INTRODUCTION

Manganese (Mn) deficiency is a major plant nutritional disorder, often caused by an alkaline soil pH, which is favoring oxidation of soluble Mn^{2+} to the plant unavailable form MnO_2. Mn deficient crops are found in many areas of the world, but are especially widespread in Australia, USA, Asia and Northern Europe including Scandinavia. A recent survey, estimated that approximately 30% of soils in China are Mn deficient, causing severe yield and quality reductions in crops (Yang et al., 2007). Likewise, Mn deficiency has become the foremost plant nutritional problem in major parts of Scandinavia (Hebbern et al., 2005; Hebbern et al., 2009; Husted et al., 2009).

The O_2 evolving complex (OEC) in photosystem II (PSII) has a metalloenzyme core containing both Mn^{2+} and Ca^{2+} ions as well as the halogen ion Cl^- (Kok et al., 1970). The reaction center protein PsbA binds the inorganic core, which has the empirical formula $Mn_4Ca_1O_xCl_{1-2}(HCO_3)_y$ and is known as the tetra-nuclear Mn cluster (Dasgupta et al., 2008). More than 80% of all Mn contained in the chloroplasts is associated with PSII (Anderson et al., 1964). Thus, Mn deficiency leads to a rapid reduction in O_2 production (Andersson and Pyliotis, 1969) and to a marked change in Chl *a* fluorescence induction kinetics (Anderson and Thorne, 1968). Continuous Mn deficiency is followed by the development of characteristic visual leaf symptoms such as intravenous chlorosis and subsequently the development of necrotic spots, which are supposed to be related to disorganization of the thylakoid system and loss of PSII reaction centers (Simpson and Robinson, 1984). Manganese deficiency leads to photoinhibition and results in accumulation of highly oxidized species such as $P680^+$, which might lead to an increased generation of singlet oxygen and subsequent oxidation of the local protein environment, thereby promoting degradation of core proteins in PSII (Apel and Hirt, 2004). Krieger and co-workers (1998) demonstrated that successive depletion of Ca^{2+}, Cl^- or Mn^{2+} in OEC from spinach, resulted in the strongest reduction in PSII activity when Mn was depleted, being closely related to degradation of PsbA. A repair cycle operates to replace the damaged subunits of PSII, especially PsbA (Aro et al., 2005) and it has been estimated that the half life of PsbA in the light is just a few minutes, depending on the Chl concentration (He and Vermaas, 1998).

Genotypes of winter barley are known to respond differently to low Mn availability (Hebbern et al., 2005), a phenomenon commonly referred to as differential Mn efficiency (Ascher-Ellis et al., 2001). Mn deficiency can be controlled using Mn-efficient genotypes during moderate Mn limitation (Hebbern et al., 2005). However, the underlying physiological mechanisms are still incompletely understood despite the fact that numerous studies have been initiated to examine the issue during the last decades. Despite the fact that Mn is a key-component of PSII, there have not been any previous attempts to examine the relationship between differential Mn efficiency and the resistance of the photosynthetic apparatus to perturbations induced by Mn deficiency. Thus, this study was undertaken in order to identify genotypic differences in the energy transfer within the PSII reaction center that may correlate with Mn efficiency. The genotypic differences were investigated by pulse amplitude modulated (PAM) fluorescence, analysis of the fluorescence induction kinetics (FIK) and quantification of selected PSII and PSI key proteins by immunoblotting. Mn, Mg, S, Cu and Fe are all important co-factors in the photosynthetic apparatus and deficiencies of the individual elements were investigated by relating fluorescence data and the composition of PSII and PSI proteins with an ICP-MS based multi-elemental analysis

of leaf tissue in order to identify any possible unique effects of Mn on PSII, relative to disturbances caused by other nutrient deficiencies.

RESULTS & DISCUSSION

Mn content in leaf tissue and thylakoids

Plants were grown in hydroponics (Pedas et al. 2008). The development of visible leaf symptoms caused by Mn deficiency could be prevented if the quantum yield efficiency of PSII (F_v/F_m) was kept above 0.55 (data not shown). Thus, the intensity of Mn deficiency in plants was controlled by measuring F_v/F_m throughout the experimental period. Control plants were successfully maintained at F_v/F_m values close to the theoretical optimum 0.83 (Björkman & Demmig, 1987) for both genotypes throughout the experiment. However, 13 days after induction of Mn deficiency, a decline in F_v/F_m in the Mn deficient plants could be observed. This decline was most pronounced in the Mn-inefficient genotype Antonia, while the Mn-efficient Vanessa was less responsive to Mn deficiency (data not shown).

The Mn concentration was analyzed in the youngest fully expanded leaf (YFEL), 44 and 50 days after the induction of Mn deficiency, respectively **(Fig. 1)** and the Mn concentration of thylakoids was analyzed prior to termination of the experiment (data not shown). These results supported that the low F_v/F_m values were caused by Mn deficiency. The Mn concentration in the thylakoids of the inefficient genotype was reduced from 0.75±0.03 to 0.48±0.10 mg Mn g^{-1} chlorophyll under Mn deficiency, whereas the similar values for the efficient line were a reduction from 0.88±0.09 to 0.61±0.02 mg Mn g^{-1} chlorophyll. Thus, clearly less Mn was found in the thylakoids of Mn deficient plants, but no significant difference was found between the genotypes. Likewise, the Mn concentrations in leaves from deficient plants were significantly below the critical threshold concentrations of 17 µg Mn g DW^{-1} for both barley genotypes (Reuter et al., 1997). However, there were no significant differences in leaf tissue Mn concentrations between the genotypes in neither the Mn deficiency treatment nor the control treatments.

Mn deficiency leads to the development of a K and D step in the Chl *a* fluorescence induction curves

Besides reducing F_v/F_m and increasing the F_o values, Mn deficiency introduced a number of distinct changes in the position of the O-J-I-P steps and the general shape of the chlorophyll *a* fluorescence induction curves **(Fig. 2).** When the degree of Mn deficiency intensified and the F_v/F_m value was reduced below 0.67, a new step at 0.2-0.4 ms appeared in the fluorescence induction curves, designated as the K step by Strasser et al. (2004). This was accompanied by an additional and pronounced decline later in the curve, after the I step at 75-90 ms, designated as the D step by Strasser et al. (1995). The measurements on Mn deficient leaves from the genotype Antonia showed the greatest changes in the curves, representing a more extreme Mn deficient condition than was the case with Vanessa. It is important to note that the shape of the fluorescence induction curves with similar F_v/F_m values differed markedly between Antonia and Vanessa (*e.g.* compare curve #3 and #4 in Fig. 2), indicating that more severe disturbances in the functioning of the photosystems took place in the inefficient relative to the efficient genotype.

Mn deficiency affects the ability to perform state transitions

Plants have the ability to adjust the energy input to PSII and PSI by moving a mobile pool of the light harvesting complex (LHCII) proteins (Lhcb1, Lhcb2 and Lhcb3)

between the photosystems by a state transition process. The mobility of LHCII between photosystems is controlled by phosphorylation (see for instance Bellafiore et al., 2005). When LHCII is bound to PSII, plants are in state 1 and when LHCII is associated with PSI they are in state 2 (for a review see Haldrup et al., 2001). State transitions were determined 38 and 50 days after induction of Mn deficiency with similar results (**Fig. 3**). It is clearly seen that the baseline fluorescence (F_s) was stable and that no significant curvature occurred over time, indicating stable analytical conditions and accurate state transition measurements. When performing the state transition analysis, it was repeatedly noted that the background fluorescence level in Mn deficient leaves were more than doubled relative to the control (Compare **Fig. 3A** and **Fig. 3B**), which indicates an increased fluorescence emission from detached antenna proteins.

In the control plants (**Fig. 3A)**, the maximum fluorescence in state 1 (F_{m1}) was clearly higher than in state 2 (F_{m2}), indicating that the ability to adjust to PSI and PSII light was intact. The percentage change in state transitions ($(F_{m1}-F_{m2})/F_{m1}$) was measured to 9-11% in healthy control plants and was not significantly different between the control samples of both genotypes. In **Fig. 3B,** the effect of Mn deficiency on state transition in the Mn-inefficient genotype Antonia is illustrated and it is clearly seen that the ability to perform state transitions has been reduced significantly. It could be calculated that the percentage change in state transition for Mn deficient leaves was reduced to less than 1% of the Mn-inefficient genotype Antonia, whereas Mn deficiency did not cause any significant effect on state transitions in the Mn-efficient genotype Vanessa.

The quantum yield efficiency (F_v/F_m) was determined immediately before the state transition experiments and despite a striking difference in the ability to perform state transitions, at both 38 and 50 days after Mn deficiency induction, no significant differences in F_v/F_m values between Mn deficient Vanessa and Antonia were observed at both occasions (data not shown).

Immunoblotting showed that there was no significant difference in the amount of Lhcb1 between control and Mn deficient leaves, but a marked decrease in the phosphorylated form of LHCII (P-Lhcb1 and P-Lhcb2) was observed when plants were exposed to Mn deficiency **(Fig 4B)**. Likewise, a significant reduction in the Chl *a* concentration was observed in Mn deficient plants, whereas the Chl *b* concentration remained stable (**Fig. 4C**). The Chl measurements suggest a significant reduction in the amount of functional PSII reaction centers (PSII RC) relative to the peripheral antenna under Mn deficiency, which subsequently was confirmed by the immunoblot based quantification of PsbA (**Fig. 4D**). Generally, Mn deficiency lead to a 75% reduction in the amount of PsbA (representing PSII), whereas PSI-F (representing PSI) under the same conditions was reduced with approximately 45% in both genotypes. Time of analysis is a very important parameter to consider when nutrient deficiency studies are undertaken, as the impact of the physiological perturbations change with age and intensity of the deficiency. However, the trends described above were found to be highly consistent and was confirmed by repeating parts of the experiments several times, using plants of different physiological age and severity of Mn deficiency (data not shown). Thus, Mn deficiency resulted in a clear preferential degradation of the PSII RC relative to PSI. However, it is important to note that no significant genotypic effects in composition of RC and antenna proteins could be observed and apparently there is no simple linkage between protein expression and the differential reduction in state transition observed for Antonia under Mn deficiency (Compare **Fig. 4A** with **Fig. 4B, C and D**).

Influence of Mg, S, Fe, Cu and Mn deficiency on the photosynthetic apparatus

In order to compare the stability of PSII under Mn deficiency with the response observed when plants were exposed to other relevant nutritional disorders, a multi-elemental deficiency study was conducted, using the Mn-inefficient genotype Antonia. The induction of the different nutrient deficiencies was followed by visual inspection of leaf symptom development and by measuring Chl *a* fluorescence on the youngest fully expanded leaves, two to three times per week. Plants were sampled for analysis when the first leaf symptoms appeared (for Mg, S, Fe and Cu) or if no distinct symptoms developed, when F_v/F_m was reduced to 0.55 (for Mn). Very distinct leaf symptoms were noticed on Mg, S, Cu and Fe deficient plants before any significant decline in F_v/F_m was observed, whereas no symptoms developed for Mn even at a F_v/F_m value at 0.55 (data not shown). Cu and S deficiency resulted in a marked reduction in shoot growth and Cu deficiency resulted in the development of necrotic tips commonly referred to as "yellow tip" whereas S deficiency resulted in anthocyanosis of lower stems (data not shown). The chlorophyll content was reduced significantly in plants exposed to Mg-, S- and Fe-deficiency, but only marginally in Cu deficient plants despite a very low Cu concentration **(Table I)**.

Mn deficiency was the only nutritional disorder, which did not result in a decreased content of Chl nor any visible leaf symptoms and yet the F_v/F_m was reduced to 0.55 when plants were exposed to Mn deficiency **(Table I)**. The successful induction of the individual nutrient deficiencies (Mg, S, Fe, Cu and Mn) were confirmed by an ICP-MS based multi-elemental leaf tissue analysis. The Mg, S, Mn, Cu and Fe treatments were found to be well below the reported critical threshold limits for the development of deficiencies, being 1300, 1500, 17, 5 and 35 $\mu g\ g^{-1}$ dry weight (DW), respectively (**Table I**).

The impact of Mg, S, Fe, Cu and Mn deficiency on selected key-proteins in PSII and PSI was examined and compared with the control treatment (**Table II**). The amount of the central core protein of PSII, PsbA, was significantly reduced for Mn and Fe deficient plants compared to the control treatment, resulting in a 63% and 37% reduction, respectively. However, Mg, S and Cu deficiency did not affect the content of PsbA significantly. To further confirm damage to the central core of PSII, an antibody directed against PsbP, which is a subunit of the OEC, was used. The relative decrease in PsbP followed that of PsbA under Mn deficiency, however, it should be noted that the reduction in PsbP was insignificant under Fe-deficiency (**Table II**). The amount of Lhcb1, a subunit of the major light harvesting complex LHCII was insignificantly changed in Mg, S, Mn and Cu deficient plants. However, Fe-deficient plants appeared to have considerably less Lhcb1 compared to the control treatment (**Table II)** and there was a tendency towards an increase in the amount of Lhcb1 for the S and Cu deficient plants. Likewise, the amount of the PSI core subunit PsaF was significantly increased in the S and Cu deficient plants by 69% and 87% respectively.

CONCLUSIONS

The increased vulnerability of the Mn-inefficient genotype Antonia to Mn limitation appears to be influenced by photochemical disturbances. When the two genotypes were supplied equally with low additions of Mn to induce latent Mn deficiency, the leaf tissue and thylakoid concentrations were similar. However, the Chl *a* fluorescence induction curves were markedly different between the genotypes. The inefficient genotype Antonia had a reduced ability to perform state transitions whereas the energy transduction in the efficient genotypes was much less affected. This observation might reflect a higher

requirement for Mn in photosynthesis for Antonia, so that Mn becomes rate limiting at a higher physiological Mn concentration than in Vanessa. Alternatively, differences in the PsbA repair cycle, *viz*. timely re-supply of Mn to the photosystems, or differences in photo-protective mechanisms and susceptibility to damage from oxidative stress, may also be important processes.

The results presented in this paper show that Mn deficiency in barley plants primarily is targeting the OEC and affecting the donor side of PSII, primatrily due to loss of PSII core proteins (PsbA + PsbP). Thus, it can be concluded that even mild Mn deficiency without any distinct leaf symptoms may induce severe damages to PSII and consequently limit harvest yields, adaptability and survivability of plants under field conditions.

Table I. Maximum quantum yield efficiency of PSII (F_v/F_m) from dark adapted leaves, total chlorophyll content and the elemental composition of leaf tissue from plants exposed to different nutrient deficiencies. Values marked in bold were below the reported normal range for the individual nutrients (Reuter et al., 1997). Plants were sampled and analyzed when the first leaf symptoms appeared (Mg, S, Cu and Fe) or if no symptoms appeared when Fv/Fm <0.55 (See Materials and Methods). Data are presented as mean values ± SE (n=3). Values marked with the same letter for individual elements were not significantly different (P>0.05).

Treatment	Quantum yield efficiency (F_v/F_m)	Total Chl (mg chl g^{-1} DW	Normal leaf tissue concentration (µg g^{-1} DW)	Elemental concentration Mg	S	Mn (µg g^{-1} DW)	Cu	Fe
Control	0.819 ± 0.003[a]	11 ± 1.0[a]		1985 ± 32[bc]	3660 ± 90[b]	53 ± 2[ab]	11.8 ± 0.7[b]	67 ± 3[a]
-Mg	0.775 ± 0.007[ab]	4.3 ± 0.4[b]	1300 - 3000	**431 ± 24[d]**	2981 ± 107[c]	42 ± 3[b]	7.6 ± 0.4[c]	41 ± 3[b]
-S	0.781 ± 0.016[ab]	4.9 ± 0.3[b]	1500 - 4000	1762 ± 101[c]	**1152 ± 94[d]**	72 ± 16[a]	8.4 ± 0.9[c]	42 ± 2[b]
-Mn	0.512 ± 0.029[c]	9.1 ± 0.4[a]	17 – 100	2407 ± 109[b]	4340 ± 127[a]	**3.0 ± 0.2[c]**	15.6 ± 1.3[a]	74 ± 7[a]
-Cu	0.787 ± 0.005[a]	9.4 ± 1.1[a]	5-15	1779 ± 31[c]	3770 ± 169[b]	62 ± 9[ab]	**1.2 ± 0.1[d]**	75 ± 2[a]
-Fe	0.731 ± 0.024[b]	4.4 ± 0.9[b]	35-100	3075 ± 345[a]	4655 ± 250[a]	76 ± 3[a]	14.7 ± 1.8a[b]	**27 ± 2[c]**

Table II. The relative amounts of the PsbA, Lchb1, PsaF and PsbP proteins in Antonia thylakoids for various nutrient deficiencies using the control treatment as reference. Data are given as mean values ± SE (n=3). Values marked with the same letter were not significantly different (P>0.05).

Treatment	PsbA	Lhcb1	PsaF	PsbP
Control	1.0 ± 0.2^{ab}	1.0 ± 0.2^{ab}	1.0 ± 0.2^{b}	1.0 ± 0.1^{a}
-Mg	1.0 ± 0.3^{ab}	0.6 ± 0.1^{bc}	1.1 ± 0.2^{b}	0.9 ± 0.2^{a}
-S	0.8 ± 0.2^{bc}	1.4 ± 0.1^{a}	1.7 ± 0.5^{a}	1.0 ± 0.2^{a}
-Mn	0.4 ± 0.1^{d}	0.8 ± 0.1^{bc}	1.1 ± 0.1^{b}	0.5 ± 0.1^{b}
-Cu	1.2 ± 0.2^{a}	1.5 ± 0.2^{a}	1.9 ± 0.2^{a}	1.0 ± 0.2^{a}
-Fe	0.6 ± 0.1^{cd}	0.4 ± 0.1^{c}	0.7 ± 0.1^{b}	0.9 ± 0.1^{a}

Fig 1. Mn concentrations in leaf tissue for the Mn-efficient genotype Vanessa (Van) and the Mn-inefficient Antonia (Ant) measured 44 (black) and 50 (grey) days after planting, respectively. Mn (+) and Mn (-) indicate the Mn sufficiency and deficiency treatments, respectively. The critical threshold concentration for Mn in leaf tissue (17 µg Mn g^{-1} DM) is indicated by a dotted line (Reuter et al., 1997). Means ± SE (n = 3) with the same letter at each date are not significantly different.

Fig 2. Fluorescence induction kinetics from Mn deficient leaves of Mn-efficient Vanessa and Mn-inefficient Antonia, measured 57 days after planting. The individual phases of the transient are designated O-J-I-P. When F_v/F_m decreased below 0.67 in the Mn-inefficient genotype Antonia, the slope of the transients became negative at two distinct points, being 0.2-0.4 ms and 75-90 ms, and these additional steps were designated as the K and D steps, respectively (notice logarithmic x-axis). **Insert box:** a fluorescence transient from a control leaf, relative to all the Mn deficient (Mn-) measurements shown in the main figure.

Fig. 3. State transitions in the Mn-inefficient genotype Antonia measured 38 days after planting for **A:** control and **B:** Mn deficient plants. Dark adapted leaves were exposed to blue and red light preferentially exciting PSII (state 2) or PSI (state 1), respectively. State 2 was induced by illuminating the detached leaf for 20 min with blue light and F_m2 was determined after a saturating light pulse. Red light was then added and after 20 min F_m1 was determined after a saturating light pulse. F_o and F_m is the initial minimum and maximum PSII fluorescence in darkness and Fo' is the fluorescence minimum in light. Saturating white light pulses are indicated by arrows. For further information on the methodlogy please refer to Lunde et al. (2000) and Husted et al. (2009).

Fig. 4. A: State transition in Mn-efficient (Vanessa) and inefficient (Antonia) genotypes under control and Mn deficient conditions measured 55 days after planting. The percentage state transition was calculated using the difference in peak height of maximal fluorescence following a saturating pulse in state 1 and state 2, respectively (F_{m1}-F_{m2}/F_{m1}). Values are means ± SE (n = 3). Bars with the same letter were not significantly different. B: Immunoblotting of unphosphorylated (Lhcb1) and phosphorylated (Lhcb1 + Lhcb2) light harvesting protein when the Mn-efficient (Vanessa) and inefficient (Antonia) genotypes were exposed to control and Mn deficient conditions. Three individual preparations of thylakoid proteins from each combination of cultivar and treatment are shown. CF1-ATPase was included as a loading control. C: Chlorophyll *a* and *b* concentrations per g FW of the youngest fully expanded leaf (YFEL) in Mn-efficient (Vanessa) and inefficient (Antonia) genotypes exposed to control and Mn deficient condition. Values are means ± SE (n = 4). Bars with the same letter were not significantly different. D: Immunoblotting of PsbA and PSI-F representing PSII and PSI, respectively. The protein abundancy was quantified using chemiluminescence and expressed per unit total protein loaded. Values are means ± SE (n = 4). Bars with the same letter were not significantly different.

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