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BRIEF COMMUNICATION

Water deficit and recovery response of *Medicago truncatula* plants expressing the ELIP-like DSP22

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Abstract

In this article, we present the response of *Medicago truncatula* Gaert. cv. Jemalong plants expressing constitutively the *Dsp22* gene from *Craterostigma plantagineum* to water stress and rehydration. The *Dsp22* gene encodes an ELIP-like protein thought to protect the chloroplast against photooxidative damage during the dehydration and rehydration. The *Dsp22* transgenic homozygous *M. truncatula* plants showed higher amount of chlorophyll (Chl), lower Chl *a*/Chl *b* ratio and higher actual efficiency of energy conversion in photosystem 2 (Φ_{PSII}) after rehydration when compared to the wild type. The combined data from the Chl *a* fluorescence analysis, pigment quantification, and biomass accumulation showed that transgenic *M. truncatula* plants are able to recover from water deprivation better than wild type plants.

Additional key words: abiotic stress, barrel medic, chlorophyll content, chlorophyll fluorescence, relative water content, transgenic plants.

Improvement of plant responses to water deficit (WD) remains an open field of research especially in the legume family in which efforts have not kept pace with those described for cereals (Jeuffroy and Ney 1997). *Medicago truncatula* Gaert., commonly known as barrel medic, emerged as a model plant to investigate the basis of nitrogen fixation and nodulation, plant development, abiotic stress responses, population genetics, and structural genomics (reviewed in Ané *et al*. 2008, Rose 2008). *M. truncatula* is amenable to genetic transformation and different strategies based on genetic engineering can be set up to design lines with improved WD tolerance.

It is well known that the stomatal limitation on photosynthesis imposed by the earlier stages of WD results in a decrease of primary electron acceptors available for photochemistry (Chaves 1991). If protection mechanisms are not activated, the excess of absorbed energy may induce photooxidative damage in chloroplast structures. To cope with the environmental stresses, plants activate a large set of genes which lead to the accumulation of specific stress-associated proteins (reviewed in Hussain *et al*. 2011). Early light inducible proteins (ELIPs) and ELIP-like proteins are described as pigment-binding components of thylakoid membranes that accumulate in response to various stresses (for

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Abbreviations: Chl - chlorophyll; DSP22 - desiccation stress protein (22 kDa); ELIPs - early light induced proteins; F_v/F_m - variable to maximum Chl fluorescence ratio (maximum quantum efficiency of photosystem 2); LA - leaf blade area; LHC 2 - light harvesting complexes of the photosystem 2; PAR - photosynthetically active radiation; PSII - photosystem II; qN - non-photochemical quenching; qP - photochemical quenching; RWC - relative water content; SWC - soil water content; WD - water deficit; WDR - water deficit recovery; WW - well watered; Φ_{PSII} - actual efficiency of energy conversion in photosystem II.
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review see Zeng *et al*. 2002). They are postulated to have a protective role against photo-oxidative damage of the chloroplast apparatus. The protective roles of ELIPs and ELIP-like proteins may be associated with transient binding of chlorophylls (Chl) and carotenoids (Adamska *et al.* 1999, Alamillo and Bartels 2001), the preservation of chlorophyll-protein complexes (Montané *et al*. 1997), or increase in the dissipation of the excess of absorbed radiation energy at the photosystem (PS) II reaction center (RC_{PSII}) *via* stimulation of non-photochemical quenching (qN; Montané and Kloppstech 2000).

We decided to express the *Dsp22* gene from *Craterostigma plantagineum* (Bartels *et al*. 1992) in *M. truncatula*, aiming to investigate the protective role of this ELIP-like protein in the photosynthetic apparatus, during the dehydration and rehydration.

We obtained several T_2 homozygous transgenic lines of *Medicago truncatula* Gaert. cv. Jemalong (Araújo *et al*. 2004) and we measured non-destructive chlorophyll fluorescence in *Dsp22* transgenic (A.27) and wild type (M9-10a) plants together with leaf pigment content and biomass accumulation during dehydration and subsequent recovery. The A.27 line has a single insertion of the *Dsp22* cDNA from *Craterostigma plantagineum* (GeneBank acc. No. X66598; Bartels *et al*. 1992) under control of a CaMV 35S promoter with duplicated enhancer sequences and CaMV 35S terminator. The A.27 line T_2 progeny was 100 % kanamycin resistant after self-crossing and therefore selected as homozygous and *Dsp22* transcript accumulation was demonstrated by RT-PCR (data not shown). Seeds were disinfected and scarified as described in Araújo *et al*. (2004) and germinated on Murashige and Skoog (1962) basal medium (MS) in the dark (2 d at 4 ºC, then 3 d at 23 - 24 ºC). After a 2-week culture in a growth chamber (*Phytotron EDPA 700*, *Aralab*, Rio de Mouro, Portugal) under 16-h photoperiod, irradiance of 100 μmol m^{-2} s^{-1} (cool white fluorescent tubes), day/night temperature of 24/22 ºC, and relative humidity of 50 %, seedlings were transferred to trays containing *Vermiculite* watered with Hoagland's solution (Hoagland and Arnon 1950) and partially covered with a plastic film (*SILVEX*, Benavente, Portugal) to acclimate during 2 weeks under the same controlled conditions.

Four week-old seedlings were transferred to 0.5 dm^3 plastic pots (1 plant per pot) containing 0.4 dm^3 of *Montemor* soil (*Montemor*, Lisboa, Portugal). Plantlets were grown in a growth chamber at 16-h photoperiod, irradiance of 580 μmol m^{-2} s^{-1} (PAR; white fluorescent lamps *TLD 30W/840, Philips*, Amsterdam, The Netherlands), day/night temperature of 24/27 ºC, and relative humidity of 40/52 %. Plants were watered twice a week with tap water. Eight week-old plants were randomly assigned to 3 treatments: well watered plants (WW; control) were watered during the experimental period of 15 days every two days with approximately 0.15 dm^3. Water deficit (WD) was gradually imposed by withdrawing water supply during the experimental period of 15 d. Water deficit recovery (WDR) plants were obtained by re-watering plants that were under a WD period of 12 d with 0.15 dm^3 of tap water during 3 d. For each water treatment, three plants per line were used and were considered as three biological replications. Plants were maintained under the above mentioned growth conditions irrespective of the water treatment applied for a period of 15 d at the end of which physiological data were collected. Pot positioning was changed every 2 d to avoid growth chamber local effects. The soil water content (SWC) was measured gravimetrically as described in Almeida *et al*. (2007). Plant water status was estimated using the relative water content (RWC) of leaf blades following the methodology described by Čatský (1960).

Chlorophyll *a* fluorescence parameters were measured using a portable modulated pulse fluorometer *PAM 210* (*Heinz Walz*, Effeltricht, Germany), coupled to a computer with *DA-Teach* software. For each plant, measurements were made on the youngest well expanded leaf to ensure comparisons between measurements. Fluorescence measurements were conducted in the laboratory under the same growth temperature. Measurement conditions were as described in Almeida *et al*. (2007). The variable fluorescence ($F_v = F_m - F_{0,}$ where F_m and F_0 were maximum and basal fluorescence) and maximal PSII photochemical efficiency defined as F_v/F_m (Kitajima and Butler 1975) were measured in 5 min dark-adapted leaves. The photochemical quenching (qP), the non-photochemical quenching (qN) and the actual quantum yield of PSII (Φ_{PSII}) were determined after 5 min adaptation to low (100 μmol m^{-2} s^{-1}), moderate (580 μmol m^{-2} s^{-1}), and high (1850 μmol m^{-2} s^{-1}) irradiances. All the parameters were automatically calculated by the software *DA-Teach* using the following formulae:

$\Phi_{PSII} = (F'_m - F_t)/F'_m$ (Genty *et al*. 1989);

$qP = (F'_m - F_t)/(F'_m - F'_0)$ and

$qN = (F_m - F'_m)/ (F_m - F'_0)$ (Schreiber 1997), where F'_m and F'_0 were maximum and basal fluorescence in light adapted leaves and F_t was steady state fluorescence.

Photosynthetic pigments were extracted with absolute methanol at 4 ºC in the dark until full extraction. Chl *a*, Chl *b* and carotenoids were determined spectrophotometrically (*Ultrospec 4000*, *Amersham Pharmacie Biotech*, Cambridge, UK) according to Lichtenthaler (1987). Leaf blade area (LA) was measured using the digital acquired images of each leaf using the software *Image J* (Abramoff 2004, http://rsb.info.nih.gov/ij/). At the end of the experiment, each plant was harvested, divided in leaflets, petioles, stems (main and axillaries) ,and roots and the biomass of each plant part was scored after drying out in an oven at 75 ºC for at least 3 d.

The data collected for each studied parameter was analysed by two-factorial *ANOVA*. Means were compared using a Tukey′s test and considered significantly different when $P \leq 0.05$. Statistical analysis was done using the

Statistica 6.0 software.

In the present study, we found similar RWC in either non transgenic (M9-10a) or *Dsp22* transgenic (A.27) plants submitted to the same experimental condition (Table 1). Control (WW), A.27 and M9-10a plants had RWC around 80 - 83 % and WD plants reached RWC values of 16 - 22 % after 15 d of water withdrawn. This deficit can be considered as severe water stress as previous experiments have shown that *M. truncatula* was resistant to mild drought conditions maintaining high leaf RWC when the SWC decreased to one-half of its maximum suggesting that the plants are able to avoid leaf dehydration (Nunes *et al*. 2008, 2009). Three days of recovery were sufficient for WDR plants to reach leaf RWC similar as observed in WW plants.

WW and WDR *M. truncatula* plants had mean values of maximum potential efficiency of the PSII (F_v/F_m) of 0.79 which is similar to the reference value (0.8) for a broad range of different plant species under optimal growth conditions (Björkman and Demmig 1987). A significant ($P \leq 0.05$) decrease was observed in WD plants (values 0.746 and 0.741) but no significant differences were found between A.27 and M9-10a. In WW and WD plants, no significant differences in the actual effectivity of PSII photochemistry (Φ_{PSII}) were found between the two lines irrespective to the irradiance. Regardless to the treatment applied, *M. truncatula* plants expressing *Dsp22* do not present qP and qN values different from those observed in M9-10a plants (results not shown). The results suggest that DSP22 protein might not be involved in regulation of photochemical or non-photochemical quenching as proposed by Montané and Kloppstech (2000).

Significantly higher Φ_{PSII} values were found in WDR transgenic plants when compared to M9-10a plants namely under medium or high irradiances (Table 1). These results suggest that A.27 had an improved efficiency at which radiation absorbed by the PSII antennae was used by photochemistry (Genty *et al.* 1989).

Table 1. Water relations, maximum photochemical efficiency (F_v/F_m), actual photochemical efficiency (Φ_{PSII} measured at irradiance 100, 590, or 1850 μmol m^{-2} s^{-1}), chlorophyll and total carotenoid content and ratios, and biomass accumulation and distribution measured in non transgenic (M9-10a) and *Dsp22* transgenic *M. truncatula* plants (A.27) submitted to well watered conditions (WW), water deficit (WD), and recovery after water deficit (WDR). Data are means ± standard deviations (n = 3). For each parameter different letters indicate significant differences at $P < 0.05$ between water treatments. *Asterisk* indicates significant difference at $P \leq 0.05$ between A.27 and M9-10a.

Parameters	WW		WD		WDR	
	M9-10a	A.27	M9-10a	A.27	M9-10a	A.27
SWC [%]	44.50±3.88a	45.22±3.31a	14.80±3.24b	13.48±0.23b	46.04±3.75a	45.21±4.34a
RWC [%]	82.21±4.11a	83.39±4.34a	22.65±5.42b	16.00±3.85b	79.63±4.07a	79.55±2.85a
F_v/F_m	0.788±0.007a	0.785±0.011a	0.746±0.031b	0.741±0.029b	0.784±0.001a	0.789±0.010a
$\Phi_{PSII\ (100)}$	0.670±0.005a	0.673±0.005a	0.533±0.101a	0.512±0.099b	0.663±0.013a	0.667±0.010a
$\Phi_{PSII\ (590)}$	0.420±0.046a	0.445±0.028a	0.232±0.088b	0.237±0.066b	0.425±0.008a	0.451±0.012a*
$\Phi_{PSII\ (1850)}$	0.092±0.024a	0.104±0.018a	0.053±0.019a	0.050±0.015a	0.078±0.001a	0.116±0.005a*
Chl *a* [mg cm^{-2}]	0.027±0.003a	0.027±0.003a	0.012±0.006b	0.016±0.004b	0.023±0.003a	0.027±0.002a*
Chl *b* [mg cm^{-2}]	0.009±0.001a	0.009±0.001a	0.006±0.002b	0.009±0.001a*	0.007±0.001a	0.009±0.000a*
Car+Xan [mg cm^{-2}]	0.005±0.001a	0.005±0.001a	0.002±0.001b	0.001±0.001b*	0.004±0.000a	0.004±0.000a
Chl *a*/Chl *b*	2.920±0.140a	2.920±0.070a	2.230±0.200b	2.030±0.140b	3.010±0.130a	2.860±0.080a*
Chl *a*+*b*/(Car+Xan)	7.250±0.830a	7.160±0.340a	7.270±3.310	28.92±8.440b*	7.290±0.810a	8.210±0.820a*
Leaf biomass [g $plant^{-1}$]	0.261±0.066a	0.278±0.018a	0.090±0.002b	0.126±0.025b	0.173±0.045ab	0.207±0.032c
Petiole biomass [g $plant^{-1}$]	0.117±0.027a	0.106±0.008a	0.054±0.011b	0.064±0.014b	0.081±0.012ab	0.090±0.007a
Stem biomass [g $plant^{-1}$]	0.226±0.103a	0.229±0.029a	0.099±0.006b	0.118±0.014b	0.132±0.017ab	0.152±0.015bc
Root biomass [g $plant^{-1}$]	0.151±0.067	0.131±0.032	0.074±0.020	0.084±0.023	0.101±0.023	0.119±0.010
Total biomass [g $plant^{-1}$]	0.755±0.261a	0.743±0.074a	0.317±0.039b	0.392±0.074b	0.487±0.094ab	0.567±0.058c
Root/shoot ratio	0.243±0.044	0.213±0.048	0.301±0.061	0.268±0.033	0.263±0.032	0.266±0.030

Pigments content was expressed per unit of leaf area, as no variation was found in mean leaf area between M9-10a and A.27 under the same water treatment (data not shown). Under WW conditions, the mean Chl *a* and Chl *b* content was similar between the two lines (Table 1) with a value around 0.027 mg cm^{-2} for Chl *a* and around 0.009 mg cm^{-2} for Chl *b*. In our study, WD led to a decrease in Chl *a* content in both lines, likely as a result of the degradation of the chloroplast ultrastructure and consequent release/degradation of pigment components (Ristic and Cass 1991). As a consequence of the WD, Chl *b* content also decreased in M9-10a but remained unaltered in A.27. In WDR transgenic plants, significantly ($P \leq 0.01$) higher content of Chl *a* and Chl *b* was found when compared to M9-10a. Our results are in agreement with the transient photosynthetic pigment-

binding function postulated for ELIPs and ELIP-like proteins under disturbing environmental conditions (Adamska *et al.* 1999, Hutin *et al.* 2003). Alamillo and Bartels (2001) also proposed that DSP22 might transiently bind pigments of the xantophyll cycle. Although no differences were found in total carotenoid + xanthophyll content between the studied lines, we can not exclude a role for DSP22 in the interconversion of pigments of the xanthophylls cycle (Demmig-Adams 1990) and more studies are needed to clarify this possibility.

A significantly ($P \leq 0.01$) higher Chl *a*/Chl *b* ratio was found in WDR non-transformed plants. One of the well known strategy to reduce the excessive amount of absorbed radiation energy is the reduction of the PSII antennae size, through the reduction of the relative amounts of Chl-binding proteins of the light harvesting complex of PSII (LHCII; reviewed in Lindahl *et al.* 1997). A reduction in the size of LHCII could be monitored by the increase in the ratio between Chl *a* and Chl *b*. Montané *et al.* (1997) have found a close correlation between LHCII degradation and accumulation of ELIP, suggesting that ELIPs may have a protective role of LHC. Transgenic plants showed higher amounts of chlorophylls, a lower Chl *a*/Chl *b* ratio, and higher Φ_{PSII} values after rehydration when compared to M9-10a plants. Based on our results and previously described information, we suggest that the accumulation of the ELIP-like DSP22 may contribute to reduction of the impact of photooxidative damage on the PSII complex of *M. truncatula* resulting from WD and WDR treatments. Despite this assumption, the mechanisms by which DSP22 leads to enhanced photoxidative protection is yet not clear and further studies are necessary to support this hypothesis.

We investigated how water stress affected plant biomass accumulation and partitioning. We found that A.27 and M9-10a plants reacted in a very similar way irrespective of water treatment studied (Table 1). In the WW treatment, A.27 and M9-10a had biomass about 0.74 - 0.75 g plant^{-1}. The WD treatment caused a drastic reduction of the dry mass in both lines, the reduction was 58.1 % in M9-10a and 47.2% in A.27. Drought avoidance mechanisms usually involve the adjustment of plant growth to water availability by increasing root/shoot ratio (reviewed in Chaves *et al*. 2003). In our experiments, both *M. truncatula* lines faced WD by reducing the shoot and root biomass but root/shoot ratio remained unaltered. The 3 d of recovery were not sufficient to promote a significant increase in biomass accumulation in M9-10a plants, but biomass accumulation increased in A.27. We suggest that the expression of the DSP22 contributing to reduction of photoxidative damage may enhance photosynthetic capacity after the recovery which allows a greater biomass accumulation in A.27.

During the last decade, considerable progress in producing transgenic plants with improved tolerance to various stress conditions were achieved (Cherian *et al.* 2006). We studied deeply the physiological performance of a transgenic line A27 using a similar approach as described in Kogami *et al.* (1994) and we found that it is able to recover from water deprivation better than wild type.

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