Title

Metabolomic and Proteomic Analysis of Manganese Sensitivity and Tolerance in the Tropical Legume Cowpea (Vigna unguiculata L.)

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Introduction

Increased plant Mn availability typically occurs on acid and insufficiently drained soils particularly in the tropics and subtropics (Horst, 1980). In the tropical legume, cowpea (*Vigna unguiculata* L.), increasing Mn tissue concentrations typically results in brown spots in the epidermal layer of old leaves followed by chlorosis, necrosis and finally leaf shedding (Horst and Marschner, 1978b).

Several mechanisms promoting Mn tolerance have been suggested (El Jaoual and Cox, 1998). Evidence was provided that Mn compartmentation through Mn translocating transporters within the cell contributes to Mn tolerance (Peiter et al., 2007; Delhaize et al., 2003). In cowpea, Mn cellular compartmentation only partly explained differences in Mn tolerance (Maier, 1997). Instead, Fecht-Christoffers et al. (2003, 2006) provided evidence that apoplastic peroxidases (PODs) play a key role in Mn toxicity, suggesting the apoplast as a key site of Mn toxicity. However, the possibility that symplastic molecular events caused by excess Mn accumulation triggering apoplastic responses can not be ruled out.

Manganese toxicity in Mn-sensitive cowpea cultivars is delayed and reduced by Si supply (Iwasaki et al., 2002a, b). In Si-treated plants the additional Mn was more evenly distributed throughout the leaf (Horst and Marschner, 1978a). Moreover, Si-induced changes in apoplastic Mn-binding properties were found (Horst et al., 1999) even though they did not fully explain Si-mediated Mn tolerance.

Using a systems-biology approach integrating metabolomic, proteomic, and physiological methodologies, we have further characterized the role of apoplastic peroxidase (POD) isoenzymes and their modulation by specific phenylpropanoids in response to Mn and Si. Additionally, we have focused on the symplast of two cowpea cultivars with contrasting Mn tolerance. Results presented strongly indicate a major effect of Mn and Si on the symplastic proteome most likely triggered by impaired and altered photosynthesis. Also, remarkable constitutive and Mn-dependent differences in the metabolome and proteome between the genotypes could be found.

Materials and Methods

The metabolomic, proteomic and physiological methods have been extensively described earlier (Fecht-Christoffers et al., 2003; 2006; Führs et al., 2008, 2009).

Results and Discussion

Mn uptake

Elevated Mn supply increased the bulk-leaf Mn concentration in both the Mn-sensitive cultivar TVu 91 and the Mn-tolerant cultivar TVu 1987 independent of the Si supply, confirming that Si does not restrict Mn uptake in cowpea. The cultivar TVu 1987 showed higher Mn concentrations compared with TVu 91 from the beginning of the Mn treatment, confirming the greater Mn tolerance of TVu 1987. Increased Mn concentrations in the leaf tissue led to moderate toxicity symptoms in TVu 91 without Si after two days and in Si-treated plants after four days. The Mn-tolerant cultivar did not show any symptoms. The Si treatment slightly enhanced the Mn tissue concentrations in both cultivars, underlining the alleviation by Si of the expression of Mn toxicity symptoms.

Early Mn-induced changes in the apoplastic proteome in TVu 91

With increasing apoplastic Mn concentrations, PODs were secreted into the apoplast of TVu 91,

supporting the role of apoplastic PODs in the development of Mn toxicity. Also, proteins found to be affected by elevated Mn supply such as polygalacturonase-inhibiting proteins (PGIPs) and α-galactosidases suggest a Mn excess-induced modification of cell-wall development and function. Other proteins like acetylcholinesterase and GDSL-lipase 1 suggest coordinated changes in broad-sense signal transduction processes.

Characterization of the apoplastic antioxidative enzyme profile in response to Mn and Si supply
Due to the early response of PODs and their proposed key function in the development of Mn toxicity (Fecht-Christoffers et al., 2006), apoplastic PODs were specifically addressed. In a comparative study between the genotypes, the POD isoenzyme profile with and without additional Mn supply revealed a lower POD isoenzyme abundance in TVu 1987I independent of the Mn supply (Fig. 1A), suggesting a constitutive (apoplastic) Mn tolerance in TVu 1987 compared with TVu 91 (Führs et al., 2009).

BN-PAGE separation of wall proteins from differentially treated TVu 91 plants, with subsequent staining for guaiacol-POD activity, showed that Mn treatment led to enhanced release of PODs (Fig. 1B). This, together with the fact that Si did not affect the constitutive expression of POD isoenzymes but reduced the Mn-mediated induction of additional PODs, corroborates the key role of PODs as mediators of Mn toxicity. It is currently unclear which processes lead to the Si-mediated suppression/delay of Mn-induced POD isoenzyme release into the apoplast (Führs et al., 2009). Specific apoplastic POD isoenzymes were electroeluted and tested for their pH optima and response to specific phenols.

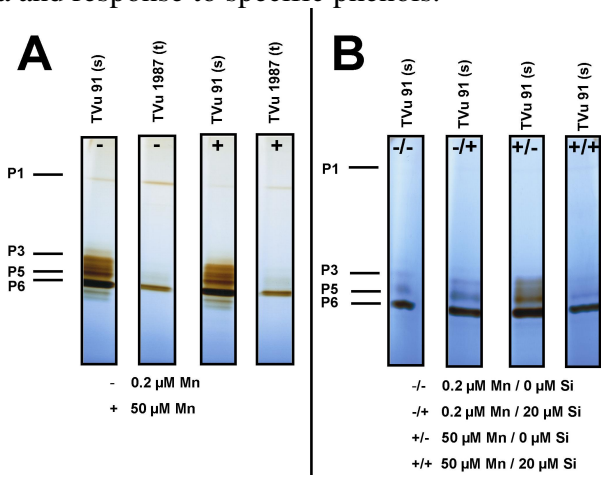

Fig. 1: (A) AWF (apoplastic washing fluid) proteins of the second oldest trifoliate leaves of the Mn-sensitive cultivar TVu 91 and the Mn-tolerant cultivar TVu 1987 and (B) AWF proteins after 0 and 4 d of Mn treatment of ±Si-treated plants of cultivar TVu 91 after separation with BN-PAGE and staining for guaiacol-POD activity. 67.5 µg (A) or 16 µg (B) protein were loaded onto each lane. Guaiacol-POD staining was done described in Führs et al. (2009).

All selected isoenzymes (P1-P6, Fig. 1) catalyzed both a H_2O_2-producing NADH-*per*oxidase and a H_2O_2-consuming guaiacol-POD activity (Führs et al., 2009) with pH optima for all isoenzymes of 5.5 and 6.5, respectively. Local apoplastic pH changes could specifically support either activity *in vivo*, but other factors than local apoplastic pH changes could additionally contribute to the induction of specific POD activities in the apoplast.

Thus, the role of phenols in the induction of particularly the NADH-*per*oxidase activity of the isoenzymes was characterized using different commercially available phenols. Among ten

phenols tested, *p*-coumaric acid and vanillic acid were the most effective cofactors for all isoenzymes. Benzoic acid hardly induced activity at higher concentrations but with a similar response pattern, whereas ferulic acid activated H_2O_2-producing activity only at a lower concentration. Other phenols did not induce NADH-*per*oxidase activity.

The potential inhibitory effect of phenols on NADH-*per*oxidase activity was studied by monitoring the effect of eight different phenols on *p*-coumaric acid-stimulated enzyme activity. Benzoic acid and vanillic acid actually enhanced *p*-coumaric acid-stimulated NADH-*per*oxidase activity. All other phenols inhibited NADH-*per*oxidase activity by about 50% to 90% for the other phenols. Apoplastic PODs activity modulation by specific phenols is important for the development of Mn toxicity symptoms in the apoplast of Mn-sensitive cultivars.

Characterization of proteomic changes in the symplast

IEF/SDS-PAGE of the water-soluble leaf proteome identified eight protein spots affected by elevated Mn supply (Fig. 2), seven in TVu 91 and one in TVu 1987 (Führs et al., 2008). Identification of the protein spots showed that in TVu 91, three proteins related to the Calvin cycle (Phosphoribulokinase, RubisCO activase and a RubisCO-binding protein) were reduced by more than 50%. Two PR proteins have been detected, one disappeared due to elevated Mn supply (PR-protein P4) and one was increased nearly 2.5-fold (PR-protein 5-1). Also a putative beta6 subunit of a proteasome was increased. An oxygen-evolving enhancer protein disappeared after additional Mn supply. In TVu 1987 another oxygen-evolving enhancer protein was increased about 4-fold after Mn treatment.

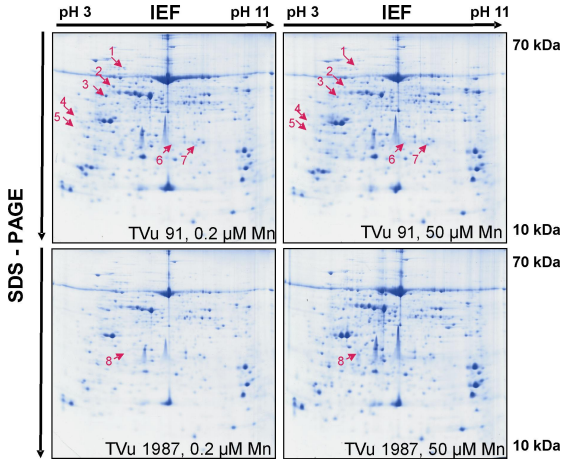

Fig. 2**:** 2-D IEF/SDS-PAGE resolution of the water-soluble leaf proteome of the sensitive cowpea cultivar TVu 91 and the tolerant cultivar TVu 1987 after treatment with 0.2 µM or with 50 µM Mn. Differentially expressed spots are marked by arrows and numbered consecutively. Adapted from Führs et al. (2008).

These changes can be divided into three classes: (1) photosynthesis (here both cultivars are concerned), (2) a directed general stress response represented by the PR-proteins, and (3) protein degradation as indicated by the change of the specific proteasome subunit.

Characterization of proteomic changes in the chloroplast

BN/SDS-PAGE of photosynthetic protein complexes revealed that Mn induced a subunit separation pattern similar to that for PSI, but with a slightly higher molecular weight (Fig. 3),

indicating a state I to state II transition of photosynthesis by binding of light harvesting complex II from PSII to PSI. This effect was greater in TVu 91 (1.7-fold) compared with TVu 1987 (1.2-fold) (Führs et al., 2008). State transitions are known to induce cyclic electron transport leading to increased ATP production at the cost of NADPH production for the Calvin cycle, which might explain the decrease of proteins of the Calvin cycle. Increased ATP production could be a response to a higher energy demand for the Mn stress response, particularly in TVu 91. Cyclic electron transport leads to a kind of redistribution of energy and also binds ferredoxin, an important reduction equivalent for many other processes in primary metabolism. This could also affect the metabolite composition.

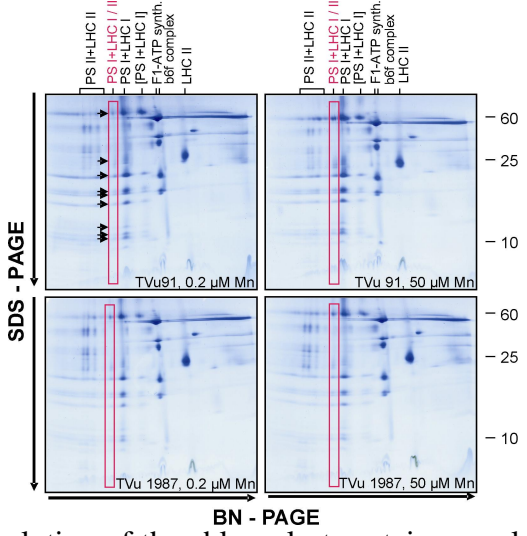

Fig. 3: Two-dimensional resolution of the chloroplast protein complexes of the cowpea cultivars TVu 91 and TVu 1987 by 2D Blue-native / SDS PAGE. Resolved protein complexes were identified on the basis of their subunit compositions according to Heinemeyer et al. (2004). Red boxed PSI + LHCI/II on the left of PSI + LHCI represents the subunit pattern of PSI after state transition. Arrows show protein subunits used for quantification. Adapted from Führs et al. (2008)

Characterization of metabolomic changes in the symplast and apoplast

A metabolite profiling study of the bulk-leaf, AWF and non-polar apoplastic fractions for both genotypes in response to Mn and Si treatments was carried out. A non-supervised Independent Component Analysis (ICA) was applied to the resulting dataset which allowed us to display treatment-dependent changes in variances due to quantitative and qualitative changes in metabolite pools (Fig. 4). In the bulk leaf the most important factor explaining variances in the experiment was the genotype. Hence, there exists a remarkable difference in the metabolome of both genotypes. The second most important factor was the Mn treatment. In Mn-control plants Si also led to sample clusters. Hence, all applied experimental conditions changed the metabolite pools. The disappearance of the Si effect in Mn-treated plants shows that Si on the one hand has a constitutive effect on the metabolome of both genotypes, but changes in the metabolome due to a Mn treatment can mask the Si-induced changes.

In the AWF the most important factor was the infiltration solution (Fig. 4B) indicating that metabolites extracted with water or NaCl solution differed not only in quantity but also quality. The second most important factor explaining the variances in the experiment could be assigned to the genotype. The additional extraction from the AWF yielding non-polar apoplastic

metabolites did not result in a clear metabolite clustering. Nevertheless, a slight clustering of specific treatments is observable here, too.

Identifying the responsible metabolites leading to such sample clusters could contribute to unraveling the basis for apoplastic Mn tolerance. Loadings (derived from ICA) weighting the contribution of each metabolite to the sample clusters allowed the identification of the most important metabolites in terms of Mn sensitivity and tolerance. The analysis of important metabolites suggests that metabolites not only in the apoplast but also in the bulk leaf are involved in the modulation of Si-mediated and genotypic Mn tolerance.

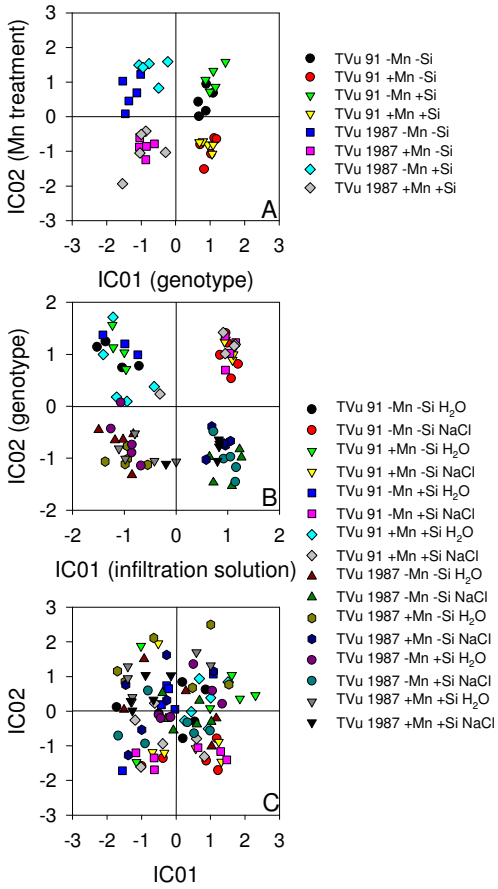

Fig. 4: ICA plot of the (A) bulk-leaf metabolites, (B) the apoplastic AWF_{H20} and AWF_{NaCl}-metabolites, and (C) non-polar apoplastic metabolites extracted from the AWF_{H20} and AWF_{NaCl} of the second oldest trifoliate leaves of TVu 91 and TVu 1987 as affected by Mn and Si treatments. Bulk-leaf, AWF- and non-polar apoplastic metabolites were extracted and measured as described (Führs et al., 2009). ICA was conducted using MetaGeneAlyse at http://metagenealyse.mpimp-golm.mpg.de.

In the apoplast, the differences in the concentrations of specific phenols between the cultivars and the response of these phenols to excess Mn and Si supply correlated with their ability to induce or inhibit the NADH-*per*oxidase activity and, therefore, with Mn-tolerance or Mn-sensitivity. The role of metabolites in stress sensing and signal transduction processes can be regarded as primary responses to Mn and Si. Organic acids function as antioxidants, scavengers of reactive oxygen species and NADH-*per*oxidase reaction intermediates, and as complexors for

Mn species. Genotypic Mn tolerance correlated with higher concentrations of antioxidants like ascorbic acid and its oxidized form. Changes in metabolites related to the primary carbon and nitrogen metabolism could be indicative of Mn stress reflecting their disturbance and need for rebalancing particularly as a consequence of changed/impaired photosynthesis.

In conclusion, a coordinated systems biology analysis of different leaf fractions allowed us to gain insights into the putative sequence of events leading to *Mn toxicity*, starting with a coordinated stress signal perception and transduction. With increasing Mn treatment duration, apoplastic peroxidases in interaction with specific phenols in the apoplast appear to be important for the development of *Mn toxicity* symptoms. Symplastically detected differences and changes in the metabolome, indicating changes in primary carbon and nitrogen metabolism seem to be indirect effects of changed/impaired photosynthesis, which in turn reflects a changed demand for energy in terms of a stress response. The *alleviative effect of Si* on Mn toxicity could be based on its ability to modulate the metabolome constitutively, particularly phenylpropanoid metabolism. Our research suggests that *Mn tolerance* in cowpea is based on several preformed genetically based mechanisms such as a higher symplastic and apoplastic proteomic and metabolomic antioxidative capacity together with lower capacities to induce oxidative stress.

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