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RESEARCH PAPER

Effects of drought stress and subsequent rewatering on photosynthetic and respiratory pathways in *Nicotiana sylvestris* wild type and the mitochondrial complex I-deficient CMSII mutant

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Abstract

The interaction of photosynthesis and respiration has been studied *in vivo* under conditions of limited water supply and after consecutive rewatering. The role of the alternative (v_{alt}) and cytochrome (v_{cyt}) pathways on drought stress-induced suppression of photosynthesis and during photosynthetic recovery was examined in the *Nicotiana sylvestris* wild type (WT) and the complex I-deficient CMSII mutant. Although photosynthetic traits, including net photosynthesis (A_N), stomatal (g_s) and mesophyll conductances (g_m), as well as respiration (v_{cyt} and v_{alt}) differed between well-watered CMSII and WT, similar reductions of A_N, g_s, and g_m were observed during severe drought stress. However, total respiration (V_t) remained slightly higher in CMSII due to the still increased v_{cyt} (to match ATP demand). v_{alt} and maximum carboxylation rates remained almost unaltered in both genotypes, while in CMSII, changes in photosynthetic light harvesting (i.e. Chl *a*/*b* ratio) were detected. In both genotypes, photosynthesis and respiration were restored after 2 d of rewatering, predominantly limited by a delayed stomatal response. Despite complex I dysfunction and hence altered redox balance, the CMSII mutant seems to be able to adjust its photosynthetic machinery during and after drought stress to reduce photo-oxidation and to maintain the cell redox state and the ATP level.

Key words: Alternative oxidase (AOX), complex I dysfunction, drought stress, mesophyll conductance, photosynthesis, recovery.

Introduction

Limited water availability impairs plant growth and is one of the main issues of future climate changes (Ciais *et al.*, 2005; Loreto and Centritto, 2008). Thus, adaptation and survival strategies are demanded from plants to persist in their current habitats. As drought stress mainly affects the plant carbon balance, in particular, photosynthesis and respiration, adjustments at the leaf level are of primary importance, while long-term adjustments at the whole plant level may then follow (Chaves *et al.*, 2003; Flexas *et al.*, 2006).

Suppression of photosynthesis during drought stress due to the closure of stomata and the contribution of leaf-internal limitations to CO_2 diffusion, particularly mesophyll conductance (g_m), has been determined in numerous studies and plant species (Flexas *et al.*, 2004, 2008; Niinemets *et al.*, 2005; Warren and Adams, 2006). Although plant species

respond differently to varying drought stress intensities, photosynthetic limitation is firstly and predominantly driven by stomata, in particular, by a decline in stomatal conductance. Further on, when stomatal conductance drops below a certain threshold (<50 mmol H_2O m^{-2} s^{-1}) limitations of non-stomatal processes become more important, in particular, decreased g_m and impaired photobiochemistry (Flexas and Medrano, 2002; Chaves *et al.*, 2003; Flexas *et al.*, 2004). Adjustment of leaf diffusion components for CO_2 is one way for plants to cope with situations of limited water supply and concurrently to improve their water use efficiency. As well as leaf internal adjustments of diffusion components during drought stress, increased thermal dissipation of excess energy and other photo-protective processes (e.g. an enhanced xanthophyll cycle) may contribute to improved stress tolerance and adaptation (Demmig-Adams and Adams III, 1996; Havaux and Niyogi, 1999; Mittler, 2002).

In parallel to these changes inside the chloroplast, respiratory pathways in mitochondria might also be altered, because of their interaction with the photosynthetic pathway. The respiratory chain is thought to dissipate excess reductants originated from chloroplasts (Raghavendra and Padmasree, 2003). Moreover, the non-phosphorylating pathways, which involve the cyanide-resistant alternative oxidase (AOX) and the type II NAD(P)H dehydrogenases, are considered to be efficient dissipation systems for these reductants, because electron flow through these pathways is not limited by adenylate control (Noctor *et al.*, 2007). Thus, the non-phosphorylating pathways may function as a mechanism of plant photo-protection, while the components of this mechanism have not been characterized in detail. Indeed, several studies have highlighted that different mutants with some impaired mitochondrial function also have a lower photosynthetic capacity (Juszczuk *et al.*, 2007; Nunes-Nesi *et al.*, 2007; Giraud *et al.*, 2008), and this includes the CMSII mutant of *Nicotiana sylvestris* (Sabar *et al.*, 2000; Priault *et al.*, 2006*a*, *b*).

Several studies on the effect of severe drought stress on respiratory pathways have revealed contrasting results, as respiration remained unaltered in soybean (Ribas-Carbo *et al.*, 2005*b*), increased in wheat (Bartoli *et al.*, 2005), and decreased in bean and pepper (Gonzalez-Meler *et al.*, 1997). However, changes in the *in vivo* activities of the cytochrome oxidase (COX) and AOX pathways, measured with the oxygen isotope fractionation technique that has been demonstrated to be the most reliable technique for the studies of electron partitioning between the two main respiratory pathways (Ribas-Carbo *et al.*, 1995; Day *et al.*, 1996), have been reported by Ribas-Carbo and colleagues (Flexas *et al.*, 2005; Ribas-Carbo *et al.*, 2005*b*). In their study on soybean (Ribas-Carbo *et al.*, 2005*b*), a decrease in COX activity was detected in leaves during severe drought stress, while AOX activity increased. Whether, and to what extent, plant species-specific factors and/or experimental conditions affect *in vivo* respiratory pathways under drought stress awaits further studies.

To examine the effect of stress-induced changes on respiratory pathways, in particular with relation to photosynthesis, plants with modified AOX expression have been intensively studied (Noctor *et al.*, 2007). Among these, the *Nicotiana sylvestris* cytoplasmic male-sterile CMSII mutant, which lacks a functional mitochondrial complex I (Gutierres *et al.*, 1997) and possesses high amounts of AOX transcript and protein (Sabar *et al.*, 2000), has received increased attention. The observed differences in activities of the photosynthetic and respiratory pathways as compared with wild-type plants have been proposed to result from alterations in the cellular redox balance (Dutilleul *et al.*, 2003*b*; Priault *et al.*, 2006*a*; Vidal *et al.*, 2007) and/or limitations of CO_2 diffusion inside the leaf (Priault *et al.*, 2006*b*). Moreover, loss of complex I function might be compensated by enhanced non-phosphorylating NAD(P)H dehydrogenases, resulting in the maintenance of cell redox balance and cross-talk between mitochondria and chloroplasts (Dutilleul *et al.*, 2003*a*, *b*; Vidal *et al.*, 2007).

Thus, the tobacco CMSII mutant offers great possibilities to study the interaction between respiratory and photosynthetic pathways, in particular, under contrasting environmental conditions (e.g. high light, temperature, and drought stress). With regard to the increasing importance of understanding plant responses to drought stress, particularly within the context of climate changes, more studies are needed to examine the plant carbon balance, especially the relationship between the carboxylation and oxygenation processes. Due to the scarcity of *in vivo* studies on photosynthesis and respiration under drought stress (Gonzalez-Meler *et al.*, 1997; Bartoli *et al.*, 2005; Ribas-Carbo *et al.*, 2005*b*; Giraud *et al.*, 2008), more research is necessary to understand the interrelation between chloroplasts and mitochondria. Furthermore, the lack of knowledge regarding the underlying and limiting processes of photosynthetic recovery from drought stress awaits further attention, as the capability to recover from drought events ensures the survival and growth of plants in their habitats (Flexas *et al.*, 2006; Galle *et al.*, 2007; Galmes *et al.*, 2007).

Apart from osmotic readjustments in *Nicotiana sylvestris* CMSII mutants (soluble sugars, proline) and wild-type plants under drought stress (R de Paepe, unpublished results), the role of respiratory pathways during drought stress induced the suppression of photosynthesis and, during photosynthetic recovery after rewatering, has been examined in more detail in the present study on CMSII and wild-type plants. The following questions have been addressed. (i) How does respiratory complex I deficiency and hence altered non-phosphorylating pathways, as related to the proposed function in the dissipation of excess energy from the chloroplasts, affect photosynthetic activity during drought stress? (ii) What are the impacts on photosynthetic recovery during rewatering, especially with regard to the possible functions of respiratory pathways? (iii) Is an efficient respiratory chain necessary for photosynthetic recovery after drought stress and what about other limiting factors?

Materials and methods

Plant material and growth conditions

Two genotypes of tobacco (*Nicotiana sylvestris*), wild-type (WT) and CMSII mutant (Gutierres *et al.*, 1997), were grown in a growth chamber (photoperiod of 12 h) at approximately 800 μmol m^{-2} s^{-1} photon flux density (PPFD) and at 26/22 °C day/night temperature. Humidity was maintained around 40–50%. Prior to the experiments, plants were kept under well-watered conditions, adding nutrient solution (Hoagland) once a week. Experiments were started with 6-week-old and 11-week-old WT and CMSII plants, respectively. This difference in age was due to the retarded growth of CMSII and the need to perform the experiment with plants of a similar developmental stage, which was considered to be achieved when plants presented a similar size and number of leaves according to Priault *et al.* (2007). Throughout the experiments, the two youngest fully expanded leaves were used for gas-exchange and respiration measurements. Drought stress was imposed by withholding water until severe drought stress was reached. Severe drought stress was considered when the stomatal conductance for water vapour dropped below 50 mmol m^{-2} s^{-1}, according to Medrano *et al.* (2002). Thereafter, plants were maintained at this intensity of drought stress for a few days by adding the amount of water they lost during the day. After this period of stress-acclimation plants were consecutively rewatered to field capacity and the recovery of photosynthetic traits was followed.

Plant water status

From the first day of withholding water until the first day of rewatering, plants were weighed at the end of the photoperiod and the total loss of water was recorded. When necessary plants were irrigated with the amount of water lost during the day.

In parallel with gas-exchange and respiration measurements (see below), a minimum of three leaf samples were collected around midday to determine the relative leaf water content (RWC_m).

$$RWC_m\,(\%) = 100\times(FW-DW)/(TW-DW)$$

where FW, TW, and DW denote for weight of fresh, turgid, and dry leaf tissue, respectively. FW was determined immediately after sampling, while TW was obtained after incubating leaf discs in distilled water for 48 h in the dark at 4 °C. DW was determined after 72 h in a drying oven at *c.* 70 °C.

Leaf gas-exchange and chlorophyll a fluorescence

Throughout the experiments, maximum net CO_2 assimilation (A_N), stomatal conductance (g_s) and chlorophyll *a* fluorescence were measured simultaneously with an open infrared gas-exchange analyser system (Li-6400; Li-Cor Inc., Lincoln, NE, USA) equipped with a leaf chamber fluorometer (Li-6400-40, Li-Cor Inc.). Under well-watered conditions (WW), on the first (S1) and last day (S4) of severe drought stress, as well as after 1 (R1), 2 (R2), and 5 d (R5) of rewatering, at least four measurements on the two youngest fully expanded leaves of WT and CMSII plants were performed under light-saturating PPFD of 1500 μmol m^{-2} s^{-1} (provided by the light source of the Li-6400 with 10% blue light). Leaves were always illuminated until A_N and g_s reached steady-state (after *c.* 20 min). The CO_2 concentration in the Li-6400 leaf chamber (C_a) was set to 400 μmol CO_2 mol^{-1} air, temperature was 25 °C and the relative humidity of the incoming air ranged between 40% and 50%. CO_2 response curves ('A_N–C_i curves') were performed in well-watered, non-watered, and rewatered plants by varying the CO_2 concentration around leaves stepwise in the range of 50–1800 μmol CO_2 mol^{-1} air. These leaves had been previously acclimated to saturating light conditions (*c.* 15–20 min at a PPFD of 1500 μmol m^{-2} s^{-1}).

From the fluorescence measurements, the actual quantum efficiency of the photosystem II (PSII)-driven electron transport (Φ_{PSII}) was determined according to Genty *et al.* (1989) as

$$\Phi PSII = (Fm' - Fs)/Fm'$$

where F_s is the steady-state fluorescence in the light (here PPFD 1500 μmol m^{-2} s^{-1}) and Fm′ the maximum fluorescence obtained with a light-saturating pulse (~8000 μmol m^{-2} s^{-1}). As Φ_{PSII} represents the number of electrons transferred per photon absorbed by PSII, the rate of electron transport (J) can be calculated as

$$J\left(\mu mol\, e^{-2}\, m^{-2}\, s^{-1}\right) = \Phi_{PSII}\times PPFD\times\alpha$$

where the term α includes the product of leaf absorptance and the partitioning of absorbed quanta between photosystems I and II. α was determined for each treatment from the slope of the relationship between Φ_{PSII} and Φ_{CO2} (i.e. the quantum efficiency of gross CO_2 fixation), which was obtained by varying light intensity under non-photorespiratory conditions in an atmosphere containing <1% O_2 (Valentini *et al.*, 1995).

From combined gas-exchange and chlorophyll *a* fluorescence measurements, the mesophyll conductance for CO_2 (g_m) was estimated according to Harley *et al.* (1992) as

$$g_m = A_N/(C_i-(\Gamma^*\times(J+8(A_N+R_d)))/(J-4(A_N+R_d)))$$

where A_N and C_i were obtained from gas-exchange measurements. A value of 37.4 μmol mol^{-1} for the CO_2 compensation point under non-respiratory conditions (Γ*) was used after Bernacchi *et al.* (2002) as determined for the related species *Nicotiana tabacum*. Other Rubisco kinetics and their temperature dependencies were also taken from Bernacchi *et al.* (2002). Dark respiration (R_d; V_t) was determined at 25 °C with an isotope ratio mass spectrometer (IRMS) at 25 °C as described by Ribas-Carbo *et al.* (2005*b*). Calculated values of g_m were used to convert A–C_i curves into A–C_c curves according to the following equation:

$$C_c = C_i - (A_N/g_m)$$

Maximum velocity of carboxylation ($V_{c,max}$) and maximum electron transport rate (J_{max}) was derived from A–C_c curves according to Bernacchi *et al.* (2002).

Corrections for the leakage of CO_2 into and out of the leaf chamber of the Li-6400 have been applied to all gas-exchange data, as described by Flexas *et al.* (2007). Due to low g_s values under severe drought stress and a possibly increased contribution of conductance via the cuticle (g_c), estimates of g_s were corrected for g_c as described elsewhere (Boyer *et al.*, 1997). In short, for each experimental condition gas-exchange was measured across the leaf with its lower side sealed with lubricant and an impermeable plastic foil to hinder any gas exchange via its cuticle and stomata. The obtained g_c was multiplied by 2 to account for the upper and lower sides of the leaf and C_i was recalculated based on the new g_s values (g_s–g_c), using the equations provided by the manufacturer (LI-6400 manual version 5, Li-Cor Inc., Lincoln, Nebraska, USA).

Respiration measurements on intact tissue

Respiration in the light (R_l) and in the dark (R_{dc}) was determined in leaves of at least four well-watered CMSII and WT plants, using a Li-6400 gas-exchange system (Li-cor Inc. Lincoln, NE, USA). R_l was determined according to the 'Laisk-method' (Laisk, 1977), using the *y*-axis intersection of three A_N–C_i curves (as described above) performed at three different light intensities (750, 250, and 75 μmol photons m^{-2} s^{-1}). In both genotypes, the apparent CO_2 compensation point (C_i*), as determined by the Laisk-method, ranged between 38.3 and 43.7 μmol mol^{-1}. R_{dc} was measured at *c.* 400 μmol CO_2 mol^{-1} air and 25 °C in attached leaves that were dark-adapted for more than 30 min.

After gas-exchange measurements (WW, S1, S4, R1, R2, R5) total dark respiration (V_t) and the activities of the cytochrome oxidase (COX) and the alternative oxidase (AOX) pathways were determined with a dual-inlet mass spectrometer system (Delta Plus, Thermo LCC, Bremen, Germany), as previously described (Gaston *et al.*, 2003; Florez-Sarasa *et al.*, 2007).

First, leaves were incubated in the dark for 30 min to avoid light-enhanced dark respiration and then used for respiration measurements. A 10 cm^2 leaf disc was cut, weighed, and immediately placed in a closed 3 ml stainless-steel cuvette, which was maintained at a constant temperature of 25 °C, using a copper plate and a serpentine around the cuvette with a temperature-controlled water bath. The values of 34/32($^{18}O_2/^{16}O_2$) and 32/28($^{16}O_2/^{28}N_2$) mass ratios were obtained from a standard and the sample air with dual-inlet analysis and four or six replicate cycles for each respiration measurement. Calculations of the oxygen isotope fractionation were made as previously described (Ribas-Carbo *et al.*, 2005*a*). The electron partitioning between the two pathways in the absence of inhibitors was calculated as described by Guy *et al.* (1989). The r^2 value of all unconstrained linear regressions between –ln f and ln(R/R_o) with a minimum of five data points was at least 0.995, which has been considered as robust and well acceptable (Ribas-Carbo *et al.*, 1997).

The electron partitioning through the AOX pathway (τ_a) was calculated as described in Guy *et al.* (1989) as follows:

$$\tau_a = \Delta n - \Delta c / \Delta a - \Delta c$$

where Δn, Δc, and Δa are the isotope fractionation in the absence of inhibitors, in the presence of SHAM, and in the presence of KCN, respectively. The Δc value of 21.3‰ obtained by Vidal *et al.* (2007) was used, as COX pathway discrimination has been shown to be constant (Ribas-Carbo *et al.*, 2005*a*). A Δa value of 30.0‰ was determined experimentally. The individual activities of the COX (v_{cyt}) and AOX (v_{alt}) pathways were determined by multiplying the total oxygen uptake rate (V_t) and the partitioning to each pathway as follows:

$$v_{cyt} = V_t \times (1 - \tau_a)$$

$$v_{alt} = V_t \times \tau_a$$

ATP production of WT and CMSII plants was calculated according to the model used by Vidal *et al.* (2007).

Pigment and protein analysis

Snap-frozen leaf discs (in liquid nitrogen) were collected daily after 8 h of light and stored at –80 °C until analysis.

Frozen leaf material was ground to fine powder and homogenized with ice-cold extraction buffer (0.5M TRIS, 10 mM EDTA, 1% Triton X-100, 5 mM DTT, and 0.25% protease-inhibitor cocktail). The leaf extract was then centrifuged at 12 500 rpm and 4 °C. The supernatant was transferred to new vials and kept on ice, while aliquots were used for the determination of total soluble protein content (TSP), photosynthetic pigment composition, and Western blot analysis.

For pigment analysis an aliquot of leaf extract was dissolved in 80% acetone, centrifuged at 12 500 rpm for 2 min and the absorbance of the supernatant was determined at 470, 646, and 663 nm in a spectrophotometer (DU 640, Beckmann Coulter Inc.). The content of chlorophyll *a*, *b* and carotenoids was then calculated according to Lichtenthaler (1987).

The TSP was determined with the Bio-Rad protein assay (Bio-Rad Laboratories, Inc.) according to the method of Bradford, using bovine serum albumin as a standard.

For SDS-PAGE, an aliquot of leaf extract was mixed 1:1 with SDS-sample buffer (0.5 M TRIS, 0.3% glycerol, 1.5% SDS, 0.15% bromophenol blue, and 5 μM DTT), after which samples were boiled in a water bath for *c*. 5 min and then kept at –20 °C for later analysis. Mixes of at least three samples (=plants) per day with equal amounts of soluble proteins were loaded per lane for SDS-PAGE, using a Mini-Protean electrophoresis system (Bio-Rad Laboratories, Inc.).

SDS-PAGE gels were blotted onto nitrocellulose membranes with the Mini-Protean system of Bio-Rad. Immunodetection of mitochondrial proteins (AOX and porin) via colorimetry was carried out with the BCIP/NBT alkaline phosphatase system according to the manufacturer's instructions (Sigma-Aldrich Co.). A 1/50 dilution of the monoclonal antibody against AOX (Elthon *et al.*, 1989) and a 1/500 dilution of the monoclonal antibody against the voltage-dependent anion channel porin (PM035, from Dr Tom Elthon, Lincoln, NE, USA) were used as primary antibodies. Densitometry quantification of AOX and porin bands were made with TotalLab Software (Nonlinear Dynamics Ltd, UK).

Results

Changes of photosynthetic traits during drought stress and recovery

Relative leaf water content (RWC_m) was reduced by 3% in WT and by 12% in CMSII, when the desired level of severe drought stress (S1) was reached (Table 1a, b), as indicated

Table 1a. Changes of photosynthetic parameters in well-watered (Control) and drought stressed (Stress) tobacco wild-type (WT) plants throughout the experiment

RWC_m, $V_{c,max}$, J_{max}, qP, and C_c denote for relative leaf water content at midday, maximum carboxylation rate, maximum photosynthetic electron transport rate, photochemical quenching of PSII, and chloroplast CO_2 concentration at the turning point of Rubisco ($V_{c,max}$) and RuBP (J_{max}) limitation to photosynthesis, respectively. Means and standard errors of at least four measurements are presented for well-watered condition (WW), first (S1), and last day (S4) of severe drought stress, as well as for the first (R1), second (R2), and fifth day (R5) of rewatering. For each parameter statistically significant differences to the control value (WW) are indicated by different letters (P <0.05).

		WW	S1	S4	R1	R2	R5
RWC_m		77.5±1.1 a	75.1±3.1 a	61.7±2.1 b	78.9±1.1 a	–	79.8±4.0 a
$V_{c,max}$	Control	166.0±28.5 a	–	138.1±26.7 a	–	–	124.0±10.4 ab
	Stress		159.7±12.5 a	112.5±9.1 ab	139.7±17.0 a	160.9±5.1 a	162.1±11.6 a
J_{max}	Control	200.3±19.1 a	–	183.2±22.1 a	–	–	170.1±10.4 a
	Stress		136.4±9.6 b	104.4±10.0 b	174.0±13.7 a	203.3±7.7 a	203.7±10.7 a
qP	Control	0.58±0.02 a	0.48±0.02 ab	0.45±0.09 ab	0.46±0.09 a	0.42±0.09 ab	0.42±0.08 b
	Stress		0.44±0.03 b	0.17±0.06 c	0.41±0.10 b	0.57±0.02 a	0.55±0.03 a
C_c($V_{c,max}$//J_{max})	Control	153.8±9.3 a	–	155.7±1.4 a	–	–	146.0±11.7
	Stress		85.4±4.6 b	72.1±6.6 b	104.3±4.8 b	124.3±8.7 ab	149.0±5.9 a

Table 1b. Changes of photosynthetic parameters in well-watered (Control) and drought-stressed (Stress) tobacco CMS mutants throughout the experiment; for details see legend Table 1a

		WW	S1	S4	R1	R2	R5
RWC_m		81.6±2.9 a	71.3±3.4 ab	58.8±2.5 c	76.5±4.1 ab	–	87.4±1.3 a
$V_{c,max}$	Control	139.9±21.9 a	–	124.0±13.7 a	–	–	108.1±3.3 ab
	Stress		184.3±18.2 a	110.7±9.5 a	135.8±13.9 a	150.8±11.4 a	167.8±8.8 a
J_{max}	Control	166.4±27.3 b	–	152.8±13.7	–	–	139.2±3.1
	Stress		146.7±13.7 b	92.8±8.6 c	189.9±9.3 ab	206.6±8.4 ab	214.6±4.8 a
*q*P	Control	0.47±0.07 a	0.32±0.07 ab	0.36±0.07 ab	0.36±0.06 ab	0.29±0.07 b	0.29±0.07 b
	Stress		0.34±0.03 b	0.08±0.01 c	0.49±0.03 a	0.44±0.09 a	0.38±0.09 a
C_c ($V_{c,max}$//J_{max})	Control	148.3±9.3 a	–	154.0±10.0 a	–	–	161.0±3.3 a
	Stress		75.8±3.8 b	72.0±4.8 b	142.1±10.7 a	148.0±18.9 a	140.7±1.9 a

by a stomatal conductance for water vapour below 50 mmol m^{-2} s^{-1} (Medrano *et al.*, 2002). After 4 d of severe drought stress (S4) RWC_m dropped by more than 20% in both WT and CMSII,. However, RWC_m was immediately restored in WT and CMSII after rewatering, indicating the rapid restoration of plant water status.

The maximum carboxylation rate ($V_{c,max}$) under well-watered conditions (Control) was slightly higher in WT than in CMS (Table 1a, b), which has been previously observed (Priault *et al.*, 2006*b*) and might be related to a higher initial Rubisco activity (Priault *et al.*, 2006*a*) or the number of catalytic sites (Dutilleul *et al.*, 2003*a*). Despite a slight decrease of $V_{c,max}$ with time in control plants, presumably due to leaf ageing, $V_{c,max}$ remained almost unaltered in stressed plants.

As in $V_{c,max}$, alterations in J_{max} were small in control plants of WT and CMSII during the experiment (Table 1a, b). However, unlike $V_{c,max}$, J_{max} decreased by about 40% during severe drought stress (S4), but it was restored to control values within 1 d of rewatering (R1) and maintained this level thereafter.

In close relation with the course of J_{max}, the photochemical quenching (*q*P; proportion of open PSII reaction centres) declined considerably with progression of drought stress, resulting in a reduction of more than 70% (Table 1a, b). Notably, the reduction of *q*P was more pronounced in CMSII under severe drought stress (S4; Table 1b). However, this reduction was completely reversed after rewatering (R1), resulting in somewhat higher *q*P values than the corresponding controls. In well-watered plants, *q*P remained almost unchanged, except for a slight decline with time (possible 'ageing effect').

The chloroplastic CO_2 concentration (C_c) at the intercept of the photosynthetic limitation by Rubisco activity ($V_{c,max}$), and the regeneration of ribulose-1,6-*bis*phosphate (J_{max}) deducted from A_N–C_c curves, was similar for WT and CMSII under well-watered conditions (Table 1a, b). Moreover, in both genotypes similar changes were determined during drought stress, as C_c values decreased from about 150 μmol mol^{-1} to 72 μmol mol^{-1} (S4). During rewatering C_c values increased in both WT and CMSII, while restoration to control values was reached within 1 d of rewatering in CMSII and after 2 d in WT.

Net photosynthesis (A_N) and stomatal conductance (g_s) were 20–30% lower in CMSII than in WT under well-watered conditions (Fig. 1a–d; Control), while stomatal density was about 10% lower in CMSII than in WT (data not shown). In both genotypes, similar values of A_N and g_s were reached under severe drought stress. Thereafter, A_N and g_s were completely restored to control values after 2 d of rewatering. Interestingly, although A_N was restored to control values by *c*. 90% in CMSII and by 'only' about 60% in WT plants after the first day of rewatering, the rate of photosynthetic recovery seemed to be very similar for both genotypes (Fig. 1a, b; compare absolute A_N values of day 10). The restoration of g_s was generally slower (lower rate) than that of A_N in both WT and CMSII, particularly during the first day of rewatering, where they reached only 40–50% of the corresponding control values. These results clearly indicate a higher intrinsic water use efficiency (*WUE*) during the initial period of rewatering in WT and CMSII (Fig. 1i, k), while highest WUE (3–4-fold increase) could be observed during severe stress.

Mesophyll conductance (g_m) of WT and CMSII plants were very similar under well-watered conditions and followed a similar trend during drought stress and rewatering (Fig. 1e, f). During severe drought stress, g_m dropped below 0.1 mol CO_2 m^{-2} s^{-1} in WT and CMSII, while it was completely restored to control values within 1 d of rewatering suggesting a negligible restriction of leaf internal CO_2 diffusion. Notably, in spite of maintained g_s (Fig. 1c, d) g_m increased after 5 d of rewatering (day 14; Fig. 1e, f). Changes in chloroplastic CO_2 concentration (C_c) were similar in WT and CMSII plants during the experiment, which resulted in a decrease of about 50% during drought stress and a progressive restoration to control values during rewatering (Fig. 1g, h).

Besides the changes in C_c (Fig. 1g, h), the relationship of internal CO_2 (C_i) to C_c remained almost unaltered throughout the experiment in stressed and control plants of both genotypes (data not shown).

Changes in respiratory traits during drought stress and recovery

In order to confirm that dark respiration (R_d, V_t) measured by the oxygen isotope technique (IRMS) can be used for

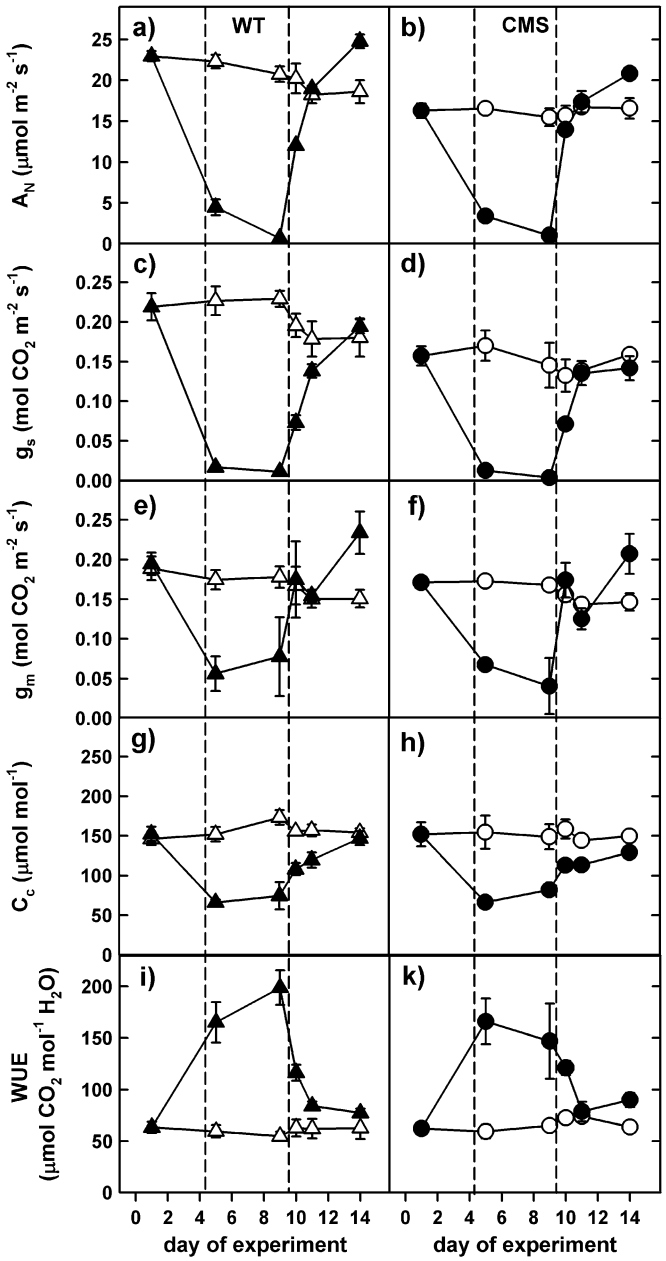

Fig. 1. Changes of leaf gas-exchange parameters during drought stress and rewatering in WT (triangles) and CMSII (circles) plants. Net photosynthetic rates (A_N), stomatal conductance (g_s), mesophyll conductance (g_m), chloroplastic CO_2 concentration (C_c). and the intrinsic water use efficiency (*WUE*) of photosynthetic carbon fixation are presented for well-watered control (open symbols) and stressed (closed symbols) plants. Means and standard errors of at least four plants are shown. The vertical dashed lines indicate the beginning and end of the severe drought period.

calculating g_m and the related parameters (i.e. $V_{c,max}$, J_{max}), as well as to check whether it was representative of leaf respiration occurring during photosynthesis, respiration in the dark (R_{dc}) and the light (R_l) were also determined with a gas-exchange system (Table 2). Besides the precautions and problems with the Laisk method used for R_l estimation, in particular during situations of drought stress (Galmes *et al.*, 2006), as well as the fundamental problem of

Table 2. Comparison of leaf respiration parameters in well-watered WT and CMSII plants, determined with the Li-6400 and IRMS system

Means and standard errors of at least four plants are shown. Asterisks indicate significant differences ($P \leq 0.05$) between WT and CMSII for each parameter. For details see Materials and methods.

Respiration parameter	WT	CMSII	Ratio CMSII/WT
R_l (μmol CO_2 m^{-2} s^{-1})[a]	0.45±0.04	0.66±0.07	1.5*
R_{dc} (μmol CO_2 m^{-2} s^{-1})[a]	1.50±0.05	1.81±0.08	1.2*
V_t (R_d) (μmol O_2 m^{-2} s^{-1})	0.89±0.06	1.30±0.10	1.5*

[a] These respiration parameters have been determined in leaves of another set of WT and CMSII plants, which were grown under similar conditions as the well-watered plants used for the V_t measurement.

measuring respiration in the light, the ratio between CMSII and WT values remained almost unaltered between 1.2 and 1.5.

Absolute values of respiration parameters ranged between 0.45 and 1.50 in WT and between 0.66 and 1.81 in CMSII, as determined by isotope (O_2) and by gas-exchange (CO_2) analysis. However, these differences only marginally affect calculations of g_m, as changes of g_m were smaller than 8% when using the most extreme values (0.45 and 1.81; data not shown). Thus, dark respiration values determined by oxygen isotope analysis were used for all calculations and as a tracer of leaf respiratory activity in each genotype.

When comparing respiratory pathways in WT and CMSII, total respiration (V_t) was significantly higher in CMSII than in WT (Fig. 2a). The higher V_t in CMSII was mainly due to increased cytochrome oxidase activity (v_{cyt}). The course of V_t, v_{cyt}, and v_{alt} (alternative pathway) during the experiment was similar in both genotypes. The reduction of V_t during drought stress was predominantly due to reduced v_{cyt}, while v_{alt} remained almost unaltered. However, when ATP production was modelled no significant differences between genotypes were detected, irrespective of being drought stressed or not (Fig. 2d).

The AOX protein level was relatively constant in WT plants during the whole experimental period (Fig. 3), while a marked reduction of AOX protein was detected in CMSII plants during severe drought stress and in the initial phase of recovery (first and second days of rewatering). However, AOX protein was more abundant in CMSII under control conditions (well-watered) than in WT, as previously reported (Gutierres *et al.*, 1997; Sabar *et al.*, 2000; Priault *et al.*, 2007). Changes in total mitochondrial protein content were negligible, as indicated by the anti-porin Western blot (Fig. 3). Control plants under well-watered conditions showed no change in AOX and in porins during the experimental period.

Changes of soluble leaf components related to photosynthesis during drought stress and recovery

Changes in contents of chlorophylls (Chl *a*+*b*) and carotenoids (Car) were only small throughout the experiment, while they increased somewhat during rewatering in CMS,

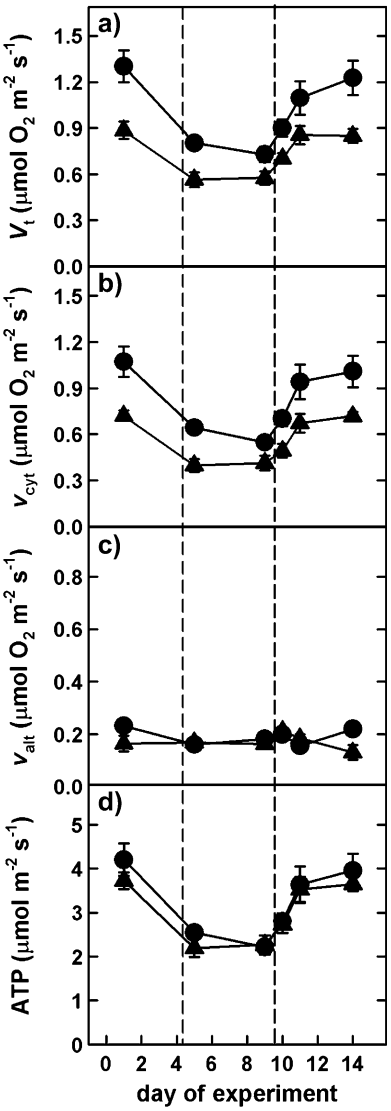

Fig. 2. Changes of total respiration (V_t), cytochrome (v_{cyt}), and alternative (v_{alt}) pathway activities, as well as modelled mitochondrial ATP production during drought stress and rewatering in WT (triangles) and CMSII (circles) plants. Values of control plants remained unaltered throughout the experiment and are therefore not depicted. Means and standard errors of at least four plants are shown. The vertical dashed lines indicate the beginning and end of the severe drought period.

but not in WT (Fig. 4*a*, *b*). Furthermore, the ratio of chlorophyll *a* to *b* (Chl *a*/*b*) was increased after rewatering in CMSII, but not in WT, indicating changes within the photosynthetic apparatus (i.e. the light-harvesting complexes). On the other hand, total soluble protein content (TSP) was increased after rewatering in WT, whereas it slightly declined in CMSII. These changes also remained similar when Chl *a*+*b*, Car, and TSP are presented on a dry-weight basis (data not shown), although specific leaf area increased during drought and initial recovery (see Supplementary Fig. 1 at *JXB* online). Under well-watered conditions Chl *a*+*b*, Car, Chl *a*/*b*, and TSP remained unaltered throughout the experimental period in both WT and

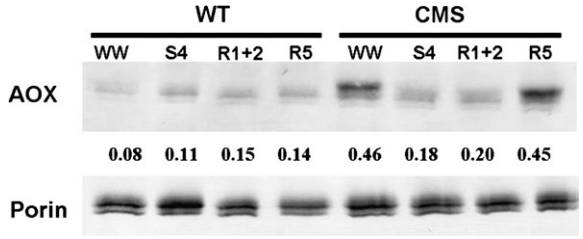

Fig. 3. Western blot analysis of mitochondrial alternative oxidase (AOX) and porin protein in WT and CMSII during drought stress and rewatering. WW, S4, R1+2, and R5 denote for the start of the experiment (well-watered), end of severe drought stress, initial phase (first and second day), and terminal phase (fifth day) of rewatering, respectively. The values below the AOX bands represent the ratio of AOX and porin quantified by densitometry. Each lane was loaded with the same amount of protein of combined leaf extracts from four plants.

CMSII. WT and CMSII also did not differ in total lipid peroxidation (data not shown) neither under drought stress nor under recovery or well-watered conditions, suggesting no or only minor effects of general oxidative stress.

Discussion

Well-watered conditions

The differences in the photosynthetic and respiratory parameters in WT and CMSII mutants under well-watered conditions, which had been described earlier (Sabar *et al.*, 2000; Dutilleul *et al.*, 2003*a*; Priault *et al.*, 2006*a*, *b*), were proposed to result from altered cross-talk between the respiratory and photosynthetic pathways (Sabar *et al.*, 2000; Dutilleul *et al.*, 2003*a*; Priault *et al.*, 2006*a*), as well as from an impaired internal CO_2 supply (i.e via mesophyll conductance, g_m) (Priault *et al.*, 2006*b*). Unlike the findings of Priault *et al.* (2006*b*), impaired CO_2 supply due to decreased g_m was not observed in CMSII plants in the present study, as g_m values, and hence C_c, did not differ between WT and CMSII (Fig. 1e–h). The growing light intensity was more than two times higher in this study than in the study of Priault *et al.* (2006*b*), which may explain the different results of g_m. As shown recently, quantity and intensity of incident light seems to affect g_m (Gorton *et al.*, 2003; Warren *et al.*, 2007). Similar g_m and C_c (and $V_{c,max}$) but different A_N in WT and CMSII might be related to differences in activation state of Rubisco (or its amount) under the prevailing growth light conditions (800 μmol m^{-2} s^{-1}), as previously shown for CMSII and WT plants growing under moderate light (350 μmol photons m^{-2} s^{-1}) and after 3 h of high light treatment of 1000 μmol m^{-2} s^{-1} (Priault *et al.*, 2006*a*). Moreover, lower stomatal density in CMSII than in WT leaves may also partially contribute to that discrepancy in A_N and g_s between both genotypes.

Drought-stress conditions

WT and CMSII plants were similarly affected by drought stress, as the values of A_N and of g_s reached almost zero

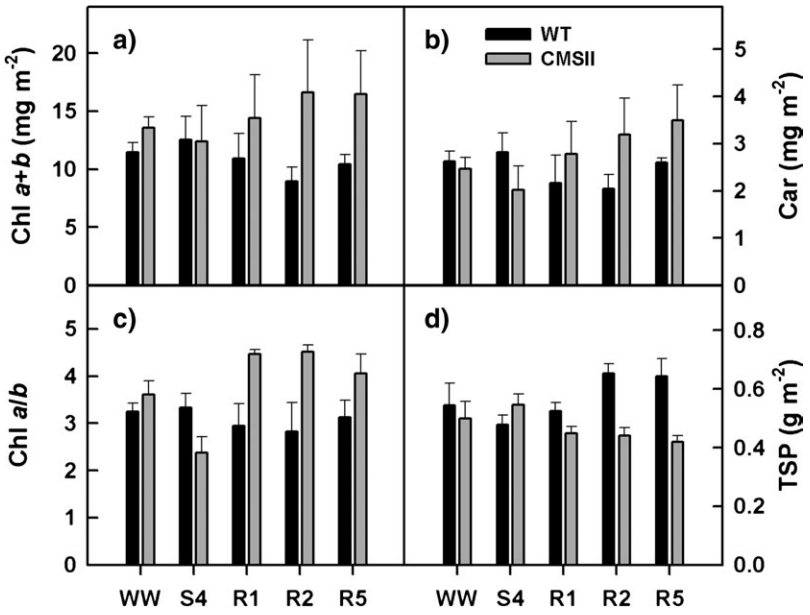

Fig. 4. Changes of photosynthetic pigments and soluble proteins in WT (triangles) and CMSII (circles) during drought stress and rewatering. The contents of chlorophylls (Chl *a*+*b*), carotenoids (Car), and total soluble proteins (TSP), as well as the ratio of chlorophyll *a* to *b* (Chl *a*/*b*) are depicted. WW, S4, R1, R2 and R5 denote for the start of the experiment (well-watered), the end of severe drought stress (day 9), the first two days (day 10, 11), and the last day of rewatering (day 14), respectively. Means and standard errors of at least four plants are shown.

during severe stress (day 9; Fig. 1), leading to a considerable drop in internal CO_2 supply (g_m and C_c). On the other hand, maximum carboxylation ($V_{c,max}$) was almost unaffected by drought stress, indicating preserved photosynthetic functioning and little metabolic limitations. However, reduced light capture via PSII antennas, as indicated by reduced ratios of Chl *a*/*b* (Fig. 4c) and fewer open PSII reaction centres (*q*P; Table 1b) were detected in CMSII mutants. Thus, the photosynthetic apparatus of CMSII seemed to be more affected by drought stress than WT.

With regard to respiration and irrespective of drought stress or not, CMSII plants always displayed a 20–30% higher respiration rate (V_t) than WT, due to increased v_{cyt} (Fig. 2), adjusting ATP production to a similar level as in WT. In fact, calculated total ATP production was almost equal in CMSII and WT and showed a similar trend under drought stress in both genotypes (Fig. 2), suggesting a strict control of respiration by ATP demand. Interestingly, although AOX activity was maintained, AOX protein content was slightly decreased during severe stress and the initial phase of recovery in CMSII. Altered redox signalling in CMSII plants has been proposed to cause differential AOX expression patterns in CMSII plants (Dutilleul *et al.*, 2003*b*; Vidal *et al.*, 2007). Nevertheless, the AOX protein level and activity are not directly related, as already discussed earlier (Millenaar and Lambers, 2003), and indicated by different correlations of AOX protein level and activity under stress (Lennon *et al.*, 1997; Ribas-Carbo *et al.*, 2005*b*; Vidal *et al.*, 2007). Moreover, it seems likely that the reduced level of AOX protein was still sufficient to maintain v_{alt} under both normal and stressful conditions. In general, the lack of mitochondrial complex I seemed to be compensated by a higher rate of respiration through v_{cyt}, which further indicates the importance of mitochondrially synthesized ATP, particularly in situations of low photosynthetic carbon assimilation (i.e. during drought stress).

Recovery during rewatering

Leaf water status was restored immediately after rewatering in WT and CMSII plants, providing full water supply to the leaves. As g_m was immediately restored to control values within the first day of rewatering, the limitation of photosynthetic recovery due to leaf internal resistances for CO_2 diffusion (i.e. g_m) can be discounted. Consequently, similar rates of photosynthetic recovery were obtained for WT and CMSII after rewatering, while complete restoration was reached after 2 d of rewatering. Photosynthetic recovery was mainly limited by stomata, as the restoration of g_s was slightly delayed. As a consequence, increased WUE during drought stress also persisted during the rewatering phase in stressed WT and CMSII plants, indicating an improved carbon fixation per loss of water. The rise of g_m at the end of the rewatering phase might also partly explain this persistence of increased WUE and the high A_N.

Although the functioning of the photosynthetic apparatus during drought stress seemed to be more affected in CMSII than in WT, photosynthetic parameters like J_{max}, *q*P, and Chl *a*/*b* were restored immediately after rewatering in the mutant, resulting in even higher ratios in the case of Chl *a*/*b* (presumably due to the increased number of light harvesting complexes and thus enhanced light capture for improved photosynthetic activity). Thus, the CMSII mutant seems to be highly flexible in adjusting its photosynthetic machinery during and after drought stress. Here, also

metabolic changes might play a role, as the carbon/nitrogen balance is affected in CMSII (Dutilleul *et al.*, 2003*a*, 2005).

Conclusions

With regard to the first two questions which have been raised in the introduction about the effect of complex I deficiency on photosynthetic activity during drought stress and recovery, photosynthesis was inhibited and restored in a similar manner in both CMSII mutant and WT. Adjustments of photosynthetic traits seemed to be involved, whereas, for example, light capture (i.e *q*P, Chl *a*/*b*) markedly changed in CMSII to minimize excess (reducing) energy. Furthermore, respiration (i.e. v_{cyt}) always remained higher in CMSII than in WT, most probably to adjust ATP production to similar levels than in WT.

Therefore, and with regard to the third question about the necessity of an efficient respiratory chain for photosynthetic recovery, a more efficient respiration chain is not required, as the CMSII mutant is capable of adjusting the activities of the photosynthetic and respiratory pathways (similar ATP level as in WT) to ensure the restoration of photosynthesis as in the WT.

The strict control of respiration by mitochondrial ATP demand under drought stress and recovery can be assumed, which emphasizes the importance of ATP production being maintained by mitochondria under limited photosynthetic carbon assimilation (i.e. during drought stress). However, the enhanced activity of NAD(P)H dehydrogenases (Rasmusson *et al.*, 1998), as well as an altered cell redox balance due to changes in antioxidant levels (Dutilleul *et al.*, 2003*b*) or alterations in the nitrogen/carbon balance (Dutilleul *et al.*, 2005) might play a role in the compensation of an impaired respiratory chain. Furthermore, the proposed role of v_{alt} in maintaining the redox balance during severe drought stress (Ribas-Carbo *et al.*, 2005*b*) still remains unclear, as v_{alt} was not altered in both CMSII and WT. A possible explanation might be that the degree of oxidative stress was not severe enough to alter v_{alt}. Further studies are needed to explore the interactions of mitochondrial non-phosphorylating pathways with photosynthetic processes and cell homeostasis under limited water supply, as well as under other stressful conditions.

Supplementary data

Supplementary data are available at *JXB* online.

Supplementary Fig. 1. Changes of specific leaf are (SLA) during the experiment in WT and CMSII plants.

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