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In the mitochondrial CMSII mutant of *Nicotiana sylvestris* photosynthetic activity remains higher than in the WT under persisting mild water stress

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ABSTRACT

Photosynthetic responses to persisting mild water stress were compared between the wild type (WT) and the respiratory complex I mutant CMSII of *Nicotiana sylvestris*. In both genotypes, plants kept at 80% leaf-RWC (WT80 and CMSII80) had lower photosynthetic activity and stomatal/mesophyll conductances compared to well-watered controls. While the stomatal conductance and the chloroplastic CO_2 molar ratio were similar in WT80 and CMSII80 leaves, net photosynthesis was higher in CMSII80. Carboxylation efficiency was lowest in WT80 leaves both, on the basis of the same internal and chloroplastic CO_2 molar ratio. Photosynthetic and fluorescence parameters indicate that WT80 leaves were only affected in the presence of oxygen. Photorespiration, as estimated by electron flux to oxygen, increased slightly in CMSII80 and WT80 leaves in accordance with increased glycerate contents but maximum photorespiration at low chloroplastic CO_2 was markedly lowest in WT80 leaves. This suggests that carbon assimilation of WT80 leaves is impaired by limited photorespiratory activity. The results are discussed with respect to a possible pre-acclimation of complex I deficient leaves in CMSII to drive photosynthesis and photorespiration at low CO_2 partial pressure.

1. Introduction

Mitochondria influence the photosynthetic activity of plants [1]. In addition, mitochondrial function also modulates the response of plant leaves to various stress conditions including drought stress [2,3]. The importance of mitochondria to modulate the leaf response to mild water stress was investigated using the mitochondrial CMSII mutant of *Nicotiana sylvestris* which displays no respiratory complex I activity as a consequence of a lack of the NAD7 subunit resulting in incomplete assembly of the complex [4]. Depending on plant age and light conditions during growth, the dark respiration of the CMSII mutant is higher and the photosynthetic activity is lower than in WT leaves [5–7]. Lower stomatal conductance (g_s) and lower mesophyll conductance (g_m) decrease the chloroplastic CO_2 molar ratio (C_c) and carbon assimilation (A_N) and increase photorespiration in CMSII [8]. This correlates with slower growth rates of the mutant [4].

CMSII mutant leaves have altered redox homeostasis and elevated contents of certain antioxidative defence mechanisms leading to improved stress-tolerance as demonstrated after ozone fumigation or infection with the tobacco mosaic virus [9]. Moreover, while severe dehydration affects photosynthetic parameters similarly in CMSII and WT leaves [10], slow dehydration retarded the decline of leaf-RWC and of photosynthetic activity in CMSII compared to WT leaves [3]. This could be explained by a lower stomatal and hydraulic conductance of well watered CMSII leaves compared to the WT, thus retarding water loss and maintaining higher photosynthetic activity [3]. However, A_N and g_s was still higher in WT compared to CMSII leaves at the same leaf-RWC of 80%. This value, higher than the RWC at turgor loss pressure, which was identical in CMSII and WT leaves (76% RWC) [3], suggests that neither WT nor CMSII leaves suffered from severe water stress under the applied conditions.

Photorespiratory activity is long known to contribute to the maintenance of photosynthetic electron flux and by this way to protect leaves from photoinhibition [11,12]. In transgenic tobacco with increased cytokinin content, higher photorespiration relative to CO_2 fixation contributed to higher drought tolerance [13]. Moreover, as the most important and rapid responses of plants to water stress are the closure of stomata and a decline

Abbreviations: A_N, net photosynthesis; C_a, C_c, C_i, external, chloroplastic, internal CO_2 molar ratios; CMSII80, CMSII at 80% leaf-RWC; $E_c(C_i, J_c/J_o)$, carboxylation efficiency (as a function of C_i or as a function of J_c/J_o); g_s, g_m, stomatal, mesophyll conductances; J_c, J_o, electron fluxes to carboxylation, oxygenation; L_S, stomatal limitation to carbon assimilation; *NPQ*, non-photochemical fluorescence quenching; *qP*, photochemical fluorescence quenching; RWC, relative water content; WT, (WT80): wild type (at 80% leaf-RWC).

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of mesophyll conductance, a decreased CO_2 supply to chloroplasts may limit photosynthesis [14–16] and increase photorespiration. Similar to transgenic tobacco with high cytokinin content, also well watered CMSII leaves increased photorespiration, compared to well-watered WT leaves [8,13]. In this context, higher photorespiration activity of the CMSII mutant might contribute to improve tolerance to persisting mild water stress.

In the present investigation the ability to maintain photosynthetic activity was compared in WT and CMSII leaves under the same mild leaf-RWC of 80%. Under these conditions, in marked contrast to results reported in plants submitted to dehydration caused by a complete arrest of water supply [3], g_s was similar and photosynthetic activity was lower in WT as compared to CMSII leaves. This tolerance is discussed with differences in mesophyll conductance, photorespiration and metabolite accumulation.

2. Materials and methods

2.1. Plant material and growth conditions

Nicotiana sylvestris L. wild type (WT) and CMSII mutant [17] plants were grown in a greenhouse in 15 cm pots on peat soil. The plants were illuminated with daylight supplemented by sodium lamps (photoperiod of 16 h) at a PFD of 350 μmol m^{-2} s^{-1} at the leaf level and at 25/20 °C day/night temperature. Plants were irrigated regularly with Hoagland nutrition solution. After reaching a comparable developmental stage (2–3 month after sowing) with similar shoot and leaf size of WT and CMSII plants [3], irrigation was stopped until the leaf-RWC was 80% (4 days in WT and 6 days in CMSII leaves [3]). Thereafter the leaf-RWC was kept at 80% for 6 up to 10 days. Therefore the total pot weight was measured in the morning and the weight loss during 1 day was equilibrated by adding water (approximately 30 ml for WT and 20 ml for CMSII plants). Simultaneous measurements of RWC showed that the leaf-RWC was maintained at 80%. The two youngest fully developed leaves of well-watered control plants (93% RWC) and plants acclimated to 80% leaf-RWC (WT80, CMSII80) were used for the experiments after the first hour of daily illumination.

2.2. Leaf RWC measurement

The fresh-weight (FW) of a 15 mm leaf disc was determined immediately after harvest. Thereafter the leaf disc was incubated in deionised water for 24 h at 4 °C and the saturation weight (SW) was measured. Afterwards leaves were dried at 80 °C for 48 h to measure leaf dry-weight (DW). The RWC was calculated as:

$$\mathrm{RWC} = 100 \times \frac{\mathrm{FW} - \mathrm{DW}}{\mathrm{SW} - \mathrm{DW}} \quad (1)$$

2.3. Gas exchange and fluorescence measurements

Leaf gas exchange and chlorophyll fluorescence was measured on attached leaves with a Licor 6400 and a portable dew point generator (Li-610) as described [6]. The leaf temperature was maintained at 25 °C, the PFD at 350 μmol m^{-2} s^{-1} and the leaf vapour pressure deficit at 1 kPa. The external CO_2 partial pressure was varied in an atmosphere with 21% oxygen and subsequently in an atmosphere with 0.5% oxygen. Experiments started by at least 30 min dark adaptation of the leaves, measurements of dark respiration under atmospheric conditions and subsequent measurements of F_o and F_m which was followed by light acclimation until photosynthesis and stomatal conductance stabilized. Light respiration (R_l) was estimated as dark respiration/2 in accordance with results obtained from well-watered WT and CMSII leaves [8]. The photosynthetic parameters under growth conditions, as shown in Tables 1 and 2, were calculated from the last 4 measurements after stabilization of A_N and g_s in the light. Chlorophyll fluorescence parameters (F_s, F_m' and F_o') were measured every 5 min during illumination by a saturating flash and a subsequent illumination with far-red light and used to calculate *qP* and *NPQ* [6]. The quantum yield of PSII (ΦPSII) was calibrated against the quantum yield of carbon assimilation (ΦCO_2) at 0.5% O_2 as shown in Fig. 3. This calibration curve was applied to recalculate total photosynthetic electron flux (J) [18] and electron flux to carboxylation (J_c) and to oxygenation (J_o) of Rubisco [19]. The ratio J_c/J_o was taken as a relative estimation of chloroplastic CO_2 partial pressure as described in Djebbar et al. [3], assuming that electrons transported by PSII are partitioned between the carboxylation and the oxygenation reaction of Rubisco and that alternative electron flux to the Mehler reaction is very low under the applied conditions [20]. It was shown previously, that A_N as a function of J_c/J_o corresponded well to results of A_N as a function of C_c in well watered WT and CMSII leaves [8], thus validating the use of J_c/J_o as a relative measure of chloroplastic CO_2 molar ratio.

The stomatal limitation was calculated according to Farquhar and Sharkey [21] as:

$$L_s = 1 - \frac{A_N}{A_o} \quad (2)$$

where A_o represents carbon assimilation in the absence of stomatal limitation.

The mesophyll conductance g_m was calculated according to Harley et al. [22] using Γ^* from previous measurements of WT and CMSII leaves [8].

$$g_m = \frac{A_N}{C_i - (\Gamma^* \times (J + 8(A_N + R_l)))/J - 4(A_N + R_l)} \quad (3)$$

Calculated g_m was used to estimate the chloroplastic CO_2 partial pressure under growth conditions according to Gallé et al. [23]:

$$C_c = C_i - \frac{A_N}{g_m} \quad (4)$$

2.4. Metabolite determinations

Metabolite contents were measured by GC–TOF.MS except for amino acid contents, which were determined by HPLC as described in Hager et al. [24].

All experiments were repeated at least for 3 times (metabolite determinations) or 4 –6 times (gas exchange measurements) and mean values and the standard errors are shown. Statistic significant differences at the 5% level were calculated with the students *T*-test using the Sigma Plot program.

3. Results

3.1. Photosynthetic responses to 80% leaf-RWC

At a growth irradiance of 350 μmol m^{-2} s^{-1} and at 25 °C, stomatal conductance (g_s) and carbon assimilation (A_N) of well-watered WT leaves were higher than in the CMSII counterpart. In contrast, while g_s and A_N declined in leaves acclimated to 80% leaf RWC in both genotypes they were higher in CMSII80 than in WT 80 leaves (Table 1). As shown previously, small variations of g_s, which parallel those of stomatal limitation to carbon assimilation (L_s), may explain significant differences of A_N (see Fig. 4a in Djebbar et al. [3]). However, stomatal limitation to CO_2 diffusion alone did not explain the difference of A_N between WT80 and CMSII80 leaves, since the internal CO_2 molar ratio (C_i) remained slightly higher in WT80 than in CMSII80. Similar to g_s, the mesophyll conductance (g_m) declined in CMSII80 and WT80 leaves compared to well-watered controls and was lowest in WT80 leaves (Table 1). From these results a slightly

Table 1
Several photosynthetic parameters measured under growth conditions (25 °C, PFD: 350 μmol m^{-2} s^{-1}, 380 ppm CO_2) in WT and CMSII leaves under well-watered conditions and after acclimation to 80% leaf-RWC (WT80, CMSII80). Net carbon assimilation A_N, dark respiration R_N, stomatal conductance g_s, limitation of assimilation by stomatal conductance L_S, mesophyll conductance g_m, intercellular CO_2 partial pressure C_i, chloroplastic CO_2 partial pressure C_c. Results are means of 4–6 independent experiments. Significant differences at the 5% level are indicated by different letters.

	WT	WT80	CMS	CMS80
A_N (μmol m^{-2} s^{-1})	14 ± 0.5^{a}	7.0 ± 0.2^{d}	11.2 ± 0.7^{b}	8.8 ± 0.4^{c}
R_N (μmol m^{-2} s^{-1})	−1.8 ± 0.06^{a}	−1.2 ± 0.07^{c}	−2.3 ± 0.19^{b}	−1.7 ± 0.23^{abc}
g_s (mol m^{-2} s^{-1})	0.32 ± 0.05^{a}	0.10 ± 0.01^{c}	0.21 ± 0.03^{b}	0.12 ± 0.01^{c}
L_S (%)	14.6 ± 2.2^{a}	28.2 ± 2.8^{b}	18 ± 2.7^{ab}	31.2 ± 4.8^{b}
g_m (mol m^{-2} s^{-1})	0.27 ± 0.02^{a}	0.08 ± 0.005^{d}	0.18 ± 0.02^{b}	0.11 ± 0.006^{c}
C_i (μmol mol^{-1})	298 ± 1^{a}	249 ± 4^{c}	277 ± 3^{b}	242 ± 4^{c}
C_c (μmol mol^{-1})	239 ± 4^{a}	160 ± 3.5^{c}	215 ± 2^{b}	169 ± 4^{c}

Table 2
Carboxylation efficiency (E_c), photochemical (qP) and non-photochemical (NPQ) fluorescence quenching and electron transport to oxygen J_o and the ratio of electron flux to carboxylation/oxygenation (J_c/J_o) of well watered leaves (WT, CMSII) and after acclimation to 80% leaf-RWC (WT80, CMSII80). E_c was calculated from the initial slope of A_N curves at 25 °C and 350 μmol m^{-2} s^{-1} PFD (corresponding to growth conditions) as a function of C_i, and as a function of the ratio of electron flux to carboxylation/oxygenation (J_c/J_o) which parallels C_c. Significant differences at the 5% level are indicated by different letters.

	WT	WT80	CMSII	CMSII80
E_c (C_i) (μmol m^{-2} s^{-1})	0.082 ± 0.006^{a}	0.047 ± 0.002^{c}	0.069 ± 0.003^{ab}	0.058 ± 0.004^{bc}
% of well watered control	100 ± 7	57 ± 2	100 ± 4	84 ± 6
E_c (J_c/J_o) (μmol m^{-2} s^{-1})	9.5 ± 0.2^{a}	7.9 ± 0.2^{c}	10.7 ± 0.4^{b}	10.5 ± 1^{ab}
% of well watered control	100 ± 2	83 ± 2	100 ± 4	98 ± 9
qP	0.89 ± 0.01^{a}	0.76 ± 0.01^{c}	0.89 ± 0.01^{ab}	0.84 ± 0.02^{b}
% of well watered control	100 ± 1	85 ± 1	100 ± 1	93 ± 2
NPQ	0.37 ± 0.01^{a}	0.91 ± 0.07^{c}	0.50 ± 0.03^{b}	0.49 ± 0.1^{ab}
% of well watered control	100 ± 3	246 ± 19	100 ± 6	98 ± 20
J_o (μmol m^{-2} s^{-1})	25 ± 1.7^{a}	28.5 ± 1.2^{a}	30.1 ± 1.2^{ab}	35.1 ± 2.6^{b}
% of well watered control	100 ± 7	114 ± 5	100 ± 4	117 ± 8
J_c/J_o	3.1 ± 0.3^{a}	1.6 ± 0.1^{c}	2.2 ± 0.1^{b}	1.7 ± 0.1^{c}
% of well watered control	100 ± 10	52 ± 3	100 ± 5	77 ± 5

higher chloroplastic CO_2 molar ratio (C_c) in CMSII80 compared to WT80 leaves was calculated according to Eq. (4) (Table 1). A significant contribution of g_m to the lower A_N of WT80 compared to CMSII80 leaves might therefore be considered.

No significant changes in total chlorophyll and total carotenoid contents and of the Chla/b ratio were measured in well-watered leaves and at 80% leaf-RWC (not shown).

3.2. Carbon assimilation as a function of C_i and J_c/J_o

In order to characterize the photosynthetic activity of WT and CMSII leaves at 80% leaf-RWC, A_N was measured as a function of internal CO_2 molar ratio at growth light and temperature. As shown in Fig. 1, A_N was lower in well-watered CMSII compared to WT leaves at low C_i but similar at high C_i. At 80% leaf-RWC, A_N as a function of C_i declined more in WT than in CMSII leaves. The carboxylation efficiency (E_c) as a function of C_i was calculated from the initial slope of A_N/C_i curves at the three lowest C_i values applied. Table 2 shows that compared to well-watered leaves $E_c(C_i)$ declined significantly in leaves of WT80 but non-significantly in CMSII80. In order to investigate whether the decline of $E_c(C_i)$ and of maximum photosynthetic activity in leaves at 80% leaf-RWC was caused by the decline of g_m, A_N was also plotted as a function of electron flux to the ratio of carboxylation to oxygenation reaction of Rubisco (J_c/J_o) (Fig. 2). This ratio is an estimation of C_c [3,16]. The E_c as a function of J_c/J_o was highest in CMSII leaves and was not changed at 80% leaf-RWC (Table 2 and insert Fig. 2). Thus, the small decline of $E_c(C_i)$ observed in CMSII80 compared to well-watered CMSII leaves is entirely caused by the decline of g_m. In contrast, $E_c(J_c/J_o)$ still declined in WT80 (83% of well-watered WT) although less as when calculated as a function of C_i (57% of well-watered WT) (Table 2). In WT80 and CMSII80 leaves the maximum measured A_N at high C_i and high J_c/J_o, was markedly lower than A_N measured at the same C_i and J_c/J_o in the corresponding well-watered leaves (Figs. 1 and 2). This indicates that factors independent of CO_2 diffusion affected the maximum photosynthetic activity at C_c values well above those in ambient air, in particular in WT80 leaves.

3.3. PSII quantum yield, chlorophyll fluorescence quenching and electron flux

The quantum yield between primary photosynthetic reactions (i.e. ΦPSII) and carbon exchange (i.e. ΦCO_2) was analysed. In the presence of 0.5% oxygen, ΦCO_2 was linearly proportional to ΦPSII with the same regression line in all varieties, showing that 8.2 photons are required for the assimilation of 1 molecule of CO_2 (Fig. 3A). In the presence of 21% oxygen however, the relation between ΦPSII

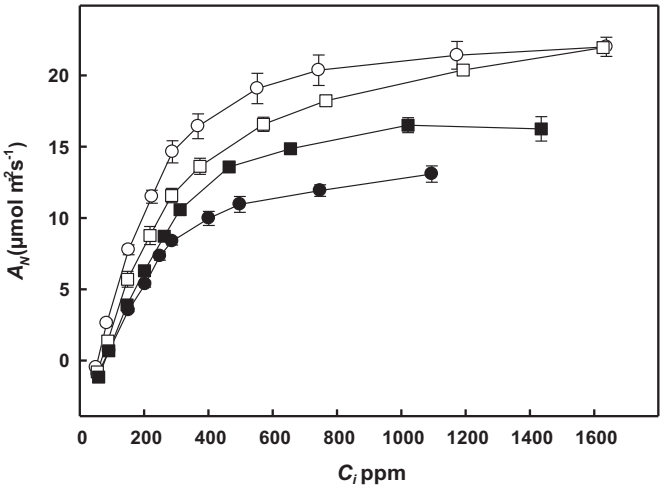

Fig. 1. Net carbon assimilation A_N as a function of internal CO_2 partial pressure in well-watered WT (○) and CMSII (□) leaves and after acclimation to 80% leaf-RWC (WT80 (●), CMSII80 (■)) at 350 μmol m^{-2} s^{-1} PFD and 25 °C and 21% O_2. Results show means of 4–6 independent experiments.

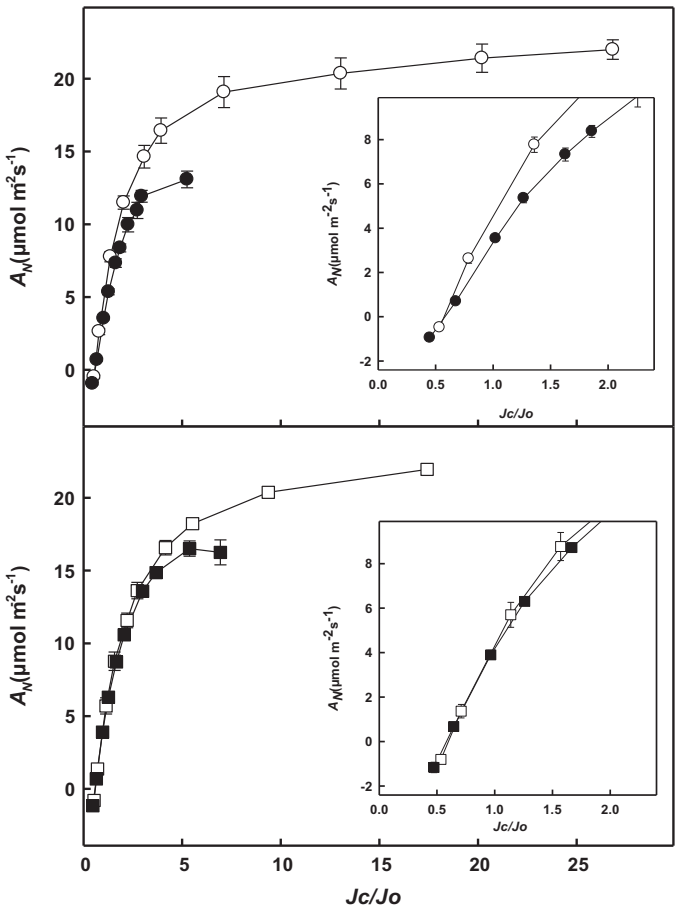

Fig. 2. Net carbon assimilation A_N as a function of the ratio J_c/J_o, which estimated C_c in well-watered WT (○) and CMSII (□) leaves and after acclimation to 80% leaf-RWC (WT80 (●), CMSII80 (■)) at 350 µmol m^{-2} s^{-1} PFD and 25 °C and 21% O_2. Insert: expansion of the scale at low J_c/J_o. Results show means of 4–6 independent experiments.

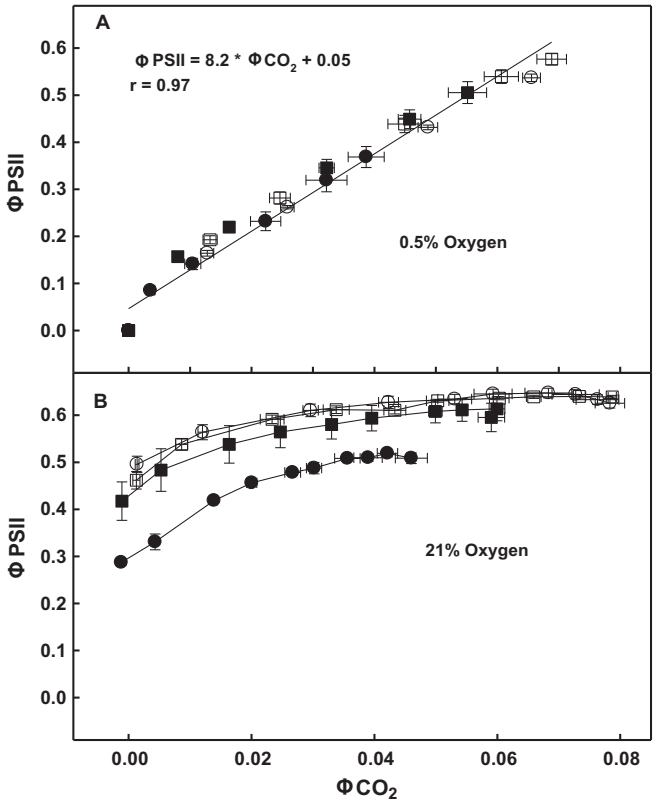

Fig. 3. Relation of quantum yield of PSII (ΦPSII) as a function of quantum yield of CO_2 assimilation (ΦCO_2) at 350 µmol m^{-2} s^{-1} PFD and 25 °C in well-watered WT (○) and CMSII (□) leaves (white) and after acclimation to 80% leaf-RWC (black) (WT80 (●), CMSII80 (■)) at 0.5% oxygen (A) and at 21% oxygen (B). Results show means of 4–6 independent experiments. The linear regression line in (A) was calculated from all values shown.

and ΦCO_2 was not linear and visibly different in WT80 leaves compared to leaves from all other conditions (Fig. 3B), suggesting that the presence of atmospheric oxygen affected particularly WT80 leaves. The analysis of chlorophyll fluorescence parameters on the basis of the same C_c as estimated by J_c/J_o in an atmosphere of 21% oxygen showed that *qP* declined markedly in WT80 leaves but only slightly in CMSII80 leaves compared to well watered leaves (Fig. 4). Even more pronounced, *NPQ* increased in WT80 but did not change in CMSII80 compared to well-watered controls (Fig. 4). The lower *qP* and the higher *NPQ* in WT80 leaves were also evident when compared to CMSII80 or control leaves measured under growth conditions at ambient C_a (Table 2). At low C_c (= low J_c/J_o), the maximum J_o declined in WT80 leaves but not in CMSII leaves compared to well-watered controls (Fig. 5). In ambient CO_2, J_o increased however, by the same extent in WT80 and CMSII80 leaves compared to well-watered leaves, but always remained higher in CMSII (Table 2).

3.4. Metabolite contents

The glycolate content of all varieties was similar and did not change at 80% leaf-RWC. In contrast, glycerate contents increased in WT80 and CMSII80 leaves by the same extent compared to well-watered leaves, and paralleled results of J_o, showing a significantly higher glycerate content in CMSII than in WT leaves (Fig. 6). Most markedly, contents of glycine and serine were much lower in CMSII80 compared to CMSII leaves without significant change in the glycine/serine ratio (CMS: 0.39 ± 0.13 CMS80: 0.61 ± 0.08). In WT80 leaves however, contents of serine declined while those of glycine increased leading to a significant higher glycine/serine ratio compared to all other treatments (WT: 0.68 ± 0.07, WT80: 1.28 ± 0.18, Fig. 6).

Dark respiration was lower at 80% leaf-RWC compared to well-watered leaves in both varieties (Table 1). Similarly, citrate and fumarate contents declined strongly in WT80 and CMSII80 leaves. However, the pyruvate content declined only in WT80 leaves while inorganic phosphate content increased (Fig. 7). Contents of total sugars and sugar alcohols increased strongly at 80% leaf-RWC compared to well-watered leaves and this increase was much higher in WT80 than in CMSII80 leaves. Saccharose contents increased in WT80 leaves only and the increase of sugar alcohols was mainly caused by an increase of myo-inositol (Fig. 8).

4. Discussion

4.1. Photosynthesis is higher in the CMSII mutant under persisting mild water stress

In contrast to well watered conditions, lack of complex I activity in the CMSII mutant of *N. sylvestris* is associated with higher photosynthetic activity during persisting mild water stress (80% leaf-RWC) as compared to the WT (Table 1 and Fig. 1). This was not observed during the complete arrest of watering, where A_N was lower in CMSII than in WT leaves at 80% leaf-RWC [3] and identical under severe water stress [10]. During dehydration, A_N is mainly controlled by stomatal opening. This was demonstrated by a same relation between A_N and g_s and between A_N and J_c/J_o in WT and CMSII leaves [3]. Increased dehydration tolerance of CMSII

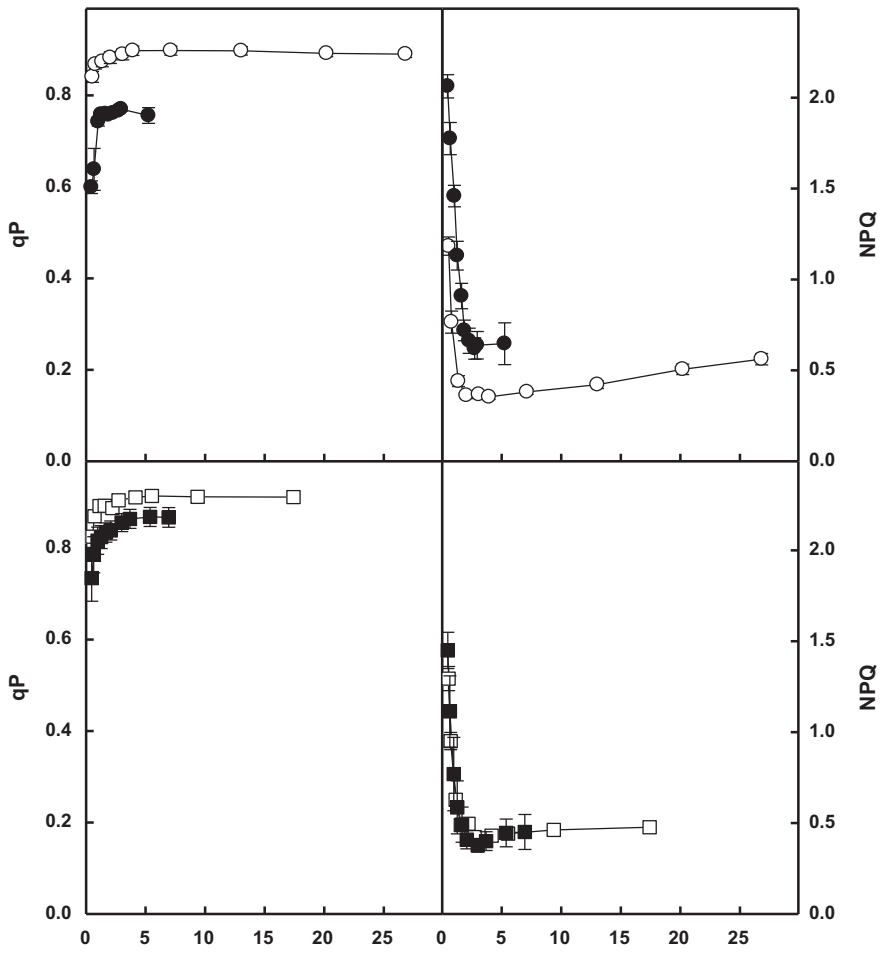

Fig. 4. Photochemical (*qP*) and non-photochemical (*NPQ*) quenching of chlorophyll fluorescence as a function of the ratio J_c/J_o in well-watered WT (○) and CMSII (□) leaves and after acclimation to 80% leaf-RWC (WT80 (●), CMSII80 (■)) at 350 μmol m^{-2} s^{-1} PFD, 25 °C and 21% oxygen. Results show means of 4–6 independent experiments.

compared to the WT leaves was therefore explained by a lower g_s and a concomitant higher leaf-RWC in CMSII leaves during the first days after the arrest of watering [3]. After extended mild water stress at 80% leaf-RWC, g_s was similar in WT and CMSII leaves, although slightly lower in the former. However, since L_S, C_i, C_c and J_c/J_o did not differ significantly between WT80 and CMSII80 leaves (Tables 1 and 2), the lower A_N in WT80 leaves is not related to a lower stomatal conductance. It is well known that patchy stomatal closure and cuticule transpiration may lead to erroneous C_i readings and erroneous conclusions regarding diffusional or biochemical limitation of A_N [14]. However, an important error for C_i calculation was only suggested at very low g_s [14], which was not the case in this study. Furthermore, the higher A_N of CMSII80 compared to WT80 leaves was consistently observed at all C_i values applied (Fig. 1) and the turgor lost pressure, indicating plasmolysis, was identical at 80% leaf-RWC in WT and CMSII leaves [3]. Finally, A_N remained even higher in CMSII80 compared to WT80 leaves when calculated at the same J_c/J_o (Fig. 2). Assuming that the specificity factor of Rubisco and the oxygen molecular ratio in chloroplasts are the same under all conditions, the J_c/J_o ratio is an estimation of the chloroplastic CO_2 molar ratio C_c [3,8]. Since the J_c/J_o ratio is independent of both, g_m and C_i, its calculation avoids pitfalls as could be induced by patchy stomatal closure under drought stress [14]. These results do not exclude that diffusional limitations are important factors affecting photosynthesis [14] in *N. sylvestris* leaves subjected to mild water stress, but highlights that additional, non-diffusive limitations affect photosynthesis in WT80 compared to CMSII80 leaves. Since A_N increased in CMSII80 leaves but decreased in WT80 leaves compared to measurements at the same leaf-RWC after the arrest of watering (Fig. 5 in Djebbar et al. [3]), CMSII obviously acclimated to this condition while the WT do not.

4.2. Lower mesophyll conductance only partially explains the lower carbon assimilation in WT80 leaves

Besides stomatal conductance, decrease in mesophyll conductance to CO_2 (g_m) is thought to limit A_N under water stress, possibly by affecting aquaporin contents and (or) activity participating in CO_2 diffusion [10,14,16,23,25–27]. It was previously shown by carbon isotope discrimination that g_m was higher in well-watered WT leaves than in CMSII leaves [8]. In this study a similar result was obtained using carbon exchange measurements although differences between CMSII and WT leaves were somehow less pronounced (Table 1). Assuming that C_i measurements were reliable at 80% leaf-RWC, g_m declined in both genotypes. In WT80 leaves however, g_m was the lowest and may be the dominant factor affecting A_N compared to CMSII80 (Table 1). The contribution of g_m to limit E_c was therefore estimated by comparing A_N as a function of C_i and as a function of C_c as determined by the J_c/J_o ratio, which is independent of possible erroneous calculations of C_i (Figs. 1 and 2). In CMSII80, E_c declined as a function of C_i but not as a function of J_c/J_o, indicating that g_m affected E_c and A_N. In contrast E_c was lowest in WT80 compared to well-watered WT leaves both, as a function of C_i and J_c/J_o (Table 2). In conclusion, lower g_m as well as lower g_s affected A_N in WT80 and CMSII80 compared to

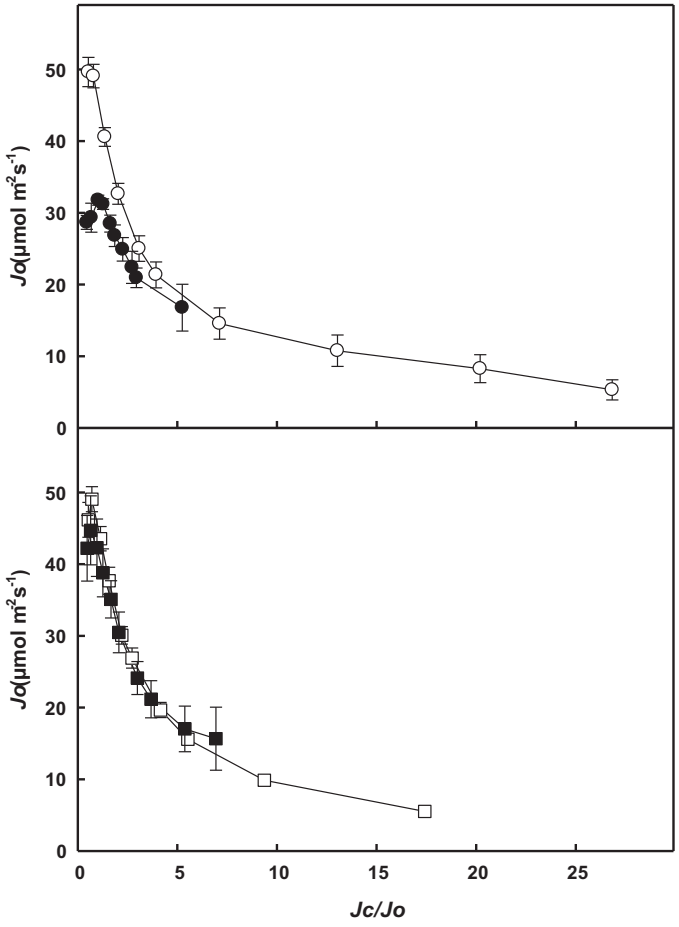

Fig. 5. Electron flux to oxygen (J_o) as a function of the ratio J_c/J_o in well-watered WT (○) and CMSII (□) leaves and after acclimation to 80% leaf-RWC (WT80 (●), CMSII80 (■)) at 350 μmol m^{-2} s^{-1} PFD and 25 °C and 21% oxygen. Results show means of 4–6 independent experiments.

well-watered leaves but A_N of WT80 leaves was also affected by other, additional, non-diffusive parameters.

4.3. Primary photosynthetic reactions decline in WT80 leaves in the presence of oxygen

The operational efficiency of primary photosynthetic reactions, estimated by plotting the quantum efficiency of PSII (ΦPSII) against the quantum efficiency of carbon fixation (ΦCO_2) in an atmosphere of 0.5% oxygen [6] was identical in leaves from all growth conditions (Fig. 3a). This demonstrates that PSII function was neither affected in WT80 nor in CMSII80 leaves. In the presence of 21% oxygen, however, the relation between ΦPSII and ΦCO_2 was not linear and markedly lowest in WT80 leaves (Fig. 3b), showing that atmospheric oxygen affected the relation between PSII activity and carboxylation efficiency in WT80 leaves. The strong decline of *qP*, indicating enhanced relative Q_A-reduction in PSII, and the marked increase of *NPQ*, indicating the build-up of a high proton gradient [28] in WT80 leaves compared to well-watered controls, confirmed an inhibitory effect of atmospheric oxygen in photosynthesis (Fig. 4 and Table 2). In CMSII80 leaves, *qP* and *NPQ* were similar to well-watered controls (Fig. 4 and Table 2). The decline of *qP* and the increase of *NPQ* in WT80 leaves correlated to results where chloroplastic ATP-synthase was repressed [29]. A water-stress-induced decrease of chloroplastic ATP synthase was reported in sunflower leaves [30]. Assuming that chloroplastic ATP availability of WT80 leaves really limits assimilation, this effect must be oxygen dependent, implying that the ATP demand by photorespiration exceeds the ATP-synthesis capacity. If true, ATP synthesis is not restricted by phosphate availability, since total free phosphate contents increased markedly in WT80 leaves (Fig. 7), contrary to previous suggestions that phosphate limitations may explain biochemical restrictions to photosynthesis under water stress [14]. However, after 5 days of dehydration, total ATP content was higher in WT compared to CMSII leaves on a fresh weight basis whereas it was lower in well watered leaves [3], questioning a possible limitation of ATPase for A_N of WT80 leaves.

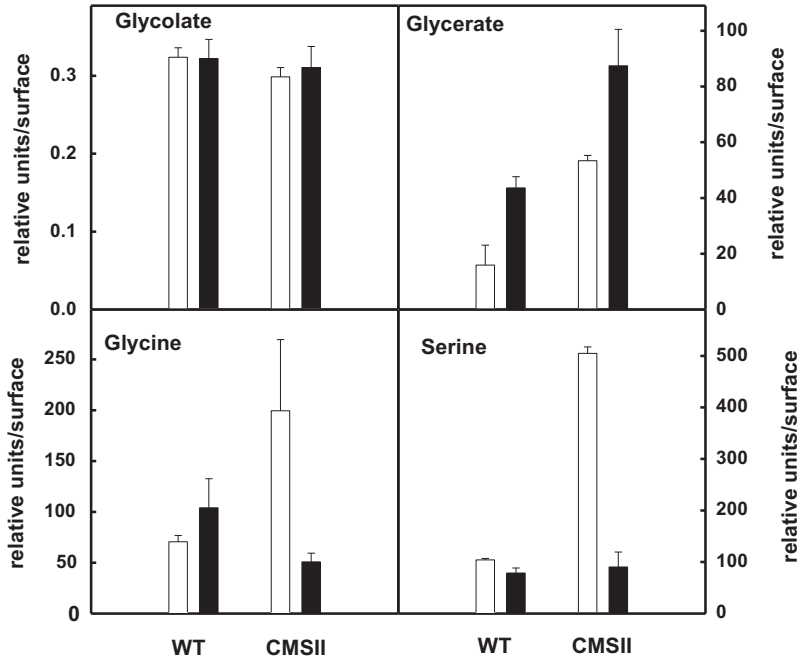

Fig. 6. Metabolites of the photorespiratory pathway in well-watered WT and CMSII leaves (white) and after acclimation to 80% leaf-RWC (black). Results show means of 3 independent experiments.

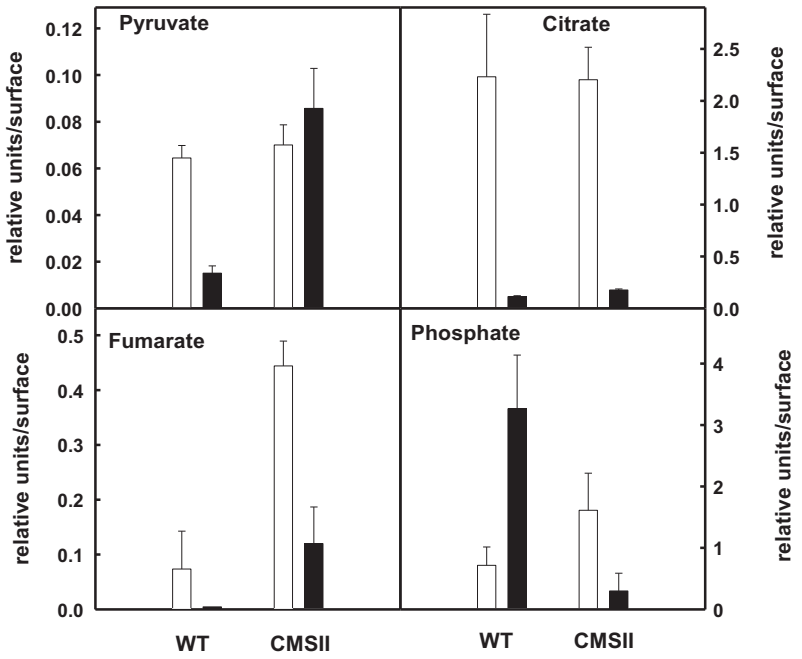

Fig. 7. Metabolites of glycolysis from the Krebs-cycle and inorganic phosphate in well-watered WT and CMSII leaves (white) and after acclimation to 80% leaf-RWC (black). Results show means of 3 independent experiments.

4.4. Photorespiration may become limiting in WT80 leaves

Photorespiration is the most important sink of electrons and of ATP under CO_2-limitation [31] and contributes to water stress tolerance in cytokinin overexpressing transgenic tobacco leaves [13]. The photorespiratory activity was estimated as electron flux to oxygen (J_o) according to Valentini et al. [19] and by monitoring glycerate contents, an end-product of photorespiration. In accordance with higher photorespiratory activity of the CMSII mutant [8], J_o and glycerate contents were higher than in the WT and increased in both by the same magnitude at 80% RWC (Table 2 and Fig. 6). However, at low J_c/J_o when CO_2 supply is strongly limiting, J_o was markedly lowest in WT80 leaves, suggesting limitation by photorespiratory capacity (Fig. 5). Glycerate and other metabolites of the photorespiratory pathway like phosphoglycolate and glyoxylate are known inhibitors of the Calvin cycle [32–35] and their accumulation may affect carbon assimilation. Since glycerate contents remained much higher in CMSII than in WT leaves under well-watered conditions as well as at 80% leaf-RWC, a glycerate-induced decline of A_N in WT80 compared to CMSII80 leaves is very unlikely. Increases of glycine and declines of serine contents in WT80 leaves (Fig. 6) suggest, however, an

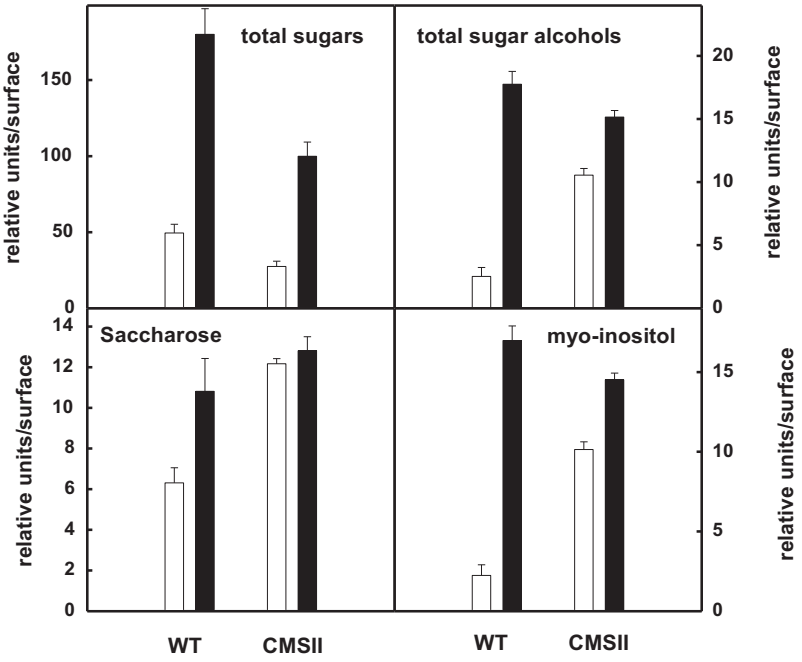

Fig. 8. Sugar and sugar alcohol contents in well-watered WT and CMSII leaves (white) and after acclimation to 80% leaf-RWC (black). Results show means of 3 independent experiments.

impairment of photorespiration at the level of glycine decarboxylation. Glycine decarboxylation requires continuous reoxidation of NADH in the mitochondrion [5]. Higher total NAD(H) contents in the CMSII mutant compared to WT leaves even under water stress [3] may improve the redox buffer capacity in the mitochondria of CMSII80 leaves. It remains therefore to be demonstrated whether phosphoglycolate, glyoxylate or other potential intermediates of photorespiration accumulate in WT80 leaves and act as Calvin cycle inhibitors.

4.5. Respiratory substrate and sugar contents

While electron flow through photorespiration increased, mitochondrial dark respiration, as measured by CO_2 release and citrate and fumarate contents decreased at 80% leaf-RWC compared to well-watered controls (Table 1 and Fig. 7). A strong decline of citrate and fumarate contents was already observed during dehydration [3] but fumarate contents and dark respiration remained higher in CMSII compared to WT leaves, probably indicating the enhanced requirement of respiratory compensation for the complex I deficiency [36]. Interestingly, pyruvate contents decreased and saccharose and inorganic phosphate contents increased only in WT80 leaves (Figs. 7 and 8), suggesting that substrates are redistributed from glycolysis to saccharose synthesis and that metabolites became largely dephosphorylated. As observed in many other investigations about drought stress [3,16,37], soluble sugar contents and, in addition, sugar alcohol contents, in particular myo-inositol, increased in CMSII80 and more markedly in WT80 leaves compared to well-watered controls (Fig. 8). The accumulation of sugars and sugar alcohols is currently interpreted to increase the cellular osmotic potential in order protect cells from dehydration [38] and results from lower sucrose export from leaves [16]. Since sugar accumulation may induce feed-back inhibition of photosynthetic carbon assimilation [16], the higher A_N of CMSII80 compared to WT80 leaves may also result from different sugar accumulation and feed-back inhibition. In addition, the synthesis of sugar alcohols may indicate a requirement for consumption of additional reducing equivalents from mitochondria and (or) chloroplasts.

It remains, however, to determine how mitochondrial complex I deficiency interacts with photorespiration. Whatever the mechanism of mitochondria/chloroplast interaction, the higher A_N of CMSII compared to WT leaves at persisting mild water stress, likely involves enhanced photorespiratory activity in the mutant, which is already pre-acclimated to cope with lower chloroplastic CO_2 supply under well-watered conditions and the results presented in this manuscript supports a major role of mitochondrial function in drought tolerance of crop plants.

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