



Genome-Wide Identification, Evolution, and Expression Profile of Aquaporin Genes in *Coffea canephora* in Response to Water Deficit

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Abstract

Aquaporins (AQPs) belong to the major intrinsic protein superfamily and play a key role in the transport of water and other solutes across cell membranes. *Coffea canephora* is an evergreen shrub used for making instant coffees. Genome analysis of *C. canephora* identified 33 putative aquaporin genes assigned to five subfamilies including seven plasma membrane intrinsic proteins (PIP), 9 tonoplast intrinsic proteins (TIP), 11 NOD26-like intrinsic proteins (NIP), 3 small basic intrinsic proteins (SIP), and 3 X intrinsic proteins (XIP). Generally, the AQPs gene structure was conserved within each subfamily, with exon numbers ranging from one to five. The prediction of the aromatic/arginine selectivity filter (ar/R) and Froger's positions indicated a noticeable difference in substrate specificity between subfamilies. Synteny analysis revealed high conservation of aquaporin genes in coffee. In silico expression analysis of the *CcAQPs* genes indicated that they were differentially expressed in various tissues. Members of *CcPIPs* and *CcTIPs* subfamilies were validated by real-time quantitative analysis in leaves of two genotypes of *C. canephora* with contrasting responses to water deficit (clone 14: drought-tolerant and clone 109A: drought-susceptible). Under severe water deficit, the relative expression of isoforms of both genes decreased in clone 14 compared with that under the irrigated condition, while clone 109A showed comparatively higher mRNA levels, with the exception of *CcPIP1;2* in the stress condition. This study was the first to characterize and validate aquaporin genes in *C. canephora* in response to water deficit, and the findings may provide insights for biotechnological approaches to increase tolerance to drought.

Keywords Robusta coffee · Water transporters · Abiotic stress · Gene expression

Key Message

•Aquaporin gene family conservation maintained in coffee plants. PIPs and TIPs isoforms expressed differentially in the contrasting genotypes of *Coffea canephora* in response to water deficit.

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Introduction

Aquaporins (AQPs) belong to the major intrinsic protein (MIP) superfamily of integral membrane proteins present in bacteria, fungi, animals, and plants. These proteins play an important role in facilitating the diffusion of water and other small molecules such as glycerol, urea, ammonia (NH₃), carbon dioxide (CO₂), hydrogen peroxide (H₂O₂), boron, and silicon through cell membranes (Gomes et al. 2009; Maurel et al. 2015; Pommerrenig et al. 2015). Many studies demonstrated the involvement of AQPs in different physiological processes such as the response to abiotic stresses (Khan et al. 2015; Zhu et al. 2019), photosynthesis, stomata and mesophyll conductance (Perez-Martin et al. 2014), and seed longevity and viability (Mao and Sun 2015). Genome-wide analyses have been used to characterize and establish the number of homolog genes in many eudicots and monocots species, e.g., 35 in *Arabidopsis thaliana* (Johanson et al. 2001); 55 in *Populus trichocarpa*

(Gupta and Sankararamakrishnan 2009); 71 in *Gossypium hirsutum* (Park et al. 2010); 47 in *Solanum lycopersicum* (Reuscher et al. 2013); 41 in *Sorghum bicolor* L. (Reddy et al. 2015); 28 in *Beta vulgaris* (Kong et al. 2017); 41 in *Zea mays* L. (Bari et al. 2018), and 34 in *Citrus sinensis* (Wei et al. 2019).

AQPs have highly conserved structures in plant, such as a molecular weight varying between 23 and 31 kDa, six transmembrane helices connected by five loops named A to E, N- and C-terminal tails both exposed to the cytosol (Törnroth-Horsefield et al. 2006). The NPA (asparagine-proline-alanine) motifs in the loops B and E are highly conserved. The conservation of specific amino acids residues in key position in aquaporin sequences denotes a signature pattern in relation to the structure, function, and substrate selectivity. These residues create the aromatic/arginine selectivity filter (ar/R) which is characterized by four residues, one in helix 2 (H2), one in helix 5 (H5), and two in loop E (LE1–LE2), and acts as a size-exclusion barrier and regulates the transport specificity for water transport and small neutral solutes (Hub and de Groot 2008; Mitani-Ueno et al. 2011). Five key positions in the protein sequence (P1–P5) in which residues are associated with function, and specific physicochemical properties for each subgroup were termed Froger’s positions (Froger et al. 1998). Thus, the analysis of these positions in protein sequences may give clues about their specificity and functionality. Based on sequence homology, the aquaporins in plants can be classified into eight subfamilies. Five of them found in higher plants: plasma membrane intrinsic proteins (PIPs), tonoplast intrinsic proteins (TIPs), nodulin 26-like intrinsic proteins (NIPs), small basic intrinsic proteins (SIPs), and uncategorized X intrinsic proteins (XIPs), the last two with still unknown function (Maurel et al. 2015). Three subfamilies: the glycerol facilitator-like intrinsic proteins (GIPs) and LIPs (large intrinsic proteins) were found exclusively in algae and mosses, respectively (Hussain et al. 2019) and the hybrid intrinsic proteins (HIPs) in lycophytes and mosses (Anderberg et al. 2012; Hussain et al. 2019).

At the molecular level, the response to water stress alters the expression of several genes, including those in the aquaporin family. In plants, aquaporins play key roles in regulating hydraulic conductance in roots and leaves, regulating water flow into and out of cells, and water transfer across membranes (Shekoofa and Sinclair 2018). The evaluation of aquaporin genes in contrasting *Phaseolus vulgaris* genotypes (i.e., tolerant and susceptible to water stress) indicated that the ability of the plant to conserve water during deficit in tolerant cultivars involves reduced expression of aquaporin-specific isoforms (Zupin et al. 2017). A global analysis of AQPs gene expression in *C. sinensis* submitted to water deficit revealed that isoforms from different aquaporin families had increased expression in leaves (Martins et al. 2015) indicating that each isoform responds specifically to the water deficit.

Despite its importance in plant physiology, little is known about the AQP superfamily in the *Coffea* genus (Santos and Mazzafera 2013; Miniussi et al. 2015). Coffee is one of the most important global agricultural export commodities. The perennial diploid specie *Coffea canephora* Pierre ex A. Froehner ($2n = 2x = 22$) represents approximately 30% of world production. Abiotic stresses, especially water deficit, are major constraints for coffee production in most producing countries (Da Matta and Ramalho 2006). Coffee breeding programs have aimed at characterizing genotypes with differential water deficit responses to study physiological parameters involved in drought tolerance. For example, *C. canephora* clone 14 characterized as tolerant, while clone 109A considered susceptible to drought (Marraccini et al. 2012; Lima et al. 2015).

The assembled sequence of the *C. canephora* genome (Denoeud et al. 2014) allows the use of these data for the identification of new genes with biotechnological potential. Therefore, in this study, we present the first genome-wide analysis of aquaporin genes in *C. canephora* (*CcAQPs*). For this, we performed an in silico identification of AQPs genes and, subsequently, a comprehensive analysis of the phylogenetic relationships, gene structure, chromosomal distribution, transmembrane domains, conserved motifs, identification and characterization of NPA motifs, ar/R selectivity filter residues, and Froger’s positions. In parallel, we analyzed and discussed the gene duplication events and synteny in coffee AQPs. Additionally, we also investigated the expression profile of selected *CcPIPs* and *CcTIPs* isoforms in response to water deficit.

Materials and Methods

Database Search for Identification of *CcAQP* Genes

Firstly, to identify AQP family members, a genome-wide search was performed using “Aquaporin” as the keyword on *C. canephora* database (<http://coffee-genome.org>; Denoeud et al. 2014). In order to perform comparative analyses, AQPs sequences were also searched in other species. The *C. arabica* and *C. eugenioides* sequences were retrieved from National Biotechnology Information Center (NCBI) database (<https://www.ncbi.nlm.nih.gov>; Altschul et al. 1997), and the *Arabidopsis thaliana*, *Solanum lycopersicum*, *Oryza sativa*, and *Solanum tuberosum* were downloaded from Phytozome v12.0 platform (<http://www.phytozome.net>; Goodstein et al. 2011). In addition, to get a better comprehension of the exon/intron structures of each *CcAQP*s gene, we compared the coding sequences (CDS) with their corresponding genomic DNA sequences using the online server Gene Structure Display Server (GSDS; <http://gsds.cbi.pku.edu.cn>; Hu et al. 2015).

Phylogenetic Analysis of AQP Proteins

Multiple sequence alignment of deduced AQPs amino acid from *C. canephora*, *C. arabica*, *C. eugenoides*, *A. thaliana*, *S. lycopersicum*, *S. tuberosum*, and *O. sativa* was performed using the ClustalW platform (<http://www.genome.jp/tools-bin/clustalw>; Thompson et al. 1994). Finally, these results were used to construct phylogenetic trees with the MEGAX software (Kumar et al. 2018) by maximum likelihood (ML) method using bootstrap procedure with 1000 replicates. The *CcAQPs* sequences were assigned to different subfamilies by comparing with other species.

Chromosomal Location and Identification of Duplication Events

The putative *CcAQPs* genes were mapped onto *C. canephora* chromosomes using the Mapchart 2.2 software (Voorrips 2002). Reciprocal BLASTN was carried out to establish paralogous relationship within *C. canephora*. The hit threshold values were set as E -value $< 1e_{-50}$, score > 200 , and positive $> 80\%$ (Huang et al. 2016). Hit threshold values were set as E -value $< 1e_{-50}$, score > 200 , and positive $> 80\%$ (Huang et al. 2016). The synonymous (Ks) and non-synonymous (Ka) substitution rates from paralogous and orthologous genes was analyzed by Ka_Ks calculator 2.0 (Zhang et al. 2006). The approximate dates of the duplication events were calculated by the Eq. ($T = Ks/2\lambda$), assuming an average value for the synonymous substitution rate (λ) of 7.41×10^{-9} (Yu et al. 2011).

Synteny Analysis of AQP Genes in Coffee Species

To analyze the synteny relationship among AQPs genes, we used the orthologous genes of the three coffee species: *C. arabica* (allotetraploid specie; $2n = 4x = 44$), *C. canephora*, and *C. eugenoides* (diploid species; $2n = 2x = 22$). The Circos software was used to represent the synteny among coffee species (<http://circos.ca/>; Krzywinski et al. 2009).

Subcellular Localization, Transmembrane Domains, and In Silico Transcriptional Profiling

The length, molecular weight (kDa), and isoelectric point (pI) of the putative *CcAQPs* proteins were calculated using the ProtParam tool (<http://web.expasy.org/protparam>). Subcellular localizations of *CcAQPs* were predicted using the Plant-mPLOC algorithms with default parameters (Chou and Shen 2010). In addition, to analyze the in silico expression of AQPs isoforms in different organs and tissues in *C. canephora*, the RPKM (reads per kilobase million) values were retrieved from the Coffee Genome Hub database (<http://coffee-genome.org>; Denoeud et al. 2014). The data were compiled into a spreadsheet, log₁₀ transformed, and

used as input data for in silico expression calculation using the Heatmapper algorithm (<http://www1.heatmapper.ca>; Babicki et al. 2016).

Motif Conservation Analysis of CcAQP Proteins

To gain more detailed information on conserved motifs, the deduced *CcAQP* protein sequences were analyzed using the MEME online program (Multiple Expectation Maximization for Motif Elicitation: (<http://meme.nbcr.net/meme/cgi-bin/meme.cgi>; Bailey et al. 2006). The optimized parameters of MEME were as follows: the optimum width of each motif ranged from six to 50, the maximum number of motifs to find was 20, and the other parameter settings were default values. The presence and number of transmembrane (TM) helices in the *CcAQPs* proteins were predicted using the TMHMM Server v.2.0 program (<http://www.cbs.dtu.dk/services/TMHMM/>). Additionally, the identification of conserved NPA motifs, air/R selectivity filter (H2, H5, LE1, and LE2), and analysis of Froger's positions were performed by visual alignment inspection compared with *AtAQPs*. Since the XIP subfamily is not present in *Arabidopsis*, the conserved motifs of this subfamily in *CcAQP* were identified by comparison with sequences of *R. communis* (Zou et al. 2015) and *G. hirsutum* (Park et al. 2010).

Plant Materials and Water Deficit Treatments

The imposition of water deficit in *C. canephora* plants was performed according to Santos et al. (2015). In summary, 12 18-month-old plants from two *C. canephora* clones characterized as being water deficit-tolerant (clone 14: drought-tolerant) and clone 109A: drought-susceptible were used in this experiment (Lima et al. 2015; Marraccini et al. 2012). The leaf water status of coffee plants was monitored by thermocouple psychrometer chambers (model C-30, Wescor, Inc., Logan, UT, USA) coupled to a datalogger (Campbell Scientific, Inc., Logan, UT, USA, model CR-7). The stress conditions were the following: irrigated (control ± -1.35 MPa), moderate stress (± -2.35 MPa), severe stress (± -4.3 MPa), and recovery (rehydrated plants 72 h after reaching the water potential established as the severe stress). These samples were collected and frozen immediately in liquid nitrogen and kept at -80 °C until RNA extraction.

Total RNA Extraction and cDNA Synthesis

Total RNA extraction from *C. canephora* leaves was performed for the water deficit experiments using the PureLink RNA Reagent kit™ (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's recommendations. The integrity of total RNA was verified by electrophoretic separation on 1% (w/v) agarose gel. RNA concentration and purity were

determined using a NanoDrop™ Lite Spectrophotometer (Thermo Fisher Scientific). Five micrograms of total RNA was used to synthesize complementary DNA (cDNA) using the SuperScript IV Reverse Transcriptase™ (Invitrogen) in a final volume of 20 µl following the manufacturer's instructions. The cDNA products were diluted 1:10 before use in RT-qPCR.

Primer Amplification Efficiency and Relative Expression

Primers were designed outside of the conserved region for each *CcAQP* isoform using Primer Express™ v3.0.1 (Applied Biosystems). RT-qPCR reactions were performed using 5 µl of SYBR Green, 0.4 µl of sense and antisense primers (5 µM), 1 µl of cDNA samples, and a final volume of 10 µl with autoclaved Milli-Q water. The amplification reaction was performed with three biological replicates (plant in each condition) and three technical replicates using StepOnePlus™ Real-Time PCR System (Applied Biosystems) following the conditions: 40 cycles of denaturation at 95 °C for 2 min, annealing at 95 °C for 30 s, and

extension at 60 °C for 30 s. The efficiency of the amplification of each pair of primers was estimated using the LinReg PCR program (Ramakers et al. 2003). Relative expression quantification was calculated by the ΔC_t method (Pfaffl 2001) where expression level of target genes (*CcAQPs*) was normalized against the *CcUBQ10* as the reference gene (Marraccini et al. 2012). The significance in expression of the *CcAQPs* genes between the different water regimes for each clone was tested using one-way ANOVA followed by the Tukey test ($p < 0.05$). Paired comparisons between the two clones (14 and 109A) within each water regime were performed by the *t* test ($*p < 0.05$).

Results

Genome-Wide Identification of the *CcAQP* Gene Family and Phylogenetic Analysis

Thirty-three members of the AQP gene family were found in *C. canephora* genome database, which were grouped into five major clusters by the phylogenetic analysis (Fig. 1). The

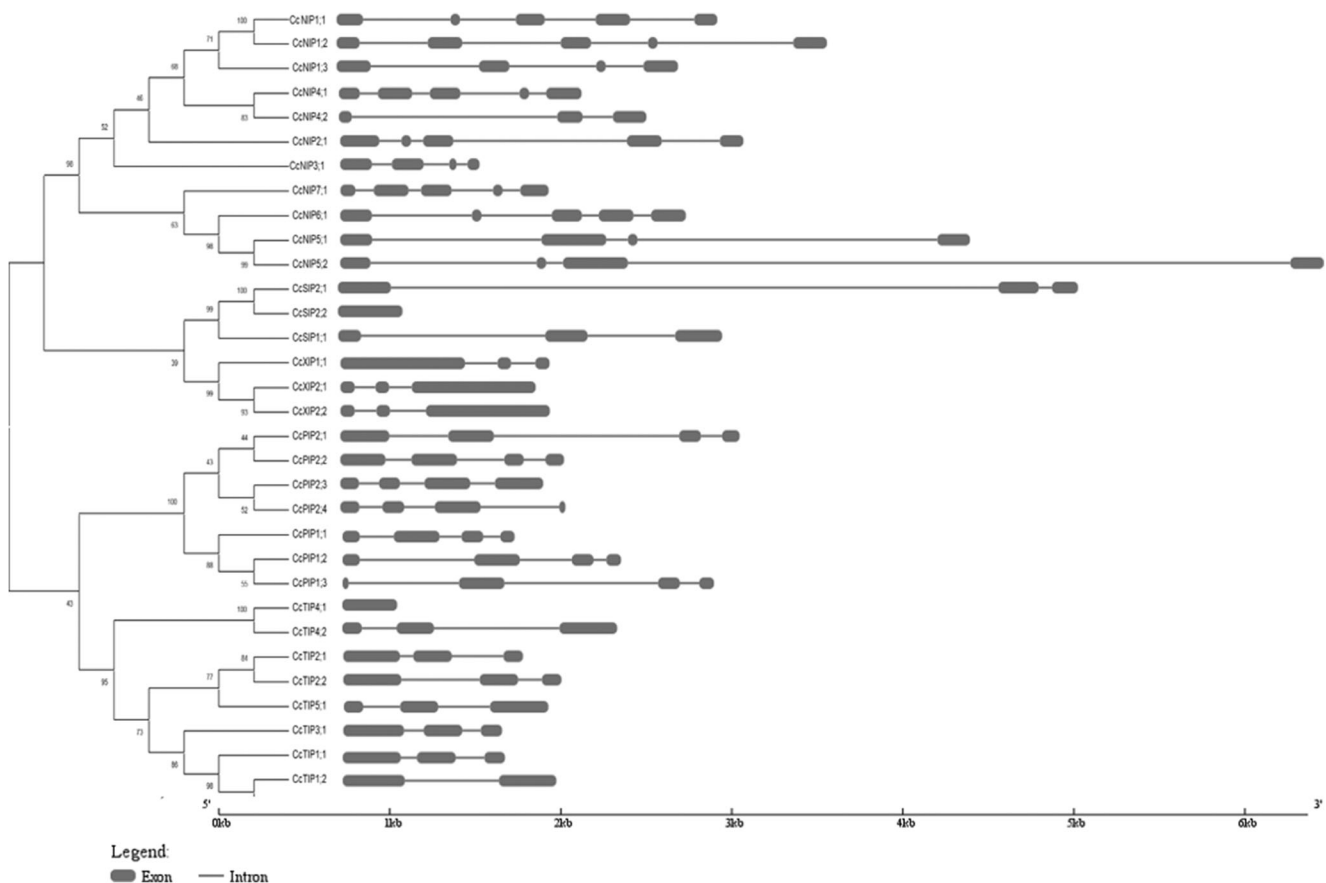


Fig. 1 Phylogenetic tree from *CcAQPs* of *C. canephora* generated by MEGAX using the ML method derived from the ClustalW alignment. Bootstrap analysis was performed with 1000 replicates; only bootstrap

values higher than 50% are indicated above the branches. AQP gene structure is represented by exons and introns as dark gray boxes and light gray lines, respectively

*CcAQP*s genes were named based on the similarity and phylogenetic relationship with *A. thaliana* as described by Johanson et al. (2001) (Fig. S1). To assess phylogenetic relationships among the *C. canephora* AQPs and to group them into the well-established subfamilies, a phylogenetic tree was generated based on multiple sequence alignments of *C. canephora*, *C. arabica*, *C. eugenioides*, *A. thaliana*, *S. lycopersicum*, *S. tuberosum*, and *O. sativa*. (Fig. 2). The 33 *CcAQP*s proteins were classified into five subfamilies: 7 PIPs, 9 TIPs, 11 NIPs, 3 SIPs, and XIPs. The subfamily XIP is not present in *Arabidopsis*. The XIP subfamily is characterized by the second NPA motif amplified to NPARC present only in this subfamily (Danielson and Johanson 2008). To verify the presence of this motif, the putative XIP sequences of *C. canephora*, *C. arabica*, and *C. eugenioides* were aligned, visually inspected, and compared with XIPs sequences from other dicotyledonous species including *R. communis* (Zou et al. 2015) and *G. hirsutum* (Park et al. 2010). All members of *CcPIPs* subfamily showed a conserved structure of four exons (Fig. 1). Most members of the NIP subfamily were characterized by four or five exons, except for *CcNIP4;2* which has only three exons. Most members of the TIP subfamily have three exons, while *CcTIP1;2* presents two exons and *CcTIP4;1* showing only one exon with 300 bp. The isoforms *CcSIP1;1* and *CcSIP2;1* contain three exons,

whereas *CcSIP2;2* has only one. The XIP subfamily presented a conserved three-exon structure (Fig. 1).

A detailed description of the physical and chemical analysis of *CcAQP*s is listed in Table 1. The deduced full-length amino acid sequence of the protein ranged from 119 to 328 amino acids. Molecular weight varied from 12.36 to 35.10 kDa, and the isoelectric point ranged from 5.12 to 9.66 pI. Most *CcAQP*s were predicted with six transmembrane domains (TMHs), while *CcNIP4;2* and *CcTIP4;1* contain only two transmembrane helices. Additionally, the amino acid sequence of the *CcAQP* indicates that they are located in the cell membrane, in the vacuole or, simultaneously, in both (Table 1).

Chromosomal Location, Duplication Events and Synteny Analysis of *CcAQP*s

CcAQP genes were mapped into 11 chromosomes of *C. canephora* (Fig. 3). Most of *CcAQP* genes were mapped on Chr02 (9); six on Chr08; three on Chr01, Chr04, and Chr07; two on Chr06 and Chr09; and only one *CcAQP* gene on Chr03 and Chr10. In contrast, no putative AQP was found in Chr05. Two *CcAQP* (*CcTIP3;1* and *CcSIP1;1*) were assigned to the unsorted sequence scaffolds denominated ChrUn (for “unknown”) (Fig. 3).

Fig. 2 Phylogenetic tree of *AQPs* including: *C. canephora*, *C. arabica*, *C. eugenioides*, *A. thaliana*, *S. lycopersicum*, *Oryza sativa*, and *S. tuberosum* generated by MEGAX using the ML method derived from the ClustalW alignment. Bootstrap analysis was performed with 1000 replicates; only bootstrap values higher than 50% are indicated above the branches. Different colors indicate different groups of *CcAQP* genes

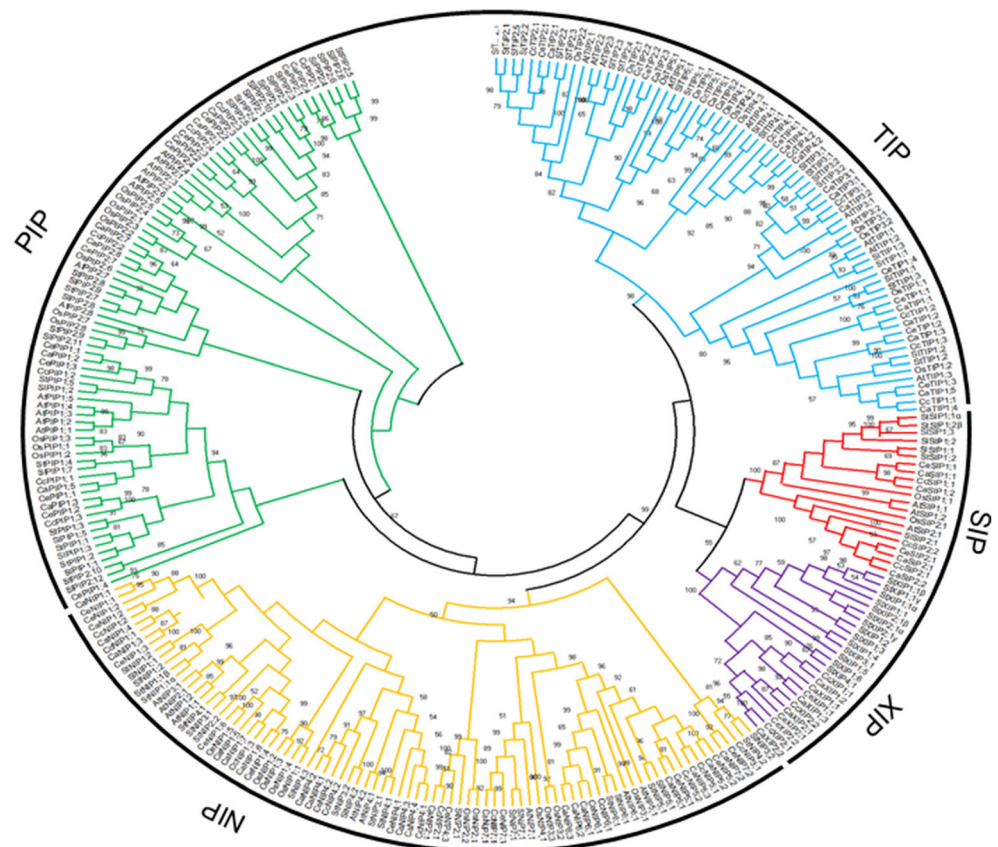


Table 1 AQPs genes identified in the *C. canephora* genome

<i>CcAQP</i> s ^a	Gene ID	Chromosome position	Protein length (aa)	Molecular weight (kDa)	TM helix ^b	pI ^c	Predicted subcellular localization
<i>CcNIP1;1</i>	Cc02_g15840	chr2:14277549..14280032	265	27.85	6	8.96	Cell membrane
<i>CcNIP1;2</i>	Cc02_g15820	chr2:14270763..14273962	284	29.91	6	8.96	Cell membrane
<i>CcNIP1;3</i>	Cc02_g15890	chr2:14390802..14393029	235	25.08	6	8.37	Cell membrane/vacuole
<i>CcNIP2;1</i>	Cc07_g03390	chr7:2365243..2367853	295	31.02	6	7.70	Cell membrane
<i>CcNIP3;1</i>	Cc10_g07150	chr10:6073710..6074611	178	19.32	4	7.67	Cell membrane/vacuole
<i>CcNIP4;1</i>	Cc07_g08140	chr7:5983226..5984796	281	30.35	7	5.73	Cell membrane
<i>CcNIP4;2</i>	Cc11_g08690	chr11:26075747..26077737	154	17.23	2	5.18	Cell membrane
<i>CcNIP5;1</i>	Cc04_g15640	chr4:25353055..25357132	299	30.56	5	9.10	Cell membrane
<i>CcNIP5;2</i>	Cc08_g17120	chr8:31492021..31498389	297	30.61	5	8.88	Cell membrane
<i>CcNIP6;1</i>	Cc04_g04990	chr4:3713193..3715430	303	30.96	6	7.75	Cell membrane
<i>CcNIP7;1</i>	Cc03_g03200	chr3:2417270..2418614	254	26.39	6	8.72	Cell membrane
<i>CcPIP1;1</i>	Cc02_g06820	chr2:5399077..5400193	213	22.44	5	9.66	Cell membrane/vacuole
<i>CcPIP1;2</i>	Cc02_g38120	chr2:52874143..52875950	250	22.53	5	9.57	Cell membrane/vacuole
<i>CcPIP1;3</i>	Cc08_g14850	chr8:29720914..29723324	190	20.38	5	9.51	Cell membrane
<i>CcPIP2;1</i>	Cc01_g11110	chr1:29854916..29857498	288	30.75	6	7.73	Cell membrane
<i>CcPIP2;2</i>	Cc09_g09880	chr9:20778382..20779828	277	29.62	6	9.13	Cell membrane
<i>CcPIP2;3</i>	Cc06_g03900	chr6:3002393..3003704	287	30.57	6	8.20	Cell membrane
<i>CcPIP2;4</i>	Cc09_g02170	chr9:1811779..1813234	199	21.08	5	9.51	Cell membrane
<i>CcSIP1;1</i>	Cc00_g09500	chrUn:81327025..81329499	240	25.41	5	9.48	Cell membrane/vacuole
<i>CcSIP2;1</i>	Cc06_g22730	chr6:35700588..35705369	256	28.15	6	9.34	Cell membrane
<i>CcSIP2;2</i>	Cc08_g05530	chr8:10009003..10009419	138	15.83	4	9.46	Cell membrane
<i>CcTIP1;1</i>	Cc02_g08360	chr2:6693536..6694585	252	25.65	7	5.78	Vacuole
<i>CcTIP1;2</i>	Cc01_g10300	chr1:28971849..28973231	257	26.53	6	6.28	Vacuole
<i>CcTIP1;3</i>	Cc01_g12260	chr1:30940570..30941579	251	25.92	6	5.35	Vacuole
<i>CcTIP2;1</i>	Cc04_g02840	chr4:2193273..2194443	248	24.95	7	6.48	Vacuole
<i>CcTIP2;2</i>	Cc08_g12280	chr8:27602314..27603735	251	25.64	7	5.12	Vacuole
<i>CcTIP3;1</i>	Cc00_g01530	chrUn:10099611..10100645	261	27.82	6	6.22	Vacuole
<i>CcTIP4;1</i>	Cc07_g20570	chr7:23586574..23586930	118	12.36	2	7.00	Vacuole
<i>CcTIP4;2</i>	Cc08_g09400	chr8:24159544..24161331	247	25.78	6	6.02	Vacuole
<i>CcTIP5;1</i>	Cc02_g04340	chr2:3431090..3432414	249	25.31	6	7.93	Cell membrane/vacuole
<i>CcXIP1;1</i>	Cc08_g04450	chr8:6507004..6508354	327	35.23	6	6.54	Cell membrane
<i>CcXIP2;1</i>	Cc02_g16640	chr2:15342830..15344090	326	35.11	7	6.30	Cell membrane
<i>CcXIP2;2</i>	Cc02_g16660	chr2:15356931..15358283	326	35.03	7	5.63	Cell membrane

^a Aquaporin from *C. canephora*^b Isoelectric point^c Transmembrane helix

We also estimated the non-synonymous (*Ka*) and synonymous (*Ks*) substitution ratios to verify whether selection pressure participated in the expansion of *CcAQP* genes. Positive selection in duplication and divergence was calculated by estimating the ratios of non-synonymous (*Ka*), synonymous (*Ks*), and substitution rates (*Ka/Ks*) for five tandem and 28 segmental duplicated gene pairs of *CcAQP* (Supplementary Table S1). The results suggest segmental duplication events

of the *CcAQP* genes in *C. canephora* from 0.74 mya (million years ago) (*Ks* = 0.02) to 10.91 mya (*Ks* = 0.27), whereas tandem duplicated gene pairs ranged from 1.67 (*Ks* = 0.24) to 9.08 mya (*Ks* = 0.24).

AQP gene synteny among the three coffee species (*C. canephora*, *C. eugenioides*, and *C. arabica*) revealed the presence of 67 syntenic relationships being (4) SIPs, (20) NIPs, (14) TIPs, (12) PIPs, and (17) XIPs (Fig. 4 and

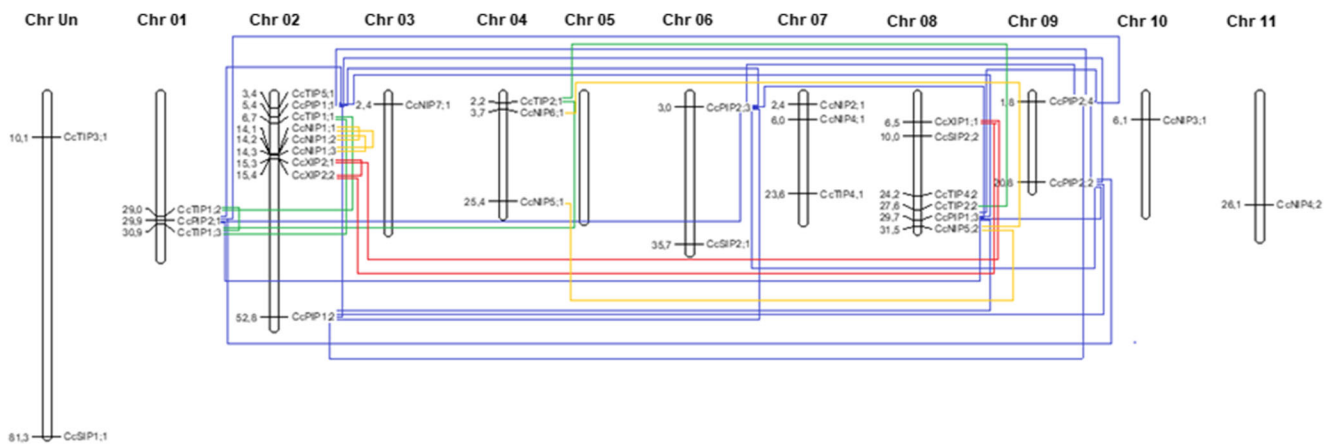


Fig. 3 Distribution of *CcAQP* genes into the 11 *C. canephora* chromosomes. Vertical bars represent the chromosomes and numbers at the left indicate gene positions (the scale on the left is in megabases, Mb). The chromosome number is indicated on the top of each chromosome (vertical bar, numbered Chr01–Chr11). The chromosome designated as ChrUn is not a true chromosome, but unsorted sequence scaffolds as

described by Denoëud et al. (2014). Blue lines are indicative of duplication events for *CcPIP* genes; green lines are indicative of duplication events for the *CcTIP* genes; red lines are indicative of duplication events for *CcXIP* genes and yellow lines are indicative of duplication events for *CcNIP* genes

Table S2). Most *CcAQP*s isoforms had a linear syntentic relationship with the AQP's in *C. arabica* and *C. eugenioides* genomes with exception *CcSIP2;1*, *CcTIP1;1*, *CcTIP4;1*, *CcNIP2;1*, *CcNIP4;1*, *CcXIP1;1*, and *CcXIP2;1* that were syntentic with the correspondent subfamily but on different chromosomes (Table S2). On the other hand, when the analysis was performed between *C. canephora* × *C. arabica*, it revealed 55 synthetic events (Table S3) and 39 between *C. canephora* × *C. eugenioides* (Table S4). From 33 *CcAQP*s identified in this work, 31 (93.93%) of them were syntentic with *CeAQP*s and *CaAQP*s (Table S3 and S4). The isoforms *CcSIP2;2* and *CcNIP3;1* showed no gene correspondence with any AQP in *C. eugenioides* or *C. arabica* (Table S2). Furthermore, the NIP family presented the largest number of syntentic events, 18 (*C. canephora* × *C. arabica*) (Table S3) and 13 (*C. canephora* and *C. eugenioides*) (Table S4). We identified that some syntentic AQP gene pairs shared the same *C. canephora* AQP member associated with *C. eugenioides* and *C. arabica*, suggesting that they may originate from a common ancestor.

The isoforms *CcTIP3;1* and *CcSIP1;1* have not yet been positioned on available *C. canephora* genome assembly v.01 and consequently were mapped on chromosome ChrUn (Fig. 3). Our synteny analysis revealed that the *CcTIP3;1* was syntentic with *CeTIP3;1*, *CaTIP3;1* and *CaTIP3;2* localized on chromosome 4, and the *CcSIP1;1* isoform was syntentic with *CeSIP1;1*, *CaSIP1;1*, and *CaSIP1;2* localized on chromosome 6 of each genome (Table S2). Therefore, we suggested that these isoforms, the *CcTIP3;1* and *CcSIP1;1* isoforms, could possibly be positioned in chromosome 4 and 6 of *C. canephora*, respectively.

described by Denoëud et al. (2014). Blue lines are indicative of duplication events for *CcPIP* genes; green lines are indicative of duplication events for the *CcTIP* genes; red lines are indicative of duplication events for *CcXIP* genes and yellow lines are indicative of duplication events for *CcNIP* genes

Motif Prediction

Fifteen conserved motifs were predicted as shown in Fig. 5. The motifs 1, 2, and 3 were widely found in the five subfamilies. The motif 4 is present in the PIP, TIP, and XIP subfamilies, and in four NIP members. Motifs 5, 6, and 7 are found exclusively in the XIP, NIP, and PIP subfamilies respectively. Motif 8 is widely present in the PIP, XIP, and NIP subfamilies. Motive 9 is widely present in the PIP and TIP subfamilies, while motif 10 is limited to the PIP subfamily (Fig. 5).

Protein Sequences Analysis

The amino acid sequences of the *CcAQP*s were aligned, and the transmembrane domains (TMHs), NPA motifs, air/R selectivity filter, and Froger's position were visually inspected to gain some insight into the physiological role and substrate specificity of each aquaporin (Table 2 and Fig. S3). The transmembrane domain number varied from 2 (*CcTIP4;1* and *CcNIP4;2*) to 7 (*CcTIP1;1*, 2;1, 2;2, *CcNIP4;1*, *CcXIP2;1*, and 2;2), with the majority of *CcAQP*s (16) presenting six TMHs (Fig. S3).

Analysis of amino acid residues (Table 2) revealed the conserved NPA motif in the first loop (LB) of all *CcNIP*s, *PIP*s, and *TIP*s. In the three *CcXIP*, the residue A (arginine) in the third position was replaced by the isoleucine (I). In the *CcSIP*s, the third position residue A (arginine) was replaced by threonine (T) for *CcSIP1;1* and a leucine (L) for *CcSIP2;1* and 2;2. The second NPA motif is conserved in *PIP*, *SIP*, *XIP*, and *TIP* subfamilies with the exception of *CcTIP4;1* in which it is absent. On the other hand, *CcNIP* subfamily showed

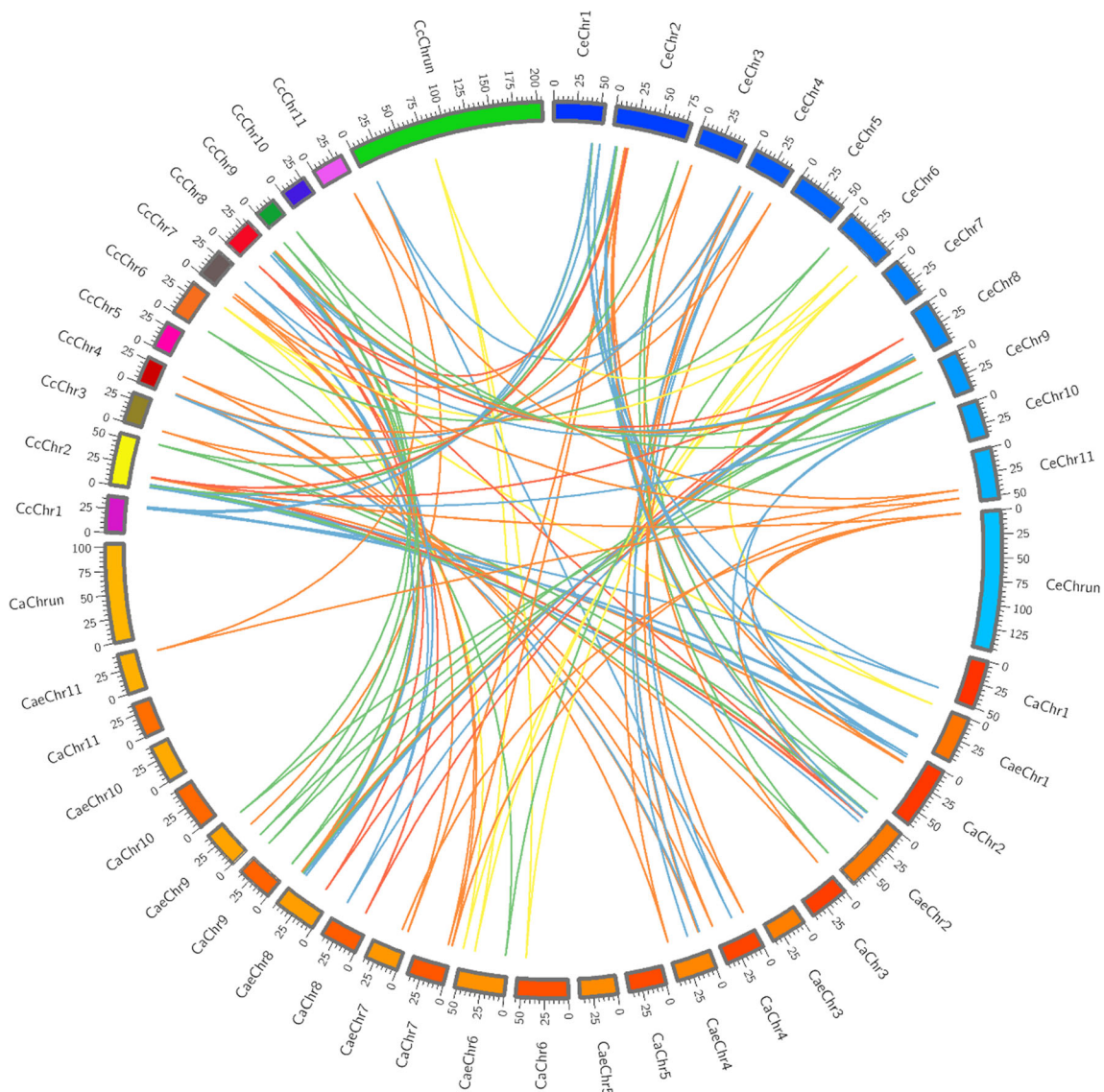


Fig. 4 Synteny analysis for AQP genes among *C. canephora*, *C. eugenioides*, and *C. arabica*. CcChr1 to CcChr11 represented the 11 chromosomes in *C. canephora*. CeChr1 to CeChr11 represented the 11 chromosomes in *C. eugenioides*. CacChr1 to CacChr11 and CaeChr1 to CaeChr11 represented the 11 chromosomes in the *C. canephora* (Ca^s)

and *C. eugenioides* (Ca^s) sub-genomes in *C. arabica*, respectively. The orthologous AQP genes were mapped onto chromosomes and linked to each other. Different colored lines in the interior represent different sub-families of AQP genes

greater diversity on the second NPA motif.

The amino acid residues on the selectivity filter (ar/R) revealed that the CcNIPs and CcTIP were highly variable compared with other families (Tables 2). In CcSIPs, the selectivity filters were different from each other with CcSIP2;2 only presenting the residue K on H2. On the other hand, CcPIPs were highly conserved (F-H-T-R), whereas CcXIPs presented variation only in H2 (V/L-F-A-R) (Table 2). Froger's positions were highly conserved within CcXIPs, CcPIPs, and CcTIPs, with the only exceptions being CcPIP1;3 and CcTIP4;1 (Table 2). The positions P1, P2, and P5 were highly variable

in CcNIPs, whereas the P3 and P4 were conserved. CcSIP1;1 and 2;1 presented variation in the first two positions, while CcSIP2;2 showed residues only in P4 (E) and P5 (F) (Table 2).

Expression Analyses of CcAQP Genes in Different Tissues

The in silico expression patterns of the different members of the CcAQP gene family in *C. canephora* was determined using RNAseq data generated from root, stamen, pistil, leaf,

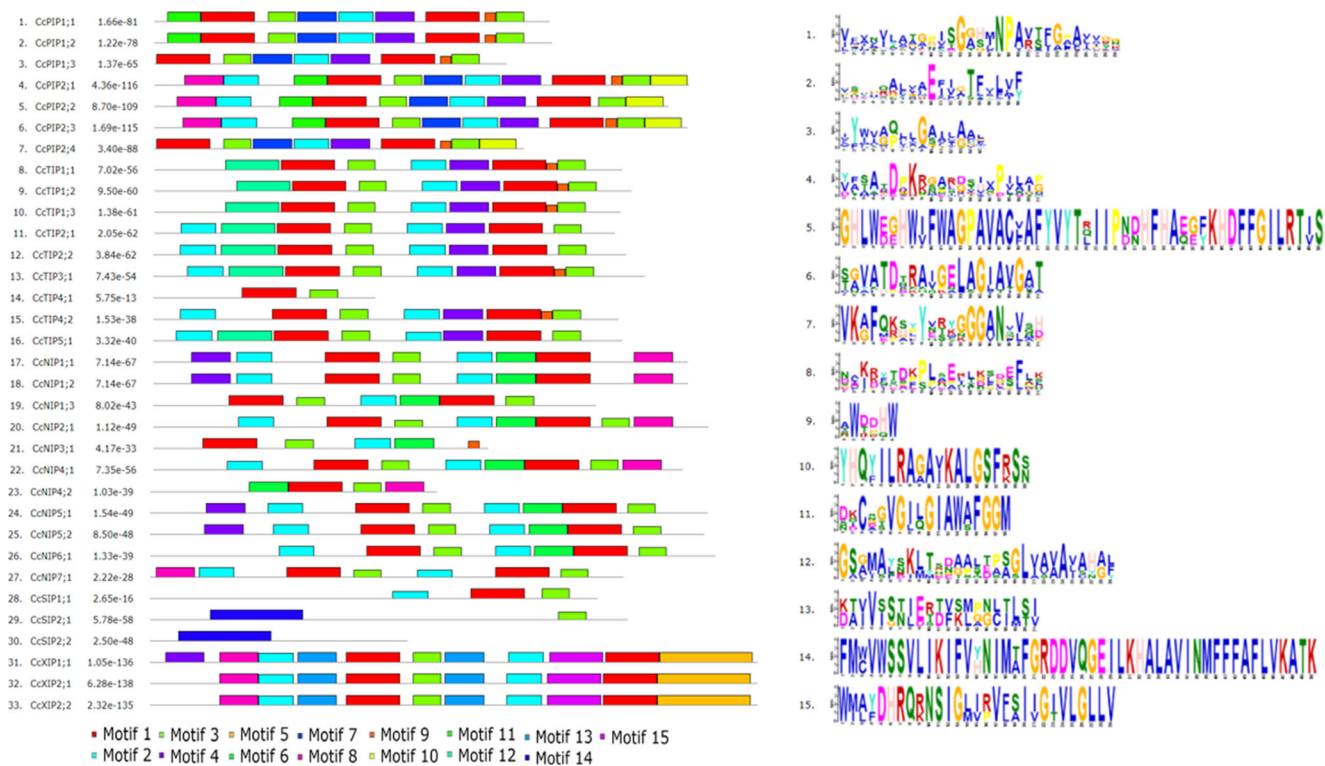


Fig. 5 Motifs conserved among the CcAQPs proteins, where each motif is represented by different color blocks as indicated at the bottom of the figure. The same color block on different proteins indicates each particular motif

perisperm, and endosperm libraries (Denoëud et al. 2014). The RPKM values of each putative gene were displayed in a heatmap (Fig. 6) to infer their transcriptional profile in the different tissues. In summary, the genes *CcPIP1;3*, *2;2*, *2;4*, and *CcTIP1;2* and *2;2* are highly expressed in all tissues, while most *CcXIPs* and *CcNIPs* show low expression levels with the exception of *CcXIP2;1* (pistils) and *CcNIP5;1* (roots), *CcNIP4;1* (stamens), and *CcNIP1;7* (perisperm). Other isoforms presented a specific pattern of expression according to the tissue analyzed, e.g., *CcTIP1;1*, *2;2*, and *5;1* showed high expression only in roots, whereas *CcTIP3;1* and *CcNIP4;1* also presented noticeable expression in stamens (Fig. 6).

Relative Gene Expression for Selected *CcPIPs* and *CcTIPs* in *C. canephora* Leaves Under Water Deficit

A total of five *CcAQPs* with high RPKM values in leaves (*CcPIP1;2*, *2;3*, and *2;4* and *CcTIP1;2* and *2;1*) were selected randomly for further RT-qPCR analysis. The dissociation curve for each primer confirmed the presence of single amplifications. Primer sequences, amplicon size, and amplification efficiency are shown in Table S5. Relative expression of each *CcAQP* was normalized against the expression of *CcUB10* ubiquitin gene (Marraccini et al. 2012).

In the leaves of plants submitted to water deficit, the relative expressions of the *CcPIP2;3* and *CcPIP2;4* isoforms were significantly higher in clone 109A compared with clone 14 under moderate and severe stress. The expression levels of the isoform *CcPIP1;2* also increased in clone 109A in severe stress and after re-irrigation (~1.5-fold) in relation to clone 14 (Fig. 7a). The expression of *CcPIP2;3* and *2;4* increased ~2.3 and 1.5-fold in plants under moderate stress and ~2 and 3.6-fold at the severe stress condition, respectively (Fig. 7b and c). The transcript abundance of *CcPIP2;4* was lower than the observed for the other PIP isoforms; however, difference in the expression level between the susceptible (clone 109A) and the tolerant genotypes (clone 14) was greater (Fig. 7c). Clone 109A also showed a significant increase in relative expression of the two *CcTIPs* isoforms (*1;2* and *2;1*) when the plants were subjected to severe water deficit and also in the re-irrigated treatment for *CcTIP1;2* (Fig. 7d and e).

Discussion

Aquaporins (AQPs) are water channel proteins that facilitate the rapid transport of water and small molecules across plant cell membranes (Maurel et al. 2015). In this study, we identified 33 AQPs in the *C. canephora* genome. The sequences

Table 2 Amino acid composition of the NPA motifs, ar/R selectivity filters (H2, H5, LE1, LE2) and Froger's positions (P1–P5) in *C. canephora* aquaporins

Subfamily	Gene	NPA ^a		ar/R selectivity filters ^b				Froger's positions ^c				
		LB	LE	H2	H5	LE1	LE2	P1	P2	P3	P4	P5
NIP	CcNIP1;1	NPA	NPA	W	V	A	R	F	S	A	Y	I
	CcNIP1;2	NPA	NPA	W	V	A	R	F	S	A	Y	I
	CcNIP1;3	NPA	NPA	W	S	A	R	F	S	A	Y	I
	CcNIP2;1	NPA	NPA	G	S	G	R	L	S	A	Y	V
	CcNIP3;1	NPA	NVI	W	S	-	R	F	F	A	Y	-
	CcNIP4;1	NPA	NPA	W	V	A	R	F	S	A	Y	I
	CcNIP4;2	NPA	NPA	-	V	A	R	-	T	A	Y	I
	CcNIP5;1	NPA	NPV	A	I	G	R	F	T	A	Y	L
	CcNIP5;2	NPA	NPV	A	I	G	R	F	T	A	Y	L
	CcNIP6;1	NPA	NPV	T	I	A	R	F	T	A	Y	L
	CcNIP7;1	NPA	NPA	A	V	A	C	Y	S	A	Y	L
PIP	CcPIP1;1	NPA	NPA	F	H	T	R	Q	S	A	F	W
	CcPIP1;2	NPA	NPA	F	H	T	R	Q	S	A	F	W
	CcPIP1;3	NPA	NPA	-	H	T	R	E	S	A	F	W
	CcPIP2;1	NPA	NPA	F	H	T	R	Q	S	A	F	W
	CcPIP2;2	NPA	NPA	F	H	T	R	Q	S	A	F	W
	CcPIP2;3	NPA	NPA	F	H	T	R	Q	S	A	F	W
	CcPIP2;4	NPA	NPA	-	H	T	R	Q	S	A	F	W
SIP	CcSIP1;1	NPT	NPA	T	V	P	N	M	A	A	Y	W
	CcSIP2;1	NPL	NPA	K	H	G	S	F	V	A	Y	W
	CcSIP2;2	NPL	NPA	K	-	-	-	-	-	-	E	L
TIP	CcTIP1;1	NPA	NPA	H	I	A	V	T	S	A	Y	W
	CcTIP1;2	NPA	NPA	Y	I	A	V	T	S	A	Y	W
	CcTIP1;3	NPA	NPA	H	I	A	V	T	S	A	Y	W
	CcTIP2;1	NPA	NPA	H	I	G	R	T	S	A	Y	W
	CcTIP2;2	NPA	NPA	H	I	G	R	T	S	A	Y	W
	CcTIP3;1	NPA	NPA	H	I	A	R	T	S	A	Y	W
	CcTIP4;1	NPA	-	H	-	A	-	T	S	A	L	Q
	CcTIP4;2	NPA	NPA	H	I	A	R	T	S	A	Y	W
	CcTIP5;1	NPA	NPA	N	I	G	Y	T	S	A	Y	W
XIP	CcXIP1;1	NPI	NPA	V	F	A	R	C	C	A	F	W
	CcXIP2;1	NPI	NPA	L	F	A	R	C	C	A	F	W
	CcXIP2;2	NPI	NPA	L	F	A	R	C	C	A	F	W

^a Asparagine-proline-alanine motifs in the loops B (LB) and E (LE)

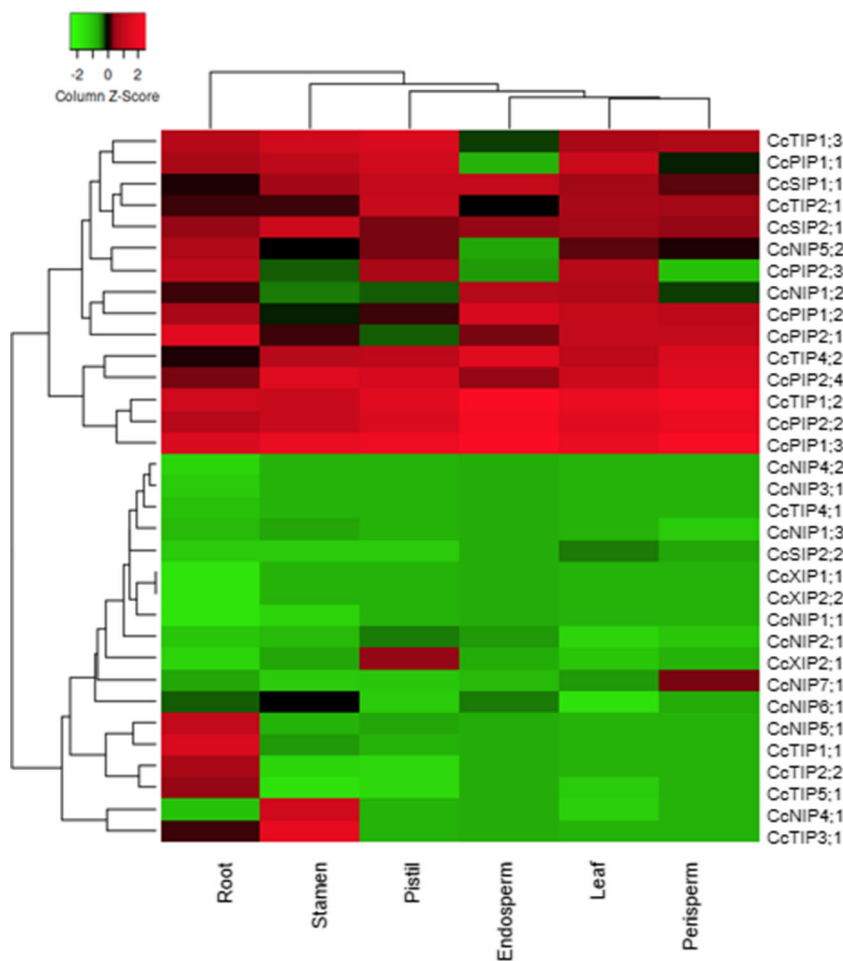
^b Aromatic/arginine selectivity filter formed by four residues in helix 2 (H2), helix 5 (H5), and two in loop E (LE1–LE2)

^c Five key positions in the protein sequence (P1–P5) associated with function and specific physicochemical properties for each subgroup

were grouped into five subfamilies: 7 PIPs, 9 TIPs, 11 NIPs, 3 SIPs, and 3 XIPs. Previous studies have reported that similar numbers of AQPs were found in *Arabidopsis* (35) and rice (33). The AQPs were classified into only four subfamilies in this species: 13 PIPs, 10 TIPs, 9 NIPs, and 3 SIPs in *Arabidopsis* (Johanson et al. 2001) and 10 NIPs, 10 TIPs, 11 PIPs, and 2 SIPs in rice (Sakurai et al. 2005). The XIP subfamily, characterized by the presence of the second motif

NPA extend to NPARC (Danielson and Johanson 2008), is absent in monocotyledons and in members of the Brassica family (Bari et al. 2018; Sun et al. 2018), as well as in *Medicago truncatula* and *Lotus japonicus* (Deshmukh and Bélanger 2016). Here, we also identified a total of 52 APQs in *C. arabica* and 37 in *C. eugenioides* that were classified into five subfamilies (Figs. S5 and S6). The larger number of AQPs found in *C. arabica* may be presumably due to their

Fig. 6 Heatmap of transcription profiling of *CcAQPs* genes in various tissues (root, stamen, pistil, leaf, perisperm, and endosperm). The heatmap was generated based on the RNA-seq (RPKM) datasets available at <http://coffee-genome.org>; Denoeud et al. 2014. Green indicates tissues with low expression and red indicates high expression



ploidy level since it is a tetraploid species originated by natural hybridization between the diploids *C. canephora* and *C. eugenioides* (Lashermes et al. 1999).

Further analysis of the *CcAQPs* genes revealed that segmental duplication (28) played a more prominent role in the expansion of *CcAQPs* genes than tandem duplication (5). According to Cannon et al. (2004), segmental duplications may have higher occurrence in plants due to polyploidization events, leading to a large accumulation of duplicated chromosomal blocks in the genome. On the other hand, tandem duplications can provide important mechanisms to adaptive resistance genes (Flagel and Wendel 2009). Some studies have reported that such events contribute to enhanced stress tolerance of plants (Fukao et al. 2006; Zou et al. 2009).

Comparison of intron and exon arrangements (number and position) between the 33 *CcAQPs* revealed wide variation in gene structure as observed in other plants, such as in *Linum usitatissimum* (Shivaraj et al. 2017), *R. communis* (Zou et al. 2015), and *Beta vulgaris* (Kong et al. 2017). The sequences *CcNIP5:1*, *CcTIP3:1*, and *CcXIP1:1* and *1:2* present molecular masses lower than

20 kDa, which has not been observed in other species (Zou et al. 2015; Kong et al. 2017). Regarding the transmembrane prediction, most of them showed the typical six transmembrane helix domains (Fig. S4). However, two transmembrane proteins were absent in sequences of *CcNIP3:1*, *CcNIP4:2*, *CcTIP4:1*, and *CcSIP2:2*. Further studies may be necessary to assess if the lack of these domains is responsible for their low expression in all tissues as detected in silico (Fig. 6).

In addition, the data from the in silico analysis showed that *CcNIP* genes have low level of expression and tissue type and/or cell specificity, as was also observed in other species (Liu et al. 2009; Alexandersson et al. 2010). *CcNIP5:1* and *CcNIP5:2* were specifically expressed in roots, *NIP1:2* in leaves and endosperm, and *NIP4:1* in stamens. Previous studies in *Arabidopsis* showed the specificity of *NIP5:1* in roots (Takano et al. 2006), while *NIP6:1* was expressed specifically within the vascular tissue from phloem of developing young shoots (Tanaka et al. 2008) and *NIP7:1* in floral tissues (Li et al. 2011). Regarding the *CcXIPs*, which have been reported to have very low participation in water transport (Bienert et al. 2011), the in silico expression analysis revealed low

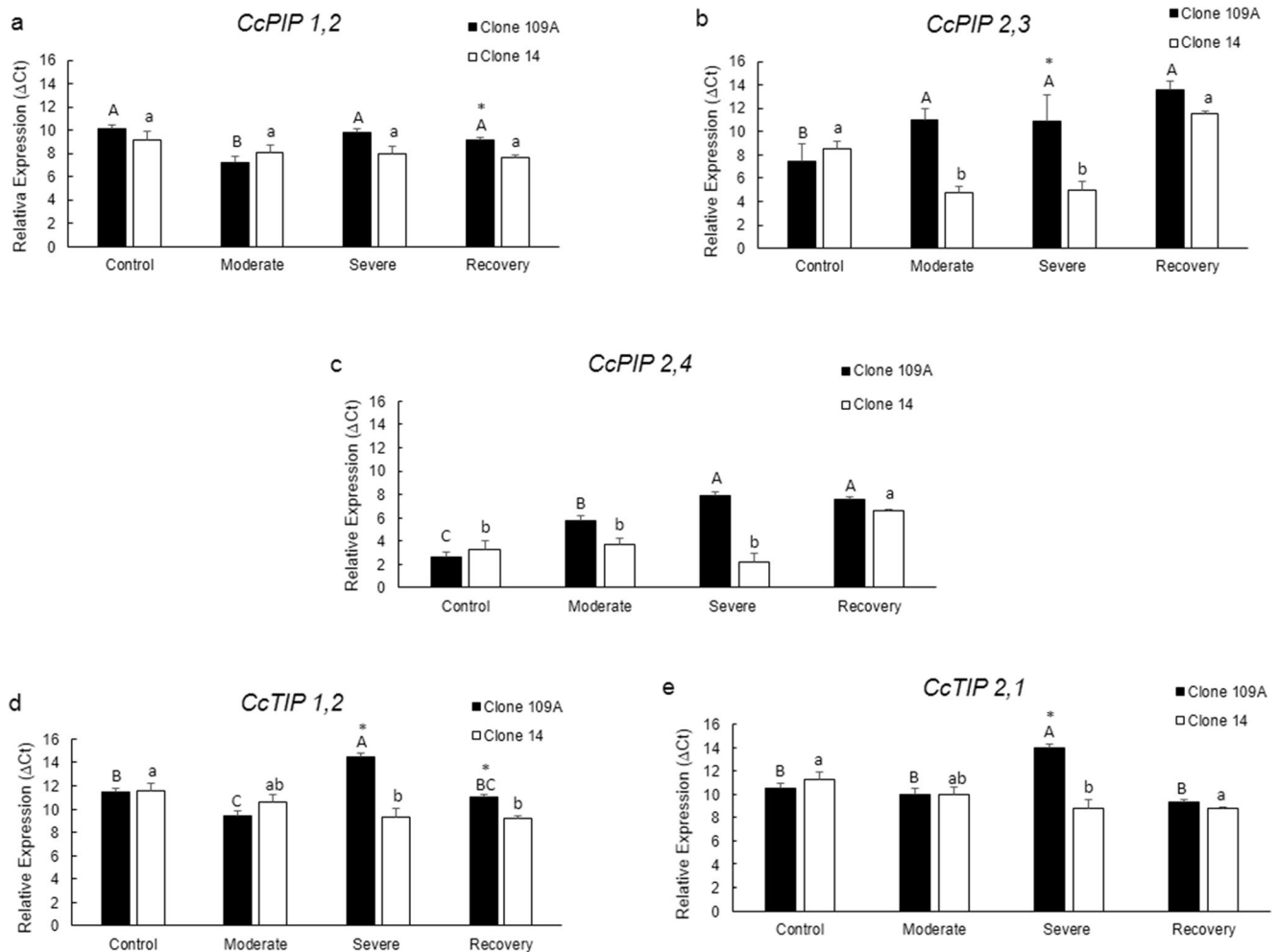


Fig. 7 Relative expression of *AQP* genes in *C. canephora* leaves under different irrigation conditions. The expression of the target genes was calculated by ΔC_t method using ubiquitin gene as internal reference. The values represent the mean (\pm SD) of three biological replicates for

each treatment. Upper- and lower-case letters indicate comparisons between the different water regimes for clone 109A and 14, respectively (Tukey's, $p < 0.05$). A paired comparison between the clones in each water regime was performed by t test ($*p < 0.05$)

abundance of the three isoforms, with the only exception of *CcXIP2;1* in pistil. The *CcPIPs* and *CcTIPs* genes showed the highest mRNA expression in silico; however, it is noteworthy to mention that *PIP1;3*, *2;2*, and *TIPs 1;2* were constitutively expressed in all tissues, in contrast to the tissue a relative specificity found for other isoforms. Members of *PIPs* and *TIPs* of *C. arbutum* were also highly expressed in different tissues and are regarded as constitutive transporters (Deokar and Tar'an 2016).

AQPs genes were highly syntenic among the three coffee species. We suggested that this fact is due to the evolutionary process. The *C. canephora* and *C. eugenioides* species diverged from a common diploid ancestral ~ 4.2 mya (Yu et al. 2011). This was evident when comparing the number of orthologous genes between these two species, circa 93% from 33 *AQPs* genes identified in *C. canephora* were syntenic with *C. eugenioides*. Only two genes, *CcSIP2;2* and *CcNIP3;1*, did not present syntenic events with other coffee species,

suggesting that these genes originated after the divergence from the common diploid ancestral or were lost during the evolutionary process. It is also worth to mention that these isoforms presented only four THMs and low in silico expression. The high number of syntenic events (55) between *C. arabica* and *C. canephora* is also likely the result of their evolutionary history. The tetraploid species (*C. arabica*) consists of two sub-genomes, and it is believed that this species appeared approximately 0.666 mya through natural hybridization between *C. eugenioides* and *C. canephora* (Yu et al. 2011).

AQPs in plants play a role in water permeability function and facilitate the transport of some small neutral molecules such as glycerol, boric acid, silicic acid, urea, NH_3 , CO_2 , and H_2O_2 (Li et al. 2014; Afzal et al. 2016). Therefore, the predicted *CcAQPs* sequences were visually inspected for the presence of characteristic and functionally important domains and motifs of the *AQP* subfamilies. NPA double motifs, ar/R selectivity filter, and Froger's positions are closely related to

the substrate specificity of AQP proteins due to atomic resolution and molecular stimulus. The duplication of NPA motif in protein is considered a basic feature of MIPS, but exceptions to this rule have already been identified in plant NIPs and SIPs (Forrest and Bhavé 2007; Danielson and Johanson 2008; Deokar and Tar'an 2016). In *C. canephora*, the third position of the first NPA motif was replaced by (T/L) in all CcSIPs members, whereas in CcNIP, only a few members showed the replacement (V/I) in the second NPA motif. However, incomplete NPA conserved motifs seems that they do not affect the functions of these proteins as water channels (Johanson and Gustavsson 2002; Yakata et al. 2007) and may be a consequence of adaptive responses to changing environments (Liu and Zhu 2010).

NPA consensus sequences, SGXHXNPAVT (Heymann and Engel 2000) and (GXXXNPAR(S/D)XG) (Zardoya and Villalba 2001), were observed in all CcPIPs, CcTIPs, and CcNIPs. Notably, the first NPA motif is markedly conserved in all NIP, TIP, and PIP isoforms in *C. canephora*, while the second NPA motif is variant in NIPs where it can be replaced by NVI or NPV. These replacements on NIP sequences are considered a signature for boron uptake (Hove and Bhavé 2011).

The ar/R selectively filters in CcPIPs were highly conserved, with the exception of CcPIP1;3 and 2;4 in which the first residue (F) was absent. PIPs are known to be excellent water channels with their selectivity filters and amino acid composition remaining invariable as a result of strong selective pressure (Hooijmaijers et al. 2012), with each amino acid discrepancy being considered a selective disadvantage for the plant (Diehn et al. 2015). The ar/R selectively filters for the CcTIP subfamily members were more variable, which may reflect the different solutes transported by these genes, such as urea (Liu et al. 2003), NH₃ (Loqué et al. 2005), and H₂O₂ (Bienert et al. 2007). The signatures SGGH(V/L/F)NPAVT, G(G/A)SMNPARS(F/L)G, and the residues TSAYW were observed in all CcTIPs and are related to NH₃ uptake and water selectivity, while the signature (T/Q/F-A/S-A-YF-W/I) at P1–P5 Froger's positions present in the most CcPIPs and CcTIPs are related to H₂O₂ transporter (Hove and Bhavé 2011). CcPIPs and CcTIPs also showed the residues SAFW at P2–P5 Froger's positions that are signatures for CO₂ and non-metal uptake (Hove and Bhavé 2011).

Plant NIPs are responsible for the transport, absorption, and translocation of various uncharged metalloids (Bienert and Bienert 2017). In plants, NIP subfamily is composed of three subgroups NIP1, NIP2, and NIP3, which differ in their selectivity to the substrate (Hove and Bhavé 2011). The residues GSGR on ar/R selectivity filter and LTAYF on the Froger's positions are signatures for silicon transporter (Hove and Bhavé 2011). Although the CcNIP2;1 presented the conserved GSGR on the ar/R selectivity filter, the motif on the Froger's positions was slightly different (LSAYV). Most CcNIPs showed the residues (FSAYI [L/V]) for the Froger's

positions related to urea and H₂O₂ transporter (Hove and Bhavé 2011).

All members of the CcXIP subfamily showed the NPI motif in the first loop (LB), while the second motif (NPA) in the loop LE was expanded to NPARC (Reuscher et al. 2013; Danielson and Johanson 2008). Despite these variations, Asn and Pro residues remain perfectly conserved, maintaining the aqueous pore stabilization and water permeation functions (Murata et al. 2000). Regarding ar/R filters, XIP in eudicotyledonous plants can be divided into four subclasses (Gupta and Sankaramakrishnan 2009), two of which have a similar ar/R signature on some plant NIPs, whereas two others are more hydrophobic. In *C. canephora*, all XIPs presented the hydrophobic residues V/LFAR, suggesting that they do not work as water channels but as transporters of more bulky and hydrophobic solutes such as urea, glycerol, and metalloids (Bienert et al. 2011). No XIP sequences of *C. arabica* and *C. eugeniodes* were found in the NCBI database, probably due to the sequences that are usually annotated based on the *Arabidopsis* genome in which this subfamily is absent. All putative XIP sequences on the three coffee species possess the second NPA motif amplified to NPARC that is the typical signature of XIP sub family (Fig. S2).

The SIPs in *C. canephora* were also highly variable and do not present a distinctive signature in the ar/R selectivity filter. In contrast, the residues at P3 to P5 (AYW) in Froger's positions were conserved in CcSIP1;1 and CcSIP2;1. These positions are highly conserved in other plants such as *Ricinus communis* (Zou et al. 2015), *Cicer arantimum* L (Deokar and Tar'an 2016), *Linum usitatissimum* (Shivaraj et al. 2017), *Sorghum bicolor* (Kadam et al. 2017), and *Citrus sinensis* (Wei et al. 2019).

Finally, we analyzed the transcriptional profiles of five aquaporin isoforms (3 CcPIPs and 2 CcTIPs) in leaves of two contrasting *C. canephora* genotypes (clones 14 and 109A, characterized as tolerant and susceptible to water deficit, respectively) (Marraccini et al. 2012; Lima et al. 2015) subjected to water deficit. Severe water deficit significantly affected RNA transcription accumulation in all five isoforms in both clones. In that condition, clone 109A showed increased expression for all isoforms while the expression in CcPIP2;3, CcTIP1;2, and CcTIP2;1 was reduced in clone 14. Similar findings were observed in two contrasting genotypes of common bean under water deficit, where isoforms of PIPs and TIPs showed lower gene expression levels in the drought-tolerant genotype (Zupin et al. 2017). In chickpea, *Cicer arietinum*, different isoforms of PIP showed contrast expression patterns. CaPIP2;4 increased in tolerant and decreased in susceptible genotypes, whereas CaPIP2;1 expression increased in susceptible genotype (Hussain et al. 2019). In *Chrysanthemum morifolium*, the isoforms PIP1 and PIP2 had the highest expression level in leaves for both genes when compared with that in roots, stem, flower, and flower buds

(Zhang et al. 2019). Nevertheless, plants under saline stress showed differential expression level of isoforms *PIP1* and *PIP2* in leaves (Zhang et al. 2019). A genome-wide analysis of the 35 aquaporin AQPs in *A. thaliana* plants subjected to water deficit revealed that most of the *PIPs* and *TIPs* genes were downregulated, suggesting that decreasing gene expression might be a way for the plant to minimize water loss and uphold turgor in leaves (Alexandersson et al. 2005, 2010). On the other hand, a genome-wide analysis in *C. sinensis* revealed no expression differences in leaves of *CsAQPs* between sensitive and tolerant cultivars during drought (Wei et al. 2019), which shows that transcriptional profiles of AQPs in plants are highly variable in response to stress depending on the isoform, plant tissue, and stress level.

AQPs in plants could play an important role in inducing the regulation of transpiration in dry soils and high vapor pressure deficit conditions, allowing plants to conserve water to sustain plant physiological processes (Shekoofa and Sinclair 2018). Studies have suggested that some AQP isoforms increase the transcription level to facilitate water transport, while others are downregulated to decrease membrane water permeability to avoid excessive water loss during the stress (Zargar et al. 2017; Zhang et al. 2019).

Conclusion

Here, we identified 33 AQP genes in the available *C. canephora* genome. The evolutionary analysis revealed that the AQP genes are highly conserved among the three coffee species. Water deficit affected the transcriptional level of three *CcPIPs* and two *CcTIPs* isoforms in leaves of contrasting *C. canephora* genotypes (clone 14-tolerant and clone 109-susceptible to water deficit stress). Particularly, our findings suggest that the differential expression patterns of the *CcPIP2;3*, *CcTIP1;2*, and *CcTIP2;1* between tolerant and susceptible *C. canephora* clones make these genes potential candidate for further studies on their effects on the plants' response to water deficit conditions. In addition, the analysis of the *CcAQP* amino acid sequences allows the construction of various hypotheses with agronomical relevance regarding the function of the *CcAQPs* in plant growth and yield. Therefore, the identification and full characterization of the differentially regulated AQPs genes, and their isoforms, in response to water deficit are relevant for developing drought-tolerant cultivars using biotechnological tools in *C. canephora*.

Data Archiving Statement

All data used in this work to perform the bioinformatics analyses are public. The genes accessions numbers are available

on the supplementary Table S6. The present study did not produce itself any raw data.

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