J. Plant Biotechnology *Vol. 1. pp.61~68*

Journal of Plant Biotechnology

Transgenic Strategy to Improve Stress Resistance of Crop Plants

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Key words : alfalfa, tobacco, ferritin, NADPH-dependent aldose/aldehyde reductase

Abstract

Rapid accumulation of reactive oxygen species (ROS) and their toxic reaction products with lipids and proteins significantly contributes to the damage of crop plants under biotic and abiotic stresses. We have identified several stress activated alfalfa genes, including the gene of the alfalfa ferritin and a novel NADPH-dependent aldose/aldehyde reductase enzyme. Transgenic tobacco plants that synthesize alfalfa ferritin in vegetative tissues-either in its processed form in chloroplast or in the cytoplasmic non-processed form-retained photosynthetic function upon free radical toxicity generated by paraquat treatment and exhibited tolerance to necrotic damage caused by viral and fungal infections. We propose that by sequestering intracellular iron involved in generation of the very reactive hydroxyl radicals through a Fenton reaction, ferritin protects plant cells from oxidative damage. Our preliminary results with the other stress-inducable alfalfa gene (a NADPH-dependent aldo-keto reductase) indicate, that the encoded enzyme may play role in the stress response of the plant cells. These studies reveal new pathways in plants that can contribute to the increased stress resistance with a potential use in crop improvement.

EMBLNEW Accession Number: X97059

Introduction

Under abiotic and biotic stress conditions rapid accumulation of reactive oxygen species (ROS) and their toxic reaction products significantly contributes to the damage of crop species [9, 11, 14, 30]. Production of the most harmful ROS, the hydroxyl radical (OH*), depends on the presence of free iron in the living cells [13]. Hydrogen peroxide (H_2O_2) can undergo the Fenton reaction, which gives rise to hydroxyl radicals in the presence of free Fe^{2+}. The iron-mediated Fenton oxidants can cause damage to all classes of biologically important macromolecules [15]. Because intracellular iron catalyzes oxidative reactions, the control of the concentration of free iron could be a potential way to reduce oxidative damage. Intracellularly most of the nonmetabolic iron is sequestered in ferritin. This iron-storage protein is widely distributed in living organisms from bacteria to mammals [32]. The plant ferritins, which show significant amino acid sequence homology and structural similarity to their mammalian counterparts are preferentially localized in the chloroplast, their synthesis is transcriptionally controlled in response to iron and influenced by the plant stress hormone, abscisic acid [23]. Under normal growth conditions, the amount of ferritin is low in vegetative organs, it accumulates in seeds during embryo maturation. Involvement of ferritin in the oxidative stress response is supported by experiments with mammalian cells that show stimulation of ferritin synthesis during oxidative damage [5, 7, 36].

The postulated role of the alfalfa ferritin in the cellular defence mechanisms during oxidative stress en-

couraged us to assess the protection afforded transgenic tobacco plants expressing the this gene after oxidative stress.

Materials and Methods

Cloning of an alfalfa ferritin cDNA. RNA prepared from *Medicag sativa* L. RA3 cell culture (treated with 10 mg/l 2,4-D for embryo induction) was used to construct a library in ZAP II vectors (Stratagene). This and another cDNA library made from *Medicago sativa* L. RA3 somatic embryos (kindly provided by H. Hirt, Vienna) were used in the experiments. Based on Northern hybridization data we have identified several stress induced clones showing elevated level of expression in somatic embryos as well. The selected clones were sequenced by the dideoxynucleotide-chain-termination method as double stranded DNA using Sequenase kit (USB). The sequence of *MsFer* was submitted to the EMBL databank under the accession number: X 97 059.

RNA Isolation and Gel Blot Analysis.

Total RNA was extracted from control and stress-treated alfalfa A2 cell suspension and from leaves of control SR1 and transformant tobacco plants according the method of Maes [25]. For RNA gel blot analysis, samples of 20 μg total RNA were electrophoresed in a 1.2% agarose-formaldehyde gel and transferred to a nylon filter (Hybond-N, Amersham). To estimate whether equal amounts were loaded, the RNA was visualized on the filter by ethidium bromide staining. Hybridizations were carried out at 65℃ in Rapid-hyb hybridization buffer (Amersham) using 32P-labelled coding region of the *MsFer* cDNA.

Preparation of ferritin antibodies and Western blotting.

A portion of the MsFer cDNA (without the transit peptide) was amplified with upper 5'-*Eco*RI primer: GAA TTC ATG TTA ACA GGT GTT ATC; lower T7 primer: TAA TAC GAC TCA CTA TAG GGC oligonucleotide primers and cloned into pGEX 4T-1 bacterial expression vector (Pharmacia). Recombinant GST-MsFer fusion protein was purified on glutathione-Sepharose 4B columns according to the manufacture's recommendations. 50μg aliquots of the fusion protein were used for the immunization of BALB/C mice. For detection of ferritin with anti-ferritin antibodies, proteins were isolated by the method described by Nechustai and Nelson [26]. In Western hybridization experiments rabbit anti-mouse IgG peroxidase conjugate (A 9044, Sigma) was used as secondary antibody. Plastids from leaves of transgenic and control SR1 plants were isolated using continuous non-linear Percoll gradient according to the method of Journet [17]. 10μg extract run on SDS-PAGE gel, transferred onto nitrocellulose membrane and probed with FLAG antibody according to the company protocol (IBI, Kodak).The antigen-antibody complex was detected using Super Signal CL-HRP Substrate System (Pierce), according to the manufacturer's recommendation.

Construction of Ti vectors for overexpression of ferritin in tobacco plants.

Rok 2 Ti vector carrying the CaMV 35S promoter and Rok 8 Ti vector with the Rubisco promoter was kindly provided by Anthony Kavanagh (Trinity College, Dublin). A full length cDNA of *MsFer* identified in the library screen was cloned into the BamHI-KpnI site of Rok 8. To generate FLAG tagged protein the cDNA was cloned into the EcoRI-KpnI site of pFLAG-ATS vector (Scientific Imaging System Kodak) by the PCR method and a BamHI-KpnI fragment was inserted into the Rok 2 vector. The plasmid constructs were introduced into *Agrobacterium tumefaciens* EHA105 (kindly provided by MOGEN, Leiden, The Netherlands) by three-parental mating. Tobacco plants (*Nicotiana tabacum* v. Petit Havanna line SR1) were infected and co-cultivated with the *Agrobacterium* suspension and kanamycin resistant plants were regenerated according to Horsch et al. [16]. Expression of the *MsFer* gene in the transgenic tobacco plants was analysed by Northern and Western hybridizations.

Photosynthetic

paramcters. Measurement of chlorophyll *a* fluorescence emitted by green plants is a very efficient, non-invasive tool to assess photosynthetic activity. In dark adapted leaves when the components of the photosynthetic electron transport chain, in particular the Photosystem II complex, are all open to utilize the absorbed light energy in driving photosynthesis, the fluorescence yield is min-

imal (F_o). After saturating photosynthesis by strong illumination the fluorescence yield increases to the maximal level (F_m), which represents a closed state of the electron transport chain in which a high proportion of the absorbed light energy is lost to fluorescence and heat dissipation. The fluorescence parameter $F_v/F_m = (F_m-F_o)/F_m$ represents the maximal yield of the photochemical reaction in Photosystem II, and indicates the efficiency of the whole photosynthetic process. Light-induced changes in the chlorophyll fluorescence yield were measured as described by Vass et al. [35] using a PAM fluorimeter (Walz). For data acquisition and analysis, the FIP software of Q_A data (Turku, Finland) was used. The chlorophyll content of the leaves was estimated according to Arnon [1].

Plants inoculation with TNV.

Seeds of non-transformed tobacco (*Nicotiana tabacum* L. cv. Petit Havana SR1 line) and genetically transformed tobaccos carrying the ferritin gene were sown in soil and grown under normal greenhouse conditions (18-23℃; supplementary light: 160 mE m^{-2} s^{-1} for 8 h per d; relative humidity: 75-80%). For each experiment 50-60 day old plants were used.

The third and fourth true leaves (3rd and 4th leaf position above hypocotyl) of tobacco plants were inoculated with a suspension of TNV. The virus was maintained in bean (*Phaseolus vulgaris* cv. Red Kidney) plants. Leaves of plants showing typical disease symptoms of TNV were ground (1 g in 10 ml 10 mM Na-phosphate buffer, pH 7. 0) in a mortar and the homogenate was used for inoculation of tobacco leaves.

Altenaria alternata 1-B-1 isolate, kindly supplied by Professor K. Kohmoto (Tottori University, Japan), and *Botrytis cinerea* „Tokaj" isolate were grown for 14 days on Czapek or potato dextrose agar media, respectively. Discs 5 mm in diameter were cut from 14-day-old cultures of the fungus and placed on the surface of tobacco leaves. Leaves were cut and put on wet filter paper in a Petri dish and held at 24℃ in continuous light. Symptoms were evaluated by determining the diameter of necrotized leaf area.

Results

Overproduction of ferritin promotes paraquat and iron resistance in transgenic plants.

The deduced protein sequence of the full-length alfalfa ferritin gene (*MsFer*, EMBLNEW accession number: X97059) comprises 251 amino acids and shares sequence homology with ferritins of different origins; 39-49% identity with the human H and the horse L ferritin and 89% identity with pea ferritin [22]. The first 53 amino acids in the alfalfa ferritin correspond to a chloroplast transit peptide, in this region the pea and alfalfa proteins share only

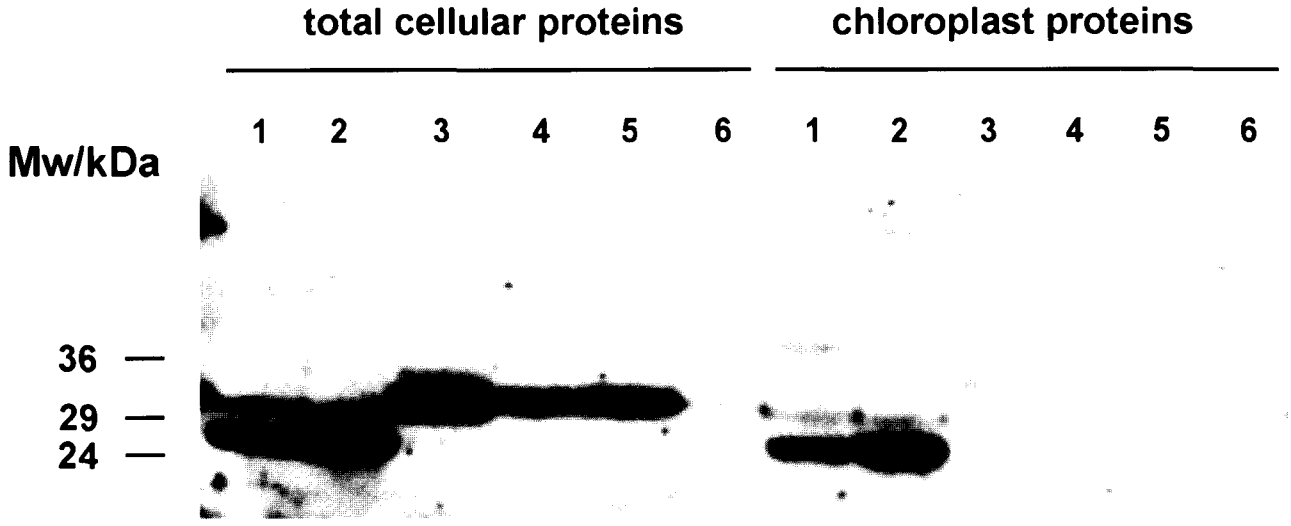

Figure 1. Transgenic tobacco lines accumulate alfalfa ferritin in different intracellular compartments. Western blot analysis of total cellular protein extracts and proteins isolated from isolated chloroplast of different transgenic tobacco lines overexpressing the *MsFer* gene. Lane 1-2: Rok 8 Rubisco promoter constructs RubF2 and RubF3, lane 3-5: Rok 2 CaMV 35S promoter-FLAG constructs CaMVF4, CaMVF5 and CaMVF9. lane 6: SR1 control.

47% of amino acid identity.

The full-length *MsFer* cDNA was cloned into an *Agrobacterium* transformation vector with either the viral CaMV 35S or Rubisco small subunit gene promoter and at least 10 independent transformants were identified and selfed. Northern blot analysis of kanamycin-resistant R1 progeny showed that transformants from both promoter combinations accumulated significant amounts of ferritin mRNA in their leaves and stems (data not shown). Subcellular location of the alfalfa ferritin in transgenic tobacco plants was analysed using antibodies raised against the glutathione-S-transferase-ferritin fusion protein. These antibodies recognize the chloroplast localized processed form of ferritin lacking the leader sequence that exhibits 22.5 kDa molecular mass in transformants carrying the Rubisco vector construct (Figure 1). The FLAG-tagged alfalfa ferritin cDNA was expressed by the CaMV 35S promoter. Because of the effect of the charged FLAG motif on the chloroplast transport of the tagged ferritin, in these transformants the nonprocessed 30 kDa ferritin was detected by the antiferritin antibodies only in the total cellular extracts (Figure 1). These analyses demonstrate the ectopic synthesis of the mature ferritin in the chloroplasts and accumulation of a precursor form in the transformed tobacco plants.

Transgenic tobacco plants producing the alfalfa ferritin were tested for the resistance against oxidative damage by applying the herbicide paraquat (Pq). The electrons produced during the photosynthetic electron transport reduce the Pq, and free radicals are formed [2]. Cells containing high intracellular level of superoxide dismutase are more resistant to Pq, supporting the idea that the Pq is an inducer of oxidative stress [29]. Total leaves from control and transformed plants were exposed to 10μM Pq. Functional damage was monitored by measuring continously the light-induced chlorophyll fluorescence yield changes as described in the Materials and Methods. Figure 2 displays the Fv/Fm values representing the maximal yield of photochemistry in the photosynthetic process during paraquat treatment. Transformed lines exhibited considerable tolerance to the damage caused by paraquat treatment, while the control SR1 leaves completely lost their photosynthetic function. Similar results were obtained using extreme high dose of iron (500μM Fe(III)-EDTA) in leaf disc experiments [8].

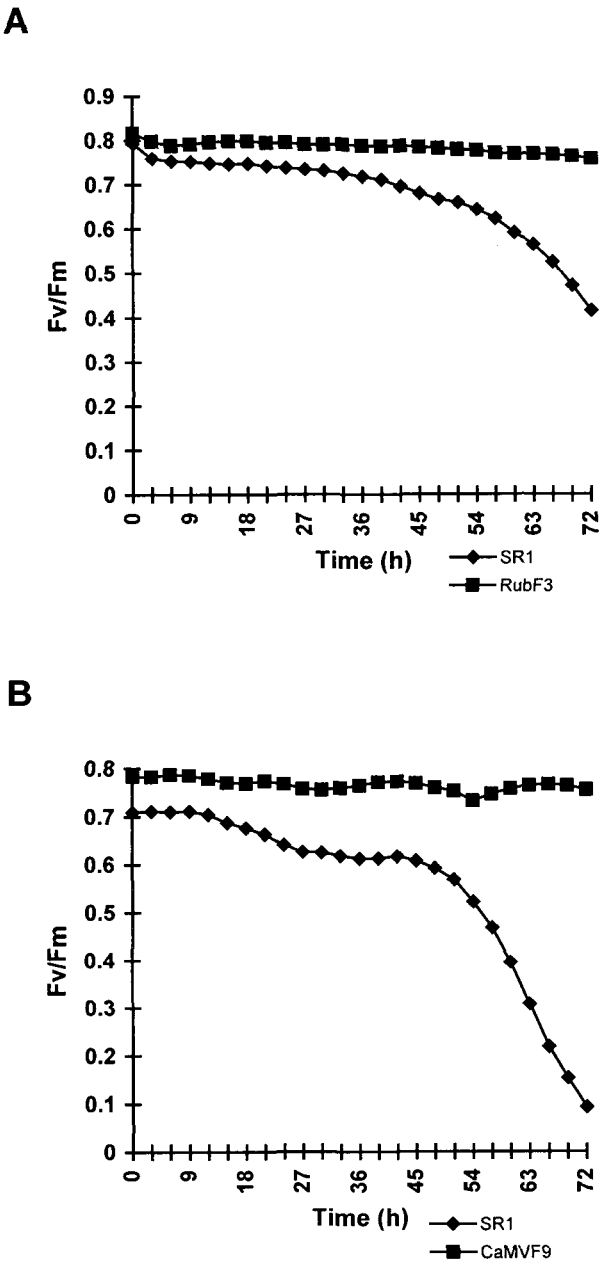

Figure 2. Light-activated fluorescence in transformed and control (SR1) tobacco plants during paraquat treatment. Light induced fluorescence was measured on 10 M paraquat-treated whole leaves of wild type and transgenic tobacco plants (**A**: SR1 and Rubisco promoter construct RubF3, **B**: SR1 and CaMV 35S-FLAG construct CaMVF9).

Reduced symptoms after infection with diffent necrotrophic pathogens in ferritin producing tobacco transformants.

ROS are produced by plants upon recognition of invading pathogens and have been suggested to be involved in signal transduction [24], limitation of pathogen ingress [18, 27, 30], and induction of plant tissue necrotization [9, 10, 19, 31]. Thus, elevation of antioxidant capacity of plants should increase their tolerance to cell and tissue necrosis caused by pathogens. Given the key role of iron in generation of damaging ROS, we have tested whether the previously demonstrated antioxidant effect of ferritin can exert a protective function against plant pathogens that cause necrotic symptoms. Seven-week-old control (SR1) and transgenic tobacco plants grown in the greenhouse were inoculated with tobacco necrosis virus (TNV). The number of necrotic lesions was significantly reduced in transformants expressing the alfalfa ferritin cDNA under the control of both promoters regardless of the cellular localization of the ectopically synthesized protein (Figure 3). All transformed plants exhibited increased tolerance to necrotization caused by *Alternaria alternata and Botrytis cinerea* as well [35].

Transgenic plants overproducing ferritin and grown in soil under greenhouse conditions did not show any visible alteration in morphology or growth rate. As iron is an essential component of photosynthetic pigments, it is important that the fluorescence studies did not indicate any detrimental effect on photosynthetic function (Figure 2).

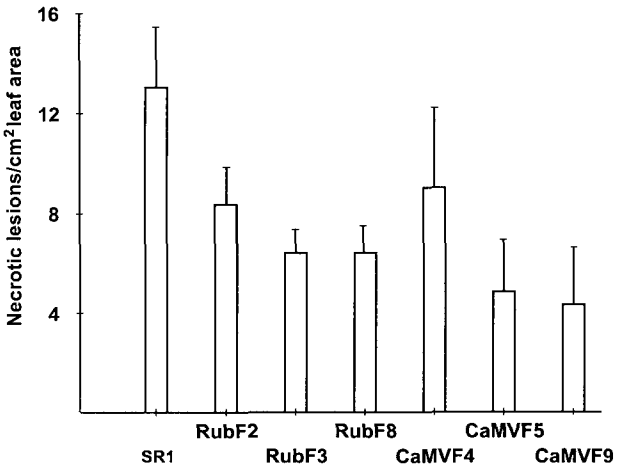

Figure 3. Necrotization by tobacco necrosis virus (TNV) infection is reduced in leaves of transgenic tobacco plants overproducing ferritin. The number of lesions was counted 7 days after virus inoculation. Data show a representative example from three independent experiments of similar results with eight replicates on four plants from each transgenic and control tobacco line.

Discussion

Our results demonstrate that ectopic synthesis of an iron-binding protein and storage protein, ferritin, in vegetative tissues increases the tolerance of tobacco plants toward both abiotic and biotic agents that are expected to cause damage by the production of ROS, particularly OH* through the Fenton reaction: $Fe^{2+} + H_2O_2\ Fe^{3+} + OH^- + OH^*$. Because ROS are produced by the herbicide Pq, excess iron and as a result of plant-pathogen interactions, we challanged the tobacco transformants synthesizing ferritin with paraquat, iron and necrotrophic pathogens. Transformed plants show Pq and iron resistance and reduced necrosis after viral or fungal infections. The protective function of ferritin against oxidative stress may result from an increased iron sequestering capacity in cells of the transformants. Although further work is needed to identify the molecular mechanisms responsible for the observed suppression of tissue necrotization, our results are in accordance with those of Zer et al. [37], who demonstrated that desferroxamine, the highly specific ironchelator can reduce Pq toxicity in pea. Recent results of Van Wuytswinkel et al. that both cytoplasm and plastid targeted ferritin can decrease the paraquat dependent reactive aldehyde formation in transgenic tobacco also supported our results [33].

Ferritin-overproducing transformants also provided a new way to test the potential role of iron-activated ROS in pathogenesis. ROS production shows a characteristic profile during plant-pathogen interaction [3]. Since the oxidative burst is expected to be a key component during necrotization, we challenged the transformants with necrotrophic pathogens and demonstrated that transformed plants were more resistant than wild type. Further experiments are in progress to clarify, whether these phenomena are due to reduced formation of ROS, enhanced oxidative capacity in the transgenic plants, or both.

Based on the detection of alfalfa ferritin in total cellular and in purified chloroplast protein extracts of transgenic tobacco leaves, we can conclude, that in transformants carrying the Rubisco vector constructs the

alfalfa ferritin was properly processed, and after elimination of the leader sequence this protein is accumulated in the chloroplast. In contrast, the transformants carrying the CaMV 35S vector construct exhibited 30 kDa ferritin molecules in the cytoplasm. During the construction of this expression vector the FLAG tag was introduced into the 5'-end of the leader sequence. Considering the size of the synthesized protein, we have to postulate that the addition of the charged amino acids of FLAG epitope prohibited the correct processing of ferritin. Nevertheless, we could not recognize essential differences between the two classes of tobacco transformants in responses to paraquat, iron excess or pathogens (Figure 2, 3). Therefore, we propose that both forms of this iron-binding protein can reduce cellular damage.

Plant development and the efficiency of crop production are highly dependent on iron-containing photosynthetic proteins. It is important, therefore, that ferritin overproduction did not change the photosynthetic activity (Figure 2) or chlorophyll content (data not shown) in the transformed plants.

Recently we isolated the cDNA of a NADPH-dependent aldo-keto reductase gene from alfalfa. The involvement of this gene in a wide range of stress responses was demonstrated by elevated accumulation of the mRNA in cultured alfalfa cells exposed to osmotic shock caused by polyethylene glycol (PEG, 15%) solution, heavy metal toxicity (250μM $CdCl_2$) and the stress hormone, abscisic acid (ABA, 75μM) (Figure 4). In mammalian cells the different members of the aldo-keto reductase superfamily can catalyze production of sorbitol from D-glucose, so they can serve as osmoregulators [4], and they play role in detoxification processes [34]. Up to now plant aldose reductase homolog genes were cloned only from monocot species, in cultured bromegrass cells elevated level of the gene expression is associated with the induction of freezing tolerance [20], the expression of wild oat aldose reductase homologue can be significant in maintaining seed dormancy or longevity [21]. The best characterized barley aldose reductase gene is expressed during the desiccation phase of embryogenesis and in mature embryos [6]. Recent results of Guillén et al. [12] suggest the role of the members of this enzyme family in specific detoxification processes in plants. Further experiments are in progress to evaluate the role of the alfalfa aldo-keto reductase enzyme in stress response using transgenic tobacco plants.

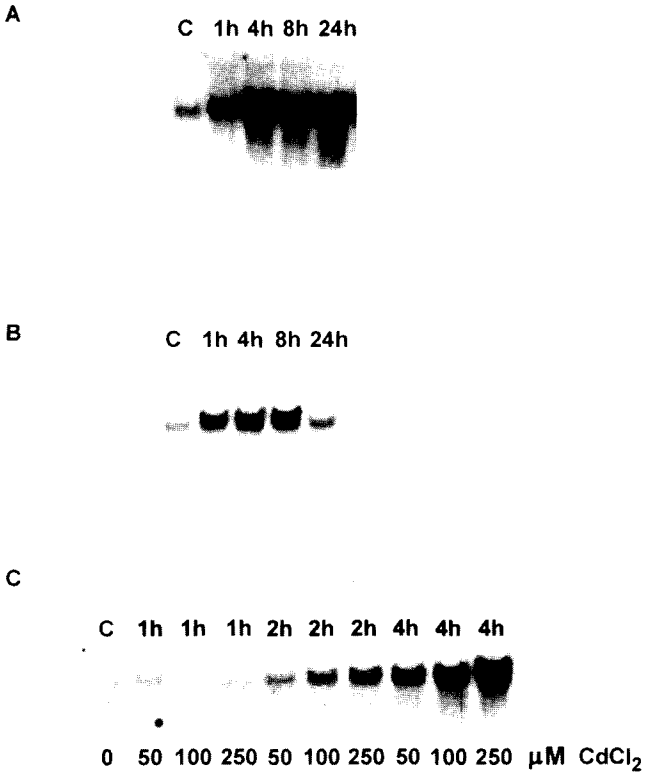

Figure 4. Wide range of stress factors can trigger the synthesis of the alfalfa aldose/aldehyde reductase mRNA. Northern blot analysis of total RNA isolated from control and abscisic acid (75μM ABA) treated alfalfa A2 cells, osmotic (15% PEG) and heavy metal (250 mM CdCl2) stressed alfalfa A2 cell suspension. The samples (20μg per lane) were taken at the indicated time points. The radiolabelled full length *MsALR* cDNA was used as a probe.

The improved stress tolerance observed in alfalfa ferritin producing transgenic tobacco plants provides basis for further application of this approache in crop improvement.

Acknowledgement

This work was supported by the Krber Foundation, Hamburg, Germany.

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