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Water-stress-induced thermotolerance of photosynthesis in bean (*Phaseolus vulgaris* L.) plants: The possible involvement of lipid composition and xanthophyll cycle pigments

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ABSTRACT

A common feature in plants that are exposed to gradual stressful environmental factors is the development of a level of resistance to such constraints, which sometimes protects against different stressful environmental conditions. The aim of this study was to assess a possible cross-resistance between water stress and high-temperature stress and to gain a better knowledge regarding the physiological basis for heat resistance. The study was performed in two bean varieties; Orfeo INIA (OI) and Arroz Tuscola (AT) are a stress-resistant and stress-sensitive variety, respectively. An increased heat resistance in OI but not in AT results from water stress as revealed by the oxygen-evolution rate at 38 °C and the thermal threshold for non-reversible damage that was assessed by the temperature-dependent increases in basal fluorescence (F_0); higher values of both parameters were observed in the leaves of water-stressed plants when compared to the control OI plants. The heat-shock proteins HSP70, HSP60 and HSP24 do not seem to be involved in the water-stress-induced resistance to high temperature because no difference in their contents was found between the water-stressed and control plants. The important features in the water-stressed OI plants, which can resist higher temperatures, are the maintenance of the xanthophyll pigment contents compared to the control plants and in contrast to the sensitive AT plants, an increase in phosphatidylglycerol and a reduction in the unsaturation level of the thylakoid fatty acids. The results from the comparative analyses of the xanthophyll, lipid and fatty acid compositions in the chloroplasts of well-watered and water-stressed AT and OI plants are discussed in terms of their possible involvement in conferring resistance to high temperature in water-stressed bean leaves.

1. Introduction

Plants are frequently exposed to environmental constraints, such as drought and high temperature, which are both considered to be among the most important environmental factors that limit net photosynthesis worldwide (Berry and Björkman, 1980; Yordanov et al., 1986; Lawlor and Uprety, 1993; Sinsawat et al., 2004) and are known to occur simultaneously (Craufurd and Peacock, 1993; Jiang and Huang, 2001). Drought limits photosynthesis through stomatal and non-stomatal limitations (for a review, see Lawlor and Cornic, 2002; Chaves et al., 2003). Under mild water stress, a reduction in stomatal and leaf mesophyll conductance has been observed; this can lead to the restrictive diffusion of CO_2 from the air into the carboxylation sites (Flexas and Medrano, 2002a). As water stress progresses, photosynthetic limitations have been reported either as a consequence of damage to the photosystems (Havaux et al., 1986), which results from the light that is absorbed in excess of the CO_2-reduction capacity (Navari-Izzo and Rascio, 1999), or from impairments in photophosphorylation, Rubisco activity and/or the regeneration of Ribulose-bisphosphate (Boyer and Younis, 1983; Havaux et al., 1988; Cornic and Massacci, 1996; Medrano et al., 1997; Tezara et al., 1999; Lawlor, 2002). The lower CO_2 fixation rate that is associated with water stress can decrease the demand for NADPH and ATP in the chloroplast stroma, which down-regulates photosynthetic electron transport. Therefore, some adaptations that have led to water-stress resistance at the photosynthetic level in plants involve changes in the pigment content of the photosystems to (i) reduce the extent of the light that is absorbed in excess of the CO_2-reduction capacity, (ii) increase the capacity for energy dissipation as heat and (iii) adapt the photosystem stoichiometry (He et al., 1999; Flexas and Medrano, 2002b; Yuan et al., 2005).

Temperatures that are above the optimum for plant growth, however, cause important reductions in net photosynthesis (for a

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review, see Wahid et al. (2007)). Under moderately elevated temperatures, CO_2 assimilation is reduced as a result of the decrease in the Rubisco activation state (Weis, 1981a,b; Crafts-Brandner and Salvucci, 2000; Salvucci and Crafts-Brandner, 2004a), which primarily occurs due to the heat inactivation of Rubisco activase (Salvucci et al., 2001; Haldimann and Feller, 2004; Salvucci and Crafts-Brandner, 2004b). Under severe high temperatures, the photosynthetic apparatus has been long considered to be the primary site of damage. It has been suggested that PSI is more resistant to heat than PSII (Pearcy, 1978; Sayed et al., 1989; Havaux, 1993; Venkataramanaiah et al., 2003; Hu et al., 2004) because an increase in the PSI cyclic electron flow occurs upon heat treatment (Havaux et al., 1991; Boucher and Carpentier, 1993; Sayed et al., 1994; Pastenes and Horton, 1996). PSII, however, has been shown to be a sensitive component to different stresses, and it is especially sensitive to heat (Berry and Björkman, 1980; Mamedov et al., 1993, Havaux, 1993). Indeed, increased temperatures can impair the PSII oxygen-evolving complex (Nash et al., 1985; Enami et al., 1994; Yamane et al., 1998; Büchel et al., 1999) and cause alterations to the thylakoid membrane fluidity (Raison et al., 1982; Havaux, 1993), which destabilise protein-lipid interactions and, therefore, PSII organisation and function (Vijayan et al., 1998).

Together, drought and high temperature are responsible for worldwide crop yield reductions, particularly in beans (Johansen et al., 1992; Slinkard et al., 1992; Monti et al., 1992), and both factors directly and/or indirectly affect the photosynthetic process. Therefore, it is likely that both environmental constraints, which often occur simultaneously, trigger common pathways for defence and resistance in plants. In fact, the proteomic analysis of leaf samples that were separately exposed to drought and heat resulted in the overexpression of 11 proteins; these proteins were primarily related to photosynthesis and the scavenging of reactive oxygen species (He et al., 2008). Drought and chilling, for example, have been argued to share redox signals in the plant responses to both stresses; this allows plants that have been previously exposed to one environmental constraint to adapt or acclimate to the other (Pastori and Foyer, 2002). Therefore, specific environmental cues may induce plant responses that lead to resistance to a range of different stresses. The study of cross-resistance is a potential tool for the identification of molecular, biochemical and physiological bases for the resistance to specific environmental constraints. This study is useful in plant breeding, and it is particularly relevant in a global climate-change scenario.

The aim of the present study was to assess the possible induction of resistance to high temperature by water stress in two bean varieties; OI is a stress-resistant and AT is a stress-sensitive variety (Silva et al., 1999; Pastenes et al., 2000; Lizana et al., 2006; Wentworth et al., 2006; Martinez et al., 2007). The pigment composition, heat-shock-protein content, thylakoid lipid and fatty acid composition and chloroplast ultrastructure of leaves from water-stressed and well-watered plants have been characterised to reveal physiological traits that confer resistance to high temperature.

2. Materials and methods

2.1. Plant material and growth conditions

The bean plants were grown in 3.5 L pots in a growth chamber with 500 μmol photons m^{-2} s^{-1} light from 400 W lamps under a 12 h photoperiod, 40–60% relative humidity and a thermal regime of 28/18 °C day/night. Until the plants displayed the first fully expanded trifoliate leaf, they were watered and fertilised on a regular basis according to water demand with a Hoagland II solution. After that time, the water-stressed plants were irrigated with 1/6 of the water volume of the control plants (well watered) for approximately 14 days.

2.2. Plant water status

The leaf water potential was assessed using a portable Schölander-type pressure chamber (Schöllander et al., 1965). The middle foliate of the first leaf, which was randomly taken from four to five plants from each water treatment, was measured at midday. The leaf lamina was enclosed inside the chamber, and the pressure was increased with a compressed nitrogen cylinder until free sap was visible at the petiole outside of the chamber. The relative leaf water content (RWC) was assessed as previously described by Smirnoff (1993) using the equation $RWC = 100 \times (FW - DW)/(TW - DW)$, where FW is fresh weight, TW is turgid weight after re-hydrating the leaves for 24 h at 4 °C, and DW corresponds to the weight of oven-dried leaves for 24 h at 80 °C.

2.3. CO_2 assimilation

The CO_2 assimilation rate and stomatal conductance were determined using an IRGA (ADC-Pro, UK). The measurements were taken at 25 °C and recorded with a thermocouple beneath the leaf at 500 μmol photons m^{-2} s^{-1} light and 370 μL L^{-1} CO_2. The measurements were recorded after an equilibration time of 60–90 s, as soon as the steady-state assimilation was reached for every measurement. Ten replicates were measured per water regime 14 days after the beginning of the differential water regimes.

2.4. Oxygen-evolution

The O_2 evolution rate was determined at different temperatures (15 °C, 20 °C, 25 °C, 30 °C, 35 °C and 38 °C) using a modified O_2 electrode (S-101 Qubit Systems, Inc., Canada). The attached leaves were placed into a closed chamber over a capillary-matting surface that was wetted with CO_2 buffer, pH 9 ($NaHCO_3$–Na_2CO_3) to maintain a high concentration of CO_2 as previously described by Walker (1990). The chamber temperature was maintained with a circulating water bath. A thermocouple was placed beneath the leaf and inside the chamber. Twelve replicates were measured for each treatment 14 days after the beginning of the differential water regimes.

2.5. Threshold temperature for non-reversible damage

The attached first trifoliate leaves were enclosed in a temperature-controlled dark chamber over a surface that was wetted with CO_2 buffer, pH 9 ($NaHCO_3$–Na_2CO_3). The temperature was increased at a rate of 1 °C/2 min from 20 to 52 °C by means of a circulating water bath, and the temperature was recorded with a thermocouple that was placed beneath the leaf as described before (Pastenes and Horton, 1999). F_0, which is the minimal fluorescence, was measured with a Hansatech (FMS 2, UK) fluorometer according to Seeman et al. (1984). The thermal limit of irreversible damage to the photosynthetic apparatus was assessed by extrapolating the linear portion of the fluorescence-temperature course above and below the threshold at which fluorescence raises to a point of intersection in the temperature axis. In addition, a similar experiment was performed, but it imposed 3 s far-red light pulses along the temperature-dependent F_0 course. The resulting value was plotted as $F_{0'}$.

2.6. Chlorophyll fluorescence

F_v/F_m, Φ_{PSII}, $F_{v'}/F_{m'}$ and non-photochemical quenching (NPQ) of chlorophyll fluorescence were determined according to van Kooten

Table 1
Water potential, relative water content (RWC), stomatal conductance (gs) and CO_2 assimilation rate (A) in the leaves from OI and AT water-stressed and control plants. Each value represents the mean ± SE of six replicates.

Variety	Arroz Tuscola		Orfeo INIA	
Treatment	Control	Water stress	Control	Water stress
Water potential (MPa)	−0.347 ± 0.067	−1.195 ± 0.041*	−0.353 ± 0.109	−1.192 ± 0.033*
RWC (%)	95.00 ± 2.10	76.99 ± 0.62*	95.12 ± 1.28	76.93 ± 0.79*
gs (mol H_2O m^{-2} s^{-1})	0.190 ± 0.041	0.018 ± 3.07e−3*	0.112 ± 0.035	0.016 ± 4.15e−3*
A (μmol CO_2 m^{-2} s^{-1})	11.52 ± 1.35	2.52 ± 0.27*	9.04 ± 1.07	1.98 ± 067*

* Significant differences between the water regimes for each variety ($P \leq 0.05$).

and Snel (1990) in the bean leaves. The leaves were dark adapted using leaf clips at midday with six replicates per treatment. The initial (F_0) and maximal (F_m) fluorescence levels were measured on the dark-adapted leaf segments using a modulated fluorometer (Hansatech FMS1, UK) and were used to calculate F_v/F_m. Next, the leaves were illuminated with 500 μmol photons m^{-2} s^{-1} actinic light at 25 °C and were recorded with a thermocouple that was placed beneath the leaf. $F_{m'}$ was assessed by means of a 1 s saturation pulse of 3500 μmol m^{-2} s^{-1}. To estimate $F_{0'}$, the actinic irradiance was turned off for 3 s, and the leaf was illuminated with far-red light. The fluorescence parameter that describes the quantum yield of PSII electron transport (Φ_{PSII}) was calculated according to Genty et al. (1989) as follows: $F_{v'}/F_{m'} \times qP$.

2.7. Carotenoids and chlorophyll content

Carotenoids were assessed according to Färber et al. (1997). Leaf discs were taken from leaves 14 days after the beginning of the differential water regimes; the discs were fast frozen in liquid N_2 and extracted by grinding into 100% acetone. The pigment extract was placed on ice and left in the dark for 30 min before it was centrifuged at 15,000 × g for 5 min to remove any cell debris. The samples were filtered and then loaded and run on a Waters HPLC system. The pigments were separated using a C18 LiChroCART® 250-4 (Merck) column, and the peaks were detected at 450 nm using a Waters spectrophotometer. The carotenoids were normalised to the chlorophyll content, which was determined according to Lichtenthaler and Wellbur (1983). The pigments were extracted with 80% ethanol at −20 °C and centrifuged at 5000 × g for 5 min at 4 °C; then, the supernatant was read at 665 and 649 nm in a spectrophotometer (UV-1601, Shimadzu, Japan).

2.8. SDS–PAGE

The leaves were exposed to 40 °C for 4 h before the intact chloroplasts were isolated on Percoll gradients as described by Leegood and Walker (1993). The chloroplasts were homogenised in a 100 mM Tris buffer, pH 8 that contained 10 mM EDTA, 1 mM PMSF, 10 mM ascorbate, 10 mM DTT, 1 mM aminocaproic acid, 1 mM benzamidine, 1% SDS, 10% glycerol, 2% PVP and 0.05% bromophenol blue. The homogenates were centrifuged at 10,000 × g for 15 min at 4 °C. The chloroplast protein samples (30 μg/lane) were separated on 15% polyacrylamide SDS gels (Laemmli, 1970) and blotted onto nitrocellulose (0.45 μM, Amersham Life Science, USA). The blots were probed for 1 h with a rabbit polyclonal antibody that was prepared against HSP70 and HSP24, which were kindly supplied by Dr. Kenneth Keegstra and Dr. Scott A. Heckathorn, respectively, and commercial anti-Rubisco (Agrisera, UK) and anti-HSP60 (Sigma, USA) antibodies. The blots were then were developed by immunodetection using alkaline phosphatase-conjugated secondary antibodies (Sigma, USA).

2.9. Lipid determination

The lipids were extracted from the isolated chloroplasts by the method of Bligh and Dyer (1959), and the lipid classes were separated on thin-layer chromatography (Christie and Dobson, 1999). Each lipid fraction was identified by staining with a 0.01% primuline solution in acetone-water (60:40, v:v) and was visualised under UV light. The separated lipids were recovered by scraping the corresponding spot. For the fatty acid determinations, whole samples or separated molecular species were transmethylated, and the fatty acid methylesters were analysed by gas liquid chromatography (Hewlett Packard, 5890 Serie II, Detector – TCD, Integrator 3396, Germany).

2.10. Electronic microscopy

Two to five millimetre-wide leaf segments were cut in water with a new razor blade from a freshly excised leaf and fixed in 3% glutaraldehyde and cacodylate buffer, pH 7.2 for a minimum of 6 h at room temperature according to Epstein and Holt (1963). These segments were washed in two changes of cacodylate buffer for 1 h and then dehydrated through graded alcohol solutions (50%, 70%, 90% and 100%) for a minimum of 1 h per solution. The samples were immersed in epoxide resin (Embed 812, EM Sciences, Fort Washington, PA) and left overnight at 60 °C for polymerisation. The unpolymerised resin was removed from the block by rinsing briefly in 70% alcohol and air drying. Sections of 80 nm thickness were cut using a Sorvall MT2-B microtome and a diamond knife; they were collected over water and stained in 4% uranyl acetate (Epstein and Holt, 1963) and subsequently in 0.1% lead citrate. The mounted sections were used for chloroplast ultrastructure observations using a Philips Model Tecnai 12 transmission electron microscope. The images were revealed on 6.5 × 9.0 Kodak SO163 film.

3. Results

Two weeks of water stress in fully expanded first trifoliate leaf plants resulted in a water potential value of −1.2 MPa and 77% RWC values, which were significantly lower than the control plants of both bean varieties, which displayed −0.35 MPa and 95% RWC (Table 1). This moderate water-stress condition strongly inhibits stomatal conductance and reduced the CO_2 assimilation rate to 22% of the control values in OI and AT (Table 1).

Under non-photorespiratory conditions, bean leaves increased their oxygen-evolution rate; the oxygen evolution increased from 3 μmol O_2 m^{-2} s^{-1} at 15 °C to 14 μmol O_2 m^{-2} s^{-1} at 35 °C in the AT control plants and from 1 μmol O_2 m^{-2} s^{-1} to 11 μmol O_2 m^{-2} s^{-1} at 15 °C and 35 °C, respectively, in the water-stressed plants of the same variety (Fig. 1). As the temperature increases from 35 to 38 °C, oxygen evolution in the leaves from the control plants decreases to values that are lower than those that were observed in the leaves of the stressed plants (Fig. 1). For OI, temperature increases from 15 °C to 35 °C enhance the oxygen-evolution

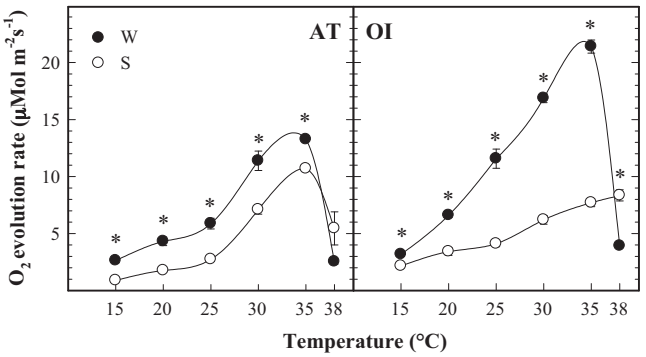

Fig. 1. O_2-evolution rate vs. leaf temperature in well watered (W, closed circles) and water stressed (S, open circles) bean leaves from AT (left panel) and OI (right panel). Bars represent SE, * represent significant differences ($P \leq 0.05$) between treatments for each variety and leaf temperature.

rate, from 3 μmol O_2 m^{-2} s^{-1} to 22 μmol O_2 m^{-2} s^{-1}, in the control plants (Fig. 1). A moderate increase in oxygen evolution is observed in the leaves of the stressed OI plants; the level reached up to 8 μmol O_2 m^{-2} s^{-1} at 35 °C, and this value was maintained as the temperature increased up to 38 °C. Conversely, the leaves from the control OI plants displayed a reduced oxygen-evolution rate of 4 μmol O_2 m^{-2} s^{-1} as the temperature increases from 35 °C to 38 °C; this value is well below the value that was observed in the leaves of water-stressed plants (Fig. 1).

According to Seeman et al. (1984), the thermal limit of irreversible damage to the photosynthetic apparatus can be assessed in dark-adapted leaves by extrapolating the linear portion of the fluorescence-temperature course above and below the threshold at which fluorescence raises to a point of intersection in the temperature axis (Fig. 2). Such limits are set to approximately 45.5 °C and 46.5 °C in the leaves from the control and stressed AT plants, respectively (Fig. 2). In OI, such limits were set to approximately 44.5 °C in the control and 47 °C in the leaves of water-stressed plants; the latter was significantly higher than the former (Fig. 2).

The temperature-dependent rise in F_0 values and $F_{0'}$ for the two bean varieties and water regimes, which were recorded after a 2 s far-red light pulse, are shown in Fig. 3. In both bean varieties, F_0 and $F_{0'}$ display similar kinetics upon temperature increase in the leaves of water-stressed and control plants (Fig. 3), which suggests that the increase in F_0 is related to the stability of PSII rather than shifts in the redox state of the PSII electron acceptor side.

The average recovery of F_v/F_m after 30 min of dark adaptation, which was measured at midday and on the same foliate for two weeks, which started at the beginning of the water treatments, was lower in the AT leaves of stressed plants than in leaves of the control plants, and the differences were significant on day 12 (Fig. 4). For OI, no differences in this parameter were observed between the leaves of control and stressed plants. A similar trend is observed in the parameters $F_{v'}/F_{m'}$ and Φ_{PSII}, which were measured under the same light intensity in the growth chamber. The leaves of water-stressed AT plants displayed lower values compared to the leaves of control plants, and the leaves of water-stressed plants also reached significantly higher values for NPQ. This is in contrast to the OI plants, in which no significant differences between the treatments are observed (Fig. 4).

Photoprotective pigments content, such as neoxanthin, xanthophylls and lutein, were assessed after two weeks of water treatment in the first foliate of the bean plants. As observed in Fig. 8A, the leaves of water-stressed plants contained significantly lower pigment concentrations per chlorophyll content when compared to the leaves of control plants. Conversely, in the OI plants, the pigment composition of the leaves of control and stressed plants are similar. As expected from the NPQ values in both bean varieties (Fig. 4), the de-epoxidation state of the leaves of stressed plants was significantly higher than the leaves of control plants (Fig. 5B).

To determine whether the water-stress-induced photosynthetic heat resistance is related to heat-shock proteins, the content of HSP70, HSP24 and HSP60 were assessed in isolated chloroplasts that were obtained from leaves of the water-stressed and control plants of plants of both varieties that were previously maintained at 25 °C or heated at 40 °C for 4 h (Fig. 6). HSP60, which is a Rubisco chaperone protein, was induced only in the heated leaves of AT plants irrespective of the water treatment (Fig. 6D); however, the Rubisco content was significantly reduced in leaves from water stressed plants heated to 40 °C when compared to the leaves of watered plants (Fig. 6A). Conversely, no differences in Rubisco were observed between the leaves of water-stressed and control AT plants at 25 °C, and no differences were observed between the leaves of water-stressed and control OI plants in either temperature condition (Fig. 6A). The ubiquitous HSP70 protein was observed in both bean varieties from 25 °C- and 40 °C-treated leaves, and there were no significant differences between the water regimes (Fig. 6B). Although HSP24 was only observed in the leaves of both varieties that were previously heated at 40 °C (Fig. 6D), its abundance was lower in the water-stressed leaves than in the control leaves in AT and OI (Fig. 6C).

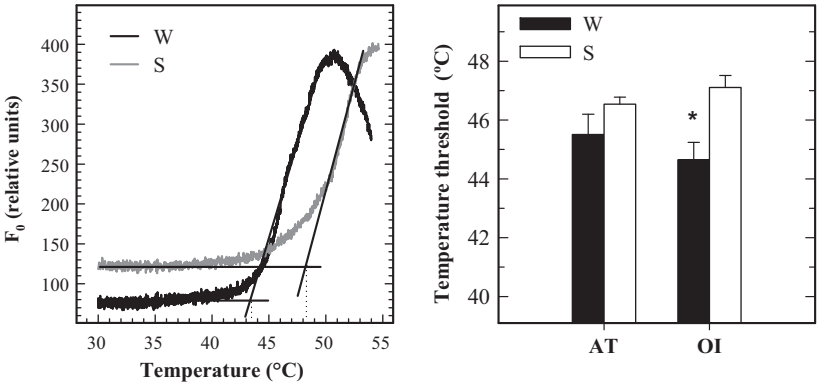

Fig. 2. Temperature threshold for non-reversible damage was assessed by means recording the changes in F_0 (relative units) upon increases in temperature at a rate of 0.5 °C min^{-1}. (A) F_0 changes upon temperature increase in well watered (W) and water stressed (S) OI plants. Dotted vertical lines represent the threshold of non-reversible damage by heat. (B) Temperature threshold for non-reversible damage in AT and OI, for W and S plants. Bars represent SE, * represent significant differences ($P \leq 0.05$) between treatments for each variety.

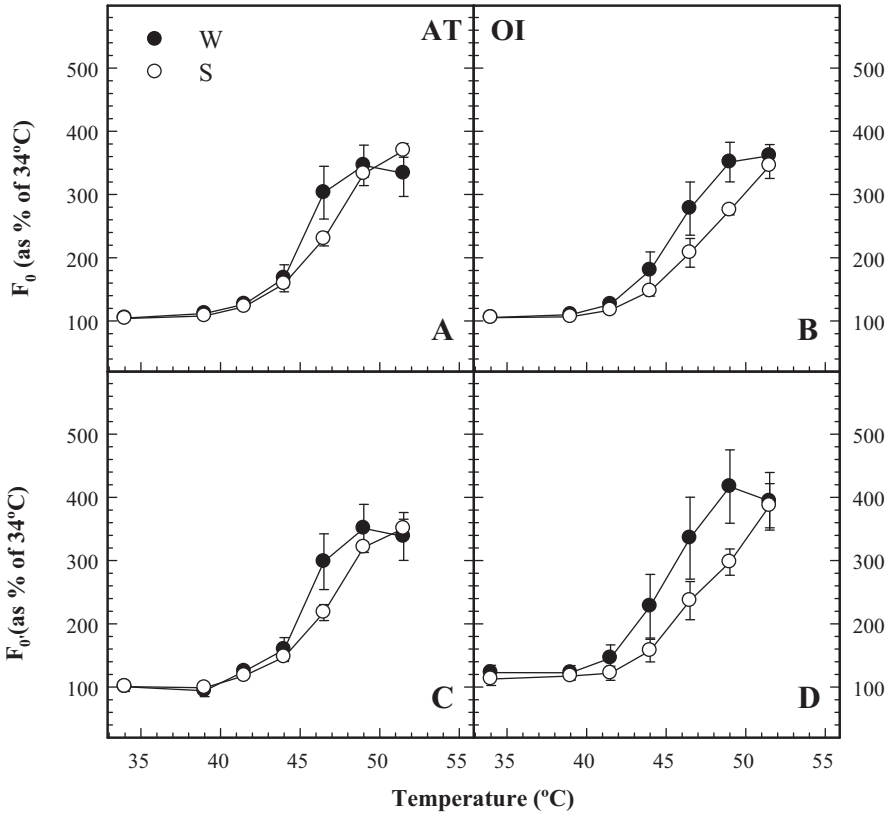

Fig. 3. F_0 (A and B) and $F_{0'}$ (C and D) vs. leaf temperature in control (W, closed circles) and water stressed (S, open circles) plants of AT (left panels) and OI (right panels). F_0 was assessed as in Fig. 2A. $F_{0'}$ was assessed by means of regular pulses of far-red light for 3 s on the temperature dependent changes of F_0. Bars represent SE, and * represent significant differences ($P \leq 0.05$) between treatments for each variety.

The most abundant lipid within the chloroplast is monogalactosyldiacylglycerol (MGDG), and its content reached 40–50% of total lipid content in AT and OI (Fig. 7). In AT only, water stress induces a nearly 10% increase in the MGDG content. Digalactosyldiacylglycerol (DGDG), which is the second most abundant lipid in chloroplasts, reaches from approximately 25% to 31% in both varieties, and the proportion does not change in the water-stressed plants when compared to the well-watered plants (Fig. 7). Phosphatidylglycerol (PG) accounted for a minor proportion of the anionic lipid in AT, and there were no differences between the stressed and control plants; this is contrary to the result that was observed in the OI plants, in which a 60% increase in PG was observed in the water-stressed plants compared to the control plants (Fig. 7). Sulfoquinovosil-diacylglycerol (SQDG) reached 20% of the total lipid content in AT and 14% in OI, and its composition did not significantly change after the AT and OI plants were water stressed (Fig. 7).

The fatty acid composition of the lipid species, which is shown in Fig. 8, is as follows: 16:0, palmitic acid; 16:3, 7,10,13-hexadecatrienoic acid; 18:0, stearic acid; 18:1, oleic acid; 18:2, linoleic acid; 18:3, linolenic acid. The chain length was modified during water stress in both plant varieties (Fig. 8). 7,10,13-Hexadecatrienoic acid, which is the most abundant fatty acid in all of the lipid species of bean chloroplasts, was reduced in content to nearly the half in the AT MGDG lipid content upon water stress (Fig. 8A). In addition, for the same lipid and variety, linolenic acid was increased by water stress nearly three-fold, but no significant differences in the fatty acid composition of DGDG, PG and SQDG were observed in the AT chloroplasts (Fig. 8A). Conversely, in the OI bean chloroplasts, differences were observed in the chain length of the fatty acids and in the saturation degree (Fig. 8B). MGDG lipids are composed of 16:3, 18:1, 18:2 and 18:3 fatty acids. No significant differences in their proportion were observed between the control and water-stressed OI chloroplasts; however, stearic acid was detected only in the stressed OI plants (Fig. 8B). Similarly, for the DGDG lipids, no significant fatty acid composition differences were observed between the water-stressed and control plants; however, palmitic acid was exclusively present in the stressed plants and was the most abundant fatty acid for this restrictive growth condition (Fig. 8B). For the PG anionic lipid, major differences were observed between the OI plants; upon water stress, there was a reduction in 16:3 and an increase in the proportion of 18:0 and 18:3 fatty acids (Fig. 8B). For the SQDG lipids, no significant differences were observed between the control and stressed plants; however, the 16:3 fatty acids are reduced by water stress in the OI chloroplasts (Fig. 8B).

Because water stress was applied to mature leaves, no major differences were observed in leaf morphology between the control and water-stressed plants of both bean varieties (data not shown). At the chloroplast level, the starch content was reduced in the leaves of water-stressed plants when compared to the control plants; this is shown in the transmission electron microscopy pictures (Fig. 9). This result is consistent with the lower CO_2 assimilation rates that were observed under water stress conditions in both varieties (Fig. 1). In the chloroplasts of water-stressed plants, some small, round electron-passing dense bodies, whose morphology suggests that they are plastoglobules, were observed. These putative plastoglobules are darker and more frequent in the OI variety than in the AT variety, and they average 6.3 and 3.7 per chloroplast, respectively (Fig. 9C–F). In a close-up observation of the grana stacking in the water-stressed AT samples, the thylakoid was more disorganised than in the control

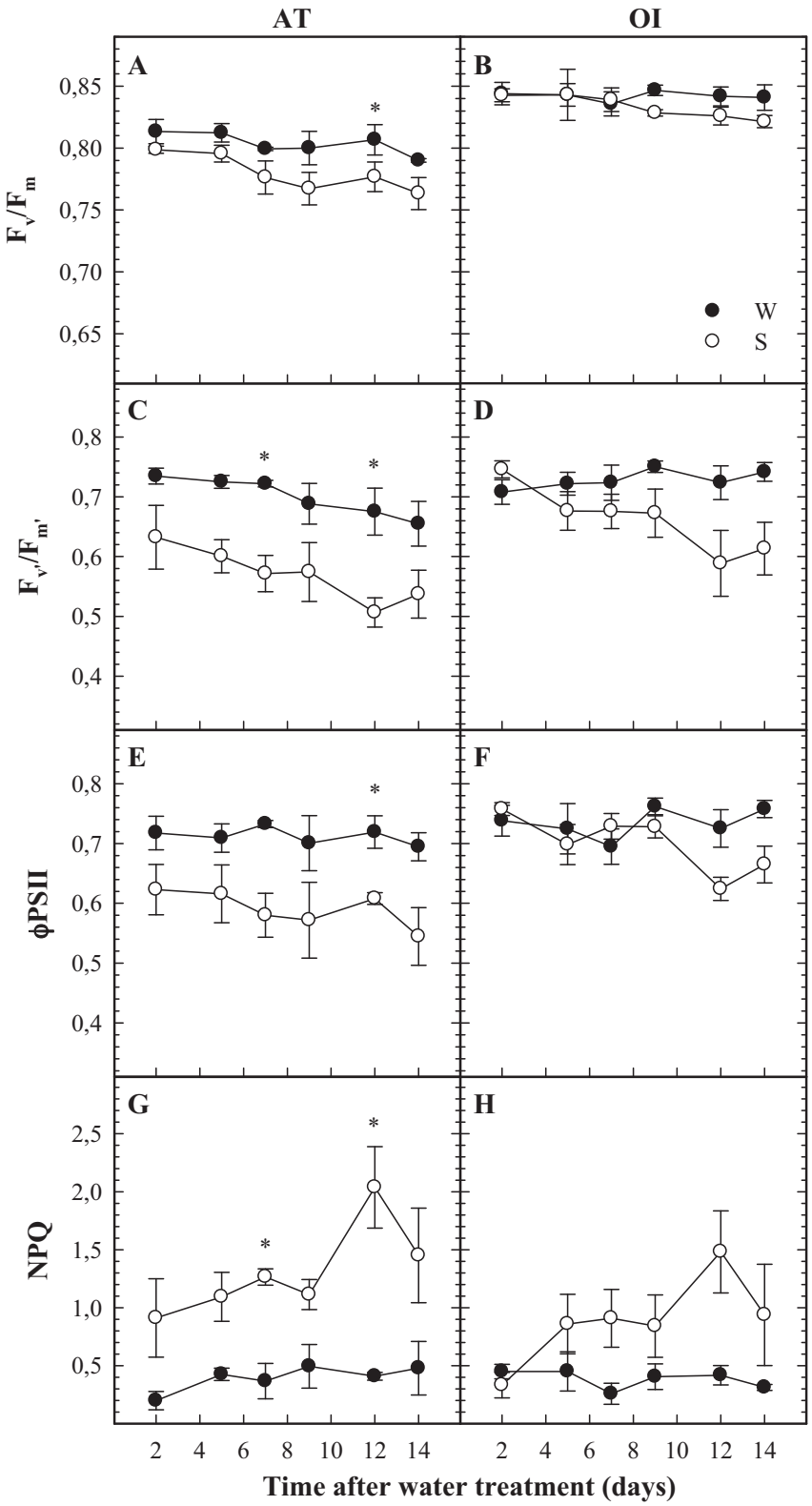

Fig. 4. F_v/F_m, $F_{v'}/F_{m'}$, Φ_{PSII}, and NPQ measured along the time of differential water regimes in AT (left panel) and OI (right panel) in control (closed symbols) and stressed (open symbols) plants. F_v/F_m was assessed after 30 min dark adaptation at midday. $F_{v'}/F_{m'}$, Φ_{PSII}, and NPQ were assessed upon 500 μmol m^{-2} s^{-1} actinic PAR light. Bars represent SE and * indicate significant differences ($P \leq 0.05$) between treatments for each day and bean variety.

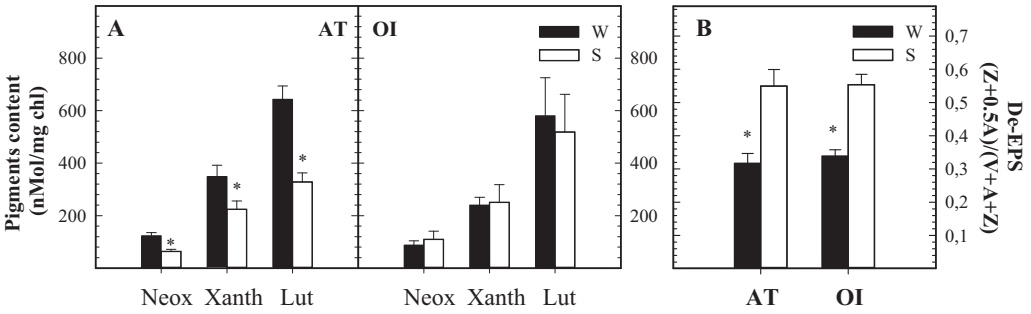

Fig. 5. (A) Xanthophylls content: neoxanthin (neox), xanthophyll cycle carotenoids (xanth) and luteine (lut) expressed on a chlorophyll bases in control (W) and stressed (S) AT (left panel) and OI (right panel). (B) De-epoxidation state of the xanthophyll cycle carotenoids calculated as $[(Z+0.5A)/VAZ]$ in OI and AT (symbols as in (A)). Leaves were taken from the growth chamber after 14 days of differential water regimes, at midday, and immediately frozen for analysis. Bars represent SE and * indicates significant differences between treatments ($P \leq 0.05$).

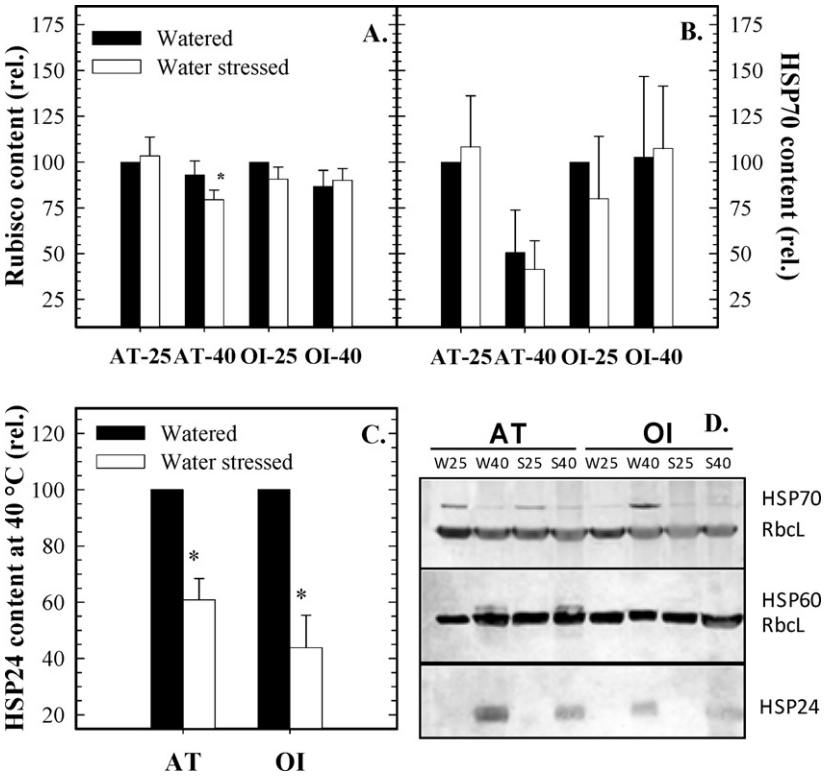

Fig. 6. Rubisco and HSP content in chloroplasts isolated from OI and AT bean leaves. Well watered and water stressed leaves were maintained either at 25 °C (control) or 40 °C for 4 h. Isolated chloroplasts were western blotted for Rubisco major subunit (A), HSP70 (B), HSP24 (C). (D) Representative blot with HSP60. Bars represent SE and * indicates significant differences between water regimes for each bean variety ($P \leq 0.05$).

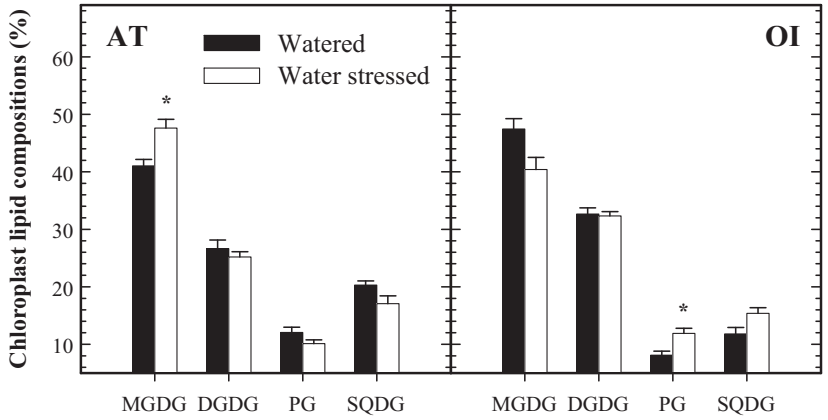

Fig. 7. Chloroplast lipid composition in AT (left panel) and OI (right panel) from watered (filled bars) and water stressed (open bars) plants, assessed by means of thin layer chromatography. MGDG, monogalactosyldiacylglycerol; DGDG, digalactosyldiacylglycerol; PG, phosphatidylglycerol; SQDG, sulfoquinovosyl diacylglycerol. Bars represent SE and * indicates significant differences between water regimes ($P \leq 0.05$).

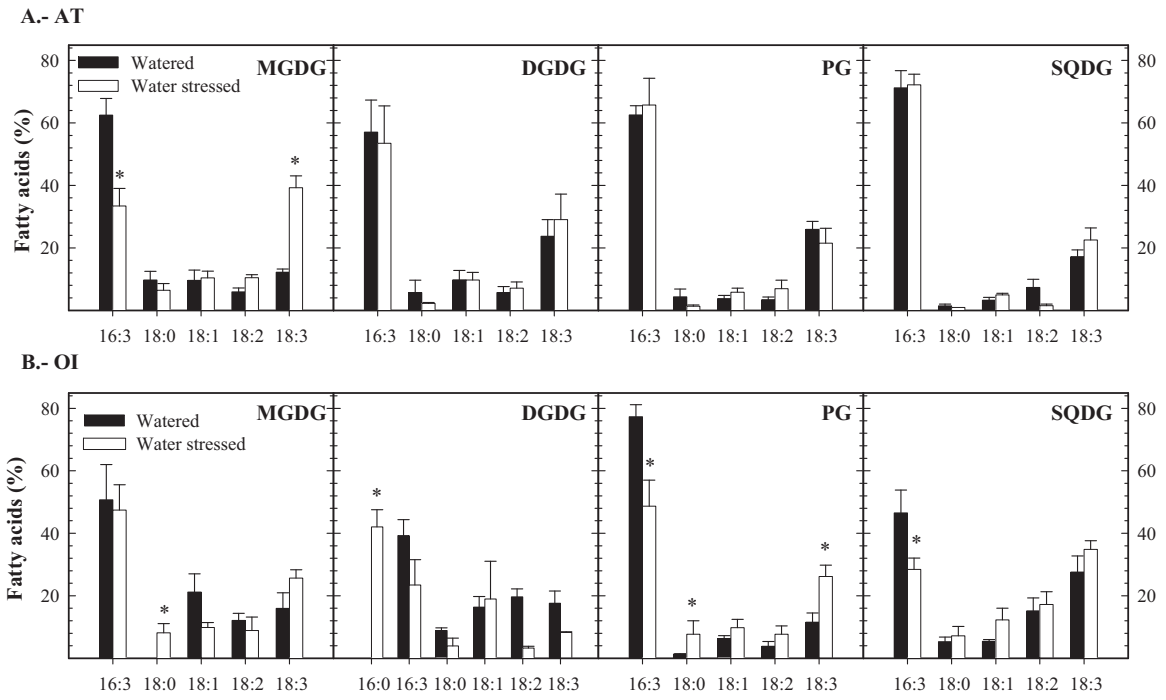

Fig. 8. Fatty acid composition of each lipid from isolated chloroplasts in Arroz Tuscola (A) and Orfeo INIA (B) plants grown in well watered and water stressed conditions. 16:0, palmitic acid; 16:3, 7,10,13-hexadecatrienoic acid; 18:0, estearic acid; 18:1, oleic acid; 18:2, linoleic acid and 18:3, linolenic acid. Bars represent SE and * indicates significant differences between water regimes ($P \leq 0.05$).

chloroplasts (Fig. 10A and B). Moreover, a higher proportion of empty spaces, which are easily bypassed by electron flow, were observed in these chloroplasts (Fig. 10A and B). Conversely, no differences were observed in the thylakoid density or organisation in the OI chloroplasts in both water conditions (Fig. 10C and D).

4. Discussion

Water stress is known to result in the absorption of light energy that exceeds the capacity for CO_2 reduction due to stomatal closure, which is shown in AT and OI (Table 1); this leads to oxidative stress and concomitant reductions in the quantum yield of

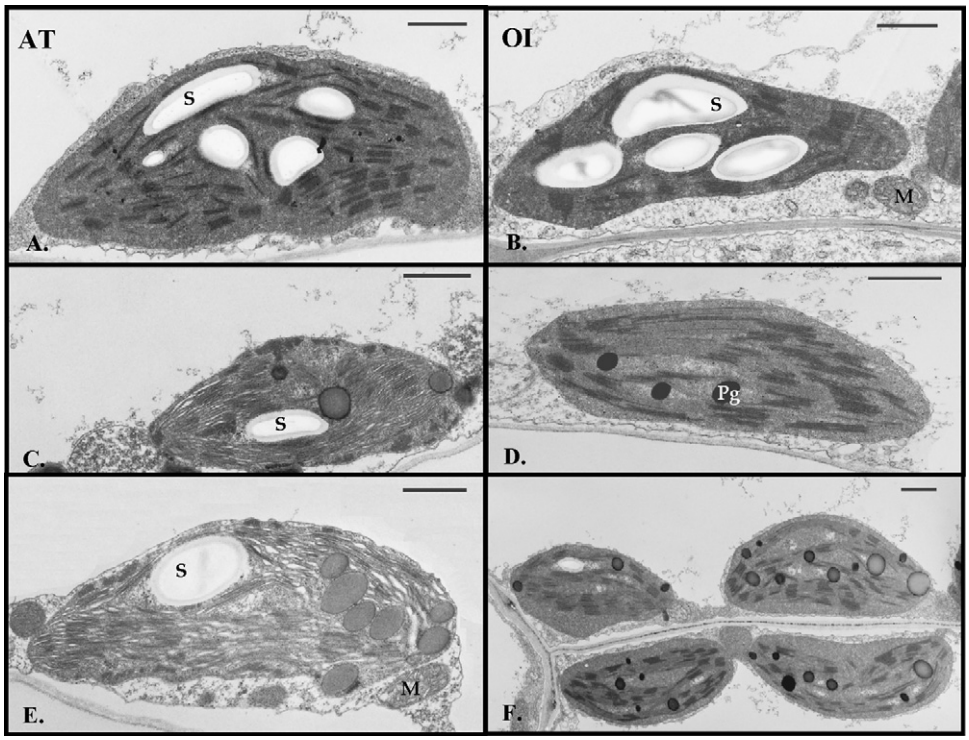

Fig. 9. Transmission electron microscopy of Arroz Tuscola (A, C, and E) and Orfeo INIA (B, D, and F) leaves of control (A and B) and water stress (C–F) plants. S, starch; M, mitochondria; Pg, plastoglobulin.

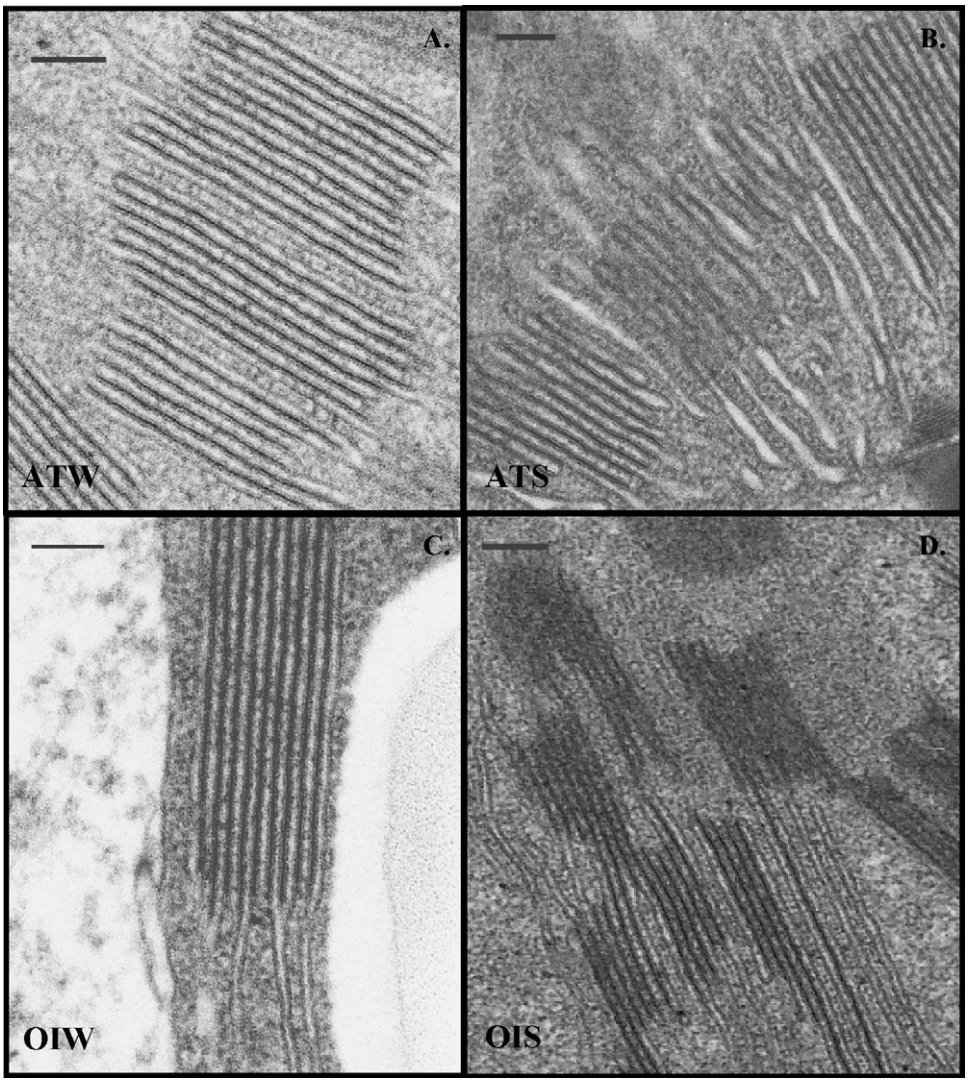

Fig. 10. Chloroplast ultrastructure analysed by transmission electron microscopy. (A) Arroz Tuscola control; (B) Arroz Tuscola water stress; (C) Orfeo INIA control; and (D) Orfeo INIA water stress.

photosynthesis (Navari-Izzo and Rascio, 1999). In the present study, the water stress only slightly affected the quantum efficiency, which was measured as F_v/F_m; this was particularly true for the AT plants (Fig. 7), and it suggested that oxidative damage does not occur. However, the photosynthesis under non-photorespiratory conditions in water-stressed plants at a temperature range from 15 °C to 35 °C was consistently lower than in the control plants, which suggests the occurrence of non-stomatal limitations (Fig. 1). ATP synthase has been proposed to be a site of water-stress-induced damage (Lawlor, 2002); however, this is proposed under more negative water potential values than those that were found in the stressed AT and OI plants (Table 1). Therefore, the previously reported effects of water stress on the Calvin cycle, such as Rubisco activation and/or RubP regeneration (Boyer and Younis, 1983; Tezara et al., 1999, Lawlor, 2002) may be responsible for the lower oxygen-evolution rate that was observed in the stressed plants in temperatures of up to 35 °C. It is noteworthy that although AT is known to be stress sensitive and OI is known to be stress resistant, the oxygen-evolution rates from 15 °C to 35 °C in the water-stressed AT plants are less reduced when compared to their control than those that are observed in OI (Fig. 1). It is unclear whether this result implies that the susceptibility of AT plants to stress is due to a lower capacity to respond to environmental constraints or that this variety is capable of higher photosynthetic rates upon water stress in the range of temperatures from 15 °C to 35 °C.

As the temperature rises from 35 °C to 38 °C, AT leaves reduce their capacity for oxygen evolution in non-photorespiratory conditions, and this phenomenon is irrespective of the water status of the plants (Fig. 1). However, in the OI water-stressed leaves, oxygen evolution is not reduced as the temperature rises from 35 °C to 38 °C; this result differs from the control plants, in which oxygen evolution drops to levels that are well below those of the leaves of stressed plants at 38 °C (Fig. 1). Subsequent temperature increases in the leaves of stressed OI plants results in strong reductions in the oxygen evolution (data not shown).

The analysis of increases of F_0 upon temperature increases (i.e., the fluorescence that is emitted by dark-adapted PSII centres when all of the reaction centres are open) has been widely used to estimate the threshold at which the heat effect on photosynthesis is non-reversible (Schreiber and Berry, 1977; Schreiber and Armond, 1978; Downton and Berry, 1982). When observing the thermal threshold for non-reversible damage, a strong detrimental effect of heat on the control was observed when compared to the water-stressed plants, and lower values were observed for the former

when compared to the latter (Fig. 2). The rise in F_0, results from the severe damage of the PSII structure and function, which suggests that the observed water-stress-induced resistance to extremely high temperatures in both bean varieties resides, at least partially, at the PSII level. PSII complexes have long been considered to be a heat-sensitive component of the photosynthetic apparatus. It has been reported that the oxygen-evolving complexes, which are located on the luminal side of PSII, are impeded by moderately high temperatures (Thomas et al., 1986; Enami et al., 1994), and this is accompanied by manganese release (Nash et al., 1985). Additionally, the temperature-induced increases in minimal fluorescence have been correlated to a blockage in the PSII reaction centres, the dissociation of the major antenna complexes (Armond et al., 1978; Gounaris et al., 1984; Sundby et al., 1986), and a shift in the redox-state equilibrium of PSII (Ducruet and Lemoine, 1985; Bukhov et al., 1990). According to Fig. 3, the $F_{0'}$ kinetics upon temperature changes in the leaves of water-stressed plants are the same as those of F_0 for each bean variety. This result suggests that any possible shift in the PII redox state cannot originate from a reduction in the PSII electron acceptor side.

To assess whether the water-stress-induced acclimation to high temperature resulted from the temperature increases that were caused by the observed stomatal closure in the water-stressed leaves (Table 1), the leaf temperature was recorded during the experimental period. No significant differences in leaf temperature between the water-stressed and watered plants were observed (data not shown). This result was very likely because of the stronger light-avoiding paraheliotropic movement of the leaves of the water-stressed bean plants (data not shown), which has been previously reported (Pastenes et al., 2005). Not surprisingly, the F_v/F_m parameter that was measured after 30 min of dark adaptation and has been correlated to photoinhibition that results from the absorption of light that exceeds the capacity for carbon reduction is very similar in the stressed and control plants (Fig. 4). Because the leaves from the stressed plants were exposed to actinic illumination, lower average values for the $F_{v'}/F_{m'}$ and Φ_{PSII} parameters were observed in OI and AT when compared to the control plants; on some days, there were significant differences for the AT plants (Fig. 4). Both the lower quantum yield efficiency and the quantum yield of PSII electron transport in stressed plants correlates with the higher proportion of non-photochemical energy dissipation that was observed during the two-week stressing period (Fig. 4). These results confirm the protective effect of paraheliotropism in stressed leaves (Pastenes et al., 2004, 2005).

Carotenoids have previously been correlated to the resistance of photosynthesis to environmental constraints. Specifically, xanthophylls act as modulators of non-photochemical energy dissipation. The protonation of the chloroplast lumen activates violaxanthin de-epoxidase, which catalyses the de-epoxidation of violaxanthin and antheraxanthin to zeaxanthin (Demming-Adams and Adams, 1992). It has been proposed that instead of zeaxanthin itself, the de-epoxidation state of the xanthophylls are important for the allosteric regulation of NPQ (Horton et al., 2005; Johnson et al., 2008; Pérez-Bueno et al., 2008). As expected, in both bean varieties, an increased de-epoxidation xanthophyll state (Fig. 5) and the concomitant higher NPQ values (Fig. 4) are observed in the water-stressed leaves compared to the control plants. Lutein has recently been proposed as a xanthophyll pigment that is involved in NPQ and acts as a direct quencher from chlorophyll *a* (Ruban et al., 2007), while zeaxanthin has been reported as an allosteric regulator of non-photochemical energy dissipation (Johnson et al., 2009) and as preventing the formation of reactive oxygen species. Furthermore, it has been suggested that during photo-oxidative stress, zeaxanthin is released from the V1 site of the antenna complexes and interacts with membrane lipids to prevent oxidative damage and decrease the thylakoid membrane fluidity (Tardy and Havaux, 1997; Havaux, 1998; Havaux and Niyogi, 1999; Baroli et al., 2003; Li et al., 2002; Dall'Osto et al., 2006). In AT, the xanthophyll cycle pigments, neoxanthin and lutein are significantly reduced upon water stress. This result is different from that observed for the OI plants, in which no significant differences in the leaf pigment concentrations were observed between the stressed and control plants. This may be a distinctive, important feature of leaves of OI-stressed plants, which leads to a higher heat resistance. Havaux (1998) suggested that zeaxanthin plays an important role in the thermotolerance of PSII because of the tight interactions between this xanthophyll and the lipid phase of the thylakoid membrane; these interactions enhance PSII thermostability and decrease lipid peroxidation under high temperatures. However, lipid peroxidation is likely to occur under light, and in our study, the threshold temperature for non-reversible damage to PSII by heat was assessed in nearly complete darkness. Therefore, the increased heat resistance of OI is related to the higher stability of its PSII complexes rather than its resistance to oxidative damage. In addition, polyphasic fluorescence transient studies have previously suggested that the endurance of the oxygen-evolving complexes, which are induced by mild and severe drought, is responsible for the acclimation to high temperatures in wheat (Lu and Zhang, 1999). A possible involvement of xanthophylls in the stabilisation of PSII complexes by buffering the heat effect on thylakoid membrane fluidity, which was previously reported (Havaux, 1998), may cause the higher resistance to heat after water-stress acclimation of the OI plants compared to the AT plants.

HSPs expression is well correlated with the acquisition of thermotolerance in a time- and temperature-dependent manner, and it has been hypothesised that they are an essential component of a process that prevents heat damage. In addition, some HSPs are selectively localised in cellular organelles upon heat exposure. Considerable attention has been paid to the roles of HSPs regarding the protection of the PSII complex against heat stress (Waters et al., 1996; Swan, 1997; Heckathorn et al., 1998, 2002; Török et al., 2001) and other abiotic stresses (Heckathorn et al., 2004; Barua and Heckathorn, 2006). HSP24 has been reported to be rapidly induced under high temperatures and transported into the chloroplast to protect the thermolabile PSII complexes (Heckathorn et al., 1998; Preczewski et al., 2000), and it has also been detected in the leaves of plants that are subjected to other abiotic stresses, such as high light intensities (Barua and Heckathorn, 2006) and heavy metal exposure (Heckathorn et al., 2004). However, its presence was only detected in the heat-treated leaves for both water conditions in the two varieties (Fig. 6C). Therefore, HSP24 is not induced at room temperature by water stress alone. A similar result was observed for HSP60; the protein was induced only in heated AT leaves irrespective of the water treatment (Fig. 6D).

HSP60 has been shown to act as a Rubisco chaperone (Goloubinoff et al., 1989), and it can also chaperone other relevant proteins in the mitochondria and chloroplasts (Mendoza et al., 1991; Zeilstra-Ryalls et al., 1991). In the present study, water stress at an ambient temperature was not found to induce the protein expression. In fact, the protein was detected only in the heated AT leaves, and it was not detected in OI. Although Rubisco is regarded as a heat-resistant protein (Eckardt and Portis, 1997), its levels were reduced in the heat-exposed AT leaves when compared to the control (Fig. 6A). In OI, which is the resistant bean variety, neither HSP60 nor Rubisco was induced under any conditions in our study (Fig. 6D). However, the Rubisco content is not reduced upon heat exposure (Fig. 6A). HSP70, however, is known to bind to denatured proteins and to avoid aggregation by forcing them to refold into their native conformations (Georgopoulos and Welch, 1993; Schöffl et al., 1998; Feder and Hofmann, 1999). Apparently, HSP70 is more strongly induced by heat when the plants have been acclimated to extremely low temperatures (Reyes et al., 2003), and it

is not necessarily involved in the protection of the photosynthetic apparatus (Preczewski et al., 2000). In our experiments, HSP70 was found in ambient and heat treated bean leaves, and no significant differences were observed between the control and water-stressed leaves at each temperature. Clearly, the HSPs that were assessed in the present study are not responsible for the water-stress-induced resistance to high temperature in OI.

Many authors have discussed the roles of the thylakoid membrane lipids in high-temperature (Raison et al., 1982; Gounaris et al., 1984; Gombos et al., 1991, 1994) and water-stress acclimation (Monteiro de Paula et al., 1993; Gigon et al., 2004; Torres-Franklin et al., 2007). To assess the effects of water stress on the most favourable thylakoid composition for high-temperature stress, the chloroplast lipid and fatty acid composition were analysed after two weeks of water treatments in both bean varieties.

The DGDG/MGDG ratio, which has been shown to be relevant to chloroplast membrane stability (Dörmann and Benning, 2002), was higher in the water-stressed OI leaves than the control, but this was not observed in AT (Fig. 7). Under water and thermal stresses, a higher DGDG/MGDG rate may be important for the maintenance of the membrane bi-layer conformation and the retention of the biological function of the thylakoid membrane, such as photosynthetic electron transport, proton translocation and photosystem activities (Bruce, 1998; Chen and Li, 1998; Dörmann and Benning, 2002). The content of the lipid species that were found upon water stress in the present study are likely the result of the water-stress effect on enzyme functioning, which involves lipid degradation and synthesis. In Arabidopsis, water stress induces DGDG synthase expression, and it inhibits MGDG synthase transcript accumulation; this leads to a decrease in the MGDG content (Gigon et al., 2004). In the present study, however, the resulting changes in the DGDG/MGDG ratio in the water-stressed leaves of both bean varieties were due to an increase in the MGDG content in AT and a reduction in OI, but no changes occurred in the DGDG content (Fig. 7).

Strong reductions in DGDG lead to abnormal phenotypes and altered fluorescence patterns (Kelly et al., 2003) because of the structural alterations of the PSII electron donor side (Steffen et al., 2005). DGDG plays an important role in the stability of extrinsic proteins in the oxygen-evolution complex (Sakurai et al., 2007b) and is involved in thermal tolerance in *Arabidopsis thaliana* (Chen et al., 2006). In this study, a constant proportion of DGDG in the chloroplasts of both bean varieties under water stress was observed (Fig. 7). Further, because AT was the most sensitive variety (at least at temperatures higher than 35 °C) this feature may be less important in conferring heat resistance to the photosynthetic stability when compared to the DGDG/MGDG ratio.

For the anionic lipids in the current study, the sensitive AT variety maintains the proportions of both PG and SQDG when the plants are exposed to water-stressing conditions, whereas in the resistant OI variety, the water-stressed leaves contained an increased proportion of PG when compared to the well-watered plants (Fig. 7). Although it is not possible to be certain that the changes in OI determine its increased resistance to high temperature at the PSII site (Fig. 2), it is well known that in addition to its role in PSII dimerisation (Sakurai et al., 2003), PG is required for both the PSII electron-acceptor side at the QB site and the donor side, where it binds extrinsic proteins in relation to the Mn cluster (Frentzen, 2004; Gombos et al., 2002; Sakurai et al., 2006, 2007a). Furthermore, PG is known to play a crucial role in the stabilisation of the LHCII oligomeric state (Nußberger et al., 1993; Hobe et al., 1995; Trémolières and Siegenthaler, 1998). In fact, the grana structural organisation and stacking rely on the interaction between the stable trimeric LHCII, RC and some membrane proteins (Páli et al., 2003; Standfuss et al., 2005). Therefore, the more than two-fold increase in PG in the stressed compared to the control leaves of OI (Fig. 7) may contribute to PSII stability under heat (Fig. 2), and it may also maintain a higher thylakoid organisation in water-stressed plants when compared to the stressed AT samples (Figs. 9 and 10).

In addition to the lipid composition, the fatty acid chain length and saturation affect the stability of thylakoids upon high temperatures. Many authors have suggested that fatty acid saturation in thylakoid membrane lipids enhances PSII thermal stability (Pearcy, 1978; Raison et al., 1982; Xu et al., 2003), although unsaturation is required for the damaged D1 repair cycle (Sippola et al., 1998; Zhang and Aro, 2002). Water stress induces an increase in fatty acid chain length in AT, which primarily occurs in MGDG lipids, maintains the saturation level of fatty acids through a reduction in 7,10,13-hexadecatrienoic acid, and induces an increase in the proportion of linolenic acid (Fig. 8A); the latter been described to be convenient for drought-stress tolerance (Gigon et al., 2004). Conversely, in OI, water stress induces changes in the fatty acid chain length and saturation and in the composition of all of the lipid species (Fig. 8B). Together, there is a clear increase in the saturation level of the thylakoid fatty acids in OI water-stressed plants compared to the control plants, and this feature is known to be convenient for situations in which oxidative stress is typically triggered, such as during water stress (Apel and Hirt, 2004). The higher temperature threshold for non-reversible damage (Fig. 2), which is assessed in darkness, is not related to oxidative stress, but it could be a convenient feature in light conditions, in which, at extremely high temperatures, stressed OI leaves are capable of maintaining oxygen-evolution rates (Fig. 1). In addition, plastoglobules are more prominent in water-stressed OI leaves compared to AT leaves. Plastoglobules are lipoprotein structures that are rich in α-tocopherol, plastoquinones, triacylglycerols, DGDG and MGDG (Greenwood et al., 1963; Kessler et al., 1999; Vidi et al., 2006) and are thought to be relevant for the removal of protein catabolites during thylakoid turnover (Smith et al., 2000).

In addition to contributing to the increased stability of the PSII complexes (Fig. 2), the water-stress-induced pigment, lipid and fatty acid composition in the stress-resistant OI variety may be involved in maintaining the chloroplast ultrastructure, which is shown in Figs. 9 and 10. It is important, however, that the resistance of the photosynthetic function and structural stability in plants does not depend solely on chloroplast and cell adaptations. More subtle mechanisms that involve whole-plant physiology may be important. For example, the transmission electron microscopy images from Fig. 10C exhibit the accumulation of prominent starch granules in the sensitive AT variety; rather than resulting from photosynthetic dysfunction during the short time, they may have been derived from a reduced demand for photoassimilates in the sink tissues or limitations in the assimilated export capacity from the leaves (Jiao and Grodzinski, 1996). These results could reduce the capacity for CO_2 reduction in light conditions as seen in Fig. 1.

5. Conclusions

From Figs. 1 and 2, it is clear that the previously reported stress-resistant OI variety (Lizana et al., 2006; Wentworth et al., 2006) is capable of a better photosynthetic performance in light at 38 °C when it is gradually exposed to water scarcity, and the non-reversible damage to PSII in these plants occurs at higher temperatures than in the leaves of well-watered plants. In AT bean leaves, which are known to be stress sensitive, no significant differences in oxygen evolution at 38 °C or at the threshold temperature for non-reversible damage are observed between the stressed and control leaves. The physiological significance of this resistance and particularly for the threshold for non-reversible damage to PSII is uncertain because bean leaves in the field seldom experience such heat. The higher resistance to heat in the water-stressed OI leaves correlates with the maintenance of grana stacking compared to

the water-stressed AT chloroplasts (Fig. 9). The capacity of OI to maintain the lutein and xanthophyll pigment contents in water-stressed leaves (Fig. 5), the ability to increase the concentration of PG (Fig. 7), and the ability to modify fatty acids by shortening carbon chains and reducing unsaturation levels in all of the thylakoid lipid species (Fig. 8) are likely important for conferring heat resistance. The results from the comparative study on the stress-resistant and stress-sensitive varieties may promote the search for physiological markers for the mechanism behind high-temperature resistance in photosynthesis.

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