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Physiological and molecular responses to drought in *Petunia*: the importance of stress severity

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Abstract

Plant responses to drought stress vary depending on the severity of stress and the stage of drought progression. To improve the understanding of such responses, the leaf physiology, abscisic acid (ABA) concentration, and expression of genes associated with ABA metabolism and signalling were investigated in *Petunia* × *hybrida*. Plants were exposed to different specific substrate water contents (θ = 0.10, 0.20, 0.30, or 0.40 $m^3 \cdot m^{-3}$) to induce varying levels of drought stress. Plant responses were investigated both during the drying period (θ decreased to the θ thresholds) and while those threshold θ were maintained. Stomatal conductance (g_s) and net photosynthesis (A) decreased with decreasing midday leaf water potential (Ψ_{leaf}). Leaf ABA concentration increased with decreasing midday Ψ_{leaf} and was negatively correlated with g_s (r = –0.92). Despite the increase in leaf ABA concentration under drought, no significant effects on the expression of ABA biosynthesis genes were observed. However, the ABA catabolism-related gene *CYP707A2* was downregulated, primarily in plants under severe drought (θ = 0.10 $m^3 \cdot m^{-3}$), suggesting a decrease in ABA catabolism under severe drought. Expression of *phospholipase Dα* (*PLDα*), involved in regulating stomatal responses to ABA, was enhanced under drought during the drying phase, but there was no relationship between *PLDα* expression and midday Ψ_{leaf} after the θ thresholds had been reached. The results show that drought response of plants depends on the severity of drought stress and the phase of drought progression.

Key words: abscisic acid, acclimation, automated irrigation, soil moisture sensor, stomatal conductance, substrate water content.

Introduction

Drought is a common abiotic stress and considered to be the most limiting environmental factor for plant growth (Boyer, 1982). Over the last few decades, many studies have reported physiological, molecular, and biochemical plant responses to drought (Chaves *et al.*, 2003). However, plant responses to water deficit may vary depending on the severity of the drought stress, the process of drought development, and the duration of drought stress (Bray, 1997; Chaves *et al.*, 2003; Kim and van Iersel, 2011), and the application of the drought treatments varies considerably across studies (Jones, 2007). Inadequate descriptions

of how the drought treatments are imposed complicate the interpretation of many previous studies (Pinheiro and Chaves, 2011). A commonly used approach is withholding irrigation until plants wilt, a method referred to as progressive drought (Kawaguchi *et al.*, 2004; Harb *et al.*, 2010). Other approaches for studying drought stress include the addition of an osmoticum such as mannitol (Kreps *et al.*, 2002) and stress induction through desiccation (Seki *et al.*, 2002). A comparison across these approaches indicated that only a small subset of the transcriptome was commonly regulated among these methods, indicating that plant

responses to drought are critically dependent on the method of drought-stress imposition (Bray, 2004).

Several recent studies have underlined the importance of the method of drought-stress imposition. The 'PHENOPSIS' system was used to automate the maintenance of soil water content at predetermined levels across different *Arabidopsis* accessions, thereby enabling the identification of an *Arabidopsis* accession altered in its response to water deficit (Granier *et al.*, 2006). Harb *et al.* (2010) studied responses of *Arabidopsis* to moderate drought stress (employed by maintaining the substrate at 30% of field capacity) and progressive drought, at the whole plant and the molecular levels, and identified different stages of plant responses to drought stress. These studies further highlight the importance of precise methods for controlling drought-stress imposition.

The severity of drought stress is also a critical factor that determines plant response. In loblolly pine (*Pinus taeda* L.), mild stress resulted in photosynthetic acclimation while severe stress inhibited it (Watkinson *et al.*, 2003). Additionally, expression of drought-related genes was dependent on the level of drought stress (Watkinson *et al.*, 2003). Under moderate stress, three stages of plant responses – pre-conditioning, acclimation, and post-acclimation homeostasis – were identified in *Arabidopsis* (Harb *et al.*, 2010). These studies indicate that plant responses to drought stress that are generally evident under one level of stress (mild to moderate) may be absent under another level (severe stress). Monitoring and controlling the soil moisture status and frequent measurement of plant responses, both during the development of the drought and after a steady drought level has been imposed, may allow for the quantification of plant responses to different drought severities. Additionally, such analyses may allow a better understanding of the temporal changes in response to drought stress. Further, integration of changes in gene expression with whole plant responses may lead to a more comprehensive understanding of physiological responses to drought (Bray, 1997; Jones, 2007; Harb *et al.*, 2010).

Abscisic acid (ABA) is the primary chemical signal for drought, increasing in concentration under drought stress and inducing stomatal closure to minimize water loss. ABA also alters the expression of a multitude of drought stress-related genes (Bray, 2004). This study focused on ABA-related mechanisms, since its anabolism and catabolism are well understood, key genes in its metabolic and regulatory pathways have been identified, it can be readily quantified, and its physiological effects at the leaf level are easily measured (Schachtman and Goodger, 2008; Schwartz and Zeevaart, 2010).

The endogenous ABA concentrations are controlled by ABA biosynthesis, catabolism, and conjugation mechanisms (Seiler *et al.*, 2011). A key regulatory step in ABA biosynthesis is the cleavage of 9-*cis*-epoxycarotenoids by 9-*cis*-epoxycarotenoid dioxygenase (NCED) to form xanthoxin (Nambara and Marion-Poll, 2005). The *NCED* genes belong to a multigene family, and have been identified in many species (Thompson *et al.*, 2000). In a subsequent step within the ABA biosynthesis pathway, abscisic aldehyde oxidase (AAO) converts the abscisic aldehyde derived from xanthoxin to ABA. In *Arabidopsis*, *AAO3* was identified as the key gene encoding this enzyme (Seo *et al.*, 2000; Nambara and Marion-Poll, 2005). ABA 8'-hydroxylases, members of the cytochrome P450 monoxygenase family (CYP707A), catalyse the ABA catabolic pathway by enabling the conversion of ABA to the inactive phaseic acid (Umezawa *et al.*, 2006). ABA can also be inactivated and stored in the conjugated ABA glucosyl ester (ABA-GE) form, which may be subsequently reactivated by specific β-glucosidases (Lee *et al.*, 2006).

ABA signalling in plants mediates stomatal opening and closing (Kim *et al.*, 2010). Phospholipase Dα (PLDα) may play an important role in ABA signalling by mediating the effects of ABA on stomatal closing and opening. PLDα1 cleaves phospholipids to generate phosphatidic acid, a molecule with dual roles in promoting stomatal closure and inhibiting stomatal opening (Zhang *et al.*, 2004; Mishra *et al.*, 2006). Phosphatidic acid binds to a negative regulator of ABA responses, ABI1/PP2C (ABA insensitive/protein phosphatase 2C), thereby promoting ABA-induced stomatal closure (Zhang *et al.*, 2004). Additionally, PLDα1 and phosphatidic acid also interact with the GTP-binding proteins (G-proteins), and may mediate ABA-dependent inhibition of stomatal opening (Mishra *et al.*, 2006). *ZPT2-3* encodes a Cys2/His2-type zinc finger protein, a class of transcription factors, and is upregulated in response to various abiotic stresses including drought in *Petunia* (Sugano *et al.*, 2003). Overexpression of *ZPT2-3* in *Petunia* increased drought tolerance and survival under drought (Sugano *et al.*, 2003). Similar studies in *Arabidopsis* showed that *ZPT* homologues were upregulated under drought stress and aided in enhancing drought tolerance, indicating that it may be an important regulator of transcriptional responses to drought (Sakamoto *et al.*, 2004; Shu-Jing *et al.*, 2010).

To measure plant responses to well-controlled, specific drought conditions, this study controlled substrate water content (θ, v/v) using an automated irrigation system based on soil moisture sensor readings (Supplementary Fig. S1, available at *JXB* online). This system irrigates when θ drops below a specific threshold θ and allows for maintenance of different θ levels for prolonged periods (Nemali and van Iersel, 2006). This system has been previously used to quantify plants' physiological responses to the rate of drought-stress imposition (Kim and van Iersel, 2011). The current study gradually exposed *Petunia*s to different levels of drought by allowing the substrate to dry until specific θ thresholds had been reached, and maintained them at these thresholds. The objectives were to investigate the responses of *Petunia* to different severities and temporal stages of drought, from the level of gene expression to whole plant physiology, focusing on stomatal regulation through ABA biosynthesis, catabolism, and signalling. Plant responses were separated into two stages: (1) the period where the θ decreased to the θ threshold (drying period); and (2) the period after the threshold θ was attained, where the θ was stable.

Materials and methods

Plant materials and growth conditions

Eight *Petunia × hybrida* 'Apple Blossom' seedlings per experimental unit were transplanted into 16 8-l trays filled with soilless substrate (Fafard 2P, peat/perlite 60:40, Conrad Fafard, Agawam, MA, USA) mixed with a controlled-release fertilizer (Osmocote 14-14-14, N/P/K 14.0:6.2:11.6, The Scotts, Marysville, OH, USA) at a rate of 7.7 kg·m^{-3}. Plants were grown for 3 weeks (from 23 March to 14 April 2010)

in a greenhouse at the University of Georgia using a soil moisture sensor-based automated irrigation system (Nemali and van Iersel, 2006), which maintained θ at 0.40 m³·m⁻³. During the growing period, the average daily temperature and relative humidity in greenhouse were 21.0 ± 1.0 °C and $53 \pm 10\%$, and the daily light integral averaged 26.9 ± 12.2 mol·m⁻²·d⁻¹ (mean ± SD).

Drought treatments

The automated irrigation system (Nemali and van Iersel, 2006) was modified for this research (Supplementary Fig. S1). Two capacitance soil moisture sensors (EC-5, Decagon Devices, Pullman, WA, USA) were placed in each tray and connected to a datalogger (CR10, Campbell Scientific, Logan, UT, USA) via a multiplexer (AM16/32, Campbell Scientific) to monitor and control θ. The soil moisture sensors were excited with 2.5 V and substrate-specific calibration was used to convert the measured voltage to θ (θ = 1.7647×sensor output (V)–0.4745, $r^2 = 0.95$). When the average reading of the two sensors dropped below θ threshold for that tray, a datalogger opened a solenoid valve using a relay driver (SDM-CD16 AC/DC controller, Campbell Scientific) to irrigate the tray for 20 seconds (approximately 90 ml per application), maintaining θ at the specific level. Each tray was watered with tap water using a custom grid, designed to apply the water as uniformly as possible, with two pressure compensated emitters (8 l h⁻¹, Netafim USA, Fresno, CA, USA). The θ was measured every 10 minutes, and averages were logged hourly. The different θ thresholds, 0.40 (well-watered), 0.30 (mild drought), 0.20 (moderate drought), and 0.10 m³·m⁻³ (severe drought), were initiated at midnight, and irrigation was withheld until a tray θ reached its threshold θ. Once, the threshold θ was attained, the automated irrigation system maintained θ close to the threshold θ ($\pm$ 0.02 m³·m⁻³, Fig. 1). Control plants were maintained at a θ of 0.40 m³·m⁻³ throughout the experiment, and the drought treatments reached their threshold θ of 0.30, 0.20, and 0.10 m³·m⁻³ after 2.5, 3.7, and 8.8 d, respectively. The collected data were separated into two different phases of drought stress: the drying period during which θ decreased to the threshold θ and the period after the threshold θ had been reached and θ was maintained at a stable level.
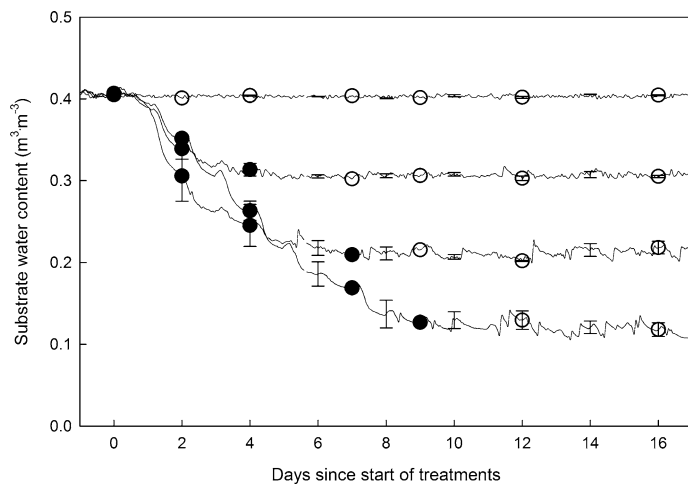

Fig. 1. Substrate water contents (θ) changes of *Petunia × hybrida* with soil moisture sensor-based automatic irrigation over a 16-d period. A datalogger monitored θ using soil moisture sensors and maintained the θ in four treatments (θ = 0.40, 0.30, 0.20, and 0.10 m³·m⁻³). The drought treatments reached their threshold θ of 0.30, 0.20, and 0.10 m³·m⁻³ after 2.5, 3.7, and 8.8 d, respectively. Closed circles indicate data collected during the drying period and open circles indicate data collected after θ reached the thresholds. Error bars indicate the standard error ($n = 4$).

Leaf water potential and gas exchange measurement

Leaf physiological measurements and sampling were performed every 2 or 3 d from the start of the drying treatment. The uppermost, fully expanded leaves were used for data collection. Leaf discs for midday Ψ_{leaf} measurements were sampled at noon using leaf cutter thermocouple psychrometers (Model 76, J.R.D. Merrill Specialty Equipment, Logan, UT, USA), and midday Ψ_{leaf} was measured after equilibration of the psychrometers in a water bath at 25 °C for 4 h. Stomatal conductance (g_s), CO₂ exchange rate (A), and quantum yield of PSII (Φ_{PSII}) were measured with a leaf photosynthesis system (CIRAS-2, PP Systems, Amesbury, MA, USA) equipped with a red/blue LED light source and a chlorophyll fluorescence module. Measurements were taken at a photosynthetic photon flux of 1000 μmol·m⁻²·s⁻¹ and at a CO₂ concentration of 388 μmol·mol⁻¹ and were started approximately 5 hours after sunrise. Φ_{PSII} was calculated as $(F'_m - F_t)/F'_m$ (Maxwell and Johnson, 2000). Leaf samples for RNA extraction and ABA assays were collected at noon by excising the leaf at the petiole using a razor blade and were immediately frozen in liquid N₂ and stored at –80 °C.

A/Ci curves, relative water content, leaf size, and shoot dry mass at harvest

At 16 d after initiation of the drought treatments, CO₂ response curves (A/Ci curves) were collected on attached uppermost, fully expanded leaves using the CIRAS-2 system, by changing the CO₂ concentration from 0 to 1200 μmol·mol⁻¹ in 200 μmol·mol⁻¹ increments. Measurements were started at 5 hours after sunrise and it took approximately 7 hours to collect all A/Ci curves. The CO₂-saturated assimilation rate (A_{max}) was calculated using empirical A/Ci curve analysis (Photosyn Assistant, Dundee Scientific, Dundee, Scotland, UK). At noon, the uppermost, fully expanded leaf samples were collected and fresh weight of the leaves was measured immediately after excision. Fully turgid fresh weight of the leaves was obtained after floating the samples on deionized water at 4 °C for 6 h, and dry weight was determined after drying the sample at 60 °C for 24 h. Relative water content was calculated as (fresh weight – dry weight) / (turgid weight – dry weight) × 100%. Leaf size was measured on eight uppermost fully expanded leaves per tray using a leaf area meter (LI-3100, LI-COR, Lincoln, NE, USA) and shoot dry weight was obtained after drying samples in an oven at 70 °C for 4 d.

Identification of genes associated with ABA biosynthesis, catabolism, and signalling from Petunia

Petunia expressed sequence tags (ESTs) coding for ABA- and drought-related genes were identified from *Petunia* EST databases (http://biosrv.cab.unina.it/454petuniadb/) (Zenoni *et al.*, 2011). All genes used in this study, except *ZPT2-3* and the reference genes, were identified based on sequence similarity after TBLASTX analysis with related genes from tomato, potato (Solanaceae) and *Arabidopsis*. Primers were manually designed based on the EST sequences. Candidate sequences were further confirmed by sequencing after PCR amplification using the same primers. The *Petunia* homologues of *NCED*, *NCED1* (contig PETIN084121) and *NCED2* (contig PETIN041019), were identified based on sequence similarity with the tomato *NCED*, Le*NCED1* (Taylor *et al.*, 2000). The putative *Petunia NCED1* displayed 93% identity with *LeNCED2* while the putative *NCED2* shared 86% identity with Le*NCED1*. Putative homologues of *AAO31* (contig PETAX039740) and *AAO32* (contig PETIN023342) were identified based on similarity with *AAO3* from *Arabidopsis* (Seo *et al.*, 2000). Also, the putative *AAO31* and *AAO32* had 80% and 90% identity with potato *AAO* (accession DQ206634.1), respectively. *CYP707A1* (contig PETIN061494) and *CYP707A2* (contig PETIN048226) were identified based on similarity to the tomato ABA catabolism gene, Sl*CYP707A1* (Nitsch *et al.*, 2009), and also shared 91% identity with potato *CYP707A1* (DQ206630.1) and 95% identity with potato *CYP707A2* (DQ206631.1), respectively. *PLDα* (contig PETIN026822) was identified based on similarity to tomato Le*PLDα1* (AF154425.1)(Bargmann *et al.*, 2009) with which it

displayed 79% identity. The genes for cyclophilin-2 (*CYP*) and elongation factor 1α (*EF1α*) were used as reference genes in quantitative real-time PCR (qRT PCR). These genes were suggested as stably expressed reference genes based on previous research in *Petunia* (Mallona *et al.,* 2010). Primers used for qRT PCR are presented in Supplementary Table S1.

RNA extraction, cDNA synthesis, and qRT PCR

RNA was extracted from ground, frozen leaf tissue using the guanidium isothiocyanate method (Chomczynski and Sacchi, 1987). Approximately 1 g of ground sample was used for RNA extraction in 7 ml of extraction buffer (38% acid phenol, 0.8 M guanidine thiocyanate, 0.4 M ammonium thiocyanate, 0.1 M sodium acetate, and 5% glycerol). After centrifugation, the supernatant was extracted with chloroform/isoamyl alcohol (24:1, v/v). The aqueous supernatant was precipitated with isopropanol and a salt solution (0.8 M sodium citrate and 1.2 M NaCl). After centrifugation, the RNA was washed with 70% ethanol and dissolved in DEPC (diethylpyrocarbonate)-treated water. This mixture was extracted with 3 M sodium acetate and chloroform/isoamyl alcohol, and the aqueous supernatant was precipitated in 70% ethanol overnight at –20 °C. After centrifugation, the RNA was washed with 70% ethanol, dissolved in DEPC-treated water and stored at –80 °C. RNA quality was analysed by gel electrophoresis and RNA quantification was performed using Nanodrop 8000 (Thermo Fisher scientific, Waltham, MA, USA).

RNA (1 μg) was treated with DNase (Promega, Madison, WI, USA) to remove genomic DNA contamination, according to the manufacturer's instructions and used for reverse transcription. Reverse transcription was performed using oligo-dT (Promega) and ImPromII reverse transcriptase (Promega) in a 20-μl volume. Subsequently, the cDNA was diluted five times with autoclaved distilled water and stored at –20 °C until further analysis.

The qRT PCR analyses were performed on the Stratagene Mx3005P real-time PCR system (Agilent Technologies, Santa Clara, CA, USA) using 1 μl of diluted cDNA in a 14 μl reaction volume with 2×SYBR Green Master Mix (Applied Biosystems, Foster City, CA, USA). The reaction parameters were: 95 °C for 10 min; 40 cycles of 95 °C for 30 s and 60 °C for 1 min. The melting curve analysis indicated single, distinct peaks for each of the amplicons analysed indicating the amplification of a single product. Controls without the template were run to ensure the absence of non-specific amplification. Normalization of gene expression was performed using the geometric mean of expression of the reference genes, *CYP* and *EF1α*, according to Pfaffl (2001). The normalization factor determined from the geometric mean of the reference genes generally changed less than 2-fold among the samples, indicating that these genes were effective for normalization. All analyses were performed using four biological replicates.

Leaf ABA content determination

Leaf tissue was ground in liquid nitrogen and extracted in darkness in an ABA extraction buffer (80% methanol with butylated hydroxytoluene at 100 mg·l^{-1}, and citric acid at 500 mg·l^{-1}) for 16 h with constant shaking at 4 °C. The supernatant was collected after centrifugation and diluted 10-fold with TBS buffer (50 mM TRIS, 1 mM MgCl$_2$, 150 mM NaCl, pH 7.8). Subsequently, the ABA concentration was quantified using ELISA with the Phytodetek ABA test kit (Agdia, Elkhart, IN, USA) following the manufacturer's instructions.

Experimental design and statistical analysis of data

A randomized complete block design with four blocks was used in this experiment. Physiological data and relative gene expression data were analysed with the general linear models procedure using repeated measures at α = 0.05 (proc glm, SAS 9.2, SAS Systems, Cary, NC, USA). ABA concentration was analysed using log-transformed [ABA] data. To quantify relationships between midday Ψ_{leaf} and physiological and gene expression responses during the drying period, regression analysis was performed and normality

of the residuals was tested using the Shapiro-Wilk test at α = 0.05 (SigmaPlot 11.1, Systat, Chicago, IL, USA). When the normality assumption was violated, data were log transformed or analysed using non-parametric Spearman's rank order correlation (SigmaPlot 11.1). The regression analysis between g_s, ABA concentration, midday Ψ_{leaf}, and the interaction between ABA concentration and midday Ψ_{leaf} was performed using the data from all sampling times after log transformation of ABA concentration and g_s (SAS 9.2). Leaf size, leaf relative water content, and shoot dry weight were analysed using the analysis of variance procedure (proc anova, SAS 9.2). Mean separation for the harvest data was done using Fisher's protected least-significant-difference procedure.

Results

Leaf physiological responses to specific θ

Withholding irrigation reduced g_s to 20% (~150 mmol·m^{-2}·s^{-1}) of that of the control plants at 2 d after drought imposition (Fig. 2A). The g_s of plants at θ of 0.30 and 0.20 m^3·m^{-3} partially recovered after thresholds of 0.30 and 0.20 m^3·m^{-3} had been reached (50–70% and 30–40% of control g_s for 0.30 and 0.20 m^3·m^{-3}, respectively). However, plants at 0.10 m^3·m^{-3} maintained a low g_s from day 4 until day 16 (< 50 mmol·m^{-2}·s^{-1}), less than 5% of the g_s of control plants. Drought decreased A to 50–30% of the control at 2 d after drought initiation (Fig. 2B). After the θ thresholds had been reached, plants at θ of 0.30 and 0.20 m^3·m^{-3} maintained A at ~67 and ~50% of control plants until the end of the experiment. However, A of plants at θ of 0.10 m^3·m^{-3} subsequently decreased to 1 μmol·m^{-2}·s^{-1}, approximately 5% that of control plants. Only the 0.10 and 0.20 m^3·m^{-3} treatments significantly decreased the quantum yield of photosystem II (Φ_{PSII}) (Fig. 2C). In contrast to the patterns of g_s and A, the 0.30 m^3·m^{-3} treatment did not show a significant decrease in Φ_{PSII} compared to control plants and maintained Φ_{PSII} at ~90% of that of control plants throughout the experiment. CO$_2$-saturated assimilation rate (A_{max}) was lower as the threshold θ decreased (Supplementary Table S2), indicating that non-stomatal limitations contributed to the decreased A under drought.

Similar to g_s, A, and Φ_{PSII}, midday Ψ_{leaf} decreased within 2 d of withholding irrigation (Fig. 2D). In general, midday Ψ_{leaf} was lower at lower θ levels, with the largest differences at 9 d after the start of the treatments. At harvest, midday Ψ_{leaf} of the plants at θ < 0.40 m^3·m^{-3} were similar among the three drought treatments, but lower than that of the well-watered control. At harvest, plants in lower θ treatments had smaller leaves and lower shoot dry weight. Only a θ of 0.10 m^3·m^{-3} reduced leaf relative water content (Supplementary Table S2).

Effects of θ on ABA concentrations

Control plants maintained the leaf ABA concentration at ~0.15 nmol·(g fresh weight)$^{-1}$, while all drought treatments resulted in a ~3-fold increase in leaf ABA concentration after 2 d of drought (Fig. 3). Plants at a θ of 0.30 m^3·m^{-3} maintained a 2–3-fold higher ABA concentration for a week, but had ABA concentrations similar to that of the 0.40 m^3·m^{-3} treatment from 9 to 16 d after the treatments. Plants in the 0.10 m^3·m^{-3} treatment gradually increased their ABA concentration up to 1.2 nmol·(g fresh weight)$^{-1}$ (8-fold that of control) as the substrate dried and
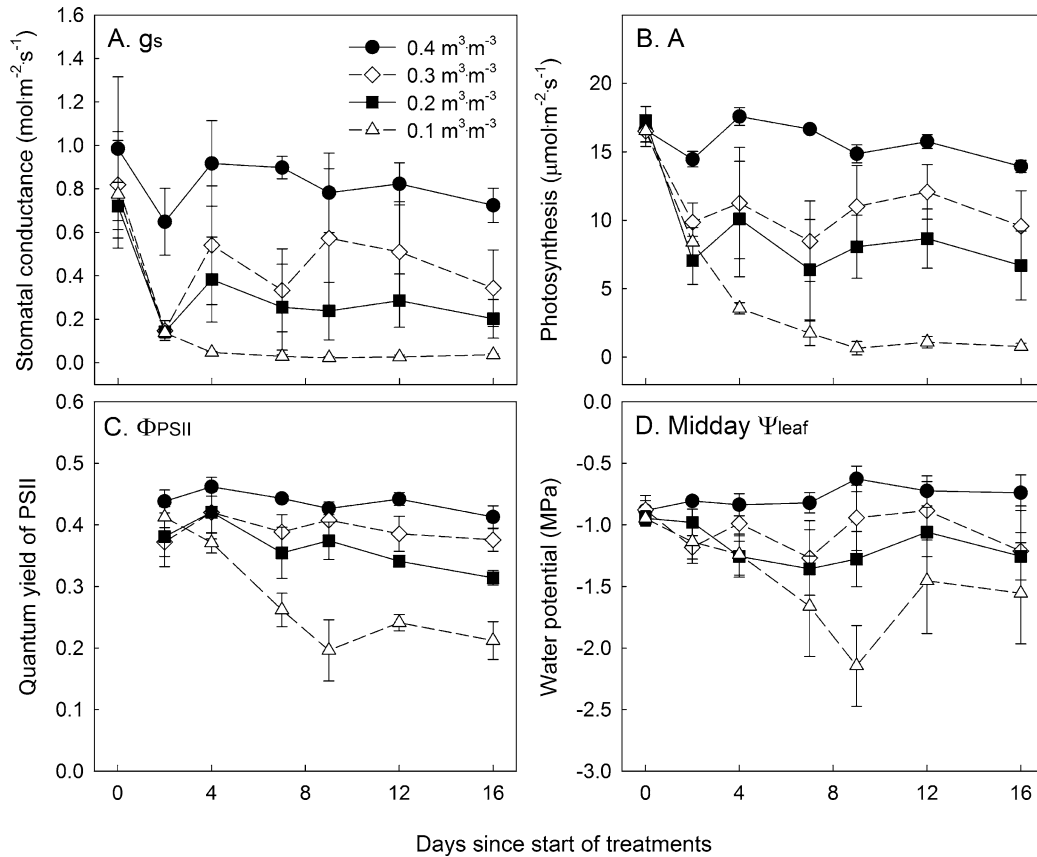
Fig. 2. Leaf physiological responses of *Petunia × hybrida* in response to substrate water content treatments (θ = 0.40, 0.30, 0.20, and 0.10 $m^3·m^{-3}$) over a 16 d period. Stomatal conductance (g_s, panel A), photosynthesis (A, panel B), and quantum yield of PSII (Φ_{PSII}, panel C) were measured by a leaf photosynthesis measurement system and midday leaf water potential (Ψ_{leaf}, panel D) was measured using thermocouple psychrometers. All the measurements were conducted at noon. The drought treatments reached their threshold θ of 0.30, 0.20, and 0.10 $m^3·m^{-3}$ after 2.5, 3.7, and 8.8 d, respectively. Error bars indicate the standard error (*n* = 4).
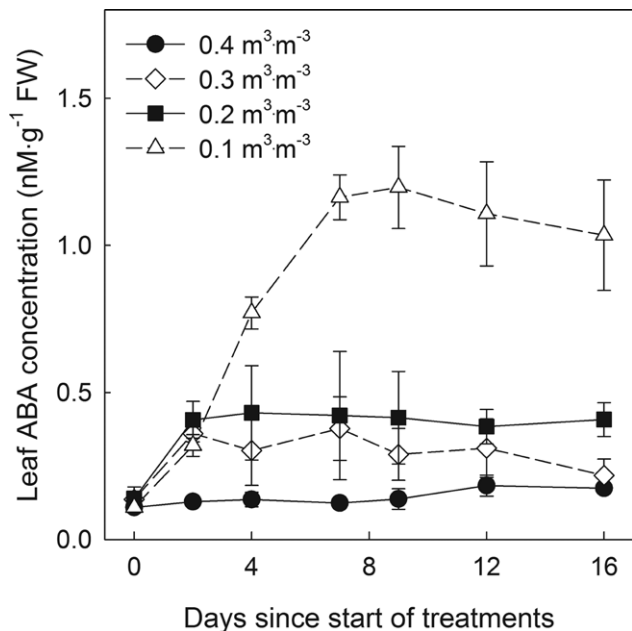

Fig. 3. Changes in leaf abscisic acid (ABA) concentration of *Petunia × hybrida* under drought with different substrate water content (θ = 0.40, 0.30, 0.20, and 0.10 $m^3·m^{-3}$) over a 16 d period. Error bars indicate the standard error (*n* = 4).

subsequently maintained a high leaf ABA concentration until the end of the experiment.

Expression of genes associated with ABA biosynthesis, catabolism, and signalling

Drought did not affect the expression of the ABA biosynthesis-related *NCED* and *AAO3* genes in *Petunia* leaves (Figs. 4A–D). However, expression of the ABA catabolism-related gene *CYP707A2* in the 0.10 $m^3·m^{-3}$ treatment was ~3-fold lower than in the control and 2-fold lower than in other drought treatments as θ reached the threshold and when it was stable at 0.10 $m^3·m^{-3}$ (Fig. 4F), suggesting a decrease in ABA catabolic activity.

Relative expression of *PLDα* in *Petunia* leaves increased rapidly during the drying period (Fig. 4G). All drought treatments displayed a ~2-fold increase in the expression of *PLDα* after 2 d. The relative expression of *PLDα* in plants at a θ of 0.30 $m^3·m^{-3}$ decreased at 4 d after treatment, when the θ reached the threshold level, and a similar pattern was observed with the 0.20 $m^3·m^{-3}$ treatment. Plants in the 0.10 $m^3·m^{-3}$ treatment displayed ~2.5-fold higher relative expression of *PLDα* than control plants and maintained higher expression rates than other treatments until θ reached the threshold. Withholding irrigation
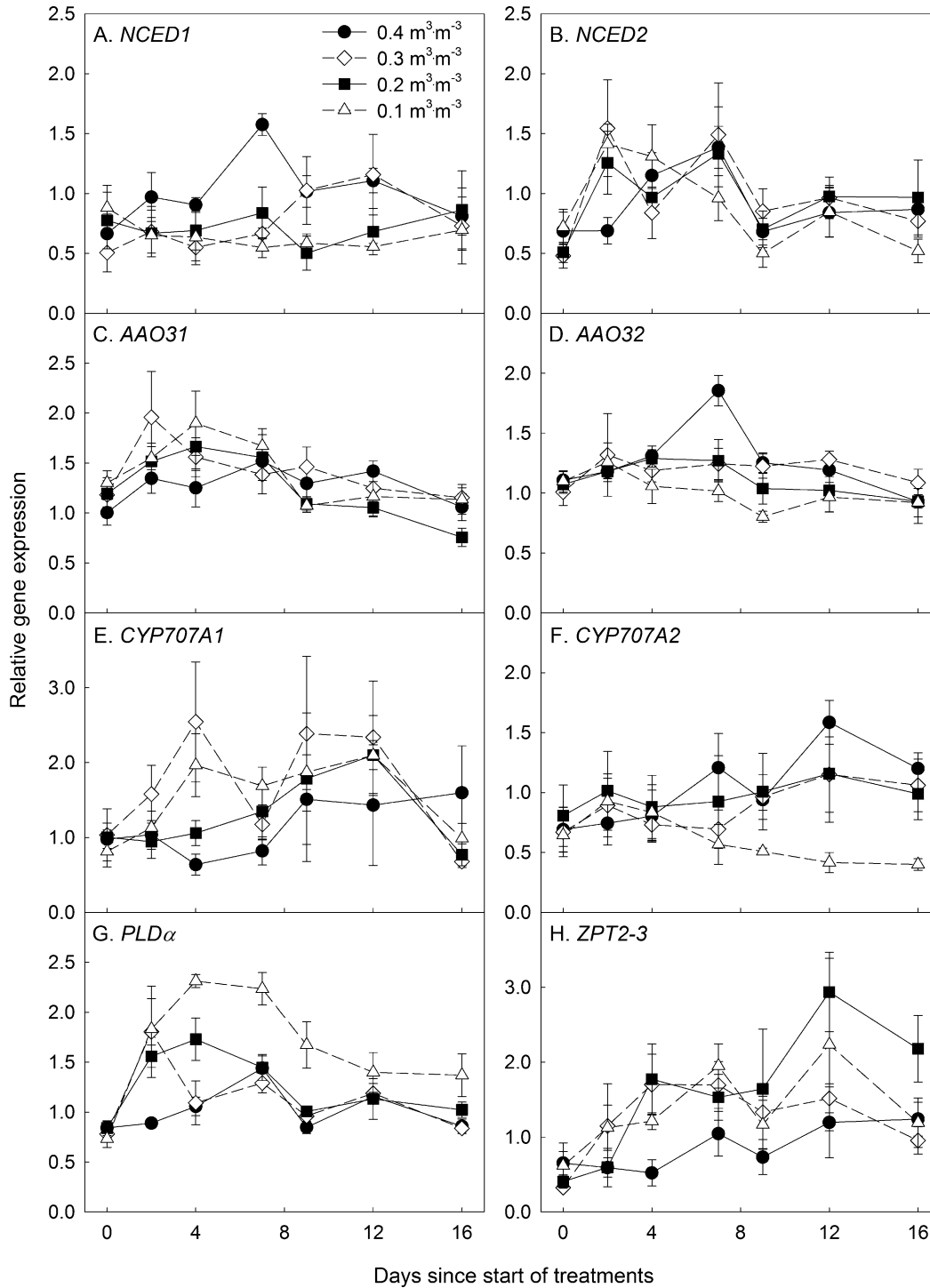
Fig. 4. Relative expression of abscisic acid metabolism-, signalling-, and drought tolerance-related genes (A, *NCED1*; B, *NCED2*; C, *AAO31*; D, *AAO32*; E, *CYP707A1*; F, *CYP707A2*; G, *PLDα*; H, *ZPT2-3*) in response to various substrate water contents (θ = 0.40, 0.30, 0.20, and 0.10 m³·m⁻³) during a 16 d period in *Petunia × hybrid* leaves. The drought treatments reached their threshold θ of 0.30, 0.20, and 0.10 m³·m⁻³ after 2.5, 3.7, and 8.8 d, respectively. Error bars indicate standard error (*n* = 4). Expression data were normalized using *EF1α* and *CYP*.

increased the expression of *ZPT2-3* within 4 d in all the drought treatments, but only plants at a θ of 0.20 m³·m⁻³ maintained higher expression levels than control plants throughout the study (Fig. 4H).

Relationships between ABA metabolism- and signalling-related genes and leaf ABA concentration

Although leaf ABA concentration was increased in all three drought treatments, the relative expression of ABA
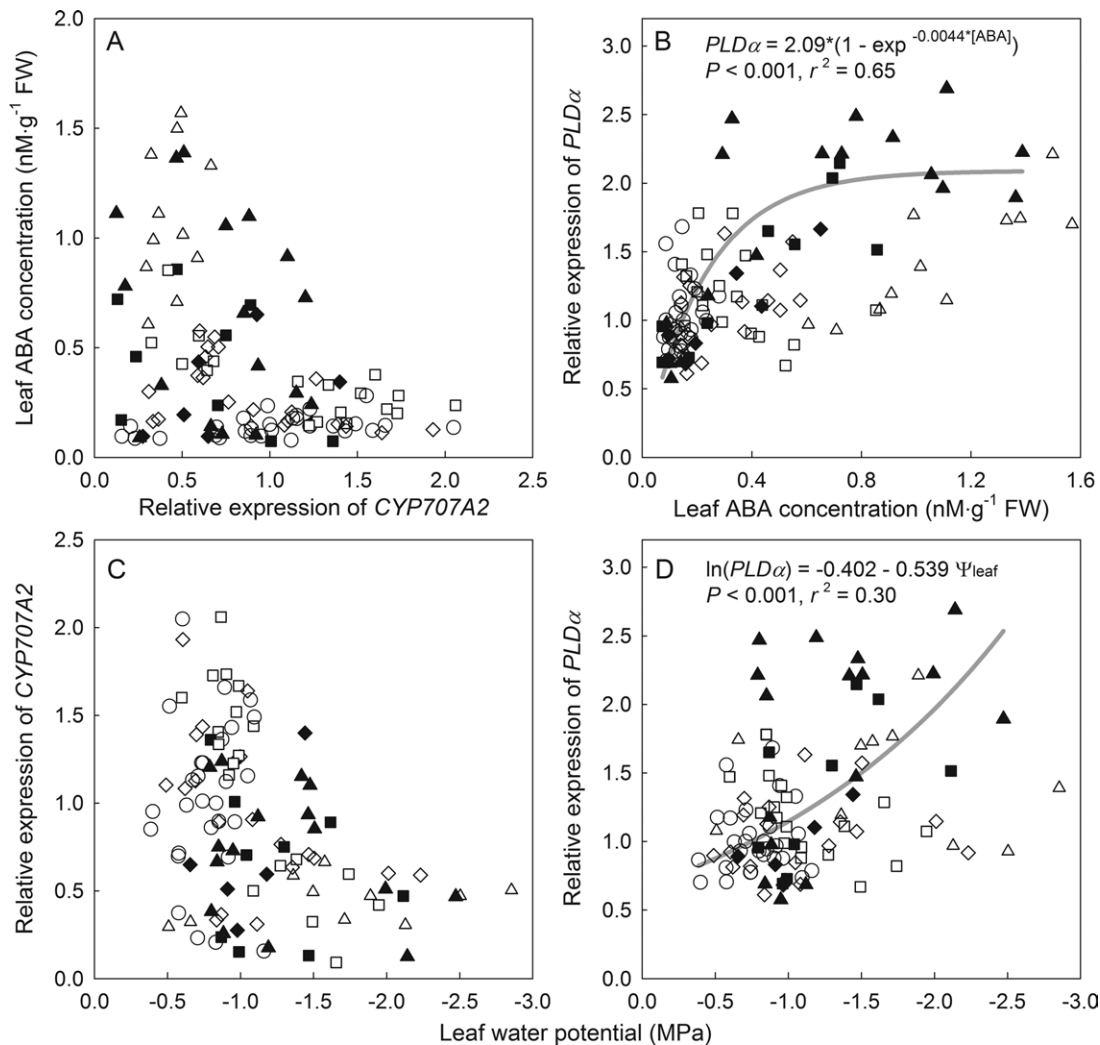
Fig. 5. Relationships between relative expression of *CYP707A2* and *PLDα* in *Petunia* leaves and leaf abscisic acid (ABA) concentration (A and B) and midday leaf water potential (Ψ_{leaf}; C and D). Substrate water content treatments: 0.40 (circles), 0.30 (diamonds); 0.20 (squares), and 0.10 $m^3 \cdot m^{-3}$ (triangles). Closed symbols indicate data collected during the drying period and open symbols indicate data collected after substrate water content thresholds had been reached. Regression curves indicate significant relationships between gene expression and leaf ABA concentration/midday leaf water potential during the drying period.

biosynthesis genes (*NCED1*, *NCED2*, *AAO31*, and *AAO32*) did not display an apparent relationship with either θ or leaf ABA concentration. However, when midday Ψ_{leaf} was low, the expression of *CYP707A2* was low, and this was associated with high concentrations of ABA in the leaves (Fig. 5A and 5C).

As ABA concentrations increased during the drying period, the expression of *PLDα* increased as well, suggesting that drought imposition not only increased leaf ABA concentrations, but also ABA-dependent signalling mechanisms associated with stomatal closing/opening (Fig. 5B). *PLDα* expression during the drying period was negatively correlated with midday Ψ_{leaf} ($P < 0.001$). However, expression of *PLDα* decreased in all drought treatments after the θ reached the threshold levels and was no longer correlated with midday Ψ_{leaf} (Fig. 5D).

Discussion

Physiological responses depend on the severity of drought stress

Stomatal conductance, A, Φ_{PSII}, and midday Ψ_{leaf} decreased during the drying period, but g_s and A of plants exposed to mild and moderate drought partially recovered after the θ reached the threshold levels (Fig. 2). This partial recovery of g_s and A suggests that the drought stress became less severe after the θ thresholds of 0.20 and 0.30 $m^3 \cdot m^{-3}$ had been reached. This may be related to the physical properties of soilless substrates. Moisture release curves for soilless substrates are hysteretic (Wallach, 2008). The frequent addition of small amounts of irrigation water after θ thresholds had been reached may have resulted in a higher substrate Ψ than that at the end of the drying period, despite similar θ, thus partially alleviating the drought stress. Therefore,

θ may have limitations as a quantitative indicator of the drought severity.

Leaf water potential is commonly used for determining the severity of drought stress (Jones, 2007), but there is no agreement about the best time of day to collect these data. Jones (2007) indicates that predawn measurement are less sensitive to environmental fluctuations and provide a better integrated short-term measure of plant water status than midday Ψ_{leaf}. However, Williams and Araujo (2002) found that predawn, midday Ψ_{leaf}, and midday Ψ_{stem} were all highly correlated with each other and that they were equally viable methods for assessing plant water status and physiological responses of grape (*Vitis vinifera* L.). Shackel *et al.* (1997) also concluded that midday Ψ_{stem} was a reliable method to quantify drought stress of fruit trees. The current results show clear relationships ($P < 0.001$) between midday Ψ_{leaf} and leaf responses to drought (Fig. 6), confirming that midday Ψ_{leaf} is a useful indicator of drought-stress severity. Stomatal conductance, A, and Φ_{PSII} decreased with decreasing midday Ψ_{leaf} ($P < 0.001$), while leaf ABA concentration increased with lower midday Ψ_{leaf}

($P < 0.001$). These results suggest that midday Ψ_{leaf} is a better indicator of drought-stress severity than θ, but it is important to realize that correlations between midday Ψ_{leaf} and other physiological responses do not indicate a causal relationship. There is no evidence that regulatory systems in plants are controlled by Ψ_{leaf}, and cell volume or turgor may be the signal perceived by plants (Jones, 2007). Quantifying the severity of the stress in drought studies is important and these results show that maintenance of θ at specific thresholds results in differences in midday Ψ_{leaf} and can be used to achieve different severities of drought. The differences in midday Ψ_{leaf} were achieved by controlled changes and maintenance of θ at specific thresholds, indicating that θ can be used to achieve different severities of drought. Further, these results indicate that quantifying the severity of stress in drought studies is important.

Severity of drought stress, leaf ABA, and g_s

A clear and major increase in ABA concentration was observed in all the drought treatments. As with the physiological responses,
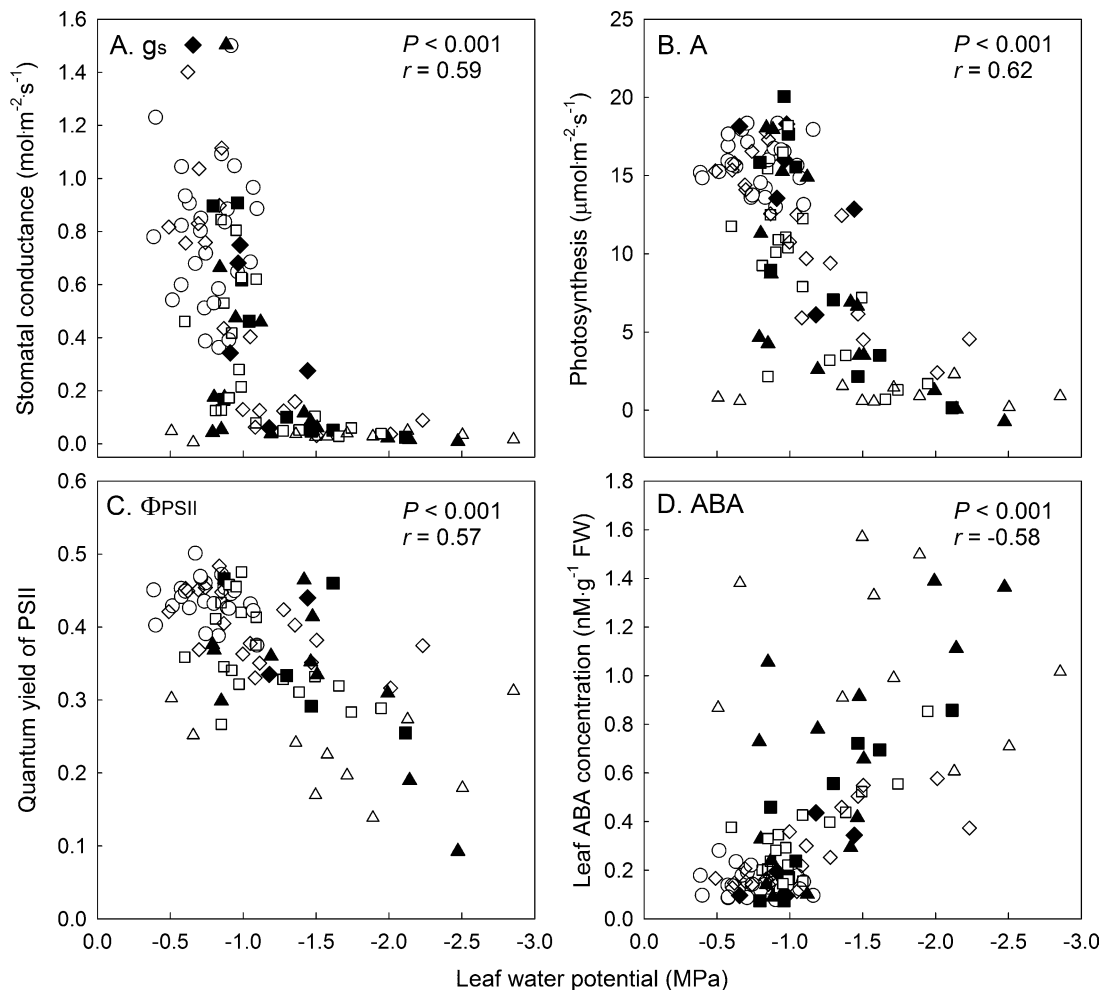

Fig. 6. Leaf physiological responses of *Petunia* as a function of midday leaf water potential (Ψ_{leaf}). Stomatal conductance (g_s, panel A), photosynthesis (A, panel B), and quantum yield of PSII (Φ_{PSII}, panel C) were measured by a leaf photosynthesis measurement system and midday leaf water potential (Ψ_{leaf}, panel D) was measured using thermocouple psychrometers. Substrate water content treatments: 0.40 (circles), 0.30 (diamonds); 0.20 (squares), and 0.10 m³·m⁻³ (triangles). Closed symbols indicate data collected during the drying period and open symbols indicate data collected after substrate water content thresholds had been reached. *P*- and *r*-values indicate the results from Spearman's rank order correlation performed on the combined data.

the extent of increase in ABA concentrations depended on the severity of drought, and more specifically on midday Ψ_{leaf} (Fig. 6), with severe drought resulting in the highest increase in ABA concentrations.

Previous research suggested that g_s is better correlated with xylem ABA concentration than with leaf ABA concentration (Tardieu and Davies, 1993; Heilmeier *et al.*, 2007; Jiang and Hartung, 2008). However, the current results show a strong correlation between leaf ABA concentration and g_s in *Petunia*, regardless of θ or time, suggesting that g_s is controlled by leaf ABA concentration (Fig. 7). Tardieu and Davies (1993) also reported that the effect of xylem ABA on g_s depends on midday Ψ_{leaf}. Using multiple regression analysis, the current study found that g_s was correlated with both leaf ABA concentration and midday Ψ_{leaf}, but their interaction was not significant. The partial r^2 of ABA and midday Ψ_{leaf} in the regression were 0.84 and 0.02, respectively, indicating that most variation in g_s could be explained by changes in leaf ABA concentration, with only a minor contribution of midday Ψ_{leaf}.

Expression of ABA-related genes responds to the severity of drought stress

Despite the increase in leaf ABA concentration under drought, there were no significant changes in the expression of the ABA biosynthesis-related genes *NCED* and *AAO3* in *Petunia* leaves. Previous studies indicated that *NCED* (Thompson *et al.*, 2000;
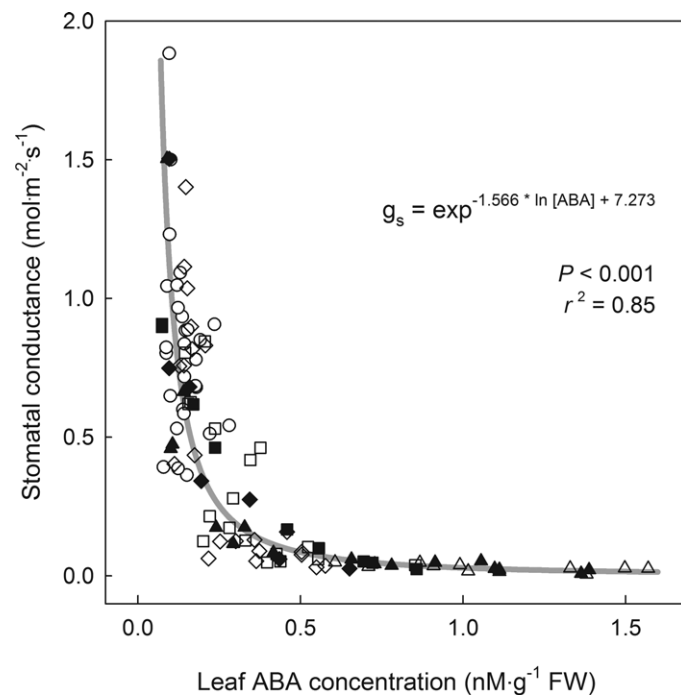

$$g_s = \exp^{-1.566 * \ln[ABA] + 7.273}$$

$P < 0.001$
$r^2 = 0.85$

Fig. 7. Leaf ABA concentration effects on stomatal conductance. Data from all treatments and the entire study period are combined. Substrate water content treatments: 0.40 (circles), 0.30 (diamonds); 0.20 (squares), and 0.10 $m^3 \cdot m^{-3}$ (triangles). Closed symbols indicate data collected during the drying period and open symbols indicate data collected after substrate water content thresholds had been reached.

Harb *et al.*, 2010) and *AAO3* (Seo *et al.*, 2000) are key regulatory genes involved in ABA biosynthesis (Nambara and Marion-Poll, 2005). This suggests that *de novo* ABA synthesis may not be a significant contributor to the increase in ABA concentrations in *Petunia* leaves during drought. It also is possible that the putative *NCED* genes identified in the current study, *NCED1* and *NCED2*, do not regulate ABA biosynthesis in response to drought, but may have primary roles in maintaining normal ABA concentrations. It is possible that other, as yet unidentified, members of the *NCED* family in *Petunia* may specifically regulate ABA biosynthesis under drought. In *Arabidopsis*, at least three *NCED* genes have been identified, among which primarily At*NCED3* was responsive to drought stress (Iuchi *et al.*, 2001; Harb *et al.*, 2010).

Interestingly, the relative expression of the ABA catabolism-related gene *CYP707A2* was related to the ABA concentration. *Petunia* leaves with low midday Ψ_{leaf} had lower expression of *CYP707A2* and higher ABA concentration than those at higher midday Ψ_{leaf} (Fig. 5A and 5C), suggesting that severe drought decreases ABA catabolism. The results of the expression of the putative *CYP707A2* agree with previous findings, implicating it in regulating ABA homeostasis (Umezawa *et al.*, 2006). These data suggest that low ABA catabolic activity may be important in maintaining high ABA concentrations in *Petunia* leaves during drought.

PLDα was upregulated in *Petunia* leaves as θ decreased with the highest expression noted under severe drought. Further, the increase in *PLDα* expression was evident primarily during the drying period and was correlated with g_s (Fig. 5B), suggesting that decreasing θ enhanced the expression of *PLDα* thereby promoting stomatal closing. Harb *et al*. (2010) also reported an increase of *PLDα1* as an early response in *Arabidopsis* leaves under moderate drought stress (employed by maintaining the substrate at 30% of field capacity). Phosphatidic acid generated through PLDα1 activity binds to ABI1/PP2C, a key component of ABA signalling involved in stomatal regulation in *Arabidopsis* (Mishra *et al.*, 2006). Hence, increase in *PLDα1* expression and subsequent PLDα1 activity may therefore mediate ABA-dependent stomatal closure during the drying phase of drought imposition. *PLDα* expression decreased in all drought treatments after the θ thresholds were reached, which may be related to a partial alleviation of the drought stress.

ZPT2-3 was upregulated under cold, drought, and heavy metal stress (Sugano *et al.*, 2003). Consistent with this report, in the current study, *ZPT2-3* expression increased in the drought treatments during the drying period. Overexpression of *ZPT2-3* and its homologues enhanced drought tolerance in *Petunia* and *Arabidopsis*, respectively (Sugano *et al.*, 2003; Sakamoto *et al.*, 2004). Hence, increase in *ZPT2-3* expression during the drying period may result in the transcriptional regulation of downstream genes associated with mechanisms that mediate drought tolerance. Higher levels of *ZPT2-3* expression at later stages in the moderate drought treatment further support a role for this gene in regulating drought tolerance mechanisms.

In conclusion, by precisely controlling θ and conducting temporal analyses of physiological and molecular responses, this study demonstrates differences in plant responses to varying severity of drought stress. All *Petunia* plants at θ < 0.40 $m^3 \cdot m^{-3}$ displayed an increase in leaf ABA concentrations, but no

significant changes in the relative expression of ABA biosynthesis-related genes were observed. There was a significant decrease in the expression of *CYP707A2*, a putative ABA catabolic gene, in plants under severe drought stress. This suggests that maintaining high ABA concentration in leaves of plants under severe drought is at least partially associated with decreased ABA catabolism. The results also show a close correlation between leaf ABA concentration and g_s, regardless of stage of drought or θ, suggesting that g_s was regulated by leaf ABA concentration. Higher expression of *PLDα* during the drying period observed in all the drought treatments may promote ABA-dependent stomatal closure. Leaf physiological responses (g_s, A, and Φ_{PSII}) and ABA concentration were correlated with midday Ψ_{leaf}. The results indicate that the drought response of plants varies depending on the severity of drought stress and that this severity can be manipulated by controlling θ. Accurate descriptions of the severity and method of imposing drought stress deserve more attention, since they can greatly affect plant responses.

Supplementary data

Supplementary data are available at *JXB* online.

Supplementary Fig. S1. Schematic diagram of the soil moisture sensor-based irrigation system.

Supplementary Table S1. List of genes and sequence of the primers used for qRT PCR.

Supplementary Table S2. Morphological and physiological changes of *Petunia × hybrida* 'Apple Blossom' in response to various substrate water contents at 16 d after the start of the drying treatment.

Acknowledgements

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