

RESEARCH PAPER

Differentially expressed genes and proteins upon drought acclimation in tolerant and sensitive genotypes of *Coffea canephora*

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Abstract

The aim of this study was to investigate the molecular mechanisms underlying drought acclimation in coffee plants by the identification of candidate genes (CGs) using different approaches. The first approach used the data generated during the Brazilian Coffee expressed sequence tag (EST) project to select 13 CGs by an *in silico* analysis (electronic northern). The second approach was based on screening macroarrays spotted with plasmid DNA (coffee ESTs) with separate hybridizations using leaf cDNA probes from drought-tolerant and susceptible clones of *Coffea canephora* var. Conilon, grown under different water regimes. This allowed the isolation of seven additional CGs. The third approach used two-dimensional gel electrophoresis to identify proteins displaying differential accumulation in leaves of drought-tolerant and susceptible clones of *C. canephora*. Six of them were characterized by MALDI-TOF-MS/MS (matrix-assisted laser desorption-time of flight-tandem mass spectrometry) and the corresponding proteins were identified. Finally, additional CGs were selected from the literature, and quantitative real-time polymerase chain reaction (qPCR) was performed to analyse the expression of all identified CGs. Altogether, >40 genes presenting differential gene expression during drought acclimation were identified, some of them showing different expression profiles between drought-tolerant and susceptible clones. Based on the obtained results, it can be concluded that factors involved a complex network of responses probably involving the abscisic signalling pathway and nitric oxide are major molecular determinants that might explain the better efficiency in controlling stomata closure and transpiration displayed by drought-tolerant clones of *C. canephora*.

Key words: *Coffea canephora*, differential expression, drought acclimation, proteomic, real-time PCR, candidate gene.

Introduction

Coffee is one of the commodities most exchanged in the largest production (Lashermes *et al.*, 2008). It is international markets, with Brazil being the country with cultivated in >80 countries and ~25 million people depend

Abbreviations: 2-DE, immobilized pH gradient 2-dimensional gel electrophoresis; CG, candidate gene; DA, drought acclimation; qPCR, quantitative polymerase chain reaction; SD, standard deviation.

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on coffee for their livelihoods in Latin America, Africa, and Asia. The annual world production (134 million bags of 60 kg coffee beans in 2010, www.ico.org) is always subjected to regular oscillations mainly explained by the natural biennial cycle (larger production in one year and lesser production in the next year) and adverse effects of climatic conditions. Periods of drought and high temperature are key factors affecting coffee plant development and production (DaMatta and Ramalho, 2006). In the case of severe drought, flowering can be affected, leading to abortion of developing fruits, and under extreme conditions it may even cause plant death. As a consequence of global warming, coffee-growing geographical regions could also suffer delocalization (Assad *et al.*, 2004), leading to important environmental, economic, and social problems. In such a context, the generation of drought-tolerant coffee varieties has now turned into one of the priorities of many coffee research institutes.

The coffee genus contains >100 species (Davis *et al.*, 2006), with *Coffea arabica* and *C. canephora* being the two most cultivated species. Concerning drought tolerance, it is well known that genetic variability exists within the *Coffea* genus, as the Kouillou group appears to be more tolerant to drought than Robusta (currently defined as the SG2 group) types of *C. canephora* (Montagnon and Leroy, 1993; Montagnon, 2000). Under severe drought, Boyer (1965, 1969) showed that plants from the Kouillou group maintained stomatal opening, and consequently an active photosynthesis, while stomata of Robusta plants were completely closed under such conditions. The same author also reported more efficient root water absorption for the Kouillou plants that could explain its drought tolerance despite its maintenance of stomatal opening. Among the strategies displayed by coffee plants to cope with drought, leaf folding and inclination that reduce the leaf surface, water loss by transpiration, and exposure to high irradiance were commonly observed for Guinean and SG1 genotypes (Montagnon and Leroy, 1993). Leaf abscission is then reduced, favouring a rapid recovery of vegetation with the return of the rains. Such a trait can be considered as a selective advantage when compared with the dramatic leaf abscission that characterizes SG2 genotypes.

In addition to affecting coffee productivity, shape, and bean defects (Carr, 2001; Silva *et al.*, 2005), large variations in rainfall and temperature also influence the biochemical composition (e.g. reducing sugars, proteins, and caffeine) of beans (Mazzafera, 2007) and consequently the final cup quality (Camargo *et al.*, 1992). For example, bean caffeine content was shown to decrease with irrigation and temperature (Silva *et al.*, 2005). In the same work, high protease and polyphenol oxidase activities were correlated with higher temperature and lower rainfall. Nevertheless, before analysing the interaction between quality and drought adaptation, the molecular control of drought tolerance in terms of physiological behaviour needs to be unravelled.

The identification and selection of *C. canephora* clones combining drought tolerance with other agronomic traits (e.g. efficient flowering, productivity, and vigour) is of particular interest to generate new varieties better adapted

to climate changes. During the last decade, several drought-tolerant clones of *C. canephora* var. Conilon have been characterized as vigorous plants with high productivity throughout years under drought stress (Ferrão *et al.*, 2000; Fonseca *et al.*, 2004). Fingerprint analyses also revealed that these Conillon clones belong to the SG1 group of *C. canephora* (Lambot *et al.*, 2008). Physiological analyses suggested that drought tolerance could be a direct consequence of better root development (Pinheiro *et al.*, 2005). In another study, Lima *et al.* (2002) suggested that enhanced activity of antioxidant enzymes might also be involved in the drought tolerance mechanism. A key role of ascorbate peroxidase (APX) was postulated to allow clone 14 to cope with potential increases of H₂O₂ under drought conditions, as an increased (38%) activity of this enzyme was found for this clone upon drought stress (Pinheiro *et al.*, 2004). When looking for activities of sucrose-metabolizing enzymes [e.g. acid invertase, sucrose synthase, and sucrose phosphate synthase (SPS)] as well as for contents of hexoses, sucrose, starch, and amino acids in leaves, Praxedes *et al.* (2006) observed a maintenance of SPS activity with the decrease of pre-dawn leaf water potential (Ψ_{pd}) for the drought-tolerant clone 120 but not for the drought-sensitive clones. According to DaMatta *et al.* (2003), the better crop yield of a drought-tolerant clone compared with a drought-sensitive clone is mainly associated with the maintenance of leaf area and tissue water potential that are consequences of reduced stomatal conductance (g_s). Taken together, these observations suggest the existence of different biological mechanisms conferring drought tolerance in *C. canephora*, the molecular nature of which is still largely unknown.

Simkin *et al.* (2008) demonstrated that drought affected the leaf expression of several gene-encoding enzymes of the carotenoid biosynthesis pathway, therefore suggesting that changes in carotenoid content and activation of the xanthophyll cycle may play some role in drought tolerance, as also emphasized in previous studies (Davison *et al.*, 2002; Du *et al.*, 2010). Up-regulation of genes (*CaGolS*) encoding galactinol synthase upon drought was also observed (dos Santos *et al.*, 2011). In that case, no concomitant accumulation of galactinol was observed, probably because this sugar was used as a substrate for the biosynthesis of high molecular weight polysaccharides. In a recent publication, differential gene expression from both subgenomes of *C. arabica* was studied through a coffee-specific microarray and showed preferential expression of *C. canephora* homologues at higher temperatures and expression of *C. eugenioides* homologue genes at lower temperatures (Bardil *et al.*, 2011; Privat *et al.*, 2011).

The recent advances in coffee genomics including expressed sequence tag (EST) sequencing projects (Lin *et al.*, 2005; Poncet *et al.*, 2006; Vieira *et al.*, 2006; Vidal *et al.*, 2010; Mondego *et al.*, 2011), the construction of bacterial artificial chromosome (BAC) libraries (Noir *et al.*, 2004; Leroy *et al.*, 2005), and genetic maps (Crouzillat *et al.*, 2004; Lefebvre-Pautigny *et al.*, 2010; Leroy *et al.*, 2011), as well as the improvement of coffee genetic transformation techniques (Alpizar *et al.*, 2008; Ribas *et al.*, 2011) now open the way to study the molecular and genetic

determinism of drought tolerance and other important agronomic traits, leading to the identification of molecular markers that could be used to speed up coffee breeding programmes (Lashermes *et al.*, 2008; De Kochko *et al.*, 2010).

The search for candidate genes (CGs) for drought tolerance in coffee has now been initiated by an *in silico* analysis of the Brazilian Coffee Genome database (Vieira *et al.*, 2006; Mondego *et al.*, 2011). As part of this project, two EST libraries from leaves of plants of *C. arabica* var. Catuaí (considered as drought sensitive) and *C. canephora* clone 14 (drought tolerant) submitted to drought were generated. The plant materials of *C. arabica* and *C. canephora* were obtained from field and pot trials, respectively. More than 15 000 clones were sequenced and, after trimming and clustering, resulted in 10 924 reads grouped on 6141 unigenes (1779 contigs and 4362 singlets). The approaches used to identify CGs underlying drought acclimation (DA) in coffee consisted of an *in silico* analysis of ESTs generated from drought-exposed and untreated leaf cDNA libraries of *C. canephora* plants (Mondego *et al.*, 2011), transcription profiling by macroarrays and quantitative real-time polymerase chain reaction (qPCR) analyses of drought-submitted and control tissues, as well as protein profiling by two-dimensional gel electrophoresis (2-DE) coupled with tryptic peptide identification by matrix-assisted laser desorption-time of flight-tandem mass spectrometry (MALDI-TOF-MS/MS). These integrated analyses resulted in the identification of >40 candidate drought-responsive genes. Results showing differential gene expression in leaves of sensitive versus tolerant clones of *C. canephora* cultivated in the greenhouse and submitted or not to water limitation are presented and discussed.

Materials and methods

Plant material

Clones of *C. canephora* Pierre var. Conilon tolerant (14 and 120) and susceptible (22) to drought were obtained as rooted stem cuttings from the Institute for Research and Rural Assistance (Incaper, Vitoria, Espírito Santo, Brazil) (Ferrão *et al.*, 2000; Fonseca *et al.*, 2004). These clones were grown in greenhouse (Federal University of Viçosa-UFV, Minas Gerais, Brazil) conditions [25 °C, 70% relative, average mid-day photosynthetic photon flux (PPF) of 900 $\mu\text{mol m}^{-2} \text{s}^{-1}$] individually in pots of 12 litres of a mixture of soil, sand, and manure (3:1:1, v/v/v). After 6 months, plants of each clone were separated into two groups: the first one received regular irrigation (control) while watering was suspended for the second group until plants reached nearly -3.0 MPa (DA; e.g. clone 22, 6 d of DA; clones 14 and 120, 12 d of DA) (Marraccini *et al.*, 2011). For both conditions, six plants (biological repetitions) were analysed. Fresh leaves (fully expanded leaves corresponding to the third pair in plagiotropic branches) were used for physiological and molecular analyses. In that case, leaves were collected in the daytime (at ~10:00 h), immediately frozen in liquid nitrogen, and further stored at -80°C for protein and RNA extractions.

Physiological analyses

DA was evaluated by measuring Ψ_{pd} with a Scholander-type pressure chamber. Ψ_{pd} was regularly followed to reach near -3.0 MPa for

drought-acclimated plants (Silva, 2007; Silva *et al.*, 2010). The net CO_2 assimilation rate (A), stomatal conductance to water vapour (g_s), and internal to ambient CO_2 concentration ratio (C_i/C_a) were measured between 10:00 h and noon under artificial and saturating PPF with a portable open-system infrared gas analyser (LCpro+, Analytical Development Co. Ltd, Hoddesdon, UK) under a relative humidity of ~80%. The chlorophyll *a* fluorescence parameters were measured using a portable pulse amplitude modulation fluorometer (FMS2, Hansatech, King's Lynn, Norfolk, UK). The maximum photochemical efficiency of PSII (F_v/F_m), the coefficient of photochemical quenching (q_P), the quantum yield of photosystem II (PSII) electron transport (Φ_{PSII}), the non-photochemical quenching (NPQ), and the fraction of PPF absorbed in PSII antennae and used neither in photochemistry nor dissipated thermally (P_E) were measured as previously described (DaMatta *et al.*, 2002; Lima *et al.*, 2002; Pinheiro *et al.*, 2004). For each parameter, values represent the mean \pm SD of five replicates (Table 1).

In silico identification of candidate genes

For *in silico* expression analysis, contig and singlet frequencies across the libraries were obtained from the data set derived from the CAP3 assembly. A first set of CGs corresponded to genes exclusively expressed in the leaf cDNA library of drought-acclimated clone 14 (SH3) from *C. canephora*. Other CGs came from (i) the comparison of the cDNA library from leaves of drought-acclimated plants of clone 14 (SH3) with the leaf cDNA library (LF1) of the BP409 variety (Lin *et al.*, 2005) of *C. canephora* and (ii) the comparison of the cDNA library from leaves of plants submitted to drought (SH2) with well-watered plants conditions (LV8 and LV9) for *C. arabica* var. Catuaí (Vieira *et al.*, 2011). In all cases, *CcUBQ10* transcripts were used as an internal control of expression. Fisher's statistical test (Fisher, 1922) was performed, with a threshold of 4 indicating a significant variation in expression. Sequence homologies were detected by BLAST analyses (Altschul *et al.*, 1990) against the GenBank and SGN (SOL Genomics Network; Mueller *et al.*, 2005) databases.

RNA extraction

Samples were ground into a powder in liquid nitrogen and total RNAs were extracted as described previously (Marraccini *et al.*, 2011). RNA quantification was performed using a NanoDrop™ 1000 Spectrophotometer (Waltham, MA, USA).

Northern blot experiment

A 15 μg aliquot of total RNA was fractionated on a 1.2% (w/v) agarose gel containing 2.2 M formaldehyde in MOPS buffer. The homogeneity of the amount of RNA deposited was checked according to the abundance of 26S and 18S rRNA on gels stained with ethidium bromide. Total RNA was transferred to Hybond N+ membranes which were hybridized at 65 °C in modified Church and Gilbert buffer (7% SDS, 10 mM EDTA, 0.5 M sodium phosphate pH 7.2). Probes corresponding to the *CcCABI*, *CcRBCS1*, *CcACBP1*, *CcELIP1*, *CcMET1*, *CcMPR1*, *CcUNK10*, *CcGRP1*, and *CcUBQ10* genes were amplified by conventional PCR using universal primers from a plasmid harbouring the EST sequences GT647358, GT649534, GT646045, GT647659, GT650680, GT648734, GT648004, GT650953, and GW488515, respectively (Vidal *et al.*, 2010; Mondego *et al.*, 2011), and labelled by random priming with [α - ^{32}P]dCTP (Amersham Biosciences, São Paulo, SP, Brazil) as previously described (Geromel *et al.*, 2006). Washes were performed at 65 °C in 2 \times SSC (1 \times SSC=150 mM sodium chloride and 15 mM sodium citrate, pH 7.0)-0.1% SDS (2 \times 15 min) with a final stringent wash in 0.1 \times SSC, 0.1% SDS (2 \times 15 min). Membranes were exposed with BAS-MS 2340 IP support and data were acquired using a Fluorescent Image Analyzer FLA-3000 (Fujifilm Life Science, Woodbridge, CT, USA).

Table 1. Effects of the drought on leaf pre-dawn water potential (Ψ_{pd} in MPa), rate of decrease of Ψ_{pd} (RDPWP in MPa d⁻¹ m⁻²), net CO₂ assimilation rate (A in $\mu\text{mol m}^{-2}\text{s}^{-1}$), stomatal conductance (g_s in $\text{mmol m}^{-2}\text{s}^{-1}$), internal to ambient CO₂ concentration ratio (C_i/C_a), maximum photochemical efficiency of PSII (F_v/F_m), quantum yield of PSII electron transport (Φ_{PSII}), photochemical (q_p) and Stern-Volmer non-photochemical (q_N) quenching coefficients, and the fraction of PPF absorbed in PSII antennae and used neither in photochemistry nor dissipated thermally (P_E) of clones 14, 22, and 120 of *C. canephora*

Different upper case letters denote significant differences among means of the two genotypes in irrigated conditions. Different lower case letters represent significant differences among means of the genotypes submitted to drought by the Newman-Keuls test at $P \leq 0.05$ (clone effect). Means for plants submitted to drought marked with an asterisk differ from those of control plants by F -test at $P \leq 0.05$ (treatment effect).

Parameters	Drought-tolerant clone (14)		Drought-sensitive clone (22)		Drought-tolerant clone (120)	
	Control	Drought	Control	Drought	Control	Drought
Ψ_{pd}	-0.02±0.01 A	-3.02±0.12 a*	-0.03±0.00 A	-3.01±0.11 a*	-0.03±0.01 A	-3.1±0.15 a*
RDPWP	-	0.67±0.04 c	-	1.01±0.04 a	-	0.84±0.03 b
A	9.40±0.34 A	2.62±0.27 a*	9.35±0.17 A	0.95±0.23 b*	9.62±0.61 A	2.57±0.14 a*
g_s	60.00±5.00 B	13.00±3.50 a*	105.00±9.50 A	5.00±3.00 a*	72.00±7.00 B	10.00±1.00 a*
C_i/C_a	0.520±0.040 B	0.380±0.040 b*	0.670±0.040 A	0.520±0.040 a*	0.520±0.050 B	0.320±0.050 b*
F_v/F_m	0.840±0.011 A	0.842±0.011 a	0.831±0.011 A	0.800±0.011 b*	0.830±0.001 A	0.850±0.004 a
Φ_{PSII}	0.455±0.049 A,B	0.287±0.050 a,b*	0.472±0.049 B	0.210±0.050 b*	0.577±0.048 A	0.395±0.046 a*
q_p	0.713±0.051 A,B	0.495±0.051 a,b*	0.697±0.051 B	0.362±0.051 b*	0.815±0.033 A	0.615±0.055 a*
q_N	0.665±0.056 A	0.717±0.057 a	0.642±0.056 A	0.543±0.057 a	0.560±0.067 A	0.652±0.021 a
P_E	0.252±0.040 A	0.425±0.040 a,b*	0.242±0.039 A	0.508±0.039 a*	0.152±0.027 A	0.332±0.048 b*

Real-time qPCR assays

To eliminate contaminant genomic DNA, samples were treated with RQ1 RNase-free DNase according to the manufacturer's instructions (Promega, Madison, WI, USA) and RNA quality was verified by agarose gel electrophoresis and visual inspection of the rRNA bands upon ethidium bromide staining. Synthesis of the first-strand cDNA was done by treating 1 μg of total RNA with the ImProm-IITM Reverse Transcription System with oligo(dT)₁₅ according to the manufacturer's recommendations (Promega). The absence of contaminating genomic DNA in the cDNA preparations was checked by conventional PCR using the SUS10/SUS11 primer pair that spans introns 5–9 of the *CcSUS1* gene (AJ880768) encoding isoform 1 of the sucrose synthase (Leroy et al., 2005; Geromel et al., 2006). In that case, PCR was carried out with 1 μl of synthesized cDNA with a PTC-100 Thermocycler (MJ Research), using GoTaq DNA polymerase according to the supplier's instructions (Promega) under the following conditions: initial denaturation 94 °C, 2 min; followed by 40 cycles of 94 °C, 30 s; 55 °C, 30 s; and 72 °C, 3 min, and a final extension step of 72 °C, 6 min. In such conditions, the amplification of a 667 bp fragment characterized the *CcSUS1* cDNA and the absence of the corresponding genomic sequence is indicated by the lack of an amplicon at bp 1130 (data not shown).

Real-time qPCR assays were carried out with the synthesized single-stranded cDNA described above and using the protocol recommended for the use of 7500 Fast Real-Time PCR Systems (Applied Biosystems, Foster City, CA, USA). cDNA preparations were diluted (1/25 to 1/100) and tested by qPCR using CG primer pairs (Table 3) designed employing the Primer Express software (Applied Biosystems) and preliminarily tested for their specificity and efficiency against a mix of cDNA or using cloned ESTs (data not shown). The qPCR was performed with 1 μl of diluted single-stranded cDNA and 0.2 μM (final concentration) of each primer in a final volume of 10 μl with SYBR green fluorochrome (SYBR-Green qPCR Mix-UDG/ROX, Invitrogen). The reaction mixture was incubated for 2 min at 50 °C (uracil DNA-glycosylase treatment), then for 5 min at 95 °C (inactivation of uracil DNA-glycosylase), followed by 40 amplification cycles of 3 s at 95 °C, 30 s at 60 °C. Data were analysed using the SDS 2.1 software (Applied Biosystems) to determine the cycle threshold (Ct) values. The

specificity of the PCR products generated for each set of primers was verified by analysing the T_m (dissociation) of amplified products. PCR efficiency (E) was estimated using absolute fluorescence data captured during the exponential phase of amplification of each reaction with the equation $(1+E)=10(-1/\text{slope})$ (Ramakers et al., 2003). Efficiency values were taken into account in all subsequent calculations. Gene expression levels were normalized to expression levels of *CcUBQ10* or *CcGAPDH* as a constitutive reference (Barsalobres-Cavallari et al., 2009). For the *CcUNK10* gene expression study, relative quantification was also normalized with *CcADP1*, *CcACT1*, *CcCYN1*, and *CcCTSI* as alternative housekeeping genes (Fig. 4). Expression was expressed as relative quantification by applying the formula $(1+E)^{-\Delta\Delta Ct}$, where $\Delta Ct_{\text{target}} = Ct_{\text{target gene}} - Ct_{\text{reference gene}}$ and $\Delta\Delta Ct = \Delta Ct_{\text{target}} - \Delta Ct_{\text{internal calibrator}}$, the internal calibrator always being the 14I sample with relative quantification equal to 1.

Screening of macroarrays

The inserts of 3388 clones of a unigene set based on clustering ESTs from drought-acclimated cDNA libraries from *C. arabica* var. Catuai (SH2) and *C. canephora* clone 14 (SH3) (Vieira et al., 2006; Mondego et al., 2011) were PCR amplified with the universal M13 reverse and forward primers. The concentration and quality of PCR products were checked on agarose gels. Aliquots of 50 μl each ($\pm 250 \text{ ng } \mu\text{l}^{-1}$) were transferred to 384 plates. An equal volume of dimethylsulphoxide (DMSO) was added to each well. Spotting of the PCR products on nylon Hybond-N+ membranes (GE Healthcare) was performed using a Q-Bot (Genetix Inc.). Each PCR product was spotted in duplicate, in a 3×2 format, totalling 6912 spots including 68 controls. After spotting, nylon membranes were treated with denaturing and neutralizing solutions, according to the manufacturer. DNA was fixed onto membranes by UV cross-linking. Membranes were further hybridized independently with probes corresponding to single-stranded cDNAs obtained by reverse transcription of 30 μg of total RNA extracted from leaves of *C. canephora* clones 22 and 14 grown with (I) or without (NI) irrigation. Labelling was performed by random priming with [α -³³P]dCTP. After overnight hybridization, nylon membranes were washed and revealed as described for northern

blot experiments. Differentially expressed genes were identified using the ArrayGauge software (Fuji).

Proteome analysis by 2-DE

Proteins were extracted from leaves of clones 22 and 14 of *C. canephora* by a modified phenol/SDS method (Ramos *et al.*, 2007) followed by 2-DE. The first dimension (isoelectric focusing) was carried out using 13 cm IPG strips (pH 3–10 or pH 4–7) in an IPGphor system (GE Healthcare), and the samples (500–1000 µg of proteins) were loaded and separated at 20 °C for 12 h. The second dimension was in an SDS-PAGE (11%) using the Hoefer SE600 Ruby system (GE Healthcare) with a 15 mA gel⁻¹ for 45 min and a 30 mA gel⁻¹ for 180 min at 12 °C. Gels were stained with Coomassie Blue G-250 and R-350, digitalized using an UMAX Image Scanner, and analysed with ImageMaster 2D Platinum 6.0 software (GE Healthcare). Protein spots differentially expressed were removed manually from gels and analysed by mass spectrometry using a MALDI-TOF/TOF (Auto-flex, Bruker) mass spectrometer.

Protein identification by MS

The proteins were identified by PMF (peptide mass fingerprinting) using PiumsGUI2.2, and MS/MS ion search using the software X!Tandem. The obtained sequences were screened against the SOL Genomics Network and other coffee sequences available in public databases. The packages Trans-Proteomic Pipeline (TPP) and Scaffold were used to analyse protein data. Results were further verified by *de novo* sequencing data using the FlexAnalysis software (Bruker Daltonics).

Results

Physiological characteristics of *C. canephora* clones

A leaf Ψ_{pd} close to zero confirmed the untreated condition of irrigated plants (Table 1). In addition, values of A , F_v/F_m of PSII, Φ_{PSII} , q_N , and P_E were equal for clone 22 (drought-sensitive) and the drought-tolerant clones 14 and 120 under irrigation. The main differences between these clones concerned the g_s values that were ~2-fold higher for clone 22 than for clones 14 and 120. Under irrigation, the q_P of drought tolerance was similar for all the clones.

After suspension of watering, clone 22 showed a faster decline [rate of decrease in the pre-dawn leaf water potential (RDPWP)] of Ψ_{pd} than the drought-tolerant clones to reach drought conditions (–3.0 MPa). Regarding the drought-tolerant clones, it is worth noting that the decrease of RDPWP was faster for clone 120 than for clone 14, but both reached –3.0 MPa 12 d after suspension of watering. The delay observed for clone 120 compared with clone 14 in initiation of its reduction of Ψ_{pd} explains the differences observed between these two drought-tolerant clones (Marraccini *et al.*, 2011). When established, DA led to decreases in both g_s and A , 80% and 73% for clone 14; 72% and 86% for clone 120; and 95% and 90% for clone 22, respectively. Independently of the clones, the g_s reduction was greater than the decline of A , and was accompanied by a decrease in the C_i/C_a ratios. Altogether, these data clearly indicate that the inhibition of photosynthesis by drought tolerance is probably related to stomatal closure.

Regarding the maximum photochemical efficiency of PSII (evaluated by the F_v/F_m ratio), similar values were observed

under irrigated (control) and non-irrigated conditions for the drought-tolerant clones 14 and 120. Even if a slight decrease in the F_v/F_m ratio was observed for the drought-sensitive clone under drought conditions, F_v/F_m values were close to that considered as an optimum (~0.80), therefore indicating that no photoinhibitory damage occurred for clone 22. For all the clones, the reduction of A under DA was not accompanied by an increase of q_N . However, all clones showed a reduction of q_P , demonstrating that PSII excitation pressure increased. This indicates that a fraction of closed PSII reaction centred to a decrease in Φ_{PSII} observed during DA.

Selection of candidate genes

Based on the Brazilian Coffee Genome database, a first set of five CGs exclusively expressed in the leaf cDNA library (SH3) of drought-tolerant clone 14 of *C. canephora* subjected to drought was selected (Fig. 1A). It contained the genes encoding a WRKY transcription factor (*CcWRKY2*) and a psaL subunit of PSI (*CcPSALI*); two putative early light-induced proteins (*CcELIPI* and *CcELIP2*) that shared >99% amino acid identity, and a ‘no hit’ protein (*CcUNK1*; with UNK for unknown) were also identified.

A second set of CGs corresponded to reads overexpressed in leaf cDNA libraries from drought-acclimated plants of *C. canephora* (SH3) but also present in other libraries. This was the case for genes coding for a catalase (*CcCAT1*), an acyl-CoA-binding protein (*CcACBPI*), chlorophyll *alb*-(CAB) binding protein (*CcCABI*), a metallothionein-like protein (*CcMET1*), a GLB2-like non-symbiotic haemoglobin (*CcNSHI*), a glycine-rich protein (*CcGRPI*), a mannose 6-phosphate reductase (*CcMPRI*; EC 1.1.1.224), and an unknown protein (*CcUNK10*), all of them presenting higher *in silico* expression in the SH3 library of *C. canephora* than in the SH2 library of *C. arabica* var. Catuai (Fig. 1B).

Identification of CG genes by the screening of macroarrays

Another set of CGs was selected by screening macroarray membranes containing ESTs representing coffee unigenes expressed in SH2 and SH3 cDNA libraries, with cDNA probes corresponding to RNA extracted from leaves of *C. canephora* clones 22 and 14 subjected or not to drought conditions. This allowed the selection of 28 CGs that showed differential expression between the two clones of *C. canephora* and the water treatments. Hybridization results, presented for some of them (Fig. 2), concerned genes coding for a TRAF-like [tumour necrosis factor receptor (TNFR)-associated factor] protein (*CcTRAF1*), a pre-phenate dehydrogenase (*CcPDHI*; EC 1.3.1.12), an unknown protein 8 (*CcUNK8*), a dehydrin identical to the *CcDH3* protein (Hinninger *et al.*, 2006), an EDR1 (enhanced disease resistance)-like mitogen-activated protein kinase kinase (MAPKK) kinase protein (*CcEDR1*) previously reported to function as a negative regulator of

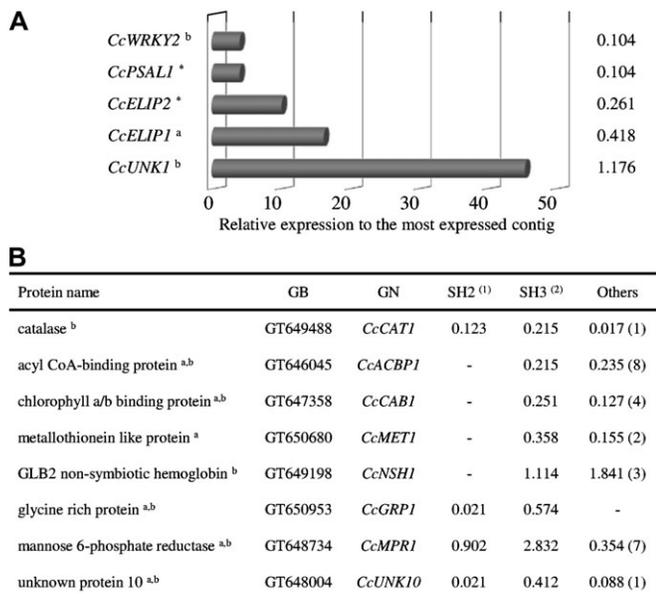


Fig. 1. *In silico* analysis of coffee ESTs overexpressed under drought conditions. (A) ESTs exclusively overexpressed in the leaves of drought-tolerant clone 14 of *C. canephora* var. Conilon subjected to drought (SH3 cDNA library). The numbers indicate the percentage of reads in the library (n total=5743 reads). Results of TBLASTX against the non-redundant protein sequence database (NR; E-value cut-off of $1e^{-10}$) were as follows: *CcWRKY2* encoding a WRKY transcription factor (*Ricinus communis*, XP002529048, E-value $1e^{-115}$), *CcPSAL1* (GT646615) encoding the photosystem I subunit XI (*Nicotiana attenuata*, AAO85557, E-value $2e^{-103}$), *CcELIP2* (GT647647) encoding isoform 2 of a putative early light-induced protein (*Ricinus communis*, XP002517068, E-value $3e^{-59}$), and *CcELIP1* encoding isoform 1 of a putative early light-induced protein (*Camellia sinensis*, ACB20694, E-value $3e^{-63}$). No hits were found for the *CcUNK1* (DV689820) gene. (B) ESTs from *C. canephora* mainly overexpressed in the SH3 cDNA library (results of Fisher's test). Values indicate the percentage of ESTs in the corresponding cDNA libraries. Numbers of other cDNA libraries containing reads are indicated in parentheses. Total reads in SH2 [⁽¹⁾ 5053] and SH3 [⁽²⁾ 5743]. GenBank accession numbers (GB) and gene names (GN) of coffee ESTs are also given. ^a, Gene expression analysed by northern blot; ^b, gene expression analysed by qPCR; *, gene expression not determined.

disease resistance and ethylene-induced senescence (Tang *et al.*, 2005), a small heat shock protein (*CcHSP1*), and the previously identified *CcMPR1*. The expression of these genes was further tested by qPCR.

Protein expression pattern in response to drought

Finally, a 2-DE approach was also used to search for other CGs. The 2-DE of soluble proteins extracted from leaves of *C. canephora* clones 14 and 22 (under both I and NI conditions) usually led to the identification of 700–1000 well resolved spots (data not shown). Comparisons between these gels permitted selection of ~40 intense proteins showing differential accumulation between coffee clones

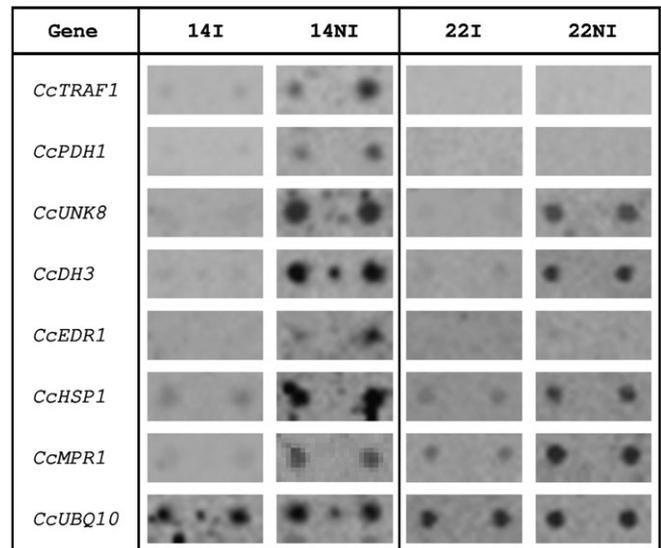


Fig. 2. Identification of candidate genes by the screening of macroarrays (reverse northern). Membranes were hybridized with cDNA probes representing RNA extracted from leaves of *C. canephora* clones 22 and 14 grown with (I) or without (NI) irrigation. Hybridization results are shown for the *CcTRAF1*, *CcPDH1*, *CcUNK8*, *CcDH3*, *CcEDR1*, *CcHSP1*, and *CcMPR1* genes, and also for the constitutive *CcUBQ10* gene. For all these genes, expression was analysed by qPCR.

and with drought conditions applied to the plants. Some of these proteins were further sequenced by MALDI-TOF-MS/MS (Table 2). Among them, a chloroplastic carbonic anhydrase-like protein (CcCA1: EC 4.2.1.1) was identified in higher amounts in clone 14 than in clone 22 under untreated (I) conditions and decreased with drought (NI) for both clones (Fig. 3). For a type-2C protein phosphatase (CcPP2C: EC 3.1.3.16), a decrease with drought was observed in leaves of clone 22 while the opposite was observed in clone 14. 2-DE profiles also showed differential accumulation of extrinsic proteins of PSII involved in the oxygen-evolving complex (OEC), such as CcPSBO and CcPSBP proteins, with a higher amount in clone 22 than in clone 14 and increased accumulation under drought for both clones. For CcPSBQ, an increased amount of protein was found for both clones upon drought. A small heat shock protein (*CcHSP1*, described before) accumulated with DA in leaves of the drought-susceptible clone 22 but not in those of the drought-tolerant clone 14. For all these proteins, corresponding coffee SGN (Mueller *et al.*, 2005) contig or EST (Lin *et al.*, 2005; Vieira *et al.*, 2006) sequences with high similarity were identified and used to design specific primers to analyse the effects of drought on gene expression by qPCR (Table 3).

Expression profiles of candidate genes

As described before, CGs were selected by either electronic northern, screening of macroarrays, or after the comparison of 2-DE gels. This list was also increased by adding several

Table 2. Identification of leaf coffee proteins presenting differential accumulation during drought acclimation

Protein names and corresponding peptide sequences obtained by MALDI-TOF-MS/MS are indicated and validated statistically by Scaffold Score expressed as a percentage (%). The observed isoelectric point (pI) and molecular weight (MW) were calculated from the 2-DE gels by ImageMaster Platinum 6.0 Software. Homology searches were done with the TBLASTN program (Altschul *et al.*, 1990) using an E-value cut-off of $1e^{-10}$. GenBank (GB) and SGN (<http://solgenomics.net>, Mueller *et al.*, 2005) accession numbers of ESTs and contigs containing the sequenced peptides are indicated.

Protein name	Gene name	Sequences	Scaffold Score (%)	Accession nos	pI/MW
Carbonic anhydrase	<i>CcCA1</i>	EKYETNPALFGQLAK	91	GT008701	6.85/23
		YMIVACADSR	85		
		VNPSLILSLQPGDAFIVR	80		
		SVANLVPPYDQLK	90		
		HSEFGAAIEFAVLHLK	70		
		VENIVIGHSACGGIK	60		
		GLMSIPEDGTTSTDFIEDWVK	90		
		VKTEHGSKPLPEQIVLAE	95		
Type-2C protein phosphatase (PP2C)	<i>CcPP2C</i>	KTSTSLMALSSPQLR	90	GW435032 SGN-U631279	5.26/22
		EEMEDDVIIVR	95		
		ALEEAFESADMK	85		
		EAGGWISNGR	85		
Oxygen-evolving complex PSII (psbO)	<i>CcPSBO</i>	KDGIDYAAVTVQLPGGERV	90	GT647517 SGN-U629535	5.46/33
		RLTYDEIQSK	74		
Oxygen-evolving complex PSII (psbP)	<i>CcPSBP</i>	WNPSKEVEFFGQVLR	90	GW488995	5.76/29
		YEDNFDSNSSVSVIIIPCDKK	50		
		SITDYGPPEEFLSK	95		
		VDYLLGK	52		
		TEAEGGFEPNTVATANILEQETPIV	55		
TADGDEGGKHQLIR	90				
Oxygen-evolving complex PSII (psbQ)	<i>CcPSBQ</i>	RFFIQALPPAGAAARA	90	GT645658 SGN-U637442	10.01/15
		DLDLPLKDR	74		
		LKAELLR	62		
		STPEAEKYYAATVSTLNDVLSK	95		
		YDLNTVISAKPK	90		
Small heat shock protein (sHSP)	<i>CcHSP1</i>	SAPVAPIGVLDLRFPTAR	95	GW447897	5.87/22
		TVQQMMETMER	91		
		NEDGEEEEKNEWSAK	90		
		LMEDPFAYSGGWPSPLAPDTGGYSR	60		
		GRTPWEIK	60		
		ALPENAQF	70		

other CGs as yet not reported in the literature [e.g. Responsive to Dehydration (RD)-type genes) to respond upon drought conditions. Expression profiles of CGs were further checked in leaves of *C. canephora* clones 14, 22, and 120 by classical northern blot and qPCR experiments. In some cases, expression profiles were also assessed by both methods. These analyses led to the identification of differentially expressed genes under irrigated and non-irrigated conditions. These results are detailed below in separated sections.

Analysis of *CcUNK10* gene expression standardized with different housekeeping genes

Even though it codes for an unknown function, the study of *CcUNK10* is of a particular interest because this gene was

preferentially expressed in the drought-sensitive clone 22, mainly under untreated condition, but was also highly induced by drought. Because the accuracy of qPCR always requires the use of a reference gene to normalize expression levels, several housekeeping genes were tested to standardize *CcUNK10* expression (Fig. 4). It is worth noting that *CcUNK10* expression profiles were similar when using the *CcUBQ10* (ubiquitin), *CcADPI* (ADP ribosylation factor), *CcACT1* (actin), and *CcGAPDH* (glyceraldehyde-3-phosphate dehydrogenase) genes as references to standardize the results of relative quantification. In these cases, the *CcUNK10* expression levels were always conserved [22NI>22I=120NI≥14NI>14I=120I] but differed from those that used *CcCYN1* (cyclophilin) and *CcCTS1* (cathepsin) as references. Therefore, qPCR analyses of all identified CGs were performed using *CcUBQ10* or *CcGAPDH*.

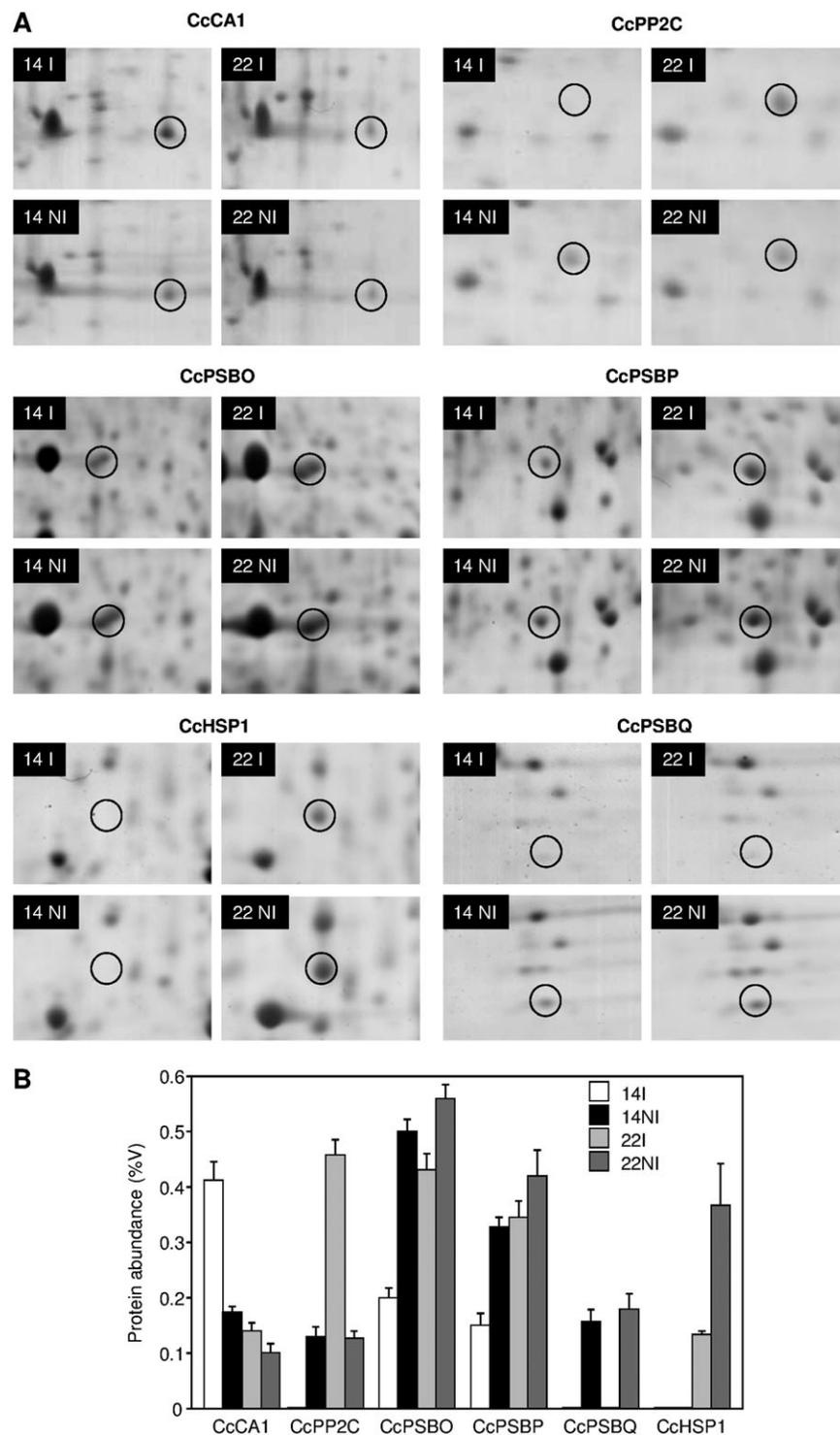


Fig. 3. Protein responses in coffee leaf exposed to drought. (A) Plants were irrigated (I) or not irrigated (NI); leaf soluble proteins were extracted and separated by two-dimensional gel electrophoresis (2-DE), stained by Coomassie Brilliant Blue, and scanned. The results shown are representative of at least three repetitions. Spots corresponding to CcCA1 (carbonic anhydrase) and CcPP2C (type-2C protein phosphatase) proteins were analysed using strips with an immobilized linear pH gradient of 3–10. Spots corresponding to CcPSBO (PSII oxygen evolving complex protein, OEC), CcPSBP (OEC), and CcHSP1 (small heat shock protein) proteins were analysed using strips with an immobilized linear pH gradient of 4–7, and CcPSBQ (OEC) with pH gradient 6–11. Proteins were further characterized by MALDI-TOF-MS/MS (see Table 2). (B) Normalized protein abundance of the coffee leaves extracted from clones 14 and 22 of *C. canephora* subjected (NI) or not subjected (I) to drought. Protein abundance was deduced from the 2-DE using ImageMaster Platinum 6.0 software and is expressed as a percentage of volume (%V) which was calculated from the gel images as the volume of a specific spot divided by the sum of the volume of all other spots present in the gel multiplied by 100. Volume percentage averages were calculated from 2-DE gels loaded with three biological replicates.

Table 3. Candidate genes and corresponding primers used for qPCR experiments

Coffee sequences were selected (E -value $\leq 1e^{-10}$) from public databases with the BLAST programs (Altschul *et al.*, 1990). Gene names were assigned based on the best BLASTX hit obtained by comparing the selected coffee ESTs with public databases. GenBank (GB) accession numbers of coffee EST sequences are also given. Primers were designed using the Primer Express software (Applied Biosystems).

Protein name	Gene name	GB numbers	Primer name	Primer sequence	bp	CG
Ubiquitin	<i>CcUBQ10</i>	GW488515	BUBI-F BUBI-R	5'-AAGACAGCTTCAACAGAGTACAGCAT-3' 5'-GGCAGGACCTTGGCTGACTATA-3'	104	R*
Glyceraldehyde-3-phosphate dehydrogenase	<i>CcGAPDH</i>	GW445811	GAPDH-F GAPDH-R	5'-TTGAGGGCGGTGCAAA-3' 5'-AACATGGGTGCATCCTTGCT-3'	59	R*
Cathepsin	<i>CcCTS1</i>	GW442860	CATHEP-F CATHEP-R	5'-CCCATTATCGTTCTGGTGTTTACAA-3' 5'-ACCCCATCCAATCAGCTTTACA-3'	80	R
Actin	<i>CcACT1</i>	GW428479	ACTIN-F ACTIN-R	5'-GGATTTCCAGGGCTGAATATGA-3' 5'-CGATACGATAGTAGCAGCTTAAAAGC-3'	80	R
ADP-ribosylation factor (ARF)	<i>CcADP1</i>	GW450736	ADP-F ADP-R	5'-ATGAATGCTGCTGAAATAACTGATAAG-3' 5'-GCACAGGTGCTTTGGATATACCA-3'	80	R
Cyclophilin	<i>CcCYN1</i>	GW488988	CYCLO-F CYCLO-R	5'-TCTTCATCTGCACCGACAAGA 3' 5'-CACATCCATACCCTCCGTGAT-3'	80	R
WRKY transcription factor	<i>CcWRKY2</i>	GT647873	18089-F 18089-R	5'-CATGATCCCAAAGCAGTTATTACG-3' 5'-TTCTGGCAGTTGGTACATCATGAT-3'	67	1
Photosystem I subunit XI	<i>CcPSAL1</i>	GT646615	ND			1
Early light-induced protein (ELIP)	<i>CcELIP1</i>	GT647659	**			1
Early light-induced protein (ELIP)	<i>CcELIP2</i>	GT647647	**			1
Unknown protein 1	<i>CcUNK1</i>	DV689820	182052-F 182052-R	5'-TATAGTGTTTATGGTGTGGCTTTCAGT-3' 5'-GTACCACCGTAGGGAGACGTATG-3'	79	1
Catalase	<i>CcCAT1</i>	GT649488	18297-F 18297-R	5'-TGACAGAACCTGCAGTAAAGACAGTATT-3' 5'-AACCAACCAAATGAACGAACAAT-3'	146	2
Catalase	<i>CcCAT2</i>	GT648692	SH3055G111-F SH3055G111-R	5'-GATACCCAGCGACACCGTCTT-3' 5'-GATGAGCACACTTGGGAGCAT-3'	70	5 <i>CcCAT1</i>
Acyl-CoA-binding protein	<i>CcACBP1</i>	GT646045	9158-F 9158-R	5'-AATACTACCAATGCAAGCAAGCTTA-3' 5'-CCTTCCATGCATCCCACCTT-3'	128	2
Chlorophyll <i>a/b</i> -binding protein	<i>CcCAB1</i>	GT647358	18230-F 18230-R	5'-CTCTGAACTTCACCAGCTCTTCAA-3' 5'-GAGCTCGTGTCCAATGAAGA-3'	140	2
Metallothionein like protein	<i>CcMET1</i>	GT650680	**			2
GLB2-like non-symbiotic haemoglobin	<i>CcNSH1</i>	GT649198	RBCS3'-F RBCS3'-R	5'-CTAAGGGATACGGATGAAATCCA-3' 5'-CTCTCGCAGCTGCACTACTGATT-3'	102	2
Glycine-rich protein	<i>CcGRP1</i>	GT650953	GC182-F GC182-R	5'-AAGCCAGCTTCTAGCTATGTATGA-3' 5'-GATCCAGCACITATTTGAGATCACA-3'	100	2
Mannose 6-phosphate reductase	<i>CcMPR1</i>	GT648734	LPSH3069F05-F LPSH3069F05-R	5'-AATCAGCAATTACAGCGTTTTGC-3' 5'-AGTGACACAGATGCCGTGCTT-3'	100	2,3
Aldose-phosphate reductase	<i>CcAPR1</i>	GT649483	LPSH3060G02-F LPSH3060G02-R	5'-TGAAGCCAGCTGTGAATCAACT-3' 5'-GTGTGGCAGTGACACAGATG-3'	100	5 <i>CcMPR1</i>
Unknown protein 10	<i>CcUNK10</i>	GT648004	D18240-F D18240-R	5'-TAGCCTTGTTCTTTAGGGAGCTTATC-3' 5'-AGAGCTTCGTCCAGGAAGAAGA-3'	134	2
TNF-associated factor (TRAF)	<i>CcTRAF1</i>	GT652792	LP-12677-F LP-12677-R	5'-GACTCTGGGCACATCGTATAG-3' 5'-TGCGAAGGGAAGAATGGAA-3'	100	3
Prephenate dehydrogenase	<i>CcPDH1</i>	GT655248	LP175502-F LP175502-R	5'-GCAGGGACATGGACGAATTT-3' 5'-TGAACGGCATGGACTTGAC-3'	100	3
Unknown protein 8	<i>CcUNK8</i>	DV695331	LP18100-F LP18100-R	5'-CTCGCGTGGCCGAGATT-3' 5'-CCCTCACATTTCCACGTGAAT-3'	100	3
Dehydrin	<i>CcDH3</i>	DV689895	18390-F 18390-F	5'-TTAATAGCAGCTTTCCAGTGTGTCA-3' 5'-GATCCCCCAAAAAGCAGAAAA-3'	100	3
EDR1-like MAPKK kinase	<i>CcEDR1</i>	GW457961	LPSH3054B02-F LPSH3054B02-R	5'-TGTCAAATTGATGAAAAGCGAAGA-3' 5'-AAGGAAAAGTAGGAAATCAGCCAAA-3'	100	3
EDR1-like MAPKK kinase	<i>CcEDR2</i>	DV681462	EDR13-F EDR13-R	5' CGGCATAAGAGCAGTGGAA 3' 5' ATGCAATCGCTGGTGTAGAAAA 3'	70	6
Small heat shock protein (sHSP)	<i>CcHSP1</i>	GW447897	LPSH3056B04-F LPSH3056B04-R	5'-GGTAGGACGCCATGGGAGAT-3' 5'-CCTCAACCCACACCTTCACAT-3'	100	3, 4
Carbonic anhydrase	<i>CcCA1</i>	GT008701	HRAM1-F	5'-AAGGCCATTGTGGACTTCA-3'	100	4

Table 3. Continued

Protein name	Gene name	GB numbers	Primer name	Primer sequence	bp	CG
Type-2C protein phosphatase (PP2C)	<i>CcPP2C</i>	GW435032	HRAM1-R	5'-TTGTTTGCAACTCTGCAGTGATT-3'	101	4
			HRAM2-F	5'-CGAAGAAATCAGGCGTATCAGAG-3'		
Oxygen-evolving complex PSII (psbO)	<i>CcPSBO</i>	GT647517	HRAM2-R	5'-TAAACCGCACGTCACCAAAG-3'	101	4
			HRAM12-F	5'-TGAATTTCTCGTGCCATCATACA-3'		
Oxygen-evolving complex PSII (psbP)	<i>CcPSBP</i>	GW488995	HRAM12-R	5'-CTCCAGCAGGCAATGCAACT-3'	100	4
			HRAM7-F	5'-AGCTGTCGATTCCTCAAAATG-3'		
Oxygen-evolving complex PSII (psbQ)	<i>CcPSBQ</i>	GT645658	HRAM7-R	5'-GAGACTGCTGTGTATCGAA-3'	102	4
			HRAM36-F	5'-CAGGGCAATCAAGGTTGGA-3'		
Rubisco small chain (RbcS)	<i>CcRBCS1</i>	GT649534	HRAM36-R	5'-CGGTCTTGAGTGCCAAATC-3'	169	6
			18244-F	5'-CACCAACTGAAAAGTTGAAGAA-3'		
Subtilisin-like serine protease	<i>CcSDD1</i>	GT659840	18244-R	5'-TATCCCGGTGACCTGTGGTATT-3'	101	6
			SDD1-F	5'-GAGCCCGATTGATCTTCTG-3'		
Glucosyltransferase arbutin synthase	<i>CcGAS1</i>	GT721173	SDD1-R	5'-ACTCAGCCCAAAAGGGTTAA-3'	80	6
			ARBU09-F	5'-AAAGCGTTGCGAGGACAAGA-3'		
Abscisic acid receptor	<i>CcPYL3</i>	GT720590	ARBU09-R	5'-CTGTCCCCTGAGCCCATCT-3'	80	6
			PYL31-F	5'-CGGTGACGACTGTCCATGAG-3'		
Abscisic acid receptor	<i>CcPYL7</i>	DV704550	PYL31-R	5'-CGGCACGTCAAAGTATACG-3'	80	6
			PYL72-F	5'-GAGAAGCATTCTTGGGATCAA-3'		
Amidase (IAA synthesis)	<i>CcAMI1</i>	GT651356	PYL72-R	5'-GGATGCACGTAAGGATGGA-3'	80	6
			HD141612-F	5'-AGATGTCTGCCACAGTGTGAAGA-3'		
RD29-like protein	<i>CcRD29</i>	GT660256	HD141612-R	5'-GGAGTGCAAGGATGCCAAAA-3'	100	6
			RD29-F	5'-TGATGATCAAGATCCCCAACAC-3'		
AP2/ERF DREB-like	<i>CcDREB1</i>	GW463524	RD29-R	5'-CTTCGCTTTCGCCTTCACTT-3'	80	6
			DREBA09-F	5'-CAATGCCTGCAAAGCCAATTA-3'		
NAC-RD26-like	<i>CcRD26</i>	GT003652	DREBA09-R	5'-TTTTCTGCCTGCACGTTTC-3'	98	6
			NACRD26-F	5'-TTTGGCCCTGCGCTCTAGT-3'		
MYB-type 2 transcription factor	<i>CcMYB1</i>	GT689406	NACRD26-R	5'-AAGCGGGTCAGTTTCTCGAA-3'	100	6
			MYB61-F	5'-CCCGCAATCTTCCAGCTA-3'		
Caffeoyl-CoA 3-O methyltransferase	<i>CcCCoAOMT1</i>	GT715419	MYB61-R	5'-TCAAGCGTGGCAACTTCACT-3'	100	6
			CCoAOMT-F	5'-GACACACCAGCCCTTTCGAT-3'		
Clp protease ATP-binding subunit	<i>CcCLP1</i>	GT651512	CCoAOMT-R	5'-CAGCTCTCGCTCTTCCAGAAG-3'	80	6
			CLP1-F	5'-CCACCCAGTAGGACCAGAAA-3'		
Ascorbate peroxidase	<i>CcAPX1</i>	GT697455	CLP1-R	5'-CATTGCTGCTGCTGCTGTTG-3'	70	6
			ASCPER1-F	5'-GACCTGAACAATGCCAGAAG-3'		
Ascorbate peroxidase	<i>CcAPX2</i>	EE193467	ASCPER1-R	5'-CGTAAATGAGCAGCAGGTGATG-3'	80	5
			ASCPER6-F	5'-AGACCGTGTCTCAAACCGACTAC-3'		
Leucine zipper (ABA signalling)	<i>CcABI5</i>	GT645781	ASCPER6-R	5' GTTGATCTGTTGCCCCAAGA 3'	99	6
			ABI52-F	5'-GCCGCTGCAGCCTCTATTT-3'		
Leucine zipper (ABA signalling)	<i>CcAREB1</i>	EE198828	ABI52-R	5'-AGCTGAGCGTTGTTCCCTATCT-3'	100	6
			AREB11-F	5'-TGTTGTAAGGGAGGATGCTCAA-3'		
Homeobox leucine zipper hypothetical protein	<i>CcHDZ1</i>	GT724869	AREB11-R	5'-TCCAAACCCAAAAGCCTGAT-3'	100	6
			HD68272-F	5'-GACGTTGACGGACGAGAACA-3'		
Homeobox leucine zipper hypothetical protein	<i>CcHDZ2</i>	GW447493	HD68272-R	5'-TGTAGCCGCTGGCAACTG-3'	100	6
			HD111162-F	5'-AGTGCCAGGGAAAAGAATTCC-3'		
			HD111162-R	5'-TCCGCTGTCTCTTCTCATG-3'		

R, reference genes tested, with *CcUBQ10* and *CcGAPDH* being used as reference genes in qPCR experiments (*, **, gene expression tested by northern blot but not by qPCR); ND, gene expression not determined. The size of the amplicon in bp is also indicated. The selected candidate genes (CGs) corresponded to (1) genes exclusively overexpressed in the leaf SH3 cDNA library of drought-tolerant clone 14 of *C. canephora* var. Conilon subjected to drought (Fig. 1A); (2) genes mainly overexpressed in the SH3 cDNA library (Fig. 1B); (3) genes identified by the macroarray experiment (Fig. 2); (4) genes encoding proteins showing differential accumulation by 2-DE (Fig. 3); (5) genes homologous to other CGs; and (6) genes selected from the literature. CGs are ordered as presented in the text.

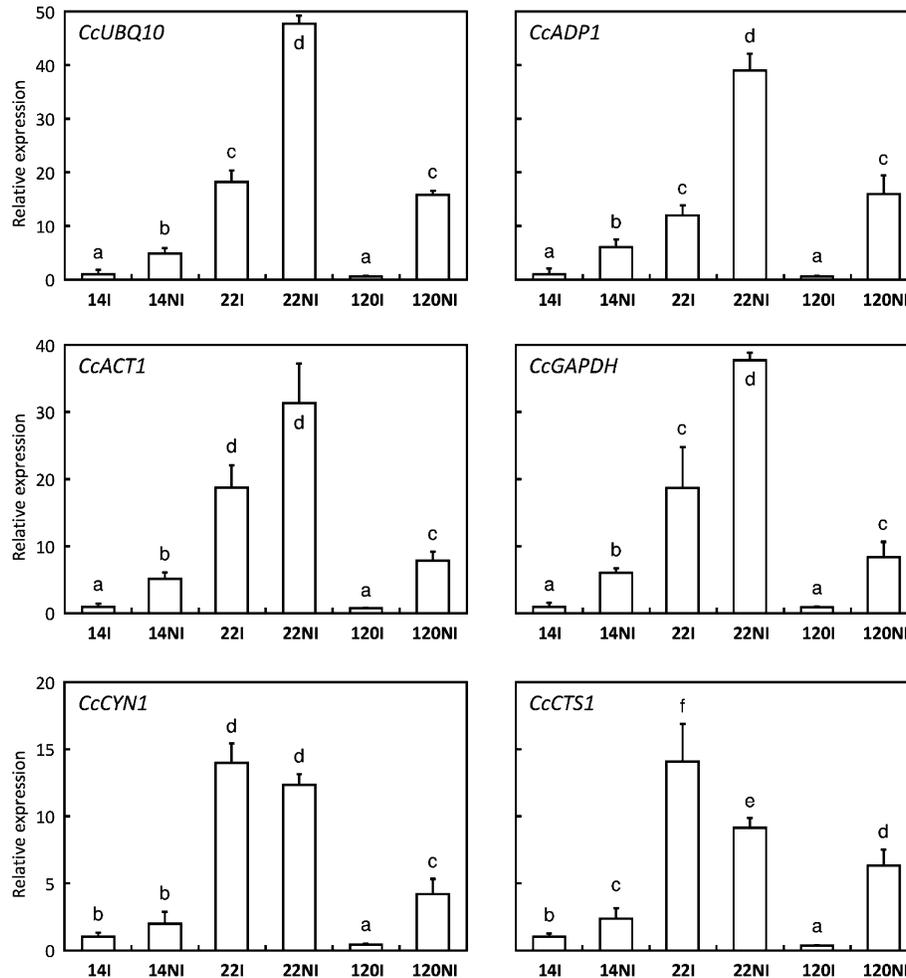


Fig. 4. Relative expression of the *CcUNK10* gene standardized with different reference genes. Transcript abundances were analysed by qPCR in leaves of drought-tolerant (14 and 120) and drought-susceptible (22) clones of *C. canephora* grown with (I) or without (NI) irrigation and standardized independently with the *CcUBQ10* (ubiquitin), *CcADP1* (ADP ribosylation factor), *CcACT1* (actin), *CcCYN1* (cyclophilin), *CcCTS1* (cathepsin), and *CcGAPDH* (glyceraldehyde-3-phosphate dehydrogenase) genes as references. Values are the mean of at least three technical repetitions \pm SD. The significance of expression level differences between the treatments was evaluated using the pairwise Wilcoxon rank test (non-parametric test). Treatments sharing the same letter are not significantly different.

Genes with reduced expression under drought acclimation

Expression of genes coding for CAB-binding protein (*CcCABI*), the small subunit (*CcRBCS1*) of ribulose 1,5-bisphosphate carboxylase (Marraccini *et al.*, 2003), and an acyl-CoA-binding protein (*CcACBP1*: EC 3.4.17.3) was studied by northern blot and qPCR experiments (Fig. 5A). For all clones of *C. canephora* tested, expression of *CcCABI* and *CcRBCS1* genes decreased greatly with drought. For both genes, expression levels were higher under irrigated conditions for clone 120 as compared with clones 14 and 22. For *CcACBP1*, expression upon drought and irrigated conditions was higher in clone 22 than in both tolerant clones 14 and 120. The decrease under the NI condition was higher in the tolerant clones. For all these genes, expression profiles obtained by northern blot and qPCR were identical. Northern blot experiments also showed that drought caused the down-regulation of

CcELIP1 and *CcMET1* gene expression for all *C. canephora* clones tested (Fig. 5A).

QPCR experiments also demonstrated that expression of the genes *CcCAI*, *CcCAT1*, *CcSDD1* (coding for a subtilisin-like serine protease), *CcGAS1* (coding for a glucosyl-transferase arbutin synthase, EC 2.4.1.218), and *CcPP2C* declined with drought, except for clone 120 in the case of *CcCAT1* where this decline was not statistically different (Fig. 5B). In the same way, expression of *CcPSBO*, *CcPSBP*, and *CcPSBQ* genes encoding OEC proteins of PSII was also greatly reduced under DA (Fig. 5B). Down-regulated expression of *CcAMH1* (coding for an amidase involved in indole acetic acid synthesis), *CcCCoAOMT1* coding for the caffeoyl-coenzyme A 3-*O* methyltransferase, (Lepelley *et al.*, 2007) and *CcAPX2* (coding for an ascorbate peroxidase) was also observed upon drought (Fig. 5B). Interestingly, for *CcPYL3* [coding for an abscisic acid (ABA) receptor], down-regulation was clearly observed only for clone 120 (Fig. 5B).

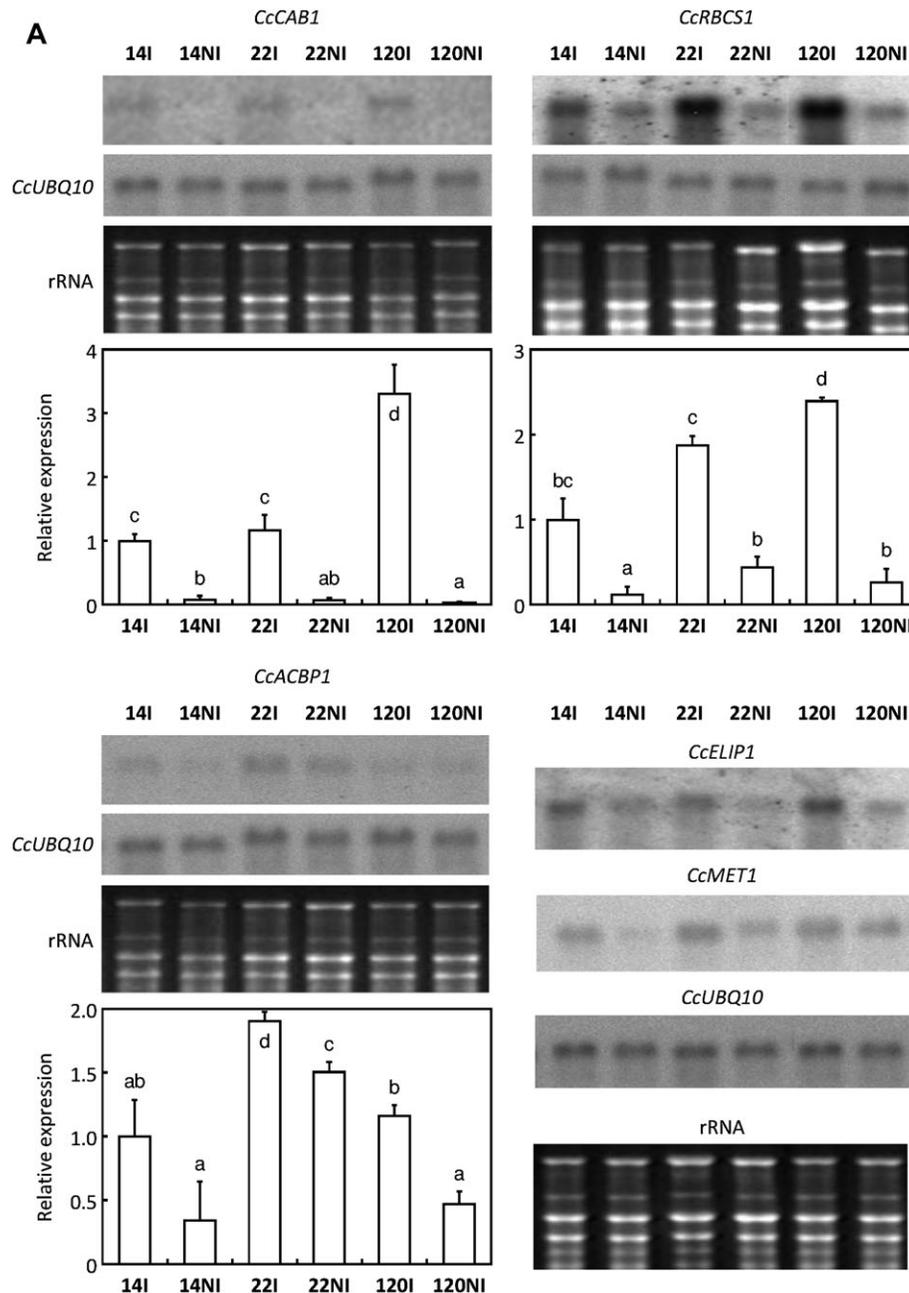


Fig. 5. Expression profiles of genes down-regulated during drought acclimation. Gene expression was analysed in leaves of clones 14, 22, and 120 of *C. canephora* subjected (NI) or not (I) to drought. (A) Abundances of specific transcripts were analysed by northern blot and qPCR for *CcCAB1*, *CcRBCS1*, and *CcACBP1*. Transcript abundances of *CcELIP1* and *CcMET1* genes were monitored only by northern blot. For this analysis, total RNA stained with ethidium bromide was used to monitor the equal loading of the samples. Constitutive expression of the *CcUBQ10* gene is also shown. (B) For other genes, transcript abundances were analysed by qPCR. The expression of the *CcUBQ10* gene was used as a reference to measure the relative quantification that corresponds to the mean of three technical repetitions \pm SD. The gene names are indicated in the histograms. Results are expressed using 14I as an internal calibrator. In each case, values of relative quantification correspond to the mean of at least three technical repetitions \pm SD. Significance of expression level differences between the treatments was evaluated using the pairwise Wilcoxon rank test (non-parametric test). Treatments sharing the same letter are not significantly different.

Genes with increased expression under drought acclimation

As demonstrated by northern blot and qPCR analyses, drought increased the expression of *CcMPRI*, *CcUNK10*, and *CcGRP1* genes (Fig. 6A). On the other hand, water

limitation also increased the expression of *CcRD29*, *CcDREB1*, and *CcRD26* genes coding for RD29-like protein, and the AP2/ERF DREB-like and NAC-RD26-like transcription factors, respectively (Fig. 6B). It is worth noting that higher levels of induction upon drought were

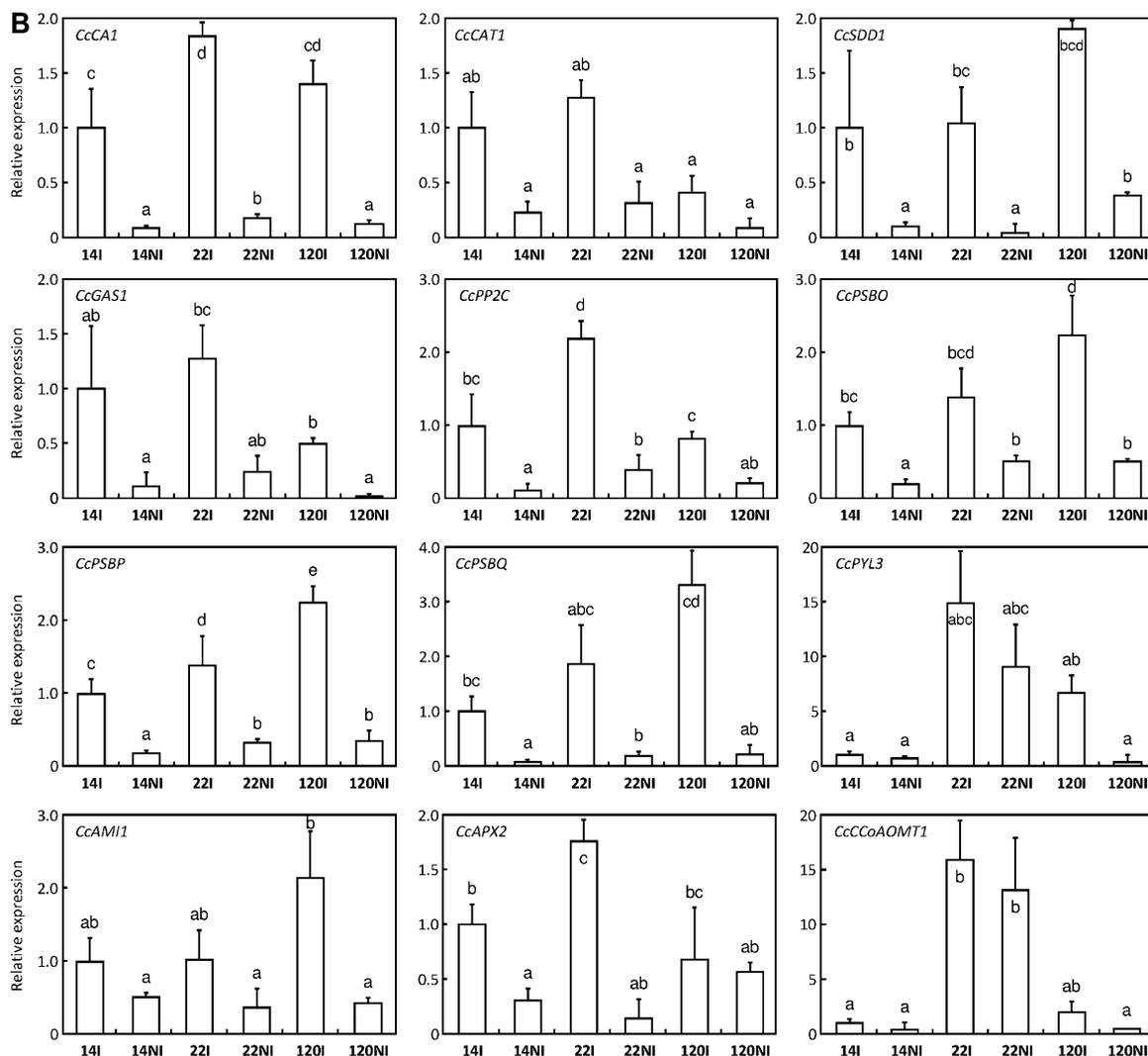


Fig. 5. Continued

observed for *CcDREB1* and *CcRD26* in the drought-tolerant clone 14 as compared with the other clones tested under the same conditions. A similar differential pattern was also observed for the regulatory genes *CcABI5*, *CcAREB1*, and *CcHDZ1* (Fig. 6B). Furthermore, the *CcHSP1*, *CcDH3*, *CcAPR1*, and *CcCAT2*, as well as the *CcUNK8* expression profile followed a similar pattern (Fig. 6B). In summary, the results show that for all these genes mentioned above, clone 14 displays a clear stronger induction upon DA (12 d of DA for both clones) than clone 120, suggesting that different mechanisms may account for their drought tolerance phenotype.

In addition, the expression patterns displayed by other regulatory genes such as *CcERD2*, *CcHDZ2*, and the ABA receptor-encoding gene *CcPYL7*, whereby induction upon DA was observed for clone 14 and down-regulation was observed for clone 120, also corroborates this hypothesis (Fig. 6B). Other genes such as *CcCLP1*, *CcUNK1*, and *CcAPX1* also follow the same pattern, but in the case of *CcTRAF1*, clone 120 displayed unchanged levels upon drought (Fig. 6B).

The expression of *CcNSH1*, *CcPDH1*, and *CcWRKY2* was also increased by drought, at higher levels in the drought-sensitive clone 22 than in both tolerant clones 14 and 120 (Fig. 6B). Similarly, higher expression levels of the *CcMYB1* gene coding for a helix–turn–helix MYB-type 2 transcription factor was observed for clone 22 as compared with both tolerant clones, but in this case induction upon DA was not statistically different. Finally, transcript accumulation upon DA was also observed for *CcEDR1* in all clones tested (Fig. 6B).

Discussion

The analyses of physiological parameters performed under irrigation showed higher values of g_s and C_i/C_a for the drought-sensitive clone 22 compared with the drought-tolerant clones 14 and 120. For the latter, the results presented here were identical to those previously reported for the same clone by Pinheiro *et al.* (2004). Taken together, these analyses suggested that the drought-sensitive clone 22

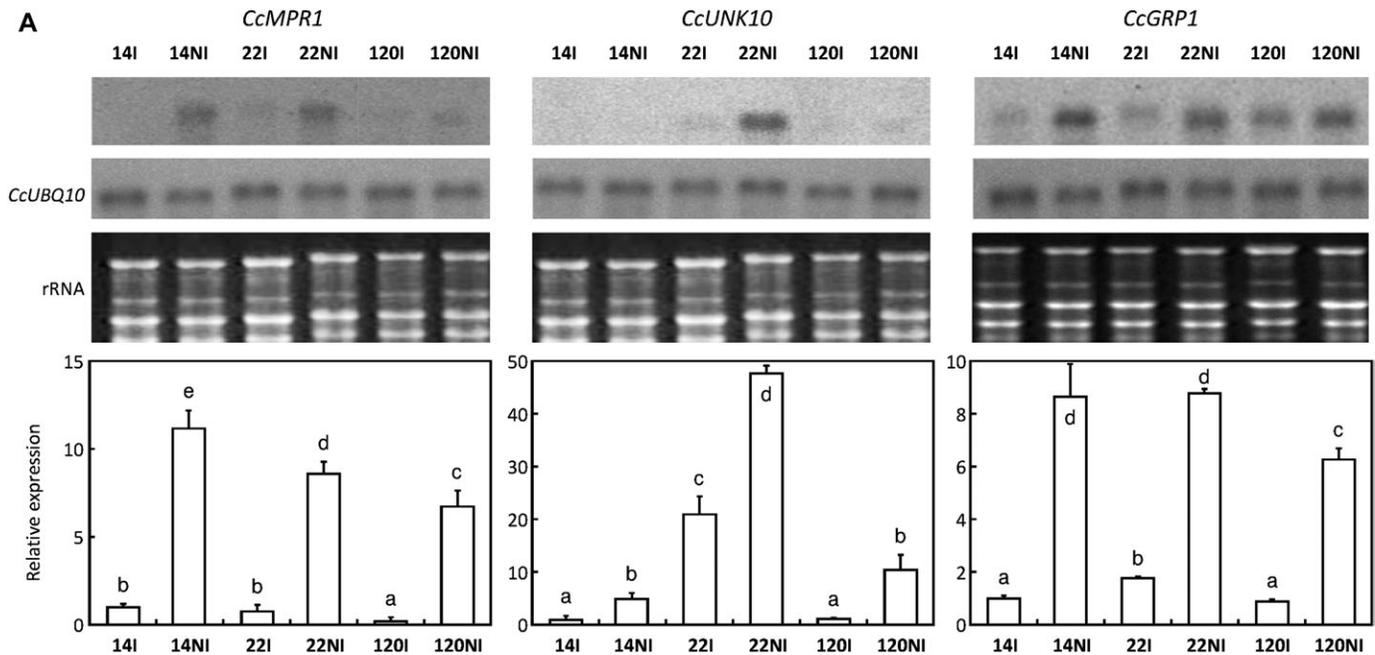


Fig. 6. Expression profiles of genes up-regulated during drought acclimation. Gene expression was analysed in leaves of clones 14, 22, and 120 of *C. canephora* subjected (NI) or not (I) to drought. (A) Abundances of specific transcripts were analysed by northern blot and qPCR for *CcMPR1*, *CcUNK10*, and *CcGRP1*. For northern blot analyses, total RNA stained with ethidium bromide was used to monitor the equal loading of the samples. Constitutive expression of the *CcUBQ10* gene is also shown. (B) For other genes, transcript abundances were analysed by qPCR. The expression of the *CcUBQ10* gene was used as a reference to measure the relative quantification, except for the genes marked with an asterisk that used the expression of the *CcGAPDH* gene as reference. The gene names are indicated in the histograms. Results are expressed using 14I as an internal calibrator. In each case, values of relative quantification correspond to the mean of at least three technical repetitions \pm SD. Significance of expression level differences between the treatments was evaluated using the pairwise Wilcoxon rank test (non-parametric test). Treatments sharing the same letter are not significantly different.

had a lesser efficiency in controlling stomatal closure and transpiration than the drought-tolerant clones 14 and 120, a situation that could explain its faster decline of Ψ_{pd} and greater reduction of A under drought. Because the stomatal closure reduced transpiration more than photosynthesis, the drought-tolerant clones 14 and 120 should have higher water use efficiency than the drought-sensitive clone 22 as previously observed by carbon isotope discrimination for other clones of *C. canephora* (Lima *et al.*, 2002; DaMatta *et al.*, 2003). The data presented here also suggested that the photosynthetic inhibition was mainly due to stomatal closure. However, non-stomatal limitation of photosynthesis, possibly associated with the decrease of Rubisco regeneration and carboxylation, could also occur (Kanechi *et al.*, 1996; DaMatta *et al.*, 1997, 2002). The reduction of Φ_{PSII} observed under DA, for the drought-tolerant clones 14 and 120, was not accompanied by variations of F_v/F_m , probably being associated with the inhibition of PSII. Such a reduction should reflect a mechanism of photo-protection under DA, by adjusting the rate of electron transport to the rate reducing power, for example through photorespiration.

In order to study the molecular changes that occurred during DA, an integrated analysis was performed to identify proteins and CGs presenting differential expression profiles

under drought stress, using different approaches. For all the CGs identified, a qPCR analysis was used to ascertain the expression pattern upon DA. The accuracy of qPCR experiments requires a reference gene to normalize expression levels, such as *CcUBQ10* that was used as a reference gene in coffee (*C. arabica* L. cv. Laurina) to standardize expression levels of a large set of genes during fruit development (Salmona *et al.*, 2008; Joët *et al.*, 2009). Recently, Cruz *et al.* (2009) recommended using *CcUBQ10* and *CcGAPDH* genes as internal references because of their stable expression in drought-acclimated leaves of *C. arabica* cv. Catuaí vermelho. In addition, the use of *CcGAPDH* as a reference gene was also recommended to realize accurate and reliable normalization of tissue/organ-specific gene expression in *C. arabica* (Barsalobres-Cavallari *et al.*, 2009). In the present work, expression levels of the *CcUNK10* gene in leaves of *C. canephora* were identical using either *CcUBQ10* or *CcGAPDH* as reference genes, and the former was chosen to standardize most of the qPCR experiments performed.

The results presented here indicated that a number of genes were down-regulated and others up-regulated under drought. Furthermore, some genes displayed a clear differential pattern of expression between the clones tested. Based on the biological functions of these genes, the results demonstrate that different categories of the

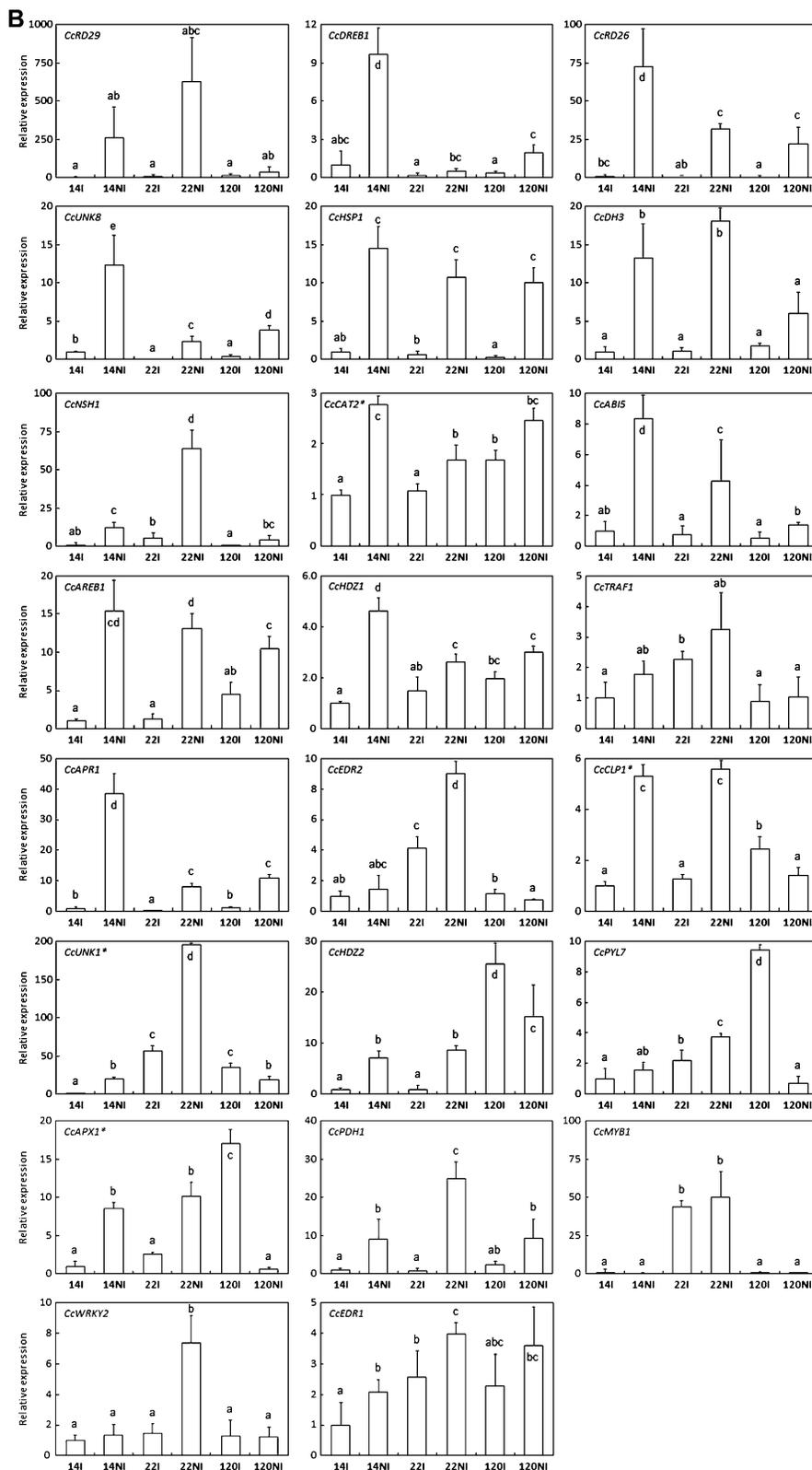


Fig. 6. Continued

signalling pathways of drought response in coffee have been identified; and these are discussed below, focusing mainly on the responses displayed by the drought-tolerant clones.

Core components and modulators of ABA signalling

Results of qPCR assays of two coffee homologues of the recently identified PIR/PYL/RCAR type of ABA receptors

(Ma *et al.*, 2009; Cutler *et al.*, 2010; Hubbard *et al.*, 2010) indicated that *CcPYL3* and *CcPYL7* expression was higher in clone 120 than in clone 14 under irrigated conditions and its expression decreased upon DA. On the other hand, *CcPYL7* expression was induced by drought only in clone 14, suggesting a probable involvement of the ABA signalling pathway in coffee responses to drought. This result is in agreement with data presented by Sun *et al.* (2011), where expression of some PYL-encoding genes from tomato was down-regulated by DA and some other genes showed an increase in expression.

In coffee, Silva (2007) showed that severe drought ($\Psi_{pd} \sim -3.0$ MPa) effectively led to an increase of ABA quantified in leaves of both drought-tolerant (clone 120) and sensitive (clone 109A) clones of *C. canephora* var. Conilon. According to the newly developed model for early ABA signalling, a core mechanism is based on a three-component system made up of three protein classes: PIR/PYL/RCARs, protein phosphatase 2Cs (PP2Cs), and SNF1-related protein kinase 2s (SnRK2s) (Hauser *et al.*, 2011). Data presented here for *CcPP2C* indicated a decreased expression of this gene upon DA and lower expression for both drought-tolerant clones under irrigated conditions as compared with clone 22. This result also supports a probable active ABA signalling pathway in coffee in response to drought and is in agreement with the newly established model for ABA signalling, in which the PYR/RCARs act as ABA receptors, the PP2Cs act as negative regulators of the pathway, and SnRK2s act as positive regulators of downstream signalling (Ma *et al.*, 2009; Park *et al.*, 2009). In that way, a double-negative regulatory pathway is established, whereby ABA-bound PYR/RCARs inhibit PP2C activity and PP2Cs inactivate SnRK2s (Park *et al.*, 2009; Umezawa *et al.*, 2009; Vlad *et al.*, 2009). Thus, in the absence of ABA, the PP2Cs are active and repress SnRK2 activity and downstream signalling. In the presence of ABA, PYR/RCARs interact with PP2Cs and inhibit phosphatase activity, allowing SnRK2 activation and phosphorylation of target proteins. Furthermore, in addition to the lower gene expression, the protein content of *CcPP2C* was lower for clone 14 under irrigated conditions as compared with clone 22, pointing to a potentially more active ABA signalling pathway in clone 14. Based on these results, and considering that coffee PP2Cs function as a negative regulator of ABA signalling, the down-regulation of this gene under drought would probably sensitize coffee plants to ABA and its downstream responses.

Secondary messengers

A clear difference in the expression pattern of *CcNSHI*, encoding a non-symbiotic haemoglobin, was observed between the clones studied. Under irrigated conditions clone 22 showed a higher expression of *CcNSHI* (>5-fold) than both drought-tolerant clones, and a 7-fold increased expression was also observed for clone 22 upon DA as compared with the drought-tolerant clones 14 and 120 under the same conditions. Non-symbiotic haemoglobins have been shown to be involved in scavenging of nitric

oxide (NO) molecules, which play a key role in oxygen sensing/balancing in plants and animals (Dordas *et al.*, 2003; Perazzolli *et al.*, 2006). Therefore, the results suggest that NO levels might be elevated in the drought-tolerant clones which could be due to the lower scavenging capacity of *CcNSHI* observed for these genotypes. In recent years, NO has emerged as an important endogenous plant signalling molecule that mediates many developmental and physiological processes (Neill *et al.*, 2008). In leaves, NO mediates ABA-induced stomatal closure (Desikan *et al.*, 2002, 2004; Garcia-Mata and Lamattina, 2002), modulates the rate of leaf expansion (Leshem and Haramaty, 1996), and may affect metabolic processes such as photosynthesis (Hill and Bennett, 1970) and respiration (Zottini *et al.*, 2002).

Furthermore, ABA also stimulates the generation of H₂O₂ in guard cells, and pharmacological and genetic data demonstrate that NO accumulation in these cells is dependent on such production. Recent data have extended this model to maize mesophyll cells where the induction of antioxidant defences by drought and ABA required the generation of H₂O₂ and NO in addition to the activation of a MAPK. The present results demonstrate that there is an increased expression of *CcEDR1* (similar to the ENHANCED DISEASE RESISTANCE 1 from *Arabidopsis*) upon drought for all clones tested. In contrast, *CcERD2* displays a decrease in expression upon DA in clone 120. *EDR1* encodes a CTR1-like kinase which was previously reported to function as a negative regulator of disease resistance and ethylene-induced senescence. Tang *et al.* (2005) reported that the *edr1* mutant displays enhanced stress responses and spontaneous necrotic lesions under drought conditions in the absence of pathogen, suggesting that EDR1 is also involved in stress response signalling and cell death regulation. MAPKs are abundant in all eukaryotes and phosphorylate a wide range of target proteins, including other kinases and/or transcription factors (Colcombet and Hirt, 2008). Diverse stimuli, including pathogens, abiotic stress, H₂O₂, ABA, and phytohormones, activate MAPKs. A role for MAPKs in ABA signalling has already been demonstrated (Colcombet and Hirt, 2008) as well as a negative regulation of MAPK pathways by PP2Cs (Schweighofer *et al.*, 2004).

Published data suggest that drought and salinity induce NO generation which activates cellular processes that afford some protection against the oxidative stress associated with these conditions. Thus, the data suggest an emerging model of stress responses in which ABA has several functions, including the rapid induction of stomatal closure to reduce transpiration water loss and the activation of antioxidant defences to combat oxidative stress, processes involving NO as a key signalling intermediate (Neill *et al.*, 2008).

Transcription factors

Differential patterns of expression between clones 22 and the drought-tolerant clones 14 and 120 were also observed for genes involved in transcriptional regulation. The qPCR

results presented here demonstrated that water deficit induced differential effects between the two drought-tolerant clones on the expression of regulatory genes such as *CcABI5*, *CcAREB1*, *CcHDZ1*, *CcHDZ2*, *CcDREB1*, and *CcRD26*. Upon drought, clone 14 displayed a higher increase in mRNA levels for *CcABI5*, *CcAREB1*, *CcHDZ1*, *CcHDZ2*, *CcDREB1*, and *CcRD26* as compared with clones 120, indicating that different mechanisms may account for their drought tolerance phenotype. In agreement with this hypothesis, in the case of *CcHDZ2*, clone 120 even showed a decrease in expression upon DA. Those genes comprise components of the ABA-dependent (*CcABI5* and *CcAREB1*) and ABA-independent (*CcHDZ1*, *CcHDZ2*, *CcDREB1*, and *CcRD26*) signalling pathways. In plants, it is well known that two pathways of signal transduction exist in the control of stress-related genes: one dependent on ABA and another independent of this phytohormone (Yamaguchi-Shinozaki and Shinozaki, 2005). In this scheme, it was demonstrated that transcription factors belonging to the ERF/AP2 family (e.g. *DREB*), but also NAC/HD-ZIP transcription factors, controlled the expression of the *RD29A* gene, encoding a late embryogenesis abundant (LEA)-like hydrophilic protein, co-operatively or separately in an ABA-independent manner (Qiang *et al.*, 2000). The very high expression of *CcRD29* observed in the leaves of clones 14 and 22 upon drought is noteworthy.

The results obtained by qPCR for *CcMYB*, encoding a MYB protein displaying high similarity to MYB4 from *Arabidopsis*, shows a very different pattern of expression between the coffee clones tested. The expression of *CcMYB1* is almost absent in the drought-tolerant clones 14 and 120, and a 50-fold difference in expression is observed when compared with the results obtained for clone 22. In *Arabidopsis*, a group of MYB transcription factors have been reported to regulate the biosynthesis of secondary metabolites (Stracke *et al.*, 2001), and MYB4 acts as a transcription repressor inhibiting expression of the cinnamate 4-hydroxylase (*C4H*) gene, which encodes a key enzyme in hydroxycinnamate ester biosynthesis. In a *myb4* knockout mutant, hydroxycinnamate esters accumulate, resulting in tolerance of the mutant plant to UV-B (Jin *et al.*, 2000). In that sense, due to the absence of negative regulation performed by *CcMYB1*, the drought-tolerant clones might have a higher content of secondary metabolites such as the antioxidant chlorogenic acid, a hydroxycinnamic acid synthesized by the same pathway. A higher content of antioxidant would certainly contribute to drought tolerance. However, a pattern of expression similar to that of *CcMYB1* (e.g. higher expression in clone 22 and almost null expression for the drought-tolerant clones 14 and 120) was observed for *CcCCoAOMT1*, the first enzyme in the phenylpropanoid pathway. Further analysis of the metabolite content of these clones is needed to clarify this point.

From the set of transcription factor genes tested, only *CcWRKY2* had unchanged expression upon drought for both drought-tolerant clones tested. However, a significant induction of *CcWRKY2* was observed for the drought-sensitive clone 22 under drought. Recently, the *Arabidopsis*

WRKY2 transcription factor has been shown to mediate seed germination and post-germination arrest of development by ABA (Jiang and Yu, 2009). Analysis of the *WRKY2* expression level in ABA-insensitive and ABA-deficient mutants further indicated that ABA-induced *WRKY2* accumulation requires ABI5, ABI3, ABA2, and ABA3. Furthermore, the authors demonstrated that the ABA hypersensitivity of the *wrky2* mutants is attributable to elevated mRNA levels of *ABI5*, *ABI3*, and *ABI5-induced Em1* and *Em6* in the mutants (Jiang and Yu, 2009). The results presented here also show a similar pattern of expression as, upon drought, both tolerant clones displayed negligible expression of *CcWRKY2*. On the other hand, increased expression of *CcABI5* was observed for both clones under the same drought condition.

Photosynthesis-related genes

As previously reported in *Arabidopsis* (Seki *et al.*, 2002), a clear down-regulated expression of *CcCABI*, *CcPSBO*, *CcPSBP*, *CcPSBQ*, and *CcRBCS1* was observed in leaves of *C. canephora* clones under drought. Drought also repressed the expression of *CcCAI* coding for the carbonic anhydrase (CA) supplying CO₂ for Rubisco (Badger and Price, 1994). In barley, CA activity increased in response to elevated ABA concentration, and in wheat CA activity increased during the first period of drought in flag leaves of drought-resistant genotypes but was shown to decrease at the last stage of vegetation (Popova *et al.*, 1996; Guliyev *et al.*, 2008). Northern blot analysis shows that *CcELIP1* was also down-regulated by drought, for all clones evaluated. The early light-induced proteins (ELIPs) belong to the multigenic family of light-harvesting complexes, which bind chlorophyll and absorb solar energy in green plants. Reports indicate that ELIPs fulfil a photoprotective function that could involve either the binding of chlorophylls released during turnover of pigment-binding proteins or the stabilization of the proper assembly of those proteins during high light stress (Hutin *et al.*, 2002). Taken together, these results clearly demonstrated that leaf expression of several photosynthetic genes was greatly affected by DA in *C. canephora*.

Furthermore, results of a 2-DE experiment indicated identical responses for *CcCAI*-encoded protein, with a reduction under DA in leaf protein extracts for both clones 14 and 22 of *C. canephora*. Other differential responses between gene expression and protein accumulation were also clearly observed for *CcPSBP*, *CcPSBQ*, and *CcPSBO*, with reduced gene expression under DA and a higher amount of corresponding proteins under drought than under the irrigated condition. This situation, similar to that observed for the Rubisco protein (Marraccini *et al.*, 2011), could be explained by (i) the action of chaperones that could prevent the protein degradation under DA; (ii) the up-regulation of other gene alleles to compensate the down-regulation of those measured during DA; and (iii) the protein translation that occurred from mRNA transcribed during the night. In that case, nocturnal accumulation of mRNAs could

participate in maintaining the high daytime amount of the corresponding protein even under a sharp reduction in gene expression. Then, this should favour a quick recovery of photosynthetic capacity under favourable environmental conditions and help coffee plants to cope with drought.

Drought-responsive structural genes

Genes involved in DA such as that encoding a small heat shock protein (*CcHSP1*), a *CcDH3* coding for a coffee dehydrin, and *CcCLP1*, coding for an ATP-dependent calpain protease, also displayed differential responses upon drought and between the drought-tolerant clones. Heat shock proteins, dehydrins, and ATP-dependent calpain proteases have been previously reported to be involved in protein folding and protein turnover upon DA (Close *et al.*, 1993; Zagdanska and Wishniewski, 1998; Demirevska *et al.*, 2008). The results of protein analysis by a 2-DE experiment also confirmed the up-regulation of *CcHSP1*, particularly for clone 22. For clone 14, the same explanation given above for photosynthesis-related genes could apply to explain the differences between gene expression and protein accumulation. The importance of narrowing down the right protein isoform or allele is further supported by the results obtained here, whereby upon drought, the catalase-encoding gene *CcCAT1* is down-regulated and *CcCAT2* (encoding another catalase) is up-regulated. Similar results were obtained for the ascorbate peroxidase genes *CcAPX2* (down-regulated) versus *CcAPX1* (up-regulated). Furthermore, in the case of *CcAPX1*, a clear differential response was observed between the drought-tolerant clones 14 and 120, as it is induced upon DA in clone 14 and down-regulated in clone 120. The involvement of another APX in drought responses in clone 120 has still to be investigated. In the case of the oxidoreductase genes *CcAPRI* and *CcMPRI*, up-regulation upon drought was observed for all clones tested. However, higher induction for clone 14 was observed compared with clone 120. Therefore, the results obtained for *CcCAT2*, *CcAPX1*, and *CcMPRI* indicate a stronger induction of the coffee antioxidant and osmo-protection systems upon DA in clone 14 as compared with the other clones.

Pinheiro *et al.* (2004) reported that clone 14 displayed increased activity (38%) of APX, an enzyme which has high affinity for H₂O₂, as a result of drought (Smirnoff, 1995; Polle, 2001; Mittler, 2002). The authors concluded that APX might have a key role in allowing clone 14 to cope with potential increases in H₂O₂ under drought conditions. In the same vein, the present qPCR results for *CcAPX1* show a clear induction of this gene upon drought. In addition, a clear difference in expression between clones 14 and 120 was observed, as *CcAPX1* decreases upon DA, suggesting a role for *CcAPX1* in the drought tolerance phenotype in clone 14. However, clone 22 displayed a similar pattern of induction of *CcAPX1* as clone 14 and, therefore, further studies aiming at characterizing other APX-encoding genes from coffee and their expression upon DA might be needed.

The results presented here also identified DA-inducible expression of the *CcPDHI* gene coding for a prephenate dehydrogenase protein and down-regulation of the *CcGASI* gene encoding arbutin synthase (Hefner *et al.*, 2002) with water limitation in *C. canephora*. The first enzyme catalyses the aromatization of prephenate to form tyrosine precursors and subsequently phenylalanine, and might affect the synthesis of several secondary metabolites (Warpeha *et al.*, 2006; Urano *et al.*, 2009), and *CcGASI* is involved in the synthesis of arbutin (4-hydroxyphenyl-β-D-glucopyranoside), a phenolic glucoside compound that accumulates in leaves of some drought- and cold-tolerant plant species such as *Myrothamnus flabellifolia* where it could represent 25% of leaf dry weight (Bianchi *et al.*, 1993; Hinch *et al.*, 1999). As previously mentioned for *CcCCoAOMTI*, to address the role of arbutin and other phenolic compounds to prevent cell damage under abiotic stresses in coffee, further analysis of the metabolite content of these clones should be performed.

Down-regulated expression of the *CcSDD1* gene encoding a subtilisin-like serine protease (*SDD*: stomatal density and distribution) involved in stomata development was also observed under drought. In *Arabidopsis*, *sdd* mutants showed increased stomata number, indicating that this gene is a negative regulator of guard cell formation (Von Groll *et al.*, 2002). Compared with the *CcUBQ10* expression level, the expression level of *CcSDD1* reported here in *C. canephora* leaves was low. This is expected considering that expression of this gene is mainly observed in stomata precursor cells. For this reason, the analysis of *CcSDD1* expression in meristem cells of stressed and unstressed coffee plants should be of particular interest.

As already reported before in other plants (Dubos *et al.*, 2003; Le Provost *et al.*, 2003; Rodriguez *et al.*, 2006; Wang *et al.*, 2009), stress-inducible expression of the *CcGRP1* gene coding for a glycine-rich protein was also observed. Currently, the role of GRPs in abiotic stress in plants is still unknown, but these proteins were localized in the cell wall of many higher plants where they may play a role in its maintenance, reinforcement, and repair during the dehydration–rehydration process (Harrak *et al.*, 1999; Ringli *et al.*, 2001; Le Provost *et al.*, 2003; Mousavi and Hotta, 2005). Some drought-inducible genes were also found whose functions in relation to drought are still poorly understood. Results have identified three novel genes (*CcUNK1*, *CcUNK8*, and *CcUNK10*) highly induced upon drought. The functions of these genes are not yet known, and further research is needed to characterize their function.

In many cases, ABA was shown to control the expression of several stress-responsive genes (Seki *et al.*, 2002) such as HSP- and dehydrin-encoding genes, among several others. Due to the oxidative burst caused by drought and the protective role of HSPs, dehydrins, catalases, APXs, and oxidoreductases, the overexpression of their corresponding genes reported here is in accordance with responses usually observed in other plant species submitted to water limitations (Shinozaki and Yamaguchi-Shinozaki, 1997).

The leaf ABA contents of clones 14 and 22 analysed in this study are unknown but should increase under drought,

as already shown for clone 120 of *C. canephora* var. Conilon (Silva, 2007). The results found for CGs differentially expressed upon DA and the comparison between the responses of drought-tolerant genotypes, presented here, lead to the first insight on the molecular players involved in drought responses in coffee. The identified CGs belong to different classes of genes, such as ABA perception and signalling, transcriptional regulation, and drought-responsive structural genes. Photosynthesis-related genes have also been identified. Based on the obtained results, it can be concluded that a complex network of responses probably involving the ABA signalling pathway and NO might be the major molecular determinants to explain the better efficiency in controlling stomatal closure and transpiration displayed by the drought-tolerant clone 14 of *C. canephora*.

Some of the identified CGs have recently been mapped (Leroy *et al.*, 2011), and for several of these genes work is now in progress to analyse their corresponding genomic copy and promoter regions to verify if single nucleotide polymorphisms (SNPs) and/or insertion/deletions (INDELs) could be related to the differential expression profiles observed for these genes in drought-tolerant and sensitive clones of *C. canephora*. Such an approach will be facilitated with the forthcoming sequencing of the whole *C. canephora* genome (De Kochko *et al.*, 2010).

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