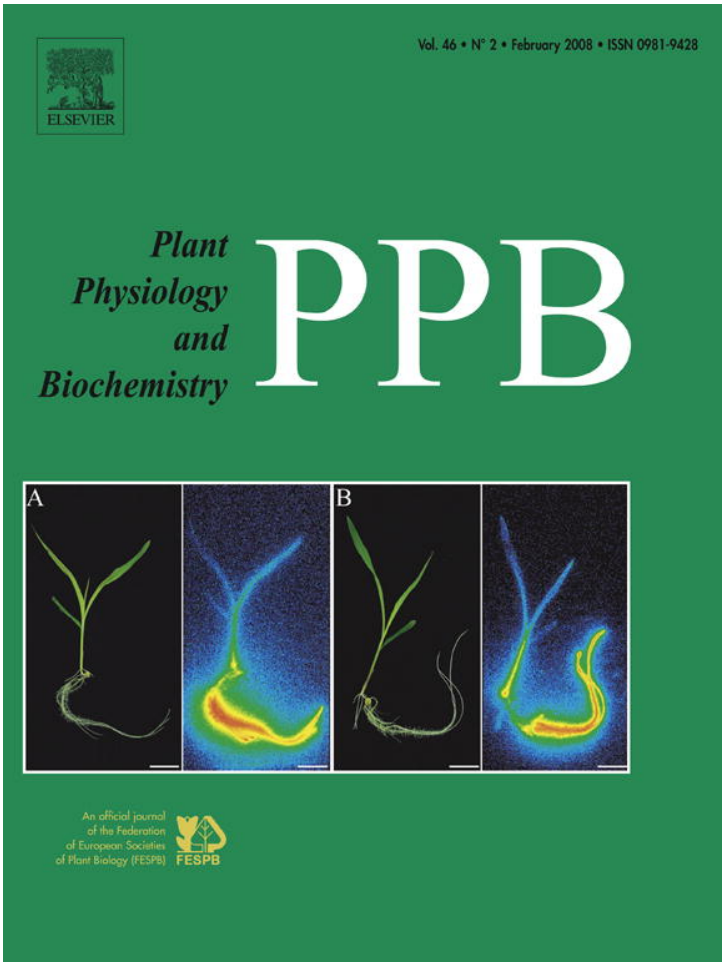

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Research article

The drought response of *Theobroma cacao* (cacao) and the regulation of genes involved in polyamine biosynthesis by drought and other stresses

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Abstract

Drought can negatively impact pod production despite the fact that cacao production usually occurs in tropical areas having high rainfall. Polyamines (PAs) have been associated with the response of plants to drought in addition to their roles in responses to many other stresses. The constitutive and drought inducible expression patterns of genes encoding enzymes involved in PA biosynthesis were determined: an ornithine decarboxylase (*TcODC*), an arginine decarboxylase (*TcADC*), an *S*-adenosylmethionine decarboxylase (*TcSAMDC*), a spermidine synthase (*TcSPDS*), and a spermine synthase (*TcSPMS*). Expression analysis using quantitative real-time reverse transcription-PCR (QPCR) results showed that the PA biosynthesis genes were expressed in all plant tissues examined. Constitutive expression of PA biosynthesis genes was generally highest in mature leaves and open flowers. Expression of *TcODC*, *TcADC*, and *TcSAMDC* was induced with the onset of drought and correlated with changes in stomatal conductance, photosynthesis, photosystem II efficiency, leaf water potential and altered emission of blue-green fluorescence from cacao leaves. Induction of *TcSAMDC* in leaves was most closely correlated with changes in water potential. The earliest measured responses to drought were enhanced expression of *TcADC* and *TcSAMDC* in roots along with decreases in stomatal conductance, photosynthesis, and photosystem II efficiency. Elevated levels of putrescine, spermidine, and spermine were detected in cacao leaves 13 days after the onset of drought. Expression of all five PA associated transcripts was enhanced (1.5–3-fold) in response to treatment with abscisic acid. *TcODC* and *TcADC*, were also responsive to mechanical wounding, infection by *Phytophthora megakarya* (a causal agent of black pod disease in cacao), the necrosis- and ethylene-inducing protein (Nep1) of *Fusarium oxysporum*, and flower abscission. *TcSAMDC* expression was responsive to all stresses except flower abscission. *TcODC*, although constitutively expressed at much lower levels than *TcADC*, *TcSAMDC*, *TcSPDS*, and *TcSPMS*, was highly inducible by the fungal protein Nep1 (135-fold) and the cacao pathogen *Phytophthora megakarya* (671-fold). The full length cDNA for ODC was cloned and characterized. Among the genes studied, *TcODC*, *TcADC*, and *TcSAMDC* were most sensitive to induction by drought in addition to other abiotic and biotic stresses. *TcODC*, *TcADC*, and *TcSAMDC* may share signal transduction pathways and/or the stress induced signal induction pathways may converge at these three genes leading to similar although not identical patterns of expression. It is possible altering PA levels in cacao will result in enhanced tolerance to multiple stresses including drought and disease as has been demonstrated in other crops.
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Keywords: Polyamine; *Theobroma cacao*; Drought; Nep1; Wounding; *Phytophthora*; ODC; ADC; SAMDC; SPDS; SPMS

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1. Introduction

Theobroma cacao (cacao) is a tropical tree typically grown in areas of high annual rainfall. Although somewhat counter-intuitive, cacao production is prone to periodic drought due to seasonal rainfall patterns that often include prolonged dry cycles. *Theobroma cacao* (cacao) is intolerant of drought [1,11,48,69], although very little research has been directed towards the identification and development of drought tolerant germplasm [10]. The limited drought tolerance of cacao is a growing concern in cacao production areas due to inconsistent rainfall patterns [1,10,11,48,69]. Cacao production is often limited by other abiotic stresses, such as nutrient deficiencies, and by biotic stresses, such as disease and insects [69].

Polyamines (PAs) are ubiquitous in both eukaryotes and prokaryotes (reviewed in [16,35]). The most common PAs in higher plants are putrescine (Put), spermidine (Spd) and spermine (Spm). PAs have important roles in plant physiological and developmental processes, such as cell division, regulation of morphogenesis, embryogenesis, floral initiation and development, flower and fruit development and ripening, leaf senescence, root growth, and tuberization. In plants, PAs are commonly associated with responses to biotic and abiotic stresses and have been shown to function in drought and chilling tolerance in some situations (reviewed in [12,30]).

In higher plants, there are two main pathways for PA biosynthesis (Fig. 1, modified from [27]). In eukaryotic cells, Put is synthesized directly from ornithine, through the activity of ornithine decarboxylase (ODC). In plants, there is an alternative pathway for Put synthesis, via the activity of arginine decarboxylase (ADC) and *Arabidopsis* lacks a sequence for ODC [21]. Spd and Spm are synthesized by spermidine synthase (SPDS) and spermine synthase (SPMS), respectively.

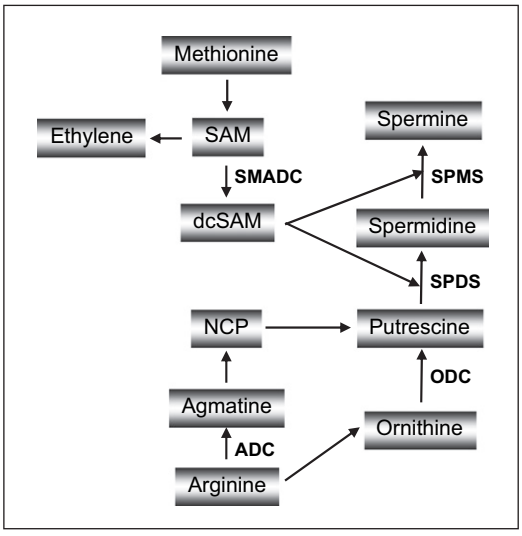

Fig. 1. Polyamine biosynthetic pathway in plants (modified from [27]). SAM, *S*-adenosylmethionine; SAMDC, *S*-adenosylmethionine decarboxylase; dcSAM, decarboxylated *S*-adenosylmethionine; NCP, *N*-carbamoylputrescine; ADC, arginine decarboxylase; SPMS, spermine synthase; SPDS, spermidine synthase; ODC, ornithine decarboxylase.

Spd and Spm are also synthesized by the successive addition of aminopropyl groups derived from decarboxylated *S*-adenosylmethionine (dcSAM) that is generated by *S*-adenosylmethionine decarboxylase (SAMDC).

In some cases the genes involved in PA biosynthesis are regulated developmentally and may show tissue specificity [17,24,25,46,51,59]. In addition, both ODC and ADC are known to be induced by various stresses, such as disease [18,71], chilling [24], osmotic stress [43,47,59], acidic pH [43,50], and nutrient deficiency [43]. SAMDC also plays an important role in plant developmental and physiological processes, as well as in plant responses to environmental stresses [25,54,64].

The study of PAs and the associated genes involved in their biosynthesis is limited in *Theobroma cacao*. Understanding the regulation of PA biosynthesis may aid in the development of stress management strategies and the selection of stress tolerant cacao. We have identified ESTs encoding ODC (*TcODC*), ADC (*TcADC*), SAMDC (*TcSAMDC*), SPDS (*TcSPDS*), and SPMS (*TcSPMS*) in cacao. The primary objectives of this research were to determine the tissue specific and developmental expression patterns of these five ESTs encoding enzymes involved in polyamine biosynthesis and to determine the relationship between their expression and physiological measures of the drought response in cacao. In addition, their inducibility by ABA and their responsiveness to multiple stresses were studied.

2. Methods

2.1. Plant materials and drought treatment

Open pollinated cacao seeds (*Theobroma cacao* variety comun, Lower Amazon Amelonado type) were harvested by Alan Pomella from the Almirante Cacau, Inc. farm (Itabuna, Bahia, Brazil) and shipped to Beltsville, MD. After removing the seed coat, seeds were surface sterilized in 14% sodium hypochlorite for 3 min followed by 3 washes in sterile distilled water. Three sterile seeds were placed on 1.5% water agar plate (100 mm in diameter) and the plates were sealed with parafilm. The plates were incubated under fluorescent lights at 22 °C for 3 days until germinated.

For the drought experiments, germinated seeds were planted 3-cm deep in sterile soilless mix (2:2:1, sand/perlite/promix), in double Magenta boxes (77 × 77 × 194 mm; Chicago, IL), in which the sterile soilless mix was added up to 9 cm. Four holes (0.5 cm in diameter) were made on the bottom of the magenta boxes and the holes were taped. Sterile distilled water (20 ml) was added to the soilless mix after planting and seedlings were grown in a controlled environment chamber (model M-2, EGC Corp., Chagrin Falls, OH) for 3 weeks with 12-h light/12-h dark photoperiod at 25 °C. The irradiance was 50 μmol m^{-2} s^{-1} photosynthetically active radiation (PAR). Relative humidity was not controlled in this experiment, but relative humidity was always above 50%. After 14- or 16-day growth in the double magenta box system, the upper boxes and tape on the box bottoms were removed.

The soilless mix was saturated with distilled water every other day. After 32 days growth in the magenta box system, watering was stopped for 13 days, while control plants were watered every other day. Six replications were carried out for each treatment/time combination. The replications were split between 2 experiments of 4 and 2 replications using different seed shipments. Seedling roots and the largest leaves were harvested for quantitative real-time reverse transcription PCR (QPCR). The second largest leaves were harvested for PA measurement.

For wounding, *Phytophthora megakarya*, and Nep1 treatments, seeds were planted in 15.2-cm pots filled with soilless mix (2:2:1, sand/perlite/promix). Seedlings were grown under greenhouse conditions with ambient light for up to 9 months or longer. The temperature was maintained between 20 °C and 29 °C, and humidity was maintained above 75% during daylight hours using a misting system (Atomizing Systems, Ho-Ho-Kus, NJ).

Leaves from greenhouse grown cacao seedlings (described above) were used to study the effects of development on gene expression. Cacao leaf development was divided into four stages [8]: stage (1), unexpanded leaves (UE), less than 1 cm long with limited pigmentation; stage (2), young red leaves (YR), 5–10 cm long and pliable; stage (3), immature green leaves (IG) 10–20 cm long and pliable; and stage (4), mature green leaves (MG), 10–20 cm and rigid.

Cacao flowers were harvested from mature cacao trees (more than 8 years old) grown under greenhouse conditions. Cacao flower development was divided into 5 stages: stage (1), elongated unopened green flower (EG), less than 0.2-cm long with only green sepal; stage (2), unopened white small flower (US), less than 0.5-cm long with green sepal and white petal; stage (3), unopened white large flower (UL), less than 1-cm long with green sepal and white petal; stage (4), open white flower (OW); stage (5), abscised flower (AB). Cacao seeds were incubated on water agar plates under fluorescent lights at 22 °C for 1 day and then whole seeds were harvested.

2.2. Stress treatments

Leaf disks (8.5 cm in diameter) were cut from detached MG leaves from greenhouse-grown plants and placed in petri-dishes abaxial side up on sterile no. 2 Whatman paper moistened with sterile distilled water. The 8.5-cm leaf disks were inoculated with 20 drops of 20 μl zoospores of *P. megakarya* strain GWH 252 (3×10^4 zoospores ml^{-1}), while 20 drops of 20 μl distilled water were applied for control as previously described by Bailey et al. [7]. The petri-dishes were sealed with parafilm and incubated at 25 °C for up 3 days under dim light. Leaf disks were harvested 0–3 days after inoculation for three replications.

For mechanical wound treatment, a Groomax™ hard slicker brush (Pacific Coast Distributing Inc., Phoenix, AZ) was used over selected leaves backed with Styrofoam [8]. Controls were unwounded leaves on separate seedlings. MG leaves from greenhouse-grown plants were harvested 0, 0.25, 4, and 20 h after wounding for three replications.

Necrosis- and ethylene inducing peptide 1 (Nep1) from *Fusarium oxysporum* (5 μg ml^{-1} plus 0.2% v/v Silwet-L77) was applied to MG leaves of greenhouse-grown plants at a rate of 46 ml m^{-2} between 10:00 and 11:00 a.m., a time point when stomata were consistently open [7]. Silwet-L77 (0.2% v/v) alone was applied as a control. Seedlings were maintained under greenhouse conditions and leaves were harvested at 0, 0.5, 4, and 24 h after spray application for three replications.

2.3. Quantitative real-time reverse transcription PCR (QPCR)

Total RNA was isolated from cacao seedlings (root, stem and leaf), seed, pod and flower, and the extracted RNA was treated with DNase I as previously described by Bailey et al. [8]. Procedures for cDNA synthesis, QPCR conditions, and data analysis were as described by Bae et al. [6]. *ACTIN* (CF973918), a constitutively expressed gene, was used as an expression control. Primer sequences for the ESTs being studied were obtained from cacao accessions in GenBank and are presented in Table 1. Threshold cycle (C_T) values for all genes of interest ($C_{T.GOI}$) were normalized to the C_T values of *ACT* ($C_{T.ACT}$) for each replication [$\Delta C_T = (C_{T.ACT}) - (C_{T.GOI})$]. Relative transcript levels of each gene were normalized with respect to cacao *ACTIN* transcript levels (% of *ACTIN*). The fold changes in gene expression were then obtained from the equation [Fold change $= (E) \times \Delta C_T$] as described previously by Pfaffl [53]. Mean values were obtained from 3 to 4 biological replications, and the error bars indicate the standard error of the mean.

2.4. 5′ RACE (rapid amplification of cDNA ends)

In order to clone the full length of *TcODC*, 5′ RACE was performed using the RACE system (Invitrogen, Carlsbad, CA). The PCR product was separated by electrophoreses in an 0.8% agarose gel, purified by using a QIAEX II Gel Extraction System (Qiagen, Valencia, CA), cloned into pCR2.1-TOPO vector (Invitrogen, Calsbad, CA), and prepared for sequencing. DNA sequences were determined by the University of Maryland, DNA Sequencing Facility (http://www.umbi.umd.edu/~cbr/dna.html).

2.5. Measurement of leaf gas exchange, chlorophyll fluorescence, and leaf water potential

A portable photosynthesis system (LI-6400; LI-COR, Inc., Lincoln, NE, USA) with a leaf chamber fluorometer (LI6400-40) was used to determine net CO_2 assimilation rate (A), stomatal conductance (g_s), and the light adapted photochemical efficiency of photosystem II (Φ_{PSII}). Gas exchange and chlorophyll fluorescence measurements were made at steady-state with the following conditions: photosynthetically active radiation (PAR) = 300 μmol m^{-2} s^{-1}; leaf temperature = 25.9 °C; vapor pressure deficit = 1.13 kPa; $[CO_2] = 400$ μmol mol^{-1}. Water potentials were determined using leaf discs excised from the second largest leaf from each plant. Leaf discs were sealed immediately after excision and placed in insulated

Table 1
Primer sequences used for QPCR analysis in cacao

Gene encoding	Accession no.	Sequence (5′ to 3′)	Expected size (bp)	Identity (%)/E-value (species)
Ornithine decarboxylase (*TcODC*)	DW246134	F: GTGTTAACCTGACAACTTTCGACTC R: AATGTGGAACGAGACTCCTGTAAC	218	68/2E−150 (*Nicotiana tabacum*)
Arginine decarboxylase (*TcADC*)	AF045666	F: AGAAGAGGAGGTGGATATGGTTATT R: GATTTGGGATCCAATATGGAAGTG	235	80/0.0 (*Arabidopsis thaliana* ADC2)
S-Adenosyl methionine decarboxylase (*TcSAMDC*)	CF974649	F: ATAATCTAATGGAGTCTAAAGGCGG R: AAACAACAGCTGATTGACCACTAC	216	100/9E−14 (*Daucus carota*)
Spermidine synthase (*TcSPDS*)	CA796906	F: TACCAGAATGTTATGGTCTTCCAGT R: CTCAACAAGAAGAATGACGAGAAAC	214	78/8E−54 (*Arabidopsis thaliana* SPDS2)
Spermine synthase (*TcSPMS*)	CA797160	F: AACTCTCACTGACATTAACACCAGA R: AGAGGAAGACTGATATTCCGAGAAC	244	67/6E−29 (*Arabidopsis thaliana* SPMS3)
Apoplastic quiacol peroxidase-like (*TcPER-1*)	CK144296	F: GATCCGAACCTGAACACTACATACT R: GCTGTTTGATTAGCACTAAACCTGT	233	87/4E−64 (*Gossypium hirsutum*)
Photosystem I 24 kDa protein-like (*TcLHCA-1*)	CK144297	F: TAACGAGGTGAAGAATGGGAGAT R: ATCAAGAAAGGACAAGAACGAGAG	228	91/2E−19 (*Nicotiana tabacum*)
Chitinase (*TcCHIB*)	CF973685	F: AACAGCTCCATAGTTGTAGTTCCAG R: GGTAAGCTTATGCAAATGAAGGAG	246	75/32E−66 (*Gossypium hirsutum*)
Actin (*TcACT*)	CF973918	F: CAGACTTTGAGTTCACTTGACACAG R: AGTGTCTGGATTGGAGGATCTATCT	200	100/42E−15 (*Gossypium hirsutum*)

F, forward; R, reverse.

Wescor C-52 sample chambers (Wescor, Inc, Logan, UT, USA). Water potentials were determined after equilibration periods of up to 2.5 h, using a Wescor HP 115 water potential system. Leaf gas exchange, chlorophyll fluorescence, and water potentials were measured near mid-photoperiod of 0, 7, 10, and 13 days after watering with 7 or 8 plants in each treatment.

2.6. Multispectral fluorescence imaging system

The imaging system and method used in this study were described by Kim et al. [34]. Four longwave UV-A fluorescent lamps were used as the excitation light source (12-W; Model XX-15A, Spectronics Corp., New York, USA). The intensity of UV excitation was 0.33 mW cm^{-2} with an emission maximum at 360 nm at the target area. To eliminate wavelengths greater than 400 nm, the radiation from the UV lamps were filtered with Schott UG-1 glass. The fluorescence images from cacao leaves were captured using a back-illuminated, thermoelectrically cooled CCD camera (PixelVision, OR, USA). A Nikon f 1.4/35 mm lens was coupled to a common aperture multispectral adapter and the coupled lens were used to collect the fluorescence emissions (MSAI-04, Optical Insights, AZ, USA). Two interference filters were used in this study: a blue filter at 450 nm with a 25 nm full width at half maximum (FWHM) and a green filter at 530 nm with a 25 nm FWHM.

2.7. Measurement of PA content

Polyamines were determined by an HPLC procedure using pre-column derivatization by the AccQ Taq method (Waters Corp., Milford, MA, USA). Cacao leaf tissue was ground to a fine powder under liquid N and lyophilized. Approximately 50 mg DW of each sample was homogenized at room temperature with 2 ml of 70% methanol containing 0.1 N HCl. The homogenized samples were left standing at room temperature for 1 h and were then incubated for 15 min at 45 °C in a H_2O bath. The extracts were then centrifuged for 15 min at 5800 × *g* at 15 °C. The resultant pellets were washed twice with 1 ml of the acidified solvent and centrifuged as above. The extracts were evaporated to dryness under a stream of N_2 at 37 °C. The dried samples were resuspended in 0.5 ml of 20 mM HCl and then centrifuged through a 0.22 μm Ultrafree-MC membrane filter unit (Millipore Corp., Bedford, MA). Tissue extracts and standards were derivatized with the AccQ Fluor kit (WAT070200) from Waters according to the manufacturer's instructions. Separations were performed essentially as described by Merali and Clarkson [45] using a Waters 600E Multisolvent Delivery System equipped with a 4.6 × 150 mm C_{18} (5 μm) column (Phenomenex, Torrance, CA). Column flow was 1 ml min^{-1} and the elution gradient was prepared with eluent A (140 mM sodium acetate and 17 mM triethanolamine) and eluent B (acetonitrile). The column was equilibrated with 83% A and 17% B before injecting 10 μl samples. This was followed by a linear gradient ending with 70% A and 30% B at 10 min, a linear gradient ending with

60% A and 40% B at 20 min and a linear gradient ending with 40% A and 60% B at 30 min. The final step was held for 1 min before regenerating the column. Detection was with a Gilson model 121 fluorometer using excitation and emission wavelengths of 250 and 395 nm, respectively. The output of the detector was monitored using Empower2 software from Waters.

2.8. Data analyses

Biology WorkBench (http://workbench.sdsc.edu/) was used to analyze DNA and protein sequences. A phylogenetic tree was generated based on amino acid sequences, using Mega3 software (http://www.megasoftware.net/).

3. Results

3.1. Tissue and organ specific expression of PA biosynthesis genes

To examine steady-state expression patterns in various plant organs and tissues, total RNA was extracted from 4 leaf stages and 5 flower stages as well as from stems, roots, seeds, and green pods. Quantitative real-time reverse transcription PCR (QPCR) results (Fig. 2) showed that *TcADC*, *TcSAMDC*, *TcSPDS* and *TcSPMS* transcripts were highly expressed, while *TcODC* was the least abundant transcript in all tissues examined (approx. 100 times less than *TcADC*). The expression patterns of transcripts encoding putative peroxidase (*TcPER-1*), light harvesting complex protein in photo system II (*TcLHCA-1*), and chitinase (*TcCHIB*) were monitored as controls. *TcPER-1* was most highly expressed in expanding leaves (YR and IG), seed, and open flowers. *TcLHCA-1* was most highly expressed in mature green leaves, and *TcCHIB* showed the least dependence on tissue type or developmental stage. Each transcript exhibited different expression patterns in the tissues and organs examined. The highest level of *TcODC* transcript was detected in mature green leaves, stems, roots, and open white flowers. *TcADC* transcript accumulated mainly in the unopened large flowers and opened flowers, mature green leaves, and stems. *TcSAMDC* was highly expressed in mature green leaves and open flowers. *TcSPMS* was highly expressed in mature green leaves with generally similar levels of expression in all other tissues, while *TcSPDS* was highly expressed in mature green leaves, all flower stages, and seed.

3.2. Induction of gene expression in response to drought

During drought treatments, we monitored photosynthesis, stomatal conductance, photosystem II efficiency, and leaf water potential (Fig. 3). After withholding water for 7 days, values for net photosynthesis, stomatal conductance, and photosystem II efficiency were reduced compared to watered controls at the same time point and decreased for the duration of the experiment. Leaf water potential decreased after withholding water for 10 days and continued to decrease out to 13 days.

Increased leaf fluorescence was observed in the blue (F450, data not shown) and green (F530) spectral ranges starting 10 days after withholding water (Fig. 4). Fluorescence emission was greater for the drought-treated leaves than for control leaves 10 days after withholding water (Fig. 4). Blue fluorescence was higher in the vascular tissues than in the inter-veinal regions. Pairwise comparisons of the mean relative intensities for each fluorescence band (the blue band at 450 nm and the green band at 530 nm) showed a significant difference at $\alpha = 0.05$ after 10 days withholding water (data not shown).

Free Put accumulated in response to drought, reaching 4-fold higher concentrations in mature green leaves of cacao seedlings 13 days after withholding water (Fig. 5). Free Spd and Spm concentrations also increased in response to drought.

Leaves from seedlings responding to drought treatment showed similar induction patterns for *TcODC*, *TcADC* and *TcSAMDC*, while *TcSPDS* and *TcSPMS* transcript levels were not changed (Fig. 6). The induction of *TcODC* (induction 10.7-fold), *TcADC* (induction 4.0-fold), and *TcSAMDC* (induction 4.9-fold) was observed after withholding water for 10 days and increased out to 13 days. In these same leaves, *TcPER-1* was induced by drought up to 4.4-fold and *TcCHIB* was repressed by drought as much as 74%. *TcLHCA-1* was repressed by drought after 10 and 13 days as much as 57%.

In the root tissues, *TcADC* was rapidly induced by drought reaching near maximum expression after 7 days without water (Fig. 7). *TcSAMDC* was induced after 7 days without water and reached maximum expression after 10 days. *TcODC* was induced and reached maximum expression after 10 days without water. Although less than 2-fold induced, *TcSPDS* and *TcSPMS* tended to be expressed at higher levels in seedling roots after water was withheld. Only limited changes were observed in the expression of *TcPER-1*, *TcLHCA-1*, and *TcCHIB* in roots responding to drought.

The observed changes in expression of *TcSAMDC* in the leaves were highly correlated (correlation coefficient = −0.72) with changes in leaf water potential (Table 2) and expression of *TcODC* in the leaves was the second most highly correlated (correlation coefficient = −0.65) with changes in water potential. Changes in *TcADC* expression in the roots were most highly correlated (correlation coefficient = 0.75) with the drought treatment (plus or minus drought) followed by *TcSAMDC* expression in the roots (correlation coefficient = 0.59).

3.3. Induction of PA biosynthesis genes by ABA

Expression of all five ESTs associated with polyamine biosynthesis was enhanced by treatment with ABA (Table 3). In general, the induction levels were between 1.5- and 3-fold in leaves and roots 1 and 3 h after ABA treatment. The primary exception was *TcODC* which was not induced in roots until after 12 h of treatment. ABA treatment for 12 h was associated with a reversal in leaf transcript levels to levels 40–50% below that found in controls for *TcODC*, *TcADC*, *TcSAMDC*, and *TcSPDS*. ABA treatment repressed the control genes *TcPER-1*, and *TcCHIB* in roots and did not alter their expression in leaves. *TcLHCA-1* was unchanged after ABA treatment in roots and induced 1.7-fold in leaves after 12 h.

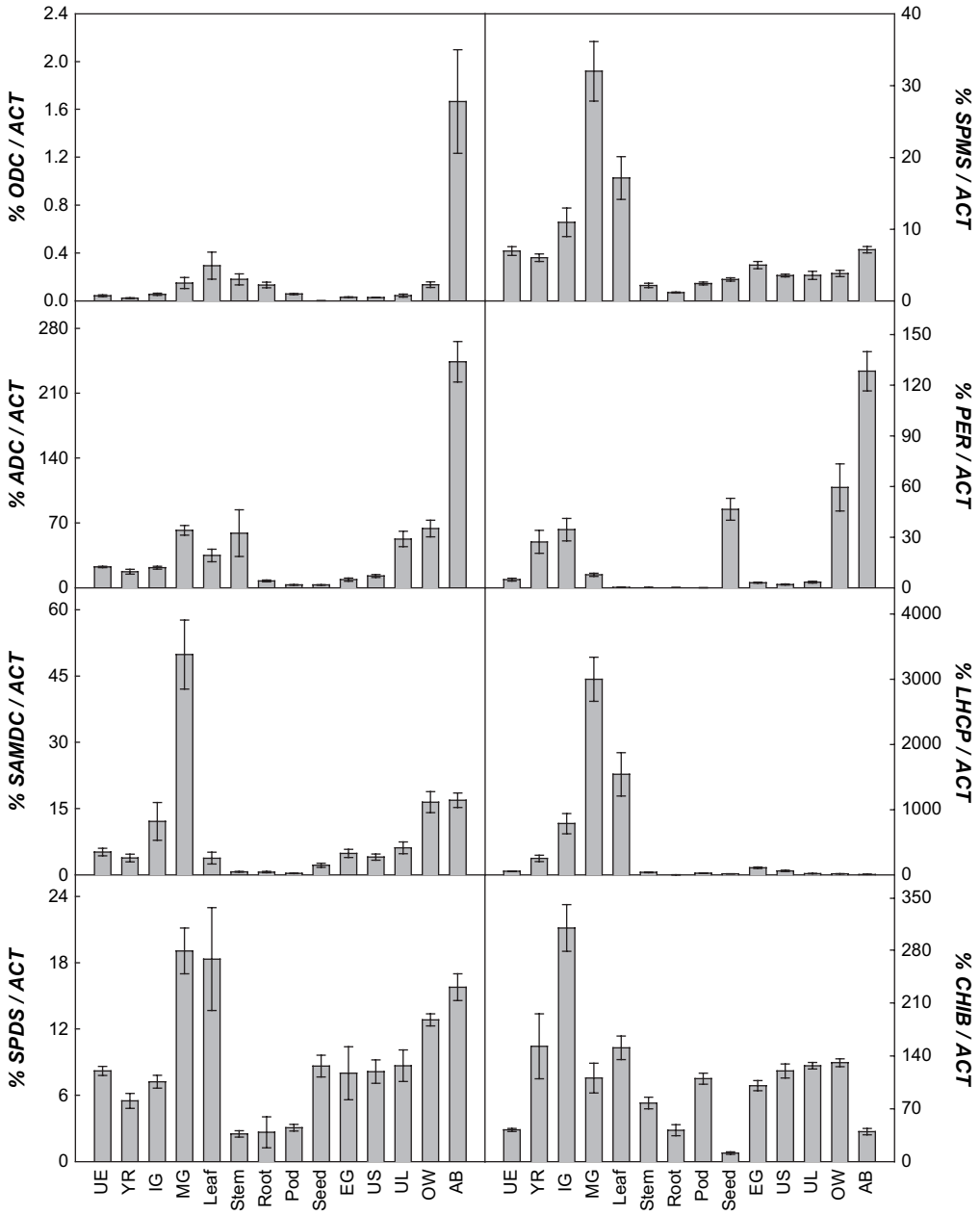

Fig. 2. Expression pattern of genes involved in polyamine biosynthesis in various cacao plant organs and tissues at different developmental stages. Relative transcript levels were measured using QPCR. UE, unexpanded leaf; YR, young red leaf; IG, immature green leaf; MG, mature green leaf; Leaf, seedling mature leaf; Stem, seedling stem; Root, seedling root; Pod, young green pod; Seed, mature seed; EG, elongated green flower; US, unopened white small flower; UL, unopened large flower; OW, open white flower; AB, abscised flower. Relative mRNA level was calculated with respect to that of *ACTIN*. Three other genes (*PER*, peroxidase; *LHCA*, light harvesting complex protein in photo system II, and *CHIB*, chitinase) were monitored as controls. Bars show means ± standard error ($n = 4$).

3.4. Induction of PA biosynthesis genes by biotic and abiotic stresses

We treated mature green leaves from mature trees with various stresses including *Phytophthora megakarya* infection (detached leaves), the necrosis inducing protein Nep1 from *Fusarium oxysporum* (attached leaves), and mechanical wounding (attached leaves). *P. megakarya* is the causal agent of black pod disease in cacao. *TcODC*, *TcADC* and *TcSAMDC* began accumulating in detached leaves 1 day after inoculation with *P. megakarya* zoospores (Table 4). A maximum induction of 671-fold for *TcODC* was observed 48 h after inoculation, while *TcSPDS* and *TcSPMS* showed transient induction of 2.3- and 3.6-fold 24 h after inoculation. *TcADC* and *TcSAMDC* were maximally induced 8.3- (after 24 h) and 11.5-fold (after 48 h), respectively, after inoculation.

Nep1-like-proteins are produced by at least two pathogens of cacao, *Phytophthora megakarya* [5] and *Moniliophthora perniciosa* [20]. Nep1 treatment induced *TcODC*, *TcADC* and *TcSAMDC* 4 h after treatment, while *TcSPMS* and *TcSPDS* were unchanged after this time period (Table 4). *TcODC* was highly induced by Nep1 treatment (135-fold) while *TcADC*

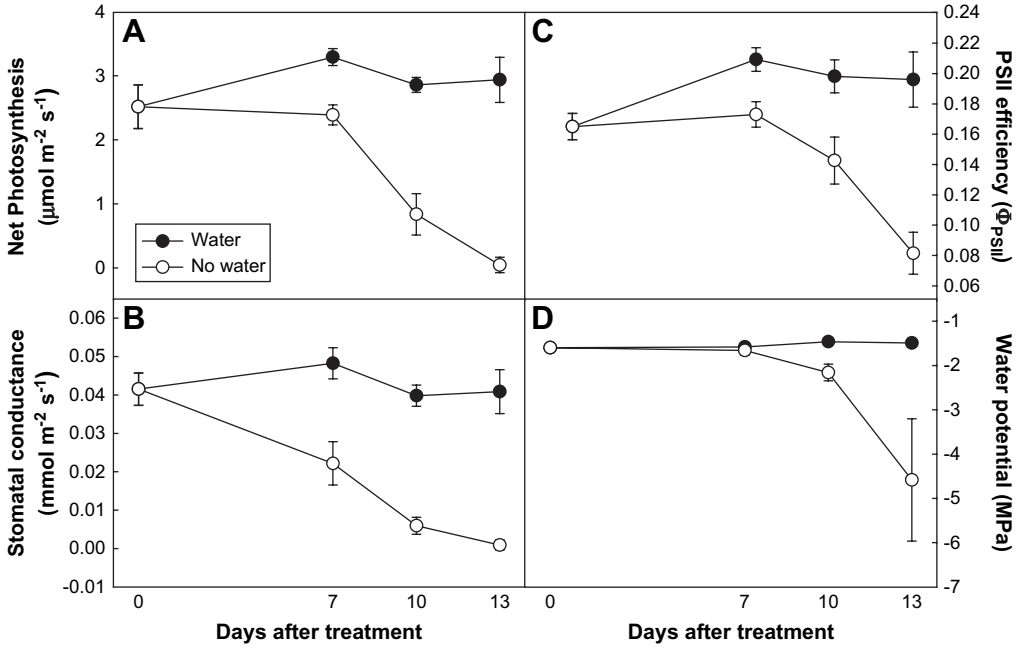

Fig. 3. Leaf gas exchange and water potential. (A) net photosynthesis, (B) stomatal conductance, (C) PSII efficiency of light adapted leaves (Φ_{PSII}), and (D) water potential. Each value was measured at midday. Environmental conditions are as follows: PAR, 300 μmol m^{-2} s^{-1}; [CO_2], 400 μmol mol^{-1}; air temperature, 25 °C; VPD, 1.2 kPa. Treatments were: closed circles, seedlings were watered every 2 days; open circles, watering was withheld starting at day 0. Bars indicate mean ± standard error ($n = 6$).

and *TcSAMDC* were up-regulated 2.7- and 8.4-fold, respectively by Nep1 treatment.

All 5 of the genes that encode PA biosynthesis related enzymes responded to mechanical wounding 15 min after treatment (Table 4). Expression of *TcODC*, *TcADC*, and *TcSAMDC* remained high 4 h post-treatment, whereas *TcSPDS* and *TcSPMS* transcript levels initially increased 15 min after wounding and then declined to levels near those observed in unwounded leaves 4 h after treatment.

TcODC and *TcADC* were highly expressed in abscised flowers (Fig. 2). In contrast, the expression of *TcSAMDC*, *TcSPDS*, and *TcSPMS* was minimally changed between opened attached flowers (OW) and abscised flowers (AB).

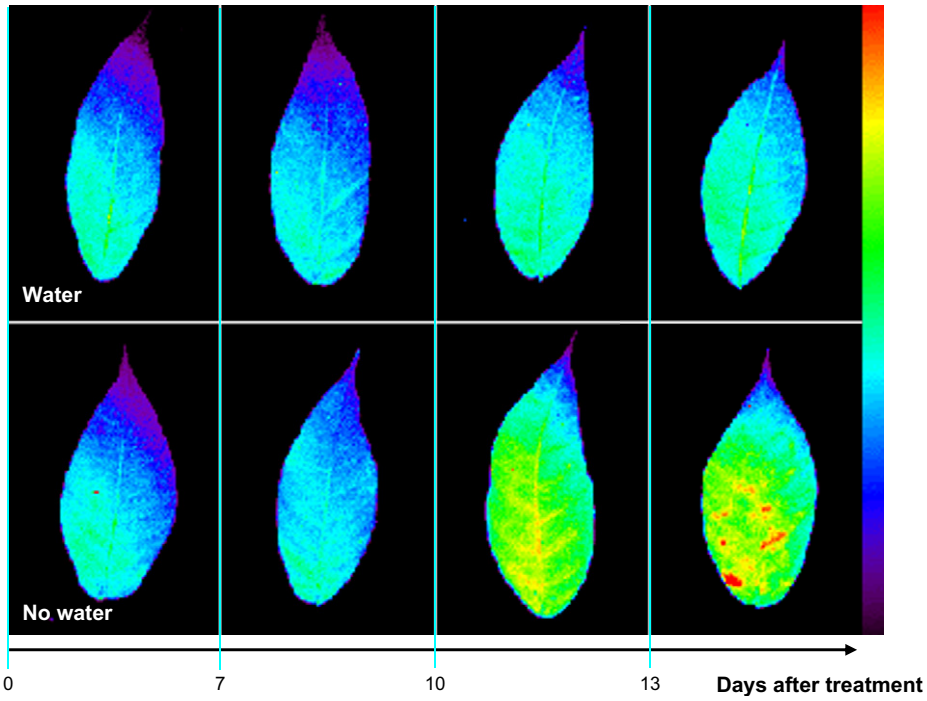

Fig. 4. Representative fluorescence responses of cacao leaves during drought treatment. Fluorescence emissions of abaxial surfaces of cacao leaves at 530 nm (F530) were measured using four biological replications. Relative fluorescence intensity is given in vertical color scales on the left.

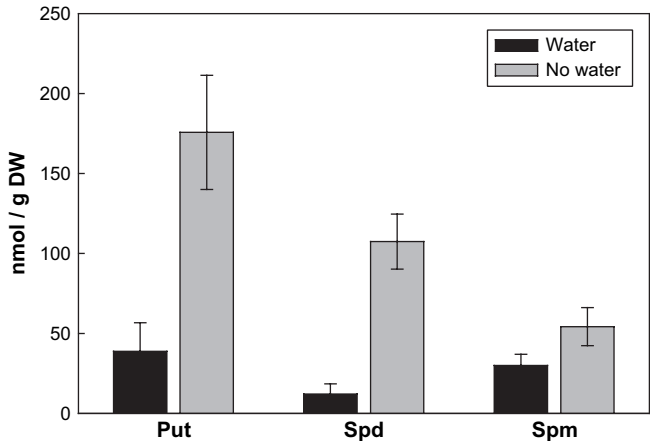

Fig. 5. Effects of drought on individual polyamine levels in leaves of cacao. Polyamines were extracted from lyophilized leaves of 13-day drought treated and control plants and quantified by HPLC. Put, putrescine; Spd, spermidine and Spm, spermine are shown. Bars are means ± SE ($n = 4$).

3.5. Isolation of full length TcODC

TcODC was constitutively expressed at very low levels yet was the most highly inducible of the PA associated ESTs being studied. In order to isolate a full-length *TcODC*, 5′ rapid amplification of cDNA ends (RACE) was performed. A full-length copy of *TcODC* (1745 bp) consisting of a 1236 bp open reading frame (ORF) encoding 411 amino acids was obtained. The full-length *TcODC* was deposited in the Genbank database under the accession number EF122792.

The deduced amino acids of the full length *TcODC* sequence aligned with other known plant ODCs (Fig. 8). The deduced amino acid sequences of *TcODC* showed highest identity with the ODC of *Populus nigra* (71%). Important amino acid residues associated with catalytic activity were conserved in *TcODC*. For example, Lys69 and Cys360 of mouse ODC (Lys77 and Cys356 in *TcODC*) are conserved in all plant ODCs. These residues participate in binding both the cofactor, pyridoxal 5′-phosphate (PLP), and a specific inhibitor of ODC, D,L-adifluoromethyl-ornithine (DFMO), respectively [55]. The residues Lys115, Lys169 and His197 of mouse ODC (Lys123, Lys178 and His206 in *TcODC*, respectively) that participate in active site formation are also conserved [63]. In addition, the hh(D/N)hGGGh(G/T) motif, where h represents a small hydrophobic residue, is found in all eukaryotic and prokaryotic decarboxylases [46] and was also conserved in the *TcODC* translation product.

4. Discussion

4.1. PA biosynthetic genes show developmental and tissue specific expression

TcODC showed the lowest level of constitutive expression in comparison to the other four PA related ESTs studied here. Although detected in all tissues tested, constitutive expression of *TcODC* was highest in mature tissues. This was in contrast to studies on other plant species where *ODC* transcripts were detected in tissues with active cell division, such as shoot tips (not tested here) and whole flowers [2,46,66,71].

TcADC transcript was very highly expressed in all the tissues studied. *Arabidopsis ADC2* was mainly detected in siliques and cauline leaves, while *ADC1* was expressed ubiquitously [59]. In apple trees, high transcript levels of *ADC* were observed in young tissues and organs of rapidly dividing cells [24]. A low level of the *ADC* transcript was detected in pea roots [51]. Although *TcADC* transcript was also detected at lower levels in cacao roots compared to most other tissues, it was still highly expressed in comparison to *TcODC*.

The highest transcript level of *TcSAMDC* was detected in MG leaf tissue and in mature flowers. In apple, *SAMDC1* transcript was more abundant in young leaves compared to mature leaves, while higher *SAMDC2* transcript levels were detected in mature leaves, indicating that the two transcripts may function differently [25]. SAMDC activity is a rate-limiting step in PA biosynthesis in plants (reviewed in [16]). Apple *SAMDC1* was proposed to be involved in fruit development and cell growth [25].

In cacao, *TcSPDS* transcripts were detected in all tissues and organs, while the highest level of transcripts was detected in mature leaves. In *Arabidopsis*, an embryo lethal double mutant of SPDS was complemented by the wild-type *Arabidopsis SPDS1* gene, indicating a critical role of SPDS in embryo development. *SPDS1* and *SPDS2* expression was observed in all organs of *Arabidopsis* with the highest expression being observed in root tissues [23]. *SPDS2* transcript was detected at low levels in the upper stems and mature siliques of *Arabidopsis* [64]. Two *SPDS* genes were also reported in pea and the transcript levels were higher in shoot tips, young leaves, stems and flowers than in roots and adult leaves [3]. The two pea *SPDS* genes were up-regulated in different patterns during early fruit development [3]. Similar to *SPDS*, *TcSPMS* transcripts were highly detected in the MG leaves of cacao.

4.2. The drought response of cacao and expression of ESTs encoding PA biosynthetic enzymes

The earliest responses to drought in cacao leaves were decreases in net photosynthesis, PS II efficiency, and stomatal conductance 7 days after withholding water (Fig. 3). Of particular significance is the 50% drop in stomatal conductance indicating the stomata were closing in an attempt to maintain the water status of the plant. An increase in the blue-green fluorescence (BGF) emission was noted after 10 days of withholding water (Fig. 4) along with a decrease in leaf water potential (Fig. 3) indicating severe drought stress.

BGF is emitted by plant pigments that have been excited by the absorption of UV radiation (reviewed in [40]). The emission of BGF has been attributed to cell-wall-ferulic acid, flavonoids and other simple phenols, such as *p*-coumaric acid [41,49]. During the process of water loss, the plasma membrane is compressed and solutes are concentrated in the cells, which result in the changes to BGF. The induction of BGF under stress conditions has been reported in plant species

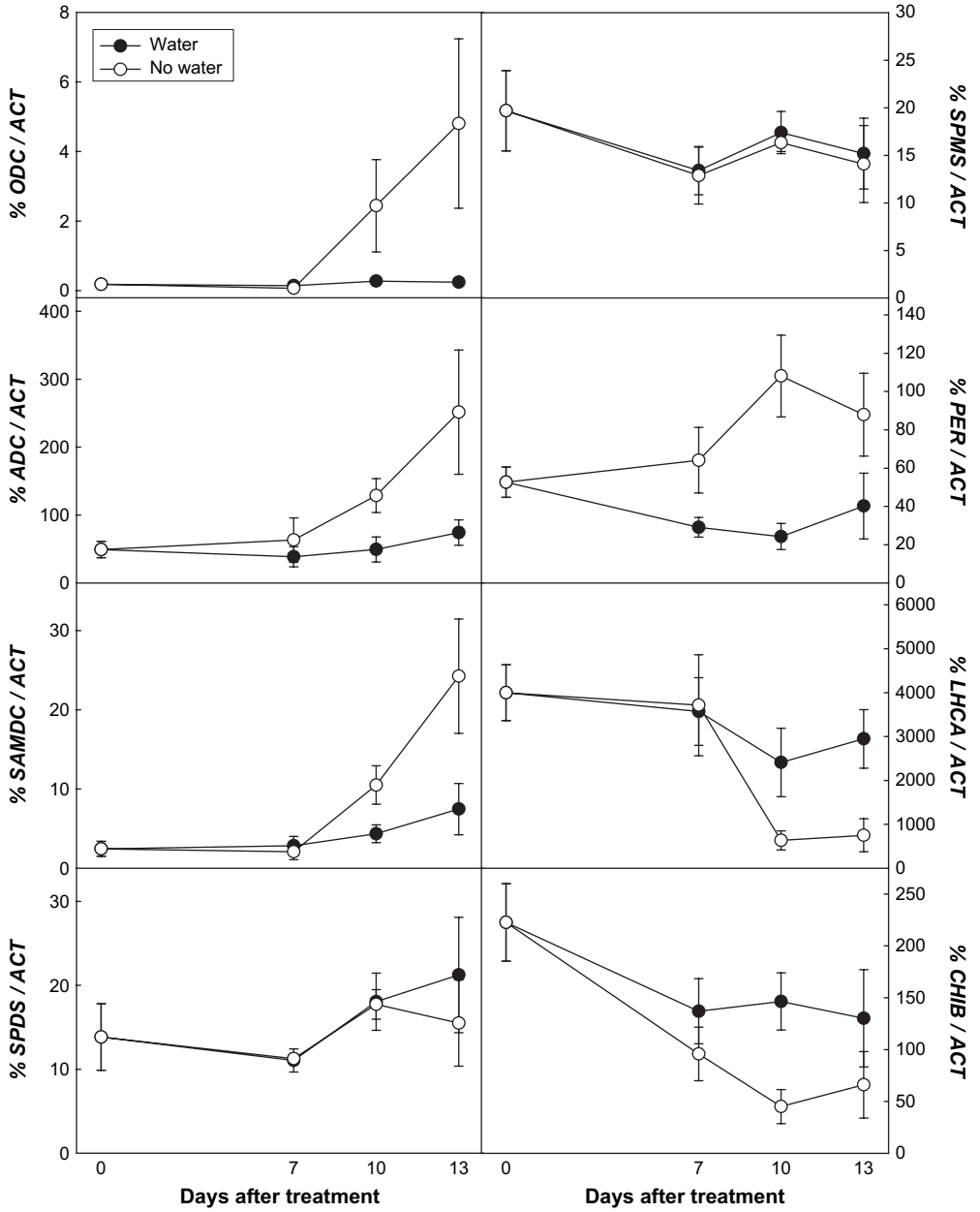

Fig. 6. Expression patterns of genes involved in polyamine biosynthesis in water stressed leaf tissue. Relative transcript levels were measured using QPCR. Thirty-two-day-old, Magenta box-grown cacao seedlings were used for drought treatment. The largest leaves were harvested 0, 7, 10, and 13 days after the last watering. Relative mRNA levels were calculated with respect to *ACTIN* transcripts. Treatments were: closed circles, seedlings were watered every 2 days; open circles, watering was withheld starting at day 0. Bars show means ± standard error ($n = 6$).

(reviewed in [40]). In addition, drought may stimulate the accumulation of polyphenolics [60], which might result in enhanced BGF.

TcODC, *TcADC*, and *TcSAMDC* were induced by 10 days of drought in leaves but their induction was a relatively late response compared to observed changes in stomatal conductance, photosynthesis, and photosystem II efficiency which was observed after 7 days of drought. Enhanced expression of *TcODC* and *TcSAMDC* in leaves was closely correlated with the observed changes in leaf water potential, a change first observed after 10 days of drought. *TcSPDS* and *TcSPMS* were not responsive to drought in leaves. The enhanced expression of PA biosynthesis associated transcripts in drought stressed leaves was paralleled by altered expression of *TcPER-1* (induced) and *TcLHCA-1* (repressed), and *TcCHIB* (repressed). The only PA associated transcripts showing induction at 7 days were *TcADC* and *TcSAMDC* in the roots. Expression of *TcADC*, which was maximally induced at 7 days, was most closely correlated with the drought treatment itself. *TcODC* and *TcSAMDC* were maximally induced in roots after withholding water 10 days. Expression of *TcSPDS* and *TcSPMS* was slightly up-regulated in droughted roots. Since PAs are associated with root development, it is possible the induction of PA biosynthesis genes in roots participates in altering the

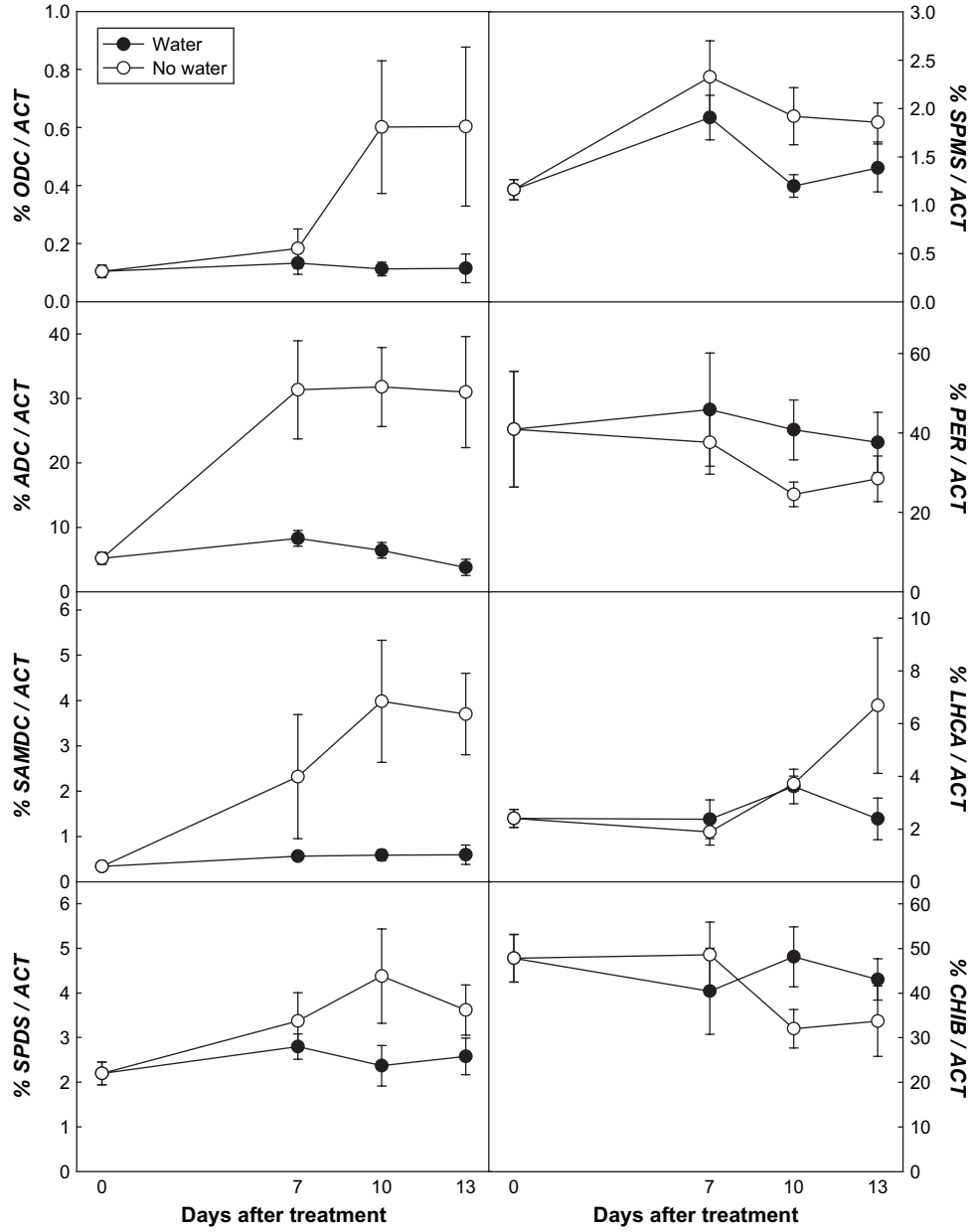

Fig. 7. Expression patterns of genes involved in polyamine biosynthesis in water stressed roots of cacao. Treatments were: closed circles, seedlings were watered every 2 days; open circles, watering was withheld starting at day 0. Other details were as in Fig. 6.

root architecture in response to stress as has been proposed in other crops [26].

4.3. ABA treatment and expression of ESTs encoding PA biosynthetic enzymes

The low level induction of all 5 ESTs associated with PA biosynthesis by ABA (100 μM solution applied to soil) is similar, in some cases, to patterns of induction observed in other plant species. In hydroponically grown rice (*Oryzea sativa* L.), *SAMDC1* was induced 2-fold by ABA (20 μM) after 3 h [39]. Interestingly, in an observation similar to ours, a decrease in *SAMDC1* occurred 12 h after treatment of rice with ABA. Li and Chen [39] attributed the decline in transcript to changes in mRNA stability. The similar induction level caused by ABA for all 5 ESTs in cacao leaves and roots is inconsistent with the expression patterns observed in response to the other stresses studied suggesting the regulation of these genes is influenced by other factors in addition to ABA. *ADC2* was highly induced by ABA (50 μM) treatment in *Arabidopsis* using a plant liquid culture system [52]. Alcazar et al. [4] observed that *ADC2*, *SPMS*, and *SPDS1* were highly induced by drought (removal from soil in contrast to natural soil drying) in *Arabidopsis*. *ADC2*, *SPMS*, and *SPDS1* induction by drought was greatly reduced in ABA insensitive mutants but induction, at least for *ADC2*, was not abolished.

Table 2
Correlation coefficients of gene expression (% relative to *ACTIN* expression) with water potential (range −1 to −5 MPa) or the presence of absence of drought (1, no drought; 2, drought)

Gene	Root		Leaf	
	WP	+/− drought	WP	+/− drought
TcODC	−0.33	0.45	−0.65	0.41
TcADC	−0.26	0.75	−0.33	0.43
TcSAMDC	−0.40	0.59	−0.72	0.40
TcSPMS	−0.12	0.46	0.36	−0.14
TcSPDS	−0.31	0.43	0.19	−0.06
TcPER-1	0.09	−0.26	−0.28	0.58
TcLHCA-1	−0.60	0.23	0.42	−0.43
TcChiB	0.30	−0.20	0.27	−0.50

For WP (water potential) correlation coefficients were calculated between gene expression and water potential values for each sample disregarding time and drought treatment. For +/− drought correlation coefficients were calculated between gene expression and whether or not the sample was drought treated disregarding time.

4.4. TcODC, TcADC, and TcSAMDC are responsive to diverse biotic and abiotic stresses

The disease black pod is one of the most important biotic stresses of cacao. Black pod is caused by several *Phytophthora* species, including *P. megakarya* Brasier & Griffin, *P. palmivora* (Butl.) Butler, *P. citrophthora* (R.H. Sm.& E.Sm.) Leonian, and *P. capsici* Leonian. Symptoms of black pod include seedling blights, stem cankers, and pod rots [7,69]. *TcODC*, *TcADC* and *TcSAMDC* were induced in cacao leaves by *P. megakarya* infection (Table 3). *TcODC* was highly up-regulated by *P. megakarya* treatment (maximum induction = 671-fold). The involvement of PAs in plant–microbe interactions has been suggested based on results of several studies. For example, in hot pepper, *CaODC1* accumulated in response to TMV-P0 and *Xanthomonas campestris* pv. *vesicatoria* (*Xcv*) inoculation [71]. It was suggested that *CaODC1* might be involved in the hypersensitive reaction (HR) to pathogenic microbes. In a previous study, *TcODC* was induced in cacao seedlings 2–10-fold in response to colonization by endophytic *Trichoderma* species [9]. In wheat, stem rust fungus induced the accumulation of PAs and activities of ODC [18]. Similar to the stem rust/wheat interaction [18], the interaction between *P. megakarya* and cacao discussed here is a susceptible interaction [7].

Table 3
Fold induction (QPCR) of genes involved in polyamine biosynthesis after treatment of cacao seedlings with abscisic acid

Tissue	Gene	Fold induction		
		1 h	3 h	12 h
Leaf	*TcODC*	2.7	2.3	0.6
	TcADC	1.6	1.5	0.5
	TcSAMDC	1.7	1.5	0.6
	TcSPMS	1.0	1.6	1.0
	TcSPDS	1.8	2.6	0.6
Root	*TcODC*	1.3	1.0	1.8
	TcADC	1.9	3.0	1.1
	TcSAMDC	2.0	1.6	1.4
	TcSPMS	1.5	1.6	1.5
	TcSPDS	2.0	1.6	1.2

Seedlings, maintained under continuous light, were watered with a solution of 1.0 mM ABA or water (controls). QPCR was performed on total RNA extracted from leaves and roots harvested 1, 3, and 12 h after treatment. The fold induction indicates expression levels in ABA treated seedling as compared to expression levels observed in controls at the same time point.

Table 4
Fold induction of genes (QPCR) involved in polyamine biosynthesis after *Phytophthora megakarya* inoculation, necrosis- and ethylene-inducing peptide 1 (Nep1) treatment, and wound treatment

Treatment	Gene	Fold induction		
		24 h	48 h	72 h
P. megakarya[a]	*TcODC*	14.4	671.3	182.4
	TcADC	8.3	2.9	4.9
	TcSAMDC	3.2	11.5	3.9
	TcSPMS	2.3	0.9	0.8
	TcSPDS	3.6	1.7	1.3
		0.5 h	4 h	24 h
Nep1[b]	*TcODC*	1.4	134.6	111.6
	TcADC	0.6	2.7	2.1
	TcSAMDC	1.2	8.4	0.7
	TcSPMS	1.5	0.7	0.7
	TcSPDS	0.4	0.7	1.1
		0.25 h	4 h	20 h
Wounding[c]	*TcODC*	16.0	36.7	4.9
	TcADC	6.4	6.2	1.4
	TcSAMDC	6.9	5.4	1.8
	TcSPMS	2.1	0.8	1.5
	TcSPDS	2.5	1.1	1.1

All treatments were applied to mature green cacao leaves.
[a] Leaf discs (8.5 cm in diameter) were inoculated with 20 drops of 20 μl zoospores of *P. megakarya* strain GWH 252 (3×10^4 zoospores ml^{-1}).
[b] Nep1 from *Fusarium oxysporum* (5 μg ml^{-1} plus 0.2% v/v Silwet-L77) was applied to leaves of greenhouse-grown plants at a rate of 46 ml m^{-2}.
[c] For mechanical wound treatment, a Groomax™ hard slicker brush was used over selected leaves backed with Styrofoam.

TcODC, *TcADC* and *TcSAMDC* were induced in cacao leaves by treatment with Nep1 (Table 4). As was observed in the *P. megakarya* treatment, *TcODC* was highly up-regulated by Nep1 treatment (maximum induction = 135-fold). Nep1-like-proteins are produced by at least two pathogens of cacao [5,20] in addition to many pathogens of other plants [40,53,62], Nep1-like-proteins have been shown to be important in disease development in several plant/microbe interactions [42,56,65]. In contrast, cryptogein, a protein produced by some *Phytophthora* isolates, elicits HR in tobacco leading to incompatibility in tobacco [61] and induces ROS production. PA oxidation was shown to be critical for ROS induction by cryptogein [70]. Nep1 induced the accumulation of reactive oxygen species (ROS) in plants [6,29] but the role of PAs in that process has not been studied.

Mechanical wounding resulted in the rapid induction (within 15 min) of all 5 of the PA biosynthesis associated genes. *TcSPDS*, and *TcSPMS* transcript levels deceased to near normal levels by 4 h after wounding, whereas *TcODC*, *TcADC* and *TcSAMDC* remained elevated at 4 h, decreasing to near normal levels by 20 h after wounding. During the

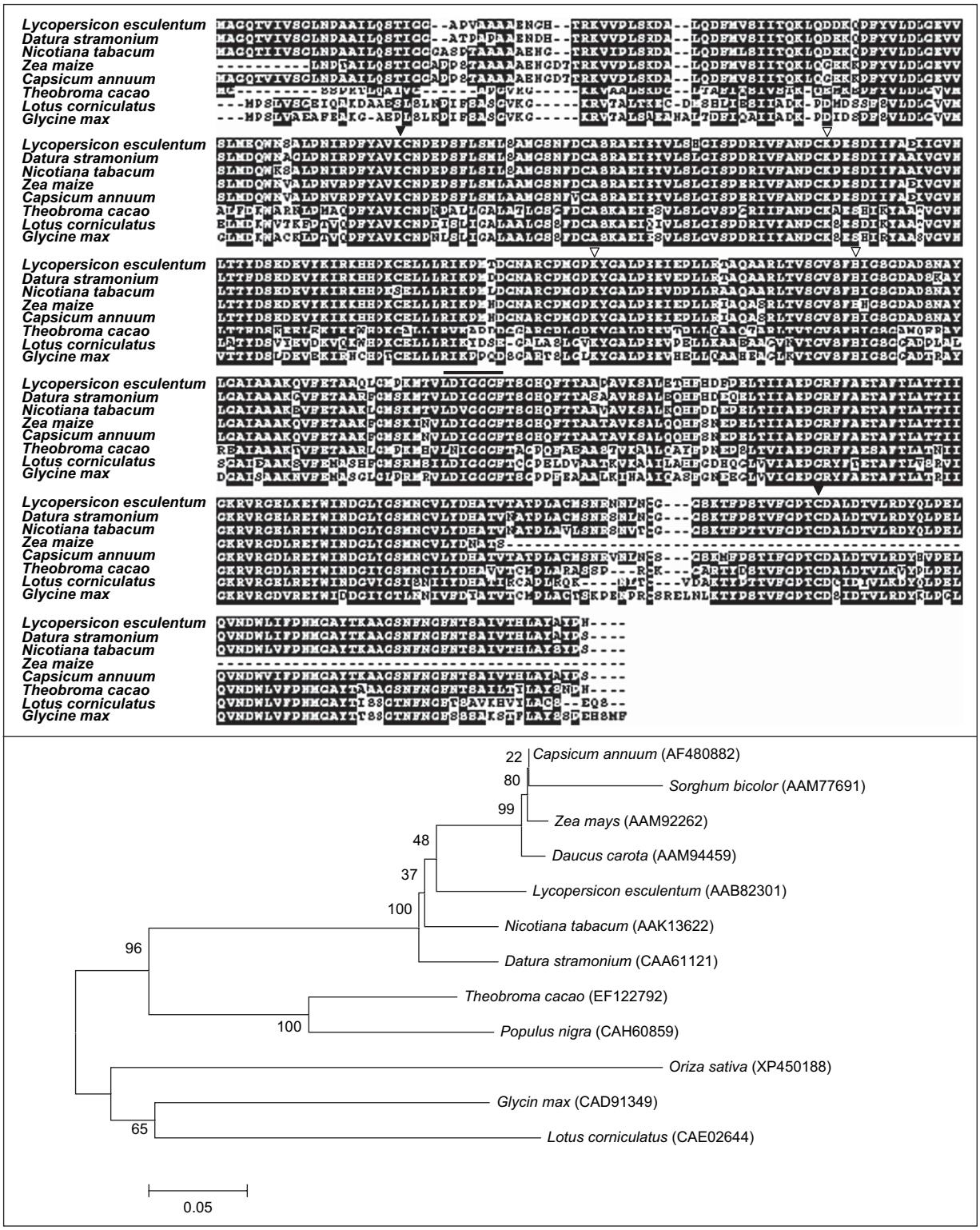

Fig. 8. Alignment of the predicted amino acid sequences and phylogenetic tree. (A) The predicted amino acid sequences of *TcODC* were aligned with other plant sequences using ClustalW and BOXSHADE sequence alignment program (http://seqtool.sdsc.edu/CGI/BW.cgi%21%23). Identical amino acids are highlighted with black boxes. Dashes indicate gaps that were introduced to optimize the alignment. Lys77 and Cys356 of *TcODC* (marked with a closed triangle) have been found to participate in binding the cofactor, pyridoxal 5′-phosphate (PLP) and a specific inhibitor of the murine enzyme, D,L-adifluoromethyl-ornithine (DFMO). Residues Lys115, Lys169 and His197 of mouse ODC (Lys123, Lys178 and His206 in *TcODC*), known to participate in active site formation, were also conserved. In addition the hh(D/N)hGGGh(G/T) motif, where h represents a small hydrophobic residue, was conserved in all ODC sequences and is marked with a line. (B) Phylogenetic analysis of ODC sequences. The phylogenetic tree was constructed from amino acid sequences by the neighbor-joining method of ClustalW and Mega2 (http://www.megasoftware.net/). Numbers at the nodes are bootstrap values from 100 replications and represent weighted amino acid substitutions, with the scale bar representing 10% weighted sequence divergence. GenBank accession numbers are as follows: *Lycopersicon esculentum* (AAB82301), *Datura stramonium* (CAA61121), *Nicotiana tabacum* (AAK13622), *Zea mays* (AAM92262), *Capsicum annuum* (AF480882), *Theobroma cacao* (EF122792), *Lotus corniculatus* var. japonicus (CAE02644), and *Glycine max* (CAD91350).

process of wound signaling, complex interactive pathways are involved in the wound defense response, including biosynthesis of plant hormones jasmonic acid (JA) and ABA, and physical processes, such as water stress and altered electrical impulses (reviewed in [38]). Similar to the results seen here, the *Arabidopsis ADC2* gene was transiently increased by mechanical wounding as early as 15 min after wounding, returning to normal level at 24 h [52].

It was in flowers that the PA associated ESTs demonstrated their most disjointed expression patterns (Fig. 2). Cacao flowers remain attached for only 1 day after opening. Expression of *TcADC* increased in maturing unopened flowers (UL) and remained stable as flowers opened, while *TcODC* and *TcSAMDC* expression increased in open attached flowers (OW). *TcODC* and *TcADC* were both up-regulated in abscised flowers while the *TcSAMDC* was unaltered. *TcSPDS* and *TcSPMS* were relatively insensitive to changes in flower development. Evidence suggests PAs are involved in the flowering process, including flower induction and floral organ development [31,33]. High Put and Spd concentrations were detected during the early stages of flower development, followed by a decline and then an increase at anthesis [36,58]. However, the roles of PAs in flower development have not been clearly explained and, as far as we are aware, this is the first study of the expression of genes associated with PA biosyntheses in abscised flowers.

We identified 3 genes, *TcODC*, *TcADC*, *TcSAMDC*, involved in the PA biosynthetic pathway that are responsive to multiple biotic and abiotic stresses including drought. *TcSPDS* and *TcSPMS*, although responsive to multiple stresses, tended to be less responsive to stresses in general when compared to *TcODC*, *TcADC*, and *TcSAMDC*. *TcODC* and *TcADC* represent points in the two major pathways for PA biosynthesis (Fig. 1) suggesting PAs are being produced in cacao from arginine by way of both ornithine and NCP. Both ODC and ADC are known to be induced by various stresses, such as disease [18,71], chilling [24], osmotic stress [43,47,59], acidic pH [43,50], and nutrient deficiency [43]. Although *TcADC* is constitutively highly expressed, *TcODC* is expressed at very low levels except in induced tissues. In addition to its roles in stress responses, *TcADC* may be more important to PA biosynthesis in cacao under normal growing conditions. On the other hand, *TcODC* showed the greatest inducibility by multiple stresses. After cloning and sequencing the full length cDNA for *TcODC*, sequence comparisons confirmed the predicted *TcODC* protein shared common characteristics with other ODCs and was most closely related to an ODC from the tree Black poplar (*Populus nigra*). *TcODC* tends to be highly induced by stress in cacao and may have a more focused function in the cacao response to stress. SAMDC represents a major regulatory point for PA biosynthesis in plants (reviewed in [16]). The observed induction of *TcSAMDC* in cacao leaves in response to stress was similar to that of soybean *SAMDC1*, which was induced by multiple stresses including drought, salt, and cold [62]. SAMDC plays an important role in plant developmental and physiological processes, in addition to its role in responses to environmental stresses [25,54,64].

There are likely additional copies of all of the genes we have studied in cacao as has been observed in other plant species [19,22,23,25,28,59,67,68,71]. Often the different copies are under different developmental and regulatory control. Although showing some differential expression due to developmental stage and tissue specificity, the genes encoded by *TcODC*, *TcADC*, *TcSAMDC*, *TcSPDS* and *TcSPMS* were constitutively expressed in all cacao tissues studied.

Altering PA biosynthesis by genetic manipulation can result in enhanced tolerance to abiotic stresses, including drought. A transgenic *Arabidopsis* that over-expressed *SPDS* of *Cucurbita ficifolia* under the 35S promoter, showed enhanced tolerance to various stresses, such as drought, chilling, freezing, salinity, hyper-osmosis, and paraquat toxicity [32]. Similarly, transgenic rice plants expressing *Datura* ADC produced much higher levels of PAs under stress resulting in drought tolerance [14]. It may be possible to enhance tolerance of cacao to drought and other stresses by altering PA levels by selection or genetic alteration using these genes, their promoter, or PA biosynthesis genes from other species as has been demonstrated in other crops. Although transgenic techniques have been developed for cacao [44], their use is presently being discouraged due to consumer concerns. Modern molecular selection techniques including marker assisted selection (MAS) and quantitative trait loci (QTL) mapping are presently being employed in cacao breeding programs [13,15,37,57]. As a subset of genes known to function as major points of regulatory control of PA biosynthesis in plants, *TcODC*, *TcADC*, and *TcSAMDC* have the potential to positively impact the response of cacao to multiple stresses. Understanding aspects of their regulatory control as described in this research may prove critical to reaching that potential.

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