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Physiological and proteomic characterization of manganese sensitivity and tolerance in rice (*Oryza sativa*) in comparison with barley (*Hordeum vulgare*)

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- **Background and Aims** Research on manganese (Mn) toxicity and tolerance indicates that Mn toxicity develops apoplastically through increased peroxidase activities mediated by phenolics and Mn, and Mn tolerance could be conferred by sequestration of Mn in inert cell compartments. This comparative study focuses on Mn-sensitive barley (*Hordeum vulgare*) and Mn-tolerant rice (*Oryza sativa*) as model organisms to unravel the mechanisms of Mn toxicity and/or tolerance in monocots.
- **Methods** Bulk leaf Mn concentrations as well as peroxidase activities and protein concentrations were analysed in apoplastic washing fluid (AWF) in both species. In rice, Mn distribution between leaf compartments and the leaf proteome using 2D isoelectric focusing IEF/SDS–PAGE and 2D Blue native BN/SDS–PAGE was studied.
- **Key Results** The Mn sensitivity of barley was confirmed since the formation of brown spots on older leaves was induced by low bulk leaf and AWF Mn concentrations and exhibited strongly enhanced H₂O₂-producing and consuming peroxidase activities. In contrast, by a factor of 50, higher Mn concentrations did not produce Mn toxicity symptoms on older leaves in rice. Peroxidase activities, lower by a factor of about 100 in the rice leaf AWF compared with barley, support the view of a central role for these peroxidases in the apoplastic expression of Mn toxicity. The high Mn tolerance of old rice leaves could be related to a high Mn binding capacity of the cell walls. Proteomic studies suggest that the lower Mn tolerance of young rice leaves could be related to Mn excess-induced displacement of Mg and Fe from essential metabolic functions.
- **Conclusions** The results provide evidence that Mn toxicity in barley involves apoplastic lesions mediated by peroxidases. The high Mn tolerance of old leaves of rice involves a high Mn binding capacity of the cell walls, whereas Mn toxicity in less Mn-tolerant young leaves is related to Mn-induced Mg and Fe deficiencies.

Key words: Apoplast, compartmentation, *Hordeum vulgare* ‘Baroness’, Mn sensitivity, Mn tolerance, *Oryza sativa* var. *japonica* ‘Guara’, proteome, photosynthesis.

INTRODUCTION

Manganese (Mn) excess represents an important factor limiting growth and crop yields particularly on acid and insufficiently drained soils with low redox potential (Foy, 1984; Schlichting and Sparrow, 1988). Soil amelioration such as liming and soil drainage is often not economic and only partly successful. Therefore, crop genotypes with high Mn resistance have to be developed. Large differences in Mn tolerance exist between plant species and cultivars within species (Heenan and Carter, 1976; Foy *et al.*, 1978; Horst, 1980). Barley is among the most Mn-sensitive crop species (Vlamiš and Williams, 1962; Vlamiš and Williams 1964), whereas rice is one of the most Mn-tolerant crops (Nelson, 1983; Lidon, 2001).

Presently, there are two major lines of evidence that provide insight into the basis of Mn toxicity and Mn tolerance in plant

species other than rice. Based on the assumption that cytosolic Mn²⁺ activity has to be kept low to avoid Mn²⁺ interfering with essential metabolic functions, pumping of Mn²⁺ from the cytosol into other cell compartments has been postulated to confer Mn tolerance (Wang and Evangelou, 1995; Pittman, 2005). It has been reported that in Mn-hyperaccumulators Mn tolerance is mainly due to the sequestration of Mn in the vacuoles (Fernando *et al.*, 2006; Dou *et al.*, 2008). There is convincing evidence from molecular studies on cation/Mn²⁺ transporters that this mechanism is important for Mn tolerance in yeast and a number of plant species (Hirschi *et al.*, 2000; Schaaf *et al.*, 2002; Wu *et al.*, 2002; Delhaize *et al.*, 2003, 2007; Peiter *et al.*, 2007; Li *et al.*, 2008). In conclusion, these studies strongly suggest that compartmentation of Mn in cellular compartments particularly in vacuoles and the endoplasmic reticulum (ER)/Golgi is an important feature of Mn plant tissue tolerance in

some plant species. This suggests that Mn toxicity is due to accumulation of Mn in Mn-sensitive cell compartments (cytosol and/or chloroplasts and mitochondria).

The second line of evidence for the mechanism of Mn toxicity and tolerance in plants is based mainly on work with cowpea (*Vigna unguiculata*). Typical Mn stress-induced toxicity symptoms develop primarily on older leaves as distinct brown spots located in the leaf apoplast of the epidermis (Horst, 1982). The brown spots consist of oxidized Mn and oxidized phenolic compounds (Wissemeier and Horst, 1992). This, and greatly enhanced activities of H₂O₂-producing and H₂O₂-consuming peroxidases (PODs) particularly in the apoplastic washing fluid (AWF), suggests that the leaf apoplast is the decisive compartment for the development or avoidance of Mn toxicity in cowpea (Fecht-Christoffers *et al.*, 2006, 2007). Proteome analysis of the AWF confirmed an enhanced release of PODs into the leaf apoplast, but in addition revealed the release of several other apoplastic stress response proteins in response to advanced Mn stress (Fecht-Christoffers, 2003b). In subsequent studies we provided evidence suggesting that apoplastic H₂O₂-producing POD-enhancing and inhibiting phenols in addition to Mn play a major role in modulating genotypic and silicon (Si)-enhanced differences in Mn tolerance (Fecht-Christoffers *et al.*, 2006; Fühns *et al.*, 2009). Results from combined proteomic/transcriptomic/physiological approaches clearly indicate that toxic Mn supply affects symplastic functions particularly related to photosynthesis (Fühns *et al.*, 2008). Consistent with a decrease in the electron transfer rates (ETRs), a state I to state II transition of photosynthesis was observed in Mn-stressed cowpea leaves after 72 h of elevated Mn supply. Also, chloroplastic proteins important for CO₂ fixation and photosynthesis were of lower abundance upon Mn stress. The relationships between these early Mn toxicity-induced lesions in the chloroplast and the above-described apoplastic reactions leading to the typical Mn toxicity symptoms remain to be elucidated.

Paddy rice is cultivated in soils with a highly reduced state and thus very high Mn availability. Since oxidation of Mn²⁺ requires a higher redox potential than Fe²⁺ (Mansfeldt, 2004), the maintenance of an oxidized zone at the root surface successfully oxidizing Fe²⁺ (Zhang *et al.*, 1998; Kirk, 2003) does not immobilize Mn²⁺ sufficiently. Thus rice accumulates and tolerates high concentrations of Mn in the plant tissue, particularly leaves. Whereas Mn concentrations of >150 µg g⁻¹ d. wt lead to Mn toxicity symptoms in barley (Vlamiš and Williams, 1964; Demirevska-Kepova, 2004), rice can tolerate up to 5000 µg g⁻¹ d. wt without showing any toxicity symptoms (Vlamiš and Williams, 1964). Lidon and co-workers (Lidon and Teixeira, 2000; Lidon, 2001; Lidon *et al.*, 2004) concluded that the high leaf Mn tolerance of rice is not due to Mn accumulation and inactivation in vacuoles but to the enhanced binding of Mn in a chloroplast-localized protein which showed superoxide dismutase (SOD) activity. Nevertheless, the general scarcity of physiological and, particularly, molecular information on Mn tolerance in rice makes additional work necessary to elucidate the high Mn tolerance of this plant species.

The present study investigates Mn toxicity and Mn tolerance mechanisms comparing the most Mn-sensitive and Mn-tolerant plant species barley and rice, respectively, as

model organisms. The comparison of the two species sets the baseline of our ongoing approaches to identify Mn tolerance genes in rice which could be expressed in barley as a proof of function. This approach has previously been convincingly applied to demonstrate the decisive role of the *TALMT1* gene in Al resistance (Delhaize *et al.*, 2004). Proteomic and physiological approaches previously applied to unravel Mn toxicity and tolerance in legumes were used in order to verify whether the same mechanisms also act in cereals.

MATERIALS AND METHODS

Plant cultivation

Rice (*Oryza sativa* 'Guara') and barley (*Hordeum vulgare* 'Baroness') were grown hydroponically in a growth chamber under controlled environmental conditions at 25/20 °C day/night temperatures, 75 ± 5 % relative humidity and a photon flux density of 150 µmol m⁻² s⁻¹ photosynthetic active radiation (PAR) at mid-plant height during a 16 h photoperiod. After germination in 1 mM CaSO₄ for 10–12 d, seedlings were transferred to nutrient solution with 20 or 30 plants of barley and rice in 5 L pots, respectively. The nutrient solution was constantly aerated only for barley. The composition of the nutrient solution was (µM): Ca(NO₃)₂ 1000 for barley or 500 Ca(NO₃)₂ and 500 NH₄NO₃ for rice, KH₂PO₄ 100, K₂SO₄ 375, MgSO₄ 325, FeEDDHA 20, NaCl 10, H₃BO₃ 8, CuSO₄ 0.2, ZnSO₄ 0.2, Na₂MoO₄ 0.05. Barley plants additionally received 0.2 µM and rice plants 1 µM MnSO₄ according to their Mn requirements for optimum growth (preliminary experiments). Half of the plants received additional Mn (50 µM, this Mn supply has proved to be most appropriate for the study of Mn toxicity in previous and preliminary studies) for up to 4 d when the fifth leaf was fully developed (approx. 34–38 d after transfer to nutrient solution), whereas the other plants received 0.2/1 µM continuously. During pre-culture the nutrient solution was changed every 2–3 d in order to prevent nutrient deficiencies. During the Mn treatment the nutrient solution was changed twice a day to maintain a constant Mn supply.

Harvesting of plant material

Leaf material for physiological and proteomic analyses was harvested from the older (second, fourth) and younger (fourth, sixth) leaves and immediately frozen in liquid nitrogen. Until further analysis the material was stored at -80 °C. For mineral analysis leaf material was dried at 65 °C.

Apoplastic washing fluid

Apoplastic washing fluid was extracted from leaves using a vacuum infiltration/centrifugation technique. Leaf blades were excised from the plants and cut in the middle. Leaf halves were washed in chilled double-demineralized water (ddH₂O) in order to reduce cytosolic contamination. Leaf halves from 20 leaves were combined and infiltrated with chilled ddH₂O by reducing the pressure four times for 2.5 min followed by a slow relaxation. The AWF was recovered by centrifugation

of the blotted cuttings at 4000 g and 4 °C for 5 min in 500 mL centrifugal devices (Nalgene, Nalge Nunc). The AWF was immediately frozen in liquid nitrogen and, until further analysis, stored at -80 °C. In order to estimate the apoplastic volume leaves were weighed before and after infiltration.

Mineral analysis

For Mn determination the dried and milled plant material was dry-ashed overnight at 480 °C. Samples were prepared and measured as described by Führens *et al.* (2009).

Total Si was determined following the protocol described by Iwasaki *et al.* (2002a, b) using 10 mg of dried plant material.

For determination of Mn concentrations in the AWF, 50 µL samples were filtered through centrifugal filter devices (0.2 µm, GHP Nanosep Mt centrifugal device, PAL Lifesciences, Ann Arbor, MI, USA) for 10 min at 4000 g. Filtrates were diluted 1 : 50 with ddH₂O and measured using inductively coupled plasma-optical emission spectroscopy (ICP-OES; Spektro Analytical Instruments GmbH, Kleve, Germany).

Isolated chloroplasts were dry-ashed overnight at 480 °C. The ash was dissolved in 300 µL of 6 M HCl supplemented with 1.5 % (w/v) hydroxylammonium chloride. Samples were diluted 1 : 10 with ddH₂O before analysis using ICP-OES. For the analysis of Mn in the individual chloroplast isolation fractions, 300 µL sub-samples were filtered through centrifugal filter units (see above) and centrifuged for 10 min at 4000 g. Filtrates were diluted 1 : 10 with ddH₂O and measured using an ICP-MS (7500CX, Agilent, Böblingen, Germany). Results were expressed as a percentage of the total Mn concentration in the leaf fresh matter.

Chlorophyll fluorescence

Chlorophyll fluorescence of the older leaves was determined using a Mini-PAM fluorometer (Waltz, Germany). Measurements were made on dark-adapted plants using the light induction curve program: 1 min after a first saturation pulse actinic light was turned on and from then every 30 s a new saturation pulse was applied over a period of 6.5 min. The yield, ETR, nP, nQ and NPQ values were calculated using the included software. All measurements were repeated four times and submitted to statistical analysis.

Determination of the chlorophyll content

Prior to harvest of the leaves their chlorophyll contents were determined according to Schulte auf'm Erley *et al.* (2007) using a portable chlorophyll meter (SPAD). The means of five measurements over the whole leaf blade of the fourth and sixth leaf are shown.

Determination of protein contents

About 100 mg of frozen leaf fresh matter was homogenized in 1 mL of chilled ddH₂O in a swinging mill (MM200, Retsch, Germany) with a frequency of 30 s⁻¹ for 2 min. The homogenate was centrifuged at 5000 g for 1 min at 4 °C, and the supernatant was used for protein quantification using the 2D Quant

Kit (GE Healthcare, Piscataway, NJ, USA) according to the manufacturer's instructions.

Protein in the AWF was quantified according to the method described by Bradford (1976).

For 2D isoelectric focusing IEF/SDS-PAGE protein was quantified after resolubilization of precipitated proteins using the 2D Quant Kit (GE Healthcare, USA) according to the manufacturer's instructions.

Determination of the phenol concentration in the AWF

The phenol concentration in the AWF was measured according to the method described by Fecht-Christoffers *et al.* (2006).

Determination of H₂O₂-consuming guaiacol-POD and H₂O₂-producing NADH-POD activity in the AWF

Both H₂O₂-consuming guaiacol-POD and H₂O₂-producing NADH-POD activities were determined as described by Führens *et al.* (2009).

Determination of the cytosolic contamination of the AWF using malate dehydrogenase (MDH) as marker enzyme

The MDH activity was determined according to the method described by Bergmeyer and Bernt (1974) including the preceding generation of oxalacetate from aspartate and α-ketoglutarate using glutamic-oxalacetic-transaminase (GOT). The decrease in absorption due to NADH oxidation was determined spectrophotometrically at λ = 340 nm (Uvikon 943 Double Beam, UV/ VIS Spectrophotometer, Kontron Instruments) for 1.2 min. Results were expressed as a percentage of the activity obtained from freshly prepared leaf homogenates.

Isolation of chloroplasts

Chloroplasts were isolated according to Tanaka *et al.* (2004) with minor modifications. Leaf material was homogenized in 100 mL of 'homogenization buffer' per 10 g of fresh matter [350 mM sorbitol, 2 mM EDTA (pH 8.0), 25 mM HEPES-NaOH, pH 7.0] using a blender for 4 × 3 s. The homogenate was filtered through four layers of gaze (50 µm mesh size) and centrifuged at 1200 g for 5 min at 4 °C. The resulting pellet was resuspended in 12–15 mL of 'homogenization buffer' containing 0.35 M sorbitol, 2 mM EDTA and 25 mM HEPES (pH 7.0), and loaded onto a discontinuous gradient formed by 10, 30 and 80 % Percoll. The gradient formation and separation of chloroplasts was done by centrifugation at 2600 g for 40 min at 4 °C. Intact chloroplasts were removed from the 30%/80 % Percoll boundary layer and subsequently centrifuged at 1800 g for 5 min at 4 °C. The resulting pellet contained the 'residue Mn' fraction. The supernatant was removed and the resulting pellet was resuspended and centrifuged at 1800 g for 5 min at 4 °C. After weighing the pellet containing the 'chloroplastic Mn', it was resuspended in homogenization buffer with a final concentration of 100 mg mL⁻¹. Sub-samples were taken from each washing step in order to determine the labile bound Mn fraction during the chloroplast

isolation procedure ('soluble Mn'). Isolated chloroplasts and sub-samples were immediately frozen in liquid nitrogen and stored at -80°C until further analyses.

2D IEF/SDS-PAGE of soluble leaf homogenate and soluble chloroplastic proteins

Proteins from the leaf homogenates were extracted according to Hurkman and Tanaka (1986) with three biological replicates for both Mn-control and Mn-treated plants. Each replicate comprised a pool of 11 individual plants.

For investigation of the water-soluble chloroplastic proteome, isolated chloroplasts were frozen in liquid nitrogen. After thawing and centrifugation, the supernatant, containing the soluble chloroplastic proteins, was precipitated and used for IEF/SDS-PAGE as described by Fühns et al. (2008). The recovered protein had to be combined into one sample due to the low chloroplast yields of the isolation procedure in rice.

2D Blue native BN/SDS-PAGE of photosynthetic protein complexes from thylakoid membranes

Proteins from thylakoid membranes of 10 mg of isolated chloroplasts were prepared and photosynthetic protein complexes were separated by 2D BN/SDS-PAGE as described by Heinemeyer et al. (2004).

Staining of 2D gels

Gels of 2D IEF/SDS-PAGE and 2D BN/SDS-PAGE were stained with colloidal Coomassie blue (Neuhoff et al., 1985, 1990).

2D IEF/SDS-polyacrylamide gels made from water-soluble chloroplastic proteins were stained with silver nitrate using an adapted method described by Heukeshoven and Dernick (1988).

Visualization of gels of 2D IEF/SDS-PAGE and 2D BN/SDS-PAGE

Coomassie colloidal-stained gels were scanned with 300 dpi (Epson Expression 1600 Scanner) and stored as .tiff files. For analysis of 2D IEF/SDS-polyacrylamide gels the files were transformed into .mel files. Subsequently the spots were detected, quantified and the three biological replications statistically analysed using Imagemaster™ 2D PLATINUM Software 6.0 (GE Healthcare, USA). The quantification parameter was the relative spot volume (%Vol, spot volume of one spot in relation to the sum of all detected spots on the gel) in order to eliminate protein loading differences. Only protein spots with a minimum spot volume of 0.01 % were included. Spots of significantly changed volumes (at least $P < 0.05$ and a spot ratio between the treatments of at least < 0.5 or > 2.0) were sequenced.

Mass spectrometric protein analysis and data interpretation

Mass spectrometric protein analysis and data interpretation were performed as described by Fühns et al. (2008, 2009).

Statistical analysis

Statistical analysis, if not mentioned otherwise, was carried out using SAS Release v8.0 (SAS Institute, Cary, NC, USA) or the Multi Experiment Viewer MeV 4.1 (Saeed et al., 2003). Results from analysis of variance and correlation analysis are given according to their level of significance as ***, ** and * for $P < 0.001$, 0.01 and 0.05, respectively. Results of the mean comparisons are given at $P < 0.05$ (Tukey).

RESULTS

Comparative response of barley and rice to elevated Mn supply

Mn uptake and expression of toxicity symptoms. Elevated Mn supply for 2–4 d led to an increased Mn uptake in both barley and rice as represented by higher Mn concentrations in the leaf dry matter (Fig. 1A, B). Also apoplastic Mn concentrations increased at least in tendency in both plant species (Fig. 1C, D). In contrast to barley, rice had 30-fold higher Mn concentrations in both the bulk leaf and the AWF at the end of the Mn treatment without showing any symptoms, emphasizing the high Mn tolerance of rice.

Toxicity symptoms in the form of typical brown spots appeared in barley on older leaves, particularly at the leaf tip. Typical symptoms are shown in Fig. 2 (top). Surprisingly, in contrast to observations made by Wang et al. (2002), brown spots in rice developed only on younger leaves (Fig. 2). Whether the brown spots are similar in nature to those in cowpea and barley on old leaves (accumulations of MnO_2 and oxidized phenols) is not yet known.

The role of the apoplast. Previous studies on Mn toxicity focusing on legumes revealed a strong relationship between the occurrence of typical toxicity symptoms in the form of brown spots and an increase in apoplastic POD activities (Fecht-Christoffers et al., 2006, 2007; Fühns et al., 2009). Only in barley was a strong relationship between the appearance of brown spots and the Mn tissue content and the guaiacol-POD activity found (Fig. 3A, B), emphasizing the key role of apoplastic PODs in Mn toxicity development in barley.

Both H_2O_2 -consuming guaiacol-POD and H_2O_2 -producing NADH-POD activities were an order of magnitude higher in the AWF of barley than in rice (Fig. 4). Whereas the former increased with increasing Mn concentrations in the leaves in both species (Fig. 4A, B), the NADH-POD activity was strongly enhanced by Mn in barley but remained at a low level in rice (Fig. 4C, D).

Understanding Mn tolerance of rice

The results of the comparison of barley and rice are consistent with the assumption derived from studies of cowpea (see Introduction); the expression of Mn toxicity as revealed by the formation of brown spots on older leaves is related to the activities of apoplastic PODs. Since the activities of PODs are low in rice, Mn can accumulate in leaves to high concentrations without the appearance of symptoms of Mn toxicity. However, this requires other Mn tolerance mechanisms particularly in the leaf symplast. In this respect the appearance

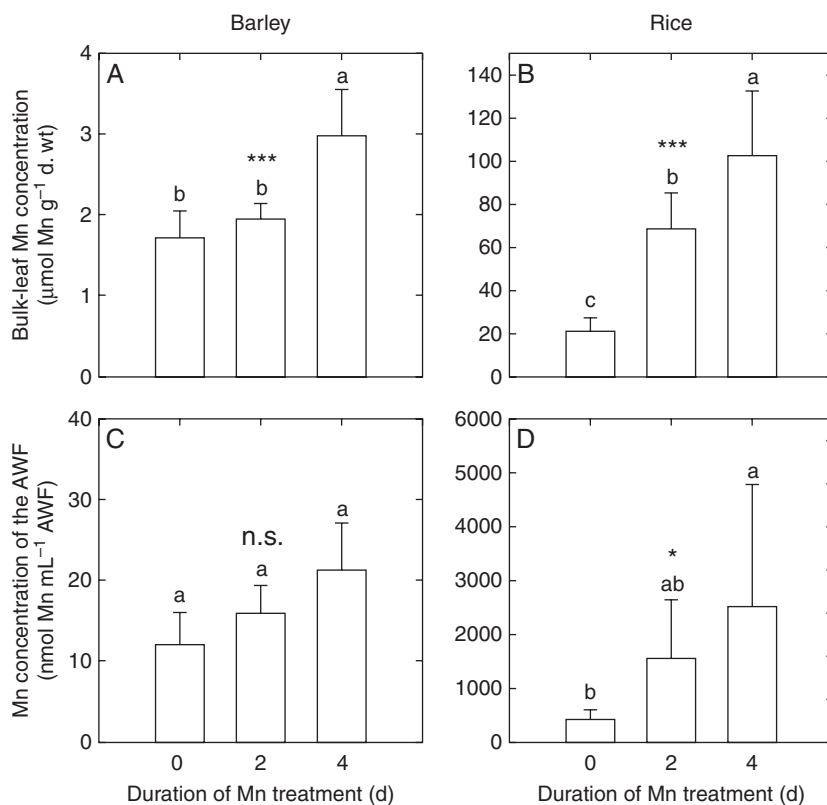


FIG. 1. Bulk leaf Mn concentrations and Mn concentrations of the AWF of the second oldest leaves of barley and rice. After pre-culture at $0.2 \mu\text{M}$ Mn, the Mn supply was increased to $50 \mu\text{M}$ for 0, 2 and 4 d, or plants received $0.2 \mu\text{M}$ Mn continuously. Results of one-factorial ANOVA are given as ***, * and n.s. for $P < 0.001$, 0.05 , and not significant, respectively. Means with different letters indicate significant differences at $P < 0.05$ (Tukey test).

of Mn toxicity symptoms in rice in young but not in old leaves (see Fig. 2) seems particularly intriguing. Thus, old and young leaves were systematically compared in the following studies. The appearance of Mn toxicity symptoms on young leaves cannot be explained by higher Mn concentrations, which were significantly lower than in old leaves (Supplementary Data Fig. S1, available online). Also, both guaiacol-POD and NADH-POD activities were lower and not affected by Mn supply in the young, Mn-sensitive leaf (Supplementary Data Fig. S2), casting doubt on whether the mechanism of Mn toxicity operating in old leaves of barley is the same as that in young leaves of rice. Mn toxicity in young leaves is also characterized by a contrasting response with respect to chlorophyll content (measured as SPAD) of young vs. old leaves (Fig. 5, highly significant $\text{Mn} \times \text{leaf age} \times \text{treatment}$ duration interaction). The increase in SPAD values with age in young leaves did not occur at elevated Mn supply, suggesting Mn excess-induced leaf chlorosis. However, photosynthesis as characterized by gas exchange and leaf chlorophyll fluorescence was not affected by Mn, either in young or in old leaves (not shown).

Compartmentation of Mn in rice leaf blades. Studies by Lidon and co-workers (see Introduction) suggested that the binding of Mn to a chloroplast-localized protein is involved in the high Mn tolerance of rice leaves. Also previous work in cowpea indicated that the chloroplast was a primary target of Mn at toxic Mn supply (Führes *et al.*, 2008). Therefore, in a

first approach, we characterized the proteome of chloroplasts. A chloroplast isolation method (Tanaka *et al.*, 2004) was adapted for proteomic characterization of thylakoid membrane protein complexes and water-soluble proteins of rice leaf chloroplasts. A 2D BN/SDS-PAGE separation of thylakoid membrane protein complexes as described by Heinemeyer *et al.* (2004) and Führes *et al.* (2008) did not reveal differences between Mn treatments and leaf ages. Therefore, an Mn-enhanced state 1 [photosystem II (PSII)] to state 2 (PS I) transition of light harvesting complex II, as has been observed in cowpea (Führes *et al.*, 2008), could not be detected either in old or in young leaves (Fig. 6).

However, the postulated Mn-binding protein could also be present in the water-soluble, hence not membrane-bound protein fraction of the chloroplast. Therefore, in an additional experimental approach we submitted water-soluble proteins, which remained in the supernatant during the chloroplast-protein complex isolation, to a 2D IEF/SDS-PAGE (Fig. 7). After separation, the gels were stained with silver nitrate to visualize as many proteins as possible. Comparative visual inspection of the 2D resolutions led to the identification of at least seven proteins (arrows) which were changed in abundance due to elevated Mn supply for 72 h. However, the protein quantities were too small to identify the proteins by nano liquid chromatography-tandem mass spectrometry (LC-MS/MS), and thus their quantitative significance in Mn binding/detoxification is questionable.

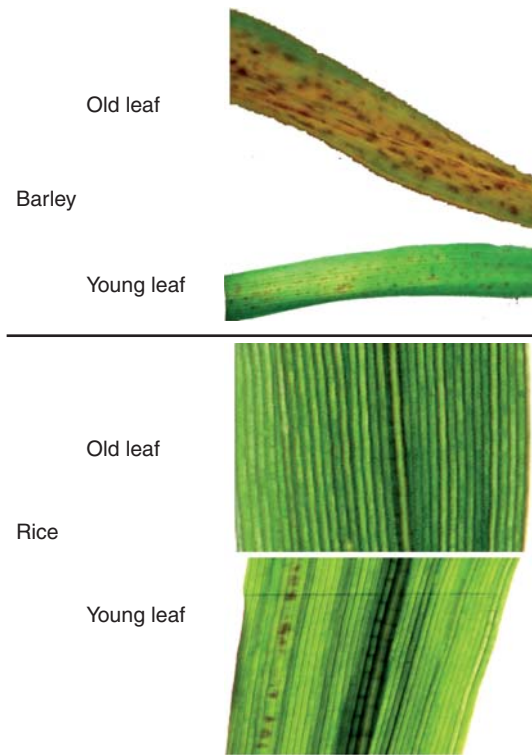


FIG. 2. Close-up views of leaf blades of old and young leaves of barley (top) and rice (bottom) after 4 d of elevated Mn supply. In barley, symptoms in the form of brown spots appear on leaves of both ages, whereas only in some replicates of rice did brown spots appear on younger leaves. In barley, old leaves additionally showed chlorosis, suggesting enhanced leaf senescence.

By determining the Mn contents in the different fractions during the chloroplast isolation procedure, we were able to calculate roughly the distribution of the bulk leaf Mn between the 'chloroplastic', the 'soluble' and the 'residue' Mn (Fig. 8). The results clearly show that with increasing bulk leaf Mn content, in Mn-treated plants in old as well as in young leaves, the contribution of chloroplastic Mn to the total bulk leaf Mn decreases. This does not support the view that sequestration

of Mn in the chloroplast contributes to Mn tolerance. It rather appears that the sequestration of Mn in the leaf residue (mainly cell walls) is more important.

Changes in the total water-soluble leaf proteome. In an attempt to identify leaf proteins which are involved in the response of leaves to high Mn supply, we submitted old and young leaves to a systematic proteomic analysis. The total soluble protein concentration was, at least in tendency, decreased by Mn treatment more in the young than in the old leaves (Supplementary Data Fig. S3, available online). A thorough evaluation of gels after 2D IEF/SDS-PAGE separation of the bulk leaf extracts using Image Master™ 2D PLATINUM Software 6.0 (GE Healthcare, USA) allowed us to identify a range of proteins affected in abundance in response to Mn treatment and leaf age (Fig. 9). Close-ups of gel regions containing proteins differing in abundance and alignments of peptide sequences are given in Supplementary Data Figs S4–S7 and Tables S1–S4, respectively.

Subsequent peptide sequencing of the identified proteins and BLAST search at the NCBI homepage identified the proteins listed in Table 1. All BLAST hits could be linked to the genus *Oryza*. In the Mn-treated older leaf, three proteins were significantly >2-fold more highly abundant compared with control plants: a putative inorganic pyrophosphatase, a probenazole-inducible protein (PBZ1) and a protein belonging to a universal stress protein (Usp) family. Younger leaves responded to elevated Mn supply with decreased abundance of a chloroplast translational elongation factor (Tu). The 50S ribosomal protein L11 completely disappeared at elevated Mn supply, indicating a change in protein synthesis. A comparison of older and younger leaves at optimum Mn supply revealed lower abundance of an enolase involved in cytosol-localized glycolysis. Two other proteins were >2-fold increased in abundance: an oxygen-evolving enhancer protein 1 (chloroplast precursor) and a ribonuclease T2 family protein.

Independently of the occurrence of toxicity symptoms, the greatest number of Mn treatment-affected proteins (seven) have been identified when young and old leaves of

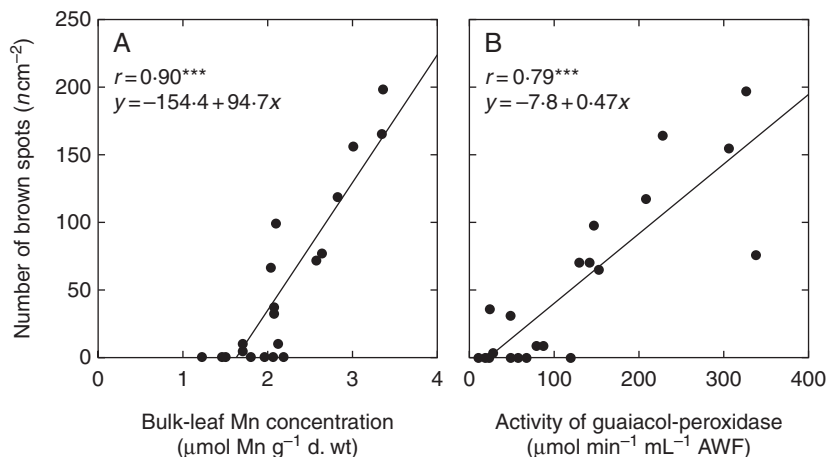


FIG. 3. The number of brown spots on the second oldest leaves of barley in relation to the bulk leaf Mn concentration (A) and the activity of the guaiacol-POD in the AWF (B). After pre-culture at 0.2 μM Mn, the Mn supply was increased to 50 μM for 0, 2 and 4 d, or plants received 0.2 μM Mn continuously. Results of the regression analysis are given as *** for $P < 0.001$.

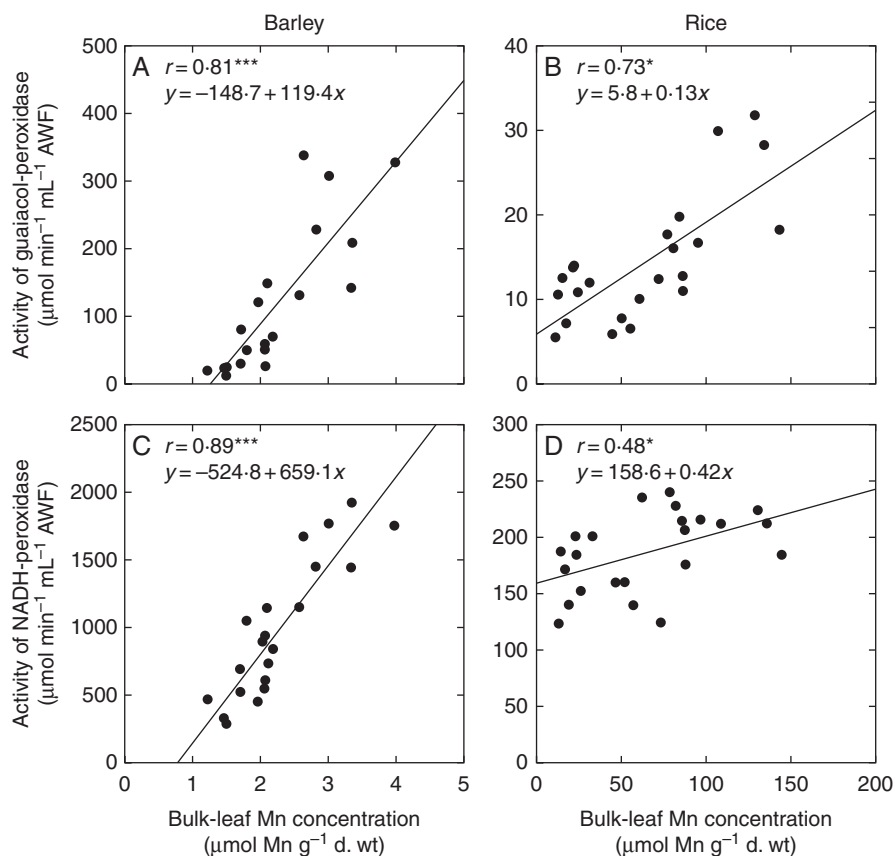


FIG. 4. Activities of guaiacol-POD (A, B) and NADH-POD (C, D) in the second oldest leaves of barley (A, C) and rice (B, D) related to the Mn bulk leaf tissue concentration. After pre-culture at 0.2/1.0 μM Mn, the Mn supply was increased to 50 μM for 0, 2 and 4 d, or plants received 0.2/1.0 μM Mn continuously. Results of the regression analysis are given as ***, * for $P < 0.001$ and 0.05, respectively.

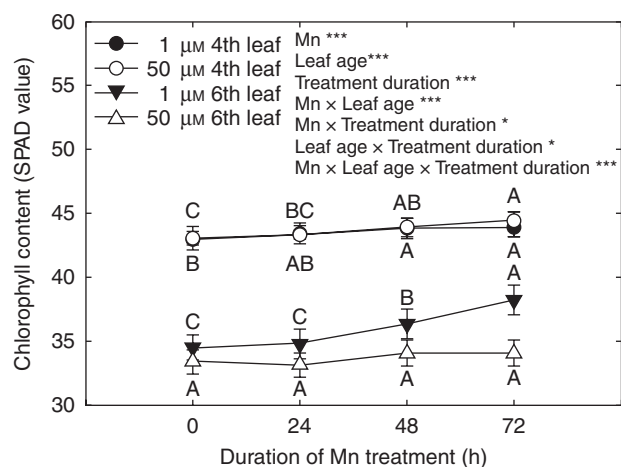


FIG. 5. Effect of leaf age and Mn treatment duration on the development of chlorophyll contents (SPAD values) of rice leaf blades. Results of the three-factorial ANOVA are given as *** and * for $P < 0.001$ and 0.05, respectively. Different letters indicate significant differences ($P < 0.05$) between treatment durations for each Mn treatment (1.0 or 50 μM) for the fourth and the sixth leaves, respectively.

Mn-treated plants were compared. An adenosine 5'-phosphotransferase 2 and a ferredoxin-1 (chloroplast precursor) were of significantly decreased abundance in younger

leaves, whereas four ribosomal proteins [50S ribosomal protein L11, 40S ribosomal protein S12, 60S acidic ribosomal protein P3 (P1/P2-like) and ribosomal protein L31 family protein] and again the probenazole-inducible protein PBZ1 were of higher abundance in the younger leaves or even only appeared in these leaves.

DISCUSSION

Expression of Mn toxicity in leaves of barley compared with rice

Fecht-Christoffers *et al.* (2006, 2007) and Fühns *et al.* (2009) described a possible interplay in the leaf apoplast of (a) class III PODs producing and consuming H_2O_2 ; (b) increased Mn concentrations; and (c) specific phenols finally leading to the typical toxicity symptoms, namely brown spots. The density of these brown spots was indicative of the severity of Mn toxicity (Horst, 1980; Fecht-Christoffers *et al.*, 2003a, b). The results presented here suggest that in barley, Mn toxicity is expressed in a similar way. The high Mn sensitivity of barley (Vlamiš and Williams, 1962) is confirmed, since bulk leaf Mn concentrations (Fig. 1A) as low as 2 $\mu\text{mol g}^{-1}$ d. wt and Mn concentrations in the AWF (Fig. 1C) of 20 nmol mL^{-1} AWF led to typical Mn toxicity symptoms and enhanced POD activities (Figs 3 and 4A, C). In contrast, Mn concentrations in the bulk leaf (Fig. 1B) and the AWF

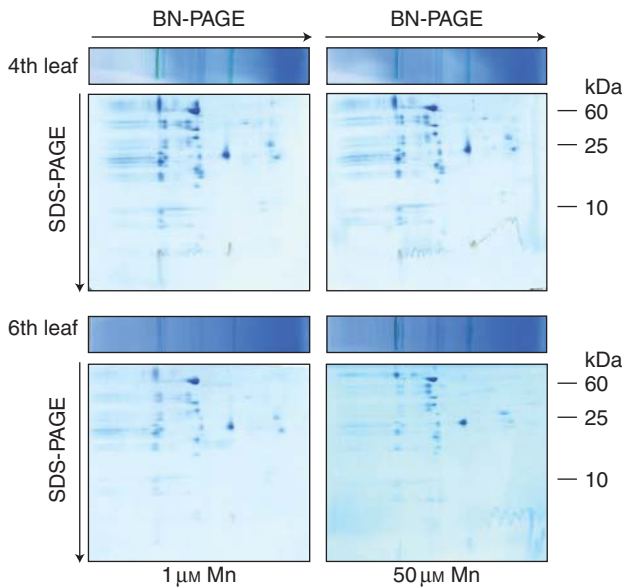


FIG. 6. Resolution of the chloroplast protein complexes by 2D BN/SDS-PAGE. Chloroplasts were isolated using an adapted method described originally by Tanaka *et al.* (2004) from rice leaf blades of the fourth leaf (top) and the sixth leaf (bottom) treated with 50 μM Mn for 3 d (right) or with 1 μM Mn continuously (left), and thylakoid membrane-localized photosynthetic protein complexes were solubilized using digitonin and submitted to BN-PAGE as described by Heinemeyer *et al.* (2004) and Führs *et al.* (2008). Afterwards, gel slices were cut and placed horizontally on a second SDS-containing gel dimension in order to separate the protein complexes into their subunits.

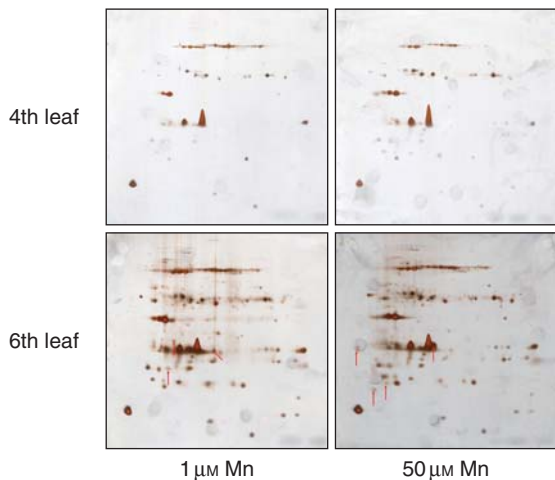


FIG. 7. IEF/SDS-PAGE resolution of water-soluble chloroplast proteins of the fourth (top) and sixth (bottom) rice leaf without (left) and with (right) 50 μM Mn supply for 3 d. Chloroplasts were isolated using an adapted method described originally by Tanaka *et al.* (2004). After purification, chloroplasts were frozen in liquid nitrogen. After subsequent thawing of the chloroplasts, thylakoids were centrifuged. The supernatant was precipitated and finally used for electrophoresis. After separation, gels were stained with silver nitrate. Comparative visual inspection of the resolutions led to the identification of at least seven differentially affected proteins in the younger leaf (marked with red arrows).

(Fig. 1D) of rice as high as 100 $\mu\text{mol g}^{-1}$ d. wt and 300 nmol mL^{-1} AWF, respectively, did not produce any Mn toxicity symptoms on older leaves. The POD activities (Fig. 4B,

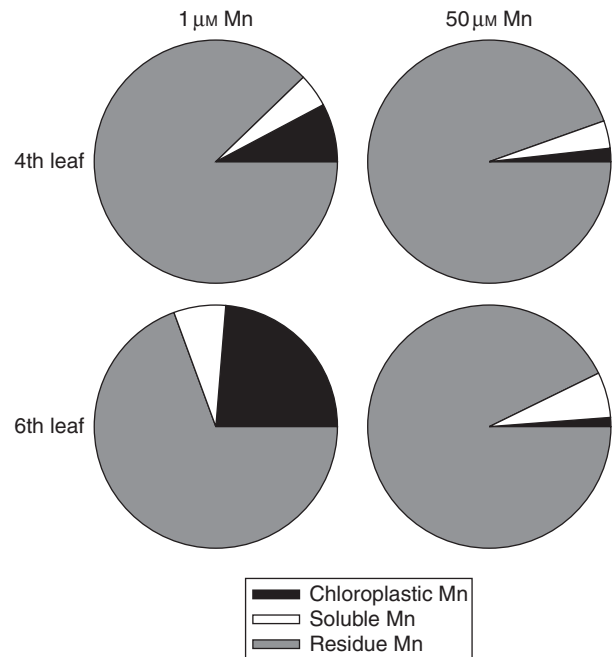


FIG. 8. Relative contribution of chloroplasmic, soluble and non-soluble residue Mn to the bulk leaf Mn content of rice leaf blades differing in leaf age and Mn treatment. Different Mn fractions were measured and calculated as described in the Materials and Methods section. Plants received either 50 μM Mn for 3 d or 1 μM Mn continuously.

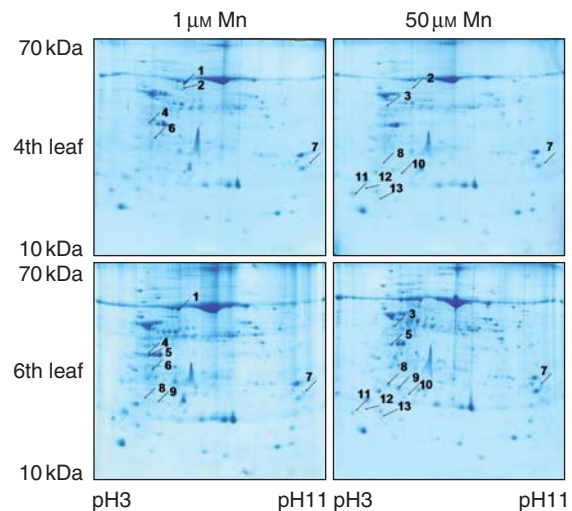


FIG. 9. Representative Coomassie-stained 2D IEF/SDS-PAGE resolution of the proteome of the older fourth (top) and the younger sixth (bottom) rice leaf blades after elevated Mn supply (50 μM) for 3 d (right) or continuous 1 μM Mn supply (left). Three biological replications of each treatment were analysed using the Imagemaster™ 2D PLATINUM Software 6.0. Significantly regulated ($P < 0.05$) protein spots (marked by arrows) were cut out and identified by nano LC-MS/MS.

D), which are lower by a factor of about 100 in the rice leaf AWF compared with barley, support the view of a central role for these peroxidases in the apoplastic expression of Mn toxicity (Horst *et al.*, 1999; Führs *et al.*, 2009).

From the data presented it cannot be excluded that at higher Mn leaf tissue concentrations than achieved here after 4 d of

TABLE 1. Identity of selected proteins extracted from rice leaves, which differ significantly in abundance between different leaf ages and Mn treatments

Protein no.*	Fold induction/reduction†	Coverage (%)‡	Accession no.§	Identity¶
Fold induction/reduction in the comparison of optimum and excess Mn- (50 µM) treated old (fourth) leaves				
2	0.44	28.40	BAD16832	Chloroplast translational elongation factor Tu
7	Only present at 1 µM Mn	29.90	NP_001048798	50S ribosomal protein L11
Fold induction/reduction in the comparison of optimum and excess Mn (50 µM) treated young (sixth) leaves				
5	2.25	34.40	BAD16934	Putative inorganic pyrophosphatase
8	2.16	67.70	NP_001066998	Probenazole-inducible protein PBZ1
9	3.40	18.00	NP_001054704	Universal stress protein (Usp) family protein
Fold induction/reduction in the comparison of old leaves (fourth) and young (sixth) leaves of plants treated with optimum Mn supply continuously				
1	0.49	48.40	Q42971	Enolase (2-phosphoglycerate dehydratase)
4	2.21	33.90	NP_001043134	Oxygen-evolving enhancer protein 1, chloroplast precursor
6	2.07	40.10	NP_001063793	Ribonuclease T2 family protein
Fold induction/reduction in the comparison of old leaves (fourth) and young (sixth) leaves of plants treated with excess Mn supply for 4 d				
3	0.47	43.70	NP_001047479	Adenosine 5'-phosphotransferase 2
7	Only present in sixth leaf	36.20	NP_001048798	50S ribosomal protein L11
8	2.64	66.50	NP_001066998	Probenazole-inducible protein PBZ1
10	2.51	14.20	NP_001058899	40S ribosomal protein S12
11	0.47	33.10	A2YQD9	Ferredoxin-1, chloroplast precursor (Ferredoxin I) (anti-disease protein 1)
12	2.31	19.30	P56724	60S acidic ribosomal protein P3 (P1/P2-like)
13	3.06	14.80	NP_001043653	Ribosomal protein L31 family protein

* Numbers of spots corresponding to Fig. 9.

† Regulation of spots between compared populations of gels based on mean relative spot volume.

‡ Sequence coverage of the identified proteins with the corresponding NCBI database hit.

§ Accession number of the identified protein in the NCBI database.

¶ Identity of the protein.

elevated Mn supply, Mn toxicity symptoms in the form of brown spots can also be induced in old rice leaves. This is suggested by a report of Wang *et al.* (2002) who used such symptoms for phenotyping of rice recombinant inbred lines (RILs) for Mn sensitivity. To our surprise, we only observed Mn toxicity symptoms in rice also in the form of brown spots on younger leaves (Fig. 2). The difference between our observations and those of Wang *et al.* could be due to the fact that we studied a particularly Mn-tolerant rice cultivar, whereas the appearance of brown spots on older leaves in the study of Wang *et al.* was characteristic of Mn-sensitive rice genotypes. The nature of the brown spots appearing on younger leaves of Mn-treated rice plants has not been clarified yet. The Mn-induced inhibition of chlorophyll synthesis in the young leaves (Fig. 5) may suggest that Mn-induced deficiencies of other (micro)-nutrients (Fe, Zn, Mg) may play a role (Foy *et al.*, 1978; Marschner, 1995). Surprisingly, the suppressed chlorophyll production did not affect photosynthesis as characterized by gas exchange and leaf chlorophyll fluorescence either in old or in young leaves (not shown), indicating an intact photosynthetic apparatus. This was additionally confirmed by 2D BN/SDS-PAGE results of photosynthetic protein complexes (Fig. 6) which were, in contrast to results published for cowpea (Führens *et al.*, 2008), not affected by elevated Mn supply.

Localization and binding of Mn in relation to Mn tolerance of rice leaves

Obviously, barley and rice differ in Mn uptake and response to high Mn supply (Figs 1–4). After confirming the decisive contribution of the apoplast and apoplastic PODs to the Mn sensitivity of barley (Fig. 3) consistent with results published

for cowpea (see Introduction), we focused on rice in order to unravel Mn tolerance mechanisms.

It is generally accepted that high cytosolic Mn concentrations are toxic to plants (Wu *et al.*, 2008). Hence, the particularly high Mn tolerance of rice leaf tissue requires the compartmentation of Mn in cellular compartments which are less sensitive to elevated Mn concentrations and/or sequestration and detoxification of Mn by ligands. To our knowledge, there is no information available in rice on the role of cation transporters/transport facilitators in relation to cellular Mn homeostasis/Mn tolerance such as for a range of other plant species (see Introduction). Studies on rice suggest that the chloroplasts could serve as Mn storage compartments contributing to its outstanding high Mn tolerance (Lidon *et al.*, 2000; Lidon, 2001). Lidon and Teixeira (2000) reported a novel Mn protein induced by excess Mn in rice which is located in the thylakoids. This thylakoid membrane-associated protein showed an Mn : protein ratio of 1, had SOD activity and was, therefore, supposed to be involved in reducing oxidative damage caused by excess Mn directly or by reducing the negative impact on photosynthesis indirectly. Since the Mn-binding protein detected by the Lidon group was barely water soluble and supposed to be membrane intrinsic, we might not have detected it using the applied extraction techniques. Nevertheless, our proteomic studies of the water-soluble chloroplastic proteome revealed Mn-induced visual changes of at least seven proteins (Fig. 7). However, these changes were only detected in the younger leaves with lower Mn contents than older leaves (data not shown). Therefore, the proteomic changes in the younger leaves may reflect the observed decrease in chlorophyll content (Fig. 5) and thus represent a response to nutrient deficiency rather than Mn toxicity.

The bulk leaf Mn content was fractionated into a chloroplastic, water-soluble and residue fraction (Fig. 8). Since a hydrophilic chloroplast isolation procedure was applied, it cannot be excluded that part of the water-soluble Mn in the chloroplast was lost to the soluble fraction during the isolation, leading to an underestimation of the chloroplast fraction. At optimum Mn supply the chloroplastic fraction represented an important part of the total Mn content, especially in the young leaves (Fig. 8). With increasing Mn content of the leaf tissue at excess Mn supply, the contribution of the chloroplast Mn to the total leaf Mn content became negligible. Therefore, a role for the chloroplast as an Mn storage compartment contributing to Mn tolerance as proposed by Lidon (2000) appears unlikely.

The dominating role of the residue fraction particularly at elevated Mn supply rather suggests that the cell wall as a major component of this fraction might play a decisive role in the detoxification of Mn. In this respect it is reasonable to assume that Si may play a major role in detoxification of Mn in the cell walls for two reasons. (1) Rice is an Si accumulator plant species (Ma and Yamaji, 2006; Ma et al., 2006) and Si in the leaves is deposited in the cell walls (Ma and Yamaji, 2008). (2) It has been shown in cucumber (Rogalla and Römheld, 2002) and cowpea (Iwasaki et al., 2002a, b) that Si increases the Mn binding capacity of cell walls, thus enhancing Mn tolerance. However, under the present experimental conditions, Si (apart from unavoidable contaminations) was not supplied to the nutrient solution, leading to rather low Si concentrations in the rice leaves (<0.2% dry matter concentration; data not shown). Nevertheless, the Mn tolerance of these rice plants was very high, suggesting that not Si but rather other cell wall characteristics were (additionally) decisive for the observed high Mn tolerance.

Mn-induced changes in the total water-soluble leaf proteome of rice

Among the changes in the water-extractable chloroplastic proteome the total water-soluble proteome was also investigated not only between Mn treatments but also between leaf ages (Fig. 9, Table 1). In particular, primary metabolism was affected by elevated Mn supply. Significant effects on the abundance of proteins associated with translational processes were observed. While the expression of a chloroplast translational elongation factor Tu was decreased and 50S ribosomal protein L11 was not detectable in the fourth leaf treated with 50 μM Mn, the expression of ribosomal proteins (cytoplasmic and plastidal) was increased in the young leaf compared with the old leaf. It is known that Mn is able to displace magnesium (Mg) in some functions such as acting as a cofactor for enzymes in the tricarboxylic acid (TCA) cycle (Burnell, 1988) and for correct assembly of ribosomes (Cammarano et al., 1972). A substitution of Mg by Mn could, therefore, explain the changes in ribosomal subunit abundance. Since the younger leaf could be regarded as the more Mn-sensitive leaf, the observed changes in the protein biosynthesis system may be regarded more as a sensitivity- rather than a tolerance-conferring mechanism.

In the young leaf not only an increased abundance of a putative inorganic pyrophosphatase at high Mn compared with

control plants, but also a higher abundance of an adenosine-detoxifying adenosine 5'-phosphotransferase 2 compared with the old leaf at high Mn was observed. Both enzymes require Mg as a cofactor (Anderson, 1977). Mg could be substituted by Mn at the expense of activity (Miller et al., 1979).

Increased Mn supply increased the abundance of general stress response proteins in the younger, more Mn-sensitive leaf. For instance, the expression of PBZ1 protein was doubled compared with the low Mn control, but was also up to 2.6 times higher than in the old leaf treated with 50 μM Mn (Table 1). This more general response was also described for other heavy metals (for further information, see Hajdich et al., 2001) and further supports the sensitivity of the young leaf.

The decreased expression of chloroplastic ferredoxin-1 precursor in the Mn-treated young leaf compared with the old leaf was probably caused by Mn-induced Fe deficiency. Indeed, Bovy et al. (1993) observed a decrease of ferredoxin transcript abundance due to Fe deficiency in cyanobacteria, and Tognetti et al. (2007) showed that Fe deficiency-mediated reduction of ferredoxin can be partially substituted by flavodoxin overexpression. It is currently unclear whether all other described responses particularly in the younger leaf at elevated Mn supply also reflect Mn-induced Fe deficiency.

In conclusion, overall the results support the view that apoplastic PODs are involved in the expression of Mn toxicity as brown spots on old leaves. They also provide circumstantial evidence that high Mn tolerance, particularly of old rice leaves, involves a high Mn binding capacity of the cell walls. The physiological and proteomic studies suggest that Mn toxicity in less Mn-tolerant young leaves is related to displacement and thus impairment of the function of Mg and Fe. Future work aiming at elucidating the outstanding Mn leaf tolerance of rice requires a better understanding of the compartmentation of Mn at the cellular level and of the binding stage. The identification of a highly Mn-sensitive genotype appears to be a prerequisite for unravelling the molecular basis of Mn tolerance in rice.

SUPPLEMENTARY DATA

Supplementary data are available online at www.aob.oxfordjournals.org and consist of the following figures and tables. Fig. S1. Mn tissue concentration of rice leaf blades of different leaf age before and after elevated Mn supply. Fig. S2. Specific guaiacol-POD (A) and specific NADH-POD (B) activities of rice leaf blades of different leaf age before and after elevated Mn supply. Fig. S3. Protein content of rice leaf blades of different leaf age before and after elevated Mn supply. Fig. S4. Representative Coomassie-stained close-ups of regions containing Mn-influenced protein spots 2 and 7 extracted from the older fourth leaves. Fig. S5. Representative Coomassie-stained close-ups of regions containing Mn-influenced protein spots 5, 8 and 9 extracted from the younger sixth leaves. Fig. S6. Representative Coomassie-stained close-ups of regions containing protein spots 1, 4 and 6 differing in abundance between the younger sixth and the older fourth leaves. Fig. S7. Representative Coomassie-stained close-ups of regions containing protein spots 3, 7, 8, 10, 11, 12 and 13 differing in abundance between the younger sixth and the older fourth leaves.

Table S1. Identity and peptide alignment of proteins changed in abundance after elevated Mn supply in the older (fourth) leaf. Table S2. Identity and peptide alignment of proteins changed in abundance after elevated Mn supply in the younger (sixth) leaf. Table S3. Identity and peptide alignment of proteins differing in abundance between the Mn control (0.2 μM) older (fourth) and the younger (sixth) leaf. Table S4. Identity and peptide alignment of proteins differing in abundance between the Mn-treated (50 μM) older (fourth) and younger (sixth) leaf.

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