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Transcriptional memory contributes to drought tolerance in coffee (*Coffea canephora)* plants

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ABSTRACT

Water deprivation is an important limiting factor in the productivity of crops like coffee. In addition to transcription factors (TFs) and small non-coding RNAs, transcriptional memory seems to act in gene expression modulation during plant drought response. Here, a RNA-Seq approach was used to investigate the drought responses of *Coffea canephora* clones 109 and 120, which are respectively sensitive and tolerant to drought. Illumina sequencing allowed us to identify differentially expressed genes (DEG) in the tolerant (826) and sensitive (135) clones and their enriched categories. Our results indicate that the sensitive clone may trigger an oxidative stress response, possibly leading to programmed cell death, when exposed to multiple drought episodes. The acclimation of tolerant plants, on the other hand, seems to involve antioxidant secondary metabolism and the ABA response. Most importantly, 49 memory genes were identified in the tolerant clone. They were mainly linked to the ABA pathway, protein folding and biotic stress. Small RNA profiling also identified regulatory microRNAs in coffee leaves, including hundreds of putative novel ones. Our findings strongly suggest that transcriptional memory modulates the expression of drought-responsive genes and contributes to drought tolerance in *C. canephora.*

1. Introduction

Harsh environmental conditions trigger a wide range of responses in plants, from altered gene expression and cellular metabolism to changes in growth rates and crop yields (Bray et al., 2000; Cavatte et al., 2012; Krasensky and Jonak, 2012). Since drought is the most important environmental stress in agriculture and drought events are expected to be exacerbated by climate change, understanding plant responses to this stress type and the cross-talk between different stresses (Fujita et al., 2006; Atkinson and Urwin, 2012; Rejeb et al., 2014) is important to increasing crop productivity while using less water.

Drought responses depend on plant species/genotypes, water deficit severity and duration (Cavatte et al., 2012) and on the imprint that previous stress episodes have left on the plant (Walter et al., 2011; Ding et al., 2014; Wang et al., 2014; Virlouvet and Fromm, 2015; Fleta-Soriano and Munné-Bosch, 2016). The imprint, or stress memory, can be defined as the structural, genetic and biochemical modifications resulting from a stress exposure that allows plants to "remember" past environmental events (Fleta-Soriano and Munné-Bosch, 2016). These "memories" can improve plant adaptation and resistance to future stress episodes (Kinoshita and Seki, 2014; Fleta-Soriano and Munné-Bosch, 2016). Even though the mechanisms underpinning plant stress

Abbreviations: Cq, Quantification Cycles; DEG, Differentially Expressed Genes; FC, Fold Change; TF, Transcription Factor

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memory are not clearly understood, a growing body of evidence suggests that the accumulation of signalling compounds and transcription factors (TFs) (Bruce et al., 2007; Conrath, 2011; Santos et al., 2011), together with epigenetic modification (Ding et al., 2012; Kim et al., 2012; Han and Wagner, 2014; Kinoshita and Seki, 2014; Avramova, 2015; Vriet et al., 2015; Crisp et al., 2016), play key roles in this process. Ding and coworkers (2013) defined "memory genes" as those having altered expression after not only the first but also subsequent stress exposures. Genes that respond only to the first stress exposure are called non-memory, whereas those responding only to subsequent stress events are called late-response genes.

Another stress response regulation layer is promoted by microRNAs (miRNAs). These small non-coding RNAs are produced by a specialized RNA silencing pathway, generating 20- to 24-nucleotide-long RNAs that guide ARGONAUTE proteins to target coding or non-coding RNAs (Bologna and Voinnet, 2014). By regulating key TFs (Stief et al., 2014) or other stress-associated genes, miRNAs are increasingly regarded as a promising target for crop tolerance to abiotic stress (Khraiwesh et al., 2012).

Coffee, an evergreen tropical tree species, is one of the major traded commodities. Worldwide coffee production is mainly based on two species: *Coffea arabica* and *C. canephora*. In Brazil, the most important coffee-producing country, drought is the major environmental constraint to coffee production. Even though several studies have explored the coffee drought response (e.g., Lima et al., 2002; DaMatta et al., 2003; Pinheiro et al., 2004, 2005; Marraccini et al., 2012; Silva et al., 2013), all of these studies examined responses in plants subjected to a single drought event. In contrast, multiple drought episodes, which are the rule under field conditions, can alter plant drought response and acclimation (Galle et al., 2011).

Recently, Menezes-Silva and coworkers (2017) firstly demonstrated that coffee plants exposed to multiple drought events cope better with water deprivation than their counterparts exposed to just one stress event. Investigating metabolic and physiological traits, our group found evidences for an improved photosynthetic performance of C3 plants of drought-tolerant clone 120 in comparison to the sensitive clone 109. Additionally, increased activities of enzymes related to key physiological/biochemical processes like RuBisCO and antioxidant enzymes were found in C3 plants as well as higher levels of protective compounds. Differential adjustments in the shikimate pathway of C3 coffee plants, particularly for the tolerant clone, might also contribute to their better performance under drought stress through production of antioxidant compounds. Taken together, these results support the hypothesis that memory has positive effects on coffee plants acclimation to drought.

In the present study, the drought responses of *Coffea canephora* cv conilon (clones 120 and 109, tolerant and sensitive, respectively) were assessed by sequencing and analyzing the leaf transcriptomes of plants submitted to one and three drought cycles. We found that the responsive genes of the tolerant (826) and the sensitive (135) clones were enriched with categories related to antioxidant secondary metabolites. Investigation of drought transcriptional memory in the tolerant clone revealed ABA-related genes and a possible interaction between drought and biotic stress memory genes. In addition to memory, MYB proteins and miRNAs were found to modulate expression in drought response. The drought-responsive genes identified in this work constitute valuable genomic resources to ameliorate coffee cultivation and develop tolerant crops.

2. Results

2.1. RNA-Seq, read mapping and identified transcripts

Drought cycles were imposed on coffee plants as shown in Fig. S2. The physiological traits of plants kept in these conditions were analysed before collecting samples for library construction (Menezes-Silva et al., 2017). The number of Illumina reads generated for clones 120 and 109 are shown in Table S3. Each sequenced library yielded about 100 million reads of 100 nt. To evaluate the quality of the biological replicates, Pearson's correlation coefficient was calculated for the read counts of clone 120 replicates. All coefficient values calculated between replicates indicated an almost perfect positive correlation (Table S4). Overall, 80% of the reads in each library mapped to the coffee genome, and more than 50% of them mapped to exons and 10% to intergenic regions (Table S3). A total of 22,764 genes were found in clones 120 and 109. The diagrams in Fig. 1 show the number of genes found to be expressed in control, C1 and C3 plants of both clones. Only genes expressed in all clone 120 replicates of each condition were considered in this diagram. For both sequenced clones, more than 85% of the expressed genes were common to the control, C1 and C3 plants (Fig. 1). We identified 86 genes exclusively expressed in the tolerant clone, and among them, 14 belonged to three groups of functionally related genes. Interestingly, the members of these groups are also neighbouring genes in the genome (Fig. S3, Table S6). Group 1 comprises six putative disease-resistance responsive proteins located at chromosome 5 that belong to a family of proteins (PF03018) induced during disease plant response. Group 2 has three genes with unknown function located at chromosome 7. Group 3 is formed by five genes of chromosome 8 that

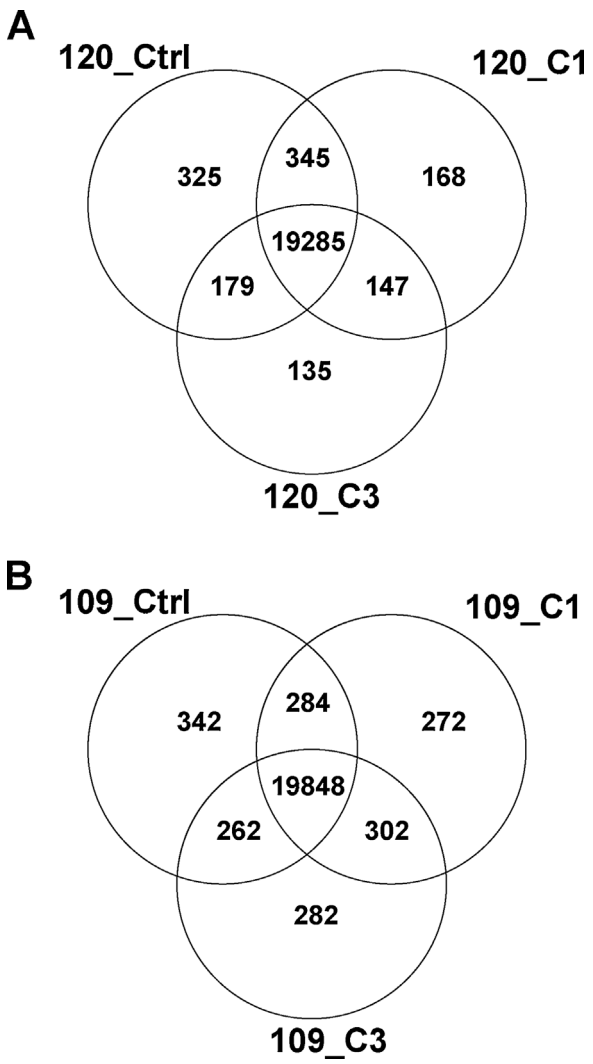
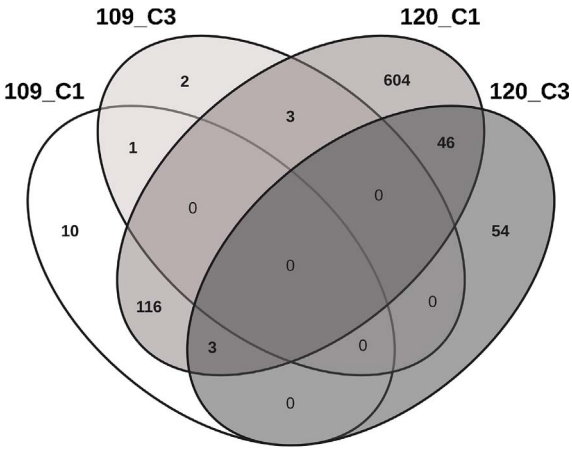


Fig. 1. Expressed genes found in clones 120 and 109.
Venn diagrams showing the overlap of genes found to be expressed in the clone 120 (A) and clone 109 (B) libraries of watered plants (Ctrl) and plants subjected to one (C1) and three (C3) drought cycles. For clone 120, only genes expressed in all replicates of each condition were considered in this diagram.



	#Regulated genes	#Up	#Down
C109	135		
C109_C1	130	26	104
C109_C3	6	6	0
C120	826		
C120_C1	772	437	335
C120_C3	103	7	96

Fig. 2. Drought-responsive genes identified in tolerant and sensitive clones. Differential expression analysis was carried out with DESeq. Venn diagram depicts the distribution of responsive genes identified in clone 120 and 109 after drought cycles. The bottom table shows the total number of drought-regulated genes as well as the number of up and down-regulated genes after one and three cycles. In clone 120, 49 drought-responsive genes were regulated after both the first and third exposures.

code for TOPLESS-related proteins (Fig. S3, Table S6).

2.2. Drought-responsive genes in clone 120 and 109

Gene expression changes in coffee clones 120 and 109 subjected to drought cycles were analysed with the DESeq package. The effects of one and three cycles were estimated by adjusting a generalized linear model. In clone 109, 135 (0.59%) genes responded to drought, 130 (96.3%) of which responded to the first cycle (Fig. 2). For this clone, genes responsive to the first drought cycle were mainly repressed (104 genes, 80%), while after three cycles, all regulated genes (6) were up-regulated. A higher number of drought-responsive genes was found in clone 120 (826 genes, 3.63%), most of which responded to the first drought cycle (772 genes, 93.46%), as observed for clone 109 (Fig. 2). The percentages of up and down-regulated genes responding to one drought cycle were similar in the tolerant clone. The drought-responsive gene diagram (Fig. 2, Table S7) shows the number of genes commonly and exclusively regulated in the two clones, although they do not necessarily have the same regulation behaviour. While 119 genes responded to the first drought exposure in both clones, no overlap was found after the third cycle (Fig. 2). The Venn diagram also revealed the coffee genes regulated after one and three drought exposures in each clone: 49 in clone 120 and only one in clone 109 (Fig. 2, Table S7).

2.3. DEG gene ontology enrichment analysis

To uncover which genes and pathways are relevant to the coffee response to drought cycles, a gene set enrichment analysis (GSEA) approach was applied on the differentially expressed genes. In the two studied clones, 104 GO categories were found to be enriched, with a higher number of enriched terms after cycle one than after cycle three (Table S8). After the first cycle, categories involved with the metabolism of flavonoids, phenylpropanoids and terpenoids were enriched in both genotypes (Fig. 3, Table S8). The category “response to oxygen-containing compound” (GO:1901700) is also enriched in both clones after the first cycle, but it remains enriched among late drought-responsive genes only in clone 109, together with “hydrogen peroxide metabolic process” and “response to hydrogen peroxide” (Fig. 3; arrow). The tolerant clone had more specific enriched categories, such as “response to abscisic acid” (GO:0009737) and “response to jasmonic acid” (GO:0009753), which are related to “response to hormones” (GO:0009725). GO terms linked to diverse abiotic and biotic stress types were enriched among coffee DEGs even after multiple drought exposures, including categories related to defence against other organisms (Fig. 3, Table S8). The specific term “response to water deprivation” (GO:0009414) is enriched only in the tolerant clone (Fig. 3; arrow). After multiple exposures, the only common enriched category between clone 120 (11 terms) and clone 109 (14 terms) was “response to high light intensity” (GO:0009644) (Fig. 3). A remarkable difference between the studied clones resided in the exclusive enrichment of categories related to “Programmed Cell Death” in clone 109 and “Heat Acclimation” in clone 120 after three drought cycles (Fig. 3; arrows).

2.4. Identification of coffee (clone 120) memory, non-memory and late response genes

We identified the tolerant clone memory, non-memory and late-response genes. There were 49 coffee memory genes, which were further split into four subtypes: [+/+], [−/−], [+/−], [−/+]. Most of them fell into the [+/−] subtype (44 genes) (Table 1), which means that they had increased levels after one drought cycle but decreased levels after the third one. Genes of the [+/+] type were not found in clone 120. The coffee memory genes |Log2FC| values ranged from 0.94 to 33.02 in CtrlxC1 comparison and 1.00 to 32.47 in C1xC3. Non-memory corresponded to the major part (87.53%) of clone 120 drought-responsive genes, while the late response (54) had a similar number of genes to the memory category (Table 1).

2.5. Coffee memory genes

We found that putative Arabidopsis homologues of seven (14.3%) coffee memory genes also exhibited drought memory behaviour (Ding et al., 2013) (Table 2). To gain further insights into their biological functions, memory genes were manually annotated and classified according to all annotation evidence (Fig. 4, Table S9). In addition to hypothetical proteins (nine genes), nine (18.4%) putative leucine rich repeat (LRR)-domain-containing and defence-related genes were found among the coffee memory genes (Table 2, Fig. 4). Another functional category that can be highlighted is “Protein modification/degradation” (Fig. 4), including heat-shock and heat-shock binding proteins.

2.5.1. Drought [−/−] memory genes

The three genes assigned to the [−/−] memory type comprised one hypothetical protein (Cc06_g18730), one putative peptide/nitrate transporter (Cc10_g09990) and one putative disease resistance protein (Cc06_g16160) (Table 2). Cc10_g09990 putative Arabidopsis orthologue (AT1G22550) encodes a membrane protein with transporter activity. Arabidopsis expression data available in Genevestigator showed that AT1G22550 was mainly down-regulated by drought (Fig. 5, Table S10). The coffee gene Cc06_g16160 (AT1G50180) is a probable LRR-domain-containing disease resistance protein (Table 3) involved in defence response. The interolog-based network constructed for clone 120 memory genes showed that this disease resistance protein directly interacts with the central [−/+] heat-shock protein (Fig. 6). The interolog-based in silico approach transfers the interaction annotation based on homology and is prone to discover interactions for the most conserved proteins (Geisler-Lee et al., 2007; Bodt et al., 2009).

2.5.2. Drought [−/+] memory genes

Two drought-responsive genes exhibited [−/+] memory. Cc02_g17500 was annotated with “regulation of transcription” by Mapman software, and its Arabidopsis putative orthologue

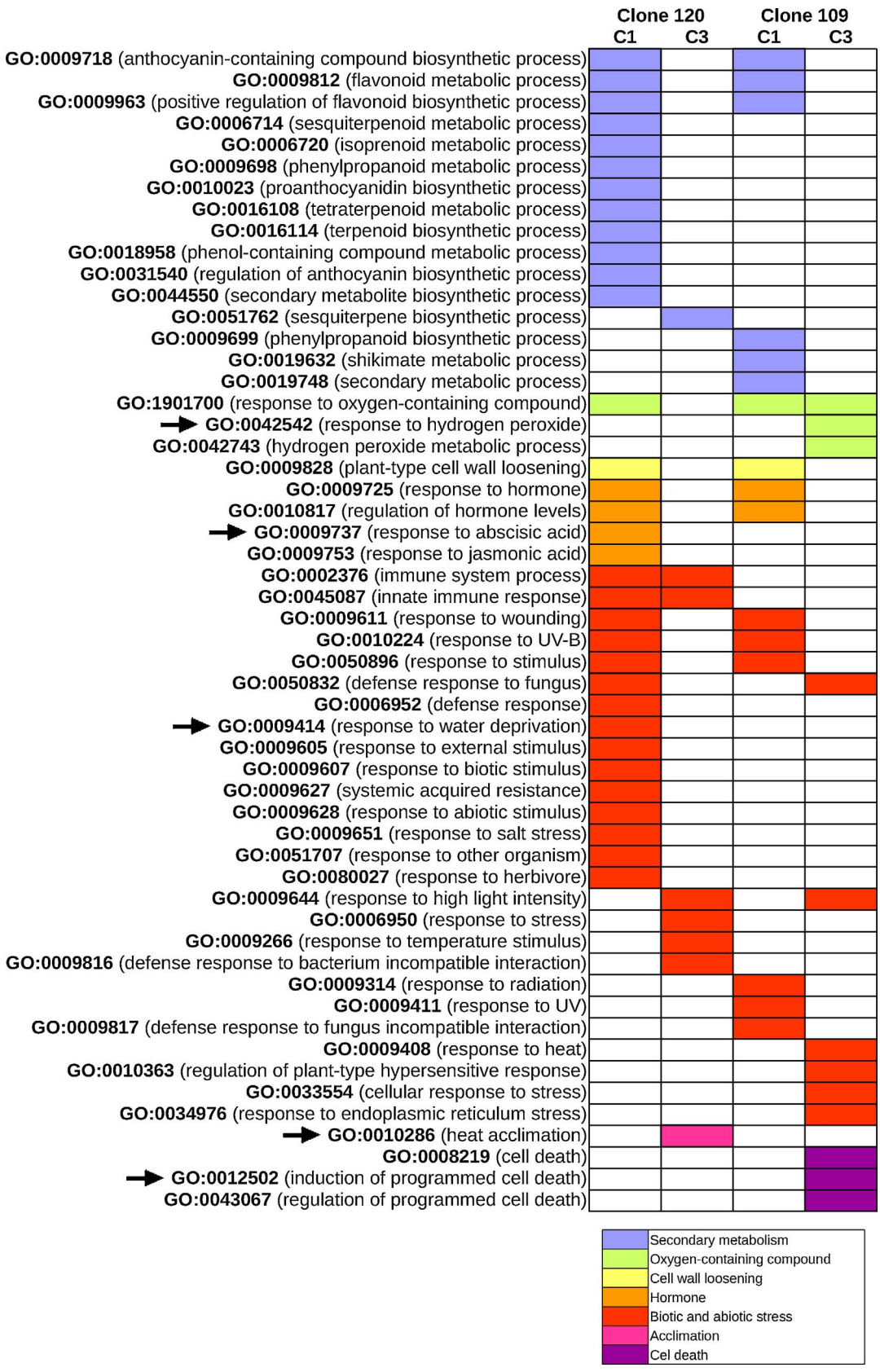


Fig. 3. Biological Process GO terms enriched in drought-responsive genes of clones 109 and 120.
Out of 104 GO terms considered significantly enriched for both clones, 54 categories related to secondary metabolism, oxygen-containing molecules, cell-wall, hormone, biotic and abiotic stresses, acclimation and cell death are shown as colour-coded. Arrows indicate specific categories exclusively enriched in each genotype and condition representing the remarkable differences between tolerant and sensitive clone responses.

Table 1
Drought-responsive and memory genes found in *C. canephora* clone 120. Memory, non-memory and late-response genes were identified by comparing the lists of drought-responsive genes of plants submitted to one (C1) and three (C3) stress cycles. (+) means induced expression, (−) means reduced expression and (=) means no significant expression differences.

	#Genes
Drought-responsive	826
Memory	49
[+/+] Ctrl < C1 < C3	0
[−/−] Ctrl > C1 > C3	3
[+/−] Ctrl < C1 > C3	44
[−/+] Ctrl > C1 < C3	2
Non-memory	723
[+/=] Ctrl > C1 = C3	393
[−/=] Ctrl < C1 = C3	330
Late response	54
[=/+] Ctrl = C1 > C3	5
[=/−] Ctrl = C1 < C3	49

(AT4G39250) is a RAD-like 1 (RL1) TF (Table S9). Furthermore, the Cc02_g17500 coded-protein was found to be a putative nuclear DNA-binding protein (data not shown). Cc02_g02350 (AT5G52640) putatively codes for a heat-shock protein, named Hsp90.1, (Table 3) and its putative Arabidopsis orthologue interacts with disease resistance signalling components and is involved in stress and defence response (Takahashi et al., 2003; Yamada et al., 2007; Meiri and Breiman, 2009). Strong *Hsp90.1* (AT5G52640) expression induction by drought stress has been observed in previous experiments (Fig. 5; Table S10). The [−/+] memory behaviour predicted in silico for Cc02_g02350 was tested and confirmed by qPCR (Fig. 7). The coffee memory network showed that Hsp90.1 protein may indirectly and/or directly interact with 15 other memory genes/proteins (Fig. 6), including high confidence interactions with several memory [+/−] LRR-domain-containing proteins and chaperones (see next section) and with the putative RAD-like TF.

2.5.3. Drought [+/−] memory genes

Almost 90% (44 genes) of coffee memory genes fell into the [+/−] subtype (Table 1, Table 2), meaning that they exhibited a reverse regulation profile in the first and third drought exposures. Both induction and repression of putative homologues of coffee [+/−] genes were previously observed in different Arabidopsis drought studies (Fig. 5; Table S10). The functional category of LRR-domain-containing proteins was the most represented in the [+/−] memory subtype with eight coffee genes (Table 3). Protein modification/degradation was also a well-represented category (eight [+/−] memory genes, 18.18%) (Fig. 4), including genes with chaperoning/protein folding activity as well as a polyubiquitin and GCN2 kinase.

Hierarchical clustering of Arabidopsis microarray log2 FC values revealed a cluster comprising mainly LRR-domain-containing-protein coding genes (Fig. 5; Table 3). The LRR-RLK Cc00_g20660 and the transducin Cc03_g15560 in this cluster are strongly regulated by drought in coffee (Table 2). AT4G08850, the homologue of Cc00_g20660 and Cc04_g15220, has been previously indicated as an ABA-specific marker (Nemhauser et al., 2006). The [+/−] coffee LRR-domain-containing proteins (Table 3) were predicted in silico to physically interact with the [−/+] heat-shock Cc02_g02350 (Fig. 6). Three of these LRR-domain-containing proteins are putative RLKs with transmembrane domains (Table 3) and are functionally related to ABA (Table 4). These RLKs formed a group of [+/−] ABA-related genes, which are indicated as square nodes in the coffee memory network (Fig. 6), including one putative isoflavone hydroxylase of the cytochrome P450 family (Cc10_g05390). Another ABA-related cytochrome P450 (Cc11_g07610) exhibited [+/−] memory (Table 2; Table 4). In this ABA group of the memory network, only the beta-glucosidase Cc02_g30420 was not found to be ABA-related according to an AHDatabase comparison (Table S11). Heat-shock and chaperones formed another cluster of co-expressed memory genes in the coffee network (Fig. 6), one of these genes (Cc03_15570) was strongly regulated in coffee after drought exposure (Table 2).

2.6. Coffee non-memory genes

Most of the clone 120 drought-responsive genes were non-memory (723 genes) (Table S12), with 55% and 45% of them up and down-regulated, respectively (Table 1). The three most represented KOG categories were “General function prediction only” (17.6%), “Secondary metabolites biosynthesis, transport and catabolism” (16.1%), followed by “Signal transduction mechanisms” (13.3%) (Fig. S4). KEGG Pathway analysis also revealed significantly enriched pathways (corrected p-value < 0.05) related to secondary metabolism, such as flavonoid and phenylpropanoid biosynthesis (Table 5). Genes involved with ascorbate and glutathione metabolism, the major cellular redox buffers, and with enzymatic antioxidant defence system were also found among non-memory genes (Table S13). The clone 120 responsive genes that take part in ROS scavenging mechanisms were mainly non-memory genes (41 genes, 85.4%) (Table S13). Genes encoding TFs were expected to be responsive to drought. Surprisingly, KOG categorization revealed only 20 non-memory probable TFs; two memory genes also code for TFs (Table 6). The clone 120 drought-responsive TFs belonged mainly to the MYB/MYB-like family (10 genes, 45.45%).

2.7. Coffee late-response genes

More than 90% (49 genes) of late-response genes were down-regulated (Table 1). As observed for memory and non-memory, functional annotation revealed several late-response genes related to biotic stress (Table S14). The most represented KOG category was “Signal transduction mechanisms”, including 16 genes (29.63%) coding for disease resistance proteins, and Mapman showed 28 (51.85%) late-response genes annotated to biotic stress pathway (Table S14). KEGG Pathway analysis revealed that the ath00130 (Ubiquinone and other terpenoid-quinone biosynthesis) and ath00480 (Glutathione metabolism) pathways were significantly enriched (Table 5). Late-response genes acting in the antioxidant system were also identified (Table S13).

2.8. qPCR expression validation

Primer efficiencies and mean Cq values for the selected genes are shown in Table S15. REST analysis showed that considering both CtrlxC1 and C1xC3 comparisons, 18 of 21 (85.71%) RNA-Seq-based predictions were confirmed by qPCR, corresponding to 17 tested genes that included the memory heat-shock gene Cc02_g02350 (Fig. 7). Moreover, C1xC3 expression changes for eight genes not predicted by RNA-Seq were considered significant by qPCR analysis (Fig. 7). Even with these possible false-negatives, only three false-positives were observed among RNA-Seq predictions, suggesting a high precision despite a lower sensitivity, especially after three cycles, which might have been affected by the generalized linear model adjustment.

2.9. miRNAs expression during drought-stress cycles

Two tolerant clone biological samples from each experimental condition were submitted to sRNA-Seq. The number of raw reads in each library ranged from 35 to 42 million, most of them having high quality Phred scores (Fig. 8, Fig. S5). To maximize the identification of miRNAs, reads from the libraries obtained here were concatenated with a previously published *C. canephora* sRNA one (Loss-Morais et al., 2014). After adapter removal, quality filtering and size selection, almost 59 million 20- to 24-nt-long reads remained and were used as input for miRNA discovery (Fig. 8). To our knowledge, this is the

Table 2
Clone 120 memory genes with their putative function and log2 FC values obtained with DESeq.

Coffee gene	Arabidopsis gene	Putative function	Log2 FC	
			CtrlxC1	C1xC3
Cc00_g08130	AT3G14470	Disease resistance protein RGA2	3.26	−5.91
Cc00_g11770[a]	AT4G35160	Tabersonine 16-O-methyltransferase	7.12	−6.54
Cc00_g12410	–	Hypothetical protein	8.15	−8.56
Cc00_g12480	AT1G52800	Gibberellin 3-beta-dioxygenase 1	3.16	−31.22
Cc00_g16440	–	Hypothetical protein	1.92	−5.78
Cc00_g20380[a]	AT5G45680	Peptidyl-prolyl *cis*-trans isomerase FKBP13 chloroplastic	7.23	−8.94
Cc00_g20660	AT4G08850	LRR receptor-like serine/threonine-protein kinase	33.02	−32.47
Cc00_g25220	AT1G52800	Gibberellin 20 oxidase 1	3.14	−8.13
Cc00_g29390	AT2G28680	RmlC-like cupins superfamily protein	4.22	−4.70
Cc00_g33210	AT1G13450	Trihelix transcription factor GT-1	2.24	−2.62
Cc01_g06280	–	Glucan *endo*-1,3-beta-glucosidase acidic isoform GI9	1.19	−3.00
Cc01_g08110	AT1G59780	Late blight resistance protein homolog R1B-14	1.50	−1.92
Cc02_g02350	AT5G52640	Heat-shock protein 83	−0.94	1.17
Cc02_g08930	AT3G01410	Pol-polyprotein	4.48	−30.71
Cc02_g10380[a]	AT4G24350	Bark storage protein A	2.75	−3.12
Cc02_g17500	AT4G39250	RAD-LIKE transcription factor	−2.50	1.00
Cc02_g30420	AT5G44640	Beta-glucosidase 11	5.24	−2.99
Cc02_g36130	–	Aldo-keto reductase yakc	30.99	−7.08
Cc02_g36150	–	Hypothetical protein	7.05	−6.43
Cc02_g36160	AT4G23540	ARM repeat superfamily protein	4.87	−29.96
Cc03_g08900	–	Hypothetical protein	6.57	−3.82
Cc03_g11800	AT3G14470	Disease resistance protein RGA3	2.08	−3.75
Cc03_g14330	–	Hypothetical protein	1.50	−3.08
Cc03_g14340	–	Hypothetical protein	1.97	−3.97
Cc03_g15550	AT4G13830	Dna-J domain protein	32.59	−7.24
Cc03_g15560	AT5G50970	Transducin family protein/WD-40 repeat family protein	32.32	−31.78
Cc03_g15570	AT4G13830	Chaperone dnaJ 20 protein	32.61	−32.06
Cc04_g15220	AT4G08850	LRR receptor-like serine/threonine-protein kinase	6.45	−4.49
Cc05_g01880	AT5G13200	GEM-like protein 5	5.16	−4.58
Cc05_g07850	AT4G35150	Tabersonine 16-O-methyltransferase	4.03	−6.81
Cc05_g13060	AT5G23960	(−)-germacrene D synthase	6.33	−4.73
Cc05_g13070	AT5G23960	(−)-germacrene D synthase	32.79	−32.24
Cc06_g02390	AT5G02160	Heat-shock dnaJ protein	1.69	−2.05
Cc06_g16160	AT1G50180	Disease resistance protein At1g50180	−1.43	−4.18
Cc06_g18730	–	Hypothetical protein	−3.06	−2.85
Cc06_g23010	AT3G03900	Adenylyl-sulfate kinase 1 chloroplastic	1.12	−1.08
Cc07_g01500	–	Hypothetical protein	33.02	−32.47
Cc07_g04140	AT3G22220	Transposase	32.79	−32.24
Cc07_g19100	AT3G59410	Serine/threonine-protein kinase GCN2	5.08	−5.84
Cc08_g05690	–	Polyubiquitin 10	4.38	−4.42
Cc08_g07730	–	Hypothetical protein	2.64	−2.77
Cc09_g08480[a]	AT1G12060	BAG5 chaperone-binding protein	4.04	−9.46
Cc10_g03440[a]	AT4G36850	Uncharacterized membrane protein YOL092W	0.94	−1.27
Cc10_g05390[a]	AT4G37370	Isoflavone 2′-hydroxylase	1.12	−1.27
Cc10_g09990	AT1G22550	Peptide/nitrate transporter At1g22550	−2.43	−27.28
Cc11_g00460[a]	AT5G24090	Chitinase	3.65	−1.69
Cc11_g02650	AT3G47570	LRR receptor-like serine/threonine-protein kinase	6.14	−5.51
Cc11_g02900	AT5G35450	Disease resistance RPP8-like protein 2	1.46	−1.17
Cc11_g07610	AT5G36110	Cytochrome P450 716B2	3.63	−3.87

[a] Indicates coffee memory genes whose Arabidopsis homologs also exhibited memory behaviour (Ding et al., 2013).

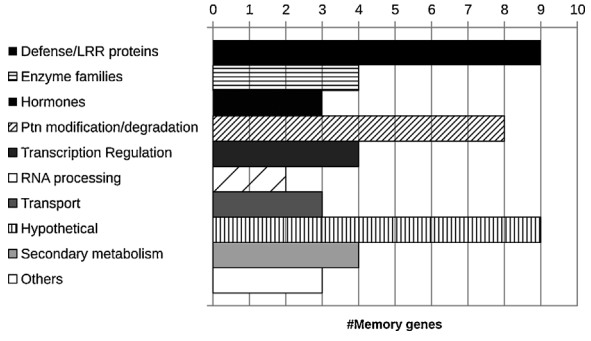


Fig. 4. Functional classification of memory genes.
Memory genes were widely annotated using the main biological databases and different tools in order to provide a detailed annotation. *C. canephora* memory genes were manually categorized in 10 classes to summarize the functional information.

deepest coverage of sRNA-enriched reads used for miRNA discovery in coffee. Most of the unique reads were 24-nt-long, as observed in coffee and other plants. The high diversity observed in this size class is most likely due to sRNAs associated with RNA-directed DNA methylation pathways (Bologna and Voinnet, 2014). However, when read counts are taken into consideration, the 21-nt sequences, which is the size most frequently associated with miRNAs in plants, were the most abundant ones (Fig. 8).

Two software packages were used to predict miRNA coffee genes, and the identified mature sequences were classified based on the miRBase database (Kozomara and Griffiths-Jones, 2014). In total, 41 conserved miRNAs were discovered among the coffee reads, most of it matching previously described *C. canephora* miRNAs (Loss-Morais et al., 2014; Chaves et al., 2015; Fernandes-Brum et al., 2017). Sequences having partial hits with miRBase entries (9) were regarded as variants of known miRNAs. Surprisingly, 198 putative novel miRNAs were identified by the software packages combined (Fig. 8, Table S16, Table

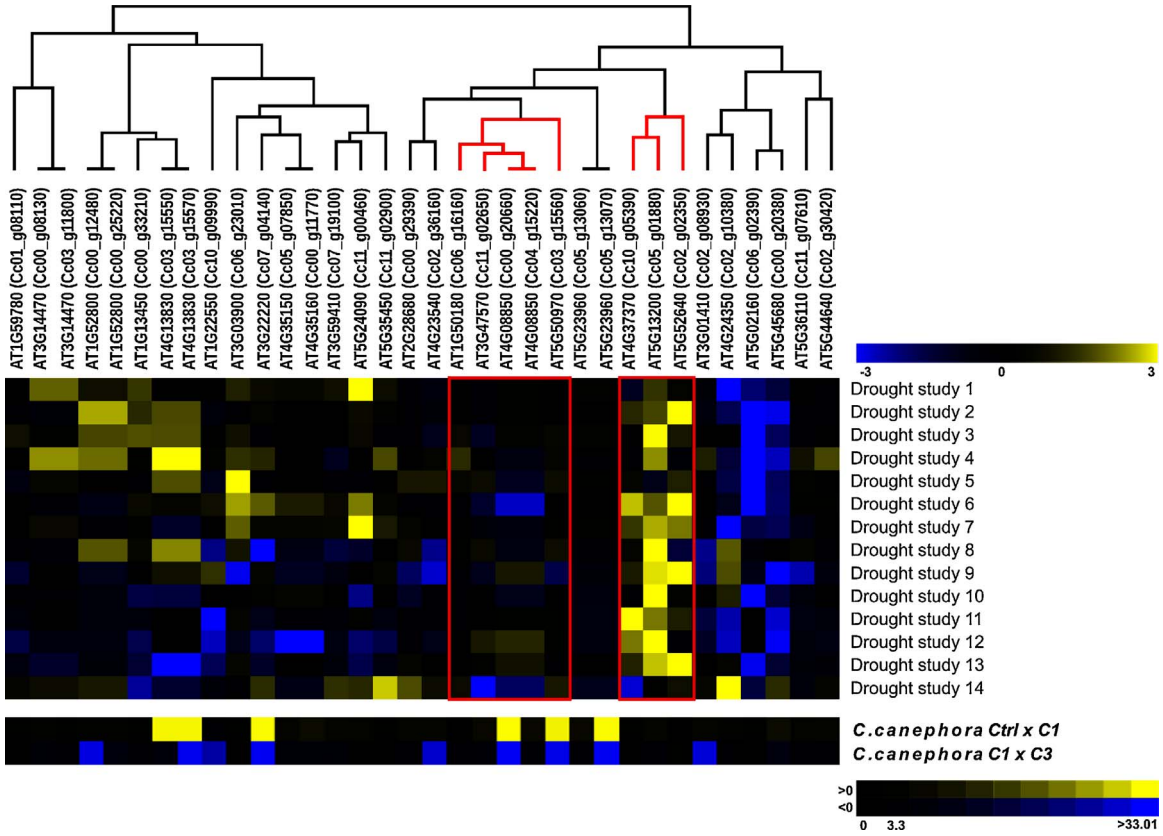


Fig. 5. Hierarchical clustering of coffee memory genes.
ATcodes assigned to clone 120 memory genes were used to get log2 FC values of 14 *Arabidopsis* microarray drought studies available in Genevestigator. A matrix with these values was used to perform hierarchical clustering. Log2 FC values of corresponding coffee genes obtained in the present work are shown below. Colour scales are shown for microarray (above) and RNA-Seq (below) heatmaps. Clusters marked in red include the central heat-shock genes and genes that appeared to interact with them in the coffee memory network, mainly the group of ABA-related genes.

S17). Our dataset therefore greatly expands the number of known miRNAs in *C. canephora*. The prediction of putative miRNA targets showed that as previously observed by Axtell and Bowman (2008), most of the conserved coffee miRNAs target TFs (Table S18). Although TFs are also predicted to be targeted by some of the putative novel miRNAs, most of them seem to regulate genes involved in other processes, such as metabolism, cytoskeleton and signal transduction (Table S18).

Differential expression analysis showed that miR398 is significantly up-regulated after the first and third drought cycles compared to the control (Table S19). Since miR398 and miR408 are frequently observed to be deregulated in different stress types (Khraiwesh et al., 2012), their expression was tested by stem-loop qPCR. The upregulation of miR398 was confirmed in the first stress cycle, but not in the third (Fig. 9). The expression of the miRNA miR408, on the other hand, showed significant upregulation in both cycles by qPCR (Fig. 9).

3. Discussion

3.1. Coffee gene expression is modulated by MYB TFs, miRNAs and transcriptional memory in response to drought stress

A plant's ability to tolerate water stress depends on extensive transcriptional reprogramming (Yamaguchi-Shinozaki and Shinozaki, 2006; Singh and Laxmi, 2015). In the tolerant coffee clone, drought-induced transcriptional changes seem to involve TFs, miRNAs and transcriptional memory. Unexpectedly, we identified only 22 (2.66%) drought-responsive TFs in the tolerant coffee clone (Table 6). Likewise, a relatively low number of responsive TFs (467, 7.10%) was reported by Ding and coworkers (2013) in their Arabidopsis dehydration memory study.

Responsive TFs in the tolerant clone belonged mainly to the MYB family (Table 6), whose members' action in drought response have been characterized (Baldoni et al., 2015). Ding and coworkers (2013) found

Table 3
The central heat-shock and LRR-domain-containing proteins predicted to interact in the coffee memory network, which was constructed by *in silico* analysis.

Coffee gene	Putative function	InterPro domains
Cc00_g08130	Disease resistance protein RGA2	LRR (IPR032675); Kinase (IPR000719)
Cc06_g16160	Disease resistance protein RGA2	
Cc03_g11800	Disease resistance protein RGA3	
Cc11_g02900	Disease resistance RPP8-like protein 2	
Cc01_g08110	Late blight resistance protein homolog R1B-14	
Cc04_g15220	LRR receptor-like serine/threonine-protein kinase	Non_Cytoplasmic, transmembrane and cytoplasmic domains; LRR (IPR032675); Kinase (IPR000719)
Cc00_g20660	LRR receptor-like serine/threonine-protein kinase	
Cc11_g02650	LRR receptor-like serine/threonine-protein kinase	
Cc02_g02350	Heat-shock protein 83	Histidine kinase-like ATPase, C-terminal domain (IPR003594)

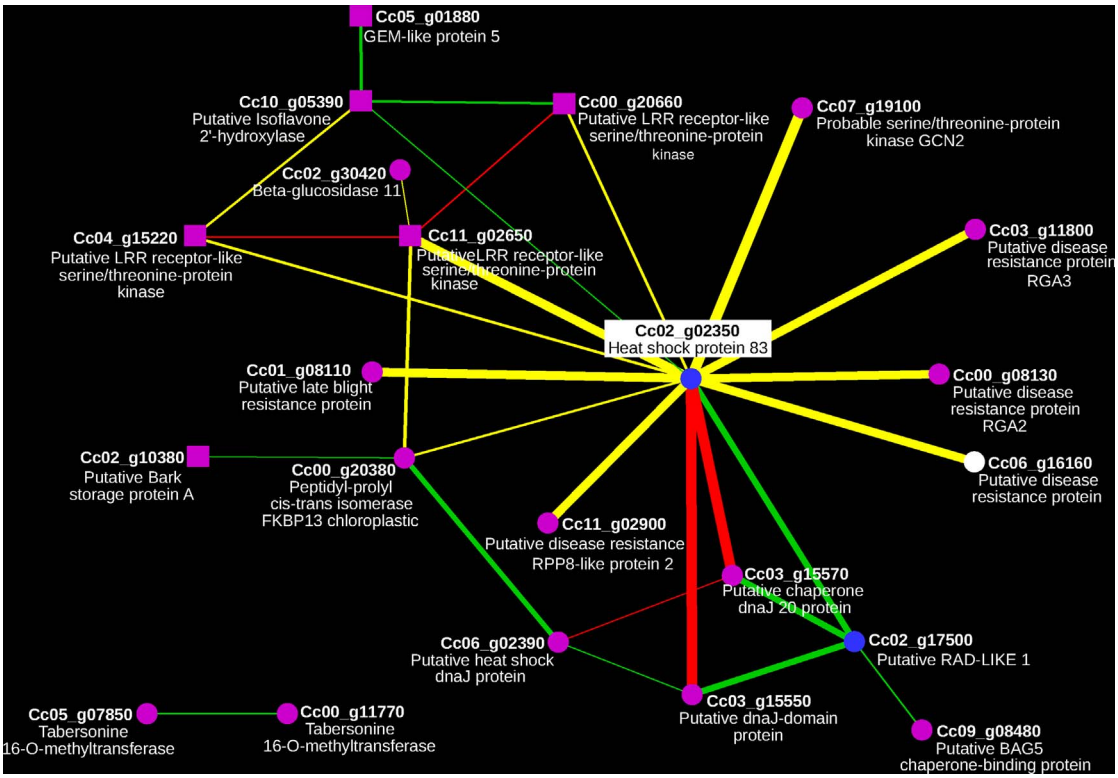


Fig. 6. Network of *C. canephora* clone 120 memory genes.
An *Arabidopsis* interolog-based network was constructed for *C. canephora* memory genes through assigned ATcodes using STRING. The coffee network combines co-expression and protein–protein interaction evidence. The type of interaction is indicated by the edge colour: yellow for protein–protein interactions, green for co-expression and red for both. Edge thickness indicates the interaction confidence: the thicker, the more reliable the interaction (the highest confidence level is 0.889). Node colours indicate the memory behaviour of the gene: [+/−] violet, [-/ +] blue, [−/−] white. Square node format means the gene was identified as ABA-related.

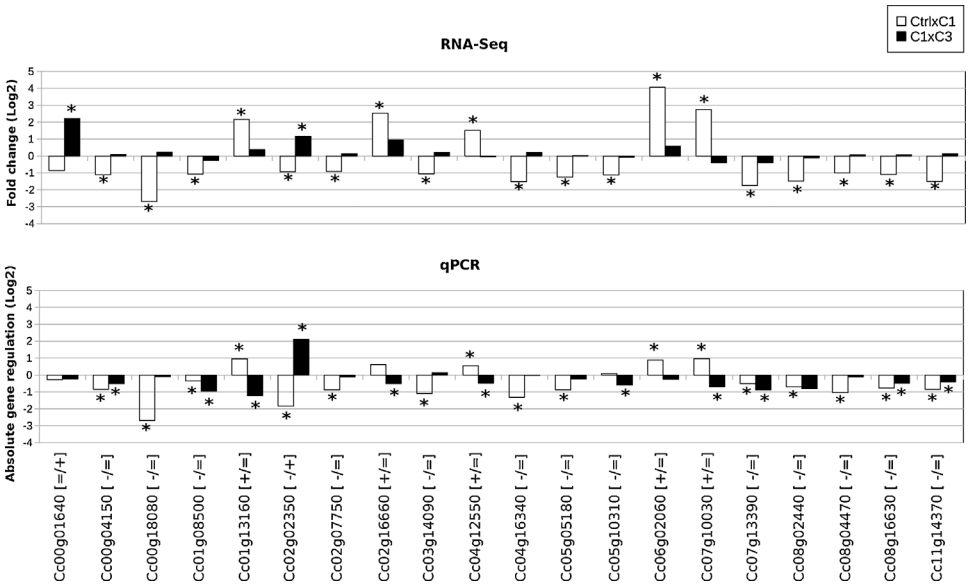


Fig. 7. Validation of RNA-Seq data by qPCR.
GADPH and S24 genes were used as internal controls in REST analysis. The qPCR results are the means of three biological replicates, each with three technical replicates. RNA-Seq responsive genes were determined by DESeq software. On the x-axis, labels comprise the gene name followed by the transcriptional profile predicted by DESeq. For both RNA-Seq and qPCR, (*) means that the gene was considered significantly regulated.

dehydration-responsive MYBs in all investigated memory categories. Moreover, they found that memory MYBs specifically clustered with ABA/abiotic stress-responding genes in Arabidopsis plants exposed to multiple dehydration events. Among the coffee responsive MYB TFs, we found one [−/+] putative RAD-like TF (Table 2, Table 6), which is a member of an MYB subfamily whose expression was already shown to be repressed by ABA (Yanhui et al., 2006) and drought (Betti et al., 2012) in other plants. As already observed for ATMYB15 (Ding et al., 2013), the expression of its putative coffee homologous gene was up-regulated by first drought exposure (Table 6). It was demonstrated that MYB15 overexpression conferred hypersensitivity to ABA and improved Arabidopsis drought tolerance (Ding et al., 2009). In contrast, the transcript abundance of MYB3 and MYB7 coffee orthologues decreased after the first drought event (Table 6). MYB7 is an R2R3-MYB protein that acts as a phenylpropanoid pathway repressor (Fornalé et al., 2014). Likewise, MYB3 was also characterized as a transcriptional repressor (Fornalé et al., 2014). Additionally, the coffee MYB Cc02_g24840 gene was identified as a possible miR159 target (Table S18). Taken together,

Table 4
Coffee memory genes linked to ABA pathway.

Coffee gene	Arabidopsis gene	Log2FC		Putative function	References[a]
		Ctrlxc1	C1xC3		
Cc00_g20660	AT4G08850	33.02	−32.47	LRR receptor-like serine/threonine-protein kinase	Nemhauser et al., 2006; Wang et al., 2011
Cc02_g10380	AT4G24350	2.75	−3.12	Bark storage protein A	Ding et al., 2013
Cc02_g36160	AT4G23540	4.87	−29.96	ARM repeat superfamily protein	Nemhauser et al., 2006; Wang et al., 2011; Ding et al., 2013
Cc04_g15220	AT4G08850	6.45	−4.49	LRR receptor-like serine/threonine-protein kinase	Nemhauser et al., 2006; Wang et al., 2011
Cc05_g01880	AT5G13200	5.16	−4.58	GEM-like protein 5	Ma et al., 2015a; Ding et al., 2013
Cc10_g05390	AT4G37370	1.12	−1.27	Isoflavone 2′-hydroxylase	Vanderauwera et al., 2007; Kreps et al., 2002; Ding et al., 2013
Cc11_g00460	AT5G24090	3.65	−1.69	Acidic endochitinase	Wang et al., 2011; Ding et al., 2013
Cc11_g02650	AT3G47570	6.14	−5.51	LRR receptor-like serine/threonine-protein kinase	Zhu et al., 2008
Cc11_g07610	AT5G36110	3.63	−3.87	Cytochrome P450 716B2	Cerekovic et al., 2015

[a] Studies with ABA and/or stress where putative *Arabidopsis* orthologous of coffee memory genes were regulated.

Table 5
Significantly enriched pathways (corrected pvalue cutoff = 0.05) for clone 120 drought-responsive genes.

	KEGG Pathway (ID)	Corrected P-Value
Non-memory	Flavonoid biosynthesis (ath00941)	1.08E-06
	Biosynthesis of secondary metabolites (ath01110)	1.93E-05
	Phenylalanine, tyrosine and tryptophan biosynthesis (ath00400)	5.43E-03
	Stilbenoid, diarylheptanoid and gingerol biosynthesis (ath00945)	3.19E-02
	Phenylpropanoid biosynthesis (ath00940)	3.19E-02
Memory	Sesquiterpenoid and triterpenoid biosynthesis (ath00909)	1.36E-02
Late-response	Ubiquinone and other terpenoid-quinone biosynthesis (ath00130)	8.54E-04
	Glutathione metabolism (ath00480)	8.54E-04

these results suggest that MYB TFs participate in gene expression modulation during coffee drought response, putatively forming a complex regulatory network that might involve miRNAs.

As observed for drought-stressed *Medicago truncatula* plants (Trindade et al., 2010), miR398 and miR408 were up-regulated by the drought cycles in coffee (Fig. 9, Table S19). Apart from drought, these genes have been reported to be regulated in other stress conditions, including ABA-, heat-, UV- and even biotic-stress events, indicating that they likely participate in a broad network of stress modulation (Zhu et al., 2011; Khraiwesh et al., 2012; Guan et al., 2013). Chickpea plants overexpressing miR408 have been recently shown to be tolerant to several stresses, including drought (Hajyzadeh et al., 2015; Ma et al., 2015b).

Transcriptional memory behaviour was observed for tolerant clone genes (Table 2), suggesting that coffee can resort to a mechanism to "remember" which genes should be modulated when the plant is newly subjected to drought stress and this modulation probably contributes to plant acclimation. Conversely, the drought-sensitive clone had only one memory gene (Fig. 2), and instead of acclimation, programmed cell death categories were enriched after the third exposure (Fig. 3). Taken together, these results suggest that transcriptional memory may contribute to coffee drought tolerance. In addition to TF accumulation (Bruce et al., 2007), epigenetic mechanisms, such as DNA methylation and chromatin remodelling, have been proposed to promote transcriptional memory (Han and Wagner, 2014; Kinoshita and Seki, 2014). One [−/=] putative methyltransferase (Cc08_g08050) with a DNA-binding domain (data not shown) and one [+/=] gene (Cc07_g06660) annotated with the "Nucleosome remodelling factor" class were found in the tolerant clone (Table S12). We hypothesize that their early regulation might contribute to creating a transcriptional memory that may remain until subsequent exposure. The role of TOPLESS (TPL) and TPL-related (TPR) neighbouring genes (Fig. S3, Table S6) in drought-induced coffee expression modulation remains to be investigated. These

Table 6
Drought-responsive putative transcription factors identified in clone 120.

KOG	Coffee gene	Memory type	Arabidopsis gene	Arabidopsis annotation
Reg Transcription (KOG0019)	Cc00_g33210	+/−	AT1G13450	GT-1 transcription factor
	Cc02_g17500	−/+	AT4G39250	RAD-LIKE 1 transcription factor
MADS box transcription factor (KOG0014)	Cc00_g02800	+/=	AT5G60910	AGAMOUS-LIKE 8
	Cc02_g37000	+/=	AT2G45660	AGAMOUS-LIKE 20
Transcription factor, Myb superfamily (KOG0048)	Cc08_g04480	−/=	AT2G16720	ATMYB7
	Cc02_g15520	−/=	AT5G16770	ATMYB9
	Cc02_g24840	+/=	AT3G60460	DUO1
	Cc05_g05740	+/=	AT3G23250	ATMYB15
	Cc00_g19890	−/=	AT3G28470	ATMYB35
	Cc04_g01370	−/=	AT5G35550	ATMYB123
	Cc06_g07950	+/=	AT3G23250	ATMYB15
	Cc04_g01380	−/=	AT1G22640	ATMYB3
	Cc03_g06560	+/=	AT3G24310	ATMYB71
	Cc02_g15530	−/=	AT2G47460	ATMYB12
Transcription factor HEX, contains HOX and HALZ domains (KOG0483)	Cc02_g01010	+/=	AT2G46680	ATHB-7
	Cc08_g16780	+/=	AT2G46680	ATHB-7
Heat-shock transcription factor (KOG0627)	Cc06_g17660	−/=	AT2G41690	AT-HSFB3
CCAAT-binding factor, subunit B (HAP2) (KOG1561)	Cc06_g16930	+/=	AT1G30500	NF-YA7
GATA-4/5/6 transcription factors (KOG1601)	Cc04_g07160	+/=	AT1G25440	BBX15
bZIP transcription factor ATF6 (KOG4343)	Cc10_g04070	+/=	AT1G45249	ABF2
–	Cc08_g04470	−/=	AT1G53910	RAP2.12
–	Cc03_g14090	−/=	AT2G31730	BHLH DNA-binding superfamily protein

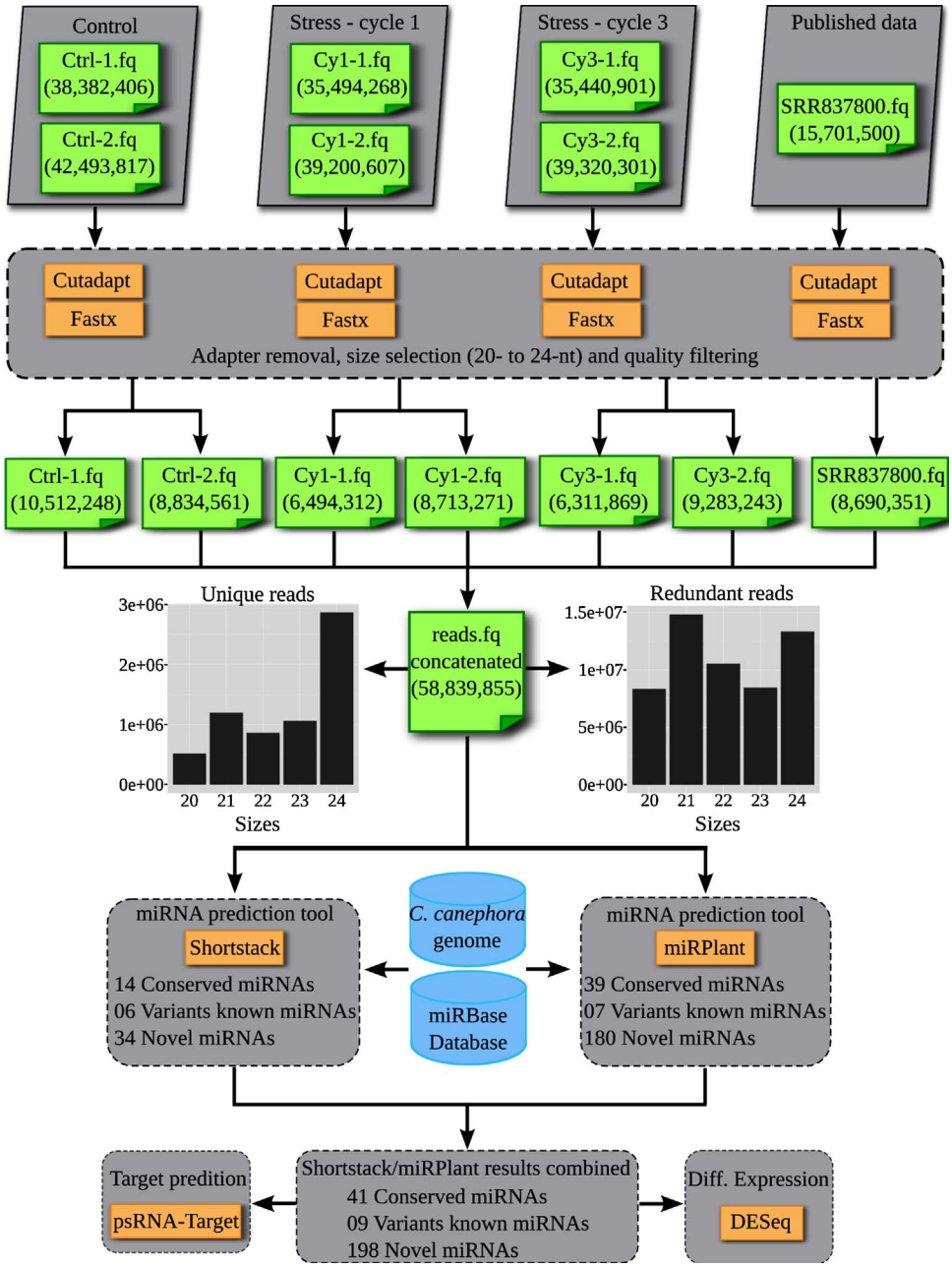


Fig. 8. Bioinformatics pipeline used for miRNA discovery.
Adaptors were removed from all libraries and sequences between 20 and 24 nucleotides (nt) were selected. Reads were concatenated before being used as input, together with the *C. canephora* genome, into two software packages: Shortstack and miRPlant. Predicted miRNAs were then classified into known or variant forms of conserved miRNAs and novel miRNAs based on the sequences available in the miRBase database. Target prediction and differential expression analysis were then performed with psRNATarget and DESeq software packages, respectively. Numbers between parentheses indicate total read counts.

proteins can act as corepressors (Causier et al., 2012) in plant defence (Zhu et al., 2010) through histone deacetylase interactions.

3.2. Interaction between LRR-domain-containing and heat-shock memory proteins may play a role in coffee drought signalling

Functional categories linked to biotic stress were enriched among the coffee drought-responsive genes of both clones (Fig. 3), indicating that an interplay may exist between coffee defence and drought responses. The interaction between plant pathogens and drought stresses, which can have detrimental or positive effects, has been reported in different species (Atkinson and Urwin, 2012; Rejeb et al., 2014). In coffee, this interaction seems to play a role in tolerant plant drought acclimation, with several defence-related genes being repressed by the third cycle (Table S14). Additionally, we found a cluster of neighbouring disease resistance-responsive genes exclusively co-expressed in the drought-tolerant clone (Fig. S3, Table S6) whose role in coffee drought and/or defence responses needs to be further investigated.

Genes coding for defence-related proteins containing LRR and kinase domains (Table 3) were highly represented among tolerant clone memory genes (Fig. 4). Recently, (Li et al., 2016) Li and coworkers (2016) showed that different kinases can be regulated by drought in a highly tolerant plant species. We found three coffee [+/−] LRR-RLKs putative membrane proteins linked to ABA metabolism (Table 3, Table 4, Fig. 6). The putative orthologue of two of them is AT4G08850, which was predicted to be an ABA-specific marker gene in Arabidopsis (Nemhauser et al., 2006). RLKs have been demonstrated to play a role in abiotic stress responses, which probably involves ABA and ROS (Marshall et al., 2012; Osakabe et al., 2013). Then, we hypothesize that coffee drought response and memory may involve signal transduction mediated by LRR-domain-containing proteins, including membrane RLKs, which can, in turn, phosphorylate targets.

The LRR-domain-containing proteins were predicted (confidence > 0.7) to physically interact with [−/+] Hsp90.1 (Fig. 6). In silico analysis also predicted physical interactions between Hsp90.1 and other memory proteins involved with protein folding (Fig. 6). These

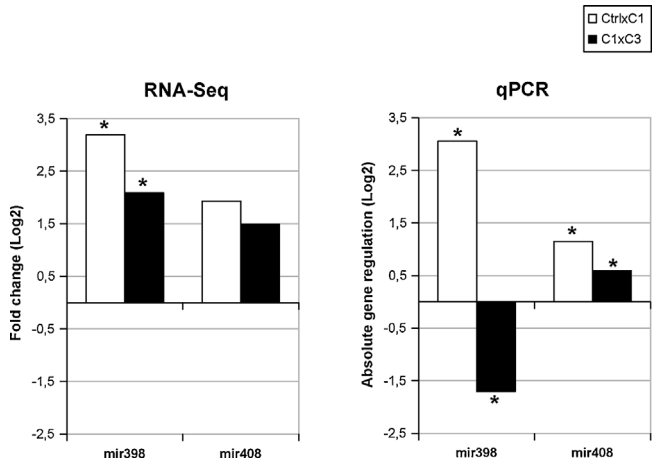


Fig. 9. Validation of sRNA-Seq data by qPCR.
GADPH and S24 genes were used as internal controls in REST analysis. The qPCR results are the means of three biological replicates, each with three technical replicates. sRNA-Seq responsive genes were determined by Shortstack and miRPlant software packages.

outcomes suggest that Hsp90.1 may assist other proteins in maintaining their proper conformation during coffee response to multiple drought stress events. Heat-shock proteins and molecular chaperones play a crucial role in protecting plants against biotic and abiotic stresses by re-establishing normal protein conformations (Wang et al., 2003, 2004; Xu et al., 2012; Jacob et al., 2017). While most of the coffee memory genes coding for defence-related proteins exhibited a [+/−] profile, the heat-shock genes displayed a [−/+] memory type (Fig. 6). Even though little is known about how plant immunity and abiotic stress tolerance are connected, evidence supports the existence of an antagonism between heat stress and plant immunity (Lee et al., 2012), which is in agreement with our results. The chaperoning activity of heat-shock proteins may provide a link in coffee and other plants.

3.3. Transcriptional memory modulates genes of ABA pathway in the drought-tolerant clone

The category “response to abscisic acid” (GO:0009737), encompassing any process resulting in changes promoted by an ABA stimulus, was enriched only in the drought tolerant clone 120 (Fig. 3). Genes related to ABA perception, metabolism and expression regulation (ABF2 TF) (Table 6, Table S11) seemed to be mainly regulated after the first drought cycle in the tolerant clone. Under stress conditions, ABA signalling and metabolism (Nambara and Marion-Poll, 2005; Kim, 2012) can be altered, triggering stomatal closure and the transcriptional regulation of ABA-inducible genes (Finkelstein, 2013; Todaka et al., 2015). Expression of eight ABA-linked coffee genes predicted to be non-memory was tested by qPCR (Fig. 7, Table S11). The qPCR results validated and complemented the DESeq predictions for three of them (Cc00_04150–auxin responsive protein; Cc01_g13160–ABA 8′ hydroxylase; Cc04_g12550–LRR-RLK), suggesting that the number of ABA-related coffee memory genes may be higher than predicted here.

Differential expression analysis also allowed us to identify ABA pathway components exhibiting memory behaviour (Table 4), indicating that this hormone may be important for drought-memory in the coffee tolerant clone. Ding and coworkers (2012) showed that ABA participates in Arabidopsis dehydration memory and has increased endogenous levels after drought exposures. The ABA level increase was also reported in double-stressed *Aptenia cordifolia* plants compared to single-stressed ones (Fleta-Soriano et al., 2015). ABA-related [+/−] genes comprising LRR-RLKs, a GEM-like protein and a isoflavone hydroxylase emerged from the coffee memory network as a group (Fig. 6). As a whole, we found coffee responsive genes putatively related to ABA signalling, metabolism and transcriptional regulation (Table S11), indicating that this hormone may play an important role in coffee drought response and memory.

3.4. Mitigation of drought-induced oxidative stress contributes to coffee acclimation to water deficit

The enrichment of drought-responsive genes with the “response to oxygen-containing compounds” category (Fig. 3) suggests an oxidative stress status induced by drought in both coffee clones. To avoid the oxidative cellular damage caused by different stresses, plants possess an antioxidant defence system comprising enzymatic and nonenzymatic components (Apel and Hirt, 2004; Sharma et al., 2012). The action of enzymatic defence mechanisms was already demonstrated in coffee plants submitted to single-event drought experiments (Pinheiro et al., 2004). Here, we found that the expression of ascorbate peroxidase (APX) and monodehydroascorbate reductase antioxidant enzymes were induced by the first drought event in the tolerant clone (Table S13). Recently, Menezes-Silva and coworkers (2017) also observed a higher activity of antioxidant enzymes, such as APX, in *C. canephora* plants submitted to drought cycles. The majority of genes linked to the antioxidant system exhibited a non-memory expression profile (Table S13), suggesting that their expression modulation preferentially occurs early after the first exposure and that their transcript levels are maintained afterwards during tolerant plant acclimation.

Nonenzymatic components of the antioxidant system also participate in coffee drought response. Drought-responsive genes were enriched with categories related to secondary compound metabolism (Fig. 3; Table 5; Table S13), such as phenylpropanoids and flavonoids, which are presumed to function as antioxidants in stressed plants (Sharma et al., 2012; Nakabayashi and Saito, 2015). The metabolic reprogramming undergone by *C. arabica* plants under high light conditions also resulted in the increase of their antioxidant capacity and flavonoid levels associated with oxidative stress avoidance (Martins et al., 2014). The results recently obtained by Menezes-Silva and coworkers (2017) suggest that coffee drought memory is associated with an orchestrated reprogramming of primary and secondary metabolism, including the increase of phenylalanine and cinnamic acid levels in clone 109 and 120, respectively, after three drought cycles. Phenylalanine is a shikimate pathway end product that yields cinnamic acid that in turn gives rise to phenylpropanoids and flavonoids (Vogt, 2010; Fraser and Chapple, 2011). Glutathione metabolism seemed to be particularly important after the third cycle (Table 5; Table S13). Taken together, our results strongly suggest that in coffee tolerant clone 120, antioxidant protective mechanisms are employed after drought exposure, allowing acclimation. Conversely, in the sensitive clone 109, the oxidative stress state seemed to persist after the third cycle and induce programmed cell death (Fig. 3, Table S8). Programmed cell death induction usually includes an increase in ROS levels (Petrov et al., 2015). Notably, we previously demonstrated that the sensitive, but not the tolerant clone, displayed evident oxidative damage when submitted to water deficit (Pinheiro et al., 2004).

4. Materials and methods

4.1. Experimental design

The experiment was conducted as described by Menezes-Silva et al. (2017). Briefly, plants of clone 120 (drought-tolerant) and clone 109 (drought-sensitive) of *Coffea canephora* Pierre ex Froehner cv conilon were grown in a greenhouse. The contrasting drought tolerance of these clones was assessed in previous studies (Pinheiro et al., 2004, 2005; Silva et al., 2013; Menezes-Silva et al., 2015). Uniform seedlings grown in pots containing a mixture of soil, sand and composted manure (4:1:1, v/v/v) were irrigated and fertilized as needed, without root development restriction. Nine-month-old plants of each clone were separated in three groups. One group received irrigation during the entire

experiment so that the soil moisture was close to the field capacity (control plants). The second group was subjected to one drought cycle (C1), while a third group was submitted to three drought cycles (C3). Each cycle consisted of two phases: dehydration and recovery. Dehydration was imposed by suspending the irrigation until the soil water content reached approximately 25% of the field capacity. Plants were kept under this condition for approximately 14 days, after which leaf samples were collected. Subsequently, the pots were rewatered until the soil reached the same water content relative to the field capacity (recovery). The recovery phase lasted approximately 10 days, i.e., the time required for the measured physiological parameters (Menezes-Silva et al., 2017) of the drought-stressed plants to attain the same values as the control plants. Additional dehydration/recovery cycles were only imposed to fully recovered plants. All samplings were made after the end of the dehydration phase. Sampled leaves were immediately frozen in liquid nitrogen and stored at −80 °C until RNA extraction.

4.2. RNA extraction, RNA-Seq library construction and sequencing

Total RNA extraction was carried out according to the Concert (Thermo Fisher Scientific) protocol, followed by phenol/chloroform purification and DNAse (Ambion) treatment. RNA amount and quality were evaluated by Nanodrop and Bioanalyser (Agilent Technologies, USA). RNA extracted from different individuals was combined to form pools for library construction. Each clone 120 sequenced library was a pool of two individuals while RNA from four individuals was combined to construct each clone 109 library (Table S1). Additionally, three biological replicates per condition were sequenced for clone 120, and one replicate was sequenced for clone 109 (Table S1). Messenger RNA purification and Illumina HiSeq 1000 paired-end sequencing were carried out by Eurofins. For the tolerant clone, the RNAs of two biological replicates from each condition was also sent for small RNA sequencing (sRNA-seq) (Table S1). All sequenced libraries in this article can be found in the NCBI SRA database under accession number PRJNA353111.

4.3. Bioinformatics analysis

Quality-checked reads were aligned to the *C. canephora* genome (Denoeud et al., 2014) from the Coffee Database (http://coffee-genome.org/) using BWA version 0.5.9 (Li and Durbin, 2009) with the default parameter values. To check the quality of clone 120 biological replicates, Pearson's coefficient correlation was calculated over raw read counts using an R script. Differential expression analysis was carried out using the Bioconductor package DESeq (Anders and Huber, 2010). DESeq tests differential expression by using the negative binomial distribution and a shrinkage estimator for the distribution's variance. Genes with FDR adjusted-pvalue < 0.05 were considered differentially expressed genes (DEG). DESeq analysis generated four DEGs lists: clone 120 C1 and C3, and clone 109 C1 and C3. Clone 120 DEG lists were crossed to determine the genes regulated after both one and three drought exposures following the memory classification proposed by Ding et al. (2013). Here, the definition of memory gene did not include fold change filter. Genes regulated only after the first or third cycles, which were respectively called non-memory and late-response genes, were also identified.

For the miRNA analysis, sRNA library adaptors were removed and read sized (20 to 24 nt) with the Cutadapt software (Martin, 2011). Reads were filtered using the fastx toolkit with −q30 and −p75 parameters (http://hannonlab.cshl.edu/fastx_toolkit/index.html). Remaining reads from all six libraries were concatenated with a previously published *C. canephora* sRNA library (Loss-Morais et al., 2014) and used as input for miRNA discovery using Shortstack, version 3.3 (Axtell, 2013) and miRPlant, version 5 (An et al., 2014). BLAST searches against the miRBase database (release 21) (Kozomara and Griffiths-Jones, 2014) were used to classify the identified miRNAs. Possible miRNA targets were identified with the psRNATarget software (Dai and Zhao, 2011). Only predicted targets with expectation (software's scoring system) lower than two were considered.

4.4. Functional annotation and GO enrichment analysis

The functional annotation available at Coffee Database was used for the identified coffee sequences. Annotations of sequences of particular interest were manually curated. Coffee sequences were also compared to Arabidopsis protein sequences (TAIR9) through blastx using an evalue of 1E-6. In addition to evalue, gap (smaller) and alignment (bigger) sizes were taken into account to select the best blast hit. To identify GO-enriched terms among DEG, a hypergeometric test was applied with a pvalue cutoff of 0.005 using an R script. Coffee protein sequences were also annotated against KOG with an 1E-6 evalue cutoff using WebMGA server (Wu et al., 2011). Mapman 3.5.1 (Thimm et al., 2004) was used to map coffee DEG to biological process diagrams. For KEGG Pathway annotation and enrichment analysis, we used KOBAS 2.0 standalone version (Xie et al., 2011), and a hypergeometric test with FDR correction was applied separately for memory, non-memory and late-response coffee genes as test sets and Arabidopsis sequences as background. Log (base 2) fold change (FC) estimates for clone 120 DEGs were compared to Arabidopsis microarray drought data from Genevestigator (Hruz et al., 2008). These Arabidopsis FC values were hierarchically clustered using Genepattern (Reich et al., 2006). Finally, an interolog-based network was constructed for coffee memory genes through their assigned ATcodes, gathering the protein–protein interaction and co-expression evidence available at STRING version 10 (Szklarczyk et al., 2014). To identify ABA-related coffee genes, a list of 4249 Arabidopsis genes was obtained from Arabidopsis Hormone Database (AHD 2.0) (Jiang et al., 2011) and was compared to clone 120 responsive genes by their ATcodes.

4.5. Quantitative real-time PCR analysis

Twenty clone 120 drought-responsive genes were selected for quantitative Real-Time PCR (qPCR) validation. The read count variability of the selected genes in each experimental condition was evaluated through average and standard deviation estimates (Fig. S1). Given its predicted role in coffee drought memory, Cc02_g02350 was included in the validation. Primers were designed using Primer3Plus (http://primer3plus.com/; Untergasser et al., 2012) and their specificity was confirmed by Primer Blast (http://www.ncbi.nlm.nih.gov/tools/primer-blast/; Ye et al., 2012). Previously validated genes GADPH and S24 (Cruz et al., 2009) were used as a reference. Mature miRNA expression was evaluated by stem-loop qPCR (Chen et al., 2005). All primer sequences are listed in Table S2. cDNA was synthesized from pools containing equal amounts of DNase-treated total RNA from each individual. Approximately 1 µg of each RNA pool was reverse transcribed using SuperScript III Reverse Transcriptase (Thermo Fisher Scientific). The amplification reactions were performed in a 7500 Fast Real-Time PCR System (Applied Biosystems) using SYBRGreen to monitor dsDNA synthesis. The reaction mixtures contained 10 µl of diluted cDNA (1:50), 0.2 µM of each primer, 50 µM of each dNTP, 1× PCR Buffer (Thermo Fisher Scientific), 3 mM $MgCl_2$, 1× SYBRGreen I (Molecular Probes) and 0.25 U of PlatinumTaq RNA Polymerase (Thermo Fisher Scientific) in a total volume of 20 µl. The miRNAs reaction mixtures contained 2.5 µl of diluted cDNA (1:50), 0.2 µM of each primer forward, 5 µl of SYBR Select Master Mix (Applied Biosystems) in a total volume of 20 µl. Three biological replicates were used, each comprising a pool of two individual plants. Additionally, each single qPCR reaction was repeated three times to make technical replicates. The efficiency and the Quantification Cycle (Cq) values generated for each qPCR reaction were estimated using Miner software (Zhao and Fernald, 2005). REST software (Pfaffl et al., 2002) was used to evaluate the significance of relative expression differences in the CtrlxC1 and

C1xC3 comparisons.

Accession numbers

All sequenced libraries of this article were deposited in NCBI SRA database under accession number PRJNA353111.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.envexpbot.2017.12.004.

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