANEV ⁷⁵ RKVKNF ⁷⁵ EQGF ⁷⁵ ITDPV ⁷⁵ V	120
Mouse	LKT ⁷⁵ PLVS ⁷⁵ SPMD ⁷⁵ TV ⁷⁵ EAGMAI ⁷⁵ AMALMGGIGF ⁷⁵ IHHNCTPEFQANEV ⁷⁵ RKVKKY ⁷⁵ EQGF ⁷⁵ ITDPV ⁷⁵ V	120
Hamst	LKT ⁷⁵ PLVS ⁷⁵ SPMD ⁷⁵ TV ⁷⁵ EAGMAI ⁷⁵ AMALMGGIGF ⁷⁵ IHHNCTPEFQANEV ⁷⁵ RKVKKY ⁷⁵ EQGF ⁷⁵ ITDPV ⁷⁵ V	120
633 protein	0
Soy	AAPSAVVEHDDAFGASPFLLVTD...TGT ⁵⁰ S...VG.KLLGYVARSDWTNQTIDKGLRVG.	165
Arab	KFP ⁵⁰ EYEITSLDAFGPSSFFVFEQ...TGT ⁵⁰ M...TTPKLLGYVTKSQWKRMYEQREMKI	163
Human	LSPSHTVGDVLEAKMRHGFSQIPITE ⁵⁰ TGTMGSKLVG.IVTSRDIDFLA ⁵⁰ EKDHTTL ⁵⁰ LS ⁵⁰ EV ⁵⁰ M	179
Mouse	LSPKDRVRDVFEAKARHGFCGIPITD ⁵⁰ TGRMGSRLVG.IISSRDIDFLK ⁵⁰ EEHDF ⁵⁰ LEEIM	179
Hamst	LSPKDRVRDVFEAKARHGFCGIPITD ⁵⁰ TGRMGSRLVG.IISSRDIDFLK ⁵⁰ EEHDF ⁵⁰ LEEIM	179
633 protein	..MAPPPKPV...PWNADLNKINEIFESEKSGAVALE ⁵⁰ EKDGEVVDLVVRE ⁵⁰ EVERV ⁵⁰ KGYPK	54
Soy	.DYMAPPKPA...PWNADLNKINEIMESEKSGAVALE ⁵⁰ ERDGEVVDLVVRE ⁵⁰ EVERV ⁵⁰ KGYPK	221
Arab	YDYMKSCDSSDYCVPWEIDFEKLEFVLEDKQKGFVVL ⁵⁰ ERDGETVNVVTK ⁵⁰ QDIQRV ⁵⁰ KGYPK	223
Human	TPRIELV ⁵⁰ VAPAGV ⁵⁰ TLKEANEILQ ⁵⁰ RSKKGKLP ⁵⁰ IVNDC ⁵⁰ ELV ⁵⁰ AIARTDLKKNRD ⁵⁰ PLASKD	239
Mouse	TKREDLV ⁵⁰ VAPAGV ⁵⁰ TLKEANEILQ ⁵⁰ RSKKGKLP ⁵⁰ IVNEN ⁵⁰ DELV ⁵⁰ AIARTDLKKNRD ⁵⁰ PLASKD	239
Hamst	TKREDLV ⁵⁰ VAPAGV ⁵⁰ TLKEANEILQ ⁵⁰ RSKKGKLP ⁵⁰ IVNEN ⁵⁰ DELV ⁵⁰ AIARTDLKKNRD ⁵⁰ PLASKD	239
633 protein	LVPATV ⁵⁰ GADGEFMV ⁵⁰ GAAV ⁵⁰ GTRE ⁵⁰ DDKERLEHLV ⁵⁰ KAGLN ⁵⁰ VVLDSSQGN ⁵⁰ SIY ⁵⁰ DLE ⁵⁰ MV ⁵⁰ YVK	114
Soy	LVPATV ⁵⁰ GADGEFMV ⁵⁰ GAAV ⁵⁰ GTRE ⁵⁰ DDKERLEHLV ⁵⁰ KAGLN ⁵⁰ VVLDSSQGN ⁵⁰ SIY ⁵⁰ QLEM ⁵⁰ VYVK	281
Arab	SGPG.TVGP ⁵⁰ DGEWMV ⁵⁰ GAAI ⁵⁰ GTRE ⁵⁰ DDKERLEHLV ⁵⁰ NVGVNA ⁵⁰ VVLDSSQGN ⁵⁰ SIY ⁵⁰ QLEM ⁵⁰ IKYVK	282
Human	SQKQLLC.....GAAV ⁵⁰ GTRE ⁵⁰ DDKY ⁵⁰ RLDL ⁵⁰ LQAGV ⁵⁰ VDV ⁵⁰ LDSSQGN ⁵⁰ SVY ⁵⁰ QIAM ⁵⁰ VYIK	291
Mouse	AKKQLLC.....GAAI ⁵⁰ GTRE ⁵⁰ DDKY ⁵⁰ RLDL ⁵⁰ LALAGV ⁵⁰ VDV ⁵⁰ LDSSQGN ⁵⁰ SIF ⁵⁰ QIN ⁵⁰ MIKYIK	291
Hamst	AKKQLLC.....GAAI ⁵⁰ GTRE ⁵⁰ DDKY ⁵⁰ RLDL ⁵⁰ LALAGV ⁵⁰ VDV ⁵⁰ LDSSQGN ⁵⁰ SIF ⁵⁰ QIN ⁵⁰ MIKYMK	291
633 protein	RVYPELDVIGGNVVTMYQAENLIQAGVDGLRVGM...GSICTTQEVCAVGRGQATAVYKVS	172
Soy	RVYPELDVIGGNVVTMYQAENLIQAGVDGLRVGMGSGSICTTQEVCAVGRGQATAVYKVS	341
Arab	KTYPELDVIGGNVVTMYQAENLIQAGVDGLRVGMGSGSICTTQEVCAVGRGQATAVYKVC	342
Human	QKYPHLQVIGGNVVTAAQAKNLI ⁵⁰ DAGVDGLRVGMG ⁵⁰ CSIC ⁵⁰ ITQEV ⁵⁰ MACGR ⁵⁰ PQ ⁵⁰ TAVYK ⁵⁰ VA	351
Mouse	EKYP ⁵⁰ SLQVIGGNVVTAAQAKNLI ⁵⁰ DAGVDALRVGMG ⁵⁰ SGSIC ⁵⁰ ITQEV ⁵⁰ LACGR ⁵⁰ PQ ⁵⁰ TAVYK ⁵⁰ VS	351
Hamst	EKYP ⁵⁰ NLQVIGGNVVTAAQAKNLI ⁵⁰ DAGVDALRVGMG ⁵⁰ CSIC ⁵⁰ ITQEV ⁵⁰ LACGR ⁵⁰ PQ ⁵⁰ TAVYK ⁵⁰ VS	351
633 protein	SIAYKSGVPVIADGGISNSGHI ⁵⁰ VKALS ⁵⁰ LGASTVMMG ⁵⁰ SFLAGSL ⁵⁰ EAPGA ⁵⁰ YVYQ ⁵⁰ ING ⁵⁰ QIRV ⁵⁰ KKY	232
Soy	LIAYKSGVPVIADGGISNSGHI ⁵⁰ VKALS ⁵⁰ LGASTVMMG ⁵⁰ SFLAGSL ⁵⁰ EAPGA ⁵⁰ YVYQ ⁵⁰ ING ⁵⁰ QIRV ⁵⁰ KKY	401
Arab	SIAAQSGIPVIADGGISNSGHI ⁵⁰ VKALV ⁵⁰ LGASTVMMG ⁵⁰ SFLAGSTEAPGGY ⁵⁰ EY ⁵⁰ TNG ⁵⁰ KIRI ⁵⁰ KKY	402
Human	EYARRFGVPVIADGGIQTVGHVVKALALGASTVMMGSLAATTEAPGEYFFSDGIRLKKY	411
Mouse	EYARRFGVPVIADGGIQNVGHI ⁵⁰ AKALALGASTVMMGSLAATTEAPGEYFFSDGIRLKKY	411
Hamst	EYARRFGVPVIADGGIQNVGHI ⁵⁰ AKALALGASTVMMGSLAATTEAPGEYFFSDGIRLKKY	411
633 protein	RGMGSLEAMTKGSDA..RYLGD ⁵⁰ TAKL ⁵⁰ IAQ ⁵⁰ VVGAV ⁵⁰ KDKGS ⁵⁰ VLN ⁵⁰ FIPY ⁵⁰ T ⁵⁰ LQAV ⁵⁰ RQ ⁵⁰ G ⁵⁰ QDI	290
Soy	RGMGSLEAMTKGSDA..RYLGD ⁵⁰ TAKL ⁵⁰ IAQ ⁵⁰ VVGAV ⁵⁰ KDKGS ⁵⁰ VLN ⁵⁰ FIPY ⁵⁰ T ⁵⁰ LQAV ⁵⁰ RQ ⁵⁰ G ⁵⁰ QDI	459
Arab	RGMGSLEAMTKGSDQ..RYLGD ⁵⁰ QTKL ⁵⁰ IAQ ⁵⁰ VVGAV ⁵⁰ ADKGS ⁵⁰ VLKL ⁵⁰ IPY ⁵⁰ TMH ⁵⁰ AV ⁵⁰ RQ ⁵⁰ G ⁵⁰ QDL	460
Human	RGMGSLDAMEKSSSSQKRYFSEGD ⁵⁰ KVK ⁵⁰ IAQ ⁵⁰ VVSG ⁵⁰ SIQ ⁵⁰ DKGS ⁵⁰ I ⁵⁰ Q ⁵⁰ KFV ⁵⁰ PLY ⁵⁰ LIAG ⁵⁰ IQH ⁵⁰ GC ⁵⁰ QDI	471
Mouse	RGMGSLDAMD ⁵⁰ KHLSSQ ⁵⁰ NR ⁵⁰ YFSEAD ⁵⁰ KIK ⁵⁰ V ⁵⁰ AQ ⁵⁰ GV ⁵⁰ SGAV ⁵⁰ QDKGS ⁵⁰ I ⁵⁰ H ⁵⁰ KFV ⁵⁰ PLY ⁵⁰ LIAG ⁵⁰ IQH ⁵⁰ SC ⁵⁰ QDI	471
Hamst	RGMGSLDAMD ⁵⁰ KHLSSQ ⁵⁰ NR ⁵⁰ YFSEAD ⁵⁰ KIK ⁵⁰ V ⁵⁰ AQ ⁵⁰ GV ⁵⁰ SGAV ⁵⁰ QDKGS ⁵⁰ I ⁵⁰ H ⁵⁰ KFV ⁵⁰ PLY ⁵⁰ LIAG ⁵⁰ IQH ⁵⁰ SC ⁵⁰ QDI	471
633 protein	GANS ⁵⁰ LQSAHDLLRSRVLRL ⁵⁰ LEVR ⁵⁰ SGAAQ ⁵⁰ VEGG ⁵⁰ IHGL ⁵⁰ VS ⁵⁰ YERILLKYETIQK	340
Soy	GASS ⁵⁰ LQSAHDLLRSRELRL ⁵⁰ LEVR ⁵⁰ SGAAQ ⁵⁰ VEGG ⁵⁰ VHGL ⁵⁰ VS ⁵⁰ YEKKYF.....	502
Arab	GASS ⁵⁰ LQSAHGLLLRSN ⁵⁰ ILRL ⁵⁰ EARTGAAQ ⁵⁰ VEGG ⁵⁰ VHGL ⁵⁰ VS ⁵⁰ YEKKSF.....	503
Human	GARSLSVLRSMYS ⁵⁰ GELK ⁵⁰ FEKRT ⁵⁰ MSA ⁵⁰ IEGG ⁵⁰ VHGL ⁵⁰ HS ⁵⁰ YEKRLY.....	514
Mouse	GAKSLTQVRAMTYS ⁵⁰ GELK ⁵⁰ FEKRT ⁵⁰ SSA ⁵⁰ QVEGG ⁵⁰ VHSL ⁵⁰ HS ⁵⁰ YEKRLF.....	514
Hamst	GAKSLTQVRAMMYS ⁵⁰ GELK ⁵⁰ FEKRT ⁵⁰ SSA ⁵⁰ QVEGG ⁵⁰ VHSL ⁵⁰ HS ⁵⁰ YEKRLF.....	514

Fig. 27. Multiple alignment for the protein coded by 633 cDNA (67% identity).

-- >50% homology; -- > 75% homology; -- 100% homology on alignment. 633 protein – protein coded by cDNA of the clone 633; Soy – translation of the soybean (*Glycine max*) mRNA for inosine monophosphate dehydrogenase (Acc. No AJ010201); Arab – Inosine-5'-monophosphate dehydrogenase (IMPDH) from *Arabidopsis thaliana* (Acc. No P47996); Human – IMPDH1 from *Homo sapiens* (Acc. No P20839); Mouse – IMPDH2 from *Mus musculus* (Acc. No P24547); Hamst – IMPDH from *Mesocricetus auratus* (Acc. No P12269).

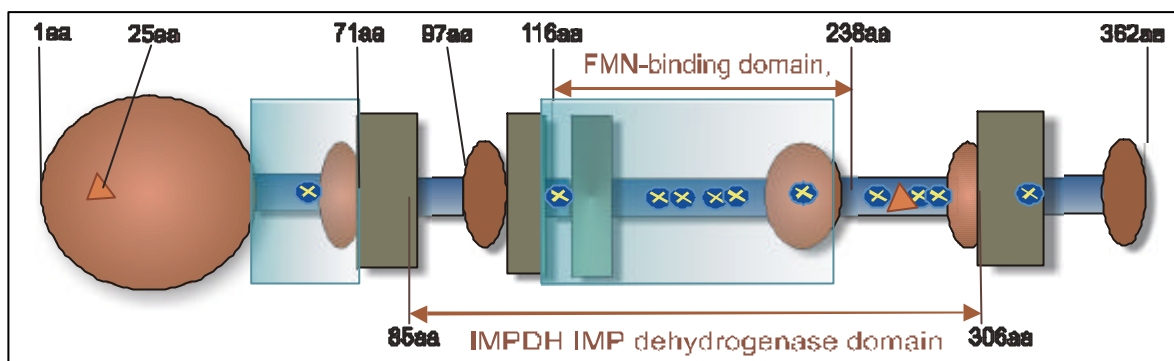
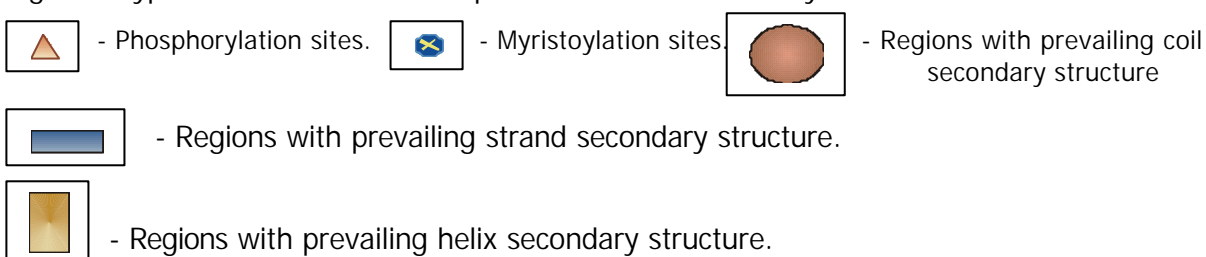


Fig. 28. Hypothetical scheme of the protein structure coded by 633 cDNA.



The semi-transparent zones indicate regions with prevailing hydrophobic amino acids. Numbers on the picture show amino acid position on the protein sequence. IMPDH-IMP domain indicates the domain characteristic for inosine-5'-monophosphate dehydrogenases.

The main feature of this protein is very intensive myristoylation. Myristoylation sites are all located in the middle of the protein, more to C-terminus. In such position (not in N-terminal part) they hardly indicate membrane association, but probably the region (-s) involved in protein-protein interactions (Fig. 28, see Thompson and Okuyama, 2000, for the review).

4.5 Al-responsiveness of transgenic yeast over expressing 318 functional gene

In order to check whether 318-cDNA fragment coding for complete functional protein improve resistance for aluminium stress, 318 full protein coding fragment was introduced into yeast cells (strain Inv Sc2) in expression plasmid under control of P gal1 promoter (in cooperation with Dr. Kunze, IPK, Gatersleben, Fig. 29).

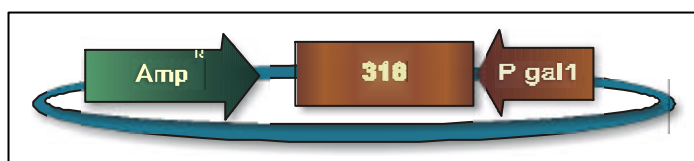


Fig. 29. Schematic representation of expression plasmid 318pYES based on yeast vector pYES2 used for introduction of 318 full protein-coding fragment into yeast under control of P gal1 promoter.

Amp^R – ampicillin resistance gene, 318 – full protein-coding fragment, p gal1 – galactose inducible promoter for expression in yeast.

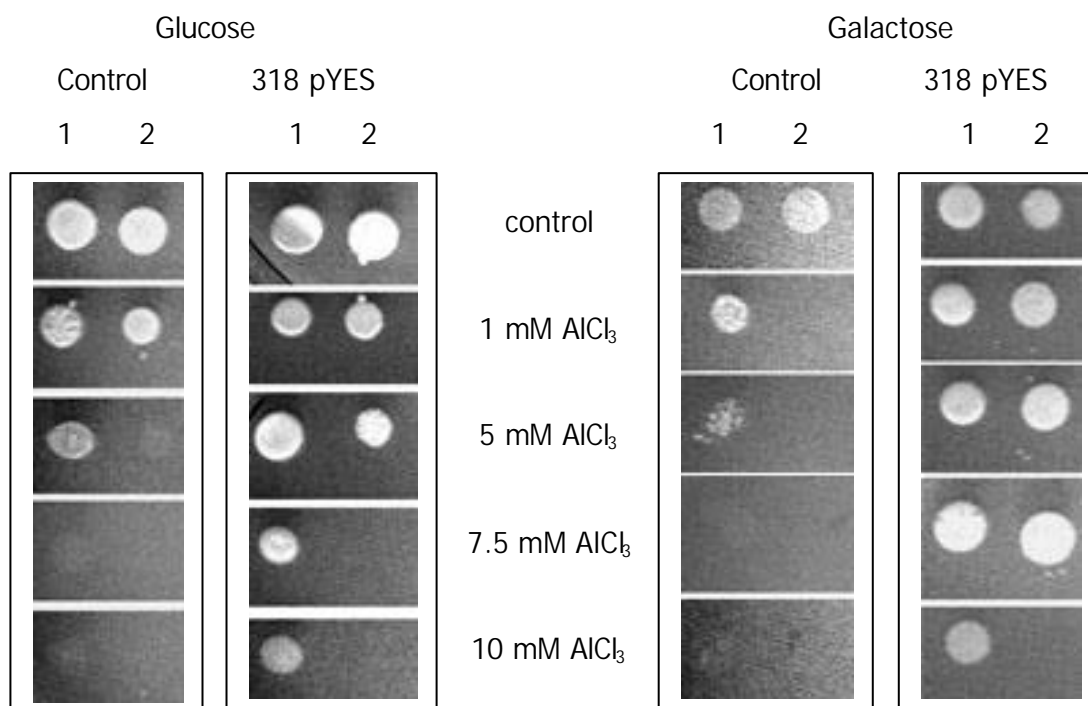


Fig. 30. Al resistance of yeast transformants over expressing the gene product encoded by 318 cDNA compared to wild type controls.

Exponential-phase cells were grown on LPM media containing glucose (non-inductive conditions) or galactose (inductive conditions) indicated as Glucose and Galactose, respectively. The cells were diluted in LPM medium and spotted on LPM agarose. The dilution ratios were: undiluted (1), 1:10 (2). The concentrations of aluminium chloride in the media reached from 1 to 10 mM as indicated.

Yeast test system was chosen since it allowed testing the effect of putative gene expression in eukaryotic cells using relatively less time and work for the experiment than propagation of transgenic plants. Transgenic yeast cells were grown on the media containing glucose or galactose supplemented with various concentrations of aluminium chloride at pH 4.0. Galactose was taken as promoter inductor. Glucose allowed “leaky” expression of 318 fragment so that effect of expressed product on Al-resistance is detectable but not in full scale as with the medium containing galactose.

On the media supplemented with galactose the difference between wild type and transgenic yeast appeared already at 1mM AlCl₃, being very dramatic at concentrations 7.5 and 10 mM. No yeast growth for wild type could be detected under such conditions, but quite intensive – for transgenics, undiluted and 1:10 diluted cells at 7.5 mM AlCl₃ and undiluted – at 10mM AlCl₃ (Fig. 30). The difference on the media containing glucose was not so profound, however, clearly detectable, which can be explained by the “leaky” expression mentioned above. At 5 mM aluminium chloride the cells diluted 1:10 were growing, which was not

observed for wild type control. On galactose containing media undiluted cells were able to grow on 7.5 and 10 mM AlCl_3 . Practically no detectable growth for wild type could be observed under the same conditions (Fig. 30). These results indicated that expression of 318 full protein coding fragment revealed enhanced resistance of transgenic yeast against aluminium stress in comparison to wild type controls, which created good basis for transgenic plant propagation.

4.6. Constructs for gene over expression in plants

In order to verify if the obtained cDNA fragments coding for the functional proteins improve plant response induced by aluminium stress, the constructs for over expression of the corresponding genes in transgenic plants have been created.

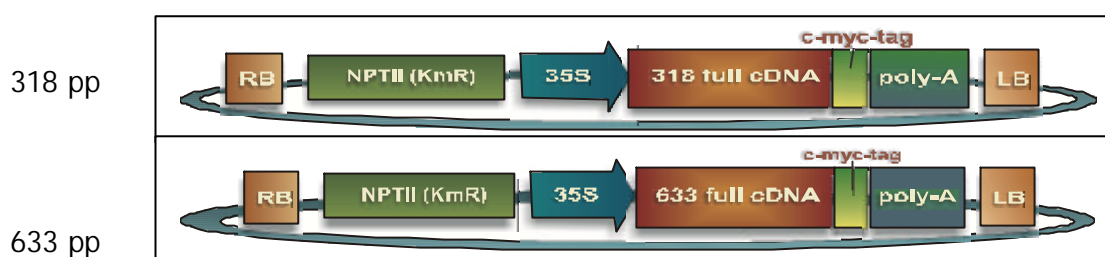


Fig. 31. The schemes of the constructs for over expression of selected Al stress up-regulated genes in transgenic plants.

318 pp and 633 pp – the constructs for plant transformation on the basis of pBIN19 plasmid vector under control of Cauliflower mosaic virus (indicated as 35S) with use of 318 and 633 structural genes, respectively; NPTII (Km^R) – the gene of kanamycin resistance for the transgenic plant selection; c-myc-tag – small peptide tag for detection of transgene expression on the protein level in plants; poly-A – poly-A part enhancing the gene expression efficiency; RB and LB – the border sequences necessary for construct insertion into plant genome.

The clones chosen for this approach were 633 and 318 full-length cDNAs. They were favoured with respect to evaluation of the expression level for corresponding genes (Fig. 18). The constructs were prepared as it is shown on Fig. 31. The expression cassettes were made under control of 35S promoter from Cauliflower mosaic virus to express the whole gene ubiquitous in the plant. Downstream from the putative structural gene poly-A part enhancing the gene expression efficiency and DNA coding for the reporter peptide from c-myc to enabling detection of gene expression on the protein level were introduced. NPTII gene was inserted to permit kanamycin selection of transgenic plants after *Agrobacterium* mediated gene transfer.

4.7. Plant transformation and detection of introduced genes in transgenic plants

The plant specie taken for the transformation was *Arabidopsis thaliana* cv Columbia. After *Agrobacterium*-mediated transformation of the foreign gene sequences in *Arabidopsis* (see *Materials and Methods*, chapter 2.2.11) kanamycin selection of transgenic plantlets was made. Plantlets of F_2 generation were verified to contain putative cDNA sequences. For this

purpose amplification was performed using the primers for appropriate cDNA and genomic DNA isolated from F_2 plants of transgenic lines. The DNA of the plasmid used for plant transformation served as a positive control. Amplification with wild type genomic DNA revealed no detectable bands, however, clear fragments could be observed in PCR with genomic DNA from all transgenic lines selected by kanamycin resistance. The sizes of these fragments corresponded to the sizes of the ones obtained in amplification with the plasmids applied for plant transformation (Fig. 32 and 33).

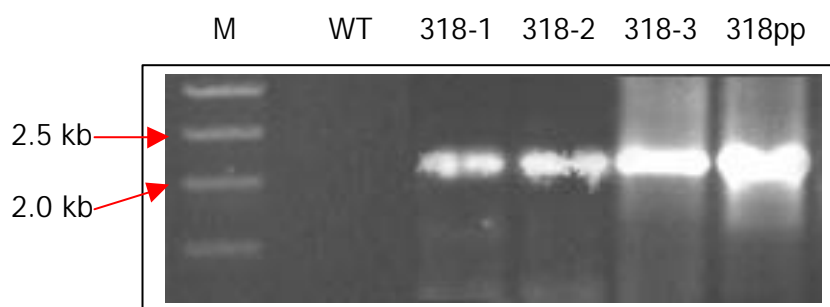


Fig. 32. Electrophoretic resolution of amplification products obtained using primers for 318 full-length cDNA and genomic DNA of transgenic plantlets.

150 ng of appropriate genomic DNA was taken for each PCR reaction. Amplification products were resolved on 1% agarose gel and visualised by ethidium bromide staining. M – mass marker, the sizes of selected fragments in kilobase pairs (kb) are indicated on the left side. WT – amplification with wild type genomic DNA as a negative control, 318-1, 318-2, 318-3 – amplification with genomic DNA of individual transgenic lines propagated as F_2 generation, 318pp – amplification with plasmid DNA of putative construct used for plant transformation as a positive control.

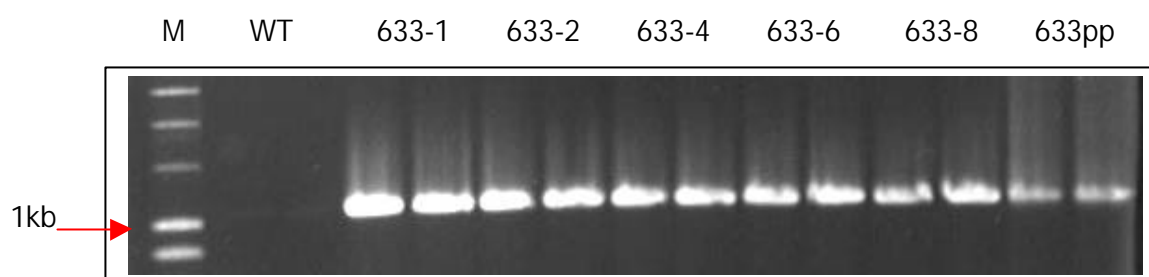


Fig. 33. Electrophoretic resolution of amplification products obtained using primers for 633 full-length cDNA and genomic DNA of transgenic plantlets.

150 ng of appropriate genomic DNA was taken for each PCR reaction. Amplification products were resolved on 1% agarose gel and visualised by ethidium bromide staining. M – mass marker, the size of the fragment in kilobase pairs (kb) corresponding to 633 cDNA is indicated on the left side. WT – amplification with wild type genomic DNA as a negative control, 633-1, 633-2, 633-4, 633-6, 633-8 – amplification with genomic DNA of individual transgenic lines propagated as F_2 generation, 633pp – amplification with plasmid DNA of putative construct used for plant transformation as positive control.

Amplification products were hybridized with appropriate cDNA fragments isolated from the constructs used for plant transformation radioactively labelled by ^{32}P -dCTP. Southern hybridizations gave clear signal with the samples from transgenic plants, which correlated with the signal from positive control (plasmid DNA of the construct used for plant transformation taken as an amplification template). No signal was detectable in wild type plants taken as a negative control (Fig. 34 and 35). The results reveal that the full protein-coding cDNA fragments used for plant transformation are really present in genomic DNA of plants from individual transgenic *Arabidopsis* lines. No hybridization signal could be detected with wild type genomic DNA.

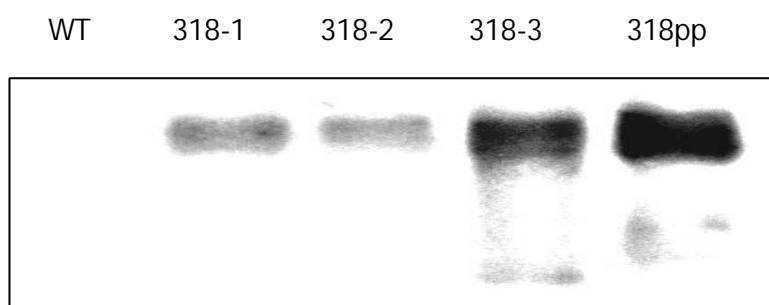


Fig. 34. Southern hybridization of the ^{32}P -dCTP labelled probe isolated from 318pp construct with the fragments obtained in amplification with genomic DNA using 318 cDNA fragment-derived primers (Fig. 32).

WT – wild type genomic DNA as a negative control, 318-1, 318-2, 318-3 – genomic DNA of individual transgenic lines propagated as F_2 generation, 318pp – plasmid DNA of putative construct used for plant transformation as positive control.

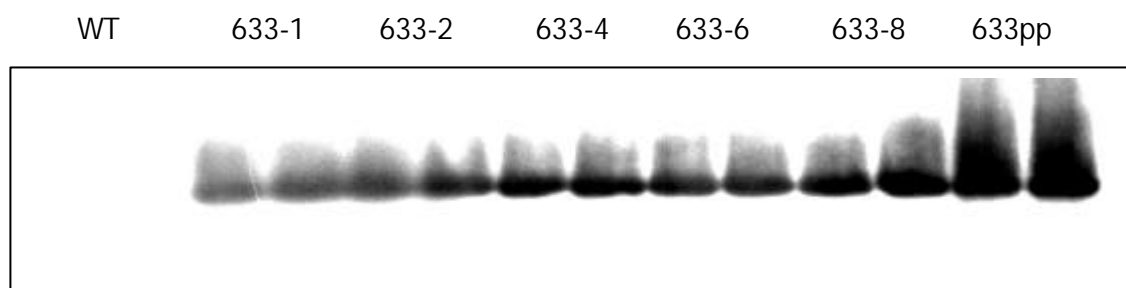


Fig. 35. Southern hybridization of ^{32}P -dCTP labelled probe isolated from 633pp construct with the fragments obtained in amplification with genomic DNA using 633 cDNA fragment-derived primers (Fig. 33).

WT – wild type genomic DNA as a negative control, 633-1, 633-2, 633-4, 633-6, 633-8 – genomic DNA of individual transgenic lines propagated as F_2 generation, 633pp – plasmid DNA of putative construct used for plant transformation as a positive control.

4.8. Characterization of transgenic plant stress response

In order to study the responsiveness of transgenic plants on Al stress, vitality and root morphology of wild type and transgenics were compared under conditions of different Al concentrations. Al penetration into roots of wild type and transgenic plants was determined after short (2h) Al treatment by using morin staining. Al toxicity in wild type and transgenics was compared by visualization of Al-elicited callose biosynthesis after longer (48h) Al stress using aniline blue staining of the roots.

4.8.1 Evaluation of transgenic plant vitality under conditions of aluminium stress

To prove Al sensitivity of *Arabidopsis* cv Columbia plants used for transformation, plantlets were treated with different aluminium concentrations such as 50, 75, 100, 200 and 300 μmol aluminium chloride in Hoagland solution, pH 4.0. One-week-old plantlets were treated for 48 h and then their survival ratio was calculated. Aluminium chloride restricted growth abilities of *Arabidopsis* plants already in concentration of 50 μmol and at 200 μmol aluminium chloride less than 10% *Arabidopsis* plantlets survived 48 h treatment (Table 3). At AlCl_3 concentration of 300 μmol practically all wild type *Arabidopsis* plants could not overcome 48 h of stress conditions. Moderate acidic stress (Hoagland solution, pH 4.0 without any aluminium) did not restrict the growth or vitality of *Arabidopsis* plants, so that the plant stress effects could be attributed directly to aluminium toxicity.

To compare Al-sensitivity of transgenic and wild type plants aluminium treatment was performed in the nutrient solution supplemented with 200 μmol AlCl_3 at pH4.0. Transgenic plants showed much higher tolerance against aluminium stress as wild type plants (Table 4). 83 to 91% of plants transformed by 318pp construct survived the treatment. The plants transformed by 633pp construct showed survival ratio of 80 (633-6 transgenic line) to 93% (633-1 transgenic line). The wild type controls had 10 times lower vitality ratio, such as 5.6 to 9% (Table 3 and 4) under identical conditions.

Table4. Aluminium responsiveness evaluation of wild type *Arabidopsis* cv Columbia plants.

Experimental conditions	AlCl_3 concentration, μmol	Total plant number	Dead plants	Survived plants	Percentage of survivors
Hoagland, pH 5.7	-	100	-	100	100
Hoagland, pH 4.0	-	100	-	100	100
Hoagland, pH 4.0	50	92	70	22	22
Hoagland, pH 4.0	75	105	81	24	20
Hoagland, pH 4.0	100	102	92	10	10
Hoagland, pH 4.0	200	105	96	9	9
Hoagland, pH 4.0	300	100	100	-	0

Table 5. Aluminium tolerance evaluation for transgenic *Arabidopsis* plants in comparison to wild type controls.

Sample	Total plant number	Dead plants	Survived plants	Percentage of survivors
WT con	100	--	100	100.0
633 con	250*	--	250	100.0
318 con	150*	--	150	100.0
WT, Al	106	100	6	5.6
633/1, Al	100	7	93	93.0
633/2, Al	100	12	88	88.0
633/4, Al	100	18	82	82.0
633/5, Al	100	11	89	89.0
633/6, Al	100	20	80	80.0
318/1, Al	100	17	83	83.0
318/2, Al	100	9	91	91.0
318/3, Al	100	11	89	89.0

The experiment was performed for 48h in Hoagland nutrient solution, pH 5.7 in all the samples marked as "con", and pH4.0 with 200 μ mol aluminium chloride in the samples marked as "Al".

* -- 50 plants were taken from each line for 318 and 633 controls. They are not extra indicated because all showed the same results.

Under the stress conditions transgenic plants showed also morphological difference in comparison to the wild type ones. During prolonged (48h) aluminium treatment their main roots did not grow as long as wild type ones, however, many secondary roots were formed, which was not observed for the wild type. Secondary roots had normal morphology and developed root hair system, which dramatically differed from the roots of wild type plants with very rare and small root hairs.

4.8.2. Investigation of aluminium penetration into the roots

In order to study aluminium penetration into the roots of wild type and transgenic plants morin staining was performed after aluminium treatment. Al treatments were performed in Hoagland solution at pH 4.0 supplemented by 200 μ mol AlCl_3 for 2 hours. After the treatment the roots were cut and immediately stained. Morin staining patterns of Al treated transgenic and wild type plants are presented on Fig. 36 and 37. The roots of wild type *Arabidopsis* plants were heavily stained indicating quite high value of aluminium penetration, whereas transgenic plant roots showed lower staining intensity and very different pattern of aluminium penetration.

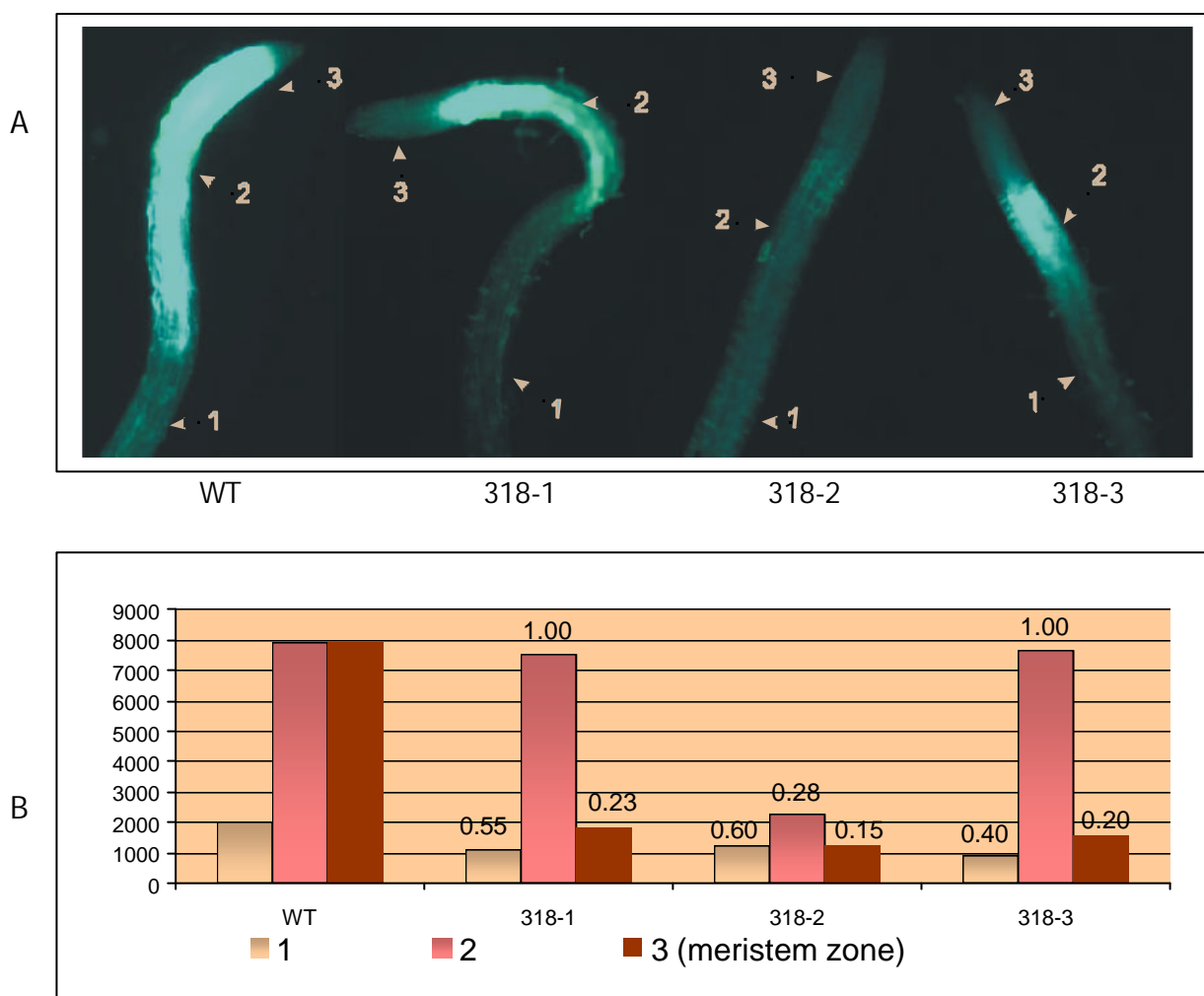


Fig. 36. Evaluation of morin fluorescence staining of roots from aluminium treated wild type (WT) and transgenic *Arabidopsis* plants transformed by 318pp construct (see Fig. 31).

A –Fluorescence patterns of wild type (WT) and transgenic plant roots of individual lines 318-1, 318-2 and 318-3. 5x magnification, DAPI-FITC-Rhodamine fluorescence filter. B – quantification of the fluorescence intensity in the zones indicated on A. The numbers on the graph show which part of wild type (WT) value constitutes the intensity of morin staining in appropriate zone.

Less significant difference was observed between transgenic lines, however, not such dramatic as with wild type controls (Fig. 36 and 37). Semi quantitative presentation of the results show that in the meristem zone of transgenic roots fluorescence intensity is up to 6.7 times lower than in wild type controls, which is the most dramatic difference. The other parts of the roots also show different aluminium concentration inside, but the difference is not such high (Fig. 36 and 37).

All three transgenic lines transformed by 318pp construct, 318-1, 318-2 and 318-3, had alike pattern of aluminium distribution in the roots, which is dramatically different from the wild type (Fig. 36). All the lines showed decreased intensity of morin staining in the meristem zone, which is 4.3 times lower as in the wild type for 318-1 line, 6.6 and 5 times – for the

lines 318-2 and 318-3, according to semi quantitative estimations (Fig 36). The best individual transgenic line with the lowest aluminium after 2h Al stress is 318-2.

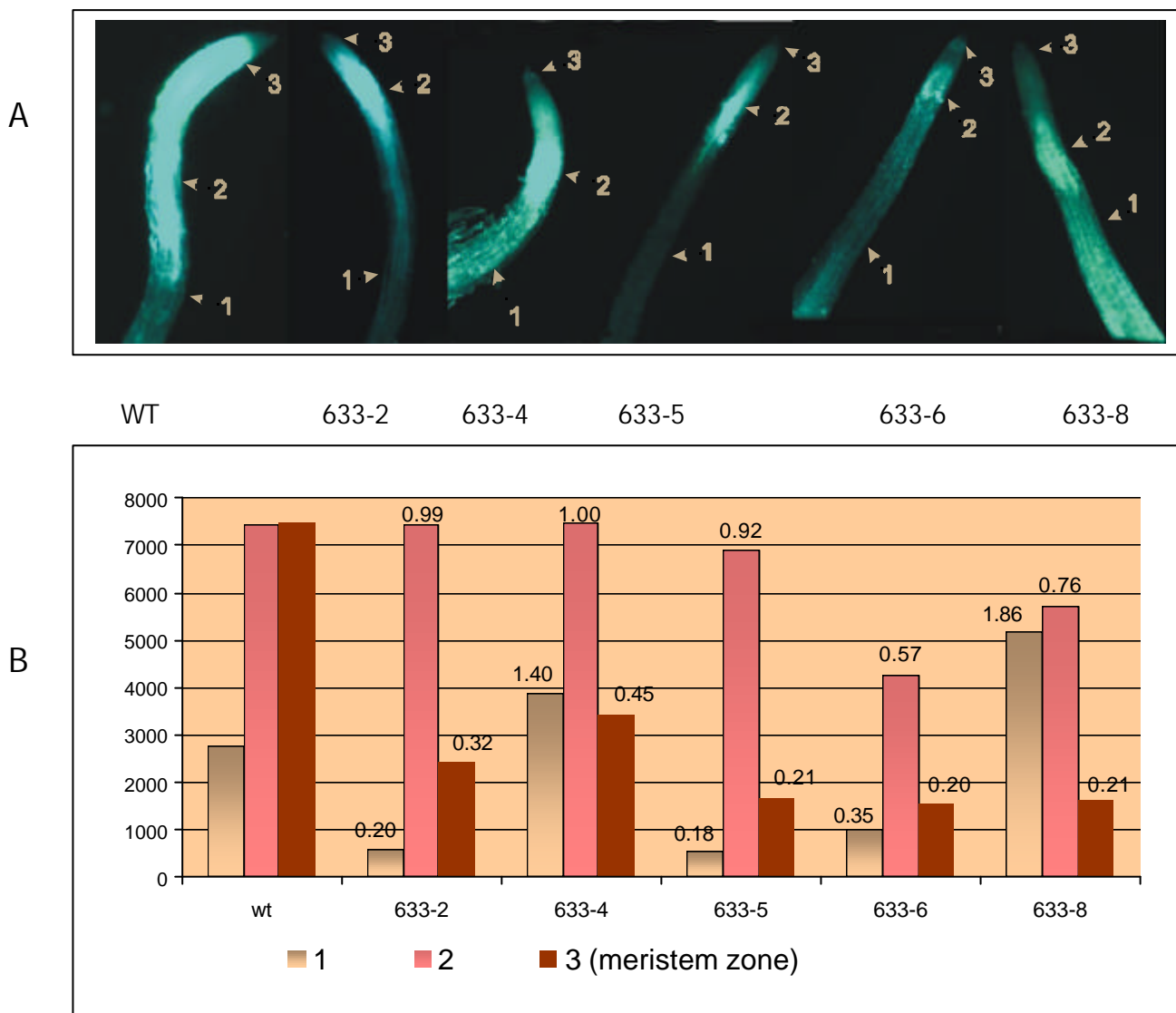


Fig. 37. Evaluation of morin fluorescence staining of roots from aluminium treated wild type (WT) and transgenic *Arabidopsis* plants transformed by 633pp construct.

A – fluorescence patterns of wild type (WT) and transgenic plant roots of individual lines 633-2, 633-4, 633-5, 633-6, 633-8. 5x magnification, DAPI-FITC-Rhodamine fluorescence filter. B – quantification of the fluorescence intensity in the zones shown on A. The numbers on the graph show which part of wild type (WT) value constitutes the intensity of morin staining in appropriate zone.

Aluminium distribution in the roots of transgenic lines containing 633pp construct was a bit different (Fig. 37), however, the main pattern was kept here also. The meristem zone contained dramatically less aluminium than wild type controls. When the intensity of fluorescence was quantified, the differences were estimated as 3 (633-2 transgenic line), 2 (633-4 transgenic line), and 5 times (633-5, 633-6 and 633-8 transgenic lines) less

aluminium-induced staining than in wild type controls. The best individual transgenic line with the lowest aluminium content after 2h Al stress is 633-6.

It should be added that 2h aluminium treatment performed before morin staining was too short to cause any difference in root morphology between wild type and transgenic plants, as it happened during prolonged stress conditions.

4.8.3. Characterization of callose biosynthesis in transgenic roots

One of the most sensitive indicators of aluminium toxicity in studied plants so far is stress-induced callose formation (SIVAGURU et al., 2000; ZHANG et al., 1995). In order to study how the roots of wild type and transgenic plants were influenced under conditions of aluminium stress, aniline blue staining was performed to visualise Al-induced callose biosynthesis. 7-day-old plantlets were subjected to 200 μ mol aluminium chloride in Hoagland solution at pH 4.0.

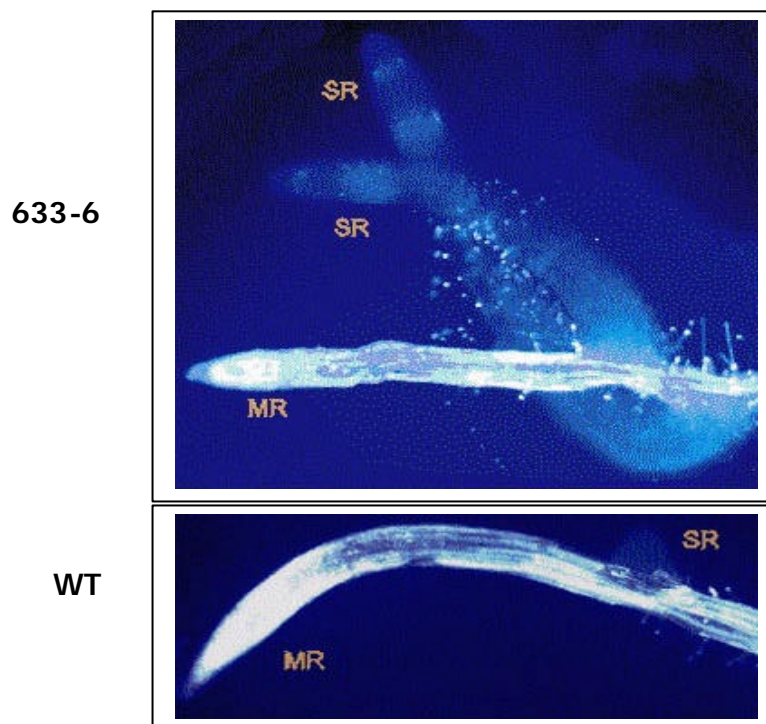


Fig. 38. Aniline blue staining of wild type (WT) and transgenic (the line 633-6) roots after 2 day aluminium treatment.

Al treatment has been performed in Hoagland solution, pH 4.0 with 200 μ mol aluminium chloride. Aniline blue fluorescence has been visualised using DAPI-FITC-Rhodamine fluorescence filter. MR – main roots, both damaged by aluminium stress. SR – secondary roots, very small and rare in dying wild type, long and vital in case of transgenic plant with dramatically lower callose content, indicating much lower degree of aluminium stress effect.

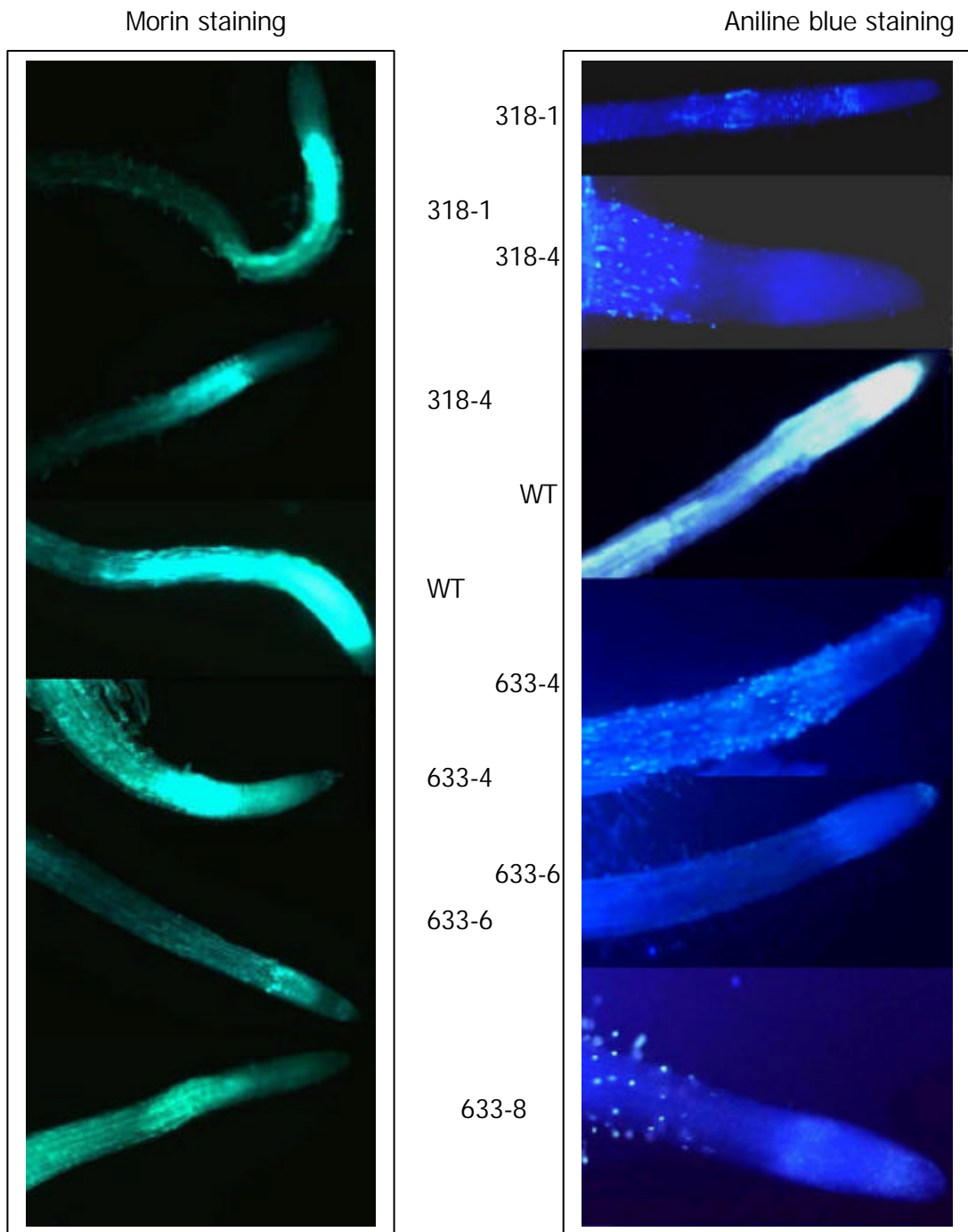


Fig. 39. Comparison of aluminium distribution and callose accumulation patterns in wild type and transgenic *Arabidopsis* lines after stress treatments.

Aluminium treatment was performed for 2h before morin staining and 48h before aniline blue staining in Hoagland solution at pH 4.0 supplemented with 200 μ M aluminium chloride. Main roots of transgenics were taken for morin staining and the secondary roots for aniline blue staining. The roots for morin staining were 2 days younger than the ones used for aniline blue staining, which explains the difference in the root thickness. WT – wild type control; 318-1, 318-4 – transgenic plants expressing ABC transporter homolog; 633-4, 633-6, 633-8 – transgenic plants expressing IMPDH homolog.

After the treatment the roots were cut, washed and immediately stained by aniline blue. The fluorescence pictures have been photographed using Zeiss fluorescence microscope AxioplanII with DAPI-FITC-Rhodamine fluorescence filter (see *Materials and Methods*, chapter 2.2.11 for the details). After 2 days of aluminium stress transgenic plantlets showed different morphology and different pattern of Al-induced callose formation in the roots compared to the wild type ones.

Such difference between wild type and 633-6 transgenic lines as an example is presented on the Fig. 38. The main roots in both cases, in transgenics and in wild type, show damaged structure. As it was mentioned above in the Chapter 3.7.2, transgenic plants had shorter main roots in comparison to the wild type controls but they form many long secondary roots with normal morphology, which was not observed for the wild type.

Aniline blue staining of secondary roots in transgenic plants was dramatically lower than in damaged main roots of wild type and all transgenic plant lines (Fig. 38). The formation of secondary roots in transgenic lines, not observed for wild type, and low degree of stress-induced morphological changes of this root type indicated much higher degree of stress resistance of the secondary roots in transgenic plants.

In order to obtain more integrated picture of stress behaviour, the patterns of morin and aniline blue staining were compared for wild type and selected individual lines of transgenic plants (Fig. 39). It could be concluded that in general aluminium distribution and callose formation patterns are alike despite the difference of aluminium treatment applied in both cases. Very intensive callose biosynthesis in wild type roots correlates with high degree of aluminium accumulation inside the roots. Dramatically lower callose content in secondary roots of transgenic plants is in agreement with much lower aluminium accumulation in the roots of transgenic lines (Fig. 36 and 37).

The root morphology and growth of transgenic plants under stress conditions show that they have dramatically better surviving potential under Al stress in comparison to the wild type ones. Formation of secondary roots under prolonged aluminium treatments; lower aluminium content after short Al stress and reduced stress-elicited callose formation inside secondary roots indicate that the studied genes imparted more aluminium resistance to the transgenic plants compared to the wild type.

Surprisingly, both genes gave alike result in terms of aluminium resistance being transformed into *Arabidopsis* plants. They showed similar survival rate under stress

conditions as compared to wild type controls, as well as similar pattern of aluminium distribution and stress-elicited callose formation.

5. Discussion

In the last years very intensive research has been invested in the understanding of molecular mechanisms of plant tolerance against stress including aluminium toxicity. Despite the fact that many genes have been found in different plants in response to Al, most of them are of generalized plant response to stress and not specifically involved in acquisition of Al stress tolerance (EZAKI et al., 2000).

5.1. Search for genes involved in soybean resistance against aluminium stress, a methodical approach

In order to find genes directly involved in the molecular mechanisms of plant resistance against Al it was necessary to directly compare gene expression patterns under stress and non-stress conditions in tolerant and sensitive soybean cultivars. There are several methods available for such purpose, for example subtractive hybridization (HARA et al., 1991), display of 3' end cDNA restriction fragments (PRASHAR and WEISSMAN, 1996) or DNA Arrays (KALLIONIEMI et al., 2001). The quickest, most visual and effective method is Differential Display RT-PCR (DD), described by LIANG et al. (1993). Using DD one can obtain resolution of amplified cDNA fragments representing RNA population of putative sample in any necessary point of time. It is possible to directly visualize and compare the gene expression patterns of different samples on the level of mRNA. In this work the aim was to study stressed and non-stressed soybean root tip cells of tolerant and sensitive cultivars by using DD approach.

The problem of the method is that the fragments obtained after DD contain several co migrating amplification products (JORGENSEN et al., 1999) so that it is necessary to find the right one among several fragments of the same size. After cloning of all PCR products contained in one differentially displayed band, it was necessary to verify a lot of clones in one blotting experiment to find the ones containing the fragments corresponding to the genes up regulated by aluminium stress. Use of Northern blotting in such situation would be extremely laborious and would take far more RNA than available. Therefore, Reverse Northern blotting (ZHANG et al., 1996) was used to prove if cloned DD products corresponded to genes up regulated during aluminium stress. Only the biggest one of the two fragments, verified by Reverse Northern blotting of size 212bp could be detected by Northern blotting, which is in agreement with the failure of DD fragments smaller than 200 bp to give visible signals on Northern blots as described by LIANG et al. (1993).

Another drawback of DD method is that amplified and visualized fragments correspond directly to 3'-end of RNA and range from 100 to approximately 500 base pairs in size. This feature hardly allow them to be used for any search for homologies in the gene banks as well as in cDNA library screening in order to get full protein coding cDNA fragments. It was not clear also if DD fragment or fragments identified after all cloning and blotting verification procedures corresponded to the genes involved in the mechanisms of plant resistance on the basis one fragment – one gene. As a basis for further work we accepted the hypothesis that one DD fragment could correspond to 3'-region of several mRNAs expressed in response to aluminium stress. To find the whole pool of mRNAs having 3'-part corresponding to putative DD fragment, the method of Marathon cDNA amplification (Marathon PCR) was chosen.

In this method double stranded replica of mRNA is ligated with adaptors to dramatically restrict unspecific amplification and enable search of putative cDNA fragments by PCR using the primers created on the basis of DD fragments. As a rule, a set of amplification experiments was giving several Marathon fragments (Fig. 12, 13 and 14). They were extracted and used in Reverse Northern blotting to verify which of them corresponded to the genes up regulated under the stress conditions, completing the next level of the search for the genes involved into aluminium tolerance.

The cDNA fragments obtained by Marathon PCR were much larger than after DD, however, not full protein coding ones. In order to complete them to the full length, cDNA library was created and screened by selected Marathon fragments used as probes. Final level of the gene search consisted of verification whether isolated full protein coding cDNAs corresponded to genes up regulated during aluminium stress with subsequent complete sequencing of selected cDNAs.

The whole procedure of gene search is summarized on Fig. 40. In the beginning DD pattern reflecting gene expression was obtained and analyzed. Short amplified cDNA fragments were extracted, cloned and sequenced. Marathon PCR was used to prolong them. cDNA bank was created with use of mRNA from Al treated root tips and *in vitro* cultivated cells of aluminium stress tolerant soybean cultivars. This bank was screened by selected Marathon fragments. Each step was followed by Reverse Northern verification and in some cases Northern verification, whether the genes corresponding to each obtained fragment were up regulated under stress conditions.

Our investigations were directed to specific response of soybean plants to aluminium stress. Therefore a range of aluminium chloride concentrations were applied to evaluate which

stress conditions were causing first stress responses, but not so heavy toxicity as to induce intensive expression of general stress genes. The concentration caused first detectable morphological changes such as swelling and changes of root meristem morphology compared to untreated control was 300 μ M AlCl₃ (Fig 1, A and B) but not heavy damage of root itself and root meristem as for higher concentrations (Fig1C). Therefore the concentration of 300 μ M AlCl₃ was taken as standard concentration for later aluminium treatments of roots.

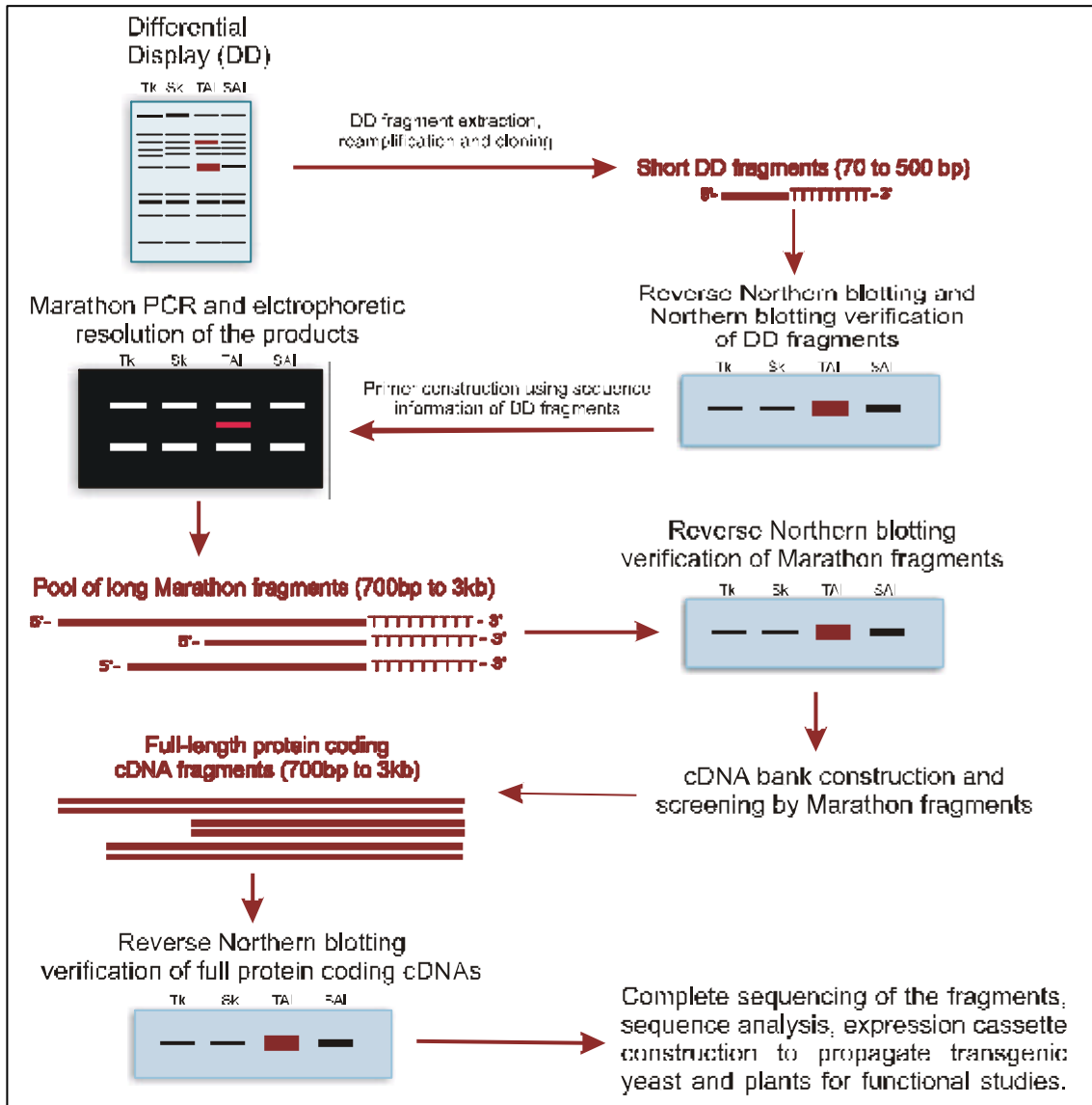


Fig. 40. Scheme of search for the genes responsible for Al resistance of plants.

The patterns of gene expression were compared in aluminium stress tolerant (Tk – for control, T Al – for aluminium treated samples) and the stress sensitive (Sk – for control, S Al – for aluminium treated samples) soybean cultivars. cDNA fragments up regulated in stress treated tolerant cultivars used in further work are shown in red.

One of the basic principles of selection for the Al responsible genes was also comparison of aluminium response between stress-tolerant and sensitive soybean cultivars. It was

considered that if some gene was involved in the general stress response, its expression should have been enhanced in both tolerant and susceptible cultivars under stress. In case of specific stress protection genes the higher expression level had to be observed in tolerant cultivars, as compared to the sensitive ones under stress where the specific genes shouldn't have been so much up regulated. That is why Northern blotting patterns as well as DD and Marathon PCR ones for different lines under stress and non-stress conditions were compared (Fig. 40).

5.2. Blotting experiments with known genes potentially involved in tolerance mechanisms

Northern blotting experiments were performed with 8 genes possibly involved in the mechanisms of plant resistance against aluminium toxicity according to the literature data (see Table 1). 7 of them did not show substantial expression difference on mRNA level between stress and non-stress conditions in tolerant as well as in sensitive cultivars (Fig. 2), which indicates that these genes probably are not involved in the mechanisms of Al stress tolerance in soybean. Therefore it can be concluded that calreticulin as the enzyme involved in the second messenger pathway, (KOCHIAN, 1995) and callose accumulation (SIVAGURU et al., 2000), glutathion-S-transferase as Al induced antioxidation enzyme (EZAKI et al., 1996), GTP-binding protein, Al-induced in tobacco (EZAKI et al., 1996) and different metallothioneins as well as malate dehydrogenase, which is involved in organic acid exudation as one of aluminium exclusion mechanisms, (SNOWDEN and GARDNER, 1993) were either not involved in the aluminium tolerance in soybean or their regulation by the stress was not realized on mRNA level.

One of studied genes, phosphoenolpyruvate carboxylase (PEPC), was up regulated in soybean root tips during aluminium stress (Fig. 3). The amount of PEPC mRNA was enhanced in root tips of both tolerant cv Tabora and sensitive cv Malabar, however, in tolerant cultivar the transcript level was 2 times higher. Higher expression of PEPC in tolerant plants is not necessarily indicating that it belongs to specific resistance genes, however, shows that it could play key role in organic acid metabolism of plants, which excrete malic (wheat, DELHAIZE et al., 1993, PELLET et al., 1996, *Arabidopsis*, LARSEN et al., 1998), citric (maize, PELLET, 1996 and *Cassia tora*, MA et al., 1997) or oxalic (MA et al, 1998, 1998a) acids to exclude aluminium from penetration in plant root. Also soybean belongs to plants exudating citric and malic acids in response to aluminium stress (YANG et al., 2000; SILVA et al., 2001).

PEPC is a key enzyme in organic acid metabolism of plants (LOPEZ-BUCIO et al., 2000). In mesophyll cells this enzyme catalyses phosphoenolpyruvate carboxylation, synthesising oxaloacetate, which is then transformed to malate or citrate (LOPEZ-BUCIO et al., 2000). PEPC is also proposed to play important role in cytosolic pH regulation by buffering OH⁻ formation during nitrate reduction. Moreover, in guard cells PEPC is implicated in generation of H⁺ via malate production (HAEUSLER et al., 1999). In C₃ plants and in non-photosynthetic tissues PEPC is active in several reactions to replenish the intermediates of Krebs cycle, provide carbon skeletons sustaining synthesis of amino acids during NH₄⁺ assimilation (SCHULLER et al., 1990). It was shown also that under conditions of iron deficiency PEPC activity was enhanced several times together with H⁺ extrusion linked to the activity of plasmalemma H⁺-ATPase and synthesis of organic acids such as malate and citrate (DE NISI and ZOCCHI, 2000).

PEPC, up regulated under aluminium stress conditions, could be involved in the processes of enhanced organic acid exudation, one of the mechanisms of plant protection (PELLET et al., 1995; MA et al., 1997; MA et al., 1998; LOPEZ-BUCIO, 2000). Other possibilities of PEPC role in aluminium protection could be maintaining of amino acid biosynthesis or some influence on H⁺-ATPase, which function is also damaged together with plasma membrane charge and function of membrane channels (KOCHIAN, 1995).

As an example of reaction pattern for a gene not involved in the process of plant defense against aluminium stress Wali 1 gene could be taken. Up regulated in wheat under aluminium stress conditions (SNOWDEN and GARDNER, 1993), it did not show ability to mediate tolerance against toxic aluminium in later studies using a biotechnological approach of Wali 1 over expression in transgenic plants (EZAKI et al., 1999). Marathon PCR with Wali1-derived primers revealed much higher amplification fragment intensity for cDNA sample from tolerant cultivar compared with non-treated control sample. No PCR results could be seen in amplification with cDNA from susceptible cultivar. Marathon PCR fragment was extracted and used in Reverse Northern hybridization. Wali1-like gene was expressed under conditions of aluminium stress about 6 times higher in tolerant soybean cultivar as compared to non-treated control. As much as 20-fold higher expression has been observed in susceptible cultivar treated in the same way (Fig. 6). Such expression pattern clearly shows that Wali1-like gene belongs to the pool of the genes generally up regulated by the stress, but not involved directly in the mechanisms of aluminium resistance. The highest transcript level was observed in sensitive cultivar under stress conditions with hardest toxicity effect.

5.3. Unknown genes up regulated by aluminium stress in soybean; DD and Marathon patterns, sequence analysis of full protein coding cDNA fragments

Expression patterns of tolerant soybean lines (Tambora and Willis) differed in general not too strongly from the ones of susceptible soybean lines (Malabar and Lumut), which can be explained by low aluminium concentration chosen for the stress experiments and, consequently, moderate stress effect. The majority of cDNA fragments showing different expression in stressed samples compared to controls were down regulated supporting the well-known phenomenon of general repression of gene activities under aluminium stress conditions (KOCHIAN, 1995).

During DD experiments we found that resolution pattern was highly reproducible and differed very slightly between controls and aluminium treated samples when the stressor concentration was 300 μ M. DD resolution pattern, however, in the samples treated by 1000 μ M AlCl₃ contained quite many down regulated bands (results not shown), which is in agreement with inhibition of DNA synthesis by aluminium stress (KOCHIAN, 1995).

Two cDNA fragments, TA52 and TA22, were chosen from 12 ones extracted after DD. Expression level of the gene corresponding to the TA52 fragment was quite low in both susceptible and tolerant controls (Fig. 7). The fragment signal intensity was enhanced already in the sample subjected to 4h Al stress as well as in the one after 24 and 48 h of stress coming to maximum at 48h of aluminium treatment in the case of tolerant cultivar but practically not different from the control in the sensitive one. In case of TA22 cDNA fragment the signal was virtually invisible on control DD patterns, however, appears on DD pattern of the tolerant cultivar after 4 h of aluminium treatment and its intensity is the highest after 48h stress treatment of tolerant cultivar. These data suggest that the genes corresponding to both DD fragments belong to the ones of relatively early Al response. The time range for the experiments was chosen according to SNOWDEN and GARDNER (1993). In this work all the genes up regulated by aluminium stress in wheat showed enhanced expression already after 4h of the stress, as well as the ones corresponding to DD fragments TA52 and TA22.

According to Reverse Northern data the expression of the gene corresponding to TA22 fragment was enhanced 7-fold (Fig. 9) and corresponding to TA52 DD fragment was enhanced 7.4-fold (Fig. 10) in tolerant cultivar under stress conditions. To obtain one more evidence for the up regulation of the genes corresponding to TA22 and TA52 DD fragments Northern hybridization experiment has been done for both of them. As it was mentioned above, in agreement with data described by LIANG et al. (1993) it was not possible to obtain

Northern blotting signal for the first of them, TA22, since its size was 71 bp only. The Northern blotting experiment with the other fragment, TA52 of size 201 bp was, however, successful. The gene corresponding to the TA52 fragment showed 7.0-fold enhancement of expression level in tolerant cultivar and 4.1-fold increase only – in the sensitive one under stress conditions as compared to control expression value. The expression level in sensitive soybean was 1.7-fold less up regulated than in tolerant one (Fig. 11), indicating that the gene may belong to the gene pool specifically involved in AI stress tolerance mechanism in soybean.

As it was predicted, Marathon PCR revealed several fragments for each starting one, which sequence was used for the primer design (Fig. 12, 13, 14). Among pool of the different amplification fragments 9 ones appeared only in reaction with cDNA from tolerant stressed cultivars, but not controls or sensitive cDNAs.

Reverse Northern Hybridization with Marathon amplification fragments have shown the enhanced level of corresponding gene expression for all Marathon fragments studied. The highest enhancement showed T5F3 and T2F2 fragments, with 234.0 and 20.0-fold increase of hybridization signal in comparison to the control sample

Not for all the candidates showing the best results in hybridization with controls, the same pattern of reaction could be found in comparison with sensitive soybean varieties. T5F3 and T2F2 showing the highest expression enhancement as compared to untreated controls were not so promising in this case, being only 2.2 and 1.8-fold up regulated, respectively, as compared with sensitive stressed cultivar values. The fragments T5pHF2 and T5pHF3 having increase of the gene expression only 10.7 and 10.0-fold as compared to control sample (Fig. 15) showed the highest up regulation (7.1 and 4.0-fold, respectively) as compared to the sensitive cultivar. That is why the last fragments were taken for cDNA bank screening.

For three full protein-coding clones obtained after the screening of cDNA library clear expression enhancement of corresponding genes has been shown in root tips under stress conditions (Fig. 18). 318 done 6.4-fold detected up-regulation of appropriate gene under stress conditions, the clones 58 and 633 – 1.6-fold and 26.8-fold up-regulation, respectively. After finishing of the gene search and verification procedure 3 cDNA sequences coding for functional proteins involved in AI stress protection in soybean corresponding to the clones 58, 318 and 633 were isolated.

5.3.1. 58 full-length cDNA clone; predicted protein sequence and potential function in AI resistance

Unlike the proteins coded by both other cDNA clones, the one coded by 58 full-length cDNA has many homologies with plant proteins. This polypeptide has very high homology with 8 Translationally Controlled Tumour Proteins (TCTP) from plants with average identity rate of 86.7% on the protein level. Maximal homology has been found with the protein from Hevea (*Hevea brasiliensis*), rice (*Oryza sativa*), pea (*Pisum sativum*) and medicago (*Medicago sativa*), being not lower than 85% with any of indicated plant proteins. This remarkable degree of protein identity indicates their highly conservative nature suggesting an important function in the plant cell.

Analysis of probable secondary structure as well as hydrophobic regions of the protein coded by the clone 58 shows that predicted protein has one relatively long hydrophobic region at C-terminal region. The rest of the protein has quite small hydrophobic regions. The predicted protein does not have many potential modification sites, for example only 5 phosphorylation sites. Potential TCTP signature motif 1 overlaps with myristoylation site also having one more site in near surrounding. Such topology of myristoylation sites indicates either probable membrane-associated region since they are located in N-terminal part of predicted polypeptide, or possible site of protein-protein interactions, in which TCTP signature 1 site can also play functional role. TCTP signature 2 overlaps with phosphorylation site, indicating potential regulation by protein kinases in this place. Other two phosphorylation sites are located near N-terminus of the protein. It is not known in the moment, however, if all potential phosphorylation or myristoylation sites are used in the protein *in vivo*.

TCTP proteins were first found in various lines of animal tumor cells as an untranslated mRNA protein particle unable to interact with the translational apparatus (YENOFSKY et al., 1983). TCTP appears to be a calcium-binding protein located in cytoplasm. Human and mouse TCTPs were shown to release histamine from human basophiles in the presence of immunoglobulin E (MAC DONALD et al., 1995). TCTPs or homologous proteins have been found in wide range of different organisms including human, mouse, rabbit, chicken, earthworm, yeast and a number of higher plants. Unlike in humans and mice, expression of TCTP-like proteins in plants is probably regulated at the transcriptional level. TCTP was induced by darkness in *Pharbitis nil* (SAGE-ONO et al., 1998) and regulated by light-stable phytochromes.

Expression of the gene coding for TCTP was identified in earthworm *Lumbricus rubellus* (STÜRZENBAUM et al., 1998) to be up regulated under conditions of heavy metal stress. The expression level was 4-fold higher under conditions of Pb/Zn/Cd pollution. In earthworms native to Cu-polluted mine up-regulation factor was as high as 335. The protein was under transcriptional control as in plants, but under translational one as it is typical for humans and animals. Probable mode of function is promotion of metal-histidine complex formation by earthworm TCTP working as a histamine-releasing factor (STÜRZENBAUM et al., 1998).

Since information on TCTP function in plants is very scarce, it is extremely difficult to suggest possible mode of function of soybean TCTP-like protein coded by 58 cDNA clone. Neither histamine releasing mechanism is not existent in plants, nor it is known, if Al can form complexes with histidine, like with copper. Therefore, it could be suggested that enzyme coded by 58 cDNA is involved in Ca homeostasis maintenance in stressed plant cells. The maintenance of essential Ca ion concentration in different cellular compartments should have an important role in minimization of the damage from exposure to nonessential enzymes, such as Al.

5.3.2. 633 full-length cDNA clone; predicted protein sequence and potential function in Al resistance

Analysis of the predicted protein sequence encoded by the clone 633 shows that it does not have hydrophobic domains, which can be surely detected. One of the main features of this predicted amino acid sequence is potentially very intensive myristoylation. There are 11 myristoylation sites, the great majority of which is located into the region of the overlap between inosine-monophosphate-dehydrogenase (IMPDH) functional domain located between amino acids 85 and 306 and FMN-binding domain between amino acids 117 and 238 (Fig. 28). Such topology of myristoylation sites hardly indicates probable membrane-associated region since they are located in C-terminal region of predicted polypeptide, and not in the N-terminal one, which is required for membrane association (THOMPSON and OKUYAMA, 2000).

The protein coded by 633 cDNA clone is homologous (average identity is 67%) to C-terminal part of inosine-5'-monophosphate dehydrogenases (IMPDH) from plants and animals. These enzymes catalyze the rate-limiting step in de novo biosynthesis of guanine nucleotides (HUBERMAN et al., 1995); have an essential role in providing necessary precursors for DNA and RNA biosynthesis and in signal transduction pathways mediating cell differentiation and transformation in humans and animals (JENKINS et al., 1993). Genomic and cDNA sequences

specific for IMPDH have been cloned from eukaryotes (HUBERMAN et al., 1995) and bacteria (TIEDEMAN and SMITH, 1985). Bacterial and eukaryotic forms are similar in size and show a high degree of amino acid sequence conservation. It has been suggested that inosine-5'-monophosphat oxidation by IMPDH might be predominant pathway leading to xantine and formation of ureides in vivo (ATKINS et al., 1985). Since this protein is relatively abundant (approximately 0.7% in cowpea nodules, ATKINS et al., 1985), it is likely that IMPDH play an important role in nitrogen fixation in some plants.

The protein coded by 633 cDNA is homologous only to the C-terminal part of IMPDHs, therefore it is not clear whether it possesses similar functions with these enzymes. Potential role of the gene corresponding to 633 cDNA fragment could involve enhancement of nucleic acid biosynthesis dramatically decreased as a part of aluminium toxicity syndrome (KOCHIAN, 1995). Another possible function could involve a role in GTP exchange (JAYARAM et al., 1999), important for the work of signal transduction pathways in stressed plant cells.

5.3.3. 318 full-length cDNA clone; predicted protein sequence and potential function in Al resistance

Analysis of predicted protein secondary structure for 318 cDNA shows that the protein could probably be membrane-bound or membrane-associated. The main protein features of predicted protein could be quite intensive phosphorylation and myristoylation, four α -helices, two ATP-GTP binding motifs and ABC-signature.

Two regions of probable intensive modifications were located in the regions around 206 and 520 amino acids, having in the centre ATP-GTP binding motif and nucleotide binding domain (P-loop), respectively. The both regions are near the border between hydrophilic and hydrophobic parts of the protein, which could be located on cytoplasmic site of membrane surface or soluble in the cytoplasm. Ploop was surrounded by probably phosphorylation sites, indicating probable regulatory activity inside the plant cell directed to this region.

The myristoylation sites were located in N- and C-terminal regions as well as in the middle of the protein near to ATP-GTP-binding site, also surrounded by potential phosphorylation sites. Such site pattern allows assuming that N-terminal and C-terminal regions of the protein may be integrated in or associated to the membrane. ABC-signature motif is flanked by phosphorylation sites, which indicates probable sites of functional regulation by protein kinases, as well as in the helix between amino acids 419 and 476, where 3 phosphorylation sites are located.

The most conservative regions with local homologies 70-90% overlap with the region of ABC-signature motif and P-loop together with ATP-GTP-binding site with flanking phosphorylation sites, indicating one more time the regions, which are most probably functionally active.

318 full-length cDNA fragment is homologous to the members of ABC transporter gene super family on the protein level. The members of this quite large and diverse gene group have ATP-GTP binding domains, so called "ATP-binding cassette", which is in good agreement with ATP-GTP-binding domain and P-loop found on predicted amino acid sequence for 318 cDNA clone. ABC transporters mediate ATP-dependent active transport processes through plasma membrane or tonoplast in plants (DAVIES and COLEMAN, 2000). These transporters have been predominantly found in plasma membrane catalyzing the efflux of various compounds out of the cell. Other ABC proteins are located in intracellular organelles such as peroxysomes, mitochondria, the endoplasmic reticulum and vacuoles; some of these proteins mediate the compartmentation of compounds into different organelles.

The ABC proteins can transport a remarkable variety of substrates including ions, carbohydrates, lipids, xenobiotics, antibiotics, pigment molecules and even large peptides. In addition, some ABC transporters can act as ion channels or regulators of ion channels, and some as membrane bound proteases (REA, 1999). These proteins are of major physiological importance because of their role in subcellular compartmentation of toxic compounds. Plant transporters have been found to be involved in herbicide detoxification, xenobiotic transport, and alleviation of oxidative damage (REA, 1999), which is especially interesting since aluminium toxicity causes lipid peroxidation of all membranes (RATH and BARZ, 2000).

The closest relative to the protein coded by 318 cDNA is GCN-20 transporter from yeast, which belongs to Cluster IV in ABC classification (DAVIES and COLEMAN, 2000). This family is characterized by having two ABC-type binding domains, but only weakly predicted membrane-spanning domains, if any, which correlates with two nucleotide binding domains on predicted amino acid sequence of 318 cDNA. Analysis of transmembrane domain existence revealed no such domains in 318 predicted polypeptide, which is also in agreement of this group features.

GCN-20 is yeast protein interacting with protein kinase GCN2, which catalyzes phosphorylation of α -subunit from translation initiation factor 2 (DE ALDANA et al., 1995). The gene corresponding to 318 clone is 42% identical to GCN20, especially in the middle part and C-terminus. The structure of both proteins as well as hydrophobicity profile as analyzed

with DNAMAN software show very strong similarities also. Walker A motif (short nucleotide binding motif, WALKER et al., 1982; HIGGINS et al., 1992), ABC-signature (HIGGINS et al., 1986) and shortly after – Walker B motif (HIGGINS et al., 1992) is maintained both in GCN20 and 318 proteins and considered to be typical for ABC transporters.

However, direct functional similarity between the protein coded by 318 cDNA and GCN20 is hardly existent. There is no any homology between two proteins in the region of first 117 amino acids in GCN20, which are absolutely critical for protein-protein interactions essential for the function of GCN20 (DE ALDANA et al., 1995).

The structure of the protein corresponding to 318 cDNA has quite lower similarities with MRP subfamily ABC transporters from plants (REA, 1999) as well as ABC transporters from *Aspergillus nidulans* (ANDRADE et al., 2000) and Sta1 mitochondrial transporter from *Arabidopsis thaliana* (KUSHNIR et al., 2001).

In the moment it is very difficult to predict, which function or which localization has the protein coded by 318 cDNA. Since there is hardly any transmembrane domain existent in the protein sequence, its function may not include direct role in any transport processes. Similarities with GCN20 suggest that it is soluble protein probably functioning in either channel activation or some process of translation regulation. It is worth noting that there are also two quite similar proteins coded by *Arabidopsis* genome, however, with no functional information available.

5.4 Over expression of 318 and 633 full protein coding cDNA fragments in transgenic yeast and plants

5.4.1. Over expression of 318 functional gene in transgenic yeast

Yeast test system was chosen since it allowed testing the effect of putative gene expression in eukaryotic cells using relatively less time and work for the experiment than propagation of transgenic plants. 318 full protein coding fragment was introduced into yeast cells (strain Inv Sc2) in expression plasmid under control of galactose activated P gal1 promoter (in cooperation with Prof. Dr. G. Kunze, IPK, Gatersleben, Fig. 29). Transgenic yeast cells were grown on the media containing glucose or galactose, as a carbon source supplemented with various concentrations of aluminium chloride at pH 4.0. Not being a promoter inductor, glucose still allowed “leaky” expression of 318 fragment so that effect of expressed product on Al-resistance was detectable but not in full scale as with the medium containing galactose.

The difference between wild type and transgenic yeast was clearly detectable for both glucose ("leaky" gene expression) and galactose (induced promoter activity) containing media. On the media supplemented with galactose the difference appeared already at 1mM AlCl_3 , where only undiluted control was growing, but 1:10 diluted transgenic yeast showed very good growth. At stressor concentrations 7.5 and 10 mM no yeast growth could be detected for wild type at all, but the normal one was observed for transgenics, both undiluted and 1:10 diluted cells at 7.5 mM AlCl_3 and undiluted – at 10mM AlCl_3 only (Fig. 30).

The only work on verification of whether the genes up regulated by aluminium really mediate aluminium tolerance of transgenic yeast was published by EZAKI et al., 1999. Virtually all the genes found to be up regulated by aluminium stress in different plants were transformed into yeast and the transformants were analysed in terms of aluminium resistance. Despite the same strain chosen for transformation, aluminium concentration differed dramatically between our work and putative work by EZAKI et al., 1999. In this publication 75 μM Al was enough to practically restrict the growth of wild type yeast. In our work wild type was growing even on the medium supplemented by 5mM AlCl_3 , which constitute as much as 1 mM Al. Another fact is that in the work by EZAKI et al. blue copper binding protein and GDP dissociation inhibitor genes enabled transgenics to survive 75 μM Al, which was 1.5-fold higher concentration than 50 μM Al, where wild type showed the same growth. In our experiments undiluted cells survived 10mM AlCl_3 or 2mM Al, which was 10 times higher as compared to 1mM AlCl_3 (0.2mM Al), where wild type control showed comparable growth.

These results indicate that over expression of 318 full protein coding fragment revealed enhanced resistance of transgenic yeast against aluminium stress in comparison to wild type controls. One can also conclude that probably the gene corresponding to 318 full cDNA is able to impart aluminium resistance on cellular basis since the transgenic yeast cells growing in the log phase have no cell differentiation or any highly organized structures.

5.4.2. Over expression of 318 and 633 functional genes in transgenic Arabidopsis plants; increased aluminium tolerance and influenced root morphology

In order to study if the genes up regulated by aluminium in soybean mediate Al tolerance, transgenic Arabidopsis plants have been created with use of 318 and 633 full protein coding cDNA fragments. 7 day old transgenic plantlets of F_2 generation showed much higher tolerance against aluminium stress as wild type plants (Table 4). 83 to 91% of plants transformed by 318pp construct survived the stress treatment. The plants transformed by 633pp construct showed survival ratio of 80 to 93%. The wild type controls had 10 times

lower vitality under identical culture conditions. Moderate acidic stress (pH 4.0, with no aluminium) did not restrict the growth or vitality of *Arabidopsis* plants, so that the plant stress effects could be attributed directly to aluminium toxicity. Under the stress conditions transgenic plants showed also phenotypic difference in comparison to the wild type ones. During long-term aluminium treatment their main roots did not grow as long as wild type ones, however, many secondary roots were formed, which was not observed for the wild type. Secondary roots had normal morphology and developed root hair system, which dramatically differed from the roots of wild type plants with very rare and small root hairs. Probably the roots form some adaptive secondary roots, which are able to survive enhanced aluminium concentrations and keep the plant alive.

5.4.2.1. Aluminium distribution in transgenic and wild type plants

After 2 hours of aluminium stress the roots of wild type *Arabidopsis* plants were heavily stained by morin indicating quite high value of aluminium penetration, whereas transgenic plant roots showed lower staining intensity and very different pattern of aluminium penetration. The three selected transgenic lines transformed by 318pp construct, 318-1, 318-2 and 318-3, had alike pattern of aluminium distribution in the roots, which is dramatically different from the wild type (Fig. 34). All the lines showed decreased intensity of Al content in the meristem zone, which was estimated to be 4.3 times lower as in the wild type for 318-1 line, 6.6 and 5 times – for the lines 318-2 and 318-3 (Fig 36). Meristem zone of the plant roots is the primary site of aluminium toxicity (RYAN et al., 1992; RYAN et al., 1995), therefore lower Al content in this zone is probably connected with enhanced Al resistance of transgenics. The other parts of the roots also show different aluminium concentration inside, but the difference is not such high. Aluminium distribution in the roots of transgenic lines containing 633pp construct was a bit different (Fig. 37), however, the main pattern was kept here also. The meristem zone contained dramatically less aluminium than wild type controls, morin fluorescence intensities were estimated as 2 to 5 times lower than in wild type controls.

Whether the proteins coded by 318 and 633 cDNA fragments directly prevent aluminium penetration into the meristem zone of transgenics or are involved in its chelation inside the cells, is unclear, however, less free aluminium in this zone should significantly diminish aluminium toxicity effect. Some tolerant plants also have lower aluminium content in roots as compared to the sensitive varieties (FOY et al., 1978; KOCHIAN, 1995; LAZOF et al., 1997), what was shown also with other methods (LAZOF et al., 1994, 1997). In which mechanisms the genes corresponding to 318 and 633 cDNA are involved is now not clear. These gene

functions could involve regulation of either channel activation or translation of certain proteins, GTP-synthesis and exchange or influencing protein synthesis machinery in general.

5.4.2.2. Biosynthesis of aluminium stress induced callose in transgenic plants

Callose is one of the most sensitive indicators of aluminium stress. Callose biosynthesis is initiated as soon as the Al signal is perceived by the cells (ZHANG et al., 1994; HORST, 1995; HORST et al., 1997), it is concentrated around plasmodesmata, affecting mineral and nutrient transport (SIVAGURU et al., 2000).

Prolonged aluminium stress (48h) causes damage in the structure of main roots from both transgenic and wild type plants. Transgenic plants had shorter main roots in comparison to the wild type controls but they formed many long secondary roots with normal morphology, which was not observed for the wild type. Callose accumulation as indicated by aniline blue staining of secondary roots in transgenic plants was dramatically lower than in damaged main roots of wild type and all transgenic plant lines (Fig. 38) indicating much lower degree of stress effect in the secondary roots of transgenics. Such different morphology gives advantage in surviving aluminium toxicity to the transgenics yielding more root surface for intensive water and nutrient uptake. As the pattern of callose accumulation shows, these secondary roots have dramatically higher capacities to overcome aluminium stress conditions.

In general aluminium distribution and callose formation patterns were very much alike. Very intensive callose formation in wild type roots correlated with high degree of intracellular soluble aluminium inside the roots. Substantially lower callose in secondary roots of transgenic plants is in agreement with much lower aluminium accumulation in the roots of transgenic lines (Fig. 36 and 37).

It is difficult to classify now probable functions of the genes corresponding to 318 and 633 cDNA fragments, as well as predict if they belong to symplastic or apoplastic types of stress response. Correlation of aluminium content and callose biosynthesis in transgenic roots can indicate the exclusion mechanism of the gene function. However, since morin reacts only with soluble, phytotoxic aluminium (LARSEN et al., 1996), lower Al content in the roots can also be a result of internal chelation (POLAK et al., 2001) inside the root cells leading to the transfer into some insoluble form invisible in terms of morin staining. The studied genes could in this case be involved in symplastic response machinery.

Enhanced aluminium resistance of transgenic yeast shows that the gene corresponding to 318 cDNA can mediate the tolerance on the cellular level. The morphological differences of transgenic roots as compared to wild type controls show, however, that the action of both genes is not limited on the cellular level only, but could be observed on tissue level also. Surprisingly, both genes showed similar pattern of stress tolerance mediation. Whether these genes are involved in the same mechanisms of resistance and whether such mechanisms belong to symplastic or apoplastic type of tolerance could not be detected by the methods used in this work and should be studied further.

Recently it was found that signal grass (*Brachiaria decumbens* Stapf cv Basilisk) had outstanding aluminium tolerance, which was not associated with external chelation of aluminium despite some exudation of organic acids, which was, however, much less than enough to enable such a high degree of resistance. Such tolerance was attributed to the internal mechanisms, such as active Al extrusion from symplasm or high symplastic resistance level (WENZL et al., 2001).

These findings together with the results on aluminium tolerance of transgenic *Arabidopsis* plants in this work show that symplastic tolerance mechanisms could also play a role of plant resistance in soybean against aluminium stress along with organic acid exudation (SILVA et al., 2001) or other apoplastic responses.

6. References

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Erklärung

Ich erkläre hiermit, dass ich mich mit der vorliegenden wissenschaftlichen Arbeit erstmals um die Erlangung des Doktorgrades bewerbe, die Arbeit selbständig und ohne fremde Hilfe verfasst, nur die von mir angegebenen Quellen und Hilfsmittel benutzt und die den benutzten Werken wörtlich oder inhaltlich entnommenen Stellen als solche kenntlich gemacht habe.

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