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Multigenic regulation in the ethylene biosynthesis pathway during coffee flowering

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Abstract Ethylene regulates different aspects of the plant's life cycle, such as flowering, and acts as a defense signal in response to environmental stresses. Changes induced by water deficit (WD) in gene expression of the main enzymes involved in ethylene biosynthesis, 1-aminocyclopropane-1-carboxylic acid synthase (ACS) and oxidase (ACO), are frequently reported in plants. In this study, coffee (*Coffea arabica*) *ACS* and *ACO* family genes were characterized and their expression profiles were analyzed in leaves, roots, flower buds, and open flowers from plants under well-watered (WW) and water deficit (WD) conditions. Three new *ACS* genes were identified. Water deficit did not affect *ACS* expression in roots, however soil drying strongly downregulated *ACO* expression, indicating a transcriptional constraint in the biosynthesis pathway during the drought that can suppress ethylene production in roots. In floral buds, *ACO* expression is water-independent, suggesting a higher mechanism of control in reproductive organs during the final flowering stages. Leaves may be the main sites for ethylene precursor (1-aminocyclopropane-1-carboxylic acid, ACC) production in the shoot under well-watered conditions, contributing to an increase in the ethylene levels required for anthesis. Given these results, we suggest a possible regulatory mechanism for the ethylene biosynthesis pathway associated with coffee flowering with gene regulation in leaves being a key point in ethylene production and *ACO* genes play a major regulatory role in roots and the shoots. This mechanism may constitute a regulatory model for flowering in other woody species.

Keywords ACC oxidase · ACC synthase · Anthesis · *Coffea arabica* · RT-qPCR · Water deficit

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Introduction

In general, coffee flowering is an asynchronous event (Alvim 1960) which results in uneven fruit ripening that reduces coffee cup quality (DaMatta et al. 2019). Water deficiency negatively affect growth and development (Joshi et al. 2016; Koch et al. 2019) and therefore reduce the productive potential of crops (Conti et al. 2019; Sah et al. 2020).In such scenario a controlled irrigation management system has always proved to be an efficient mechanism to regulate *Coffea arabica* (*C. arabica*) flowering (Alvim 1960; Magalhães and Angelocci 1976; Ronchi and Miranda 2020) and productivity (Guerra et al. 2005). Considering that coffee is the second most traded commodity in the world, it is essential to study the events and understand the mechanisms associated with its flowering (López et al. 2021).

Positive regulation in the biosynthesis pathway and increases in ethylene levels have been reported under water deficit conditions (Arraes et al. 2015; Lima et al. 2021). Changes in soil water content regulate ethylene production in coffee plants, decreasing its levels in leaves, flower buds

and roots from the wet season to the dry season and, increasing it after the rain (López et al. 2022). Thus, re-watering positively regulated ethylene biosynthesis genes and induced shoot ethylene production (Lima et al. 2021; López et al. 2022), suggesting that changes in the ethylene biosynthesis and sensitivity during drought and upon re-watering are involved in coffee anthesis promotion (Lima et al. 2021).

Ethylene plays a fundamental role in different phases of the plant life cycle (Abeles and Morgan 1992; Liu et al. 2015; Iqbal et al. 2017; Graham et al. 2018), and it also acts in modulating responses to a variety of biotic and abiotic stresses (Paudel and Bede 2015; Thao et al. 2015; Savada et al. 2017; Lima et al. 2021). Its biosynthesis results from the methionine metabolism (Adams and Yang 1979) and follows a sequence of enzymatic reactions where the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC) is converted into ethylene (Fa Yang and Hoffman 1984). The enzymes 1-aminocyclopropane-1-carboxylate synthase (ACC synthase—ACS) and 1-aminocyclopropane-1-carboxylate oxidase (ACC oxidase—ACO), which control the rate limiting steps of ethylene production (Fa Yang and Hoffman 1984) are encoded by multigene families in several species (Yamagami et al. 2003; Gallie and Young 2004; Seymour et al. 2013; Arraes et al. 2015). Even though the biological concept for the existence of multigene families are not yet defined, it has been postulated that the difference in the chemical composition of cells, tissues, and organs may influence the specificity of the expression of homologous genes (Yamagami et al. 2003; Tsuchisaka and Theologis 2004). In a previous study conducted by our group, homologs for *ACS* and *ACO* were identified in coffee plants (Ságio et al. 2014) and it was shown that they are differentially expressed depending on the organ or environmental condition (Ságio et al. 2014; Lima et al. 2021). Although the ethylene metabolic pathway is conserved among different species, significant variations in the expression pattern of different *ACS* and *ACO* homologs genes occur in different species (Tsuchisaka and Theologis 2004; Forni et al. 2017; Tombesi et al. 2018), and water deficit studies have shown that the analysis of these variations is key for understanding ethylene effects (Arraes et al. 2015; Zhang et al. 2015).

The transcriptional control of *ACS* and *ACO* gene families is one of the points of ethylene synthesis regulation (Argueso et al. 2007). Mastering the knowledge of the genes responsible for encoding the enzymes for ethylene synthesis pathway, determining the location and the stimulus for differential expression, (Ruduś et al. 2013) are extremely relevant to identify single genes that are regulated under specific stages and developmental conditions. Previously, we showed that changes in ethylene levels promoted by plant water content modulation are involved in the promotion of coffee anthesis (Lima et al. 2021; López et al. 2022). Therefore, in this work, we proposed that the space–time

expression of the *ACS* and *ACO* genes modulated by the plant water status may help to explain the ethylene involvement in coffee anthesis promotion. We have compared the expression levels of *ACS* and *ACO* genes in leaves, roots, floral buds, and open flowers expression from coffee trees under well-watered and water deficit conditions, showing that the unique or overlapping expression patterns for the three *ACS* and three *ACO* homologs depend on both the organ and the soil water condition. Soil drying repressed all *ACO* genes in roots and two *ACO* genes in leaves, with no significant changes being found in floral buds. Organ-specific regulation of these homologs controls the differential expression between roots and the shoot according to the plant water status. We believe that this is directly involved in controlling ethylene production during coffee flowering and *ACO* genes play an important role in this process.

Materials and methods

In silico and phylogenetic analyses

Genes encoding for enzymes of the ethylene biosynthesis and response pathways of *Arabidopsis thaliana* (*A. thaliana*) were retrieved from the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (Aoki and Kanehisa 2005). The protein sequences of these genes were used as input to perform similarity searches against the genomes of plant species from different orders such *Solanales, Gentianales, Brassicales, Cucurbitales, Rosales, Fabales, Malpighiales, Vitales, Poales, Amborellale, Ginkgoales,* and the fern *Selaginella moellendorffii.* These searches were performed with BLAT (version 36 × 2) (Kent 2002). The *C. arabica* sequences were obtained from predictions made in the draft of its genome available in phytozome 13 (http://phytozome.jgi.doe.gov/) version 0.5 ("The UC Davis *Coffea arabica* Genome Project, Juan F. Medrano, Dario Cantu, Amanda Hulse-Kemp and Allen Van Deynze. *Coffea arabica* UCDv 0.5. http://phytozome.jgi.doe.gov/") database. The two best results (higher number of matches) for each enzyme mapped to each genome had their genomic coordinates expanded into 1500 base pairs in both genomic directions. These genomic coordinates were then used as input in the Augustus (version 3.3.2) gene prediction tool (Stanke and Morgenstern 2005) to identify possible genes in those genomes.

All predicted protein sequences for a specific enzyme were submitted to a length filter and only proteins comprising at least 70% of the *A. thaliana* input sequence were considered for further steps. The remaining sequences and the input sequence for each enzyme were then aligned with MAFFT (version 7.427) (Katoh and Toh 2008) by the *perl* script guidance (version 2.02) (Sela et al. 2015). Only one enzyme per species was reported based on multiple

interactions of phylogenetic analysis with the guidance and phylip package (version 3.698) (Felsenstein 1993). Finally, *C. arabica* and *Coffea canephora (C. canephora)* protein sequences were aligned against plant sequences in the NCBI *NR* database by the *online* BLAST tool (Altschul et al. 1990) available at NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi). This step was carried out to verify if the predicted protein is a homolog to any deposited protein in the NCBI database.

Growth conditions and treatments

Five-year-old plants of *C. arabica* grown in 50 L pots with a mixture of soil, sand, and cattle manure (3: 3: 1 v / v / v) were kept well irrigated at field capacity for 7 days. After this period, two different treatments were implemented: well-watered (WW), where plant water loss by evapotranspiration was daily replaced by watering pots until the soil saturation, and water deficit (WD), where irrigation was suspended during the entire experimental period. The experiment was conducted in a greenhouse of the Plant Physiology Sector at the Federal University of Lavras (UFLA) in September 2019. In the greenhouse, the average relative humidity was about 56.1% and the average maximum and minimum temperatures were around 29.4 °C and 20.3 °C, respectively. Each treatment consisted of ten biological replicates in a completely randomized design.

Measurement of water potential and collection of plant material

Predawn leaf water potential (ψ_{leaf}) was used as a parameter to determine water deficit. Plant water status (ψ_{water}) was monitored, with a Scholander pressure chamber, in fully expanded leaves located between the 3rd and 5th branches in the middle third of the plant. The analysis was performed every three days, from 3:00 to 5:00 AM. Measurements were performed in ten repetitions for each treatment (WW and WD) on two or three leaves from each plant. The third leaf was only measured if there was a difference greater than 0.2 MPa between the first two measurements. When the ψ_{leaf} of the WD plants declined to a minimum of −2.5 MPa (Drinnan and Menzel 1994), the experiment was interrupted (Fig. 1). Roots, leaves, flower buds from 6.1 to 10 mm (G5 stage—light green color), and open flowers (Morais et al. 2008) were collected for Reverse Transcription quantitative Polymerase Chain Reaction (RT-qPCR) analysis using three biological repetitions from each tissue and treatment.

RNA isolation and cDNA synthesis

Total RNA was extracted using the CTAB buffer (Chang et al. 1993) with modifications for coffee tissues (Online Resource 1). RNA (5 μg) was then treated with DNase, using
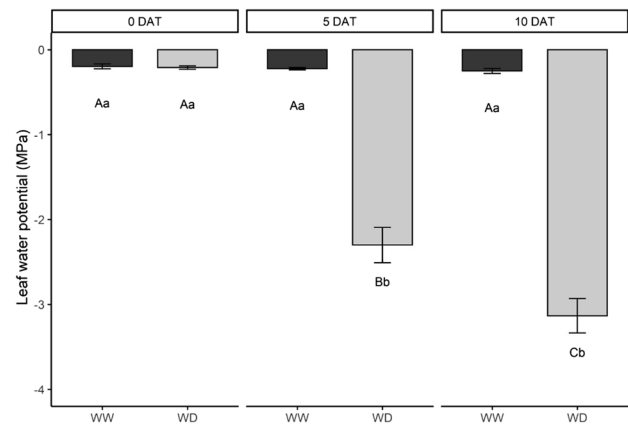

Fig. 1 Predawn leaf water potential in *Coffea arabica* under well-watered (WW) and water deficit (WD) conditions. Data are means and the standard error ($n = 3$) with different letters indicating statistical difference ($P < 0.05$). Uppercases letters compare WW and WD between each experimental period: 0, 5 and 10 days after treatment (DAT). Lowercases letters compare WW or WD inside each DAT

the Turbo DNA-free kit (Life Technologies) according to the manufacturer's recommendations to remove any residual DNA. RNA integrity was analyzed in 1% agarose gels. The quantity and quality of RNA ($OD_{260/280}$ and $OD_{260/230} > 1.8$) were checked by spectroscopy (NanoVue GE Healthcare, Munich, Germany). The synthesis of complementary DNA (cDNA) was performed with the High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, Waltham, USA), following the manufacturer's protocol, using 1 μg of total RNA (see Online Resource 2). Samples were then stored at −20 °C until RT-qPCR analysis.

Gene expression analyses

The analysis of gene expression was performed in a Rotor-Gene Q Real-Time PCR thermocycler (Venlo, Netherlands), using the SYBR® Green detection system (QuantiFast SYBR Green PCR Kit—Qiagen). The final reaction volume was 15 μL: 7.5 μL of SYBR Green, 1.5 μL of cDNA, plus the specific concentrations of primer (see Online Resource 3 for primer sequences and amplification efficiencies) and water for each gene (*CaACS1*, *CaACO1*, *CaACO4*, *CaACO5* 3.0 μL of primers and 3 μL of water were used; *AP47* 4.5 μL of primers and 1.5 μL of water *RPL39*, *CaACS3* and *CaACS7* 6.0 μL of primers). The conditions for the RT-qPCR reactions were: 5 min at 95 °C for enzyme activation, followed by 40 cycles of 5 s at 95 °C and 10 s at 60 °C, ending with a melting curve to analyze the specificity of the reaction, increasing temperature at 1 °C every 5 s, 60 °C to 95 °C. For the putative *ACS* gene of *C. arabica* (*CaACS1*), 40 cycles of sample denaturation and amplification occurred was carried out at 58 °C. Three biological repetitions from

each treatment and organs were used, with three technical replicates for each sample. The relative expression was calculated by $\Delta\Delta^{CT}$ method (Pfaffl 2001) using *AP47* and *RPL39* as reference genes (Fernandes-Brum et al. 2017). Online Resource 2 shows the RT-qPCR parameters according to the minimum requirements for publishing experiments with quantitative real-time PCR (MIQE) (Bustin et al. 2009).

Statistical analyses

The difference in ψ_{leaf} values between the WW and WD treatments was verified by an analysis of variance (ANOVA) and the means with Scott Knott's significant difference at $p < 0.05$. For the gene expression analysis, the expression ratio calculations and confidence intervals were subjected to statistical analysis with a Linear Mixed Model (Steibel et al. 2009). The model is given by the equation:

$$y_{iklm} = \mu + TG_{ik} + Il + e_{iklm}$$

where y_{iklm} is the Cq (quantification cycle) obtained from the thermocycler software for the k-th gene (reference or target) from the m-th well, corresponding to l-th plant subjected to i-th treatment (WW, WD); TG_{ik} is the effect of the combination of the i-th treatment (WW, WD) in the expression of the gene k (reference or target); $Il \sim N(0, \sigma^2 lk)$ is a specific randomized effect from the plant; $e_{ijklm} \sim N(0, \sigma^2)$ is the residual term.

Results and discussion

Phylogenetic analysis

ACS gene family

The search for *ACS* genes in the *C. arabica* genome identified nine sequences corresponding to putative *ACS* genes (*CaACS 1–4-like, CaACS 6–8-like, CaACS10-like, CaACS12-like*). The phylogenetic analysis grouped the proteins encoded by the *CaACS7-like*, *CaACS10-like*, and *CaACS12-like* genes with ACS sequences from *C. canephora* (CcACS) – one of the progenitors of *C. arabica* (Lashermes et al. 1999), whereas the other six putative CaACS protein sequences remained in a separated branch (Online Resource 4).

The *CaACS1-like* (accession no. KF975694) was identified by Ságio et al (2014). Although the ACS protein sequence identified in this study is 28% different from Ságio et al (2014), we decided to consider the ACS sequence identified by these authors, since molecular analyses have already demonstrated the existence of this gene in *C. arabica*. The putative CaACS2 protein has more than 98%

identity with CaACS1 (accession no. KF975694) and the difference among them comes from 21 amino acid residues present only in CaACS1 sequence (Online Resource 5). Interestingly, CaACS6 showed a 29% difference in amino acid residues in relation to CaACS1 (Online Resource 6), and grouped in a more distant branch in the phylogenetic tree (Online Resource 4). Together, these findings indicate that *CaACS6-like* could be a new *ACS* gene in *C. arabica*. Nonetheless, CaACS6 grouped with CcACS2 from *C. canephora*, displaying an amino acid identity of 100% with this protein (Online Resource 6). One can notice that ACS6 protein sequences from other species are grouped in the same clade of ACS1 and ACS2 (Online Resource 4), indicating high similarity levels among these sequences. Therefore, we renamed the *C. arabica* genome sequence identified as *CaACS6-like* to *CaACS2-like* (accession no. MW246819—Online Resource 7).

The proteins encoded by *CaACS3*, *CaACS4*, and *CaACS8* have grouped in the same clade, sharing more than 98% of identity, and the difference among them resides in seven amino acid residues present in CaACS3 sequence and in the gaps that exist in CaACS4 (Online Resource 8). It is interesting to observe that ACS3 from *Amborella trichopoda* (AmtACS3) grouped with ACS11 from *Ginkgo biloba* (GbACS11) (Online Resource 4), indicating similarity between these sequences, possibly due to the ancestral characteristic concerning these species. *Amborella trichopoda* and *Ginkgo biloba* are considered the most basal species within the angiosperm and gymnosperm groups, respectively (Guan et al. 2016; Villegente et al. 2017), and therefore this phylogenetic relationship suggests that *ACS3* may be more primitive than *ACS4* and *ACS8* in plant species. Considering that the process of gene duplication generates copies of identical sequences with redundant function to the parental gene (Tutar 2012), *CaACS3-like, CaACS4-like* and *CaACS8-like* from *C. arabica* (accession no. MW246813, MW246818, and MW246817, respectively, see Online Resource 7) may have originated from genomic regions so close to each other that they originated as tandem duplicate genes. Therefore, *CaACS4-like* and *CaACS8-like* were removed from the analysis.

The sequences of CaACS1-3, CaACS7, CaACS10, CaACS12 have all showed the seven conserved domains (Online Resource 9) that are found in ACS enzymes previously described in other species (El-Sharkawy et al. 2008; Muñozmu̇muñoz-Robredo et al. 2011; Lee and Yoon 2018). However, their alignment showed that the eleven amino acid residues that are authentic to ACS enzymes (Yamagami et al. 2003; Muñozmu̇muñoz-Robredo et al. 2011) are absent in CaACS10 and CaACS12 (Online Resource 9). In addition, the glutamate residue (E) in box 1, which is responsible for substrate specificity (McCarthy et al. 2001), is not present in CaACS12. The tyrosine residue (Y) in box 2 which

enables the catalytic activity of ACS converting AdoMet to ACC (Tsuchisaka and Theologis 2004; Muñozmu˜muñoz-Robredo et al. 2011), is also absent from CaACS10 and it has been replaced by serine (S) in CaACS12 (Online Resource 9). Therefore, it is expected that CaACS10 and CaACS12 catalyze reactions that do not include ethylene production (Argueso et al. 2007), but they were considered as ACS enzymes for belonging to the family of amino acid aminotransferases (Yamagami et al. 2003; Arraes et al. 2015). On the other hand, the absence of deletions in the conserved residues of the seven conserved domains from CaACS1, CaACS2, CaACS3 and CaACS7 indicates that the genes that encode these enzymes are authentic *ACS* (El-Sharkawy et al. 2008; Muñozmu˜muñoz-Robredo et al. 2011; Lee and Yoon 2018). Therefore, *C. arabica* has a small *ACS* multi-gene family composed of four members: *CaACS1, CaACS2, CaACS3*, and *CaACS7*.

We also determined, through *in silico* analysis, the possible serine (S) residues for phosphorylation in the C-terminal region of the putative ACS identified in this study (Online Resource 10). The primary sequences of CaACS2, CaACS3, and CaACS7 have grouped as type III ACS (Liu and Zhang 2004; El-Sharkawy et al. 2008; Tucker et al. 2010) and CaACS1 as type I ACS, although it does not have the three phosphorylation sites that characterize this type of enzyme (Liu and Zhang 2004). The presence of these sites in the C-terminal region is more important than the number of available serine residues, since it has been suggested that the status of phosphorylation for enzyme activity is the most important factor in regulating the stability of type I ACS (Joo et al. 2008). It is interesting to notice that the phosphorylation mechanism is essential for the catalytic activity of some ACS, but it seems unnecessary for type III ACS.

The lack of phosphorylation sites in this group of ACS suggests that these sequences encode more stable ACS proteins (Hyun and Kieber 2005).

ACO gene family

In a previous study, three *ACO* genes from *C. arabica* were identified and the corresponding sequences were deposited in the NCBI database: *CaACO1* (accession no. AHN13883), *CaACO4* (accession no. AGM48542), and *CaACO5* (accession no. KF975696) (Ságio et al. 2014). In this study, we also identified three putative ACO genes: *CaACO3, CaACO4*, and *CaACO5* (Online Resource 11). The highest identity level among the proteins encoded by these sequences was 82%. Interestingly, *CaACO1* was not found in our similarity search and, based on this, we performed a comparative analysis between the possible genes identified in this work and the genes identified by Ságio et al (2014). We found that CaACO3 has a 98% identity level with the previously CarACO1 identified by Ságio et al. (2014), and both proteins have 98.1% and 98.4% identity with CcACO3 (Online Resource 12), respectively. Ságio et al. (2014) showed in their phylogenetic analysis with homologous sequences of *Solanum lycopersicum* that *CaACO1-like* gene displayed a high similarity level with SolACO1 and SolACO3, and it grouped with *CaACO3* (Fig. 2). These isozymes formed specific clades, but SolACO1 and CaACO1 have not grouped with the ACO1 sequences from other species, suggesting a possible error in its annotation. Therefore, *CaACO1* has been renamed as *CaACO3* (Fig. 2). On the other hand, ACO4 protein sequences from both studies are 100% identical, suggesting the existence of a unique region in the genome encoding the ACO4 protein. The sequences identified as
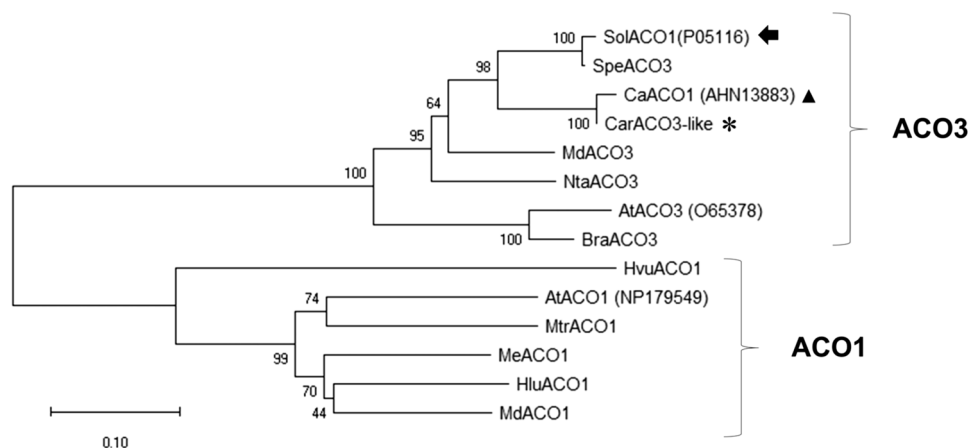

Fig. 2 Phylogenetic tree comparing the ACO1 amino acid sequence in *Coffea arabica* and the homolog sequences in other species. ACO1 and ACO3 sequences formed specific clades, but ACO1 from *Solanum lycopersicum* (arrow: accession no. P05116) and *Coffea arabica* (triangle: accession no. AHN13883 - Ságio et al (2014)) did not group with ACO1 from other species. The grouping of CaACO1 with the new sequence CarACO3 (asterisk) possibly is a mistake on its annotation. CarACO3 is CaACO3. The genetic distances are shown at the given scales

ACO5 showed less than 50% identity at the amino acid level with theACO5 sequence from Ságio et al. (2014), suggesting that there might be more than one genomic region encoding ACO5 proteins.

Recently, *ACO* genes have been identified based on similarity searches comparing the Fe-binding and 2-oxoglutarate domains (Tu et al. 2019). Here, we show that ACO polypeptides from *C. arabica* contain all 15 conserved residues at the catalytic site present in sequences functionally characterized as authentic ACO enzymes (Online Resource 13) (Dilley et al. 2013; Sun et al. 2017). The alignment highlights the essential residues for ACO activity (Online Resource 13) based on the identification of ACO1 in *Malus domestica* (*M. domestica*) (Dilley et al. 2013). Residues R244 and S246 make up the RXS motif for 2-oxoglutarate (2OG) binding and this region is conserved in all enzymes that comprise the Fe^{+2} dependent 2-oxoglutarate oxygenase/oxidase (2OGD) family (Brisson et al. 2012). The intermediate residue to the RXS motif is responsible for the classification of ACO enzymes into three different groups, where CaACO3 and CaACO4 were considered as type I ACO, since X = Methionine (Online Resource 13), whereas CaACO5 is a type III ACO, since X = Arginine (Online Resource 13) (Houben and Van de Poel 2019). Therefore, *C. arabica* does not have type II ACOs, where X = Leucine/Isoleucine. Despite the presence of the RXS motif, CaACO3, CaACO4, and CaACO5 have the residues K292, K158, F300 (Dilley et al. 2013) for the binding of ascorbate (Online Resource 13), which acts as a reducer to activate the catalysis of the ACC ring (Zhang et al. 2004). The presence of highly conserved sites for this cofactor in *C. arabica* suggests that this species may form an exclusive group within the 2OGD that does not use 2OG for its catalytic reactions, but possibly uses ascorbate for this function (John 1997; Kawai et al. 2014).

The ACO isozymes of *C. arabica* harbor the 2-His-1-carboxylate triad in the catalytic nucleus, with the carboxylate group provided by aspartate (2-His-1-Asp). It means that in this species, aspartate acts as a donor of the carboxylic acid group (Hegg and Jr 1997) and is an essential reason for anchoring the ferrous ion (Fe^{+2}) in the active site (Mirica and Klinman 2008). These amino acids are reported as indispensable sites for the activity and functionality (Zhang et al. 2004; Chen et al. 2016), since they are essential for ACC binding (Martinez and Hausinger 2015). The ACO enzyme complex is completed by anchoring the bicarbonate cofactor (HCO^{-3}) (Zhang et al. 2004; Dilley et al. 2013), which occur in the residues R175, K158, in *C. arabica* (Online Resource 13). In the absence of HCO^{-3} sites, ACO is not able to oxidize ACC and form ethylene (Rocklin et al. 2004), suggesting that the identification of these sites in *C. arabica* ensures that the active complex of these ACOs is capable of binding

HCO^{-3}, leading the oxidant to the main substrate of the reaction, the ACC.

Finally, the residues C28, T157, Q188, K199, K230 are highly conserved in all CaACO (Online Resource 13) and are associated with the stability and catalytic activity of the enzyme (Dilley et al. 2013). Glutamate residues (E) showed the highest variation rate among the species compared in this study (Online Resource 13). Residue E294 is absent in all CaACO and was detected only in *Oryza sativa*, confirming that this residue has the highest replacement rate among the 15 conserved residues (Dilley et al. 2013). Therefore, the substitution of the residue E294 does not seem to compromise the conversion of ACC into ethylene, since it has a low level of conservation in species with biochemically functional ACO enzymes (Dilley et al. 2013; Houben and Van de Poel 2019). For CaACO5, residue E297 was replaced by glutamine (Q), suggesting that glutamine can perform a similar function in this case and contribute to the stability/activity of this enzyme, as observed in *A. thaliana* (Sun et al. 2017) and *M. domestica* (Dilley et al. 2013).

Expression profiles of the ACS and ACO genes

The comparative analysis of *CaACS1, CaACS3, CaACS7, CaACO3, CaACO4,* and *CaACO5* expression levels allowed the observation of the contribution from each gene to the overall ethylene biosynthesis in leaves (Fig. 3A), roots (Fig. 3B), flower bud on the G5 stage (Fig. 3C) and open flower (Fig. 3D) of coffee plants under WW (−0.35 MPa – Fig. 1) and WD (-3.0 MPa—Fig. 1) conditions. Water deficit suppressed *CaACS1* and *CaACS7* expression in leaves (Fig. 3A). *CaACS7* showed an expression level 8 times lower in leaves from WD plants when compared to leaves from plants under WW, whereas for *CaACS1*, the expression level was 2 times lower. Therefore, *CaACS7* showed the greatest variation, indicating that this *ACS* homologous is most affected by water restriction in coffee leaves. Interestingly, a previous study by our group showed that, in coffee seedlings, *CaACS1* expression in leaves did not differ between WW and WD conditions (Lima et al. 2021). Tsuchisaka and Theologis (2004) showed that genes in the *ACS* family may be regulated at the transcriptional level according to the plant developmental stage. Therefore, the expression pattern of *CaACS1* may be under the influence of the plant's life cycle, acting on the regulation of ethylene biosynthesis during coffee flowering. The CaACS3 expression did not change between the two watering conditions (Fig. 3A), suggesting an organ-specific regulation. Thus, ACC synthesis in leaves during the dry period is supplied by the ACS3 isoform, since there was no reduction in expression, as occurred for other ACS homologs.

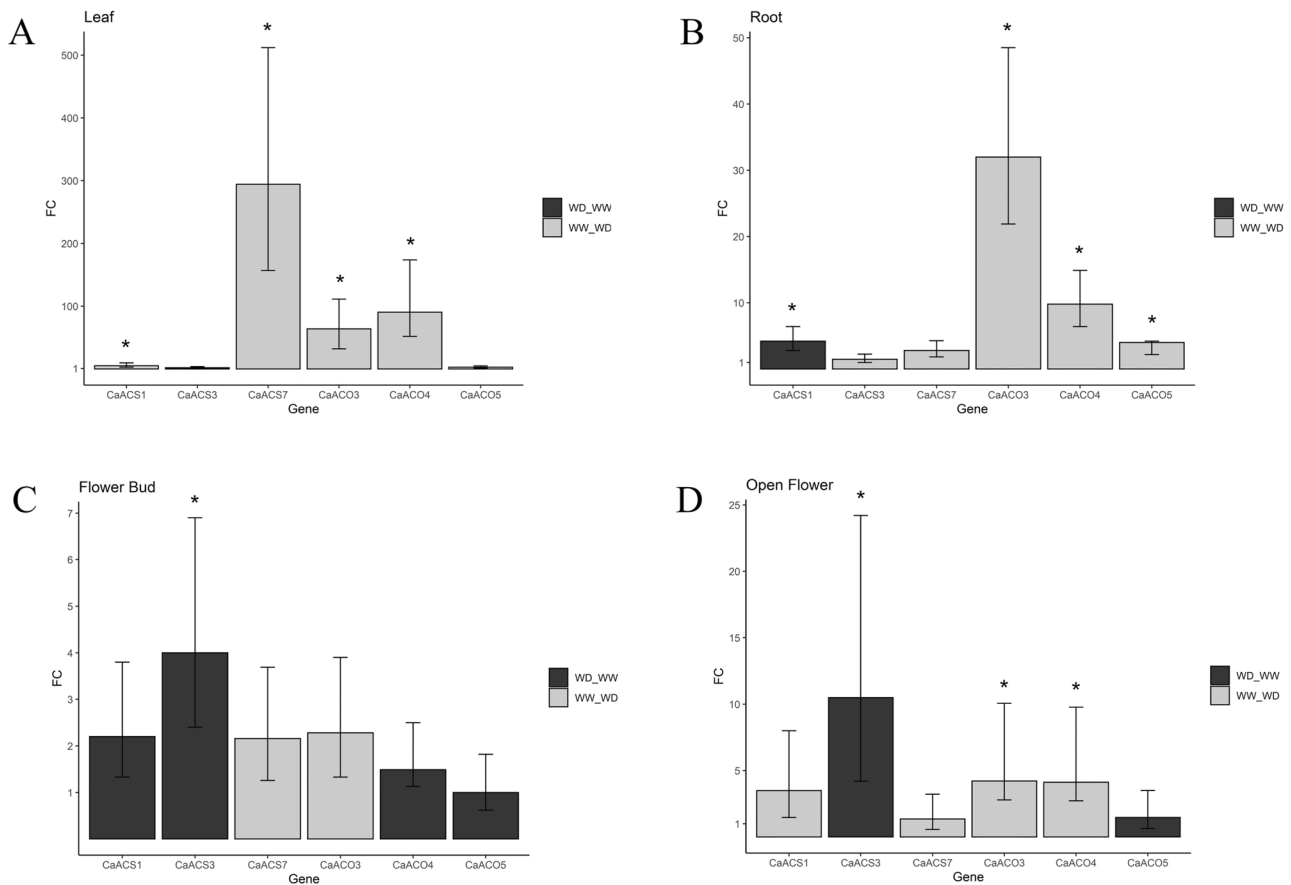Regarding the *ACO* genes, the expression of *CaACO3* and *CaACO4* in leaves from plants under WD as downregulated

Fig. 3 Fold-change (FC) estimates for the *CaACS1, CaACS3, CaACS7, CaACO3, CaACO4,* and *CaACO5* genes in (A) leaves (B) root (C) flower bud and (D) open flowers of coffee plants. Black bar represents a higher expression level under water deficit (WD) and the gray bar represents a higher expression level under well-watered (WW) conditions. The statistical difference of the FC is showed with an asterisk, indicating induction in WW or WD conditions. The segments represent the confidence interval and comparisons whose confidence intervals include the value 1 are not significant at α=5% (*n*=3)

(Fig. 3A). These genes co-expressed under WW conditions and showed an expression level 6 and 6.5 times lower under WD, respectively. Therefore, as reported for *CaACS7*, *CaACO4* is the *ACO* homolog most affected by water deficit in leaves. We also observed that the *CaACO5* expression pattern is not conditioned by the treatments, but it seems to be organ-specific (Fig. 3A), similar to *CaACS3* expression. In this case, it is suggested that leaf ethylene production during the dry period is linked to the ACO5 isoform, similar to ACS3.

In roots, *CaACS1* and *CaACS7* showed a different expression profile compared to the one observed in leaves (Fig. 3B). The *CaACS1* was induced under WD, displaying an expression level 2 times higher to the one observed in roots from WW plants. It has been reported that the concentration of ACC is directly proportional to the gene expression level (Eum et al. 2009) and, it has been shown that coffee plants with a leaf water potential of -2.5 MPa increase ACC levels (López et al. 2022). Thus, the higher

expression of *CaACS1* may be directly related to an increase in ACC levels in coffee roots. The ACC levels are modulated as a function of soil water content and, in general, it gradually increases in leaf, flower bud and root tissues during the rainy season followed by drought and rehydration (López et al. 2022). The water content did not alter *CaACS3* and *CaACS7* expression in roots (Fig. 3B). Therefore, we believe that *CaACS3* and *CaACS7* contribute to the maintenance of ACC levels in coffee plant roots under WW conditions. It is important to emphasize that, this result does not exclude the possibility of an increase in the ACC pool in roots from well-watered plants, once ACC mobility by the xylem allows this molecule to be allocated in distant regions from its synthesis site (Tudela and Primo-Millo 1992; Gómez-Cadenas et al. 1996). Increases in ACC level was greater after rehydration, in accordance with the expression of the ACC transporter gene (LHT1), especially in roots and flower bud, showing that the intracellular transport of ACC occurs

in coffee trees during flowering in response to the soil water content (López et al. 2022).

Interestingly, the three *ACO* homologs were repressed in roots under WD, showing an expression profile dependent on the water condition of the plant (Fig. 3B). Although the expression pattern is overlaid between the three genes in roots from plants under WW conditions, the amplitude of the variation in *CaACO3* expression was greater among the homologs. This gene was five times less expressed in roots from WD plants, when compared to roots from plants under WW, and similarly *CaACO4* and *CaACO5* were three and two times lower expressed in the same condition, respectively. In our experiment, the opposite expression profile of *ACS* and *ACO* genes suggests that, during the dry period, there is an increase in ACC levels in roots, possibly regulated by ACS1. Soil drying was shown to increase ACC content in roots (López et al. 2022), and a similar response has likely occurred in this experiment during the dry period, since all *ACO* genes were repressed under WD. In fact, ACO enzyme activity reduces from the wet to the dry season, resulting in an increase in ACC levels while ethylene levels are reduced (López et al. 2022). Therefore, there seem to be a positive relationship between the regulation of *ACO* gene expression and the regulation of ACO enzyme activity. Ethylene biosynthesis can be suppressed with the reduction in the expression of *ACO* genes (Eum et al. 2009), and Lima et al (2021) showed a positive relationship between *CaACO1* (here, *CaACO3*) expression and ethylene production in coffee roots under drought. Considering that ACO has been described as the limiting regulatory step for ethylene biosynthesis (Rudús et al. 2013; Van de Poel et al. 2014; Houben and Van de Poel 2019), this may be the point of the ethylene biosynthesis pathway that modulates ACC and ethylene levels during coffee anthesis related events.

In flower buds on the G5 stage, the expression level of *CaACS3* was two times higher under WD (Fig. 3C). The genes *CaACS1* and *CaACS7* were not differentially expressed and a similar result was observed for three *ACO* homologs (Fig. 3C). Based on these results, considering that the induction of *ACS* homologs favors the production of the reaction substrate (Argueso et al. 2007; Yoon 2015) and that ACO may be the key regulator in the production of ethylene (Love et al. 2009; Van de Poel et al. 2014; Chen et al. 2016), it is suggested that an ACC pool may be formed in flower buds during drought. In open flowers, *CaACS1* and *CaACS7* expression did not differ between the watering conditions, whereas *CaACS3* expression was three times higher in WD plants (Fig. 3D). In relation to *ACO* genes, *CaACO3* and *CaACO4* were two times lower expressed in plants under WD, whereas *CaACO5* expression was not affected by the treatments. Interestingly, the expression pattern observed in G5 flowers buds was maintained in flowers for the *ACS* genes. However, the expression modulation

of *ACO* homologs in flowers not show a pattern for *ACO* gene expression. The regulation *ACO* genes is dependent on the floral organ developmental stage and the plant water condition.

Based on the results of this investigation, a gene regulation model for ethylene biosynthesis in coffee trees submitted to different water conditions is provided, highlighting the response of *ACS* and *ACO* homologs for each condition and plant organ (Fig. 4). In addition, according to the proposed role for water (Crisosto et al. 1992; Guerra et al. 2005) and ethylene (Lima et al. 2021) coffee anthesis induction, in this model, we also associated the involvement of ethylene biosynthesis genes with coffee flowering. The control of coffee flowering is associated with different endogenous and environmental factors, such as phytohormones, photoperiod, water deficit, temperature and humidity (Peña Quiñones et al. 2011; Pezzopane et al. 2008; de Oliveira et al. 2014; DaMatta et al. 2019; Ronchi and Miranda 2020). A period of drought followed by rain is required to trigger anthesis (Alvim 1960; Camargo and Camargo 2001), and changes in ethylene levels and on its sensitivity in roots and shoot have recently been suggested to occur during water deficit and rehydration, which seems to be responsible for inducing anthesis in coffee trees (Lima et al. 2021). Interestingly, the application of ethylene (Ethephon® 720) promoted leaf senescence without inducing anthesis (López et al. 2022). However, connecting the gene expression profiles observed in the shoot (Fig. 3A, C, D) and roots (Fig. 3B) to the water conditions faced by the plants (WW and WD), it is likely that ACC is the signaling molecule for anthesis promotion in coffee trees (López et al. 2022), considering its capability of acting as signal molecule (Vanderstraeten et al. 2019; Mou et al. 2020).

The change in leaf water potential (Fig. 1) possibly acts as a signal to regulate ethylene biosynthesis genes in leaves, flower buds, open flowers, and roots (Fig. 3A, B, C, D). In the model (Fig. 4), WD represents the dry period that naturally occurs and precedes the rainy season, followed by the anthesis (Camargo and Camargo 2001). Considering a correlation of the transcript levels found here may be positively correlated with the enzyme activity (Eum et al. 2009; López et al. 2022), the ACC produced in WW roots would be converted locally to ethylene by ACO. Thus, constant hydration of the root system promoted by rain or irrigation would not lead to any increase in the ACC content, since *CaACS3* and *CaACS7* expression are not regulated by water content in the soil (Fig. 3B). A similar response could occur in the shoot, particularly considering the induction of two out of three genes of the three *ACO* genes in leaves from WW plants (Fig. 3A). Therefore, the flowering observed in coffee trees under constant irrigation (Crisosto et al. 1992) is possibly the result of short periods of stress in the hottest hours of the day combined with continuous availability of water and
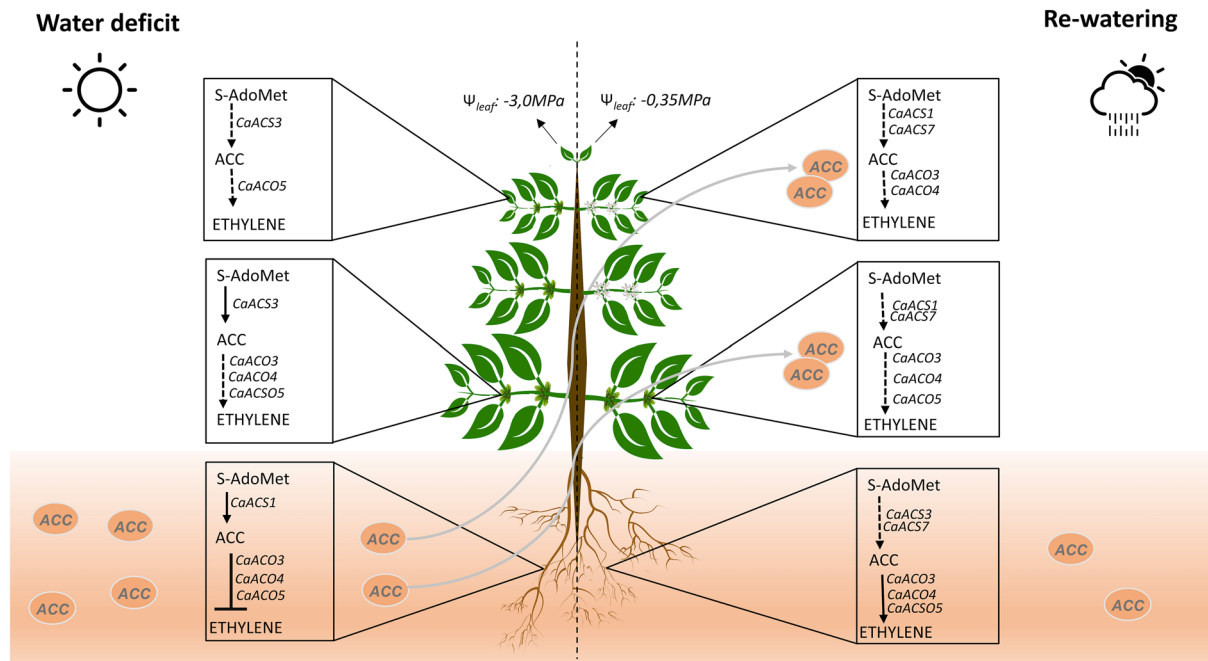
Fig. 4 Transcriptional regulation model of ethylene biosynthesis genes in coffee plants submitted to the different water conditions. During coffee flowering, an alternation between the dry period (Water deficit) and wet period (Well-watered) promotes change in leaf water potential (Ψ_{leaf}), coordinating changes in *ACS* and *ACO* genes expression. Expression profiles of the ACS and ACO homologous genes can be unique or overlapping, and their regulation can be water-dependent or water-independent in each organ. Finally, a fine adjustment in *ACO* expression regulation between roots and leaves seems to be the key point to ethylene production control in the shoot and anthesis induction. Results from this study are shown in black, and connections from previous studies found in literature and discussed in the text are shown in gray. Solid arrows denote increases, T end to the arrows indicate decreases, and dashed arrows denote no significant changes in expression or relative amount. ACC in the circle indicates ACC produced in the shoot part during WD. ACC: 1-aminocyclopropane-1-carboxylic acid; ACS: 1-aminocyclopropane-1-carboxylic acid synthase; ACO: 1-aminocyclopropane-1-carboxylic acid oxidase

associated with the elevated levels of leaf ethylene, since *ACO* genes from flower buds are not regulated by the plant water condition.

All *ACO* genes were repressed in roots during drought (WD) whereas Ca*ACS1* was induced in this condition (Fig. 3B). Similar to our results, Lima et al (2021) showed that *CaACO1* (renamed to, *CaACO3* in this work) was repressed in coffee roots under drought and the ethylene production was not responsive in dry roots. Additionally, it was shown that ACO activity is reduced during the dry period and ethylene levels do not increase proportionally to ACC levels (López et al. 2022). Collectively, these results suggest that the positive regulation of *CaACS1* and the absence of changes in coffee root ethylene levels lead to the accumulation of ACC in roots during the dry season, as observed by López et al (2022). Considering that the *ACO* genes were shown to be more affected by drought in this organ, it seems plausible to suggest that ACO enzymes regulate coffee ethylene biosynthesis in roots during WD. This information is relevant as it has been suggested that the regulation of anthesis may be under the control of the ACC in an ethylene-independent pathway (López et al. 2022). More studies are

needed to validate this new hypothesis, however the evidence indicates that the repression of *ACO* gene expression and inhibition of ACO enzyme activity contribute to the accumulation of ACC.

Correlating this result with the flowering dynamics of *C. arabica*, the ACC accumulated in roots during the dry season is probably transported to the shoot with the resumption of the rain or irrigation (Lima et al. 2021; López et al. 2022). Then, as it reaches the leaves, ACC would be converted into ethylene by the action of ACO enzymes, whose genes were shown to be positively regulated once water supply is restored (Fig. 3A). The plant water status has already proved to be an important regulator of ethylene production in coffee trees, being able to re-establish the production of this hormone in leaves and flower buds (Lima et al. 2021). Thus, it seems that ethylene displays an important function in coffee flowering, and it may help to promote coffee anthesis possibly through the activation of rehydration-responsive genes, which was shown to promote flower opening in other species, as observed in rose (*Rosa hybrida*) (Meng et al. 2014). Although additional data are needed to validate the connections described above, the results of this study

allow mapping the main points of the multigene regulation of ethylene biosynthesis triggered by drought and re-watering in coffee trees. This information helps to explain the importance of the dry season during coffee flowering and contribute to the development of future research aimed at elucidating the metabolic network associated with anthesis induction in this species, and possibly other wood species.

Conclusions

In this study, we expanded and improved the knowledge about the members of the ethylene biosynthesis pathway in *Coffea arabica*. Coffee ACS and ACO enzymes are encoded by small multigenic families and their unique or overlapping expression patterns depend on both the organ and the plant water condition. Despite the contribution of *ACS* genes, it was shown that the regulation of *CaACO3* and *CaACO4* in roots and leaves act as the main control point in the ethylene biosynthesis pathway in coffee trees during the wet and dry periods. Especially in roots, the repression of all *ACO* genes show an important regulatory role that leads to increased levels of ACC during soil drying in this organ, which seems to be important for triggering anthesis once plants are rehydrated. Therefore, these results help to explain the importance of the soil water variation during coffee flowering, in addition to provide an excellent opportunity to improve the knowledge of the root-to-shoot communication associated with coffee flowering, and possibly in other woody species.

Given the importance of ethylene for flowering and, consequently, for the ripening of coffee fruits, future studies should focus on the functional analysis of the different *ACS* and *ACO* homologous genes, using first model species as a tool for heterologous expression, and then in coffee species. This approach will certainly enable a better identification of the genes involved in ethylene regulation during anthesis, providing a new research avenue for controlling this important stage of the coffee plant life cycle, which is directly related to coffee cup quality, negatively affected by the uneven fruit ripening caused by the asynchronous flowering commonly observed in this species.

Author's contribution ISS: Conceptualization, Methodology, Validation, Investigation, Data Curation, Writing—Original Draft. THCR: Formal analysis, Investigation, Data Curation, Writing—Review & Editing. KKP de O and JO dos S: Validation, Investigation, Writing—Review & Editing. ROM: Investigation, Formal analysis. RRL and Jo dos S: Formal analysis. AAL and AC-J: Conceptualization and Writing—Review & Editing. AC-J: Supervision and Funding acquisition. All the authors read and approved the manuscript.

Data availability statement Nucleotide sequence data were deposited in the NCBI database and the accession numbers were included as supplemental material.

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

Availability of data and material Not applicable.

Code availability Not applicable.

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