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ORIGINAL ARTICLE

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Ear of durum wheat under water stress: water relations and photosynthetic metabolism

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Abstract The photosynthetic characteristics of the ear and flag leaf of well-watered (WW) and water-stressed (WS) durum wheat (*Triticum turgidum* L. var. *durum*) were studied in plants grown under greenhouse and Mediterranean field conditions. Gas exchange measurements simultaneously with modulated chlorophyll fluorescence were used to study the response of the ear and flag leaf to CO_2 and O_2 during photosynthesis. C_4 metabolism was identified by assessing the sensitivity of photosynthetic rate and electron transport to oxygen. The presence of CAM metabolism was assessed by measuring daily patterns of stomatal conductance and net CO_2 assimilation. In addition, the histological distribution of Rubisco protein in the ear parts was studied by immunocytochemical localisation. Relative water content (RWC) and osmotic adjustment (osmotic potential at full turgor) were also measured in these organs. Oxygen sensitivity of the assimilation rate and electron transport, the lack of Rubisco compartmentalisation in the mesophyll tissues and the gas-exchange pattern at night indicated that neither C_4 nor CAM metabolism occurs in the ear of WW or WS plants. Nevertheless, photosynthetic activity of the flag leaf was more affected by WS conditions than that of the ear, under both growing conditions. The lower sensitivity under water stress of the ear than of the flag leaf was linked to higher RWC and osmotic adjustment in the ear bracts and awns. We demonstrate that the better performance of the ear under water stress (compared to the flag leaf) is not related to C_4 or CAM photosynthesis. Rather, drought tolerance of the ear is explained by its higher RWC in drought. Osmotic adjustment and xeromorphic traits of ear parts may be responsible.

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Abbreviations A_{sat}: Light-saturated net CO_2 assimilation rate · A^*_{sat}: Corrected light-saturated net CO_2 assimilation rate of the ear (i.e. $A_{sat} + R_d$) · F_m and F_m': Maximum fluorescence in dark-adapted and light-adapted organs respectively · F_v/F_m: Maximum efficiency of PSII photochemistry after dark-adaptation · F_v'/F_m': Efficiency of energy capture by open PSII centres · F_o': Minimum fluorescence yield in light-adapted state · ϕ_{PSII}: Relative quantum yield of PSII photochemistry · PPFD: Photosynthetically active photon flux density · q_P: Photochemical quenching of chlorophyll fluorescence · R_d: Dark respiration · RuBP: Ribulose-1,5-biphosphate · RWC: Relative water content · ψ_w: Leaf water potential

Introduction

Durum wheat is one of the more widely cultivated crops in the Mediterranean basin, where drought is the main abiotic stress limiting its production (Royo et al. 1998). Mediterranean climate is characterised by a progressive increase in drought (combination of water stress, high temperature and excess radiation) during late spring, coinciding with the grain filling of cereal crops (Acevedo et al. 1999). Grain filling under these conditions is sustained (apart from the assimilates stored before anthesis in the stem) by the current photosynthesis of the upper parts of the plant, i.e. the flag and penultimate leaves plus the ear (Carr and Wardlaw 1965). In the past, flag leaf photosynthesis was considered the main source of assimilates for grain filling (Evans et al. 1980), even though the function of ear photosynthesis in productivity of C_3 cereals had been under discussion for years (Kriedemann 1966). However, it is now accepted that

ear photosynthesis makes a major contribution to final grain yield (Simmons 1987; Araus et al. 1993a; Abbad et al. 2004), especially in drought, when the ear may be the main photosynthetic contributor to grain filling (Evans et al. 1972; Bort et al. 1994; Sánchez-Díaz et al. 2002).

Apart from the fact that the ear is closer than the leaves to the photosynthetic sink (i.e. the grains), the ear is a greater source of assimilates because its photosynthetic performance is better under stress. Several traits of the ear have been suggested as explanations for this. These can be summarised as (1) the capacity of recycling (i.e. refixation) of respired CO_2, (2) some degree of C_4 or CAM metabolism (either constitutive or drought-induced) and/or (3) more xerophytic features (including anatomy and osmotic adjustment). Although the first explanation (i.e. refixation) is supported by experimental evidence (Kriedeman 1966; Bort et al. 1996; Gebbing and Schnyder 2001), the presence of C_4 metabolism in the ears of C_3 cereals is controversial. For instance, C_4 photosynthesis has been reported (or at least suggested) in several studies (Nutbeam and Duffus 1976; Wirth et al. 1976; Singal et al. 1986; Ziegler-Jöns 1989; Imaizumi et al. 1990). An other study, by contrast, has denied this possibility, at least for durum wheat growing in absence of water stress (Bort et al. 1995). The discrepancies between these studies could arise from the methodology used (for instance ^{14}C labelling in detached versus intact organs) and the environmental conditions of plant growth. Alternatively, CAM may occur in the ears (Blanke and Lenz 1989; Cushman et al. 2000). If CAM metabolism occurs in the spike, it would confer advantage in terms of carbon balance and water use efficiency. The ecological advantage is obvious during drought, which affects cereals at anthesis and grain filling in Mediterranean conditions. Hence, C_4 or CAM metabolisms in the ear might be induced only in response to stress (e.g. drought), but this hypothesis has not been examined.

Finally, morpho-physiological traits of the ears, such as its xeromorphic anatomy (Grundbacher 1957; Araus et al. 1993a) and osmotic adjustment (Morgan 1980), could have important adaptive advantages under, drought conditions. In fact, these characteristics may help the ear to maintain better water status under drought and, thus more photosynthetic activity than the flag leaf (Xu and Ishii 1990). However, to date, just how these xerophytic adaptations explain the better photosynthetic performance of the ear under drought has not been extensively studied.

The aim of this study was to compare the photosynthetic characteristics and water status of durum wheat ear and flag leaf in response to drought during grain filling under greenhouse and Mediterranean (i.e. field) conditions. In this context, whether C_4 or CAM photosynthesis existed in the ear in the absence of stress or its induction by drought was assessed. In this sense, we used a non-destructive (and non-invasive) technique such as modulated chlorophyll fluorescence to test the response of electron transport rate in manipulated (low O_2 and low CO_2) atmosphere.

Materials and methods

Growing conditions

Two sets of experiments were conducted with plants grown either in greenhouse or under field conditions.

Greenhouse experiment

Durum wheat seeds (*Triticum turgidum* L. var. *durum*) cv. Korifla were grown at field capacity conditions in 3-l plastic pots (one plant per pot) filled with peat-perlite-vermiculite 2:1:1 (v/v) and fertilised with nutritive Hoagland solution. Plants were cultivated in a greenhouse in experimental fields at the University of Barcelona (Barcelona, Spain). Mean day/night temperatures and maximum photosynthetically active photon flux density (PPFD) were ca. 28°C/20°C and 1,500 μmol m^{-2} s^{-1}, respectively. All plants were maintained at container capacity until heading. At this juncture, water was withheld. In water-stressed (WS) plants, the water content of the substrate was determined gravimetrically and was maintained by sub-irrigation at ca. 30% of container capacity. Well-watered (WW) plants were maintained at container capacity throughout the experiment.

We measured gas exchange and modulated chlorophyll fluorescence of the ear and the flag leaf three times: at anthesis, and 15 and 30 days after anthesis. In addition, water status and osmotic adjustment were measured during grain filling.

Field experiment

This was also conducted at experimental fields of the University of Barcelona. The experimental design was a randomised block (4.5 m^2/plot) with three replications per treatment (WW and WS). Conventional farming techniques were used. Durum wheat seeds of the same cultivar as above were sown in mid-February and the plots were periodically irrigated until anthesis. Then WS was imposed by halting irrigation, while preventing rainfall with a transparent polyvinyl chloride shelter. The shelter was only used during rainfall episodes. The control (WW) plots continued to be irrigated throughout grain filling. Mean day/night temperatures and maximum PPFD during grain filling were ca. 25°C/19°C and 1,950 μmol m^{-2} s^{-1}, respectively. Environmental conditions were monitored by a weather station (Delta-T Devices, Cambridge, UK) situated 8 m from the experimental plots.

Gas exchange, chlorophyll fluorescence and water status were measured on both organs 25 days after anthesis.

Water status determinations

Relative water content

Relative water content (RWC) was measured in ear parts (glumes, lemmas and awns) and in the blades of the flag leaf and the three leaves following the flag leaf from the top. Measurements on WW and WS plants in the greenhouse experiment were taken 20 days after anthesis. Leaf blade segments or ear parts were weighed (w_i), floated on distilled water at 4°C overnight, weighed again (w_f), and dried at 80°C for 48 h, after which, dry mass was determined (w_d). Relative water content was calculated as: $\text{RWC} = (w_i - w_d)\,(w_f - w_d)^{-1} \times 100$.

Water potential

Leaf water potential (ψ_w) was measured in the flag leaf of WW and WS plants from both the greenhouse and field experiment. Measurements were taken at midday, in anthesis and 15 days (about mid-grain filling) and 30 days after anthesis, using a pressure chamber (ARI-MAD-2, ARI Far Charuv-Water Supply Accessories, Israel) with a damp piece of paper at the bottom of the chamber to avoid excessive evaporation.

Osmotic adjustment

Osmotic potential of the flag leaf blade and ear parts (glumes, lemmas and awns) was determined at full turgor (Morgan 1984) in WW and WS plants of the greenhouse experiments 20 days after anthesis. Blades and ear parts were floated on distilled water at 4°C overnight, after which the samples were frozen in liquid nitrogen and stored at −30°C until analysis. Before the measurements, the samples were warmed at room temperature. Extraction was with a 1-ml syringe and the osmotic potential was measured with a VAPRO 5520 osmometer (Wescor, USA). The instrument was calibrated at standards of 100 mmol kg^{-1}, 290 mmol kg^{-1} and 1,000 mmol kg^{-1} (Wescor). Osmotic adjustment was defined as the difference of osmotic potential at full turgor between WW and WS plants (Babu et al. 1999).

Photosynthetic measurements

Gas exchange

Leaf gas exchange was measured, using an open IRGA LI-COR 6400 system (LI-COR Inc., Lincoln, NB, USA), at the flag leaf and the ear between 0900 hours and 1700 hours, with a mixed sequence across treatments to reduce the bias due to timing. The temperature of the photosynthetic chambers was maintained at ca. 25°C throughout the measurement period. The photosynthetic rate in the flag leaf blade was assayed with the standard cuvette (LI 6400-02). Gas exchange measurements in the ear were made with the Conifer Chamber (LI 6400-05), supplemented with a 500 W halogen lamp (model 64702, OSRAM S.A., Madrid, Spain). The lamp was placed about 30 cm above the ears and 7 cm of deionised water and 1 cm of Plexiglas in the container filtered the radiation. The lamp supplemented a PPFD of about 500 μmol m^{-2} s^{-1}. Plants were also irradiated with 500 μmol m^{-2} s^{-1} of PPFD for at least 30 min prior to photosynthesis measurements.

Dark respiration rate of the ear (R_d) was determined by darkening for at least 15 min. In the ear, corrected photosynthetic rate (A^*_{sat}) was calculated as ($A_{sat} + R_d$), where A_{sat} is the light-saturated net CO_2 assimilation rate (Araus et al. 1993a).

To test whether CAM metabolism is induced by drought, stomatal conductance and net CO_2 exchange in ears of WS plants were measured over a 24 period. Gas exchange was measured with the LI-COR 6400 system. In the light period, the ear placed in the IRGA chamber was irradiated with a PPFD ca. 1,000 μmol m^{-2} s^{-1}. To avoid alteration in circadian rhythms (Cushman et al. 2000) light and dark periods coincided with local time.

Chlorophyll fluorescence

Steady-state modulated chlorophyll fluorescence of the flag leaves and the ear was measured by means of a modulated chlorophyll MiniPAM fluorimeter (Waltz, Effeltrich, Germany) used simultaneously with gas exchange measurements. The leaf cuvette of infrared gas analyser (LI-COR 6400) was modified to accept the optical fibre from the modulated MiniPAM fluorimeter, as previously described (Nogués and Alegre 2002). For the ear, the optical fibre was introduced in the conifer chamber and obliquely oriented over the ear. The fluorescence signals were analysed as described by Andrews et al. (1993) to provide estimates of the relative quantum yield of PSII photochemistry [ϕ_{PSII}, given by $(F_m' - F_s)/F_m'$], the efficiency of energy captured by open PSII centres [F_v'/F_m', given by $(F_m' - F_o')/F_m'$] and the photochemical quenching [q_P, given by $(F_m' - F_s)/(F_m - F_o')$]. F_s and F_m' are the steady-state and maximum fluorescence yield, respectively, in light-adapted leaves and F_m is the maximum fluorescence in dark-adapted leaves. The parameter F_o' (minimum fluorescence yield in the light-adapted state) was calculated following Oxborough and Baker (1997). The maximum quantum yield of PSII photochemistry [F_v/F_m, given by $(F_m - F_o)/F_m$, where F_o is the initial fluorescence in dark-adapted leaves] was determined in flag leaf and ear after 15 min of dark adaptation (Nogués et al. 1998).

Low oxygen conditions

To examine whether the decreases in CO_2 assimilation caused by water stress were related to differential effects on photorespiration and carbon assimilation metabolism, we inhibited photorespiration in the ear and the

flag leaf by reducing the O_2 concentration of the atmosphere in which the photosynthetic parameters were measured from 21% to 2% (Nogués and Alegre 2002). Air at 2% O_2 and 98% N_2 was pumped to the air inlet port of the LI-6400. CO_2 was regulated with the CO_2 mixer of the LI-6400. Measurements at low oxygen conditions were made 15 and 30 days after anthesis.

In addition to these measurements of CO_2 assimilation rate in non-photorespiration conditions, we measured the response of ϕ_{PSII} to oxygen at several CO_2 concentrations. The change of ϕ_{PSII} was plotted versus CO_2 concentrations at both normal (atmospheric) and low (2%) oxygen.

Stomatal density

Stomatal density was measured on the flag leaf and ear parts (glume, lemma and awns) of WW plants of durum wheat. The organs were coated with a thick layer of nail polish, and the dried polish was carefully peeled off the organ and placed on a microscope slide to be counted (Teare et al. 1971). Stomatal density was determined on both sides of the flag leaf (abaxial and adaxial) and glume, lemma, and awn (dorsal or external and ventral or internal). On the dorsal side of the lemma two regions were considered: the area not covered by the glume and that covered by the glume. The dorsal or ventral side of the awn was considered according the continuation of the lemma. Each value represents the mean ± s.e. of four replicates. At least four glumes, lemmas or awns were measured in each ear. The results were averaged to give a single value for each ear.

Immunocytochemical localisation of Rubisco

Fixation and embedding

Flag leaves, glumes, lemmas and awns of WW and WS plants were sampled 20 days after anthesis. Cross-sections, 1–2 mm wide, were fixed at reduced pressure in 2% (v/v) paraformaldehyde, 0.2% (v/v) glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.4) for 24 h at 4°C. The samples were subsequently rinsed in buffer, dehydrated through a graded ethanol series and embedded in Lowikryl K4M at −20°C, as described by Carlemalm et al. (1982).

Immunocytochemical localisation

We used a polyclonal antibody prepared against spinach Rubisco raised in rabbits. The antibody was previously tested in wheat mediating western-blotting, and non-cross reactivity against other proteins was found (J.J. Guiamét, personal communication).

Immunocytochemical localisation was performed in cross-sections of the organs (ca. 1 μm). To block non-specific antibody sticking, first we incubated the cuts in 0.1 M phosphate-buffered saline (PBS) containing 1% (w/w) bovine serum albumin (BSA) for 15 min. Then, the cuts were incubated for 60 min with a dilution 1:10 (v/v) of a polyclonal antibody against Rubisco detailed above. After this, antibody solution was removed by washing the cuts with PBS-BSA several times. The cuts were incubated with a dilution 1:85 (v/v) of protein A-gold (5 nm) in PBS-BSA for 120 min. Non-specifically bound gold was removed by washing several times in PBS and finally with water. Controls included omission of the primary antiserum. Silver intensification was performed for 15 min with a Silver Enhancing Kit (British Bio-Cell International, UK) following the instructions of the manufacturer. The cuts were then immediately washed with water. The cuts were stained with methylene blue and photographed with light (reflected) microscopy (Olympus BHZ-UMA). Images were taken with a JVC TK 1270 camera.

Results

Plant water status and osmotic adjustment

In the greenhouse experiment, flag leaves of WS plants showed a steady and significant ($P < 0.05$) decrease in their ψ_w and RWC (Table 1). In WW plants, RWC and ψ_w did not significantly change throughout the experiment. In the field experiment, significant differences in ψ_w and RWC between water regimes were also observed (Table 1).

The RWC of all the (non-senescent) leaves of the culm plus various ear parts of plants grown in the greenhouse was analysed 15 days after anthesis. The RWC showed a bottom-to-top gradient in WS plants, with the higher value in the awns (Fig. 1a). In the WW plants, no relevant differences were observed in RWC of the leaves and ear parts (Fig. 1a). However, when we contrasted the RWC of several organs (lower and flag leaves, glumes, lemmas and awns) in WS plants versus the water content (expressed as percentage of fresh weight) of the same organ in WW plants, a strong negative correlation ($r^2 = 0.93$) was found (Fig. 1b). The awns showed the lowest water content under WW conditions and, as mentioned above, higher RWC in WS plants.

Osmotic potential at full turgor of flag leaves and ear parts showed significant differences ($P < 0.05$) between WW and WS plants (Table 2). In the flag leaf, the osmotic adjustment (the difference in osmotic potential between WW and WS plants) was low (6%), but in the ear parts osmotic adjustment was about 40%, 46% and 28% for glumes, lemmas and awns, respectively.

Photosynthetic performance during grain filling

Greenhouse experiment

Photosynthetic response to water stress of the flag leaf and the ear was measured at three moments during grain

Table 1 Water potential (Ψ_w) and relative water content (*RWC*) of the flag leaves in well-watered (*WW*) and water-stressed (*WS*) plants of durum wheat (*Triticum turgidum* L. var. *durum*) grown under both greenhouse and field conditions

	Treatment	Ψ_w (MPa)	RWC (%)
Greenhouse			
Anthesis	WW	-1.07 ± 0.09	90.26 ± 1.63
	WS	-1.64 ± 0.19[a]	80.91 ± 5.41[a]
15 days after anthesis	WW	-1.01 ± 0.09	88.45 ± 1.17
	WS	-1.86 ± 0.17[a]	77.34 ± 2.75[a]
30 days after anthesis	WW	-1.15 ± 0.08	85.29 ± 1.50
	WS	-2.33 ± 0.15[a]	68.06 ± 3.27[a]
Field			
25 days after anthesis	WW	-1.71 ± 0.24	85.98 ± 2.46
	WS	-2.37 ± 0.23[a]	67.49 ± 5.25[a]

In the greenhouse, measurements were made at anthesis, and 15 and 30 days after anthesis. In the field, measurements were performed 25 days after anthesisValues are the mean of four to six measurements ± SE

[a]Significant differences ($P \leq 0.05$) between WW and WS plants according to the LSD test

Table 2 Osmotic potential of flag leaf and ear parts (glume, lemma and awns) of WW and WS plants of durum wheat (*T. turgidum* L. var. *durum*) grown in a greenhouse

Organ	Osmotic potential at full turgor (MPa)		
	WW	WS	Difference (%)
Flag leaf	-1.43 ± 0.01	-1.52 ± 0.03[a]	6.3
Glumes	-1.09 ± 0.08	-1.53 ± 0.02[a]	40.4
Lemmas	-1.04 ± 0.07	-1.52 ± 0.02[a]	46.1
Awns	-1.54 ± 0.10	-1.97 ± 0.05[a]	27.9

Osmotic potential (MPa) was determined at full turgor by an osmometer 20 days after anthesis. The percentage difference in osmotic potential between WS and WW plants (i.e. osmotic adjustment) is also indicatedEach value represents the mean ± SE of four to six measurements

[a]Significant differences ($P \leq 0.05$) between WW and WS plants according to the LSD test

filling. In WW plants, the light-saturated rate of CO_2 assimilation (A_{sat}) slightly decreased in the flag leaf and the ear, mainly between anthesis and 2 weeks later (Fig. 2a). In addition, water stress led to a significant decrease (compared with WW plants) of A_{sat} in the flag leaf at the three moments of measurement (Fig. 2a). In the ear, this decrease of A_{sat} by water stress was only significant ($P < 0.05$) at mid-grain filling (data not shown). Since the A_{sat} of the ear could be strongly influenced by the CO_2 emitted by this dark respiration (grain plus heterotrophic tissues), we also calculated the corrected A^*_{sat}, as $A_{sat} + R_d$ (Araus et al. 1993a). We observed no significant differences ($P < 0.05$) in the A^*_{sat} of the ear between WW and WS plants (Fig. 2b). The dark respiration rate (R_d) in the ear showed a peak 15 days after anthesis in both WW and WS plants (Fig. 2c). At 30 days after anthesis, the R_d was similar to anthesis (Fig. 2c).

Modulated chlorophyll fluorescence measurements showed steady decay of ϕ_{PSII} in the flag leaves of WS plants, mainly 30 days after anthesis (Fig. 3a). This decrease of ϕ_{PSII} was accompanied by decreases in both photochemical quenching (q_P; Fig. 3b) and in the F_v'/F_m', the efficiency of energy capture by open PSII reaction centres (Fig. 3c). In contrast, no significant ($P < 0.05$) differences between WW and WS plants were observed in fluorescence parameters of the ear (Fig. 3a, b, c; right panels). Nor did water stress lead to any significant effect on the potential yield of PSII (i.e. F_v/F_m in dark-adapted leaves) in the two organs (data not shown).

Field experiment

The field photosynthetic responses of the flag leaf and the ear to water stress were similar to the greenhouse

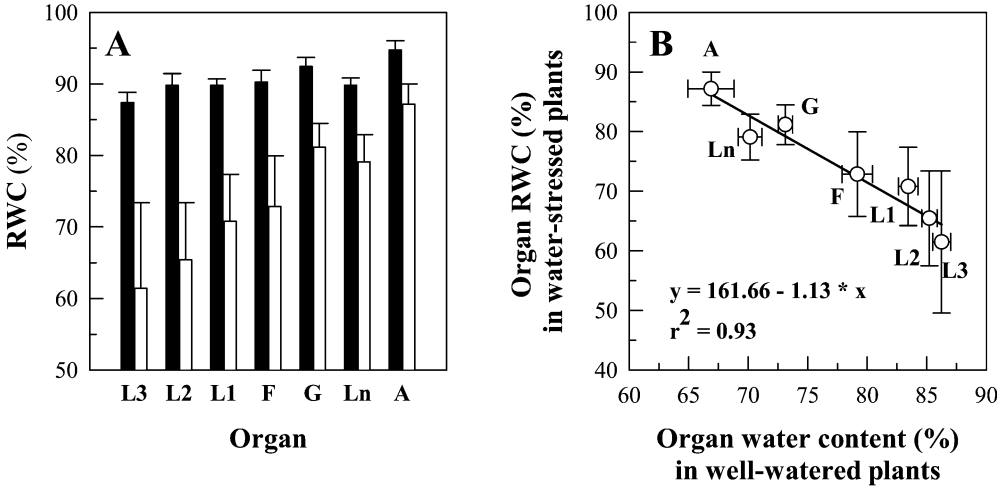

Fig. 1 **a** Relative water content (RWC) of leaves and ear parts of WW (*filled bars*) and WS (*open bars*) plants of durum wheat (*T. turgidum* L. var. *durum*) grown in a greenhouse. *F*, *G*, *Ln* and *A* correspond to the flag leaf, glume, lemma and awn, respectively. *L3*, *L2* and *L1* are the three leaves following the flag leaf, in basal direction. Each value represents the mean ± s.e. of four measurements. **b** Relationship between the water content (as percentage of fresh weight) of the leaves and ear parts in WW plants versus the RWC of the same organs in WS plants. Each point represents the mean ± s.e. of four measurements. Abbreviations as in **a**. For both panels, measurements were performed 15 days after anthesis

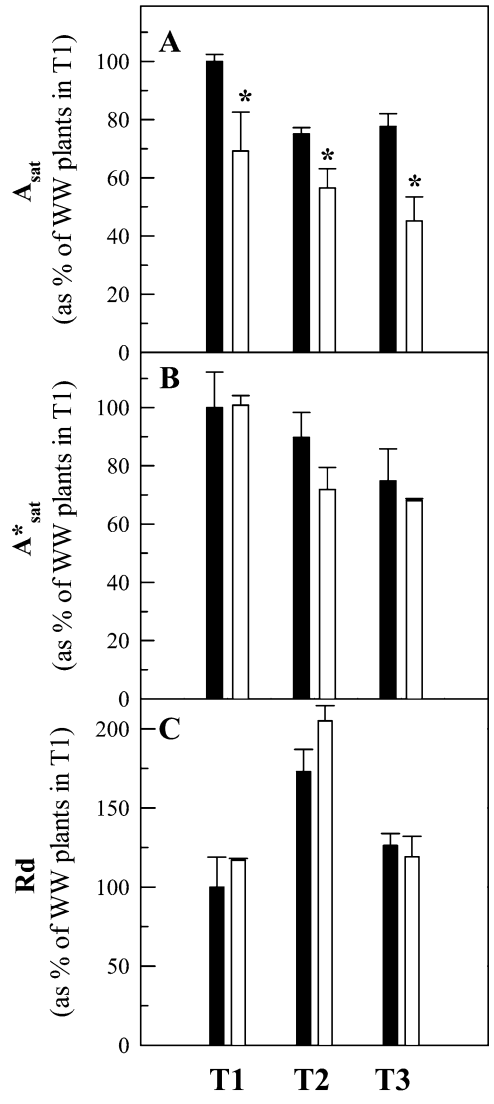

Fig. 2 Changes during grain filling in the light-saturated net photosynthetic rate (A_{sat}) of the flag leaf (**a**), and the corrected photosynthetic rate ($A^*_{sat} = A_{sat} + R_d$) and dark respiration ($R_d$) of the ear (**b, c**) of WW (*filled bars*) and WS (*open bars*) plants of durum wheat (*T. turgidum* L. var. *durum*) grown in a greenhouse. The measurements were performed at anthesis, and 15 or 30 days after anthesis (*T1*, *T2* and *T3*, respectively). Values are expressed as percentage of the measurements at anthesis (*T1*) under well-watered conditions. Each value represents the mean ± s.e. of four to six measurements. Significant differences ($P \leq 0.05$) between treatments according to the LSD test are denoted by an *asterisk*

ones. Thus, about 4 weeks after anthesis, as response to water stress, the ear showed less decrease in A_{sat} and ϕ_{PSII} than the flag leaf (Table 3).

Photosynthetic response to low oxygen

The photosynthetic response to low oxygen was measured in WW and WS plants 15 and 30 days after anthesis in the greenhouse experiment (Fig. 4). In the flag leaf, the increase of A_{sat} at low O_2 was ca. 45% in WW plants. However, this stimulation of A_{sat} was lower in flag leaves

Table 3 Comparison of decreases in the photosynthetic rate (A_{sat}) and relative quantum yield of PSII photochemistry (ϕ_{PSII}) of flag leaves and ears in WS and WW plants of durum wheat (*T. turgidum* L. var. *durum*) growing in greenhouse and field conditions

	Greenhouse (T3)		Field	
	A_{sat} (%)	ϕ_{PSII} (%)	A_{sat} (%)	ϕ_{PSII} (%)
Flag leaf	42.8	27.8	56.3	24.3
Ear	9.8 (9.0)	10.3	25.0 (5.3)	17.1

Gas exchange and fluorescence were measured simultaneously at 1,000 μmol m^{-2} s^{-1} of PPFD and 25°C, 30 days (greenhouse) and 25 days (field) after anthesis. The decrease of A^*_{sat} (i.e. $A_{sat} + R_d$) for ears is also shown (in brackets)
Values are expressed as the percentage decrease of WS compared with WW plants

in WS treatment, and decreased as water stress developed during grain filling (Fig. 4a, b; filled bars in left panels). In the ear, A_{sat} increased markedly (ca. 80% and 60% 15 and 30 days after anthesis, respectively) at low oxygen (Fig. 4a, b; filled bars in right panels). However, for the corrected photosynthetic rate (A^*_{sat}), an increase similar to that in the flag leaf was found (Fig. 4a, b; grey bars). As response to water stress, no significant decrease in the stimulation of A^*_{sat} was shown.

The ϕ_{PSII} response curves to CO_2 under photorespiratory and non-photorespiratory conditions were also analysed in WW and WS ears and flag leaves at mid-grain filling (Fig. 5). At normal atmospheric O_2 concentration, and regardless of the water treatment, both flag leaf and ear showed moderate decrease of electron transport at lower CO_2 concentrations (Fig. 5a). Under non-photorespiratory conditions, this decrease was greater (compared with normal oxygen) in the two organs of WW and WS plants (Fig. 5b).

Anatomy and stomatal density

Ear parts had typical non-Kranz anatomy. Thus, as well as the lack of a developed bundle sheath with chloroplast, in the glumes, mesophyll cells were positioned both on the dorsal and ventral sides (Fig. 6a, b), while in the lemma (at least in the area superimposed on the glume) the photosynthetic cells were distributed on the ventral side (Fig. 6c, d).

In comparison with the flag leaf, ear parts of durum wheat showed an asymmetric distribution of stomata on each side of the organ (Table 4). Although stomata are present on the ventral side (i.e. facing the grains), glumes and lemmas have the higher stomatal frequency on the dorsal side (i.e. external). On the other hand, the awns showed the higher stomatal frequency, even more than the flag leaf (Table 4).

Rubisco distribution

The immunolocalisation of Rubisco analysed by means of optical microscopy did not support any compart-

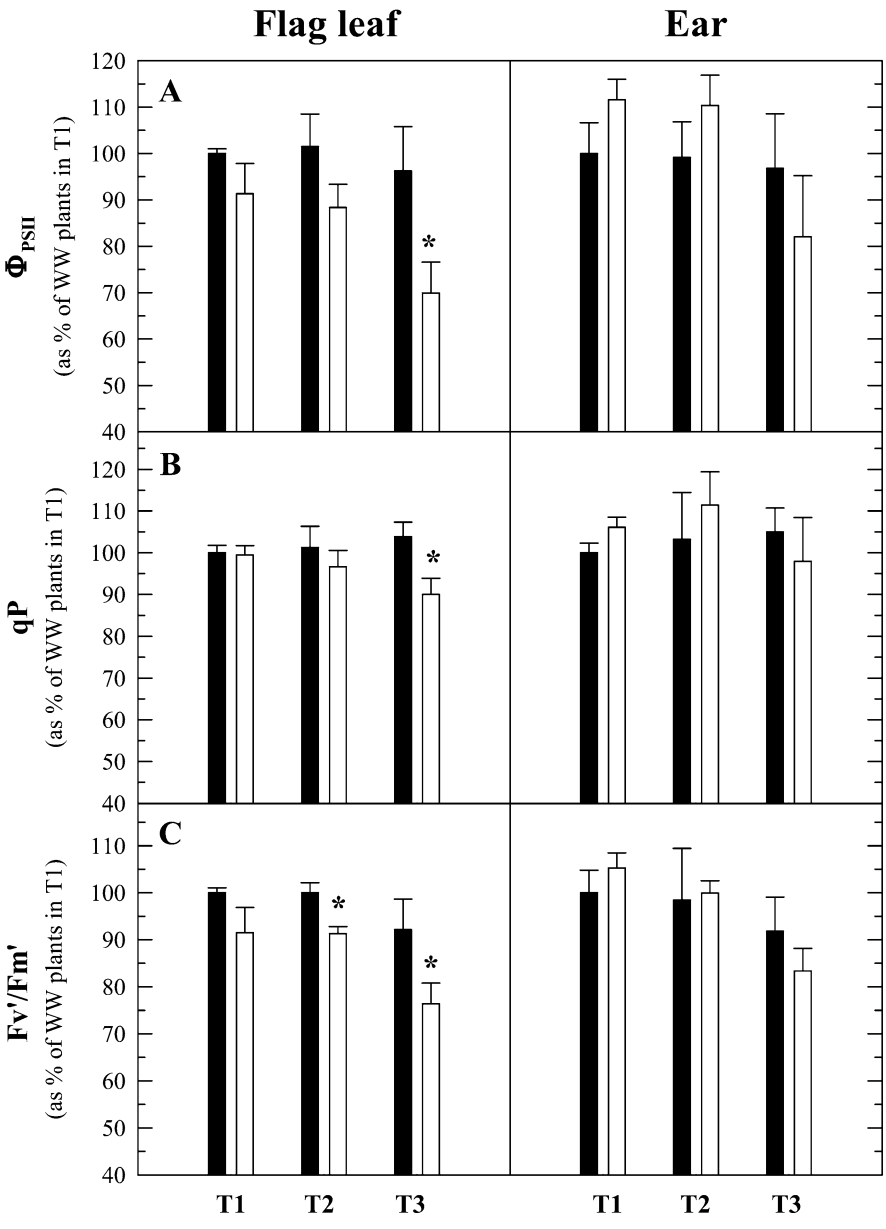

Fig. 3 Changes in effective quantum efficiency of PSII (ϕ_{PSII}, **a**), photochemical quenching (q_P, **b**) and efficiency of energy capture by open PSII reaction centres (F_v'/F_m', **c**) of flag leaves (*left side of figure*) and ears (*right side*) of WW (*filled bars*) and WS (*open bars*) plants of durum wheat (*T. turgidum* L. var. *durum*) grown in a greenhouse. The measurements were performed at anthesis, and 15 or 30 days after anthesis (T1, T2 and T3, respectively). Values are expressed as percentage of the measurements at anthesis (T1) under WW conditions. Each value represents the mean ± SE of four to six measurements. Significant differences ($P \leq 0.05$) between treatments according to the LSD test are denoted by an *asterisk*

mentalisation in the distribution of this protein in ear parts (Fig. 6). In fact, silver label (viewed as brilliant areas in the chloroplasts) was uniformly distributed in the mesophyll cells of glumes, lemmas and awns, both in WW and WS plants. In general, the label was absent from other compartments such as the cells surrounding the vessels. We also analysed the Rubisco distribution in the green layer of the pericarp of the grain. In this tissue, chloroplasts are distributed on the tangential side of the cells and were labelled against Rubisco antibody (Fig. 6g).

Microscopic observation showed the xeromorphic nature of bracts and awns, with the glume and the lemma having a dorsal epidermis and sub-epidermal sclerenchymatous tissue with thick walls (see Fig. 6a, c). In contrast, the ventral epidermis (facing the grain) of both organs had cells with thin walls.

Daily course of gas exchange

Stomatal conductance and gas exchange of the ear showed a pattern typical of non-CAM plants, i.e. stomata closing at night and opening in the light period (Fig. 7).

Discussion

Is the ear more 'drought tolerant' than the flag leaf?

The photosynthetic rate and the ϕ_{PSII}, F_v'/F_m' and q_P parameters of the ear were less affected by water stress than the flag leaf under both greenhouse (Figs. 2, 3) and field (Table 3) conditions. The better photosynthetic performance of the ear (compared with the flag leaf)

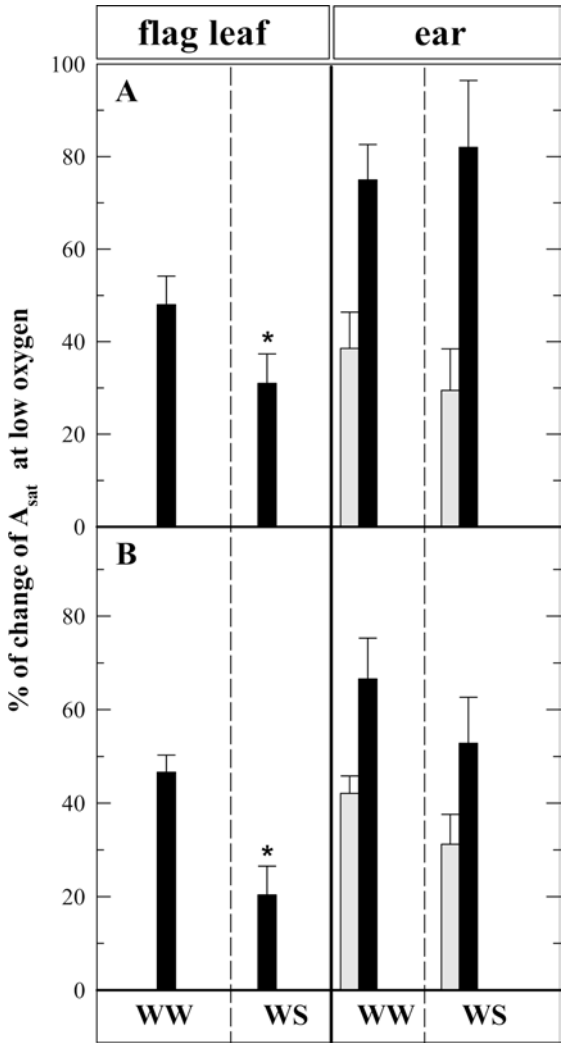

Fig. 4 Low-oxygen sensitivity of the light-saturated net photosynthetic rate (A_{sat}) of WW and WS plants of durum wheat (*T. turgidum* L. var. *durum*) grown in a greenhouse. Measurements were taken 15 days (**a**) and 30 days (**b**) after anthesis. The results (*filled bars*) are expressed as the percentage of change of the A_{sat} at low (ca. 2%) oxygen versus normal atmospheric (ca. 21%) oxygen. In the case of the ear, the response of the $A^*_{sat}(A_{sat} + R_d)$ was also calculated in a similar way (*grey bars*). Each value represents the mean ± SE of four to six measurements. Significant differences ($P \leq 0.05$) between treatments (WW vs. WS) according to the LSD test are denoted by an *asterisk*

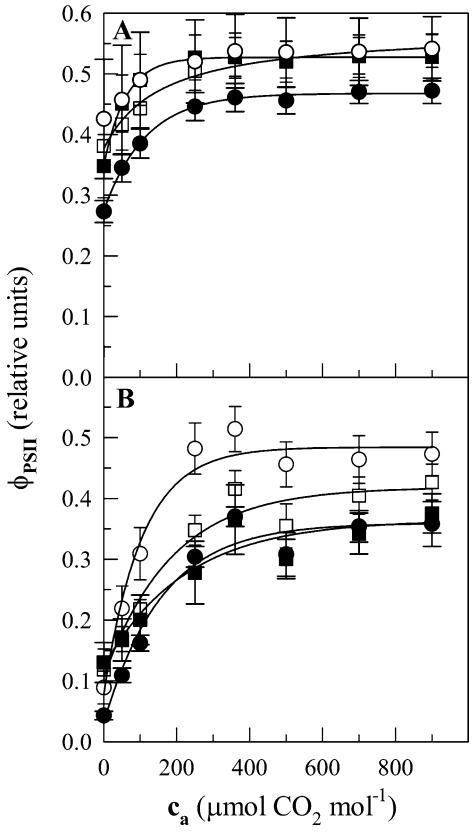

Fig. 5 Response of relative quantum yield of PSII photochemistry (ϕ_{PSII}) to CO_2 changes at normal (ca. 21%) (**a**) or low (ca. 2%) (**b**) oxygen concentration of flag leaves (*circles*) and ears (*squares*) of WW (*open symbols*) or WS (*closed symbols*) plants of durum wheat (*T. turgidum* L. var. *durum*) grown in a greenhouse. The measurements were taken 15 days after anthesis. The curves were performed at 800 µmol m^{-2} s^{-1} of PPFD. Each value represents the mean ± SE of four to six measurements

under water-stress conditions has been previously reported in some studies (Johnson et al. 1974; Xu et al. 1990), although the mechanistic basis is lacking. The greater tolerance of ear photosynthesis to water stress could be explained by an intrinsic or water-stress-induced C_4 or CAM photosynthesis, although this is not supported by our results. Alternatively, the presence of traits associated with drought tolerance, such as xerophytic structure, is discussed below.

The better photosynthetic performance of the ear than the flag leaf under water-stress conditions seems connected to its capacity to maintain higher RWC (Fig. 1). Xu and Ishii (1990) also concluded that the capacity to maintain high RWC seemed to be the main cause of higher drought resistance in the ear of bread wheat (*Triticum aestivum* L.). However, these authors measured the RWC of the whole ear (so including the growing grains) instead of the RWC of the different parts of the ear (i.e. glumes, lemmas and awns) separately. Consequently, their RWC values are strongly influenced by the high water content of the grains (Xu and Ishii 1990). Moreover, these authors reported similar water relations for glumes as for the flag leaf, and suggested that the grain is responsible for ear tolerance to water stress. We disagreed with these authors, for we found differences in RWC between the flag leaf and ear bracts (and, even more, the awns) of WS plants (Fig. 1). Recently, Wardlaw (2002) reported that the glumes of bread wheat maintained higher RWC than the flag leaf under progressive water stress. Although their study did not analyse the photosynthetic performance of the ear, its data are consistent with our results.

Greater capacity for osmotic adjustment in the different ear parts than in the flag leaf (Table 2) may contribute to the higher RWC status (Table 1; Fig. 1a) and the better photosynthetic performance (Figs. 2, 3)

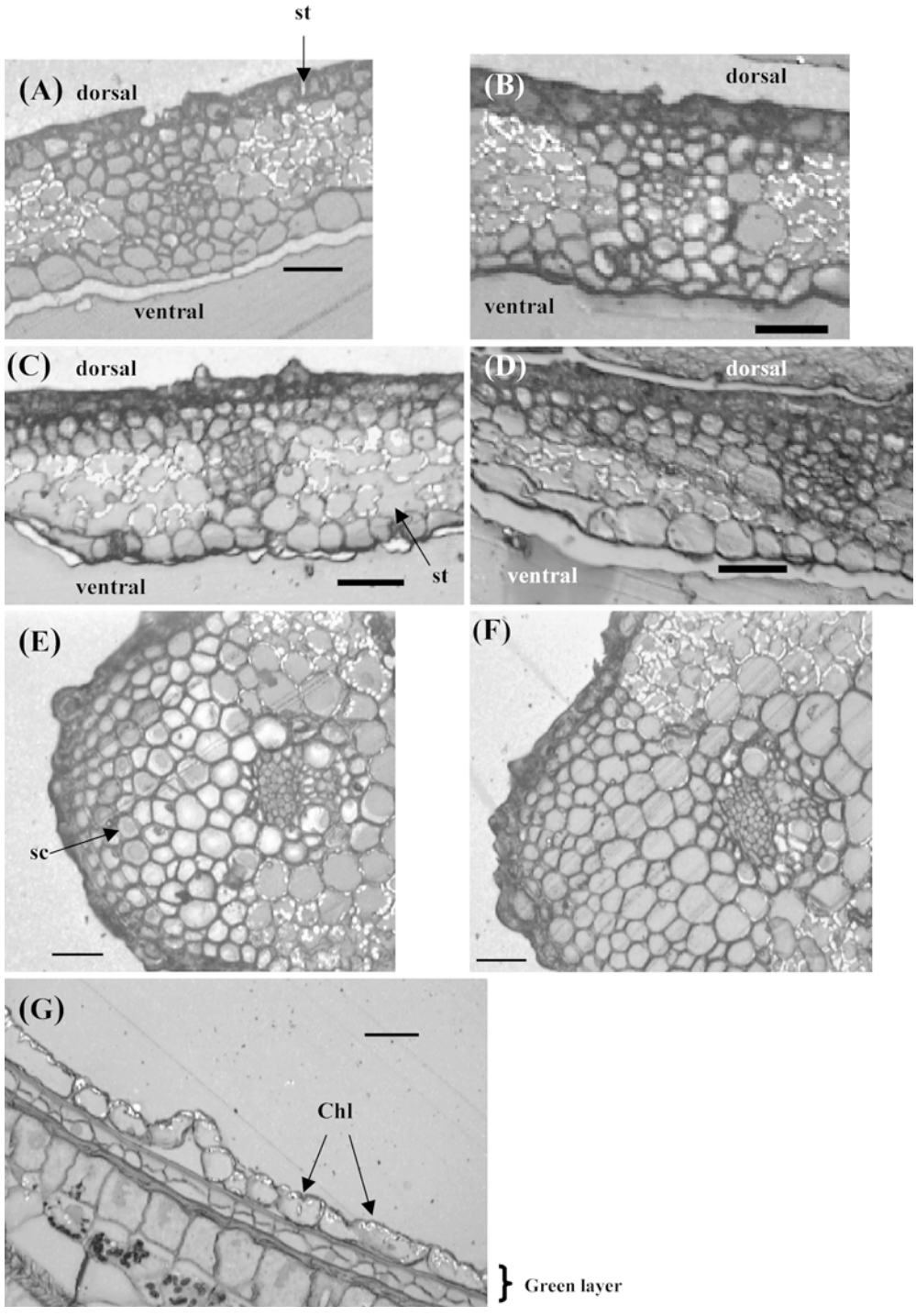

Fig. 6 Light micrographs of Rubisco immunolocalisation in cross-sections of glumes (**a, b**), lemmas (**c, d**) and awns (**e, f**) of WW (*left*) and WS (*right*) plants of durum wheat (*T. turgidum* L. var. *durum*) grown in a greenhouse. Immunolocalisation of Rubisco in cross-section of green grain pericarp in WS plants is also shown (**g**). In the lemma, the cross-sections correspond to the area superimposed on the glume (i.e. central area). Semi-thin sections were treated with specific polyclonal anti-Rubisco antibody. Immunolabelling was performed by Protein A-gold and silver intensification. Label of Rubisco is visualised as brilliant areas in the chloroplasts. *chl* Labelled chloroplasts in green layer; *sc* sclerenchyma; *st* stomata. *Bar* = 40 μm

of the ear. These results corroborate those of Morgan (1980), who reports higher osmotic adjustment in ears of *Triticum* species. Osmotic adjustment can ensure maintenance of turgor and gas exchange under water stress (Clarke 1987; Kikuta and Richter 1986; Serraj and Sinclair 2002). Although some controversy has arisen on the true role of osmotic adjustment performing drought adaptation in agronomic yield (Serraj and Sinclair 2002), in several reports osmotic adjustment has been noted as a crucial factor for drought tolerance in wheat (Morgan 1984; Morgan and Condon 1986; Sen Gupta and Berkowitz 1987; Ludlow et al. 1990; Blum et al. 1999).

In addition to osmotic adjustment, other factors could be involved in the drought response of ear photosynthesis. Vertical heterogeneity in sclerophyllous characteristics of wheat leaves (such as lower intercellular spaces, smaller and packed cells, thicker cellular walls and higher proportion of sclerenchymatous tissue) has been reported (Araus et al. 1986). In this sense, the vertical gradient of water content in several organs of

Table 4 Stomatal density of the flag leaf and ear parts (glume, lemma and awns) of WW plants of durum wheat

Number of stomata (mm^{-2})							
Flag leaf		Glume		Lemma		Awn	
Adaxial	Abaxial	Dorsal	Ventral	Dorsal	Ventral	Dorsal	Ventral
55.79 ± 5.41	40.69 ± 7.11	32.96 ± 7.54	9.93 ± 0.88	52.01 ± 8.93[a] Not found[b]	13.77 ± 1.63	78.30 ± 8.93	Not found

In the glume, lemma and awn, stomatal density was measured on the dorsal (i.e. external) and the ventral (i.e. internal) face of the organ. On the dorsal side of the lemma, two regions were considered: the covered (i.e. exposed to the air) and non-covered the by the glume. Only in the non-covered area of the lemma, the stomata are present. Dorsal or ventral side of the awn was considered according the continuation of the lemma

Each value represents the mean ± s.e. of four replicates. At least four glumes, lemmas or awns were measured in each ear. The results were averaged to give a single value for each ear. For further details, see Material and methods

[a]Non-covered area of the lemma

[b]Covered area of the lemma

WW plants, which correlated closely—and negatively—with the RWC of the same organs in WS plants (Fig. 1b), could reflect this xeromorphic tendency in upper levels of the plant and thus tolerance to water stress. Consequently, the greater capacity of the ear to maintain higher RWC than the flag leaf could also be related to sclerophyllous traits of bracts and, in particular, of awns (see Fig. 6).

C_3 versus C_4 or CAM metabolisms in the ear

The possibility that there is some degree of C_4 metabolism in the ear of C_3 cereals, such as wheat, barley and rice, has been reported (or at least suggested) in several studies (Nutbeam and Duffus 1976; Wirth et al. 1976; Singal et al. 1986; Ziegler-Jöns 1989; Imaizumi et al. 1990). However, in the present study, ear and flag leaf photosynthesis showed a typical C_3-response to low oxygen (i.e. non-photorespiratory) conditions in WW plants (Fig. 4). Thus the photosynthetic rate of both organs was stimulated by low oxygen at ca. 45%. In the flag leaf under water stress, oxygen sensitivity was lower than in WW plants, whereas in the ear the decrease was not significant. A decrease in low-oxygen stimulation of A_{sat} as response to water stress has been attributed to phosphate limitation (Sharkey 1985; Nogués and Baker 2000; Nogués and Alegre 2002). Inorganic phosphate (P_i), essential for photophosphorylation in the stroma, can be limiting if phosphorylated intermediates are not exported from the chloroplasts. In fact, O_2 insensitivity is considered a symptom of P_i deficiency in vivo in C_3 plants (Leegood 1989) and has been reported in water-stressed leaves (Sharkey 1985).

Therefore, the oxygen sensitivity of the photosynthetic rate in the ear was typical of C_3 plants, and was not significantly affected by water stress (Fig. 4). This observation is consistent with some previous reports, in which no C_4 metabolism was found in the ear of bread wheat (Bort et al. 1995). However, whereas in this latter

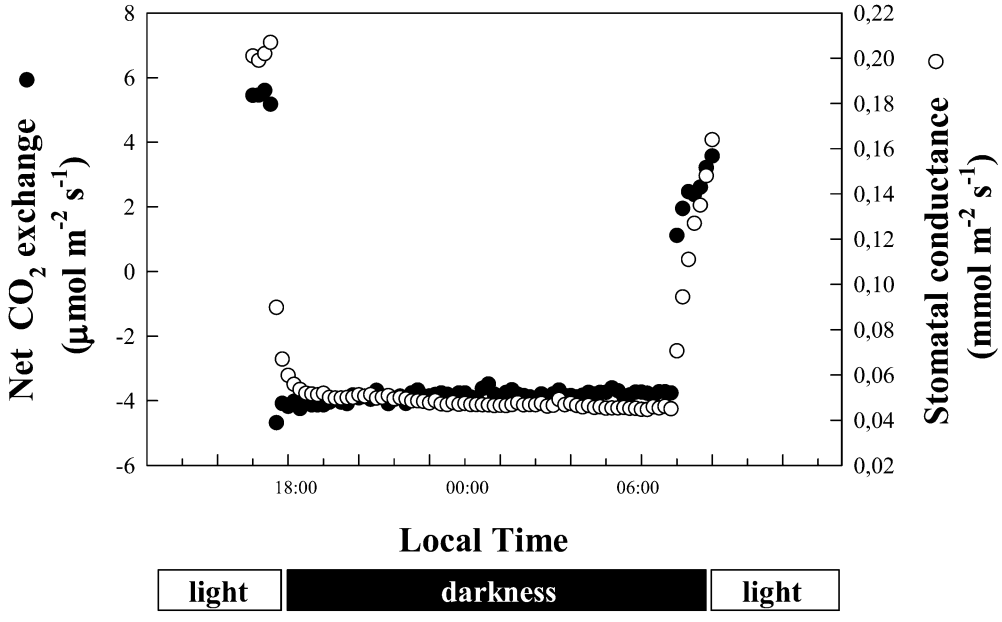

Fig. 7 Daily course of stomatal conductance and net CO_2 exchange in the ear of WS plants of durum wheat (*T. turgidum* L. var. *durum*). *Black and white bars* on the horizontal axis represent the dark and the light period respectively. In the light period, ear in the IRGA chamber was irradiated with a PPFD ca. 1,000 µmol m^{-2} s^{-1}

study only well-watered plants were analysed, our results do not support an induction of C_4 metabolism in the ears as response to water stress.

A similar conclusion can be drawn from the analysis of the ϕ_{PSII} response to decreasing CO_2 at low oxygen (Fig. 5). Decreasing the concentration of CO_2 in non-photorespiratory conditions caused the ϕ_{PSII} in the ear (as in the flag leaf) to drop markedly. This CO_2–O_2 interaction is interpreted as strong evidence for Rubisco-mediated carbon assimilation as a sink of electron transport (Lawson et al. 2002). This is further clear evidence for C_3 metabolism in the ear, which has not previously been reported.

Nor does the lack of Kranz anatomy (Fig. 6) support the existence of C_4 metabolism in the ear of durum wheat. Ziegler-Jöns (1989) reported an anatomy-like intermediate C_3–C_4 in bracts (glumes and lemmas) of bread wheat. However, the evidence for this was very indirect, and compartmentalisation of enzymes such as Rubisco or PEP carboxylase (PEPc) was not analysed. Recently, it has been demonstrated that Kranz anatomy is not essential for terrestrial C_4 plant photosynthesis (Voznesenskaya et al. 2001; Sage 2002). These authors showed that the spatial separation of the biochemical events necessary for C_4 photosynthesis occurs within a single cell in some halophyte plants. However, we did not observe any compartmentalisation in Rubisco protein, at either the histological or cellular level (Fig. 6). Thus, the evidence shown in this paper does not support the existence of C_4 metabolism in the ear of durum wheat.

Some studies report the existence of enzymes of the C_4 metabolism, such as PEPc in the ear (Wirth et al. 1976) and pyruvate orthophosphate dikinase (PPDK) (Aoyagi and Bassham 1984). However, the interpretation of their findings is not straightforward. PEPc is a ubiquitous enzyme in vegetal tissues and is non-exclusive of C_4 metabolism (Chollet et al. 1996). In fact, immunocytochemical localisation of PEPc suggests no existence of C_4 metabolism in the ear, at least in glumes of durum wheat under WW conditions (Araus et al. 1993b). Furthermore, it is known that PPDK is present in C_3 tissues (Aoyagi and Bassham 1984). As indicated by Araus et al. (1993b), the function of these enzymes seems to involve anaplerotic rather than photosynthetic CO_2 fixation, i.e. these enzymes are unlikely to be involved in photosynthesis, which is congruent with our results. Other studies giving evidence of C_4 metabolism can be reinterpreted. For instance, O_2 insensitivity reported in spikelets of rice (Imaizumi et al. 1990) could be explained in other ways (e.g. phosphate limitation), as discussed above. In addition, the carbon isotope composition of bracts and awns is higher than that of the flag leaf (Araus et al. 1992, 1993a; Gebbing and Schnyder 2001), but their value (ranging between ca. −22% and −30‰) seems to fall within the normal range of C_3 species (Pate 2001).

It is worth noting that, since gas exchange measurements only analyse the assimilation of external CO_2, we could not discard the existence of some grade of C_4 metabolism in the refixation of respired CO_2 (Bort et al. 1996; Gebbing and Schnyder 2001). However, as mentioned above, the electron transport of the ear parts studied is clearly sensitive to oxygen and CO_2 in a similar way to the flag leaf (Fig. 5b), suggesting that, despite the CO_2 source, ear photosynthesis is mainly of the C_3 type. In this respect, the mesophyll cells of the lemmas facing the grain (i.e. on the ventral side), which are probably involved in refixation of respired-CO_2 (Araus et al. 1993a), show a distribution of Rubisco typical of C_3 plants (Fig. 6c, d). Though the green pericarp of developing grains (Caley et al. 1990) was not examined in our study, its relevance as a photosynthetic part of the ear seems minor (Fig. 6g).

Because CAM metabolism is drought-inducible (Cushman et al. 2000), we examined this possibility in the ear of WS plants. However, the lack of stomatal opening at night and the CO_2 exchange pattern (Fig. 7) do not provide evidence for CAM-like metabolism. Further, studies from our team on ears of various species also show low stomatal conductance and absence of CO_2 uptake at night, in addition to a lack of daily changes in organic (i.e. malic and aspartic) acids (data not shown).

Finally, refixation of respired CO_2 seems to be a relevant process in the ear (Kriedemann 1966; Bort et al. 1996; Gebbing and Schnyder 2001). Because re-assimilation of CO_2 is independent of gas exchange with the external environment, this process can increase the overall water use efficiency WUE of the organ (Araus et al. 1993a). In accordance with Kriedemann (1966), the relative contribution to grain filling of refixation is 21% of the total assimilates. On the other hand, net fixation (i.e. assimilation of external CO_2) was only 9% in their study. However, it must be pointed out that awnless wheat was used in their work. The awns are the main photosynthetic organ of the ear, at least considering the uptake of external CO_2; thus the lack of awns in the cultivars used in Kriedemann's study could underestimate the fixation of external CO_2.

The exact site of refixation is unknown. However, green pericarp (Caley et al. 1990) and lemmas are putative loci. In fact, stomata were present on the ventral side (i.e. facing the grain) of the last organ (see Table 4). This conflicts with the findings of Teare et al. (1971), who did not find stomata on this surface of the lemmas of bread wheat. The stomatal pores on the ventral side of the lemmas could contribute to the intake of respired CO_2, although further research is necessary to elucidate this question.

Conclusions

We demonstrate that the better photosynthetic performance under water stress of the ear than the flag leaf of durum wheat is not associated with C_4 or CAM photosynthesis induction in the ear. Rather, it seems related to the maintenance of better water status in the ear than

in the flag leaf, which is at least partially explained by higher osmotic adjustment combined with a more xerophytic structure.

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