Early manganese-toxicity response in Vigna unguiculata L. a proteomic and transcriptomic study

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Complete List of Authors:	Fuehrs, Hendrik; Institute for Plant Nutrition, Faculty of Natural Sciences, Leibniz University Hannover Hartwig, Moritz; Institute for Plant Nutrition, Faculty of Natural Sciences, Leibniz University Hannover Molina, Laura; Department for Plant Genetics, Faculty of Natural Sciences, Leibniz University Hannover Heintz, Dimitri; Laboratoire de Spectrométrie de Masse Bioorganique, Unité Mixte de Recherche, Strasbourg Van Dorsselaer, Alain; Laboratoire de Spectrométrie de Masse Bioorganique, Unité Mixte de Recherche, Strasbourg Braun, Hans-Peter; Department for Plant Genetics, Faculty of Natural Sciences, Leibniz University Hannover Horst, Walter; Institute for Plant Nutrition, Faculty of Natural Sciences, Leibniz University Hannover
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Early manganese-toxicity response in *Vigna unguiculata* L. – a proteomic and transcriptomic study

Hendrik Fuehrs[1], Moritz Hartwig[1], Laura Elisa Buitrago Molina[1], Dimitri Heintz[2], Alain Van Dorsselaer[2], Hans-Peter Braun[3] & Walter J. Horst[1]

[1] Institute for Plant Nutrition, Faculty of Natural Sciences, University of Hannover, Herrenhaeuser Str.2, 30419 Hannover, Germany

[2] Laboratoire de Spectrométrie de Masse Bioorganique, Unité Mixte de Recherche 7509, 25 rue Becquerel, F-67087 Strasbourg cedex 2, France

[3] Department for Plant Genetics, Faculty of Natural Sciences, University of Hannover, Herrenhaeuser Str.2, 30419 Hannover, Germany

Corresponding author:

Prof. Dr. Walter Johannes Horst

Institute for Plant Nutrition, University of Hannover, Herrenhaeuser Str. 2, 30419 Hannover, Germany

Phone: 49511 7622627, Fax: 49511 7623611

email: horst@pflern.uni-hannover.de

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Abstract

The apoplast is known to play a predominant role in the expression of manganese (Mn) toxicity in cowpea (*Vigna unguiculata* L.) leaves. To unravel early Mn-toxicity responses after 1-3 days Mn treatment also in the leaf symplast, we studied the symplastic reactions induced by Mn in two cultivars differing in Mn tolerance on a total cellular level. Comparative proteome analyses of plants exposed to low or high Mn allowed to identify proteins specifically affected by Mn, particularly in the Mn-sensitive cowpea cultivar. These proteins are involved in CO_2 fixation, stabilization of the manganese cluster of the photosystem II, pathogenesis-response reactions and protein degradation. Chloroplastic proteins important for CO_2 fixation and photosynthesis were of lower abundance upon Mn stress suggesting scavenging of metabolic energy for a specific stress response. Transcriptome analyses supported these findings, but additionally revealed an up-regulation of genes involved in signal transduction only in the Mn-sensitive cultivar. In conclusion, a coordinated interplay of apoplastic and symplastic reactions seems to be important during the Mn-stress response in cowpea.

Introduction

Manganese (Mn) is an essential nutrient for plant growth and development [1, 2]. At the same time, high Mn availability is toxic and can limit plant growth [3]. This is especially relevant on acidic and imperfectly drained soils of the tropics and subtropics. There is a great inter- and intra-specific variability in Mn resistance [4-6]. The cultivar-specific differences in Mn resistance in the tropical legume cowpea are due to a Mn leaf-tissue tolerance as comparable leaf Mn concentrations lead to Mn toxicity in Mn-sensitive but not in Mn-tolerant cultivars [7].

In common bean as well as in cowpea, typical Mn toxicity symptoms start with brown spots on old leaves, followed by chlorosis, necrosis and finally leaf shedding [8, 9]. These Mn-induced brown spots consist of oxidized Mn and oxidized phenols mainly located in the cell wall of the epidermal layer [8, 10]. This and greatly enhanced activities of H_2O_2-producing and H_2O_2-consuming peroxidases suggest that the leaf apoplast is the decisive compartment for the development or avoidance of Mn toxicity in cowpea [11, 12]. Proteome analyses of the apoplastic washing fluid (AWF) confirmed an enhanced release of peroxidases into the leaf apoplast, but in addition revealed the induction of several other apoplastic stress-response proteins in response to advanced Mn stress [13]. We recently provided evidence suggesting that apoplastic phenols in addition to Mn play a major role in modulating genotypic differences in Mn tolerance [12].

It can be expected that changes in the proteome and metabolome of the leaf apoplast are triggered by molecular changes in the symplast and that such changes would represent a more rapid response to Mn excess. A role of the symplast in the expression of Mn toxicity is also suggested by results in other plant species that the sequestration of Mn in symplastic compartments confers enhanced Mn tolerance. In Arabidopsis, Mn tolerance is mediated by an ER-localized Ca^{2+}/Mn^{2+} pump designated ECA1 [14]. Recently, MTP11 cation diffusion facilitators from Arabidopsis and poplar were shown to confer tolerance to Mn-hypersensitive yeast mutants [15]. Promoter-GUS studies indicated a Golgi-based Mn accumulation resulting in Mn tolerance most likely through vesicular trafficking and exocytosis. In the tropical legume *Stylosanthes hamata* the ShMTP1 protein proved to be important for Mn

tolerance [16]. Expressed in Arabidopsis and yeast this protein was shown to confer Mn tolerance through inner-cellular Mn sequestration. Contradictory results were obtained for the subcellular localization of ShMTP1 in these organisms because it was found in the tonoplast membrane in Arabidopsis but in the ER membrane in yeast. Another cation transporter important for Mn translocation in Arabidopsis is the CAX2 protein. Over-expressed in tobacco this transporter conferred high Mn tolerance [17]. CAX2 was shown to be localized in the tonoplast membrane.. Mn accumulation in the vacuole was also reported in cowpea but could not be related to differences in leaf Mn tolerance owing to genotype, silicon nutrition and N form [18, 19].

There are a number of studies suggesting chloroplasts and photosynthesis as targets of Mn stress. Enrichment of Mn in the chloroplast was reported for common bean [20] and rice [21]. In common bean this was accompanied by a decrease of the chlorophyll content and by a reduction of CO_2 assimilation rates [20, 22, 23]. Reduced CO_2 assimilation upon Mn stress was also reported for tobacco [24, 25] and reduced chlorophyll content for wheat [26].

Based on our own results suggesting symplastic reactions triggering apoplastic lesions and reports on symplastic lesions of Mn toxicity we initiated a systematic investigation of Mn excess-induced changes in leaves of two cowpea cultivars differing in Mn tolerance on a cellular level using proteome and transcriptome analyses.

Materials and Methods

Plant material

Cowpea (*Vigna unguiculata* [L.] Walp. cvs TVu 91 and TVu 1987) was grown hydroponically in a growth chamber under controlled environmental conditions at 30/27°C day/night temperature, 75%±5 % relative humidity, and a photon flux density of 150 µmol $m^{-1}s^{-1}$ photosynthetic active radiation (PAR) at mid-plant height during a 16-h photoperiod. After germination on filter paper in 1 mM $CaSO_4$ for 7 days, seedlings were transferred into 5 liter pots and supplied with a constantly aerated nutrient solution with the following composition (µM): 1000 $Ca(NO_3)_2$, 100 KH_2PO_4, 375 K_2SO_4, 325 $MgSO_4$, 20 FeEDDHA, 10 NaCl, 8 H_3BO_3, 0.2 $MnSO_4$, 0.2 $CuSO_4$, 0.2 $ZnSO_4$, 0.05 Na_2MoO_4. After pre-culture for 14 days, the Mn concentration in the nutrient solution was increased to 50 µM $MnSO_4$ for 3 days, whereas control plants received 0.2 µM Mn continuously. The nutrient solution was changed two to three times a week to avoid nutrient deficiencies.

Quantification of toxicity symptoms

For the quantification of Mn toxicity symptoms, the density of brown spots was counted on a 1 cm^2 area at the base and tip on the upper side of the second oldest middle trifoliate leaf.

Mineral analysis

Manganese in the bulk-leaf tissue was determined after dry ashing (480°C, 8h) and dissolving the ash in 6 M HCl with 1.5% [w/v] hydroxylammonium chloride and diluted 1:10 [v/v] with water. Measurements were carried out by inductively-coupled plasma optical emission spectroscopy (Spectro Analytical Instruments GmbH, Kleve, Germany).

After isolation of chloroplasts (see below) the Mn content was determined as follows: 500 µl of isolated chloroplasts (0.1 g chloroplasts ml^{-1}) were centrifuged at 15000 x g for 5 min. To 500 µl of the supernatant 500 µl 6 M HCl with 1.5% [w/v] hydroxylammonium chloride was added and diluted 1:2 [v/v] with ddH_2O. The pellet was dried at 60°C and then dry-ashed at 480°C over night. The ash was dissolved in

500 µl 6 M HCl with 1.5% [w/v] hydroxylammonium chloride and diluted 1:4 [v/v] with ddH_2O. Manganese was measured by inductively-coupled plasma optical emission spectroscopy (Spectro Analytical Instruments GmbH, Kleve, Germany).

Chlorophyll fluorescence

Chlorophyll fluorescence of the second oldest middle trifoliate leaf was determined using a Mini-PAM fluorometer (Waltz, Germany). Measurements were carried out on dark adapted plants using the light induction curve-program: One minute after a first saturation pulse actinic light was turned on and from then every 30 seconds a new saturation pulse was applied over a period of 6.5 minutes. The Yield, ETR, nP, nQ and NPQ values were calculated immediately by the included software and stored. All measurements were repeated four times and evaluated by statistical analyses.

Photosynthesis rate

Photosynthesis rate was measured with four replications on the second oldest middle trifoliate leaf with the Li-Cor 6400 portable photosynthesis (LiCor Inc., Lincoln, NE, USA) system using a CO_2 curve programme with the following sequence: 400, 600, 800, 1000, 400 µmol CO_2*mol^{-1} and a flux of 500 µms. Leaves received 1500 µmol PAR*m^{-2}*s^{-1}. Photosynthesis rate was calculated immediately by the Li-Cor control software and values were submitted to statistical analysis.

Extraction of proteins from leaf

Protein extraction for 2-D gel electrophoresis (2DE) analyses was carried out as outlined by Mihr and Braun [27] using the second oldest trifoliate leaf of cowpea plants. Proteins were independently extracted three times from the pooled leaf material of two plants:
Leaf tissue (0.2 g) was ground by mortar and pestle in liquid nitrogen. Homogenized leaf powder was suspended in 750 µl extraction buffer (700 mM sucrose, 500 mM Tris, 50 mM EDTA, 100 mM KCl, and 2% [v/v] mercaptoethanol) and incubated for 10 minutes on ice. Afterwards, an equal volume of water-saturated phenol was added and incubated for another 10 min. The aqueous and organic phases were separated by centrifugation for 10 min at 5,000 x g and 4°C. The phenolic phase was re-

extracted with extraction buffer and centrifuged once more. Phenol phases were combined, supplemented with 5 volumes of 0.1 M ammonium acetate in methanol and incubated over night at –20°C. After centrifugation at 11,000 g for 3 min at 4°C, precipitated proteins were washed three times with ammonium acetate in methanol and finally with acetone. Extracted proteins were resolved in "rehydration buffer" (see below) for 2DE analysis. Protein concentration of extracts were determined in rehydration buffer using the 2-D Quant Kit© (GE Healthcare, Munich, Germany) according to the manufacturer's instructions.

Isolation of chloroplasts and protein extraction for BN/SDS-PAGE

Chloroplasts were isolated according to Heinemeyer *et al.* [28] using about 20 g of leaf tissue. Leaf tissue was homogenized in "homogenization buffer" (330 mM mannitol, 30 mM HEPES, 2 mM EDTA, 3 mM $MgCl_2$ and 0.1 % [w/v] BSA, pH 7.8) using a Waring blender for 3 x 3 seconds. Purification of organelles was based on a differential centrifugation combined with a Percoll density-gradient centrifugation (for details see [28]). Purified chloroplasts were re-suspended in "homogenization buffer" without BSA at a protein concentration of 15 mg/ml, frozen in liquid nitrogen and stored at -80°C.

2D IEF / SDS-PAGE

For IEF, the IPGphor system (GE Healtcare, Munich, Germany) and Immobiline DryStrip gels (18 cm) with a nonlinear pH gradient (pH 3-11) were used. About 500 µg protein in "rehydration solution" (8 M urea, 2% [w/v] CHAPS, 0.5% [v/v] carrier ampholyte mixture [IPG buffer, pH 3-11 NL; GE Healthcare], 50 mM dithiothreitol, 12 µl ml^{-1} DeStreak [GE Healthcare], and a trace of bromphenol blue) was loaded onto individual gel strips. Focussing was done according to Werhahn and Braun [29]. Afterwards, Immobiline DryStrip gels were incubated with equilibration solution (50 mM Tris-Cl [pH 8.8], 6 M urea, 30% [v/v] glycerol, 2% [w/v] SDS, and bromphenol blue) supplemented with (a) 1% [w/v] dithiothreitol and (b) 2.5% [v/w] iodacetamide each for 15 min. Finally, DryStrips were placed horizontally onto second dimension SDS gels and proteins were resolved according to Schägger and von Jagow [30].

2D Blue-native / SDS-PAGE

Thylakoid membrane complexes were analysed by two-dimensional Blue-native PAGE [31, 32]. Thylakoids of chloroplast fractions (about 1 mg protein) were sedimented by centrifugation for 10 minutes at 5000 x g and resuspended in 80 µl "solubilization solution" (30 mM HEPES, pH 7.4, 150 mM potassium acetate, 10% glycerol, 2 mM PMSF and 1.5% [w/v] digitonin [Fluka, Buchs, Switzerland]). Samples were centrifuged for 30 minutes; the supernatants were supplemented with "Coomassie-blue solution" (5% [w/v] Serva Blue G, 750 mM aminocaproic acid) and directly loaded onto the native gel. Gel electrophoresis conditions for the first and second dimensions were as described in Heinemeyer *et al.* [32].

Staining of protein gels and spot detection

All protein gels were stained with colloidal Coomassie-blue according to Neuhoff *et al.* [33, 34]. Spot detection and the calculation of master gels was carried out using the ImageMaster™ 2D Platinum Software 6.0 (GE Healthcare, Munich, Germany).

Mass spectrometric analysis and data interpretation

Each SDS-PAGE gel spot was dried under vacuum. In-gel digestion was performed with an automated protein digestion system, MassPREP Station (Micromass, Manchester, UK). The gel slices were washed three times in a mixture containing 25 mM NH_4HCO_3:ACN [1:1, v/v]. The cysteine residues were reduced by 50 µl of 10 mM dithiothreitol at 57°C and alkylated by 50 µl of 55 mM iodacetamide. After dehydration with acetonitrile, the proteins were cleaved in the gel with 40 µl of 12.5 ng μl^{-1} of modified porcine trypsin (Promega, Madison, WI, USA) in 25 mM NH_4HCO_3 at room temperature for 14 hours. The resulting tryptic peptides were extracted with 60% acetonitrile in 5% formic acid, followed by a second extraction with 100% [v/v] acetonitrile.

Nano-LC-MS/MS analysis of the resulting tryptic peptides was performed using a CapLC capillary LC system (Micromass) coupled to a hybrid quadrupole orthogonal acceleration time-of-flight tandem mass spectrometer (Q-TOF II, Micromass). Chromatographic separations were conducted on a Pepmap_ C18 75 µm i.d. x 15 cm length, reverse-phase (RP) capillary column (LC Packings, Sunnyvale, CA, USA)

with a flow rate of 200 nl min^{-1}, accomplished by a pre-column split. An external calibration was performed using a 2 pmol l^{-1} GFP ([Glu1]-Fibrinopeptide B) solution. Mass data acquisition was piloted by MassLynx 4 software (Micromass) using automatic switching between MS and MS/MS modes. Classical protein database searches were performed on a local Mascot_ (Matrix Science, London, UK) server. To be accepted for the identification, an error of less than 100 p.p.m. on the parent ion mass was tolerated and the sequences of the peptides were manually checked. One missed cleavage per peptide was allowed and some modifications were taken into account: carbamidomethylation for cysteine, and oxidation for methionine. In addition, the searches were performed without constraining protein Mr and pI, and without any taxonomic specifications. These searches did not always lead to a positive identification since the cowpea (*Vigna unguiculata)* genome has not yet been sequenced. In such cases, the use of a *de novo* sequencing approach was necessary for a successful identification. For this purpose, the interpretation of the MS/MS spectra was performed with the PepSeq tool from the MassLynx 4 (Micromass) software, as well as the PEAKS Studio software (Bioinformatics Solutions, Waterloo, Canada v.3). The resulting peptide sequences were submitted to the BLAST program provided at the EMBL site (http://dove.embl-heidelberg.de/Blast2/msblast.html) in order to identify them by homology with proteins present in the databases. We used the MS-BLAST specifically modified PAM30MS scoring matrix, no filter was set and the nrdb95 database was used for the searches as described by Castro *et al.* [35]. The statistical evaluation of the results and the validation of the matches was performed according to Shevchenko *et al.* [36].

Protein identifications by mass spectrometry only were carried for one of the three gel replicates, because gels obviously were very similar. Also, all analyses allowed to unambiguously identify proteins of the expected molecular mass range.

Generation of subtractive cDNA libraries enriched in transcripts induced by Mn stress

The cDNA libraries were constructed by “suppressive subtractive hybridization” (SSH) [37] as outlined in Wulf *et al.* [38]:

After 7 days of germination in 1 mM $CaSO_4$ solution cowpea plants of the Mn-sensitive cultivar TVu 91 and the Mn-tolerant cultivar TVu 1987 were transferred into 5 liter plastic pots containing nutrient solution (composition see “Plant material” section). To avoid shading and interactions between individual plants only one plant per pot was cultivated. The nutrient solution was changed every two to three days to prevent nutrient deficiencies. After 14 days of preculture under controlled environmental conditions in a growth chamber the Mn supply was either increased to 50 µM Mn for three days or kept at 0.2 µM Mn continuously. The second oldest trifoliate leaves from individual plants were harvested, petioles were removed, and remaining material was directly frozen in liquid nitrogen. Samples from five individual plants were pooled and homogenized with mortar and pestle under permanent supply of liquid nitrogen. Leaf powder was transferred into Eppendorf tubes and stored at -80°C until further analysis.

RNA was extracted with the LiCl method as described earlier [38]. Total RNA (3 µg) was used to produce cDNA using the SMART cDNA synthesis kit (Clontech, Palo Alto, CA). This cDNA was used to perform an SSH using the PCR select cDNA subtractive kit (Clontech). Amplification products were cloned into the pGEM-Teasy vector (Promega, Madison, WI, USA). Selected clones were analysed by DNA sequencing.

Statistical analysis

Statistical analysis, unless otherwise mentioned, was carried out using the SAS software package (Release v8.0, SAS Institute, Cary, NC, USA). Results from analysis of variance are given according to their level of significance as ***, **, * and $^+$ for $p < 0.001$, 0.01, 0.05, and 0.1, respectively.

Results

Effect of increased Mn supply on the Mn uptake and expression of Mn toxicity symptoms

Increasing the Mn supply from 0.2 µM (control) to 50 µM (Mn treatment) for three days led to an about 10-fold increase of Mn leaf-tissue concentrations in both investigated cowpea cultivars (Figure 1). The leaf Mn concentration of the Mn-tolerant cultivar TVu 1987 was 30% higher than of the Mn-sensitive cultivar TVu 91. Nevertheless, Mn toxicity symptoms (brown spots) were only visible on the leaves of the Mn-sensitive cultivar. The toxicity level was moderate as it was in the range of twenty spots per cm^{-2} (for a detailed evaluation of Mn toxicity symptoms see [13]). The chloroplast Mn content, which was estimated to account for about 2% of the bulk-leaf Mn after elevated Mn supply, did not show differences neither between the Mn treatments nor between cultivars (0.85 µmol Mn [g chloroplast dry matter]$^{-1}$ corresponding to 0.14 µmol Mn [g chloroplast fresh matter]$^{-1}$).

Effect of increased Mn supply on the leaf proteome

Proteome analysis was carried out to systematically identify proteins affected by Mn supply. Total proteins of both cultivars treated with 0.2 or 50 µM Mn for three days were extracted from leaves and resolved by 2D IEF / SDS PAGE (Figure 2). Careful comparison of the protein patterns on the resulting four 2D gels by visual inspection allowed identifying several differences in protein abundance between the two genotypes or the two Mn treatments. More than 25 proteins clearly exhibited differential abundance due to the Mn treatment. Protein extractions for 2D IEF / SDS PAGE were repeated three times independently using the pooled leaf material of 2 plants. The obtained gels were evaluated by the ImageMaster™ 2D Platinum Software 6.0 (GE Healthcare, Munich, Germany). Master gels were calculated for both genotypes and Mn treatments and then compared. 540 and 464 spots were included into the statistical analysis (Table S1). Using rigorous threshold parameters (spot ratio on the two compared master gels < 0.5 or > 2, p-value of the difference in abundance in individual experiments < 0.01) eight proteins were identified exhibiting differential abundance due to the Mn treatment (indicated by arrows on Figure 2 and

Figure 3). Seven of these proteins showed changes in abundance in the Mn-sensitive cultivar (five had lower and two had higher abundance, Figure 2, upper gels) and one protein showed a change in the Mn-tolerant cowpea cultivar (increased abundance, Figure 2, lower gels). Close-ups of the gel regions containing proteins of differential abundance are shown in Figure 3.

Identification of proteins affected by increased Mn supply

The eight proteins clearly affected by Mn treatment were analysed by *de novo* peptide sequencing using ESI tandem mass spectrometry and identified by sequence comparisons using the NCBI protein database (Table 1). Three of the proteins reduced at high Mn treatment in the Mn-sensitive genotype represent enzymes involved in primary carbon fixation (RubisCO-binding protein, RubisCO activase, phosphoribulokinase; Figure 2, spots 1, 2 and 3). Two proteins of changed abundance (higher, spot 7; lower, spot 5, Figure 2) in the Mn-sensitive genotype are pathogenesis-related proteins. Another protein reduced in the Mn-sensitive cultivar upon Mn treatment is homologous to an "Oxygen-evolving enhancer protein, type 1" (also termed OEC33 protein) from *Arabidopsis thaliana* (Figure 2, spot 4). Finally, the β6 subunit of the proteasome is of increased abundance in Mn-treated plants of the Mn-sensitive cowpea cultivar (Figure 2, spot 6). The only clearly Mn-affected (up-regulated) protein of the Mn-tolerant cowpea genotype represents another type of "oxygen-evolving enhancer protein (type 2)" (also termed OEC23 protein).

Physiological changes linked to increased Mn supply

Two of the Mn-affected proteins are indirectly involved in the photosynthetic water splitting process. This was assumed to have consequences for the electron transfer rate (ETR) and photosynthesis of the studied leaves. Indeed, ETR was significantly reduced in the Mn-sensitive cultivar exposed to high Mn supply as early as after one day (Figure 4). In contrast, the ETR of the Mn-tolerant cultivar was not affected by the elevated Mn supply even after 3 days of Mn treatment.

Net CO_2 fixation rate measured in parallel was not affected by Mn treatment for up to 3 days and did not differ between the Mn treatments in either cultivar (data not shown).

Since the water splitting complex forms part of the photosystem II supercomplex, two-dimensional Blue native / SDS PAGE was carried out to analyse the photosystem II subunit composition and abundance. For this approach, chloroplasts were isolated from leaves of plants of both cowpea cultivars cultivated at low (0.2 µM) or high Mn (50 µM) supply. Thylakoid protein complexes proved to have a similar structure in all four samples analysed. A representative example out of three replicates is shown in Figure 5. In particular, structure and abundance of the photosystem II was similar in both cowpea cultivars independent of Mn treatment. However, a slightly enlarged form of the photosystem I, which runs at about 650 kDa on the native gel dimension [28], was enriched 1.7-fold in the plants exposed to high Mn supply for 3 days in the Mn-sensitive cultivar TVu 91. A 1.2-fold enrichment could also be found in the Mn-tolerant cultivar TVu 1987. This larger form of the photosystem I is known to arise by attachment of trimeric LHCII during transition from state 1 to state 2 photosynthesis [40].

Manganese-induced gene expression

Changes of the cowpea-leaf proteome in response to Mn stress are assumed to be preceded by changes in gene expression. The Suppression Subtractive Hybridization (SSH) strategy [37] was chosen to systematically monitor Mn stress-induced changes in gene expression in the leaves. For this approach, total mRNA of both cowpea cultivars grown for one day in the presence of normal or enhanced Mn supply was isolated and used to generate two subtractive cDNA libraries. Enrichment of the obtained libraries in transcripts specifically induced by enhanced Mn supply was verified by Northern blotting experiments for 20 randomly selected clones (data not shown). For preliminary analyses of the two cDNA libraries, 100 clones per library were selected on a random basis and subjected to DNA sequence analyses. The corresponding genes were identified on the basis of sequence comparisons and assigned to functional categories (Figure 6). The number of transcripts involved in photosynthesis and respiration declined upon enhanced Mn supply in the Mn-sensitive TVu 91 cultivar if compared to the Mn-tolerant cultivar TVu 1987. At the same time, the number of transcripts involved in signal transduction increased in the Mn-sensitive cultivar. These preliminary results point to a rapid broad-range transcriptomic response of the Mn-sensitive cowpea cultivar upon Mn stress.

Discussion

Previous studies in cowpea revealed that the leaf apoplast represents the most important compartment involved in the expression of Mn toxicity [41] which is characterised by formation of brown spots, induction of callose formation, and an enhanced release into the apoplast of phenols, peroxidases and other stress-related proteins [11-13]. These Mn stress-induced physiological changes in the older leaves can be measured after two to three days after exposure of the plants to toxic Mn supply. It is conceivable that these apoplastic changes involved in Mn toxicity are triggered by excess Mn through molecular events in the symplast. This assumption is addressed in the present study through a transcriptomic and proteomic analysis of total leaves of cowpea genotypes differing in Mn tolerance as affected by toxic Mn supply.

Proteins specifically affected by Mn stress in cowpea

The RubisCO-binding subunit, the RubisCO activase and the phosphoribulokinase (Spots 1-3 in Figures 2, 3 and Table 1) all are essential for efficient CO_2 fixation in plants. Phosphoribulokinase catalyses the formation of ribulose-1,5-bisphosphate, which represents the acceptor molecule for CO_2 fixation of the Calvin cycle [42]. The RubisCO binding subunit is important for folding of the large subunit of RubisCO [43]. RubisCO activase promotes and maintains the catalytic activity of RubisCO [44]. It removes inhibitors from catalytic sites of RubisCO, prevents changes in conformation, and activates RubisCO. The amount of all three proteins in the Mn-sensitive cowpea genotype TVu 91 was reduced to less than half when treated with high Mn supply. Therefore, Mn stress specifically induces a down-regulation of the key enzymes responsible for CO_2 fixation of the Calvin cycle. This process might be important to save energy for the Mn-stress response. A direct regulation of RubisCO possibly would be less efficient because of the large quantities of this enzyme present in chloroplasts. However, measurable reduction of CO_2 fixation seems to be delayed compared to the reduction of the levels of RubisCO-binding protein and RubisCO activase, which most likely also is a consequence of the huge excess of RubisCO in relation to its regulating proteins.

Two cowpea proteins specifically affected by Mn-treatment form part of the “Oxygen Evolving complex” (OEC) of the photosystem II (PSII). This complex is composed of three proteins, the Psb O (OEC33), the Psb P (OEC23) and the Psb Q (OEC16) protein [45]. All three proteins are attached to the photosystem II on the luminal side of the thylakoids and are believed to be important for the stabilisation of the Mn cluster of PSII, for efficient water splitting, and overall PSII stability [46, 47]. Recently, homologous PSII subunits also were identified in cyanobacteria [48, 49]. The precise function of the three proteins in cyanobacteria and higher plants is not known. Interestingly, abundance of OEC23 (spot 8 in Figures 2, 3 and Table 1) is four-fold increased by Mn treatment in the Mn-tolerant cowpea cultivar TVu 1987. Possibly, the expression of the corresponding gene is Mn-regulated. In contrast, the Mn-stabilizing OEC33 protein (spot 4 in Figures 2, 3 and Table 1) is drastically reduced in the Mn-sensitive cultivar TVu 91 upon Mn stress. This could contribute to growth reduction of this cultivar at toxic Mn supply because the water-splitting process of photosynthesis is impeded. Indeed, electron transfer-rates (ETR) decreased in the Mn-sensitive cultivar upon Mn stress in comparison to normal Mn conditions (Figure 4). This decrease cannot be interpreted as a consequence of reduced leaf area of Mn-stressed plants due to the development of the characteristic brown spots, because the decrease in ETR occurred before these spots became visible. However, conclusions on the functional relevance of the observed changes in abundance of the OEC proteins should be treated with caution, because they are encoded by small gene families. The precise regulation of the abundances of OEC isoforms so far is unknown.

Also, a state 1 to state 2 transition of photosynthesis was observed in Mn-stressed cowpea leaves (Figure 5). During this state transition, light harvesting proteins are detached from the photosystem II and bind to the photosystem I [40]. As a consequence, light absorption at photosystem II decreases and increases at photosystem I. This leads to an overall reduction of linear photosynthetic electron transfer, but an induction of cyclic electron transfer, which is especially important for ATP synthesis in chloroplasts [50]. State I – state II transitions of photosynthesis were previously reported to form part of a plant-stress response towards varying light conditions. Both cultivars responded principally similarly to Mn treatment, although

less marked in the Mn-tolerant cultivar as calculated by ImageMaster™ 2D Platinum Software 6.0 (GE Healthcare, Munich, Germany).

The changes in the chloroplast proteome (Fig. 2, Fig. 3, Fig. 5) and in the photosynthetic performance (Fig. 4) cannot be explained by differences in the Mn contents of the isolated chloroplasts as they did not differ between Mn treatments and cultivars. However, chloroplasts were isolated in the presence of EDTA to inhibit metalloproteases. As a consequence, large amounts of free and labile-bound Mn might have been lost during the organelle isolation procedure. Indeed, Keren et al. [51] showed that they could remove a substantial labile Mn fraction by washing photosynthetic *Synechocystis* cells with EDTA. Also, using a non-aqueous isolation technique, a positive linear relationship between bulk-leaf and chloroplast Mn concentrations was shown in common bean [20]. We thus cannot exclude the possibility that the Mn-tolerant cultivar is able to exclude Mn from the chloroplasts more effectively than the Mn-sensitive cultivar.

Two further cowpea proteins affected by Mn-stress represent pathogenesis-related (PR) proteins (spots 5 and 6 in Figures 2, 3 and table 1). Both proteins only were identified in the Mn-sensitive cultivar. One PR protein belongs to the PR-4 family and is of reduced abundance in Mn-stressed plants; the other is similar to proteins of the PR-5 family and is of increased abundance. PR-like proteins were also detected by a proteome analysis of Mn-inducible proteins of the apoplast of cowpea [13] at an advanced stage of Mn toxicity. However, these proteins were not identical to the newly identified PR proteins. In general, a large number of PR proteins are known and most of them are induced by biotic and/or abiotic stress factors [52, 53]. The role of the up and down regulation of the two identified PR proteins in Mn tolerance is not yet understood.

Finally, a β-6 type proteasome subunit was specifically induced in the Mn-sensitive cowpea cultivar upon Mn stress. Proteasomes in general are responsible for the main protein degradation pathway in eukaryotic cells [54]. The substitution of α- and β-subunits of the 20S proteasome was previously suggested to be responsible for the specific proteolysis of proteins as part of defence reactions [55]. However, the specific role of the β-6 subunit in proteolysis is currently not known.

The apoplast proteins important for the Mn-stress response in cowpea [13] were not detected on the total leaf proteome level, most likely due to their comparatively low abundance. Also, Mn membrane transporters were not identified, which certainly play an important role in Mn compartmentation in plant cells (hydrophobic proteins such as ion transporters are known to be only poorly resolved during isoelectric focussing for 2D gel electrophoresis; also, enrichment of hydrophobic proteins would need a different extraction procedure than used in the current study).

Transcripts specifically affected by Mn stress in cowpea

An investigation of transcripts induced in the Mn-sensitive and the Mn-tolerant cowpea cultivars upon Mn-stress was carried out by the SSH technology (Figure 6). Using this procedure, transcripts induced by Mn stress were specifically enriched. Systematic sequencing of 2 x 100 clones of the resulting cDNA libraries on the basis of random clone selection gave first insights into the rapid (one day treatment) transcriptomic Mn-stress response: (1) Compared to the Mn-tolerant cowpea cultivar, the number of transcripts coding for proteins involved in photosynthesis, respiration and primary metabolisms were reduced in the Mn-sensitive genotype. (2) At the same time, transcripts encoding for proteins involved in signal transduction were increased. (3) All other functional categories of proteins were more or less unchanged between the Mn-tolerant and the sensitive cultivar upon Mn stress. (4) In both cultivars, several PR proteins were induced by Mn treatment. (5) Some of the induced transcripts identified in a cDNA library for the Mn-sensitive cowpea genotype after 3 days of Mn stress encode for PR proteins previously identified in this cultivar in the course of an investigation of the apoplast proteome [13, Figure 7].

Apoplastic versus symplastic Mn-stress response in cowpea

The results generally reveal an involvement of proteins of symplastic compartments in the Mn-stress response. Using stringent threshold criteria, proteins which are related to photosynthetic water splitting were shown to be clearly affected by Mn excess, CO_2 fixation, the pathogen defence-response, and the protein degradation-pathway. This can be attributed to modified transcription of genes as early as 1 day

after transfer of the plants to excessive Mn supply. This is in agreement with our expectations and appears to corroborate conclusions made by some authors (see introduction for references) that Mn toxicity primarily affects chloroplast functions in other plant species. However, it needs to be considered that in these plant species young leaves have been studied, whereas in cowpea Mn toxicity expresses strictly primarily in old leaves. This could be one of the reasons why for Mn toxicity in cowpea apoplastic lesions are more important than symplastic lesions after longer Mn treatment.

In conclusion, the Mn-stress response still is only partially understood. This is especially true for the initial symplastic molecular events induced by Mn excess which are presumed to induce signal transduction pathways leading to apoplastic stress reactions. Overall, the Mn-stress response seems to be a specific interplay of apoplastic and symplastic reactions, which in concert result in the expression of Mn toxicity and Mn tolerance. Future research will be necessary to further clarify the molecular details of the Mn-stress response in cowpea and other plant species.

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Figure Legends

Figure 1: Manganese tissue-concentrations of leaves as affected by Mn supply in the cowpea cultivars TVu 91 (Mn-sensitive) and TVu 1987 (Mn-tolerant). Plants were pre-cultured for 14 days and afterwards cultured at 0.2 µM or 50 µM Mn for 3 days continuously. * Toxicity symptoms (20 brown spots cm^{-2}) appearing only on the leaves of the Mn-treated Mn-sensitive cultivar TVu 91. Bars represent standard deviations of the means.

Figure 2: 2-D resolution of the total leaf proteome of the Mn-sensitive cowpea cultivar TVu 91 and the Mn-tolerant cultivar TVu 1987 after treatment with 0.2 µM or with 50 µM Mn. Treatment of plants was for 3 days after 14 days of pre-culture. Leaf material was homogenized in liquid N_2 and proteins were extracted as described in the Materials and Methods section. IEF was carried out on broad range pH gradient gels (pH 3-11). Differentially expressed spots are marked by arrows and numbered consecutively (see Table 1 for identities). The boxes indicate gel regions used for the detailed comparisons shown in Figure 3.

Figure 3: Close ups of regions of the 2D gels shown in Figure 2 including differentially expressed proteins of the two cowpea cultivars TVu 91 and TVu 1987. Treatment of plants was for 3 days after 14 days of pre-culture. Leaf material was homogenized in liquid N_2 and proteins were extracted as described in the Materials and Methods section. Differentially expressed spots are marked by arrows (for identifications see Table 1).

Figure 4: Electron-transport rate (ETR) of the cowpea cultivars TVu 91 (A) and TVu 1987 (B) after enhanced Mn supply relative to the ETR at optimum Mn supply. Plants pre-cultivated for 14 days were supplied with 0.2 µM or 50 µM Mn for 0 – 3 days. ETR was measured using a Mini-PAM fluorometer. Means were calculated on the basis of four independent experiments and evaluated for each cultivar separately. Results of the analysis of variance are given according to their level of significance as *** for $P < 0.001$. Bars represent standars deviations of the means.

Figure 5: Two-dimensional resolution of the chloroplast protein complexes of the cowpea cultivars TVu 91 and TVu 1987 by 2D Blue-native / SDS PAGE. Plants pre-cultivated for 14 days were supplied with 0.2 µM or 50 µM Mn for 3 days. Total leaf protein was extracted as described in the Materials and Methods section. Resolved protein complexes were identified on the basis of their subunit compositions according to Heinemeyer *et al.* [27]. Molecular masses of standard proteins are given to the right and the identities of the resolved protein complexes above the gels. Abbreviations: PS II + LHC II – supercomplex of dimeric photosystem II + light-harvesting II complexes; PS I + LHC I / II – photosystem I + light-harvesting proteins I + light-harvesting complex II; PS I + LHC I – photosystem I + light-harvesting proteins I, [PS I + LHC I] – subcomplex of PS I + LHC I; F1-ATP synthase – F1 part of the ATP synthase complex; b6f complex – cytochrome b6f complex; LHC II – light-harvesting complex II. Marked spots were analysed by ImageMaster 2D Platinum to quantify differences between

the Mn treatments within each genotype. The PS I + LHC I / II complex was enhanced at high Mn 1.7-fold and 1.2-fold in the Mn-sensitive cultivar TVu 91 and the Mn-tolerant cultivar TVu 1987, respectively.

Figure 6: Manganese-induced gene expression in the cowpea cultivars TVu 91 and TVu 1987. Treatment of plants was 0.2 µM versus 50 µM Mn for 1 day after 14 days of pre-culture. Transcripts were isolated by Supression Subtractive Hybridization (SSH) as described in the Material and Methods section. 100 clones were selected for both cultivars on a random basis, sequenced and classified into functional categories on the basis of sequence homology (for details see supplementary material, Tables S3).

Figure 7: Detailed sequence comparisons between two Mn-induced proteins of the cowpea cultivar TVu 91 identified by SSH and proteins identified by direct proteome analyses of the total leaf proteome (this study) or the apoplast proteome [13]. Stars beneath the sequences indicate identical residues. Used clones were named according to their accession numbers at the EMBL database.

Table 1. Leaf proteins of cowpea cultivars TVu 91 and TVu 1987 affected by treatment with 50 µM Mn for 3 days.

Spot[a]	Identity[b]	Acc. No[b]	MW (Da) / No. of amino acids	Fold induction / reduction[c]
1	RubisCO-binding protein, beta subunit (pea)	P08927	62984 / 595	0.38
2	RubisCO activase (rice)	Q7XXR6	51454 / 466	0.48
3	Phosphoribulokinase (pea)	P93681	39026 / 352	0.49
4	Oxygen-evolving enhancer protein 1 (*A. thaliana*)	Q9S841	35019 / 331	n.d.
5	Pathogenesis-related protein P4 (tomato)	Q04108	17439 / 159	n.d.
6	Putative beta6 proteasome subunit (tobacco)	Q93X30	20864 / 192	2.03
7	Pathogenesis-related protein 5-1 (sunflower)	Q8LSM9	23953 / 222	2.46
8	Oxygen-evolving enhancer protein 2 (*B. gymnorrhiza*)	Q9MAW2	17537 / 160	3.80

[a] The numbers correspond to numbers given in Figures 2 and 3. Spots 1-7 are from the Mn-sensitive cultivar TVu 91 and spot 8 from the Mn-tolerant cultivar TVu 1987. For statistical evaluation and peptide sequences see supplementary material, Tables S1 and S2.

[b] Identities are based on sequence comparisons using the NCBI protein database.

[c] Fold induction / reduction in plants cultivated at 50 µM Mn in relation to plants cultivated at 0.2 µM. For further details see Table S1. Spots 4 and 5 completely disappeared during Mn treatment and, therefore, could not be quantified (n.d.)

Fig. 1

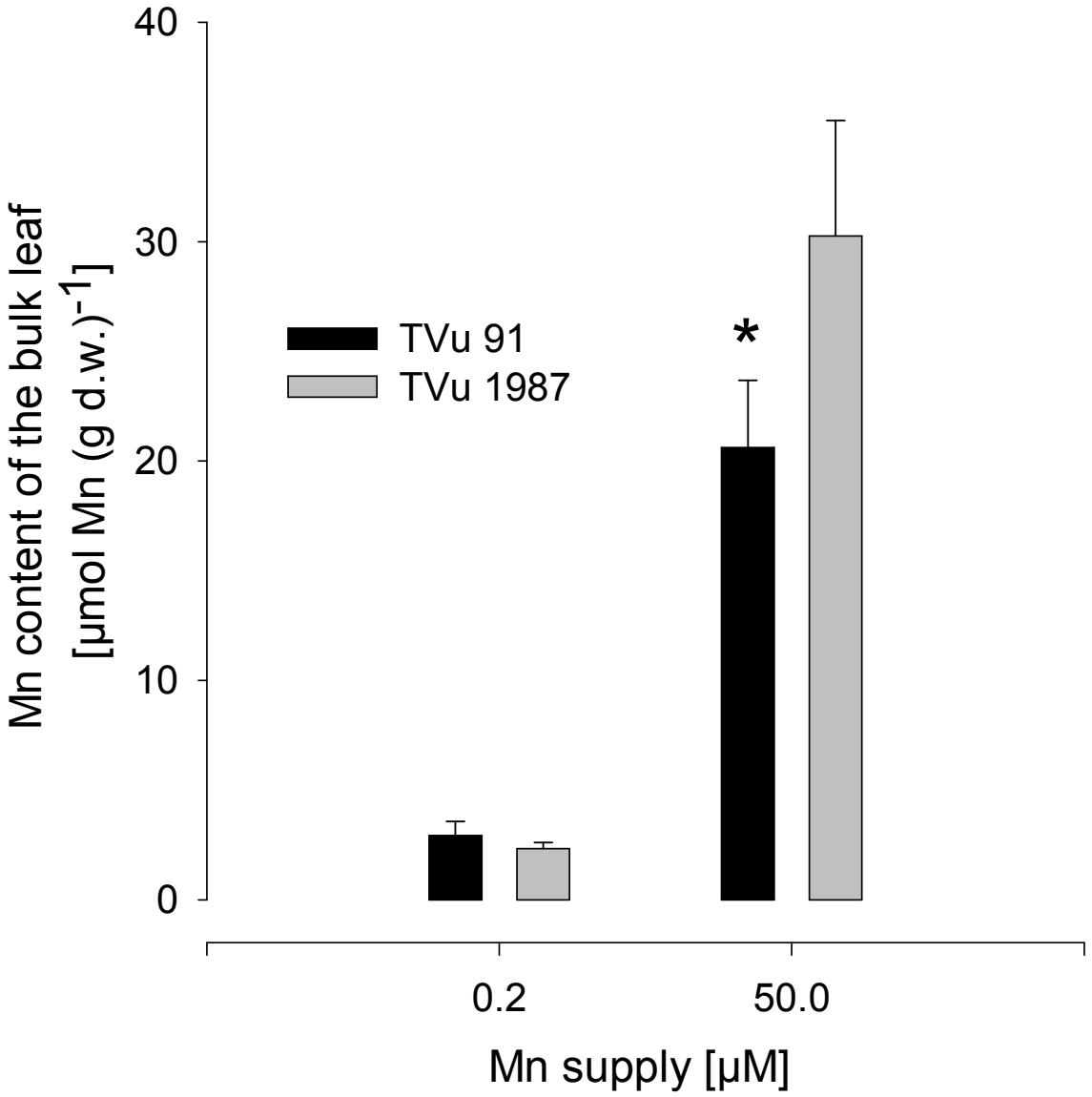

40
30
20
10
0
Mn content of the bulk leaf
[µmol Mn (g d.w.)-1]
TVu 91
TVu 1987
*
0.2
50.0
Mn supply [µM]

Fig. 2

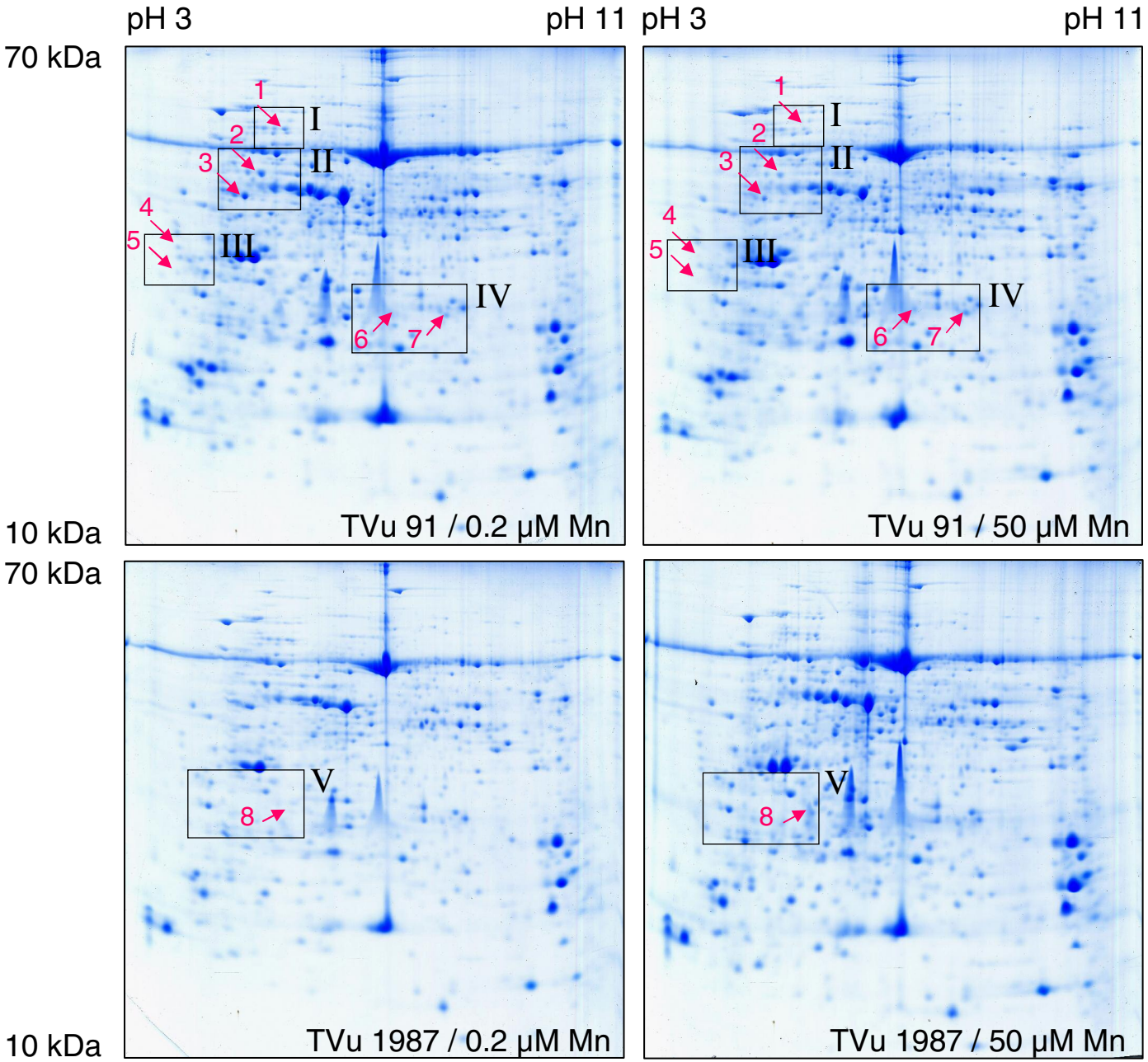

pH 3
pH 11
pH 3
pH 11
70 kDa
10 kDa
70 kDa
10 kDa
1
2
3
4
5
6
7
8
I
II
III
IV
V
TVu 91 / 0.2 µM Mn
TVu 91 / 50 µM Mn
TVu 1987 / 0.2 µM Mn
TVu 1987 / 50 µM Mn

Fig. 3

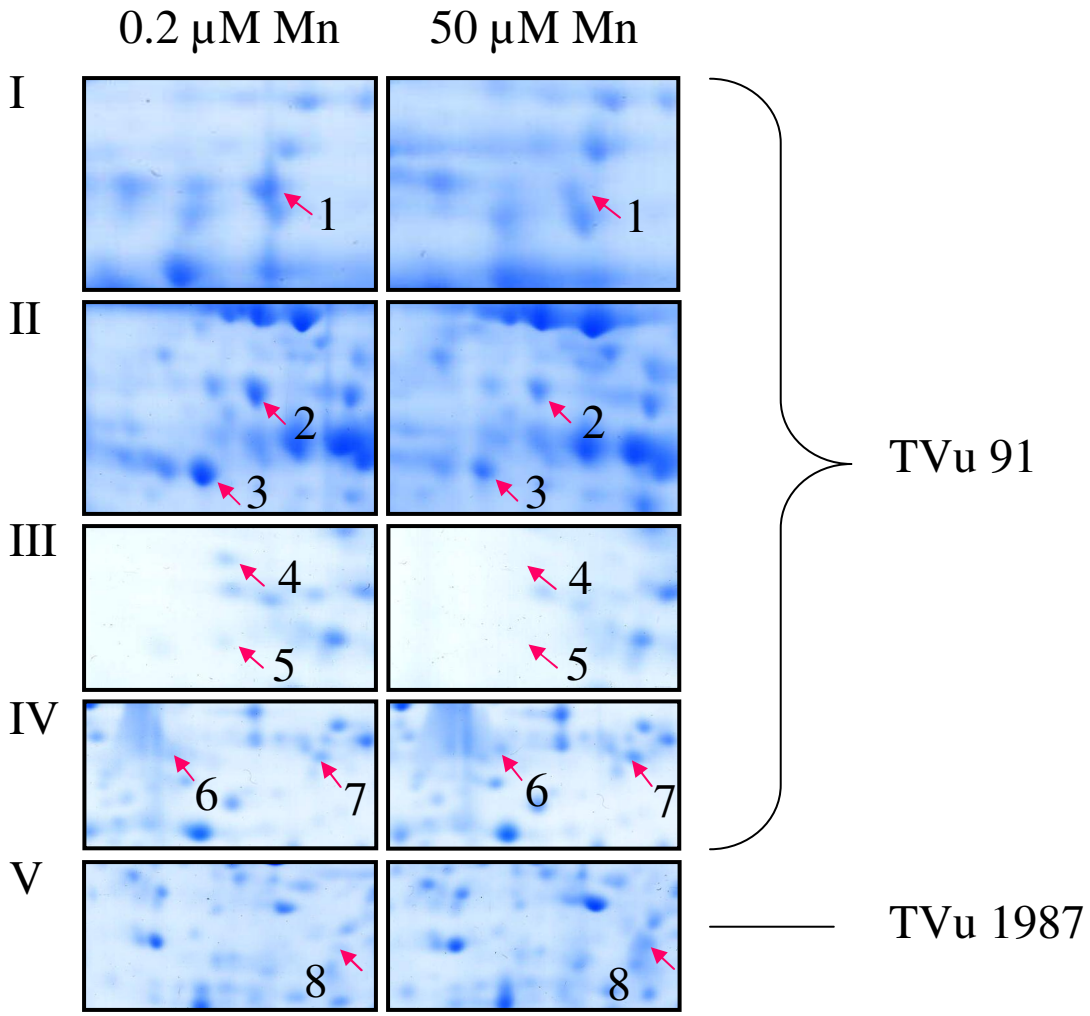


Fig. 4

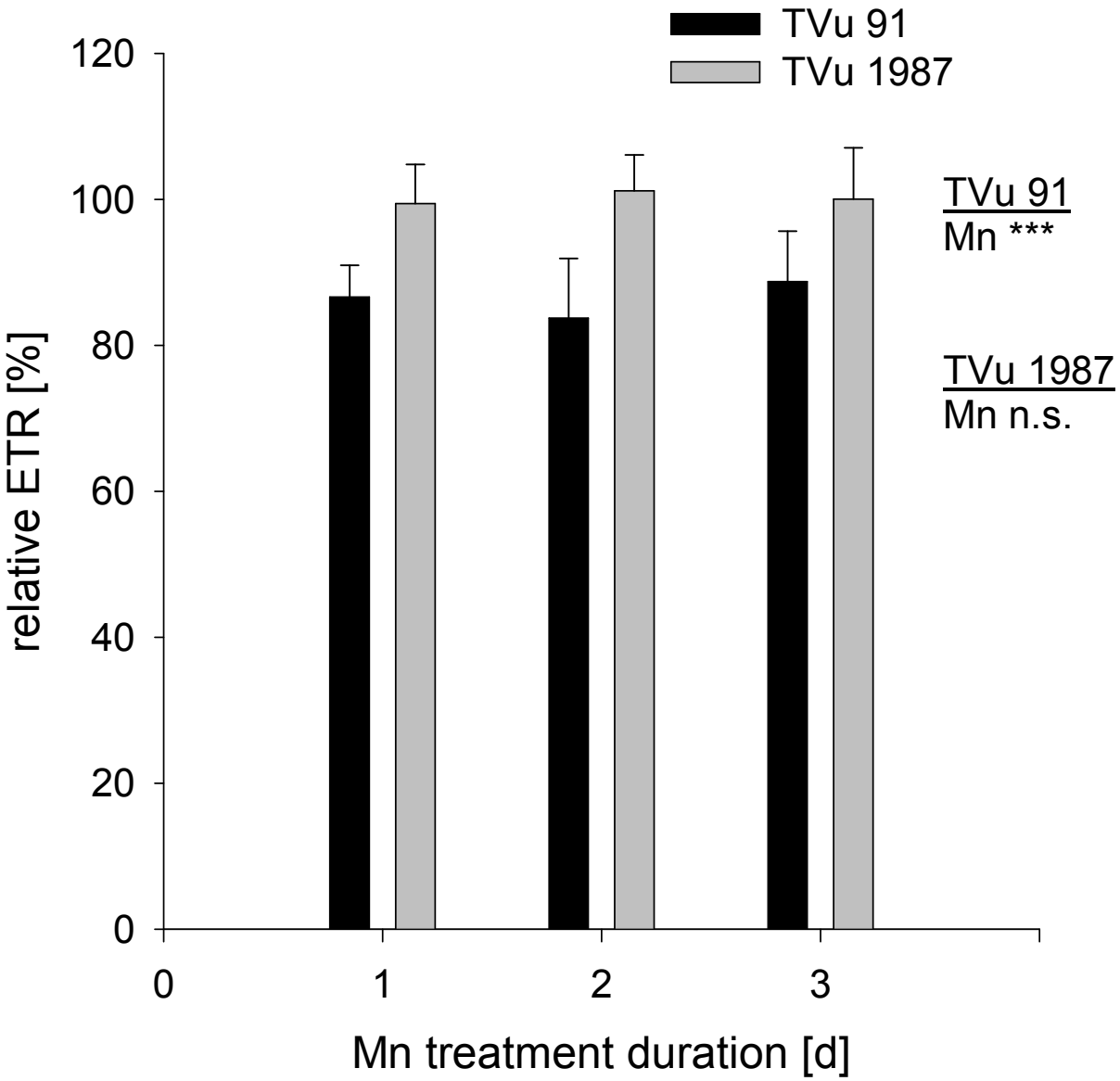

TVu 91
TVu 1987
120
100
80
60
40
20
0
relative ETR [%]
0
1
2
3
Mn treatment duration [d]
TVu 91
Mn ***
TVu 1987
Mn n.s.

Fig. 5

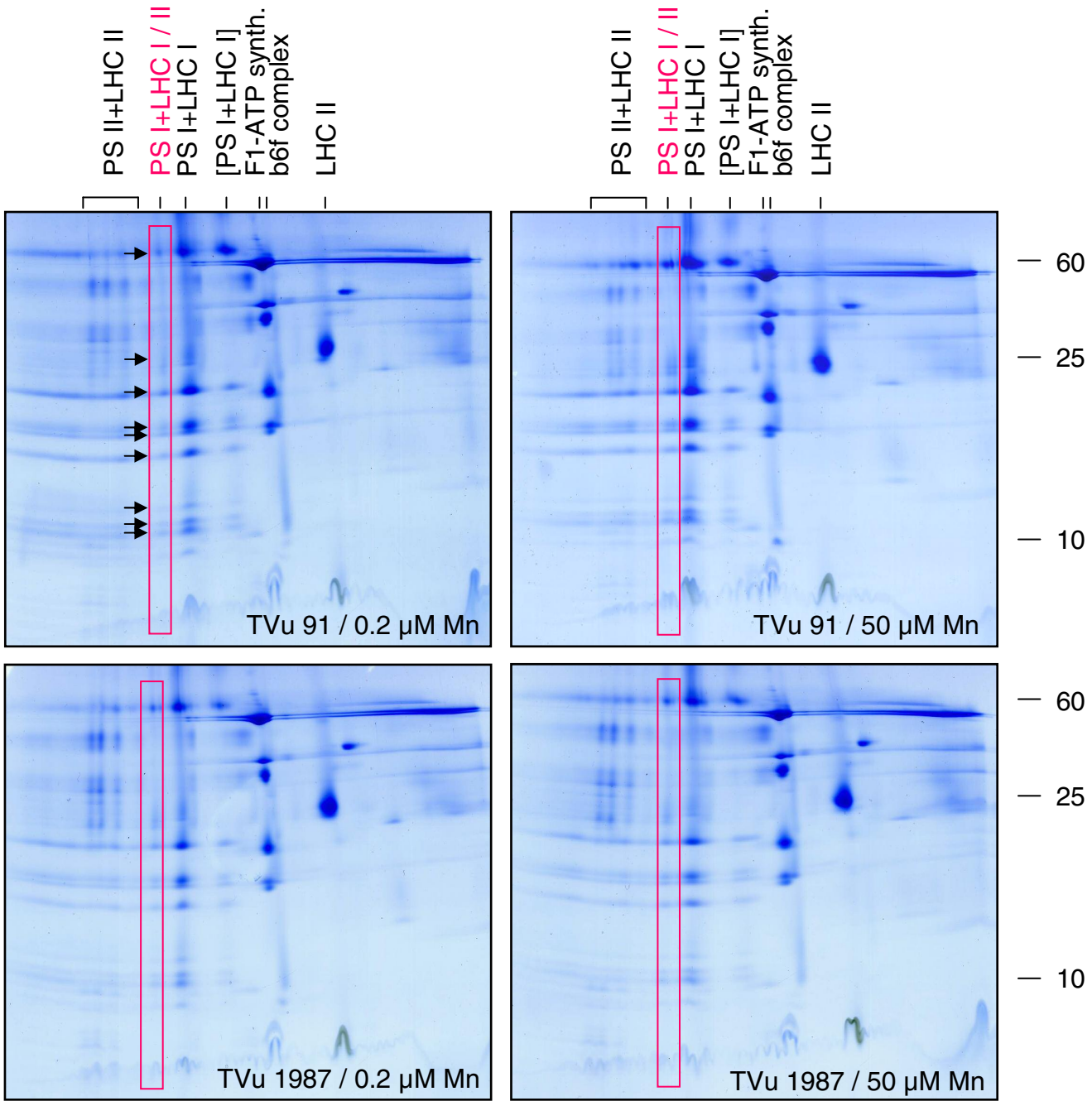

PS II+LHC II
PS I+LHC I / II
PS I+LHC I
[PS I+LHC I]
F1-ATP synth.
b6f complex
LHC II
TVu 91 / 0.2 µM Mn
TVu 91 / 50 µM Mn
TVu 1987 / 0.2 µM Mn
TVu 1987 / 50 µM Mn
60
25
10

Fig. 6

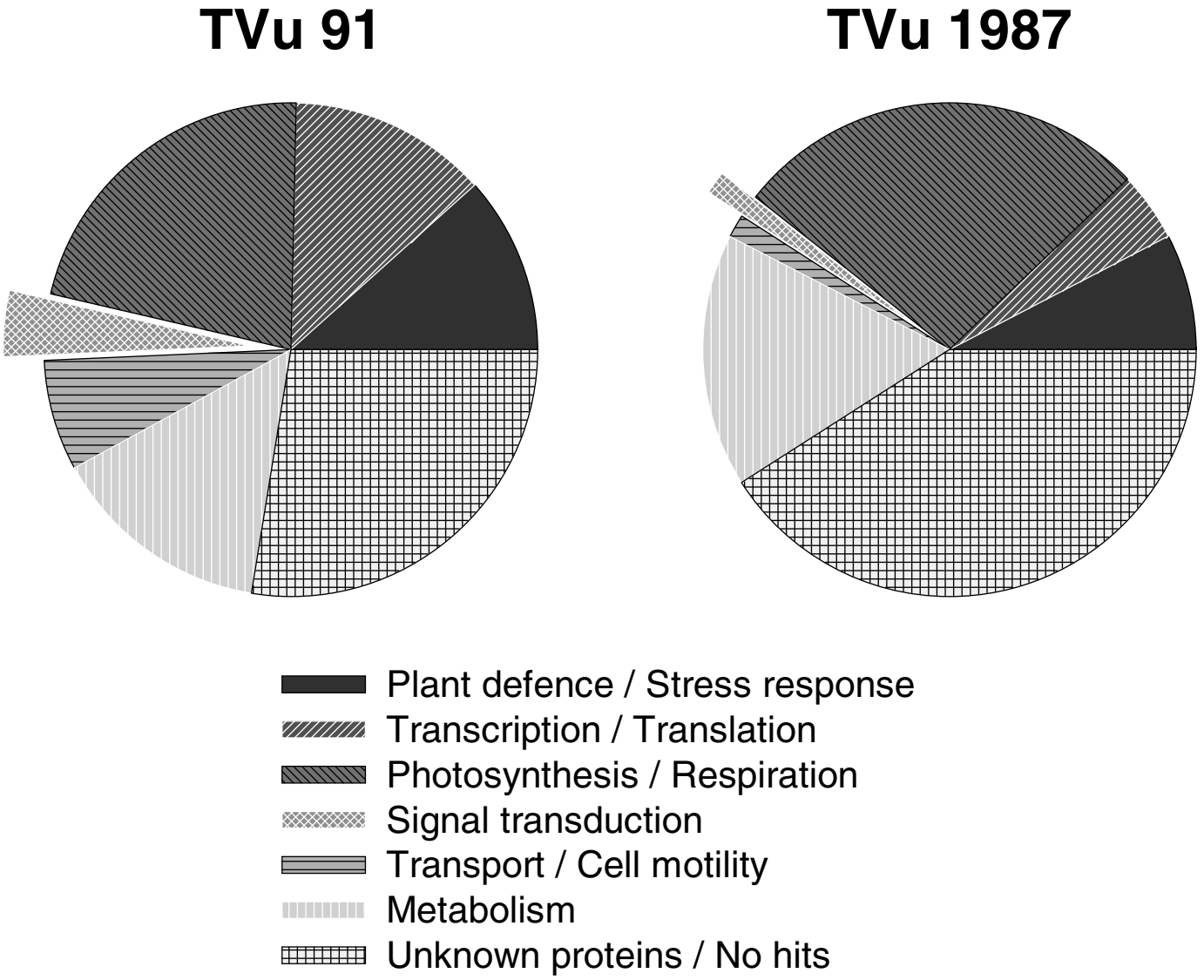
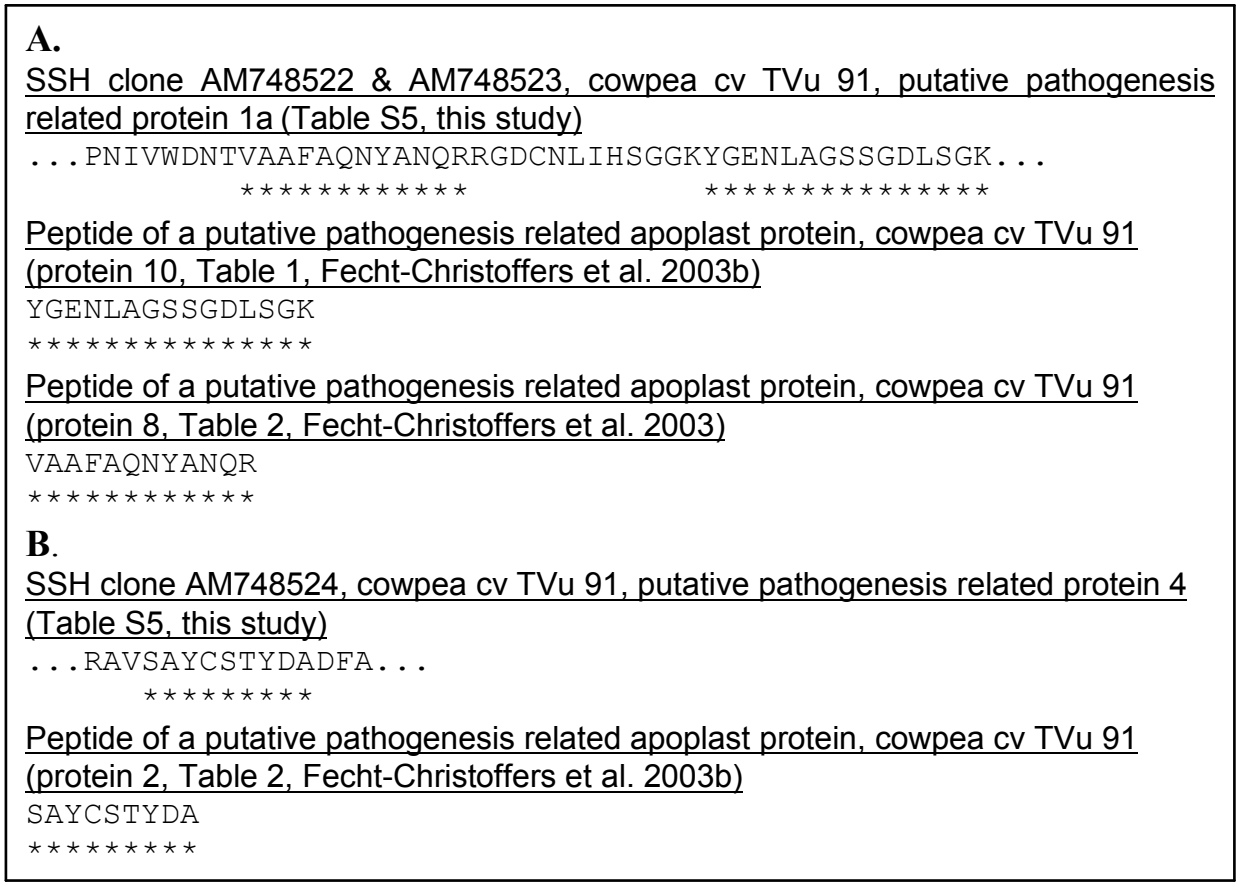

TVu 91
TVu 1987
Plant defence / Stress response
Transcription / Translation
Photosynthesis / Respiration
Signal transduction
Transport / Cell motility
Metabolism
Unknown proteins / No hits

Fig. 7


A.
SSH clone AM748522 & AM748523, cowpea cv TVu 91, putative pathogenesis related protein 1a (Table S5, this study)
...PNIVWDNTVAAFAQNYANQRRGDCNLIHSGGKYGENLAGSSGDLSGK...
Peptide of a putative pathogenesis related apoplast protein, cowpea cv TVu 91 (protein 10, Table 1, Fecht-Christoffers et al. 2003b)
YGENLAGSSGDLSGK
Peptide of a putative pathogenesis related apoplast protein, cowpea cv TVu 91 (protein 8, Table 2, Fecht-Christoffers et al. 2003)
VAAFAQNYANQR
B.
SSH clone AM748524, cowpea cv TVu 91, putative pathogenesis related protein 4 (Table S5, this study)
...RAVSAYCSTYDADFA...
Peptide of a putative pathogenesis related apoplast protein, cowpea cv TVu 91 (protein 2, Table 2, Fecht-Christoffers et al. 2003b)
SAYCSTYDA