Excess manganese differentially inhibits photosystem I versus II in Arabidopsis thaliana

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Abstract

The effects of exposure to increasing manganese concentrations (50–1500 μM) from the start of the experiment on the functional performance of photosystem II (PSII) and photosystem I (PSI) and photosynthetic apparatus composition of Arabidopsis thaliana were compared. In agreement with earlier studies, excess Mn caused minimal changes in the PSII photochemical efficiency measured as Fv/Fm, although the characteristic peak temperature of the S2/3 QB charge recombinations was shifted to lower temperatures at the highest Mn concentration. SDS-PAGE and immunoblot analyses also did not exhibit any significant change in the relative abundance of PSII-associated polypeptides: PSII reaction centre protein D1, Lhcb1 (major light-harvesting protein of LHClI complex), and PsbO (OEC33, a 33 kDa protein of the oxygen-evolving complex). In addition, the abundance of Rubisco also did not change with Mn treatments. However, plants grown under excess Mn exhibited increased susceptibility to PSII photoinhibition. In contrast, in vivo measurements of the redox transients of PSI reaction centre (P700) showed a considerable gradual decrease in the extent of P700 photoperoxidation (P700⁺) under increased Mn concentrations compared to control. This was accompanied by a slower rate of P700⁺ re-reduction indicating a downregulation of the PSI-dependent cyclic electron flow. The abundance of PSI reaction centre polypeptides (PsaA and PsaB) in plants under the highest Mn concentration was also significantly lower compared to the control. The results demonstrate for the first time that PSI is the major target of Mn toxicity within the photosynthetic apparatus of Arabidopsis plants. The possible involvement mechanisms of Mn toxicity targeting specifically PSI are discussed.

Key words: Chlorophyll fluorescence, Mn toxicity, photosystem I, PSI-associated proteins, PSII-associated proteins, redox state of P700.

Introduction

Manganese (Mn) is one of the most abundant metals in the Earth’s crust and although it is an important essential micro-nutrient for all photosynthetic organisms can be also toxic when it is present in excess (Mukhopadhyay and Sharma, 1991; Marschner, 1995). Mn is considered the second most phytotoxic element, after aluminium (Al), affecting negatively the physiological and biochemical properties of plant species (Foy et al., 1978, Foy, 1984; Millaleo et al., 2010). An
excess of this metal occurs in acid soils with low pH (<5.5) and/or under reducing conditions (Marschner, 1995; Schaaf et al., 2002), where Mn²⁺ is the predominant solution species and available ion to plant cells (Bradl, 2004). Thus, a Mn excess results in a sharp decrease in shoot height, biomass accumulation, and total leaf area of a woody species (Populus cathayana, Lei et al., 2007), a reduction of the dry weights (DW) of both shoots and roots in ryegrass cultivars (Lolium perenne, Mora et al., 2009), and Trifolium repens (Rosas et al., 2007). Furthermore, excess Mn can result in oxidative stress as indicated by the accumulation of H₂O₂ (Demirevska-Kepova et al., 2004; Lei et al., 2007), high levels of apoplastic H₂O₂-consuming peroxidases (Fecht-Christofiers et al., 2003), and high level of lipid peroxidation (Mora et al., 2009). Mn stress induced an enhancement of antioxidant enzyme activity in leaves of legumes (González et al., 1998), and it was also demonstrated in perennial ryegrass (Mora et al., 2009) and woody species (Lei et al., 2007). More recently, a proteomic and transcriptomic studies have demonstrated that chloroplastic proteins important for CO₂ fixation and photosynthesis were of lower abundance upon Mn stress of cowpea (Führs et al., 2008).

Mn has an important role in both the structure and functions of the photosynthetic apparatus (Mukhopadhyay and Sharma, 1991). Mn is a constitutive element associated with the oxygen-evolving complex of photosystem II (PSII), an important multiprotein pigment complex embedded in the thylakoid membranes (Hankamer et al., 1997; Enami et al., 2008). Therefore, the Mn cluster, together with other ions and extrinsic proteins that constitute the oxygen-evolving complex, is required to oxidize water and reduce P680, the reaction centre of PSII (Kern and Renger, 2007; Ferreira et al., 2004; Rutherford and Boussac, 2004). In conjunction with photosystem I (PSI) and linear electron transport, these reducing equivalents (electrons) are used primarily in the conversion of CO₂ into carbohydrate (Ferreira et al., 2004). In addition, Mn is indispensable as a cofactor for various enzymes involved in redox reactions such as Mn-superoxide dismutase, an essential enzyme involved in protection against oxidative stress in plants (Burnell, 1988; Bowler et al., 1994). A number of studies have suggested that chloroplasts and photosynthesis are the major targets of Mn toxicity. Indeed, increased amounts of Mn have been reported for chloroplasts isolated from Mn-stressed common bean (González and Lynch, 1999) and rice leaves (Lidon et al., 2004). Distinctive ultrastructural changes showing swelling of granal and stromal thylakoids have been also observed in the chloroplasts of Citrus volkameriana (Papadakis et al., 2007) and maize plants (Doncheva et al., 2009) under Mn excess. It has been demonstrated that high Mn accumulation is associated with inhibition of the net photosynthesis and carboxylation efficiency in various plant species. The decline of photosynthesis is considered as one of the major mechanisms constituting the toxic effects of excess Mn and is proposed as an early indicator for Mn toxicity in tobacco (Nable et al., 1988), rice (Lidon et al., 2004) and wheat (Macfie and Taylor, 1992). Reduced CO₂ assimilation induced by excess Mn was also reported for common bean (González and Lynch, 1997), deciduous broad leaved trees (Kitao et al., 1997a), and seedlings of Citrus grandis (Li et al., 2010). Interestingly, the maximum photochemical efficiency of PSII (Fv/Fm) was not substantially affected by Mn accumulation in various plant species over a wide range of leaf Mn concentrations (Nable et al., 1988; Kitao et al., 1997b; Subrahmanyam and Rathore, 2000; Hajiboland and Hasani, 2007; Doncheva et al., 2009).

The reduction in photosynthesis by excess leaf Mn has been generally attributed to modification of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco; Ohki, 1984; Houtz et al., 1988; McDaniel and Toman, 1994; Kitao et al., 1997b). It has been demonstrated that a high level of Mn affects primarily the activity rather than the amount of Rubisco (Houtz et al., 1988; Chatterjee et al., 1994) and the presence of excess Mn induces enhanced oxygenase activity (Jordan and Ogren, 1983). In addition, the decline of photosynthesis under Mn stress conditions was also ascribed to peroxidative impairment of photosynthetic enzyme activities caused by polyphenol oxidation products (Vaughn and Duke, 1984; Panda et al., 1987).

In spite of these studies, the mechanisms of Mn toxicity causing a decrease in CO₂ assimilation are still not well understood. In addition, very limited information concerning the toxic effect(s) of excess Mn on the polypeptide composition of both PSII and, especially, PSI is available. Therefore, the objective of this study was to evaluate the role of specific Mn-induced changes in the structure and function of PSII and PSI, which could help to understand the mechanisms by which Mn excess may cause a decrease of CO₂ assimilation in Arabidopsis thaliana.

Materials and methods

Plant material and growth conditions

Seeds of A. thaliana (wild type Columbia) were germinated in a substrate mix (82.5% sphagnum peat moss, 12.5% perlite, 5% vermiculite-Pro-Mix, Premier Tech Horticulture) in controlled environment growth cabinets (model GCW15, Environmental Growth Chambers, Chagrin Falls, OH, USA) with a photosynthetically active radiation (PAR) of 250 µmol photons m⁻² s⁻¹, 20/20 °C day/night temperatures, 50% relative humidity, and 8/16 light/dark cycle to prevent flowering. Water was supplied every 5 days. After 15 days, seedlings were transplanted separately in pots with vermiculite and placed in trays. Each tray containing seven pots (one plant per pot) were supplied with Hoagland nutrient solution for 2 weeks before applying the Mn treatments.

Manganese treatments

Manganese treatments included the final concentrations: 18 (control), 50, 500, 1000, and 1500 µM Mn according to Delhaize et al. (2007). Manganese was applied as MnCl₂·4H₂O. Control plants exposed to 18 µM Mn as the optimal dose for Mn for Arabidopsis (Cailliatte et al., 2010). The five Mn treatments were grown in five labelled trays, with 500 ml of Hoagland’s solution. The trays were maintained in controlled environment growth chambers under the same conditions described above. The pH was adjusted to 5.3 with diluted HCl daily and nutrient solution was changed every 5 days. After 15 days, seedlings were weighed separately in pots with vermiculite and placed in trays. Each tray containing seven pots (one plant per pot) were supplied with Hoagland nutrient solution for 2 weeks before applying the Mn treatments.

Plant growth measurements

Prior to beginning the Mn treatments, three plant samples were dried in a forced-air oven (70 °C, 48 h) and weighed to determine dry weight.
(W1) at day 0. Similarly, at the end of the experiment, plants were harvested and collected for dry weight measurements (W2). These data were used to determine mean relative growth rate (RGR) according to Fernando et al. (2009):

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RGR = \frac{(\ln W2 - \ln W1)}{t2 - t1}
\]

as g DW d⁻¹.

Manganese concentration
For Mn chemical analysis, samples of shoot and roots were dried ashed in a muffle furnace at 500 °C for 8 h and digested with 2 M HCl. Manganese was extracted as described by Sadzawka et al. (2004), and the Mn concentration was determined using a simultaneous multi-element atomic absorption spectrophotometer (model 969, Unicam, Cambridge, UK).

Thylakoid preparation, SDS-PAGE, and immunoblotting
Thylakoid membranes for SDS-PAGE were isolated as described earlier (Krol et al., 1999). Leaf material was ground in cold isolation buffer (50 mM Tricine, 0.4 M sorbitol, 10 mM NaCl, 5 mM MgCl₂, pH 7.8) in a mortar on ice, filtered through two layers of miracloth (typical pore size 22–25 μm; Calbiochem), and centrifuged for 15 min (10,000 g). The supernatant was removed and the pellet was resuspended in cold isolation buffer. Total chlorophyll concentration was measured in 90% (v/v) acetone (Arnon, 1949). For immunodetection of Rubisco, total leaf proteins were extracted as described in Rosso et al. (2009). Protein content was measured using a BCA protein assay kit (Pierce) by following the absorbance at 562 nm using a spectrophotometer (DU-640, Beckman Coulter). Proteins were separated by SDS-PAGE according to Laemmli (1970), using 15% (w/v) polyacrylamide gel in the presence of 6 M urea in the separating gel. Chloroplast thylakoids were solubilized with SDS:chlorophyll 20:1 and 15 μg chlorophyll was loaded per lane. All samples for separation of total proteins were loaded on an equal protein basis of 20 μg protein per lane (Rosso et al. 2009). Immunoblotting was performed by electrophotorechemically transferring the proteins from SDS-PAGE gel to nitrocellulose membrane (Bio-Rad) according to the method of Towbin et al. (1979). Proteins were probed with antibodies (AgriSera AB, VA, Sweden) raised against the reaction centre polypeptides of PSI: PsaA, PsaB (1:200), the major light-harvesting protein of PSI complex (LHCl1, Lhcb1 protein (1:5000), the PSII oxygen-evolving complex extrinsic protein PsbO (33 kDa, 1:2000), the harvesting protein of PSII complex (LHCII) Lhcb1 protein (1:5000), the PSII reaction centre protein: D1 and Rubisco (1:5000). As second antibodies, goat anti-rabbit IgG conjugated with horseradish peroxidase (Sigma-Aldrich) were used. Polypeptides were detected using enhanced chemiluminescence detection kit (Amersham Biosciences) and visualized by exposing the membrane to X-ray film. Densitometric scanning and analysis of X-ray films from each replicate immunoblot was performed using a Hewlett Packard ScanJet 4200C desktop scanner and ImageJ 1.41o densitometry software (Wayne Rasband, National Institute of Health, USA, http://rsweb.nih.gov/ij/).

Measurement of the redox state of P₇₀₀
The redox state of P₇₀₀ was determined in vivo, in dark-adapted (20 min) Arabidopsis leaves under growth temperature and ambient O₂ and CO₂ conditions using a PAM-101 modulated fluorometer equipped with a dual-wavelength emitter-detector ED-P7000DW unit and PAM-102 units (Klukhammer and Schreiber, 1991) as described in detail by Ivanov et al. (1998). Far-red light (λₚ₉₀₀ = 715 nm, 10 W m⁻², Schott filter RG 715) was provided by an FL-101 light source. The redox state of P₇₀₀ was evaluated as the absorbance change around 820 nm (ΔA₈₂₀–₈₆₀) in a custom-designed cuvette. Multiple turnover (MT, 50 ms) and single turnover (ST, half peak 14 μs) saturating flashes were applied with XMT-103 and XST-103 (Walz) power/control units, respectively. The relative functional pool size of intersystem electrons on a P₇₀₀ reaction centre basis was calculated as the complementary area between the oxidation curve of P₇₀₀ after either ST or MT pulse excitation (ST and MT areas) and the stationary level of P₇₀₀ under far-red excitation (Asada et al., 1993; Ivanov et al., 1998). Capacity of PSII cyclic electron (e⁻) flow was determined as the half time for the dark decay of the P₇₀₀ signal (Ivanov et al., 1998).

Modulated chlorophyll fluorescence measurements
Modulated imaging fluorometer (IMAGING-PAM, Heinz Walz, Elektor, Germany) was used for capturing the chlorophyll fluorescence images and estimation of the maximal photochemical efficiency of PSII \(F_{m}/F_{o} = (F_{m} – F_{o})/F_{m} \), effective photochemical efficiency of PSII (\(\Phi_{PSII}\)), photochemical (qP), and non-photochemical (NPQ) fluorescence quenching parameters using the nomenclature of van Kooten and Snel (1990) as described earlier (Ivanov et al., 2006a). Control and Mn-treated Arabidopsis plants were dark adapted (20 min) and all chlorophyll fluorescence measurements were performed in vivo at room temperature. Fluorescence images were captured by a CCD camera (IMAG-K, Allied Vision Technologies) featuring a 640 × 480 pixel CCD chip size and CCTV camera lens (Cosmicar/Pentax f/1.2, f = 12 mm). Light emitting diode ring array (IMAG-L) consisting of 96 blue LEDs (470 nm) provided standard modulated excitation intensity of 0.5 μmol quanta m⁻² s⁻¹ (modulation frequency 1–8 Hz) for measuring the basal \(F_{o}\) chlorophyll fluorescence and a saturation pulse of 2400 μmol quanta m⁻² s⁻¹ PAR for measuring the maximal chlorophyll fluorescence \(F_{m}\). The maximal photochemical efficiency of PSII \(F_{m}/F_{o}\) was determined as \(F_{m} – F_{o}/F_{m}\). The effective photochemical efficiency of PSII (\(\Phi_{PSII}\)) was calculated from the expression \(F_{m} – F_{o}/F_{m}\) (Genty et al., 1989), photochemical quenching (qP) was calculated as \(F_{m} – F_{o}/F_{m}\) (Schreiber et al., 1994), electron transport rate (ETR) was calculated as \(PAR × 0.5ΦPSII × 0.84\) (Genty et al., 1989), and regulated non-photochemical quenching (ΦNPQ) and constitutive photochemical quenching (ΦNcq) was determined according to Kramer et al. (2004). All measurements were performed at 0, 5, 10, 15, and 21 days in plants subjected to the different Mn treatments.

At the end of experiments (21 days), leaves of Arabidopsis plants growing under the different Mn treatments and 250 μmol m⁻² s⁻¹ PAR (normal light) were cut and exposed to high light (1000 μmol quanta m⁻² s⁻¹ PAR) at 1, 2, and 4 hours. During each time of exposition, \(F_{m}/F_{o}\) was determined.

Thermoluminescence measurements
Thermoluminescence (TL) measurements of control and Mn-treated Arabidopsis leaves were performed on a personal-computer-based TL data acquisition and analysis system as described earlier (Ivanov et al., 2001, 2006b). A photomultiplier tube (Hamamatsu R943-02, Hamamatsu Photonics, Shizuoka-ken, Japan) equipped with a photomultiplier power supply (model PS-302, EG&G Electro Optics), and a preamplifier (model C1556-03) was used as a radiation measuring set. The heating rate was 0.6 °C s⁻¹. For identifying the S₂ O₂ charge recombination peaks, dark-adapted leaf discs were subjected to two consecutive saturating microsecond flashes of white light (1.5 μs peak width at 50% of maximum) applied by a xenon-discharge flash lamp (XST103, Heinz Walz). Dark-adapted leaves (20 minutes at 20 °C) were cooled to 2 °C prior to exposing to the flashes. The nomenclature of Sane et al. (2012) was used for characterization of the TL glow peaks.

Experimental design and statistical analysis
The experimental design is a randomized split design, with 1 species × 5 Mn treatments × 7 replicates for the physiological determinations and 1 species × 5 Mn treatments × 3 replicates × 4 times (0, 1, 2, and 4 hours) for the photoinhibition measurements. Data correspond to the means of replicates for each determination as indicated above. Data were tested in their normality and equal variance by the Shapiro–Wilk test and then data were analysed by a one-way ANOVA. Significant differences between means were established by using the multiple comparisons test.
Results

A statistically significant decrease in total biomass of leaves treated with 500 and 1500 µM Mn concentrations was found compared to the control plants (18 µM Mn), although a decrease of the total leaf biomass was found at all Mn concentrations used. In contrast, root biomass exhibited significant decrease in all treatments ($P < 0.05$, Fig. 1). It should be noted, however, that while the decrease of leaf biomass was only 25% lower at the highest Mn concentration used (1500 µM), the biomass of roots was more affected and demonstrated a 2.5-fold decrease even at the lowest Mn concentration tested (50 µM). This is consistent with the RGR data, where a considerably higher reduction of root RGR was observed compared to leaves across the Mn treatments relative to control plants ($P < 0.05$, Table 1). In addition, visual symptoms of Mn toxicity (chlorosis) in leaves were observed predominantly in the highest Mn treatment (data not shown).

Analysis of the total Mn amount in plants exposed to increasing Mn concentrations have demonstrated a gradual increase of Mn in leaves and roots (Fig. 2). However, while Mn concentrations of roots reached values up to ~8100 mg kg$^{-1}$ Mn at the highest Mn treatment, which represents a 40-fold increase of Mn, the increased accumulation of Mn in leaves was much lower (15-fold) compared to roots (Fig. 2). Thus, the differential effects of Mn treatments on the total

Table 1. Relative growth rates of Arabidopsis thaliana (leaves and roots) growing at increased Mn treatments. Plants were grown under acidic conditions (pH 5.3) for 21 d. Different lower-case letters indicate statistically significant differences between the Mn treatments ($P < 0.05$).

| Mn treatment (µM) | Relative growth rate (g DW d$^{-1}$) |
|-------------------|--------------------------------------|
|                   | Leaves                               | Roots                               |
| Control           | 0.069 ± 0.001$^a$                    | 0.206 ± 0.006$^a$                   |
| 50                | 0.068 ± 0.003$^a$                    | 0.071 ± 0.005$^b$                   |
| 500               | 0.046 ± 0.006$^a$                    | 0.053 ± 0.006$^a$                   |
| 1000              | 0.059 ± 0.003$^{a,b}$                | 0.064 ± 0.007$^a$                   |
| 1500              | 0.040 ± 0.004$^a$                    | 0.057 ± 0.004$^a$                   |

Fig. 1. Total biomass (leaves and roots) of Arabidopsis thaliana plants subjected to Mn treatments at the end of experiment (21 days). Different lower-case letters indicate significant differences between the Mn treatments ($P \leq 0.05$).

Fig. 2. Mn concentration in leaves and roots of Arabidopsis thaliana plants subjected to Mn treatments at the end of experiment (21 days). Different lower-case letters indicate statistically significant differences between the Mn treatments ($P \leq 0.05$).
Manganese excess inhibits PSI of *A. thaliana*

Table 2. Maximum photochemical efficiency of PSII (Fv/Fm) in leaves of *Arabidopsis thaliana* measured at 0, 5, 10, 15 and 21 days subjected to increasing Mn treatments. Results are means ± SE of five replicates of two plants each. Different lowercase letters indicate significant differences between the Mn treatments at the same time, while different uppercase letters indicate significant differences between the times at a same Mn treatment ($P < 0.05$).

| Mn treatment (µM) | Fv/Fm | 0 days | 5 days | 10 days | 15 days | 21 days |
|-------------------|-------|--------|--------|---------|---------|---------|
| Control           | 0.803±0.004 | 0.803±0.004 | 0.803±0.004 | 0.803±0.004 | 0.803±0.004 |
| 50                | 0.806±0.004 | 0.799±0.004 | 0.800±0.004 | 0.800±0.004 | 0.800±0.004 |
| 500               | 0.803±0.004 | 0.801±0.004 | 0.804±0.004 | 0.787±0.004 | 0.791±0.004 |
| 1000              | 0.797±0.004 | 0.805±0.004 | 0.800±0.004 | 0.778±0.004 | 0.773±0.004 |
| 1500              | 0.802±0.004 | 0.798±0.004 | 0.775±0.004 | 0.769±0.004 | 0.756±0.004 |

In addition, TL measurements were used as an alternative approach for assessing the effects of Mn excess on the photosynthetic PSII-associated electron transfer reactions especially at its reducing side (Vass and Govindjee, 1996; Sane et al., 2012). Since most of the photosynthetic TL components have been assigned to arise from the reversal of light-driven charge separation in PSII, TL properties of photosynthetic apparatus provide information on the activation energies associated with the back reactions of electron acceptors (QA and QB) with the electron donors (S2 and S3) of PSII (Vass and Govindjee, 1996; Sane, 2004; Sane et al., 2012). The temperature maxima ($T_m$) of the TL peaks related to the recombination of these charge pairs reflect the activation energies and hence a measure of the redox potentials of the participating oxidized and reduced donors (de Vault and Govindjee, 1990). Typical TL glow curves representing $S_{2/3}Q_b^-$ charge recombinations of control and Mn-treated *Arabidopsis* plants obtained following excitation with two consecutive saturating flashes are shown in Fig. 3. The experimental data summarized in Table 3 indicate that treatment with a Mn dose of 500 µM did not exhibit significant differences of the TL peak position, while treatment with the highest concentration (1500 µM) induced a low temperature shift of the $T_m$ to 25.8 °C compared to control plants. Besides this, the amplitudes and the integrated areas of the TL peaks representing the $S_{2/3}Q_b^-$ charge recombination used for assessing the PSII photochemistry was not affected at 500 µM Mn excess, but the overall TL yield was significantly reduced (45%) in plants treated with 1500 µM Mn compared to controls (Table 3).

Table 3. Characteristic thermoluminescence peak emission temperatures ($T_m$) and the overall TL emission area (A) of $S_{2/3}Q_b^-$ glow peaks of control and Mn-treated (21 days) *Arabidopsis* plants. The samples (leaf disks) were dark adapted for 30 min then cooled to 2 °C and subsequently illuminated with two single turnover flashes of white light. The peak areas are presented as a percentage of the total thermoluminescence light emission in control leaves. Values are mean ± SE calculated from four independent experiments.

| Mn treatment (µM) | $T_m$ (°C) | A (%) |
|-------------------|------------|-------|
| Control           | 28.7±1.1   | 100.0 |
| 500               | 29.1±1.3   | 106.2±7.8 |
| 1500              | 25.8±0.9   | 54.5±7.9 |
leaves subjected to different Mn treatments for 21 days were exposed to high light stress (Table 4). Indeed, the photoinhibitory effect on PSII, measured as a decrease in F$_{v}$/F$_{m}$, was much stronger (49%) at the highest Mn treatment (1500 µM) compared to control plants, and 500 µM Mn treated plants exhibited only a 15% decrease in F$_{v}$/F$_{m}$ values after 4 h of exposure to high light (Table 4).

The extent of far-red light-induced absorbance decrease at 820 nm (ΔA$_{820-860}$) of Arabidopsis leaves (Klughammer and Schreiber, 1991; Ivanov et al., 1998, 2006a) was used to estimate the potential functional differences of PSI and photosynthetic electron transport pathways between plants exposed to different Mn treatments. Typical traces representing in vivo measurements of oxidation–reduction transients of P$_{700}$ in control and Mn-treated plants are shown in Fig. 4. The relative amount of P$_{700}^{+}$, measured as ΔA$_{820-860}$, gradually decreased with increasing Mn concentrations and was 30% lower in Mn-treated plants at the highest concentration used (1500 µM, Fig. 4 and Table 5). Concomitantly, kinetic measurements of dark re-reduction of P$_{700}^{+}$ after turning off the far-red light, which is thought to reflect the extent and/or capacity for cyclic electron flow around PSI (Maxwell and Biggins, 1976; Ravenel et al., 1994), indicated significantly slower (46%) re-reduction of P$_{700}^{+}$ in Mn (1500 µM)-treated plants compared to control plants (Table 5). In addition, the apparent electron donor pool size to PSI (e-/P$_{700}$) estimated by measuring single-and multiple-turnover flash-induced ΔA$_{820-860}$ under steady-state oxidation of PSI by far-red light (Asada et al., 1993; Ivanov et al., 1998) demonstrated a significant decrease in Mn-treated plants (Table 5). This indicates that the pool size of electrons that can be donated to photooxidized P$_{700}$ (P$_{700}^{+}$) from the stroma in control plants was 37% higher compared to plants treated with the highest Mn dose (13 electrons per P$_{700}$, Table 4).

The major photosynthetic components within the thylakoid membranes of control and Mn-treated Arabidopsis plants were compared by SDS-PAGE and immunodetection to quantify their relative abundance. Immunoblot analyses did not exhibit any significant Mn-stress-induced changes in the relative abundance of PSII-associated polypeptides, as revealed by the densitometry analysis of the immunoblot bands for D1 (the PSII reaction centre protein), Lhcb1 (major light-harvesting protein of LHCII), and PsbO (extrinsic protein of the oxygen-evolving complex (Fig. 5). The abundance of Rubisco was only marginally affected by the Mn treatments. In contrast, the abundance of reaction centre polypeptides of PSI (PsaA and PsaB) was significantly reduced in Mn-treated compared with control thylakoids (Fig. 5A). The densitometric analysis demonstrated that the relative abundance of PsaA and PsaB in Mn-treated Arabidopsis was only about 20 and 60%, respectively, of that observed in the control plants (Fig. 5B). Thus, the quantitative analysis of photosynthetic polypeptides clearly indicates that excess Mn has

| Irradiance exposition time (h) | F$_{v}$/F$_{m}$ Control 500 µM Mn 1500 µM Mn |
|-------------------------------|------------------------|
| 0                             | 0.815 ± 0.003$^{a}$    | 0.812 ± 0.004$^{a}$    | 0.780 ± 0.014$^{a}$   |
| 1                             | 0.761 ± 0.012$^{a}$    | 0.750 ± 0.002$^{a}$    | 0.677 ± 0.014$^{a}$   |
| 2                             | 0.752 ± 0.007$^{a}$    | 0.731 ± 0.002$^{a}$    | 0.619 ± 0.028$^{a}$   |
| 4                             | 0.685 ± 0.036$^{a}$    | 0.689 ± 0.004$^{a}$    | 0.388 ± 0.042$^{c}$   |

Table 4. Effect of high light treatments (photon flux density 1000 µmol photons m$^{-2}$ s$^{-1}$ for 1, 2, and 4 hours) on the maximal photochemical efficiency of PSII measured as F$_{v}$/F$_{m}$ in control Arabidopsis thaliana leaves and plants exposed for 21 days to different Mn doses. Results are mean ± SE of five repetitions in three leaves of two plants each. Time 0 was measured in plants subjected to 250 µmol m$^{-2}$ s$^{-1}$ photon flux density. Different uppercase letters indicate significant differences between the times of exposure at the same Mn treatment (P < 0.05).
Discussion

In agreement with a number of previous studies examining the effects of Mn stress on various plant species (Alam et al., 2006; Lei et al., 2007; Doncheva et al., 2009; Mora et al., 2009; Stoyanova et al., 2009; Khabaz-Saberi et al., 2010) Arabidopsis plants subjected to increasing Mn concentrations, also exhibited a reduction in dry weight of both shoots and roots at doses between 500 and 1500 µM Mn (Fig. 1). The decline in biomass correlated with a gradual increase of Mn concentrations in both shoot and roots of Arabidopsis subjected to excess Mn supply (Fig. 2). It should be noted that the decline of biomass was more pronounced in roots, where even at the lowest Mn treatment (50 µM) the dry weight was 2.5-fold lower compared to leaves. Similar results have been reported by Delhaize et al. (2007), where transporter proteins were implicated in the endogenous Mn tolerance of wild-type Arabidopsis. More recently, Mora et al. (2009) demonstrated that Mn-tolerant ryegrass cultivars accumulated higher Mn concentrations in roots than shoots, while Mn-sensitive cultivars exhibited a greater Mn translocation from roots to shoots. These results are also consistent with studies in legumes such as white clover (T. repens L., Rosas et al., 2007). However, in two contrasting populations of P. cathayana, acclimated to wet and dry climate exposure to excess Mn caused an increase in Mn content of plant tissues especially in leaves and a visual symptoms of Mn toxicity (chlorosis) at high Mn concentrations (Lei et al., 2007). The chlorosis observed in the present experiments (data not shown) also correspond to a decreased amounts of both Chl a and Chl b could be due to a higher Mn accumulation in leaves after exposure to Mn excess, thus suggesting a damage to the photosynthetic apparatus as reported by Demirevska-Kepova et al. (2004).

Interestingly, while reduced CO₂ assimilation induced by excess Mn has been reported in many species and is considered one of the major physiological effects of Mn toxicity (Nable et al., 1988; Macfie and Taylor, 1992; González and Lynch, 1997; Kitao et al., 1997b; Lidon et al., 2004; Li et al., 2010), the functional integrity of the photosynthetic apparatus assessed by the maximum photochemical efficiency of PSII (Fv/Fm) did not decline as a result of excess Mn treatment in Citrus species (Papadakis et al., 2007; Li et al., 2010), rice (Lidon et al., 2004), Mn-sensitive maize (Doncheva et al., 2009), and cucumber (Feng et al., 2009). Furthermore, Kitao et al. (1997b) have shown that the potential maximum photochemical efficiency of PSII (Fv/Fm) is not affected by excess Mn in white birch, although the reduction state of PSII primary electron acceptor (QA) was increased at high Mn concentrations. These results clearly indicate that the excess Mn-induced decline in CO₂ assimilation may or may not be accompanied by changes in PSII photochemistry and this response is species dependent. The experimental data presented in this study also failed to demonstrate any substantial effects of excess Mn within a wide range of Mn concentrations on the maximum photochemical efficiency of PSII in Arabidopsis plants (Table 2). Assessing the relative abundance of PSII-associated proteins also showed no changes in the immunodetectable amounts of PSII reaction centre protein D1, the light-harvesting chlorophyll-protein complex of PSII (Lhcb1), and the manganese-stabilizing 33-kDa protein of the water splitting complex of PSII (PsbO) polypeptides (Fig. 5) in plants exposed to high Mn concentrations compared to control Arabidopsis. However, the observed low temperature shift of the S2/3QB – charge recombination and much lower overall TL emission implies lower redox potential of Qb (Fig. 3 and Table 3) confirms the suggestion of altered reduction state of PSII acceptor side in Mn-stressed plants (Kitao et al., 1997b). Since QA is in quasi-equilibrium with Qb and the PQ pool, the present results imply that lowering the redox potential of Qb will decrease the probability for forward electron transfer between the two quinone acceptors by shifting the redox equilibrium between QA, Qb, and O₂Qb towards QA, Qb towards QA, Qb (Minagawa et al., 1999; Ivanov et al., 2002, 2003) in plants exposed to high Mn concentrations.

In addition to the lack of significant inhibitory effects of excess Mn on PSII photochemistry discussed above, an earlier study reported that the photochemistry of photosystem I (PSI) and the photosynthetic electron transport were not significantly affected during early development of Mn toxicity in tobacco plants (Nable et al., 1988). However, a decreased Hill activity in isolated chloroplasts was found in mungbean leaves exposed to toxic Mn concentrations (Sinha et al., 2002). More recently, Li et al. (2010) have suggested that Mn excess can effectively impair the whole photosynthetic electron transport chain, thus restricting the production of reducing equivalents and limiting the rate of CO₂ assimilation in Citrus grandis seedlings. Despite these few studies,
the potential effect(s) of excess Mn on the functional/structural integrity of PSI remains elusive. As far as is known, the results presented in this study are the first report of an *in vivo* assessment of high Mn concentrations on PSI photochemistry. In contrast to PSII photochemistry, *in vivo* measurements of the oxidation state of P700 (P700+) (Klughammer and Schreiber, 1991; Ivanov *et al.*, 1998), the primary donor of PSI demonstrated that the relative amount of oxidizable P700 (P700⁺) decreased by 30% in Mn-treated *Arabidopsis* plants at concentrations above 1000 µM Mn (Fig. 4 and Table 5). The functional impairment of PSI photochemistry by excess Mn was accompanied by a significant reduction in the abundance of PSI reaction centre polypeptides (PsaA and PsaB, Fig. 5). This clearly indicates that the major target of Mn toxicity within the photosynthetic electron transport chain of *Arabidopsis* is PSI- rather than PSII-related components. The reduced amounts of PSI reaction centre polypeptides PsaA and PsaB would imply acceptor side limitations of the photosynthetic electron transport and this could explain the increased reduction state QA in Mn-stressed plants reported earlier (Kitao *et al.*, 1997b). Moreover, the decreased expression of another Fe-containing chloroplastic protein precursor, ferredoxin-1

![Fig. 5.](image-url)
serving as a terminal electron acceptor of the photosynthetic electron transport, observed in Mn-treated young rice leaves also supports Mn-induced limitations at the acceptor side of PSI (Führs et al., 2010).

One of the major mechanisms considered for Mn toxicity involves the inhibition of other essential cations including Fe, thus suggesting that a Mn-induced Fe deficiency may play a key role in the physiological responses to excess Mn (Foy et al., 1978; Foy, 1984; Kohno et al., 1984). More recently, chloroplast alterations in maize plants exposed to excess Mn (Doncheva et al., 2009) and Mn toxicity in young rice leaves (Führs et al., 2010) have been also ascribed to Mn-induced Fe deficiency rather than to direct Mn-induced oxidative stress. Given that about 80% of the plant Fe is located in the chloroplast (Terry and Abadia, 1986) and that the functional photosynthetic apparatus requires 22–23 iron atoms, of which PSI is the most Fe-abundant component (Ferreira and Straus, 1994), it seems reasonable to assume that the observed lower abundance of PSI reaction centre polypeptides and the associated decline of PSI photochemistry in Mn-treated Arabidopsis plants were consequences of a Mn-induced moderate Fe deficiency.

Although light energy is important for photosynthetic processes in plants, an excess of light can be also harmful because it can result in photoinhibition, which can be exacerbated when it is combined with other stresses (Powles, 1984; Aro et al., 1993; Sonoike, 1996). Photoinhibition is a complex phenomenon that may cause damage to the photosynthetic apparatus reducing the photosynthetic efficiency when light conditions exceed the photon requirements for photosynthesis (Murata et al., 2007). It is considered that PSI is the main site of photoinhibition (Aro et al., 1993; Sonoike, 1996; Takahashi and Murata, 2008), being more unstable than PSI, because the D1 protein, one of the two major heterodimeric polypeptides of the PSII reaction centre complex, has a very high light-dependent turnover rate (Aro et al., 1993; Burnap, 2004; Scheller and Haldrup, 2005; Takahashi and Murata, 2008). Earlier reports have shown that the susceptibility to Mn toxicity is strongly dependent on the light intensity and exposure of Mn-treated plants to high light can exacerbate the toxic effect of Mn (Horiguchi, 1988; Nable et al., 1988; González et al., 1998; Clair and Lynch, 2004; Hajiboland and Hasani, 2007). The present results also demonstrate that Arabidopsis plants predisposed to high Mn concentrations are more susceptible to photoinhibitory damage of PSII photochemistry in a concentration-dependent manner (Table 4).

Apart from the radiation-less dissipation of excess excitation energy in the chlorophyll pigment bed of LHCCI, associated with the formation of the xanthophylls pigment zeaxanthin, which is considered one of the major protective mechanisms against photoinhibitory damage (Horton et al., 1996; Niyogi, 1999), PSI-dependent cyclic electron transport has been also suggested to play a significant role in preventing the photoinhibitory damage of the photosynthetic apparatus during exposure of plants to high light conditions (Munekage et al., 2002; Takahashi et al., 2009). Considering the increased re-reduction rate of P700* in Arabidopsis (Table 5), the higher susceptibility of plants exposed to excess Mn to photoinhibition (Table 4) might be due to lower capacity of PSI-driven cyclic electron flow under conditions of Mn toxicity.

In summary, the results presented in this research demonstrate for the first time that exposure of Arabidopsis plants to excess Mn causes specific negative effects on the abundance of polypeptides comprising the reaction centre of PSI, thus resulting in decreased PSI photochemistry and lower capacity for cyclic electron transport, which may be due to a Mn-induced Fe deficiency and may have critical physiological implications under conditions of Mn toxicity in higher plants.

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