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ORIGINAL PAPER

A method to identify early-stage transgenic *Medicago truncatula* with improved physiological response to water deficit

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Abstract Phenotypic screening after transformation experiments aiming to identify lines with the enhanced/desired trait is still a time consuming process for most agricultural crops, especially when dealing with complex physiological responses such as water deficit. In this study we evaluated the suitability of non-destructive leaf gas-exchange analysis and imaging-PAM chlorophyll *a* fluorescence to select transgenic lines of *Medicago truncatula* expressing the Trehalose-6-Phosphate Synthase 1 (*AtTPS1*) from *Arabidopsis thaliana* with altered response to water deficit (WD) and WD recovery (WDR) in the early stages of the transformation process (T_0). Primary transformants (T_0) with different expression levels of a constitutive *AtTPS1* construct were used. Additionally, we evaluated if the expression of the transgene could be correlated with the phenotype assessed. Among tested techniques and parameters measured, the net carbon assimilation (A) from gas-exchange analysis was the best parameter to early detect lines with WD and WDR improved performance, at the earliest stages of the transformation process. With this multidisciplinary approach, we selected 3 transgenic lines TPS7, TPS10 and TPS16 for further studies, which have higher or intermediate expression levels of the transgene and improved response to WD and WDR. This work will contribute to speed-up the identification of elite lines with confidence within a large number of individuals, thus reducing time, cost and labor associated with this plant improvement strategy.

Keywords Early-stage selection · Legumes · Trehalose metabolism · Physiological responses · T_0 transformants · Water deficit

Abbreviations

A	Net photosynthesis rate
Chl *a*	Chlorophyll *a*
Chl *b*	Chlorophyll *b*
MWD	Moderate water deficit
PAR	Photosynthetic active radiation
RT-qPCR	Reverse transcription quantitative PCR
RWC	Relative water content
SWC	Soil water content
SWD	Severe water deficit
WD	Water deficit
WDR	Water deficit recovery
WW	Well watered
T_0	Primary transformants
T6P	Trehalose-6-phosphate

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Φ_{PSII}	Effective quantum yield of the photosystem II
Ψ_w	Leaf water potential

Introduction

Phenotypic screening after transformation experiments is needed to identify which lines have the enhanced/desired trait, to understand how transgene expression modulate plant responses and to evaluate the overall success of the transformation events (Birch 1997). Phenotyping is a time consuming and demanding task especially when dealing with complex physiological plant responses. Such as plant reaction to water deficit, not only because a careful control of experimental conditions has to be maintained but also because a variety of different parameters can be assessed.

The challenges involved in the generation of abiotic stress tolerant plants through the manipulation of the trehalose/T6P metabolism were recently pointed out (Delorge et al. 2014). Trehalose 6-phosphate (T6P) is the intermediate metabolite of a unique two-step pathway of trehalose formation involving trehalose phosphate synthase (TPS) and trehalose phosphate phosphatase (TPP) (Wingler 2002; Paul et al. 2008; Nunes et al. 2013).T6P concentrations are known to regulate physiological and metabolic processes involving carbon storage, plant development, and stress perception and response (for review see O'Hara et al. 2012; Nunes et al. 2013; Lunn et al. 2014). For example, under water deficit T6P accumulation leads to activation of biosynthetic and growth processes, alteration in sugar content and photosynthesis regulation, as well as a decrease in the degradation of cellular compounds finally resulting in an enhanced tolerance to drought (Almeida et al. 2007; Martínez-Barajas et al. 2011; Debast et al. 2011).

Several research groups have tried to engineer trehalose accumulation by introducing genes codifying enzymes of this metabolic pathway aiming to enhance productivity and drought tolerance in several plants. Such approaches included inhibition of the trehalase activity (Goddijn et al. 1997), the constitutive or stress inducible expression of TPS-TPP constructs (Jang et al. 2003; Miranda et al. 2007), ectopic expression of TPS1 genes from one plant species into another (Almeida et al. 2005) or overexpression of an additional homologous TPS1 gene (Li et al. 2011). A common result is that resulting transgenic plants showed increased tolerance to water deficit, but displayed unwanted phenotypes, such as, decreased plant biomass, stunted growth and altered leaf morphology (Imai 2011; Delorge et al. 2014). Legumes have an essential role in improving food security, promoting the development of a range of economic, social and environmental benefits (Araújo et al. 2015). *Medicago truncatula* is a model legume, widely used in research due its relatively short life cycle, autogamy and small genome (500–550 Mbp) that is almost completely annotated and publicly available at Phytozome (Goodstein et al. 2012). Some of our previous works support the use of this model to deeply study the response of legumes to water deficit (Nunes et al. 2008; Trindade et al. 2010; Capitão et al. 2011). Since somatic embryogenesis is easily induced in the M9-10a genotype of cultivar Jemalong, an efficient regeneration-transformation protocol was long-established allowing the recovery of a large number of transgenic lines (Araújo et al. 2004).

Usually the physiological evaluation of the performance of transgenic plants is assessed in homozygous lines (Bhat and Srinivasan 2002; Nunes et al. 2009; Araújo et al. 2013). To accomplish this goal, plants are allowed to self-pollinate and progenies screened in segregating concentrations of kanamycin, a process that takes about 7–9 months. For most of the crops this approach is time demanding and requires extra labor and resources to recover few interesting lines (Mieog et al. 2013), with no warranty that lines with the desired phenotypes will in fact be recovered. Additionally, a considerable variation of transgene expression is often observed within populations of transgenic plants transformed with the same transgene construct and under identical transformation conditions (Butaye et al. 2005). Consequently, it is crucial to develop reliable methods to early detect lines both expressing the transgene and showing evidences of the desired phenotype.

Previous studies in the *M. truncatula* M9-10a genotype showed that the impact of transgene expression could be detected early in the transformation process (Confalonieri et al. 2009, 2014). In those studies, T_0 transgenic lines with enhanced DNA repair response or tripterpen saponin biosynthesis were identified as a result of the combination of molecular approaches and phenotype characterization. Concerning abiotic stress responses, the current state-of-art is limited to reliable physiological screening methods to early detect *M. truncatula* plants expressing the transgene and showing evidences of the desired phenotype (Nunes et al. 2009; Araújo et al. 2013).

This study was designed to evaluate the suitability of leaf gas-exchange analysis and imaging-PAM Chla fluorescence to selected transgenic lines of *M. truncatula* with altered response to water deficit (WD) and water deficit recovery (WDR). Primary transformants (T_0) of *M. truncatula* with different expression levels of a constitutive construct with the *TPS1* gene from *A. thaliana* (*AtTPS1*) were used. In particular, we tested the hypothesis that this multiple approach is able to discriminate between lines with different WD tolerance in the early stages of the transformation process and see if the expression of the transgene can be correlated with the phenotype assessed. To the best of our knowledge, a multidisciplinary method to early distinguish *M. truncatula* lines with improved WD and WDR response in the transformation process has never been described. The development of an early stage screening method combining

both expeditious molecular data with physiological data will be crucial to speed-up the identification of improved lines thus reducing time, cost and labor associated with this type of improvement programs.

Materials and methods

Construction of the plasmid pBIN-2x35S-AtTPS1-t35S

The 2x35S-*AtTPS1*-t35S was excised from plasmid pGreen0229-2x35S-*AtTPS1*-t35S previously constructed with the purpose to overexpress *AtTPS1* in tobacco plants (Almeida et al. 2005). This plasmid contains the cDNA sequence of trehalose-6-phosphate synthase 1 gene from *A. thaliana* (*AtTPS1*, GenBank accession number: Y08568) cloned under the control of 35S CaMV promoter. Briefly, the 4.3 kb fragment corresponding to the 2x35S-*AtTPS1*-t35S cassette was excised from pGreen0229-2x35S-*AtTPS1*-t35S using with restriction enzymes *KpnI* and *XbaI* (Invitrogen, Carlsbad, USA) and inserted in the multiple cloning site of the plant transformation vector pBINplus previously linearised with the same restriction enzymes. The pBINplus contains in its T-DNA a pNos-*NptII*-tnos cassette that encodes for the neomycin phosphotransferase II (*NPTII*) that confers kanamycin resistance. Standart cloning and plasmid manipulation procedures were made according to Sambrook et al. (1989). The integrity of gene construct was confirmed by digestion with multiple restriction enzymes. The resulting pBIN-2x35S-*AtTPS1*-t35S vector was then transferred by electroporation into the hypervirulent EHA105 *Agrobacterium tumefaciens* strain (Hood et al. 1993). The presence of the plasmid into bacterial cells was confirmed by PCR using *AtTPS*1 specific primers.

Agrobacterium-mediated transformation of *M. truncatula*

Agrobacterium-mediated transformation of *M. truncatula* was made as described in Araújo et al. (2004). Briefly, young leaf explants from in vitro grown plants of the M9-10a genotype of *M. truncatula* were infected with an EHA105 suspension carrying the pBIN-2x35S-*AtTPS1*-t35S vector. After 5 days of co-culture period somatic embryogenesis was induced under strict selective pressure with a 16/8 day/night photoperiod. As a control of the transformation process non-infected explants were also cultivated with and without selective pressure. After 3–4 months of in vitro culture well-rooted kanamycin-resistant (Kan+) regenerated plantlets were selected as putative transgenic plants (T_0 generation). To avoid sibling's recovery, only plantlets resulting from different embryogenic *calli* were chosen. To proceed with the molecular and physiological analyses, putative transgenic lines (TPS) and non-infected M9-10a plantlets were propagated on in vitro culture. Stem segments containing 2–3 internodes were inoculated into a growth-regulator-free medium MS030A as described by Neves et al. (2001). Micropropagated TPS and M9-10a plants were kept in a growth chamber (PHYTOTRON EDPA 700, ARALAB) with 16 h photoperiod of 100 μmol m^{-2} s^{-1} provided by cool white fluorescent lights and a day/night temperature of 24/22 °C.

DNA extraction and PCR detection of *AtTPS1* gene

Regenerated plants were screened for the presence of the *AtTPS1* gene by PCR amplification. Genomic DNA was isolated from putative *M. truncatula* T_0 transgenic plants and the non-transgenic M9-10a line using the DNAeasy Plant Mini Kit (Qiagen,Venlo, The Netherlands) according to manufacturer's instructions. *AtTPS1* transgene specific primers (see Table S.1, supplemental material) were used to amplify a fragment of 834 bp. PCR was carried out with 200 ng of template DNA, 50 pmol of transgene specific primers; 1.5 mM of $MgCl_2$, 1× PCR buffer and 0.5 Units of Taq polymerase (Invitrogen, Carlsbad, USA) in a final volume of 25 μl with sterile MiliQ water. PCR amplification conditions were 1 min at 98 °C for denaturation, followed by 30 cycles of 1 min at 95 °C for denaturation, 1 min at 60 °C for annealing, 1,5 min at 73 °C for polymerization and a final extension step of 8 min at 72 °C. PCR amplifications were performed using a BIOMETRA UNO II system (BIOMETRA, Göttingen, Germany). The presence of PCR products with expected size was assessed by standard electrophoresis in a 1.0 % agarose gel stained with SYBR® Safe (Life Technologies, Carlsbad, USA).

RNA extraction and RT-qPCR detection of *AtTPS1* gene

The expression of the *AtTPS1* in regenerated transgenic plants was confirmed by Reverse Transcription quantitative PCR (RT-qPCR). Total RNA was isolated from 100 mg of leaf tissue from PCR positive *M. truncatula* T_0 transgenic lines (TPS01, TPS03, TPS04, TPS07, TPS09, TPS10, TPS11, TPS14, TPS16, TPS19) and non-transgenic line (M9-10a) using the RNeasy Plant Mini Kit (Qiagen, Venlo, The Netherlands), according to the manufacturer's instructions. RNA samples from three individuals per line were treated with TURBO DNA-free kit (Ambion, Texas, USA) and pooled. One microgram of RNA was reverse-transcribed using the ImProm-IITM Reverse Transcription System (Promega, Madison, USA) and the poly-T oligonucleotide primer. cDNA samples were diluted ten-fold before moving to quantitative PCR (qPCR). PCR primers (Supplemental material, Table S.1) were designed

using the Primer 3 software (http://bioinfo.ut.ee/primer3-0.4.0/primer3/) on *M. truncatula* or *A. thaliana* target sequences available on DFCI Gene Index (http://compbio.dfci.harvard.edu/tgi/) and NCBI GenBank (http://www.ncbi.nlm.nih.gov/genbank/) databases. Primers were designed to have a size of 18–22 bp, GC content between 40 and 56 %, a melting temperature (Tm) between 55 and 67 °C and a predicted fragment size between 100 and 216 bp. Oligonucleotides were synthesized by Stabvida (Stabvida, Caparica, Portugal). Target *AtTPS1* primers were designed to amplify a non-conserved gene region, to avoid the amplification of the endogenous *MtTPS1*. qPCR reactions were performed using an iQ^{TM}5 Real-Time PCR Detection System (Bio-Rad Laboratories, Munich, Germany), by adding 10 µl of iQ^{TM} SYBR Green Supermix (Bio-Rad Laboratories, Munich, Germany), 2 µL of diluted cDNA, 0.25 µM each primer, and water to a final volume of 20 µL. After one initial incubation step at 95 °C for 3 min, amplification was performed for 40 cycles with the following cycle profile: a denaturing step at 95 °C for 30 s followed by an annealing step at 60 °C for 30 s, and an extension step of 72 °C for 1 min. Specificity of qPCR products was confirmed by performing a melting temperature analysis at temperatures ranging from 55 to 95 °C in intervals of 0.5 °C. *M. truncatula*'s reference genes were selected based on a previous study where the expression of *ACT*, *APRT*, *ATP*, *HSP70*, *L2* and *PDF2* (Table S.1, supplemental material) genes was quantified on cDNAs from putative transgenic and non-transgenic plants using the geNorm (Vandesompele et al. 2002) and NormFinder (Andersen et al. 2004) in the GenEx software (version 5, MultiD, Göteborg, Sweden). *PDF* and *ACT2* were selected as the best reference genes for the experimental conditions (transgenic vs. non transgenic) and used in this work. For all the genes studied, two cDNA samples of RNA pools from each experimental condition were amplified in technical triplicates, giving a total of 6 replicates per line. The raw, background-subtracted, fluorescence data provided by the iQ5^{TM} Optical System Software version 2.0 (Bio-Rad Laboratories, Munich, Germany) was used to estimate the PCR efficiency (E) and cycle quantification number (Cq) for each transcript quantification. The PfaffI method (Pfaffl 2001) was used for relative quantification of the *AtTPS1* transcript accumulation using *PDF* and *ACT2* as reference genes. For each studied line the relative expression was normalized against the lowest expression value.

Growth conditions and water deficit and recovery treatments

M9-10a and TPS+ *M. truncatula* lines were acclimated to ex vitro conditions during 2 weeks following the established protocol for in vitro sown seeds (Araújo et al. 2004, 2013) then transferred to 1 L plastic pots containing a mixture of commercial soil (Compo Sana S.A., Barcelona, Spain) and vermiculite (3:1, v/v). Plants were grown in a growth chamber (Fitoclima 5000 EH, Aralab, Rio de Mouro, Portugal) at 25/18 °C of day/night temperature, 16/8 h day/night, 50 % relative humidity (RH) and 300–350 µmol m^{-2} s^{-1} of photosynthetic photon flux density obtained with Osram Lumilux L 58W/840 cool white lamps and watered every other day for 4 weeks. Using this protocol, we ensured the recovery of well rooted plants, with vigorous growth in soil pots under controlled conditions and suitable for molecular and physiological characterization within 6–7 weeks. After the growing period, the plants within each line were separated into homogenous groups, both in size and number, and submitted to an experimental water deficit and recovery scheme routinely used in our laboratory to evaluate WD and WDR responses (Nunes et al. 2008, 2009; Araújo et al. 2013).The well-watered (WW) plants were kept fully irrigated and sampled throughout the experiment, while irrigation was suspended for the rest of the plants. After 4–8 days upon water withdrawal plants from each genotype were sampled. Plants with water withhold treatments with relative water content (RWC) ranging from 70 to 50 % were assigned into the moderate water deficit (MWD) group and plants with 45–30 % of leaf RWC were grouped into the severe water deficit (SWD) treatment. The WDR group was re-watered after reaching the SWD state and sampled 3 days after re-watering. Throughout the experiment, the positions of the pots inside the growth chamber were frequently randomized to avoid microclimate effects. Young fully expanded leaves were always used, either in vivo or in vitro assays.

Water relations

Soil water content (SWC) was determined as a weight fraction according to Coombs et al. (1985), with SWC (%) = (sFW − sDW)/sDW * 100, where sFW is the fresh weight of a soil portion from the internal part of each pot and sDW is the dry weight of the same portion after 2 days at 105 °C. Leaf RWC was estimated on leaf discs according to (Catský 1960) as follows: RWC (%) = (FW − DW)/(TW − DW) * 100, where FW is the fresh weight of the disc, TW represents the turgid weight of the same disc floated overnight on water, and DW would be the dry weight of the disc after 48 h at 80 °C. Leaf water potential was determined using a thermocouple psychrometer (HR 33T, Wescor, USA) equipped with a C-52 leaf chamber. Psychrometric measurements were performed in 6 mm diameter leaf discs after 20 min of equilibrium with the atmosphere of the leaf chamber, according to previous protocol used in *M. truncatula* by Nunes et al. (2008).

Leaf gas exchange analysis

Photosynthetic gas exchange measurements were performed in a non-detached well expanded leaf from each plant using a portable infra-red gas analyzer (IRGA, LCpro+ ADC BioScientific, Hertfordshire, UK), with the microenvironment in the measuring chamber set to 50 ± 5 % RH, 370 ppm CO2 and 25 ± 2 °C. Net photosynthesis rate (A), stomatal conductance (gs) and transpiration rate (E) rate were measured at growth light intensity (350 μmol m^{-2} s^{-1}) and at saturating light intensity (1000 μmol m^{-2} s^{-1}), according to Nunes et al. (2009).

Photosynthetic pigment determination

Leaf discs 0.9 mm wide were immersed in 2 mL of 99.8 % (v/v) methanol (Panreac Química S.L.U., Castellar del Vallès, Spain) and kept 4 °C in the dark until full extraction. Chlorophyll *a* (Chl *a*), chlorophyll *b* (Chl *b*) and carotenoid (Carot) concentrations were calculated according to (Lichtenthaler 1987) after spectrophotometric measurements at 470.0, 652.4 and 665.2 nm (Helios Beta UV/Vis spectrophotometer, Thermo Electron Corporation, Waltham, USA).

Modulated chlorophyll *a* fluorescence

The effective quantum yield of the photosystem II (Φ_{PSII}) was measured with an IMAGING-PAM M-series (IMAG-MIN/B, Heinz Walz GmbH, Effeltrichm, Germany). The equipment has an IMAGE-K5 1/2″ CCD camera (640 9 480 pixel resolution) with a 16 mm objective (Allied Vision Technologies GmbH, Stadtroda, Germany) and uses a Luxeon LED array (460 nm) of 12 high-power LED organized in four groups equipped with short-pass filters. Images of 2 non-detached central leaflets of each plant were acquired with a measuring pulse frequency of 8 Hz after 10 min of light adaptation at 320 μmol m^{-2} s^{-1} followed by a saturating pulse of 6000 μmol m^{-2} s^{-1} for 0.8 s. In each leaflet, two circular Areas of Interest (AOI) were selected with the largest diameter possible that would not include leaves' dark spots and Φ_{PSII} was determined. Fluorescence parameters were analyzed using the Imaging Win analytical software (Heinz Walz GmbH, Effeltrichm, Germany).

Statistical analysis

Differences in the relative quantification of the expression of *AtTPS1* between lines were analysed by One-Way Analysis of Variance (ANOVA), means values were statistically compared using a *t* Studenťs test. One- and two-way ANOVA were used for mean comparison among plant groups on SigmaPlot version 11.0 (Systat Software, San Jose, USA). Differences between individual means were further tested by post hoc multiple range tests (Tukey). GraphPad Prism® 5 software, version 5.03 (GraphPad software, San Diego, USA) calculated the linear regressions to evaluate the relation between relative water content and leaf water potential of MWD and SWD plants. In all cases, statistical significance was declared when $P < 0.05$.

Results

Plant transformation and analysis of transgenic plants

Twenty-two kanamycin resistant (Kan+) lines were recovered and screened by PCR for the presence of the *AtTPS1* cDNA sequence (data not shown). A 918 bp fragment was amplified in lines carrying the AtTPS1 gene construct (see Supplemental Fig. S.1 for an agarose gel result for representative lines). The Kan+ lines growing in greenhouse showed no morphological or developmental alterations neither growth rates out of the range of those seen by the non-transformed M9-10a plants subjected to the same regeneration process. The Reverse Transcription quantitative Polymerase Chain Reaction (RT-qPCR) analysis revealed *AtTPS1* expression in 11 of the 22 lines (Fig. 1) that could be grouped into three categories, according to the relative levels of expression: TPS4, TPS9, TPS19 had the lowest expression; TPS10 and TPS16 had the highest expression; and TPS1, TPS3, TPS7, TPS11, TPS13 and TPS14 had an intermediate level of expression of *AtTPS1*. Expression of *AtTPS1* was not detected in M9-10a non-transformed control plants. Taking in account this quantification TPS4, TPS7, TPS10, TPS14, and TPS16 were selected to further physiological analysis and comparison with the M9-10a non-transgenic line.

Water relations

No leaf RWC significant differences were recorded in plant lines subjected to the same water treatment (Table 1). TPS10 was the only line that was not able to recover its RWC to values similar to those of WW plants after imposition of SWD but this aspect is not reflected in the leaf water potential (Ψ_w) values measured for the same experimental condition (Table 2). When comparing Ψ_w between WW and recovered (WDR) plants, no significant differences were found among the different lines (Table 2) with the exception of the TPS7 line. A significant leaf Ψ_w increase was seen in TPS7 line, 3 days after recovering for SWD. Along water deficit exposure, all transgenic lines have leaf Ψ_w decreasing rates

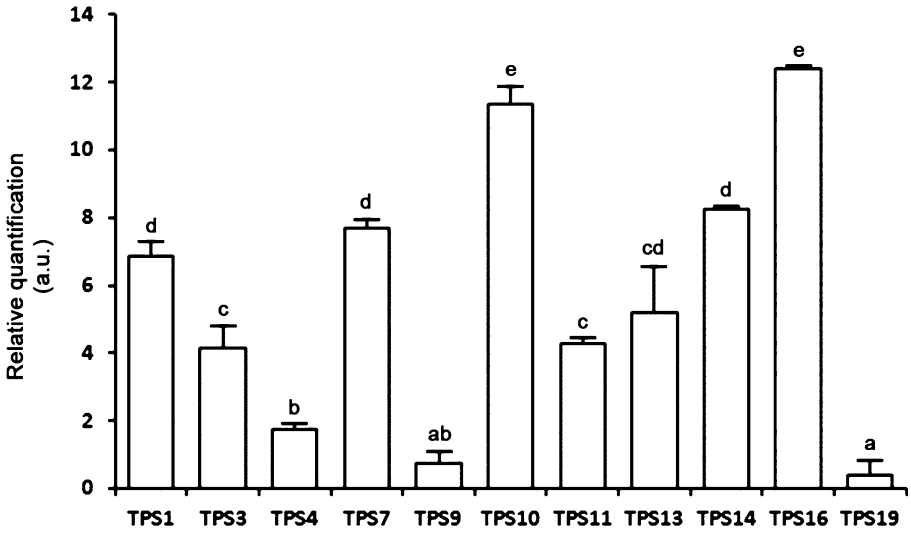

Fig. 1 qPCR quantification of *AtTPS1* gene expression in transgenic *M. truncatula* plants. Normalization was made using *ACT* and *PDF* as reference genes (Vandesompele et al. 2002). Values are average ± standard deviation (n = 3–6 measurements). *Different letters* indicate significant differences between plant lines ($P \leq 0.05$)

Table 1 Leaf relative water content (RWC) and soil water content (SWC) in non-transgenic (M9-10a) and transgenic (TPS+) *M. truncatula* plants subjected to different water status

	RWC (%)				SWC (%)			
	WW	MWD	SWD	WDR	WW	MWD	SWD	WDR
M9-10a	71.8 ± 5.2 c	58.3 ± 6.6 b	37.1 ± 4.1 a	73.7 ± 5.8 c	77.3 ± 2.5 c	31.4 ± 6.4 b	21.5 ± 2.5 a	75.2 ± 3.9 c
TPS04	75.1 ± 4.6 c	64.5 ± 5.2 b	42.2 ± 5.1 a	75.6 ± 8.1 c	76.7 ± 6.3 c	37.5 ± 6.6 b	25.6 ± 0.5 a	71.4 ± 3.9 c
TPS07	73.3 ± 5.9 c	63.3 ± 4.0 b	36.3 ± 2.8 a	70.0 ± 6.0 c	79.5 ± 0.4 c	39.4 ± 2.6 b	22.8 ± 3.7 a	70.9 ± 2.5 c
TPS10	74.3 ± 3.8 c	60.0 ± 6.3 b	40.6 ± 7.7 a	63.7 ± 3.2 b	72.4 ± 13.9 c	33.4 ± 5.0 b	22.8 ± 2.4 a	73.5 ± 5.0 c
TPS14	74.3 ± 1.5 c	55.9 ± 6.5 b	42.1 ± 5.1 a	71.7 ± 3.2 c	72.9 ± 12.5 c	31.6 ± 6.2 b	22.8 ± 3.5 a	70.8 ± 1.1 c
TPS16	76.2 ± 1.8 c	54.2 ± 0.3 b	37.7 ± 6.6 a	68.7 ± 7.1 c	78.0 ± 2.8 c	44.0 ± 7.2 b	25.1 ± 2.2 a	76.6 ± 2.4 c

Values are average ± standard deviation (n = 3–5 plants). When found, different letters indicate significant differences between water treatments ($P \leq 0.05$). No significant differences were found among plant lines within the same water treatment

WW well watered, *MWD* moderate water deficit, *SWD* severe water deficit, *WDR* water deficit recovery

Table 2 Leaf water potential (Ψ_w) in well watered (WW) and water deficit recovery (WDR) in non-transgenic (M9-10a) and transgenic (TPS+) *M. truncaula* plants

	Ψ_w (MPa)	
	WW	WDR
M9-10a	−1.29 ± 0.18 a	−1.35 ± 0.16 a
TPS04	−1.09 ± 0.03 a	−1.04 ± 0.08 a
TPS07	−1.51 ± 0.17 a	−1.05 ± 0.12 b
TPS10	−1.13 ± 0.09 a	−1.41 ± 0.25 a
TPS14	−1.48 ± 0.12 a	−1.14 ± 0.29 a
TPS16	−1.17 ± 0.35 a	−1.20 ± 0.13 a

Values are average ± standard deviation (n = 3–5 plants). Different letters indicate significant differences between water treatments ($P \leq 0.05$). No significant differences were found among plant lines within the same water treatment

similar to M9-10a (Fig. 2). However, TPS16 (Fig. 2, arrow) had always significantly higher values of leaf Ψ_w than the non-transgenic line and TPS07.

Photosynthesis responses to water treatments

Differences in net photosynthetic rates (A) of WW, SWD and WDR plants were observed among the different lines (Fig. 3). The WD effect on photosynthesis was more pronounced when measurements were made at growing light intensity (Fig. 3a–d) than at non-limiting light intensity (Fig. 3e–h). In WW conditions, the TPS04 line showed always a higher CO_2 fixation rate than M9-10a plants (Fig. 3a, e). Under MWD, a decrease in A was observed in all plants but differences between plant lines were highlighted when higher irradiance was applied. The M9-10a line had a null photosynthesis rate at both light conditions (Fig. 3b, f), but all transgenic lines maintain relevant photosynthetic rates at high irradiance (Fig. 3f). TPS04, TPS07 and TPS16 lines stood out with higher A values under non-limiting light intensity (Fig. 3f). In SWD plants, net CO_2 fixation was observed for TPS10 and TPS14 lines at high photosynthetic active radiation (PAR) intensity (Fig. 3g). No significant differences were noticed between

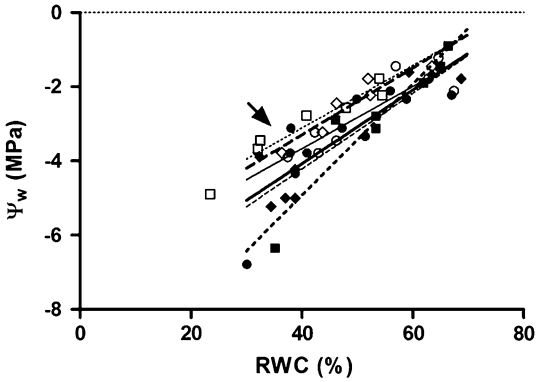

Fig. 2 Effect of decreasing relative water content (RWC) on leaf water potential (Ψ_w) in non-transgenic (M9-10a) and transgenic (TPS+) *M. truncatula* plants subjected to water deficit. *Each point* corresponds to independent measurements on a different plant. *Lines* represent linear regressions of M9-10a (*filled circle*, *solid thick line*, $\Psi_w = 0.099$RWC(%)—8.03, $R^2 = 0.654$), TPS04 (*open circle*, *solid thin line*, $\Psi_w = 0.084$ RWC(%)—7.01, $R^2 = 0.804$), TPS07 (*filled diamond*, *dashed thin line*, $\Psi_w = 0.102$RWC(%)—8.30, $R^2 = 0.864$), TPS10 (*filled square*, *dashed thick line*, $\Psi_w = 0.149$ RWC(%)—10.93, $R^2 = 0.898$), TPS14 (*open diamond*, *dashed line*, $\Psi_w = 0.090$ RWC(%)—6.90, $R^2 = 0.887$), and TPS16 (*open square*, *dotted line*, $\Psi_w = 0.084$ RWC(%)—6,48, $R^2 = 0.929$)

transgenic and non-transgenic lines under SWD. At 3 days after re-watering, all plants recovered from SWD conditions showing photosynthetic performances similar to that was observed for WW plants. Under growing light conditions, WDR TPS07 plants showed photosynthetic rates significantly higher than the non-transgenic line (Fig. 3d). On the other hand, WDR TPS10 and TPS16 plants showed, at non-limiting PAR, photosynthetic rates significantly higher than the non-transgenic line (Fig. 3h). Albeit stomatal conductance (data not shown) closely paralleled the net photosynthetic rates, small deviations resulted in different transpiration rates and consequently in differences in instantaneous water use efficiencies (WUE) values mainly seen at growth-light conditions (Fig. 4). In the WW plants, TPS16 showed higher WUE than any other line but only at growth light (Fig. 4a,b). On the other hand, WDR plants had not significantly different WUEs than WW plants, except for a higher value of TPS10 line at growth light.

Pigment content and quantum yield of photosystem II

Under WW conditions, differences were seen in pigment contents among the different lines (Table 3). TPS07, TPS10 and TPS16 lines have higher Chl *a* contents than the non-transformed line. The same occurs with Chl *b*, which results in similar ratios between Chl *a* and Chl *b* among the plant lines. The carotenoid (Carot) content of TPS16 was also increased in relation to M9-10a, a feature shared by TPS07 and TPS10 plants.

Figure 5a shows images of Chl *a* fluorescence of representative leaflets of the tested plant lines in response to the different water treatments. Plant lines can be grouped into three groups regarding plants response the treatments imposed (Fig. 5b). TPS04 and TPS07 plants showed similar values of Φ_{PSII} at WW, MWD and WDR conditions, thus only decreasing its Φ_{PSII} at SWD. In the second group, we found M9-10a and TPS16 plants with similar Φ_{PSII} under WW and WDR, which decreased its Φ_{PSII} with decreasing water availability (MWD and SWD). The remaining lines (TPS10 and TPS14) also decreased their Φ_{PSII} with WD imposition but at WDR had lower quantum yields when compared to WW plants, thus showing less ability to recover full photochemistry capacity after a SWD.

When comparing plant lines within the same treatment, TPS10, TPS14 and TPS16 have significantly higher Φ_{PSII} than the non-transgenic line under WW conditions (Fig. 5b, white bars). Under MWD conditions, all transgenic lines were able to maintain higher Φ_{PSII} than the non-transgenic line, with exception of the TPS14 (Fig. 5b, light grey bars). All SWD plants had similar Φ_{PSII} values irrespective to the treatment or line studied (Fig. 5b, dark grey bars).

Discussion

In this study we evaluated the suitability of leaf gas exchange analysis and imaging-PAM Chl *a* fluorescence to discriminate between transgenic lines of *M. truncatula* with altered response to WD and WDR in the early stages of the transformation process (T_0). To the best of our knowledge, this approach has never been exploited in *M. truncatula* transformation for improved WD response. Generally, the impact of transgene expression is studied in established transgenic homozygous lines (T_2) with stable transgene expression (Bhat and Srinivasan 2002). However, this approach is time-consuming when working with a large number of individuals (resulting from transformation experiments) with a 3–4 month life cycle (Araújo et al. 2004). Importantly, if there is some misbehavior in the plant line, for instance due to inappropriate transgene insertion, by the time of the screening a significant amount of resources and time would have been wasted.

The lines studied have different expression levels of the *TPS1* cDNA from *A. thaliana* (*AtTPS1*), a construct previously used to increase drought tolerance in tobacco (Almeida et al. 2005, 2007). Quantification of *AtTPS1* expression by RT-qPCR allowed to group transgenic lines into three categories (low, medium and high expression

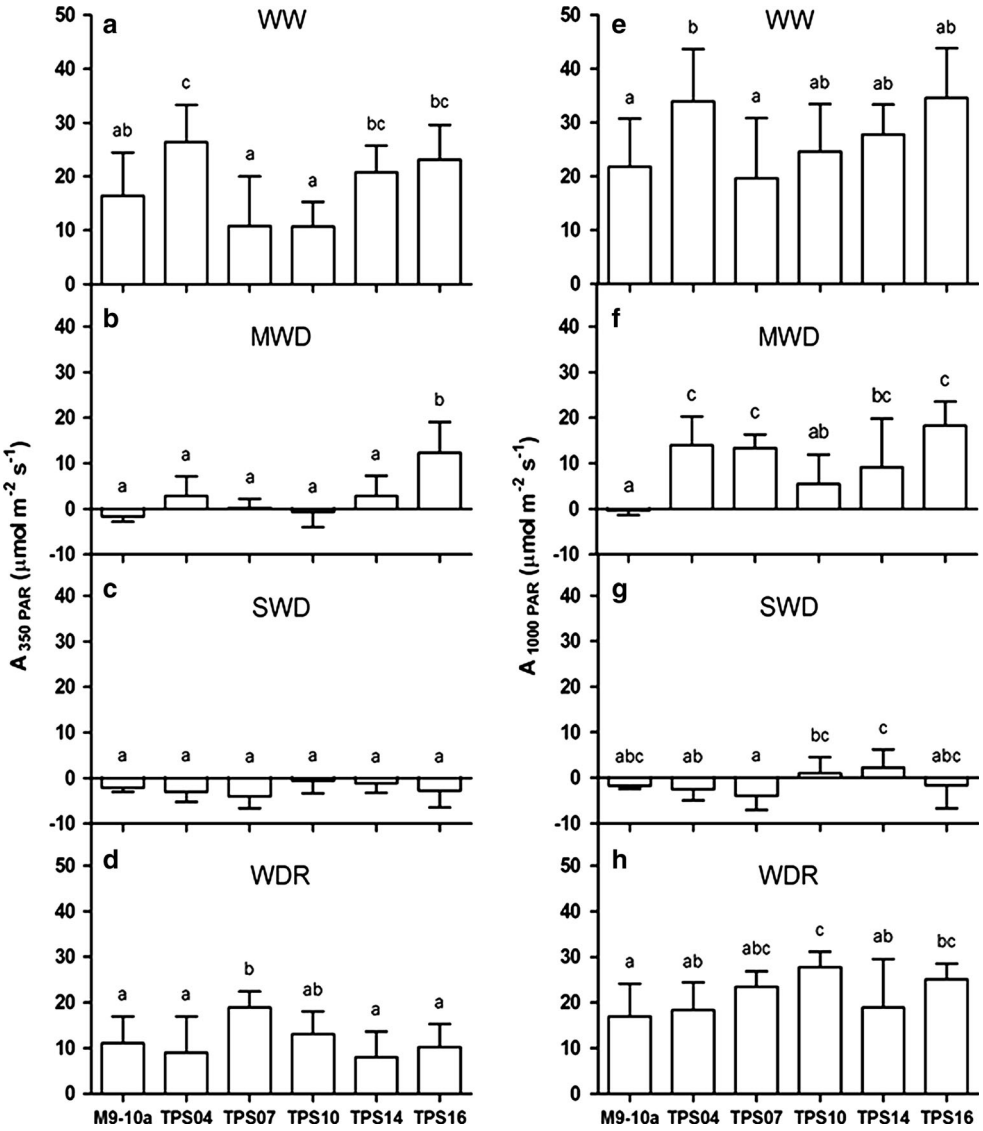

Fig. 3 Net photosynthetic rate (**a**) at growth (350 µmol m^{-2} s^{-1}, **a**–**d**) and non-limiting (1000 µE m^{-2} s^{-1}, **e**–**h**) light intensities of the tested *M. truncatula* lines (non-transgenic, M9-10a, and transgenic, TPS+) in response to different water availability treatments, such as Well Watered (WW, **a** and **e**), Moderate Water Deficit (MWD, **b** and **f**), Severe Water Deficit (SWD, **c** and **g**), and Water Deficit Recovery (WDR, **d** and **h**). Values are average ± standard deviation (n = 3–5 plants). *Different letters* indicate significant differences between plant lines ($P \leq 0.05$)

level). At least, one line representative of these groups were propagated by stem cuttings and used for phenotyping under WD and WDR in an approach similar to the one described for transgenic potato tubers expressing either *E. coli TPS* or *TPP* (Debast et al. 2011). Plantlets were acclimated to ex vitro conditions following established protocols, ensuring that we were able to recover well rooted plants, with vigorous growth in soil pots under controlled conditions and suitable for molecular and physiological characterization within 6–7 weeks.

Among the physiological parameters studied, we found that leaf gas exchange analysis was the best approach to discriminate between T_0 transgenic and non-transgenic lines. In a previous work, we observed that overexpression of the same *AtTPS1* construct in homozygous transgenic tobacco plants leads to enhanced photosynthesis under WD (Almeida et al. 2007). A similar behavior was seen in our T_0 *M. truncatula* plants, corroborating the suitability of gas exchange to discriminate the photosynthetic performance in primary transformants. Contrary to the observed in M9-10a plants, all transgenic lines were able to sustain positive net carbon assimilation rates (A) at MWD conditions when exposed to non-limiting light intensities. This suggests that the expression of the *AtTPS1* construct improved the WD tolerance of these *M. truncatula* plants. TPS07, TPS10 and TPS16 lines stood out with particular responses to the water regimes applied. TPS07 and TPS10 plants have a improved recovery after a WD episode, as seen by their higher A values under growth light. TPS16 plants show the highest A rates in MWD conditions at growth light. Their highest WD tolerance is described by two complementary processes: by maintain higher A rates when subjected to MWD or by showing the improved recovery after water deficit imposition. Interestingly, these

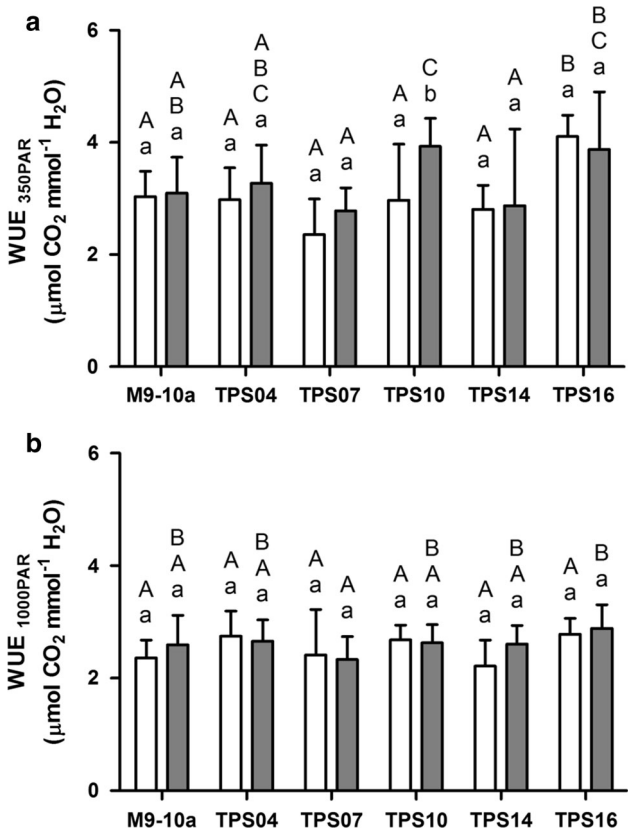

Fig. 4 Water use efficiency (WUE) at growth (350 μE m^{-2} s^{-1}, **a**) and non-limiting (1000 μE m^{-2} s^{-1}, **b**) light of the tested *M. truncatula* lines (non-transgenic, M9-10a, and transgenic, TPS) from the well watered (*white bars*) and the water deficit recovery (*grey bars*) groups. Values are average ± standard deviation (n = 3–5 plants). When found, significant differences ($P \leq 0.05$) were indicated with *different letters* between plant lines (*upper case*) and water treatments (*lower case*)

lines have the highest or intermediate relative amounts of the transgene expressed, as shown by the RT-qPCR results.

TPS07, TPS10 and TPS16 have higher Chl *a* contents than the non-transformed line under WW conditions. However we are unable to state if this tendency is maintained under WD or after WDR, since no pigment quantification was performed under these conditions. In plants under MWD, the Φ_{PSII} variation was quite similar to A, showing that the photochemical electron transport flux was mainly directed towards carbon reduction. Overall data analysis, supports that *AtTPS1* expression improves the photosynthesis performance during WD, increasing leaf light absorbance and its effective utilization, being this effect most evident in lines TPS07, TPS10 and TPS16.

Along WD imposition, all transgenic lines have leaf Ψ_w decreasing rates similar to M9-10a, which suggests that the different transformation events tested are sensing and responding to water deprivation in a very similar way to the one observed in non-transformed plants. Nevertheless, TPS16 had always significantly higher values of leaf Ψ_w than the non-transgenic line and TPS07. Another interesting feature is that a significant leaf Ψ_w increase was seen in TPS7 line, 3 days after recovering for SWD when compared to the WW group, despite the similar RWC. On the other hand, Ψ_w of TPS10 plants showed no significant difference between WW and WDR. These results suggest that WD changed the relation between cell water content and cell water potential, probably due to alterations on osmolyte concentrations and/or cell wall elasticity, as previously found in severe drought *M. truncatula* cv Jemalong plants (Nunes et al. 2008). The leaf Ψ_w alterations observed in the transgenic lines may be related to the reported influence of T6P in cellular sugar pools of transgenic plants (for review see Ponnu et al. 2011), resulting in osmotic pressure variation which influences leaf water potential (da Silva and Arrabaça 2004). These observations on the TPS07 and the TPS10 lines are probably influencing the results obtained in the photosynthesis parameters and the quantum yield of the photosystem II, respectively. Further studies are required to elucidate relevant metabolic mechanisms underlying such responses.

In conclusion, gas exchange analysis is the best approach to assess the photosynthetic performance of T_0 transgenic plants in an effective and nondestructive manner. Although this approach does not provide a deep and comprehensive knowledge, neither on the physiological mechanisms underlying *M. truncatula* responses to WD, nor on the metabolic changes induced by *AtTPS1*

Table 3 Pigment content of well watered leaf samples from *M. truncatula* lines (non-transgenic M9-10a and transgenic, TPS+)

	Chlorophyll *a* (μg cm^{-2})	Chlorophyll *a*/chlorophyll *b*	Carotenoids (μg cm^{-2})
M9-10a	26.0 ± 5.5 a	3.12 ± 0.48 a	7.03 ± 1.43 a
TPS04	30.0 ± 3.8 ab	3.28 ± 0.49 a	7.92 ± 0.93 a
TPS07	30.9 ± 4.3 bc	3.08 ± 0.48 a	8.09 ± 0.84 ab
TPS10	30.8 ± 4.3 bc	3.23 ± 0.47 a	8.02 ± 1.18 ab
TPS14	29.5 ± 4.6 ab	3.20 ± 0.47 a	7.29 ± 0.58 a
TPS16	34.9 ± 3.2 c	3.04 ± 0.45 a	9.13 ± 1.00 b

Values are average ± standard deviation (n = 15–20 plants). Different letters indicate significant differences between plant lines ($P \leq 0.05$)

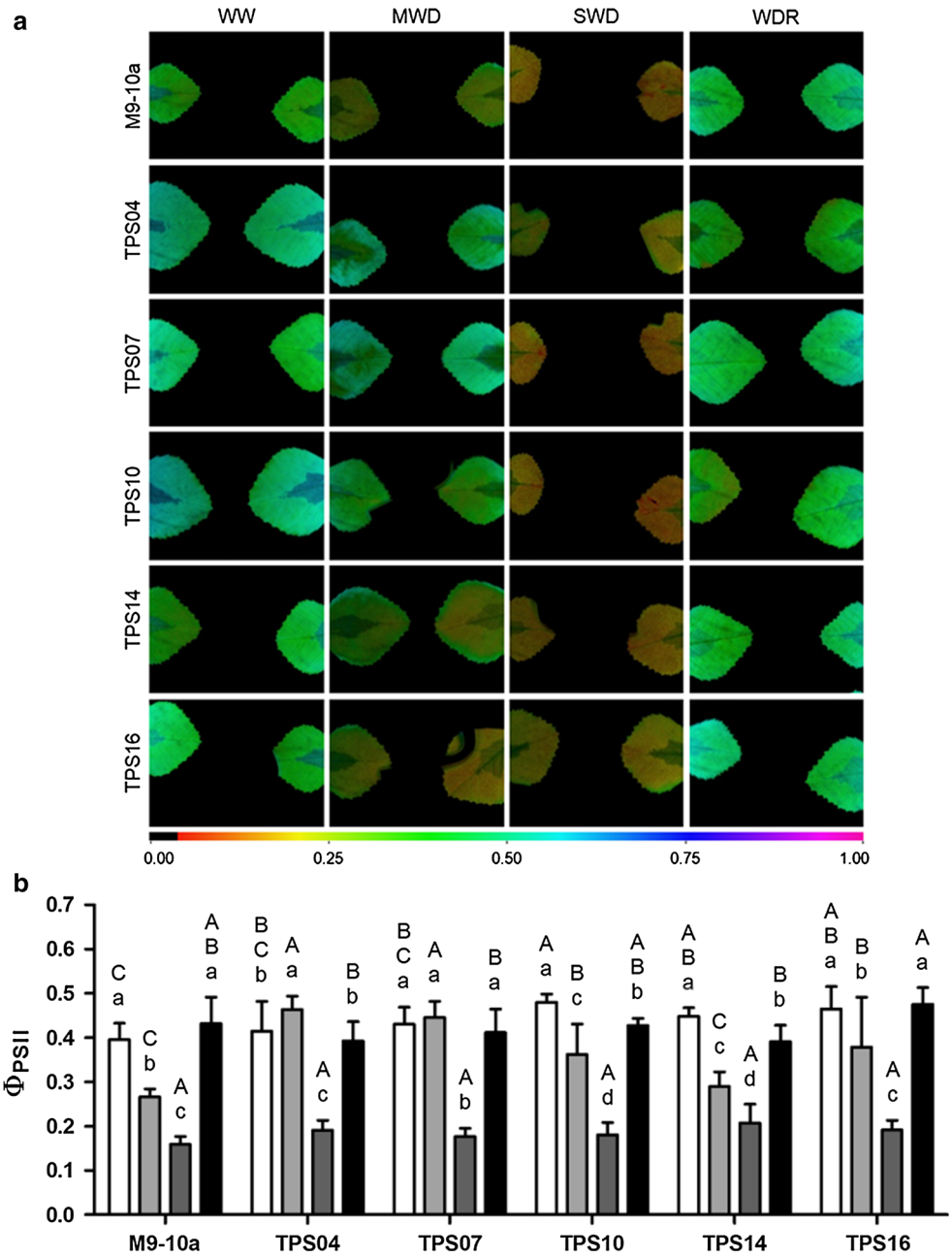

Fig. 5 Effective quantum yield of photosystem II (Φ_{PSII}) of the non-transgenic (M9-10a) and transgenic (TPS+) lines of well watered (WW, *white bars*), moderate water deficit (MWD, *light grey bars*), severe water deficit (SWD, *dark grey bars*), and water deficit recovery (WDR, *black bars*) plants: **a** chlorophyll fluorescence imaging of representative leaflets; **b** average values ± standard deviation (n = 3–5 plants). In some instances an object didn't interfere with the measurements had to be used to hold the leaflets within the focal distance of the equipment (SWD in TPS07 and TPS07, and MWD in TPS16). *Different letters* indicate significant differences between plant lines (*upper case*) and water treatments (lower case)

expression, it constitutes an effective early-stage screening tool. This study shows the added value of combining molecular data with physiological data to evaluate the effect of transgene expression on water deficit. With this multidisciplinary approach we were able to select lines TP7, TPS10 and TPS16 for further studies, which have intermediate and higher expression levels of the transgene and improved response to WD and WDR. Our approach allows to speed up a confident identification of transgenic lines with altered response to water deficit, in a large set of candidate lines. Being non-destructive it also allows maintain plant material for propagation and functional studies thus reducing time, cost and labor associated with this type of plant improvement programs.

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