SOLANGE APARECIDA SÁGIO

ANÁLISE MOLECULAR E FISIOLÓGICA DO ETILENO DURANTE O AMADURECIMENTO DE FRUTOS DE CAFÉ

LAVRAS – MG

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Tese apresentada à Universidade Federal de Lavras, como parte das exigências do Programa de Pós-Graduação em Agronomia, área de concentração em Fisiologia Vegetal, para a obtenção do título de Doutor.

Orientador

PhD. Antonio Chalfun Júnior

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RESUMO GERAL

A qualidade do café está diretamente associada ao estádios de maturação dos frutos na época da colheita, o qual é frequentemente desuniforme devido ao florescimento sequencial presente no café, elevando o custo de produção e gerando bebida de baixa qualidade. Alguns estudos sugerem que o café seja um fruto climatérico indicando que o etileno apresenta um importante papel no processo de maturação do café. As cultivares precoces geralmente apresentam um processo de maturação mais uniforme, no entanto pouco se sabe sobre os fatores genéticos que promovem a precocidade da maturação. Assim, com o objetivo de melhor entender os fatores fisiológicos e genéticos envolvidos na regulação do tempo de maturação, os perfis da produção de etileno e da respiração durante a maturação de frutos de cultivares precoce (Catucaí 785-15) e tardia (Acauã) foram analisados. Assim como os perfis da expressão de elementos das rotas de biossíntese e sinalização do etileno. As análises de respiração e de etileno mostraram diferentes comportamentos entre as duas cultivares de café. Os frutos da Catucaí 785-15 apresentaram uma típica elevação climatérica na respiração e na produção de etileno durante a maturação, enquanto que os frutos da Acauã apresentaram somente pequenas mudanças nesses parâmetros. As análises *in silico* permitiram a identificação de prováveis membros de quase todos os passos das rotas de biossíntese e sinalização do etileno. As análises de RT-qPCR demonstraram que os genes da biossíntese (*CaACS1-like; CaACO1-like; CaACO4-like* e *CaACO5*) analisados nesse estudo, foram induzidos nos estádios finais da maturação em ambas cultivares, com destaque para *CaACS1-like* e *CaACO4-like* que apresentaram maiores níveis de expressão do que aqueles encontrados em folhas e flores, indicando que estes genes possam apresentar um importante papel na maturação do café. Por outro lado, os membros da rota de sinalização do etileno apresentaram um padrão distinto daquele encontrado para os genes da biossíntese, com todos os genes, de ambas cultivares, apresentando níveis de expressão um pouco maiores nos estádios iniciais de desenvolvimento. As análises de expressão dos genes da biossíntese *CaACO1-like* e *CaACO4-like* e do receptor de etileno *CaETR4-like*, sugerem que os maiores níveis de produção de etileno nos frutos da Catucaí 785-15 possam induzir uma maior degradação do *CaETR4-like*, levando a um aumento na sensibilidade ao etileno e consequentemente à precocidade no processo de maturação desta cultivar. A produção de etileno nos frutos da Acauã pode não ser suficiente para desativar os níveis de *CaETR4-like* e assim as mudanças na maturação ocorrem em um ritmo mais lento, sugerindo que esta cultivar apresente um fenótipo climatérico suprimido.

Palavras-chave: Bioinformática. Coffea arabica. Expressão gênica. Etileno. Maturação

GENERAL ABSTRACT

Coffee quality is directly associated to the fruit ripening stage at harvest time, which is often highly asynchronous due to the sequential flowering found in coffee trees, and usually leads to a higher production costs and also a lower cup quality. Some studies suggest that coffee may constitute a climateric fruit indicating that ethylene plays an important role in the coffee fruit ripening process. Coffee early cultivars usually show a more uniform ripening process, although little is known about the genetic factors that promote the earliness of ripening. Thus, in order to better understand the physiological and genetic factors involved in the regulation of ripening time, ethylene and respiration patterns during coffee ripening of early (Catucaí 785-15) and late (Acauã) cultivars were analyzed, as well as the expression patterns of elements from the ethylene biosynthesis and signaling pathways. Ethylene and respiration analyses showed different patterns the between two coffee cultivars. Catucaí 785-15 fruits displayed a typical climacteric raise in respiration and in ethylene production during ripening, while Acauã fruits showed only a slight increased on these parameters. *In silico* analysis allowed the identification of putative members from almost every step of the ethylene biosynthesis and signaling pathways. RT-qPCR analysis of the four biosynthesis genes (*CaACS1-like; CaACO1-like; CaACO4-like* e *CaACO5*) analyzed in this study, showed that they were all induced at the final stages of fruit ripening in both cultivars, specially for C*aACS1-like* and *CaACO4-like* that showed higher expression levels than those found in leaves and flowers, indicating that these genes may play an important role on coffee fruit ripening.On the other hand, members of the ethylene signaling pathway (*CaETR1-like; CaETR4-like; CaEIN2-like; CaEIN3-like e CaERF1*) showed a distinct pattern from that observed for biosynthesis genes, with all of the genes, in both cultivars, showing slightly higher expression levels during the initial stages of development. The expression analysis of the ethylene biosynthesis genes *CaACO1-like* and *CaACO4-like* and the ethylene receptor *CaETR4-like*, suggest that the higher ethylene production levels in Catucaí 785-15 fruits may induce an enhance *CaETR4-like* degradation, leading to an increase in ethylene sensitivity and consequently an earliness in the ripening process of this cultivar. Ethylene production in Acauã fruits may not be sufficient to inactivate the *CaETR4-like* levels and thus ripening changes occur in a slower pace, suggesting that this cultivar show a suppressed climacteric phenotype.

Keywords: Bioinformatics. *Coffea arabica*. Gene expression. Ethylene. Ripening.

SUMÁRIO

PRIMEIRA PARTE

1 INTRODUÇÃO

A economia cafeeira é uma das mais importantes no cenário brasileiro, posicionando o país como o maior produtor e exportador mundial de grãos, além disso, atua na geração de milhares de empregos, diretos e indiretos. O consumo interno de café vem crescendo anualmente, cerca de 3 % sendo o Brasil o segundo maior consumidor. Segundo dados da Associação Brasileira da Indústria do Café- ABIC, este aumento no consumo interno de café, se deu pelo aumento da qualidade da bebida. A estimativa da CONAB para a safra 2012/2013 é de 55,8 milhões de saca de café, mas atualmente o Brasil tem-se preocupado não só em manter o café como uma *commodity*, como também em valorizar como um produto especial, visando à qualidade.

O café é um produto agrícola cuja qualidade final do grão beneficiado é resultado da interação de vários fatores, como as condições climáticas, adubação, tratos fitossanitários, estádio de maturação dos frutos na hora da colheita e cuidados no manuseio, secagem, beneficiamento e armazenamento. Cada vez mais a pesquisa tem se empenhado em controlar, entender e melhorar cada um dos aspectos que influenciam na qualidade da bebida do café, no entanto, nem todos estes aspectos podem ser controlados, a desigualdade na maturação dos frutos, por exemplo, é praticamente inevitável em condições naturais, já que o café apresenta também uma florada desuniforme, podendo haver mais de uma florada, dependendo das condições climáticas da região.

A diferença de maturação existente entre os frutos além de ser um fator que dificulta a colheita prejudica também a qualidade final do produto. Os frutos de café devem ser coletados, somente a partir do momento em que atinjam a maturação, pois é nessa fase que o fruto apresenta todas as características

químicas necessárias para gerar o aroma e paladar ideais. Através do melhoramento genético convencional, tem-se conseguido cultivares de cafés bastante precoces e mais uniformes quanto à maturação de seus frutos, no entanto, pouco se sabe a respeito dos fatores que influenciam para a presença desta característica.

Neste contexto, o objetivo deste trabalho foi acompanhar a maturação de duas cultivares de café, com perfis de maturação distintos: tardio e precoce. Avaliando principalmente as características ligadas a fisiologia e a expressão de genes de biossíntese e sinalização do etileno. Sabe-se que este fitohôrmonio está diretamente relacionando com o amadurecimento de frutos climatéricos. Alguns autores afirmam ser o café um fruto climatérico, mas ainda existe pouca evidência para esta afirmação. Assim, a caracterização dos aspectos fisiológicos e moleculares, durante o amadurecimento de frutos de café é um passo inicial para um melhor entendimento deste processo, embasando pesquisas futuras que visam à obtenção de frutos mais uniformes.

2 REFERENCIAL TEÓRICO

2.1 Fenologia reprodutiva do cafeeiro

O cafeeiro é uma planta bienal, que tem sua fenologia dividida em duas fases que ocorrem simultaneamente: vegetativa e reprodutiva. As plantas de café demoram dois anos para completar o ciclo, diferentemente da maior parte das plantas, que florescem e frutificam no mesmo ano fenológico.

Com o intuito de facilitar a descrição dessas duas fases, Camargo e Camargo (2001) subdividiram-nas em seis fases distintas, sendo duas delas no primeiro ano fenológico, que compreende a fase vegetativa, e as quatro últimas no segundo ano fenológico ou na fase reprodutiva, adaptadas às condições climáticas do Brasil.

No primeiro ano fenológico, a primeira fase vegetativa está relacionada à formação das gemas vegetativas e ocorre normalmente de setembro a março. Já na segunda fase vegetativa, ocorre a maturação das gemas florais, indo normalmente de abril a agosto, período durante o qual é observado um crescimento das gemas florais existentes. Após o completo desenvolvimento, entram em dormência e ficam prontas para a antese, que ocorrerá quando houver um aumento substancial de seu potencial hídrico, causado pela chuva ou irrigação. Nos dois meses finais dessa etapa, julho a agosto, as gemas dormentes produzem um par de folhas pequenas, separando o primeiro ano fenológico do segundo (CAMARGO; CAMARGO, 2001; GOUVEIA, 1984).

No segundo ano fenológico, período reprodutivo, a terceira fase inicia-se com a florada após um aumento do potencial hídrico nas gemas florais maduras (choque hídrico). Após a fecundação, ocorre o processo de formação de frutos (chumbinhos) e a expansão dos frutos. Essa etapa compreende quatro meses, entre setembro e dezembro (CAMARGO; CAMARGO, 2001).

Cafeeiros que recebem, na terceira fase, água com muita frequência têm a floração indefinida. Uma florada principal ocorre quando se verifica um período de restrição hídrica, seguido de chuva ou irrigação abundante (RENA; MAESTRI, 1985).

A quarta fase está relacionada com a granação dos frutos que ocorre entre janeiro e março, com a completa expansão dos frutos. De abril até junho ocorre o processo de maturação dos frutos (fase cinco), onde ocorre um pequeno aumento no tamanho dos frutos e pode-se perceber a mudança completa de coloração dos mesmos. Aproximadamente de 24 a 34 semanas após a antese, a maturação está completa, ou seja, as sementes estão formadas (DAMATTA et al., 2007); e finalmente ocorre a senescência (fase seis), geralmente entre os meses de julho a agosto (CAMARGO; CAMARGO, 2001).

Porém, a bienalidade do café é percebida não só na fenologia, mas afeta diretamente a produção, pois acontece o que chamamos de bienalidade de produção. A produção bienal do cafeeiro é caracterizada por produções elevadas, que acarretam na redução do crescimento vegetativo, através da exaustão de reservas, restrição da atividade dos ápices em crescimento, redução da emissão de novos ramos laterais e diminuição da atividade do sistema radicular. Esses fatores limitam a quantidade de meristemas axilares disponíveis para a formação de inflorescências.

Nos anos de grande produção, os frutos em crescimento são um forte dreno, absorvendo a maior parte da atividade metabólica da planta, reduzindo o desenvolvimento vegetativo. Assim, a energia produzida no período seguinte é mais direcionada à sua recomposição do que à produção de frutos. Como o desenvolvimento dos frutos do cafeeiro se dá na parte nova dos ramos do ano anterior há, portanto, uma produção menor no ano subsequente ao de elevada produção (GOUVEIA, 1984; MEIRELES et al., 2004).

Com relação ao desenvolvimento dos frutos, a formação das sementes é um processo longo, caracterizado por mudanças e evoluções nos tecidos. Este período pode variar de seis a oito meses após a florada e essa variação leva em consideração fatores genéticos e climáticos (DAMATTA et al., 2007).

Após a fecundação, começa o crescimento do fruto, pela divisão e elongação das células do perisperma (0 a 90 Dias Após a Florada = DAF), um tecido transitório que será substituído progressivamente pelo endosperma. O perisperma é constituído de células esclerenquimáticas, remanescentes do tecido nucelar. Com o crescimento do fruto (150 a 200 DAF), este tecido começa a dar lugar ao endosperma, que ficará envolto pelo que sobrou do perisperma o que chamamos de película prateada. O endosperma é o principal tecido de reserva ocupando o maior volume da semente, desse modo, durante a maturação este tecido endurece devido ao acúmulo gradual de proteínas de reserva, sacarose, polissacarídeos complexos e compostos fenólicos. Durante a maturação, ocorre também alteração da cor do pericarpo (CASTRO; MARRACCINI, 2006; PEZZOPANE et al., 2003).

O pericarpo é composto por endocarpo, mesocarpo e exocarpo. O endocarpo também chamado de pergaminho é uma estrutura que envolve completamente a semente e é composto basicamente por fibras e hemicelulose (SALAZAR et al., 1994). O mesocarpo ou mucilagem é uma substância gelatinosa e adocicada, rica em substâncias pécticas, enzimas e açúcares. Em frutos verdes este tecido é rígido e vai se desestruturando durante a maturação, através da ação de enzimas pectinoliticas (CASTRO; MARRACCINI, 2006).

Já exocarpo ou casca é a camada externa do fruto, composto basicamente por celulose e hemicelulose e os pigmentos clorofilados conferem a cor verde durante as fases iniciais de maturação, estes pigmentos vão sendo substituídos durante a maturação por teores de antocianina, pigmentos que conferem cor avermelhada e ou amarelada, sendo um dos fatores que caracteriza

o estádio “cereja” dos frutos (MARÍN-LÓPEZ et al., 2003). Essa coloração do fruto foi usada por Caixeta (1981) para correlacionar o estádio de desenvolvimento do fruto com o ponto de maturação fisiológica.

A maturação dos frutos de café é um dos fatores que afeta a produção, reflexo da desuniformidade desse processo, em razão do florescimento sequencial encontrado nesta espécie, dificultando a colheita e causando perdas na produção.

2.2 Fisiologia Molecular do Etileno durante a Maturação de Frutos

A maturação é o estádio de desenvolvimento dos frutos que antecede a senescência, é quando o fruto está completamente formado, com suas sementes prontas, apto para ser colhido. A sinalização através do hormônio vegetal etileno, é a via mais bem definida, que influência nas mudanças fenotípicas que ocorrem durante a fase de maturação dos frutos.

Durante o processo de maturação, os frutos passam por várias alterações, genes específicos são ativados, ocorrem mudanças na coloração e também alterações químicas e enzimáticas (CASTRO; MARACCINI, 2006). O envolvimento do etileno no processo de amadurecimento tem sido comprovado pelo estudo de plantas geneticamente transformadas, nas quais a inibição da síntese de etileno reduz ou inibe o amadurecimento (SILVA et al., 2004). Além disso, plantas com mutações, que comprometem a síntese normal de etileno, apresentam padrões anormais de amadurecimento (STEPANOVA; ECKER, 2000).

Trabalhos pioneiros relacionados à expansão dos frutos, genética da maturação, tempo de prateleira e à qualidade nutricional, tem focado o tomate (*Solanum lycopersicum*), como modelo (GIOVANNONI, 2004, 2007). Apesar dos elementos essenciais à biossíntese, percepção e transdução de sinal do

etileno se mostrar conservados em diferentes espécies, estudos têm demonstrado grande variação quanto ao número e modo de regulação destes elementos ao longo desenvolvimento dos frutos, afetando diretamente o tempo de maturação dos mesmos (ADAMS-PHILLIPS et al., 2004; BAPAT et al., 2009; TATSUKI; ENDO, 2006).

O etileno é formado a partir do aminoácido metionina via S-Adenosilmetionina (AdoMet), e o precursor imediato do etileno, denominado de Ácido-1-aminociclopropano-1-carboxílico (ACC) (ADAMS; YANG, 1979). AdoMet é sintetizada a partir da metionina por ação da enzima AdoMet sintetase e a conversão de AdoMet em ACC é catalisada pela enzima ACC sintase (ACS) (KENDE, 1993). A ação da ACS produz, além do ACC, a 5-Metiltioadenosina a qual é utilizada para a síntese de uma nova metionina através do ciclo modificado da metionina ou ciclo de Yang (MIYAZAKI; YANG, 1987). Um aumento na taxa respiratória fornece o ATP necessário para o ciclo de Yang e pode permitir que elevados níveis de etileno sejam produzidos na ausência de altos níveis intracelulares de metionina. O ACC gerado nessa etapa é então convertido a etileno, essa conversão é catalisada pela enzima ACC oxidase (ACO), gerando além do etileno, CO_2 e ácido cianídrico (HCN) (YANG; HOFFMAN, 1984).

Em tomate já foram identificados nove genes ACS (*SlACS1A, SlACS1B, e SlACS2*-8) e cinco ACO (*SlACO1-5*) (BARRY et al., 1996; HOEVEN et al., 2002; NAKATSUKA et al., 1998; OETIKER et al., 1997; ZAREMBINSKI; THEOLOGIS, 1994). A regulação da expressão desses genes durante a maturação de frutos tem sido extensivamente estudada, permitindo a constatação de que pelo menos quatro genes *ACS* e três genes *ACO* são diferencialmente expressos ao longo da maturação de frutos (BARRY et al., 1996; BARRY; LLOP-TOUS; GRIERSON, 2000; NAKATSUKA et al., 1998).

Além da importância da regulação dos genes de biossíntese na fase de maturação dos frutos, devemos também destacar a regulação que envolve os genes de sinalização, que são componentes responsáveis pela percepção e ativação das respostas promovidas pelo etileno. Estudos genéticos em espécies modelos (arabidopsis e tomate) caracterizaram diferentes famílias de genes responsáveis pela rota de sinalização do etileno, incluindo *ETR1, CTR1, EIN2 , EIN3/EILs e ERFs* (CHANG; STADLER, 2001; CHEN; ETHERIDGE; SCHALLER, 2005).

A ação do etileno, assim como para os demais fitohôrmios, é dependente de sua ligação a um receptor, o gene *ETR1* (Ethylene Receptor 1) foi inicialmente identificado em Arabidopsis, e estudos anteriores demonstraram que a família de receptores nesta espécie é composta por pelo menos cinco membros: *ETR1* (CHANG et al., 1993; HUA et al., 1995), *ERS1* (Ethylene Response Sensor1) (HUA et al., 1995), *ERS2* (Ethylene Response Sensor2) *EIN4* (Ethylene Insensitive 4) (HUA et al., 1998), e *ETR2* (Ethylene Receptor 2) (SAKAI et al., 1998). As proteínas codificadas por estes receptores se caracterizam pela presença de três domínios: o domínio sensor, localizado na extremidade N-terminal e caracterizado por abrigar o local de ligação ao etileno; o domínio GAF envolvido na interação entre os diferentes tipos de receptores (GAO et al., 2008); e o domínio histidina quinase (CLARK et al., 1998).

Com relação à maturação de frutos, os receptores de etileno constituem um regulador central deste processo em frutos climatéricos, se colocando como um importante alvo de manipulação do tempo de maturação. Em tomate foram identificados seis receptores de etileno, os quais são diferencialmente expressos (KLEE, 2002). Todos receptores apresentaram baixos níveis de expressão durante o desenvolvimento do fruto imaturo, mas durante o amadurecimento pôde ser observado um grande aumento na expressão dos receptores *LeETR3, LeETR4 e LeETR6* (KEVANY et al., 2007).

De acordo com modelo descrito da via de transdução de sinal do etileno, os receptores interagem fisicamente com a proteína CTR1 (Constitutive Triple Response 1), que regula negativamente a via de resposta ao etileno, na ausência do mesmo (CLARK et al., 1998). Embora apenas um gene *CTR1-like* tenha sido identificado em Arabidopsis, quatro foram isolados a partir de tomate, dos quais somente *LeCTR1* apresentou um aumento de expressão durante o amadurecimento (ADAMS-PHILLIPS et al., 2004; LECLERCQ et al., 2002). Atuando após o complexo formado pelos receptores e a *CTR1*, o gene Ethylene *Insensitive 2* (*EIN2*) é um regulador positivo da rota de transdução de sinal do etileno, que através do estudo de mutantes (perda de função) demonstrou um maior grau de insensibilidade ao etileno (ALONSO et al., 1999). Em frutos, foi observado que plantas de tomate com níveis reduzidos da expressão do gene *LeEIN2* apresentaram inibição da maturação, gerada possivelmente pela inibição de genes relacionados a maturação (HU et al., 2010).

No final da via de sinalização estão as famílias de fatores de transcrição *EIN3* e *ERF* (CHÃO et al., 1997; SOLANO et al., 1998). O fator de transcrição *EIN3* atua como um regulador positivo da via de sinalização de etileno e pertence a uma pequena família gênica em Arabidopsis, cujas proteínas possuem funções redundantes. Os membros desta família se ligam em motivos específicos (KOSUGI; OHASHI, 2000; SOLANO et al., 1998) presentes em genes relacionados com a senescência (ITZHAKI; MAXSON; WOODSON, 1994), maturação (BLUME; GRIERSON, 1997; MONTGOMERY et al., 1993; YIN et al., 2010) entre outros fatores de transcrição, tais como *ERF1* (SOLANO et al., 1998). Em tomate foi observado que os fatores de transcrição *EIN3* regulam a sensibilidade ao etileno, causando grande atraso na maturação em plantas que apresentam a versão antisenso para estes gene (TIEMAN et al., 2001).

Ao contrário dos *EIN3*, os genes ERFs constituem uma das maiores famílias de fatores de trancrição, com 122 e 85 membros identificados em

Arabidopsis e de tomate, respectivamente (NAKANO et al., 2006; SHARMA et al., 2010). Os genes ERF de fruto têm sido estudado em várias espécies (BAPAT et al., 2009) e desempenham um papel importante na modulação da maturação induzida pelo etileno em frutos, regulando genes relacionados com a biossíntese de etileno (ZHANG et al., 2009).

Com base na produção de etileno e na taxa de respiratória, os frutos podem ser classificados como climatérico e não-climatérico. Assim, dois sistemas de produção de etileno, foram definidos em plantas, por McMurchie, McGlasson e Eaks (1972), os quais estão associados com a fase pré-climatérica e climatérica. O sistema I é responsável pelos baixos níveis de produção de etileno presente no pré-climatérico e na produção de etileno dos tecidos vegetativos e frutos não climatéricos (ABELES; MORGAN; SALTVEIT JUNIOR, 1992; OETIKER; YANG, 1995). A fase climatérica é decorrente do sistema II da biossíntese de etileno, no qual ocorre a produção autocatalítica. O aumento da produção autocatalítica de etileno se deve ao aumento da atividade da ACC sintase (VENDRELL; PALOMER, 1997).

Alguns estudos abordando a produção de etileno e a regulação de genes envolvidos na sua biossíntese ao longo da maturação de frutos do cafeeiro tem sugerido o café como um fruto climatérico (PEREIRA et al., 2005; SALMONA et al., 2008). Além disso, outros estudos relatam um efeito positivo na antecipação e sincronização da maturação de frutos do cafeeiro pela aplicação exógena de Ethephon (CARVALHO et al., 2003; SCUDELER et al., 2004).

2.3 Espécie modelo para o estudo da maturação de frutos

A planta modelo *Arabidopsis thaliana* é a mais utilizada para o estudo de mutantes em plantas. Porém, algumas plantas de interesse agronômico tem se destacado como modelos genéticos, como o milho (*Zea mays* L.) o arroz (*Oryza*

sativa L.), a ervilha (*Pisum sativum* L.) e o tomateiro (*Solanum lycopersicum* L.). Estudos genéticos relacionados com a formação e desenvolvimento de frutos foram realizados em Arabidopsis (PINYOPICH et al., 2003), enquanto a maturação de frutos tem sido usado o tomateiro como modelo (GIOVANNONI, 2004, 2007; HONG; LEE, 1993), pois esta espécie apresenta frutos carnosos e climatérico.

O tomateiro é considerado uma planta modelo por apresentar características tais como, genoma relativamente pequeno (950 Mb), genes distribuído em 12 cromossomos e facilmente mapeados devido a uma abundância de marcadores associados a características de importância econômica e biológica, além de ser uma espécie diplóide autógama com uma ampla riqueza de germoplasma, constituída por 9 espécies selvagens do gênero Solanum seção Lycopersicon (LI; CHETELAT, 2010) que podem ser cruzadas com a espécie cultivada (STEVENS; RICK, 1986).

Além disso, o tomateiro apresenta um grande número de mutantes bem caracterizados. Já foram descritos mutantes relacionados com as principais classes de hormônios, tais como etileno, giberelinas, citocinina e ácido abscísico (BENSEN; ZEEVAART, 1990; BURBIDGE et al., 1999; CARVALHO et al., 2003; FUJINO et al., 1988; PINO-NUNES, 2005), bem como, brassinoesteroides e ácido jasmônico (LI; LI; HOWE, 2001; MONTOYA et al., 2002). Esse tipo de estudo tem possibilitado a compreensão dos mecanismos que regulam a maturação de frutos, através do estudo dos mutantes ripening-inhibitor (*rin*), nonripening (*nor*), colorless nonripening (*Cnr*), green-ripe (*Gr*), green flesh (*gf*), high pigment1 (*hp1*), high pigment2 (*hp2*), and never-ripe (*Nr*) (BARRY et al., 2008; BARRY; GIOVANNONI, 2006; LANAHAN et al., 1994; LIU et al., 2004; MANNING et al., 2006; MUSTILLI et al., 1999; VREBALOV et al., 2002).

Os locos *rin* e *Cnr* codificam fatores de transcrição MADS box e um SPBP, respectivamente, e são reguladores da maturação (MANNING et al., 2006; VREBALOV et al., 2002). O gene *Gr* interage com componentes de resposta ao etileno em frutos (BARRY; GIOVANNONI, 2006), enquanto que a mutação *Nr* tem sido caracterizada como um receptor de etileno *ERS-like,* com uma baixa capacidade para se ligar ao etileno (LANAHAN et al., 1994).

Atualmente, a cultivar Micro-Tom (MT) tem sido muito utilizado como modelo genético (MEISSNER et al., 1997) para o estudo de mutantes, por possuir porte pequeno, de 10 a 20 cm (EMMANUEL; LEVY, 2002), frutos e sementes viáveis, ciclo de apenas 70-90 dias, facilmente cultivada em laboratório e adequada para a utilização das técnicas de cultura de tecidos. Existem vários mutantes já introgredidos em MT, como o alelo *Rg1* de *S. peruvianum* que foi transferido para a cv MT (LIMA et al., 2004) o que possibilitou melhorias no processo de transformação genética, por aumentar a capacidade de regeneração (PINO et al., 2010).

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SEGUNDA PARTE - ARTIGOS

ARTIGO 1 Physiological and molecular analyses of early and late coffee cultivars at different ripening stages

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NORMAS DA REVISTA CIENTIFICA *ACTA PHYSIOLOGIAE PLANTARUM* (SUBMETIDO)

Abstract Coffee quality is strongly influenced by a great number of factors, among which the fruit ripening stage at harvest time exerts a major influence. Studies comprising ethylene production and the regulation of ethylene biosynthesis genes during the ripening process indicate that ethylene plays an important role on coffee fruit ripening. Early cultivars of coffee usually show more uniform ripening although little is known about the genetic factors that promote the earliness of ripening. Thus, in order to better understand the physiological and genetic factors involved in the regulation of ripening time, and consequently ripening uniformity, this study aimed to analyze ethylene and respiration patterns during coffee ripening, as well as to analyze ACC oxidase (ACO) gene expression, in fruits of early and late cultivars of coffee. Coffee fruits were harvested monthly from 124 days after flowering (DAF) until complete maturation. Dry matter, moisture content, color, respiratory rate and ethylene production analysis were performed. *In silico* analysis identified a coffee ACC oxidase gene (*CaACO-like*) and its expression profile was further analyzed by real-time PCR. Dry matter and relative water content constantly increased and gradually decreased during fruit ripening. Color analysis enabled the observation of the earliness in the ripening process displayed by Catucaí 785-15 and its higher fruit ripening uniformity. The results from respiration rate and ethylene production analysis and the *CaACO-like* gene expression analysis suggest that coffee ripening may differ among cultivars, and may be an ethylene-dependent process, as observed for Catucaí 785-15, or an ethylene independent, as observed for Acauã, which showed a suppressed climacteric phenotype.

Keywords: Coffea arabica. Ethylene. Respiration. Ripening.

Resumo A qualidade do café é fortemente influenciado por um grande número de fatores, entre os quais o estádio de maturação de frutos no momento da colheita exerce uma grande influência. Estudos que compreendem a produção de etileno e a regulação dos genes de biossíntese do etileno durante o processo de amadurecimento indicam que o etileno desempenha um papel importante no amadurecimento de frutos. As cultivares precoces de café geralmente apresentam um amadurecimento mais uniforme, embora pouco se sabe sobre os fatores genéticos que promovem a precocidade de maturação. Assim, a fim de compreender melhor os fatores fisiológicos e genéticos envolvidos na regulação do tempo de amadurecimento e, conseqüentemente, a uniformidade de maturação, este estudo teve como objetivo analisar padrões de etileno e da respiração durante o amadurecimento de café, bem como para analisar a expressão do gene ACC oxidase (ACO), em frutos de cultivares precoces e tardios de café. Frutos de café foram coletados mensalmente, a partir dos 124

dias após o florescimento (DAF) até a completa maturação. Foram avaliados massa seca, teor de umidade, cor, freqüência respiratória e a produção de etileno. Foram feitas análise *in silico* identificando o gene ACC oxidase (CaACO-like) em café e o seu perfil de expressão foi analisado por PCR em tempo real. Para massa seca e o teor relativo de água houve um aumento constante e diminuio gradativamente, durante o amadurecimento dos frutos. A análise de cor permitiu a observação da precocidade no processo de amadurecimento exibido por Catucaí 785-15. Os resultados da taxa de respiração, análise de produção de etileno e a análise da expressão do gene CaACO-like sugere que o amadurecimento de café pode variar entre cultivares, e pode ser um processo dependente de etileno, tal como observado para Catucaí 785-15, ou etileno independente, como observado de Acauã, que mostrou um fenótipo climatérico suprimido.

Palavras-chave: Coffea arabica. Etileno. Respiração. Amadurecimento.

1 INTRODUCTION

Coffee is one of the most valuable traded commodities in the world. It poses as an important source of income and jobs in several tropical countries, including Brazil, the largest producer and exporter of coffee.

Originated in the African continent, coffee belongs to the Rubiaceae family and its genus, Coffea, is represented by more than 100 species (Davis et al. 2006), among which only *Coffea arabica L.* and *C. canephora* Pierre ex Froehner are commercially important, representing 70% and 30% of world production, respectively (Vieira et al. 2006).

Coffee quality, among other factors, is directly associated to the fruit ripening stage at harvest time, which is often highly asynchronous due to the sequential flowering of this species, and usually leads to higher production costs and a lower cup quality.

According to the harvesting time, coffee cultivars can be classified as late, medium and early cultivars. Usually, early cultivars show a higher uniformity in the ripening process of their fruits, although little is known about the genetic factors that control this feature.

Studies comprising ethylene production and the regulation of ethylene biosynthesis genes during coffee fruit ripening (Pereira et al. 2005; Salmona et al. 2008), as well as studies reporting the positive effects of exogenous Ethephon application in fruit ripening synchronization (Carvalho et al. 2003; Scudeler et al. 2004), suggest that coffee constitute a climacteric fruit and ethylene plays an important role on its ripening process.

The plant hormone ethylene is involved in several developmental and physiological process in plants, including seed germination, shoot elongation, fruit ripening, organ abscission, and senescence (Chen et al. 2005; Jacek et al. 2011; Yu et al. 2011; Qi et al. 2012), as well as in biotic and abiotic stress

responses (Wang and Ecker 2002; Yibing et al. 2011). According to their ethylene production and respiration rates, fruits can be classified as climacteric and non-climacteric. Climacteric fruits are characterized by a rapid increase in ethylene biosynthesis, associated to an increase in respiration rate, at the beginning of the ripening process that culminates with fruit ripening. This behavior enables climacteric fruits to complete their maturation after being harvested, while non-climacteric fruits do not show any increase in ethylene production and respiration rate and must complete ripening while being attached to the plant (McMurchie et al.1972; Lelievre et al. 1997).

Although many efforts have been made to better comprehend coffee flowering (Oliveira et al. 2010; Barreto et al. 2012) and ripening (Pereira et al. 2005; Lima et al. 2011), little is known about the ethylene's role in these processes. Thus, in order to better understand the physiological and genetic factors involved in the regulation of ripening time, and consequently ripening uniformity, this study aimed to analyze the ethylene production and respiration rates during coffee fruit ripening, as well as to analyze gene expression of a key ethylene biosynthesis enzyme, ACC oxidase (ACO), in fruits of early and late coffee cultivars.

2 MATERIAL AND METHODS

2.1 Plant material

Fruits from an late, *C. arabica* cv. Acauã, and early, *C. arabica* Catucaí 785-15, coffee cultivars, grown at the Procafe Foundation Experimental Farm (21º 34' 00''E e 45º 24' 22''E) (Varginha, Brazil), were harvested monthly from 124 days after flowering (DAF) until complete maturation. After harvest, fruits were immediately separated to perform the dry matter, moisture content, color,

respiratory rate and ethylene production analysis, or snap frozen in liquid nitrogen and stored at -80°C until RNA extraction. The experimental design was completely randomized with six repetitions in a factorial scheme 2x5, with two cultivars and 5 sampling times. Results were submitted to analysis of variance (ANOVA) and a regression test was performed, using SISVAR.

2.2 Dry matter, relative moisture and color analyzes

A sample of 100 fruits, randomly collected, was used to perform the analysis of dry matter and relative moisture. Fruit color analyses were evaluated by a Minolta Colorimeter (Model CR-300, NY, USA) and each record was averaged from 30 measurements for each coffee cultivar (ten measurements for each replicate). The changes in fruit color were evaluated by the a* parameter, which is an index of red color (i.e. a high positive value means a strong red color while a high negative value means a green color). Moreover, 100 fruits, randomly collected, were used to estimate the percentage of green, yellow-green, cherry, raisin and dry fruits in order to associate fruit color with the optimal harvest time for both cultivars.

2.3 Ethylene production and respiration rate

Ethylene production and respiration rate were assayed by incubating 16 fruits in 50mL air tight flask for 1h at 22°C. For ethylene measurement, samples of 1mL of the head-space gas were withdrawn using a syringe and injected in a gas chromatograph fitted with a RT-QPLOT column at 60°C and flame ionization detector at 250°C. Ethylene was quantified with reference to a standard curve for ethylene concentration and expressed as $\mu L\ C_2H_4\ kg^{-1}\ h^{-1}$. The concentration of CO_2 in the head-space was measured using a PBI

Dansensor CheckPoint CO_2/O_2 gas analyzer and respiration was expressed in mg de CO_2 kg^{-1} h^{-1}. Ethylene CO_2 production rates were assayed for five days at each sampling time.

2.4 *In silico* analysis

In order to identify a putative coffee ACO homolog gene *(CaACO-like*) data mining in the CAFEST database (http://bioinfo04.ibi.unicamp.br), composed by 214,964 expressed sequence tags (EST) obtained from 37 libraries (Vieira et al. 2006), were carried out using plant gene (BLASTn) and protein (tBLASTn) sequences as bait, as well as key word searches. The ORF (Open Reading Frame) of the selected sequence was obtained through the ORFinder tool, from NCBI homepage (http://www.ncbi.nlm.nih.gov) and its protein sequence was generated through the translate tool found in the ExPASY protein database (http://www.expasy.ch). *CaACO-like* similarity to ACO sequences from other species was accessed through a conserved domain analysis and amino acid sequence alignments by the ClustalW program (Thompson et al.1994), using default parameters.

2.5 RNA isolation and cDNA synthesis

Total RNA from fruit samples of the five sampling times from both cultivars was extracted by the CTAB method (Chang et al. 1993), with minor alterations (Paula et al. 2012). RNA samples (5.0µg) were treated with DNase I using Turbo DNA-free Kit (Ambion) for elimination of residual DNA contamination. RNA was quantified by spectroscopy (Nanodrop® ND-1000) and its integrity was visually analyzed in 1% agarose gel. The cDNA was synthesized from 1.0µg of DNA-free RNA using the High-Capacity cDNA

Reverse Transcription kit (Applied Biosystems) following the manufacturer's protocol.

2.6 Primer design and real time quantitative RT-PCR

Real-time quantitative PCR was performed using 10ng of cDNA in a 10 μL reaction volume with SYBR Green UDG Master Mix with ROX (Invitrogen) on an ABI PRISM 7,500 Real-Time PCR thermalcycler (Applied Biosystems). CaACO-like primer (forward primer5′ACGTGGAAGCCAATGTTACC and reverse primer5′GAGGGAGAAGAAAACATCCTAGC) design was performed using the sequence obtained in the *in silico* analysis and the Primer Express v2.0 program (Applied Biosystems). RT-PCR conditions were as follow: 95°C (15 min), then 40 cycles of 95°C and 60 °C (15s), followed by 1 min at 60°C, and completed with a melting curve analysis program. Each sample was formed from cDNAs of three different biological samples and was run in three technical replicates on a 96-well plate. For each sample, a Ct (threshold cycle) value was calculated from the amplification curves by selecting the optimal ΔRn (emission of the reporter dye over starting background fluorescence) in the exponential portion of the amplification plot. Relative fold differences were calculated based on the comparative Ct method using *β-actin*, with forward primer 5′-AATTGTCCGTGACATCAAGGAA-3' and reverse primer 5′-TGAGCTGCTTTGGCTGTTC-3', and *GAPDH*, with forward primer 5′-TTGAAGGGCGGTGCAAA-3' and reverse primer 5′-AACATGGGTGCATCC-3', as reference genes (Barsalobres-Cavallari et al., 2009). To demonstrate that the efficiencies of the different gene primers were approximately equal, the absolute value of the slope of log input amount versus ΔCt was calculated for *CaACO-like*, *β-actin* and GAPDH sequences, and was determined to be <0.1. To determine relative fold differences for each sample,

the Ct value for *CaACO-like* was normalized to the Ct value for *β-actin* and *GAPDH*, and was calculated relative to a calibrator using the formula $2^{-\Delta\Delta Ct}$. The calibrator was the sample that exhibited the minimum level of transcripts in the whole experiment (Acauã fruits at 124 DAF).

3 RESULTS AND DISCUSSION

3.1 Color analysis

Values for the a* parameter clearly show the change in coffee fruit color from green (negative values) to red (positive values) for both cultivars analyzed, and it could be found a significant interaction between cultivar and ripening time (Figure 1). Catucaí 785-15 showed higher a* values than Acauã at 184 and 214 DAF, demonstrating its earliness when compared to Acauã.

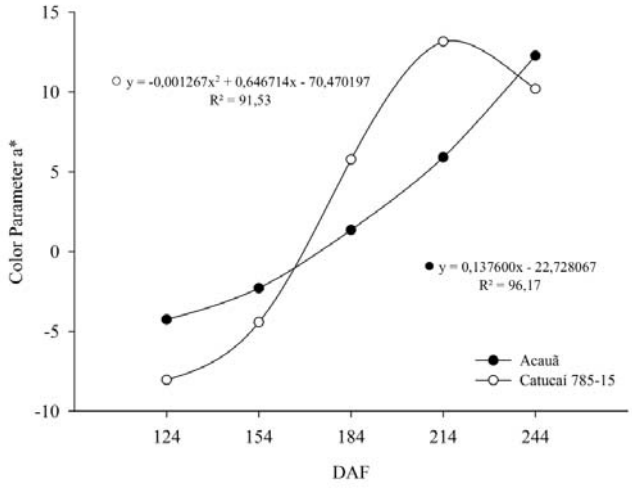

Figure 1Color a* values for Acauã and Catucaí 785-15 coffee fruits at five different ripening stages.

Coffee fruit color may be considered as an indicative parameter for the optimal time for fruit harvest, as well as for cup quality. Pimenta and Vilela (2002) observed that the low cup quality from coffee green fruits is related to potassium leaching, high acidity levels and increased levels of chlorogenic acids. Moreover, at this stage, sugar levels are still low compared to fruits at the cherry stage, where fruits have reached their maturity and provide a higher cup quality, being considered the optimal stage for fruit harvest (Carvalho and Chalfoum, 2000; Pimenta et al. 2000).

As shown in Figure 2, fruit color posed as a good parameter for indicating the optimal fruit harvest time, in which green fruits should represent less than 20% and cherry fruits make up the great majority of fruits (Nogueira et al. 2005). It also enabled the observation of the earliness in the ripening process displayed by Catucaí 785-15 and its higher fruit ripening uniformity (Figure 2). At 184 DAF, 74.1% and 24,2% of fruits from Acauã were at the cherry and green stages, respectively, compared to 84% and 13,1% for the early cultivar Catucaí 785-15, showing that this cultivar reaches its optimal harvest time one month earlier than Acauã. The a* parameter values also corroborates with these results (Figure 1).

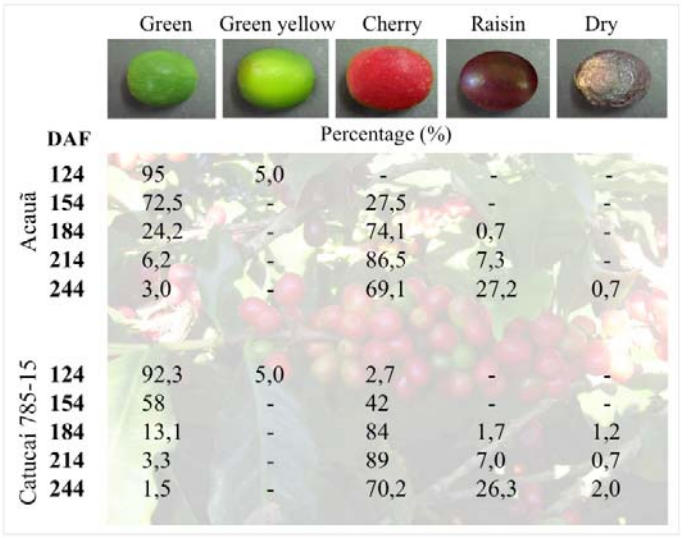

Figure 2 – Visual aspect and color percentages of green, green-yellow, cherry, raisin and dry fruits for Acaua and Catucai 785-15 coffee cultivars at five sampling times.

3.2 Dry matter and relative moisture content

A significant interaction for dry matter and relative water content were found among the sampling times, but not between cultivars (Figure 3). Dry matter constantly increased during fruit development and reached its maximum at 244 DAF with 38,55g (Figure 3), where a low and high percentage of green and raisin fruits were found (Figure 2), respectively. From 124 to 154 DAF the dry matter increase is mainly related to cell elongation and expansion and fruits reach around 80% of their final dry matter (Cunha and Volpe, 2011). Then, fruits enter in the reserve storage phase, characterized by a reduction in fruit growth rate and dry matter accumulation (Rena et al. 1994), corroborating with the results found in this study. Relative water content gradually decreased during fruit development and stabilized at 214 DAF. At this stage, fruits reach their

physiological maturity and the reduction in their water content is mainly associated to endosperm hardening and seed formation (Silva and Volpe, 2005).

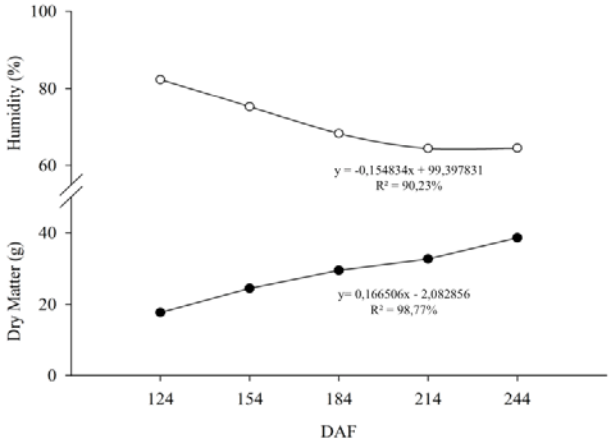

Figure 3 – Dry matter accumulation and relative water content at five different coffee ripening stages.

3.3 Respiration rate and ethylene production

The CO_2 production rate differed between the two coffee cultivars with Catucaí 785-15 showing a typical respiration climacteric that reached its maximum at 184 DAF with 22,10 mg CO_2 Kg^{-1} h^{-1} (Figure 4). This pattern of CO_2 production was not observed for Acauã fruits that showed only a slight increase on their respiration rate from 154 DAF to 184DAF, although values were statistically different among the sampling times (Figure 4).

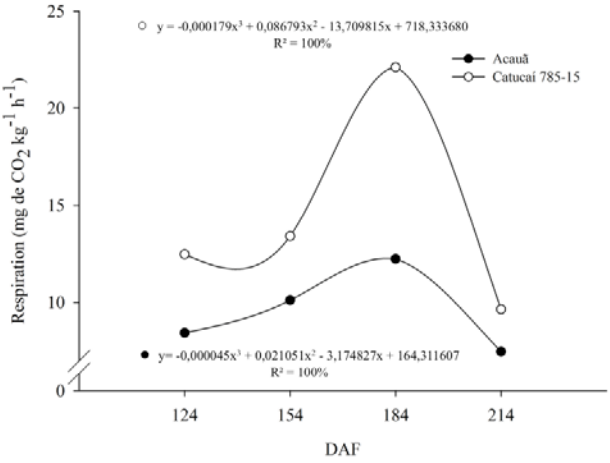

Figure 4 – Respiration rate for Acauã and Catucai 785-15 fruits at five different ripening stages.

These results diverge from those found by Pushmann (1975) on coffee fruit pericarp, where no increase in respiration rate, followed by a decrease associated to fruit senescence, both patterns typical of climacteric fruits, were found from 154 DAF to 231 DAF. Thus, this study clearly shows the climacteric phase for Catucaí 785-15 fruits and corroborates with the results found by Marin-Lopez et at (2003) where coffee fruits also displayed a respiratory climacteric pattern after harvest. However, Acauã fruits did not show a significant increase in their respiratory rate wich indicates that not every coffee cultivar display a typical climacteric phase.

The ethylene production rates showed similar patterns compared to those found for fruit respiration rates. Catucaí 785-15 fruits displayed a typical climacteric raise in ethylene production, while Acauã fruits displayed the same pattern, although in a much lesser extent (Figure 5). Although fruits are physiologically classified as climacteric or non-climacteric based on the presence of a rapid increase in the respiration and ethylene production rates at

the beginning of ripening process, some species may exhibit climacteric and non-climacteric varieties, such as melon (Périn et al. 2002) and pear (Yamane et al. 2007), and also varieties showing a suppressed climacteric phenotype, such as plum (Abdi et al. 1997; El- Sharkawy et al. 2007), what seems to be the case for Acauã (Figure 5).

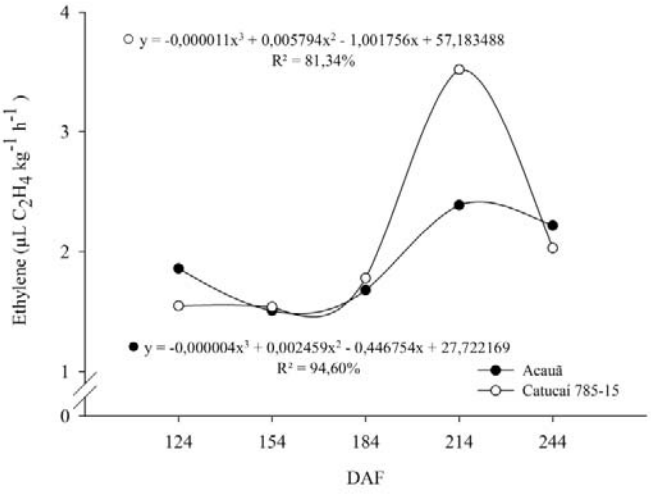

Figure 5 – Ethylene production for Acauã and Catucaí 785-15 fruits during at five different ripening stages.

According to Chitarra and Chitarra (2005), non-climacteric fruits display a slow ripening process when compared to climacteric fruits, since the increase in ethylene production induces a higher respiratory rate, which act as an indicative of the speed with which changes in fruit composition occur, and influenced by fruit composition and chemical alterations that take place during the ripening process. However, the respiratory peak (Figure 4) for coffee fruits anticipated the increase in ethylene production (Figure 5), a common pattern

found in some species that show an ethylene-dependent ripening (Biale et al. 1954; Kosiyachinda and Young 1975).

3.4 *In silico* and gene expression analyses

The annotated coffee ACC oxidase (*CaACO-like*) on GenBank showed a high similarity to previously described ACO sequences from different plant species (Figure 6). The conserved domain analysis highlighted that *CaACO-like* possess all 12 conserved residues (P4, A27, G32, H39, H186, D188, L204, Q205, G227, H243, R253, S255) that characterize the superfamily of iron-ascorbate oxidases (Tang et al. 1993; Lin et al. 1997). Moreover, *CaACO-like* showed high amino acid identity with ACO sequences from other species such as *Arabidopsis thaliana*, *Nicotiana tabacum*, *Solanum lycopersicum*, with identity values of 74%, 82% and 85%, respectively (Figure 6).

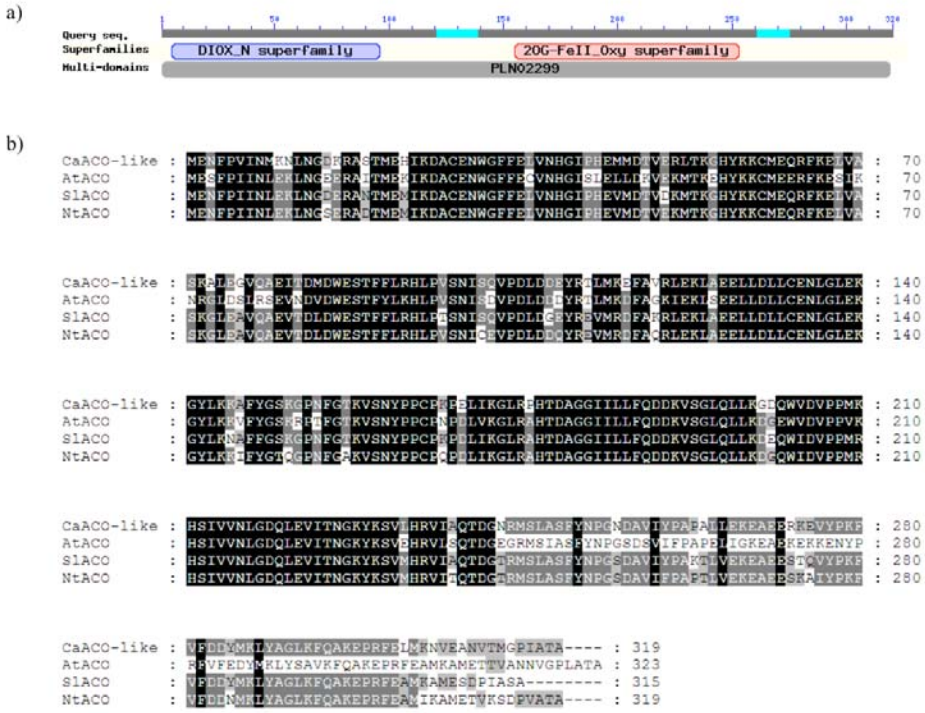

Figure 6 – Conserved domain analysis for *CaACO-like* generated by the Conserved Domains tool from NCBI (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) (a). Deduced amino acid comparison analysis of *CaACO-like* and ACO sequences from *Arabidopsis thaliana* (15220386), *Nicotiana tabacum*(5751171), *Solanum lycopersicum*(14573461). The alignment was performed by ClustalW program and displayed with GeneDoc. Identical amino acid residues in relation to *CaACO-like* are shaded in black and conserved residues are in gray. Inverted slashes indicate gaps inserted for alignment optimization. Amino acid positions are shown on the right.

CaACO-like gene expression corroborates with results obtained for ethylene production (Figure 5), and fruits from Catucaí 785-15, when compared to Acauã fruits, showing a higher expression level for this gene throughout the experiment, reaching its maximum expression level at 214 DAF (Figure 7). Acauã fruits *CaACO-like* gene expression did not show any change during the

last three ripening stages (Figure 7). Ethylene plays an important role during the ripening process of climacteric fruits triggering modifications in fruit color, through chlorophyll degradation and carotenoid and flavonoid biosynthesis, fruit texture, through alterations in cell turgor and/or cell wall metabolism, and fruit flavor, aroma and nutritional quality, modifying fruit sugars, acids and volatile profiles (Giovannoni, 2004).

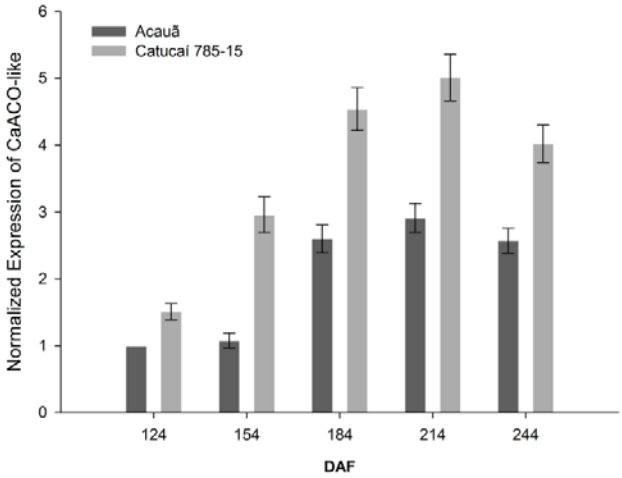

Figure 7 – Relative quantitative expression profiling of *CaACO-like* in Acauã and Catucaí 785-15 coffee fruits at five different ripening stages. Columns represent the fold difference in gene expression relative to Acauã fruits at 124 DAF. Expression values for each biological sample were obtained from three technical replicates and error bars represent the standard errors for three technical replicates. Gene transcripts were normalized by expression of two reference genes (*Actin* and *GAPDH*).

The results obtained in this study corroborates with those found by Pereira et al (2005), where ACC oxidase showed low expression levels in the beginning of the ripening process (green fruits) and high expression levels in the

following ripening stages. Moreover, a strong expression of two ACC oxidase genes, one just prior the climacteric crisis the other during the late stages of coffee fruit ripening (Salmona et al., 2008). However, the lower expression level of *CaACO-like* in Acauã fruits, suggest coffee may include both climateric and suppressed climacteric cultivars, such as plum (Abdi et al. 1997; El- Sharkawy et al. 2007). In suppressed climacteric phenotypes, ethylene production rates increase during the latter stages of the ripening process but are low when compared to climacteric cultivars, not being able to develop a climacteric (Abdi et al.1997).

Thus, the results from respiration rate and ethylene production analysis, as well as the results from *CaACO-like* gene expression analysis, suggest that coffee fruit ripening may differ among cultivars, and may be an ethylene-dependent process, as observed for Catucaí 785-15, or ethylene-independent, as observed for Acauã, which showed a suppressed climacteric phenotype with only a slight increase in ethylene production associated to ripening.

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ARTIGO 2 Identification and expression analysis of nine genetic elements of the ethylene biosynthesis and signaling pathways in early and late coffee cultivars

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NORMAS DA REVISTA CIENTIFICA *JOURNAL OF PLANT PHYSIOLOGY*

Abstract
The plant hormone ethylene is involved in several developmental and physiological processes in plants, including senescence, fruit ripening and organ abscission, as well as biotic and abiotic stress responses. Some studies comprising ethylene production and the regulation of ethylene biosynthesis genes during the ripening process, in addition to the higher fruit ripening synchronization generated by exogenous Ethephon application, indicates that ethylene plays an important role on coffee fruit ripening. Coffee early cultivars usually show a more uniform ripening process although little is known about the genetic factors that promote the earliness of ripening. Thus, this work aimed to characterize *in silico* the putative members of the coffee (Coffea arabica) ethylene biosynthesis and signaling pathways, as well as to analyze the expression patterns of nine of these members during fruit ripening of early (Catucaí 785-15) and late (Acauã) coffee cultivars. Data mining in the CAFEST database allowed the identification of members from every step of these pathways, except for the signaling molecule CTR1. The phylogenetic trees showed that coffee sequences displayed high similarity levels to tomato sequences, and the *in silico* expression profile showed that these candidate genes are expressed in different tissues, developmental stages and conditions, and indicated that ethylene may have important functions in process such as coffee flowering and ripening, as well as in abiotic and biotic stress responses. RT-qPCR analysis of the four biosynthesis genes (*CaACS1-like; CaACO1-like; CaACO4-like* e *CaACO5*) analyzed in this study, showed that *CaACO1-like* and *CaACO4-like* displayed an expression pattern typically observed in climateric fruits, being up-regulated during ripening. *CaACS1-like* gene expression was also up-regulated during fruit ripening of both cultivars, although in a much lesser extent when compared to the changes in *CaACO1-like* and *CaACO4-like* gene expression. CaACO5-like was only induced in raisin fruit and may be related to senescence processes. On the other hand, members of the ethylene signaling pathway (*CaETR1-like; CaETR4-like; CaEIN2-like; CaEIN3-like* e *CaERF1*) showed slightly higher expression levels during the initial stages of development (green and yellow green fruits), except for the ethylene receptors *CaETR1-like* and *CaETR4-like*, which was constitutively expressed and induced in cherry fruits, respectively. The higher ethylene production levels in Catucaí 785-15 fruits, indicated by the expression analysis of CaACO1-like and CaACO4-like, suggest that it promotes an enhanced CaETR4-like degradation, leading to an increase in ethylene sensitivity and consequently to an earliness in the ripening process of this cultivar. Ethylene production in Acauã fruits may not be sufficient to inactivate the CaETR4-like levels and thus ripening changes occur in a slower pace.

Keywords: Ethylene. Biosynthesis. Signaling. Maturation.

Resumo

O hormônio vegetal etileno, está envolvido em vários processos do desenvolvimento fisiológico em plantas, incluindo a senescência amadurecimento dos frutos, e abscisão de órgãos, bem como as respostas ao estresse biótico e abiótico. Alguns estudos visando compreender a produção de etileno e a regulação de genes da biossíntese durante o processo de amadurecimento, além da aplicação de Ethephon, para a sincronização da maturação, indica que o etileno tem um papel importante no amadurecimento de frutos de café. Cultivares de café precoce geralmente apresentam um processo de maturação mais uniforme, embora pouco se sabe sobre os fatores genéticos que promovem a precocidade na maturação. Assim, este trabalho teve como objetivo caracterizar, *in silico,* os possíveis membros da via de sinalização e biossíntese de entileno em café (Coffea arabica), bem como analisar os padrões de expressão de nove desses membros durante a maturação de precoce de frutos de cultivares de café precoce (Catucaí 785-15) e tardia (Acauã). A busca no banco de dados CAFEST permitiu a identificação de membros em cada etapa destas vias, exceto o CTR1 molécula de sinalização. As árvores filogenéticas mostraram que as sequências de café apresentaram níveis elevados de similaridade com sequências de tomate, e o perfil de expressão *in silico* mostraram que estes genes candidatos são expressos em diferentes tecidos, fases do desenvolvimento e condições, e indicando que o etileno pode ter funções importantes nesses processos, tais como na floração e maturação de café, bem como na resposta ao stress biótico e abiótico. A análise por RT-qPCR dos genes da biossíntese (*CaACS1-like; CaACO1-like; CaACO4-like* e *CaACO5-like)* realizados neste estudo, mostraram que CaACO1-like e *CaACO4-like* apresentam um padrão de expressão, tipicamente observado em frutos climatéricos, sendo auto regulado durante o amadurecimento. A expressão do gene *CaACS1-like* também foi auto regulada durante a maturação das duas cultivares, embora em um nível muito menor, quando comparado com as mudanças na expressão gênica de *CaACO1-like e CaACO4-like.A expressão de CaACO5-like* só foi induzida em frutos passas e pode estar relacionada com o processo de senescência. Por outro lado, os membros da via de sinalização de etileno (*CaETR1-like; CaETR4-like; CaEIN2-like; CaEIN3-like* e *CaERF1-like*) mostraram níveis de expressão ligeiramente mais elevados durante as fases iniciais do desenvolvimento (em frutos verde e verde amarelo), excepto para os receptores de etileno *CaETR1-like* e *CaETR4-like*, os quais foram expressos em frutos cereja. Os níveis mais elevados de produção de etileno em frutos da cultivar Catucaí 785-15, indicadas pela análise de expressão de *CaACO1-like* e *CaACO4-like*, sugerem que ocorre uma degradação mais eficiente de *CaETR4-like*, conduzindo a um aumento da sensibilidade de etileno e, em consequência, uma precocidade na o processo de amadurecimento dessa cultivar. A produção

de etileno na Acauã pode não ser suficiente para inactivar os níveis *CaETR4-like* e, assim, as alterações de maturação ocorrem em um ritmo mais lento.

1 INTRODUCTION

The plant hormone ethylene is involved in many aspects of plant life cycle, including organ abscission, seed germination, growth transition from vegetative phase to reproductive phase, flowering, fruit ripening, senescence, and is also involved in biotic and abiotic stress responses. Ethylene production is tightly regulated by internal and external signals during development and varies according to the tissue or organ and its developmental stage, with meristematic tissues, stress conditions and fruit ripening displaying the highest ethylene production rates (Abeles et al., 1992).

Fruit ripening is a highly coordinated, genetically programmed, and an irreversible phenomenon involving a series of physiological, biochemical, and organoleptic changes that leads to the development of a soft and edible ripe fruit with desirable quality attributes (Prasanna et al. 2007). Based on their ethylene production and respiration rates, fruits can be classified as climacteric and non-climacteric. Climacteric fruits, such as tomato, avocado, banana, peaches, plums and apples, are characterized by a rapid increase in ethylene biosynthesis, associated to an increase in respiration rate, at the beginning of the ripening process that culminates with fruit ripening. This behavior enables climacteric fruits complete their maturation after being harvested, while non-climacteric fruits, such as strawberry, grape, and citrus, do not show any increase in ethylene production and respiration rates and must complete ripening attached to the plant (Mcmurchie et al., 1972; Lelievre et al., 1997).

Two systems of ethylene regulation have been proposed to operate in plants: System 1 and System 2. System 1 operates in both climacteric and non-climacteric fruits, as well as in vegetative tissues, and is responsible for

producing basal ethylene levels. System 2 operates during the ripening of climacteric fruits and senescence of some petals when ethylene production is autocatalytic (McMurchie et al. 1972). Considering its gaseous nature, ethylene responses may be regulated by its concentration, controlled by its biosynthesis, degradation and conjugation, and sensitivity, which is associated to the presence of receptors and a signaling pathway (Davies, 2003). Pioneering work on the genetic basis of early steps in fruit formation and development were performed in the model system Arabidopsis (Pinyopich et al., 2003; Roeder et al., 2003), whereas investigations of organ expansion, maturity, ripening, shelf-life and nutritional quality have centered on the crop model tomato (Solanum lycopersicum) (Giovannoni, 2004, 2007). Although the essential elements of ethylene biosynthesis, perception and signal transduction are apparently conserved among species, family composition and regulation mode can vary substantially.

Ethylene production in plant tissues results from Met metabolism and the rate-limiting steps in fruit ethylene synthesis include the conversion of S-adenosylmethionine to 1-aminocyclopropane-1carboxylic acid (ACC) via ACC synthase (ACS) and the subsequent metabolism of ACC to ethylene by ACC oxidase (ACO). ACS and ACO are encoded by multigene families in higher plants, with tomato possessing at least nine ACS (SlACS1A, SlACS1B, and SlACS2-8) and five ACO (SlACO1-5) (Barry et al., 1996; Nakatsuka et al., 1998; Oetiker et al., 1997; Zarembinskia; Theologis 1994). Expression analysis has revealed that at least four *ACS* and three *ACO* genes are differentially expressed in tomato fruit (Barry et al., 1996, 2000; Nakatsuka et al., 1998).

Ethylene action takes place via the ethylene signaling pathway. Genetic studies of ethylene action in higher plants, especially in Arabidopsis and tomato, have established a linear ethylene signal transduction model, in which ethylene is perceived by a receptor family, and the signal is mediated downstream by

members of different gene families including *CTR1*, *EIN2*, *EIN3*/*EILs*, and *ERFs* (Chang and Stadler, 2001; Chen et al., 2005).

Five and six ethylene receptors have been identified in Arabidopsis and tomato, respectively. Although they have a very similar structure, each receptor may display a distinct expression pattern, as observed in tomato, where a subset of receptors (NR, SlETR4 and SlETR6) are strongly induced during ripening (Kevany et al., 2007). Downstream of the receptors is the Raf-like protein kinase (MAPKKK), At-CTR1 (Kieber et al., 1993). According to the model, ethylene receptors and CTR1 physically interact to negatively regulate ethylene response pathway in the absence of ethylene (Clark et al., 1998). Although only one *CTR1-like* gene has been identified in Arabidopsis, four have been isolated from tomato, with SlCTR1 being up-regulated by ethylene and during ripening (Leclercq et al., 2002; Adams-Phillips et al., 2004). Further downstream of the receptor-CTR1 complex is an *Nramp*-related integral membrane protein, EIN2, which is absolutely required for ethylene signaling (Alonso et al., 1999) and whose reduced expression level may lead to ripening inhibition, possibly due to down-regulation of ripening-related genes, as observed for the breaking cell wall enzyme Polygalacturonase (Hu et al., 2010). At the end of the signaling pathway are the EIN3 and ERF families of transcriptional factors (TFs) (Chao et al., 1997; Solano et al., 1998). EIN3 acts as a positive regulator of the ethylene signaling pathway and belongs to a small gene family that includes EIN3 and various EIN3-like (EIL) proteins (Chao et al., 1997; Tieman et al., 2001; Yokotani et al., 2003). Members of this family have been shown to directly bind to specific motifs (Solano et al., 1998; Kosugi and Ohashi, 2000) present in genes related to senescence (Itzhaki et al., 1994), ripening (Montogomery et ., 1993; Blume & Grierson, 1997; Yin et al., 2010) and other TFs, such as ERF1 (Solano et al., 1998). Unlike EIN3/EILs, ERFs constitute one of the largest TF gene families, with 122 and 85 members identified in Arabidopsis and tomato,

respectively (Nakano et al., 2006; Sharma et al., 2010). Fruit ERF genes have been isolated from several species (Bapat, 2010) and play an important role in modulating ethylene induced ripening, regulating genes related to ethylene biosynthesis (Zhang et al., 2009) and breaking cell wall enzymes (Yin et al., 2010).

According to time the fruits got ripe, coffee cultivars can be classified as late, medium and early cultivars. Usually, early cultivars show a higher uniformity in the ripening process of their fruits, although little is known about the genetic factors that control this feature coffee quality, among other factors, is directly associated to the fruit ripening stage at harvest time, which is often highly asynchronous due to the sequential flowering found in this species, and usually leads to higher production costs and a lower cup quality studies comprising ethylene production and the regulation of ethylene biosynthesis genes during coffee fruit ripening (Pereira et al., 2005; Salmona et al., 2008), as well as studies reporting the positive effects of exogenous Ethephon application in fruit ripening synchronization (Carvalho et al., 2003; Scudeler et al., 2004), suggest that coffee constitute a climacteric fruit and ethylene may be one of the genetic factors involved the regulation of ripening time, as observed in other species (El-Sharkawy et al., 2007; El-Sharkawy et al., 2008), directly affecting coffee ripening uniformity.

Although many efforts have been made to better comprehend coffee flowering and ripening (Pereira et al., 2005; Oliveira et al., 2010; Lima et al., 2011; Barreto et al., 2012), little is known about ethylene's role in these processes. Thus, in order to better comprehend coffee ethylene biosynthesis and signaling pathways, as well as ethylene's role during coffee fruit ripening, this study isolated and analyzed the expression patterns of nine components from these two pathways in fruits of early and late coffee cultivars.

2 MATERIAL AND METHODS

2.1 *In silico* analysis

Putative homolog genes of the coffee ethylene biosynthesis (*ACS* and *ACO*) and signaling (*ETR, EIN2, EIN3, ERF*) pathways, partially identified in a previous study (Lima et al., 2011), were obtained from data mining in the coffee (*Coffea arabica*) expressed sequence tag (EST) database CAFEST (http://bioinfo04.ibi.unicamp.br), composed by 214,964 ESTs distributed into 37 cDNA libraries sequenced from the 5' end (Vieira et al., 2006). Data mining in the CAFEST database was carried out using plant gene (BLASTn) and protein (tBLASTn) sequences as bait, as well as keyword searches. The sequences with significant similarity (e-value$<10^{-4}$) were selected and sent to the sequence manager and manipulation system, the GeneProject, and submitted to clustering by using the CAP3 program (Huang and Madan, 1999), forming the EST contigs and singlets. Data validation was performed by local tBLASTx and tBLASTn searches of the retrieved sequences against the GenBank database. The Open-reading frame (ORF) of the validated sequences was obtained through the ORFinder tool (NCBI).

2.2 Phylogenetic and *in silico* expression analyses

Protein sequence alignments were performed by the ClustalW program (Thompson et al., 1994), using default parameters, and phylogenetic trees were generated by the MEGA software, version 4.0 (Tamura et al., 2007), with neighbor-joining comparison model (Saitou and Nei, 1987), p-distance method and pair-wise suppression. Bootstrap values from 1000 replicates were used to assess the robustness of the trees.

In silico qualitative gene expression profiling was performed using virtual Northern blot analyses of the coffee EST database. The frequency of reads from each contig and singlet in the CAFEST libraries was calculated, and data normalization enabled the comparison of gene expression in each treatment and plant organ. Normalization consisted of multiplying each read by the ratio between the total number of reads from all libraries and the read number of the library where it was expressed. The results were plotted in a matrix and gene expression patterns among ESTs and libraries were obtained by hierarchical clustering, performed by the Cluster v.2.11 program (Eisen et al., 1999). Graphic outputs were generated by the TreeView v.1.6 software (Eisen et al., 1999).

2.3 Plant material

Fruits were harvested from coffee (*Coffea arabica*) cultivars Catucaí 785-15 and Acauã grown at the experimental farm of the Procafé Foundation (21º34’ 00’’E e 45º 24’ 22’’E) (Varginha, Brazil). These two varieties were chosen according to their maturity times, early and late, respectively. Fruits were harvested monthly from 94 days after flowering (DAF) until complete maturation, making up six sapling times: 94, 124, 154, 184, 214 and 244 DAF. At each harvest time, fruits were separated according to their ripening stage, which was based on the following fruit colors: green, yellow-green, light red, cherry and raisin. Other tissues such as young leaves, mature leaves and flowers were collected from the same coffee trees. All plant material was frozen in liquid nitrogen and stored at -80°C.

2.4 RNA isolation and cDNA synthesis

Total RNA from fruit samples, separated by their colors, of the six sampling times was extracted by the CTAB method (Chang et al., 1993), with minor alterations (Paula et al., 2012). RNA samples (5.0μg) were treated with DNase I using Turbo DNA-free Kit (Ambion) for elimination of residual DNA contamination. RNA was quantified by spectroscopy (Nanodrop® ND-1000) and its integrity was visually analyzed in 1% agarose gel. The cDNA was synthesized from 1.0μg of DNA-free RNA using the High-Capacity cDNA Reverse Transcription kit (Applied Biosystems) following the manufacturer's protocol.

2.5 Primer design and real time quantitative PCR (RT-qPCR)

Real-time quantitative PCR was performed using 10ng of cDNA in a 10 μL reaction volume with SYBR Green UDG Master Mix with ROX (Invitrogen) on an ABI PRISM 7,500 Real-Time PCR thermalcycler (Applied Biosystems). Based on the sequences obtained in the in silico analysis, primers for one putative *ACS (CaACS1-like),* three *ACO* (*CaACO1-like*, *CaACO4-like* and *CaACO5-like*), two ethylene receptors (*CaETR1-like* and *CaETR4-like*), one *EIN2* (*CaEIN2-like*), one *EIN3* (Ca*EIN3-like*) and one *ERF* (*CaERF1-like*) genes were designed using the Primer Express v2.0 program (Applied Biosystems) (Table 1). RT-qPCR conditions were as follow: 95°C (15 min), then 40 cycles of 95°C and 60 °C (15s), followed by 1 min at 60°C, and completed with a melting curve analysis program. Each sample was formed from cDNAs of three different biological samples and was run in three technical replicates on a 96-well plate. For each sample, a Ct (threshold constant) value was calculated from the amplification curves by selecting the optimal ΔRn

(emission of the reporter dye over starting background fluorescence) in the exponential portion of the amplification plot. Relative fold differences were calculated based on the comparative Ct method using *β-actin* and *GAPDH* as reference genes (Table 1) (Barsalobres-Cavallari et al., 2009). To demonstrate that the efficiencies of the different gene primers were approximately equal, the absolute value of the slope of log input amount versus ΔCt was calculated for each target gene sequence, as well as for the reference genes, and was determined to be <0.1. To determine relative fold differences for each sample, the Ct value for each target gene was normalized to the Ct value for *β-actin* and *GAPDH*, and was calculated relative to a calibrator using the formula $2^{-\Delta\Delta Ct}$. Expression levels of Acauã green fruits at 94 DAF were used as a calibrator for all genes under study.

Table 1: Real-time quantitative PCR primers.

Gene	Foward primer (5' to 3')	Reverse primer (5' to 3')
CaACS1-like	TCCTTACCATCCCACCAGAA	CCATGAATTTGTTCGCTCCT
CaACO1-like	ACGTGGAAGCCAATGTTACC	GAGGGAGAAGAAAACATCCTAGC
CaACO4-like	CGCAACTGTTTGAGATCACG	CCAATCCAAGCATTAACAAGG
CaACO5-like	GCTCTTGTATCCCGGAGGTT	GAGTTTGGGAGCCTTGTCAG
CaETR1-like	CAAAACTCCGACCTTCTGGA	CATAGCGCTTTGTTGACAGC
CaETR4-like	TTGGTCCATTCAGGAACTCG	GCATCCTGTTTTGCTTGTTG
CaEIN2-like	CTTATGGAAAGCAGGCCAGA	GGAGTTGAAGGCAAAAGCAG
CaEIN3-like	CCACGGATTTCAGGACAGAT	TGGCTGGACAAATGACTGAG
CaERF2	TTCCAACCCCAGCCTTACTA	TAAGCCCAGGAAAGATTCCA
GAPDH	TTGAAGGGCGGTGCAAA	AACATGGGTGCATCCTTGCT
β-Actin	AATTGTCCGTGACATCAAGGAA	TGAGCTGCTTTGGCTGTTC

3 RESULTADOS

3.1 Phylogenetic analysis

The search for putative homolog genes of the coffee ethylene biosynthesis and signaling pathways in the CAFEST database allowed the identification of members from every step of these pathways, except for the signaling molecule *CTR1*, and also enabled the identification of additional sequences compared to those found by Lima et al (2011), such as the ethylene receptors *CaERT3-like* and *CaETR4-like* (Figure 1).

Four and three sequences related to the ethylene biosynthesis enzymes ACS and ACO were found in the CAFEST database, respectively. Coffee ACS sequences, designated *CaACS1-like*, *CaACS2-like*, *CaACS3-like* and *CaACS4-like* encode for incomplete ORFs, 372, 693, 288 and 366bp, respectively. The phylogenetic tree indicated the high similarity levels between the putative coffee ACS enzymes with ACS from tomato (Figure 1). *CaACS1-like* and *CaACS2-like* displayed amino acid identities of 76 and 69% with SlACS1 and *SlACO4*, respectively, compared to 69 and 64% with *AtACS2*. *CaACS3-like* was more closely related to *SlACS3* and *SlACS7*, being 71 and 63% identical to these sequences at the amino acid level. The phylogenetic tree also allowed the observation that *CaACS1-like* and *CaACS2-like* belong to type 1 ACS while *CaACS3-like* was more closely related to type 2 ACS (Yoshida et al., 2005). Although *CaACS4-like* showed amino acid identities of up 80%, as found with AtACS6, it did not cluster to any of the ACS types previously described (Yoshida et al., 2005), probably due to its short sequence and for aligning at the C-terminal portion of ACS proteins.

Coffee ACO sequences, designated *CaACO1-like*, *CaACO4-like* and *CaACO5-like*, encode for complete ORFs of 960, 957 and 879bp, respectively. The

predicted proteins encoded by *CaACO1-like* and *CaACO4-like* showed amino acid identity of 82% between each other, and up 85% to among the ACO proteins from tomato, as found between *CaACO1-like* and *SlACO1* and *SlACO3*. *CaACO5-like* showed low identity level to *CaACO1-like* and *CaACO4-like* (less than 50%), being more closely related to *AtACS1*, with an identity of 67% at the amino acid level. The phylogenetic tree showed that *CaACO1-like* was more closely related to *SlACO1, SlACO2* and *SlACO3* from tomato, whose ACO sequences show high similarity levels among themselves (Anjanasree et al., 2005), and *CaACO4-like* was more similar to *SlACO4*, sharing an amino acid identity of 82% with this tomato ACO. *CaACO5-like* was found to be more distant related to the coffee and tomato ACO sequences (amino acid identity values lower than 50%), being grouped in a different clade with *AtACO1*, whose protein sequence is 67% identical to *CaACO5-like.*

The putative members of the coffee ethylene signaling pathway identified in a previous study (Lima et al., 2011), were renamed in this work (Supplementary material), according to their similarity to the members of the tomato ethylene signaling pathway, except for the coffee ERFs, usually represented by a large family of transcriptional factors (Nakano et al., 2006; Sharma et al., 2010). The phylogenetic tree comprising the putative coffee signaling members identified so far, and the signaling members from the two plant model species tomato and Arabidopsis, is depicted in figure 1 (Only the putative coffee *EIN2* and ERFs that were found to be expressed in fruit libraries (Lima et al., 2011), were included in the phylogenetic trees).

Two additional putative coffee ethylene receptors, designated *CaETR3-like* and *CaETR4-like*, were found in this work. *CaETR3-like* and *CaETR4-like* encode for incomplete ORFs of 741 and 789bp, respectively. The phylogenetic tree showed that *CaETR3-like* belong to the *ETR1-like* subfamily, displaying amino acid identities of up to 66% among *ETR1-like* ethylene receptors from

tomato, as found for *SlETR3*. *CaETR4-like* was more closely related to *ETR2-like* ethylene receptors, displaying amino acid identities ranging from 52% (*AtETR2*) to 78% (*SlETR4*) (Figure 1).

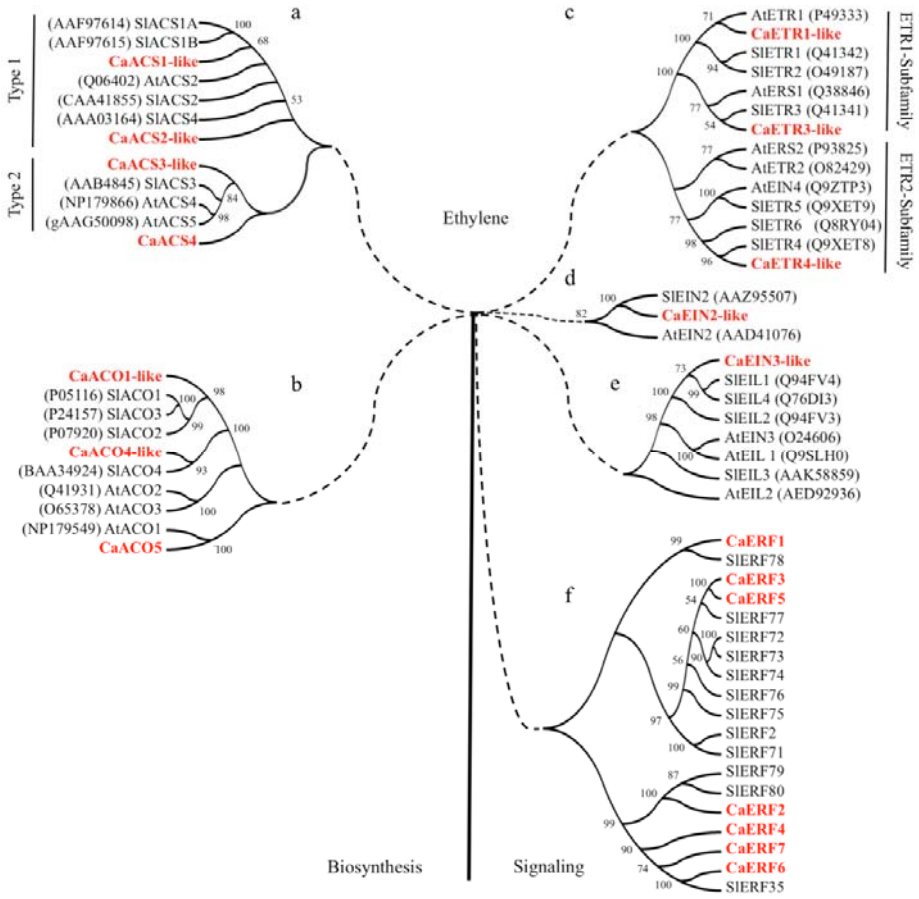

Figure 1 - Phylogeneticanalysis of putative coffee ethylene biosynthesis and signaling members and homolog sequences from Arabidopsis and tomato obtained from the NCBI database. A) ACC synthase; B) ACC oxidase; C) Ethylene receptors; D) EIN2; E) EIN3; F) ERFs. Neighbor-joining trees were built for coffee deduced amino acid and protein sequences from Arabidopsis and tomato aligned with ClustalW. Bootstrap values from 1,000 replications were used to assess the robustness of the trees. Bootstrap values lower than 50% were omitted. Only the tomato ERFs expressed in fruits tissues and most closely

related to coffee ERFs were included in the ERF phylogenetic tree. ERF sequences were obtained from Sharma et al. (2010).

The *in silico* expression profile for the putative coffee ethylene biosynthesis and signaling members, showed that the 19 candidate genes were expressed in 19 different coffee libraries (Figure 2). For the ethylene biosynthesis enzyme ACS, the electronic northern showed that none of the candidate coffee *ACS* were expressed in fruit libraries, however, *CaACS1-like, CaACS2-like* and *CaACS4-like* are probably related to coffee reproductive development since their expression were detected in flower tissues at different developmental stages (Figure 2).

The three putative coffee ACO genes were expressed in 15 different libraries, involving different stress agents, developmental stages and tissues, with *CaACO4-like* showing high expression levels in fruit tissues at different developmental stages (Figure 2). The in silico expression profile for the putative coffee ethylene signaling members, showed that members from every step of this pathway were shown to be expressed in fruit libraries, except for the *EIN3* transcriptional factors (Figure 2).

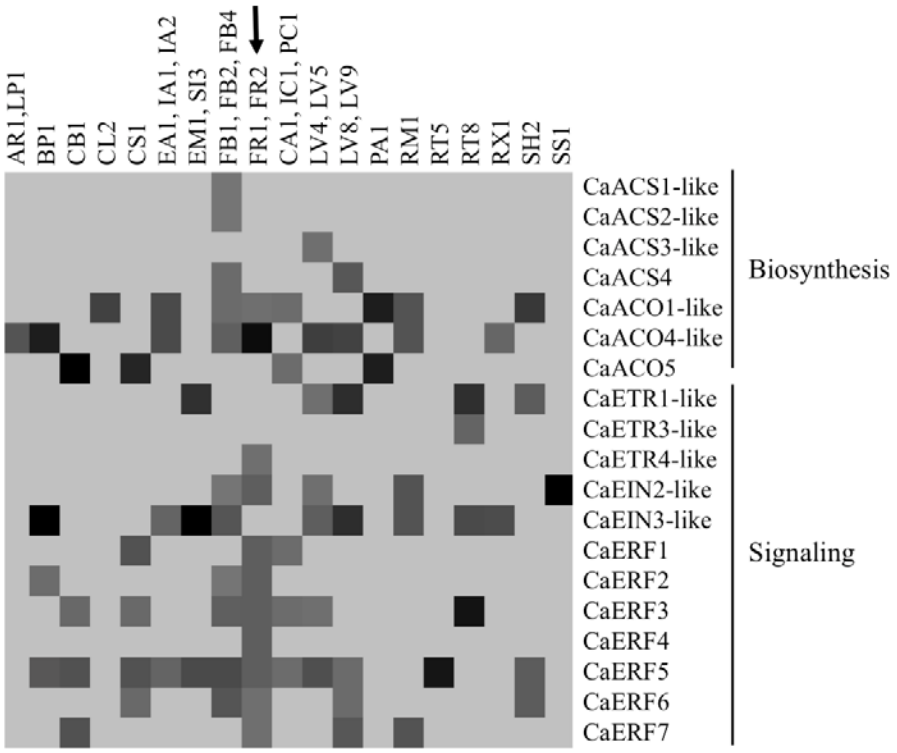

Figure 2 – In silico expression profile of putative elements of the coffee ethylene biosynthesis and signaling pathways. The normalized number of reads for the transcripts in each library are represented by grayscale, where the darker the shade, the higher the expression. Coffee libraries are as follow (Vieira et al., 2006): AR1/LP1, Plantlets and leaves treated with araquidonic acid; BP1, Suspension cells treated with acibenzolar-S-methyl; CB1, Suspension cells treated with acibenzolar-S-methyl and brassinosteroids; CL2,hypocotyls treated with acibenzolar-S-methyl; CS1, Suspension cells treated with NaCl; EA1/IA1/IA2, Embryogenic calli; EM1/SI3, Germinating seeds (whole seeds and zygotic embryos); FB1/FB2/FB4, Flower buds in different developmental stages; FR1/FR2, Flower buds + pinhead fruits + fruits at different stages; CA1/IC1/PC1, Non embryogenic calli with and without 2,4 D; LV4/LV5, Young leaves from orthotropic branch; LV8/LV9, Mature leaves from plagiotropic branches; PA1 Primary embryogenic calli; RM1, Leaves infected with leaf miner and coffee leaf rust; RT5, roots with acibenzolar-S-methyl; RT8, Suspension cells with stressed with aluminum; RX1, Stems infected with *Xylella spp*.; SH2, Water deficit stresses field plants (pool of tissues); SS1, well-watered field plants (pool of tissues). The arrow indicates fruit libraries.

3.2 Expression analysis of coffee ethylene biosynthesis genes

To understand the possible role of ethylene during coffee fruit ripening, expression analysis of four ethylene biosynthesis genes, one ACS (*CaACS1-like*) and three ACO (*CaACO1-like*, *CaACO4-like* and *CaACO5-like*), were carried out (Figure 3). CaACS1-like was up-regulated during fruit ripening of both cultivars and reached its highest expression levels in cherry fruits at 184 DAF and raisin fruits at 214 DAF in the late and early cultivars, respectively (Figure 3).

Among the three coffee ACO analyzed, CaACO4-like showed the highest expression patterns during coffee fruit ripening, showing expression values of up to 15 (Acauã green yellow fruits at 154 DAF) and 780 (Catucaí 785-15 cherry fruits at 214 DAF) times higher than those found for CaACO1-like and CaACO5-like of fruit from the same color, respectively (Figure 3). CaACO5-like showed a similar expression pattern in both cultivars, with a slight increase in raisin fruits. At 184 DAF, cherry fruits from both cultivars showed the highest expression values for CaACO1-like, and slightly decreased thereafter in raisin fruits. Catucaí 785-15 cherry fruits showed a CaACO1-like induction at least two times higher when compared to Acauã cherry fruits at 184 DAF, and the same pattern was observed in raisin fruits at 214 and 244 DAF (Figure 3). CaACO4-like was strongly induced during coffee fruit ripening, reaching its highest expression values at 214 DAF in both cultivars (Figure 3).

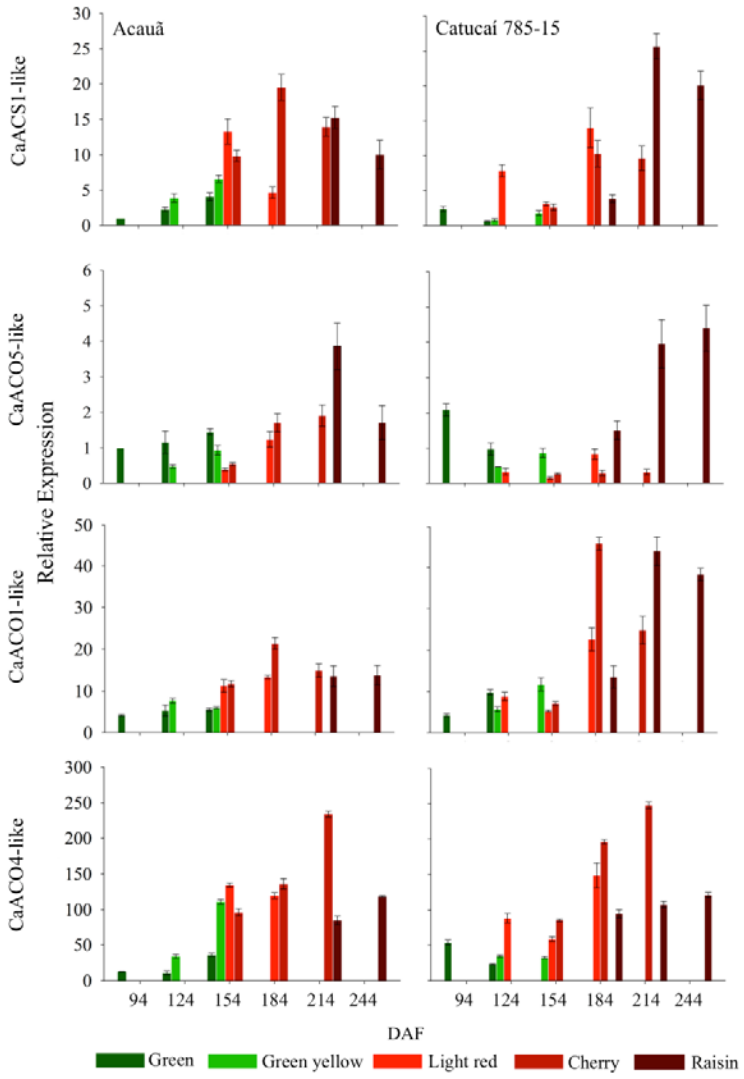

Figure 3- Relative quantitative expression profiling of the coffee ethylene biosynthesis genes *CaACS1-like*, *CaACO5-like*, *CaACO1-like* and *CaACO4-like*, in fruits of late Acauã (Left panel) and early Catucaí 785-15 (right panel) cultivars during six sampling times. Columns represent the fold difference in gene expression for green, yellow green, light red, cherry and raisin fruits, relative to calibrator sample (Acauã green fruits at 94 DAF for CaACS1-like; CaACO5-like expression level in Acauã green fruits at 94 DAF for all coffee ACO gene). Expression values for each biological sample were obtained from

three technical replicates and error bars represent their standard errors. Gene transcripts were normalized by two reference genes (*Actin* and *GAPDH*).

3.3 Expression analysis of coffee ethylene signaling genes

The quantitative expression analysis of coffee ethylene signaling members showed a distinct pattern from that observed for biosynthesis genes, with some genes being up-regulated not only during the final stages of ripening, but also at the initial stages of development and ripening (green and yellow green fruits) (Figure 4).

Coffee ethylene receptors *CaETR1-like* and *CaETR4-like* displayed similar expression patterns in both cultivars, with *CaETR1-like* showing only minor changes in expression during fruit ripening, and *CaETR4-like* showing higher expression levels in cherry fruits. Both ethylene receptors showed lower expression levels in raisin fruits (Figure 4). Expression profiling of the signaling members *CaEIN2-like*, *CaEIN3-like* and *CaERF1-like* showed that these genes displayed increased expression levels in green fruits at 124 DAF of both cultivars, also in cherry fruits from Acauã at 214 DAF (Figure 4).

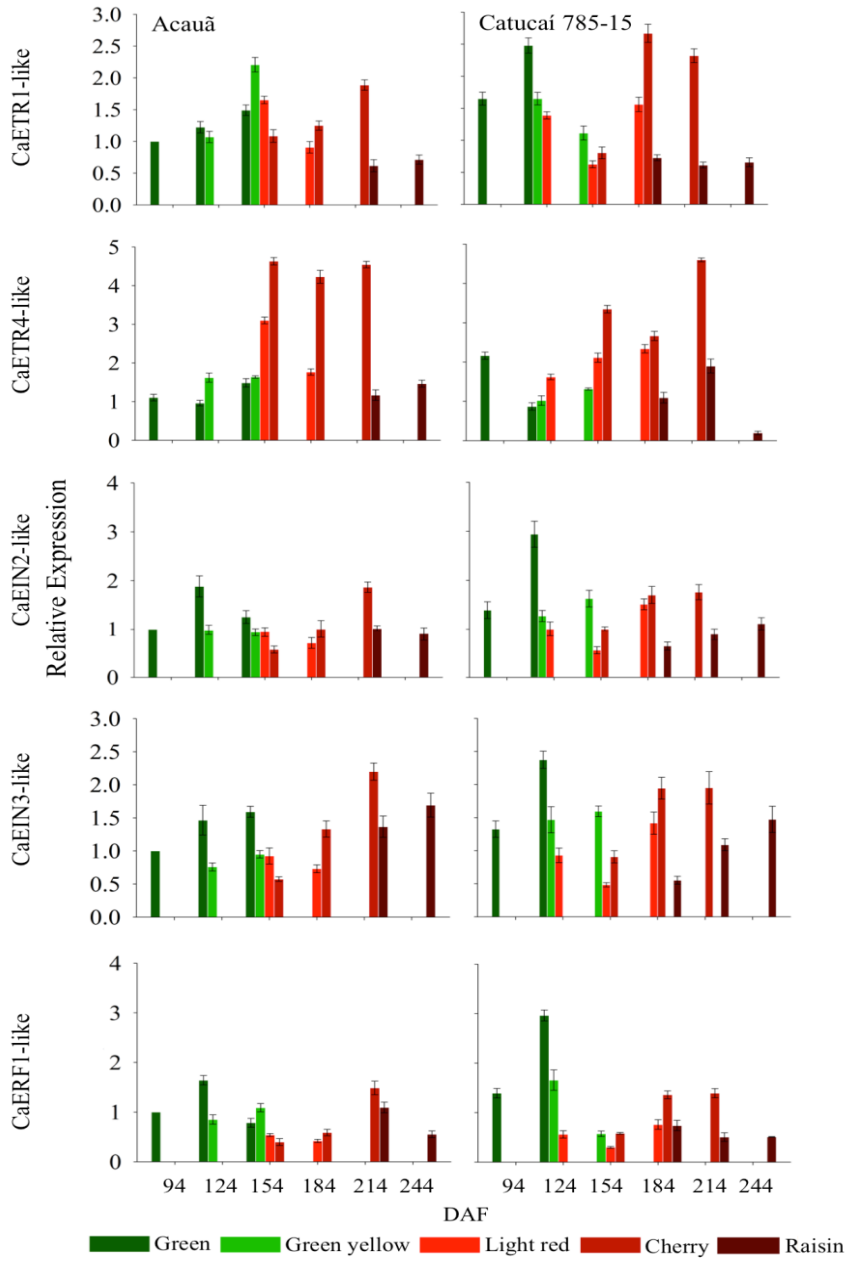

Figure 4- Relative quantitative expression profiling of the coffee ethylene signaling genes *CaETR1-like*, *CaETR4-like*, *CaEIN2-like*, *CaEIN3-like* and *CaERF1-like*, in fruits of late 'Acauã' (Left panel) and early 'Catucaí 785-15'

(right panel) cultivars during six sampling times. Columns represent the fold difference in gene expression for green, yellow green, light red, cherry and raisin fruits, relative to calibrator sample (Acauã green fruits at 94 DAF for *CaEIN2-like*, *CaEIN3-like* and *CaERF1-like*; *CaETR1-like* expression level in Acauã green fruits at 94 DAF for both ethylene receptors). Expression values for each biological sample were obtained from three technical replicates and error bars represent their standard errors. Gene transcripts were normalized by two reference genes (*Actin* and *GAPDH*).

3.4 Average gene expression analysis

Average gene expression analysis for each color enabled the observation that all coffee ethylene biosynthesis genes were induced at the final stages of fruit ripening, specially for *CaACS1-like* and *CaACO4-like* that showed higher expression levels than those found in leaves and flowers, indicating that these genes may play an important role on coffee fruit ripening (Figure 4). It also allowed the observation that *CaACO1-like* and *CaACO5-like* showed higher expression levels in leaves, compared to fruits and flowers (Figure 5). For the coffee ethylene signaling genes, this analysis showed that all genes of this pathway showed higher expression in fruit tissues than in leaves and flowers, except for *CaEIN2-like* (Figure 4). It also allowed the observation that *CaERF1-like* showed the highest expression levels from signaling genes studied, especially in green fruits (Figure 5).

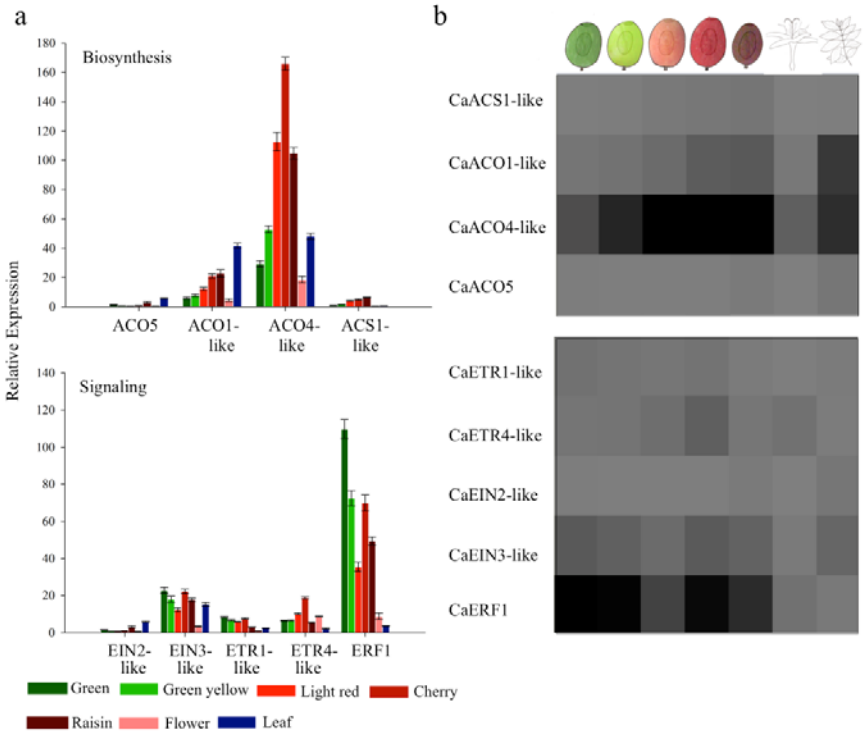

Figure 5 – Average gene expression analysis for green, yellow green, light red, cherry and raisin fruits, of four coffee ethylene biosynthesis genes *CaACS1-like*, *CaACO5-like*, *CaACO1-like* and *CaACO4-like*, and five coffee signaling genes, *CaEIN2-like, CaEIN3-like*, *CaETR1-like, CaETR4-like* and *ERF1*, compared to their expression in leaves and flowers (a). Columns for each fruit color represent an average of the expression values from both cultivars at the six sampling times. CaACO5-like and CaEIN2-like gene expression values from Acauã green fruits at 94 DAF, were used as calibrator samples of coffee ethylene biosynthesis and signaling genes, respectively. Schematic representation of the graph results from the average gene expression analysis (a) in color scale from gray to black, where closer to black color the higher the expression level (b).

Thus, the results from the quantitative expression analysis of coffee ethylene biosynthesis and signaling proteins, suggest that *CaACO4-like* and *CaERF1-like* may display essential roles during coffee fruit ripening, given their

high expression levels at different ripening stages and low expression levels in other tissues, such as leaves and flowers.

4 DISCUSSION

4.1 Phylogenetic analysis

The searches for putative coffee ethylene biosynthesis and signaling genes on the CAFEST database, were highly representative, enabling the identification of members from almost every step from these two pathways.

The search for genes related to the ethylene biosynthesis enzyme ACS, allowed the identification of four sequences related to this enzyme and, although they all encode for small ACS fragments, their sequence analysis enabled the identification of some conserved regions commonly found in ACS from other species. The multiple alignment comprising putative ACS found in this study and ACS from other species, allowed the observation of the seven conserved boxes found in ACS from Arabidopsis, tomato and other plant species (Yamagami et al., 2003; El-Sharkay et al., 2008), with *CaACS1-like* presenting boxes 1 and 2, *CaACS2-like*-3 boxes 3 to 6, *CaACS3* and *CaACS4* with box 7. For *CaACS1-like,* it was also possible to observe the presence of a glutamate residue in box 1, which is directly associated to ACS substrate specificity (Mccarthy et al., 2001). According to Yoshida et al. (2005), ACS enzymes can be classified into three types according to some features of their C-terminal region. *CaACS3-like* aligned in the C-terminal portion of ACSs from other species, allowing the identification of the 'WVF' motif, just before the 'RLSF' motif, which is followed by a short tail (nine amino acids) rich in basic and acidic amino acids that characterize type 2 ACS. According to this observation, *CaACS3-like* was classified as type 2 ACS (Figure 1).

For the second ethylene biosynthesis enzyme, the search in the CAFEST database identified three putative coffee ACO sequences, designated *CaACO1-like*, *CaACO4-like* e *CaACO5-like*. Multiple alignments comprising coffee ACO sequences and ACOs from other species allowed the identification of the 12 residues (P4, A27, G32, H39, H186, D188, L204, Q205, G227, H243, R253, S255) that characterize the iron-ascorbate oxidases superfamily (Tang et al. 1993; Lin et al. 1997), in all coffee sequences. As observed in tomato (Anjanasree et al., 2005), two coffee ACOs (*CaACO1-like* and *CaACO4-like*) displayed high similarity levels between each other, and also compared to other species, as found for *CaACO4-like* and *SlACO4*. However, their 3' and 5' UTR sequences were shown to be unique, enabling the identification of these three coffee ACO.

Previous studies in Arabidopsis and tomato have identified five and six different ethylene receptors, respectively, which according to their sequence and structural similarities, were separated into two subfamilies: *ETR1-like* and *ETR2-like* (Hua et al., 1998; Klee 2002). CaETR1-like was shown to belong to *ETR1-like* subfamily (Lima et al., 2011), and this study identified two additional ethylene receptors, *CaETR3-like and CaETR4-like*, which were shown to belong to the *ETR1-like* and *ETR2-like* subfamilies, respectively. Ethylene receptors from *ETR1-like* subfamily are characterized by having three transmembrane domains at N-terminal portion and a conserved histidine kinase domain at the C-terminal portion of their proteins. *ETR2-like* ethylene receptors differ from *ETR1-like* receptors by a fourth transmembrane domain at the N-terminal portion and a degenerated histidine kinase domain at the C-terminal portion of their proteins. Moreover, members of each subfamily may not present a receptor domain at the C-terminal portion, whose function has not been elucidated yet (Hua et al., 1998; Zhou et al., 2006). *CaETR3-like* and *CaETR4-like* encode for fragments of a conserved and degenerated histidine kinase domain, respectively,

and the high amino acid identity of these receptors to *SlETR3* and *SlETR4,* which was confirmed in the phylogenetic tree (Figure 1), suggest that *CaETR3-like* and *CaETR4-like* may be putative homolog genes of these tomato ethylene receptors.

Phylogenetic analysis of putative coffee signaling members from steps downstream the ethylene receptors have already been discussed in a previous study (Lima et al., 2011) and will not be covered in this study.

4.2 *In silico* expression profile

The *in silico* expression profile for the putative coffee ethylene biosynthesis and signaling members, showed that these genes were expressed in different tissues, developmental stages and conditions, and indicated that ethylene may have important functions in process such as coffee flowering and ripening, as well as in abiotic and biotic stress responses (Figure 2). Except for ethylene receptors, members from every step of the ethylene biosynthesis and signaling pathways showed expression in flower libraries (FB1, FB2 and FB4), suggesting that ethylene may display an important role in coffee flowering (Figure 2). As found in this work, several members of the ethylene biosynthesis and signaling pathways from tomato have been shown to be expressed in flower tissues (Tieman et al., 2001; Yokotami et al., 2003, Sharma et al., 2010), and ethylene has been shown to be directly involved on the control of floral transition via DELLA-dependent regulation of floral meristem-identity genes (Achard et al., 2007).

Considering the climacteric nature of coffee fruits, expression of putative ethylene biosynthesis and signaling members in fruit libraries (FR1 and FR2), as found for the ACO biosynthesis enzyme and for members from every step of the signaling pathway, except for the EIN3 transcriptional factors,

corroborates with the notion of an important role of ethylene during coffee fruit development and ripening. Ethylene plays an important role during the ripening process of climacteric fruits triggering modifications in fruit color, through chlorophyll degradation and carotenoid and flavonoid biosynthesis, fruit texture, through alterations in cell turgor and/or cell wall metabolism, and fruit flavor, aroma and nutritional quality, modifying fruit sugars, acids and volatile profiles (Giovannoni, 2004). Ethylene's role in tomato fruit ripening have been extensively studied, and different works indicate that ethylene biosynthesis and signaling genes are differentially regulated during fruit ripening and play essential regulatory roles during this process (Barry et al. 1996; Barry et al. 2000; Nakatsuka et al. 1998; Kevany et al., 2007; Hu et al., 2010; Tieman et al., 2001; Li et al., 2007).

The electronic northern also showed that a great number of genes were expressed in libraries involving abiotic stresses, such as aluminum (Al) and water stresses, and biotic stresses, such as those caused by Xyllela (RX1) and leaf miner (RM1). Al is the most abundant mineral in soils and becomes phytotoxic to plants when is solubilized to phytotoxic Al^{3+} species under acidic conditions. Inhibition of root elongation is one of the most distinct and earliest symptoms of Al toxicity and is caused by an increase in ethylene biosynthesis triggered by Al (Sun et al., 2007). Water stress positively regulates the synthesis and xylem transport from roots to shoots of the ethylene precursor ACC (Sobeith et al., 2004). Ethylene is also involved in defense responses against biotic stresses and its production stimulated pathogen attack, leading to the up-regulation of defense-related genes through a cascade of events in which the penultimate stage is the activation of ERF-type transcriptional factors (Broekaert, 2006). The most studied ethylene-induced defense related effector molecules are the so-called pathogenesis-related (PR) proteins, which contain the GCC-box present in their gene promoter sequences, a cis-acting ethylene

response element that is necessary and sufficient for ERF interaction (Broekaert et al., 2006). Many studies in different species have shown that several ERFs are up-regulated under pathogen attack, and transgenic plants overexpressing these transcriptional factors have improved their tolerance to biotic stresses (Zhang et al., 2009; Meng et al., 2010).

The *in silico* expression profile also suggest that ethylene may also be involved in developmental process, such as seed germination (EM1, SI3) and cellular differentiation (EA1, IA1, IA2, CA1, IC1, PC1). Ethylene is directly involved in seed germination, promoting the formation of the apical hook, which protects the delicate apical tissues of the growing meristem from injury while the stern is emerging from the soil into the atmosphere (Guzman; Ecker, 1990). Moreover, seeds that present an endosperm limited germination process, such as coffee seeds, endosperm softening is necessary to allow germination (Silva et al., 2004), and ethylene may be involved in the up-regulation of breaking cell wall enzymes, such as polygalacturonase, pectin methylesterase and expansins (Nascimento et al., 1999; Budzinski et al., 2011). Breaking cell wall enzymes gene expression may be regulated by EIN3 and ERF transcriptional factors, since cis-elements of these transcriptional factors have been identified in the promoter regions genes encoding for these enzymes (Yin et al., 2010).

4.3 Expression analysis of coffee ethylene biosynthesis and signaling members

The RT-qPCR analysis demonstrated that two of the four coffee ethylene biosynthesis genes studied, *CaACO1-like* and *CaACO4-like*, displayed an expression pattern typically observed in climacteric fruits, being up-regulated during ripening (Figure 3). *CaACO1-like* and *CaACO4-like* exhibited similar expression levels in green and yellow green fruits in both cultivars, and the

higher expression of these genes in Catucaí 785-15 light red and cherry fruits at 184 DAF may be associated to a higher ethylene production in these fruits, leading to a faster ripening program. The average gene expression analysis showed that *CaACO4-like* and *CaACO1-like* showed the higher expression levels during fruit ripening among the ethylene biosynthesis genes studied in this work (Figure 5), and this result corroborates with their in silico expression profiles (Figure 2). Moreover, the expression pattern observed for *CaACO4-like* matches the ethylene production pattern of coffee fruits during ripening process, with increased ethylene levels in red and cherry fruits, and lower levels in raisin fruits (Pereira et al., 2005). Several studies in different species have shown the up-regulation of ethylene biosynthsis genes during fruit ripening of climateric fruits (Barry et al., 1996; Ruperti et al., 2001; Anjanasree et al., 2005;Wiersma et al., 2007). In tomato fruit, three *ACO* genes, *SlACO1*, *SlACO3* e *SlACO4*, are expressed in fruit tissues. These genes are expressed at low levels in green fruit that are in a system 1 mode of ethylene synthesis. At the onset of ripening, as the fruit transition to system 2 ethylene production, *SlACO1*and *SlACO4* are strongly up-regulated (Barry et al., 1996; Anjanasree et al., 2005). The results found in this study indicate that *CaACO1-like* and *CaACO4-like*, and *SlACO1*and *SlACO4* are regulated in a similar manner and, in accordance with the phylogenetic analysis (Figure 1), may constitute homolog genes.

CaACS1-like gene expression was also up-regulated during fruit ripening of both cultivars (Figure 4), although in a much lesser extent, if compared to the changes in *CaACO1-like* and *CaACO4-like* gene expression (Figure 5). The results also suggest that this gene may be differentially regulated in raisin fruits of the two cultivars analyzed, and may also be related to fruit senescence processes in Catucaí 785-15 fruits (Figure 4), as observed for *CaACO5-like* in both cultivars.

The RT-qPCR analysis for the five coffee ethylene signaling members showed that all genes displayed higher expression levels in green fruits of both cultivars, except for CaETR4-like, which was up-regulated in cherry fruits (Figure 4). Only slightly changes in CaETR1-like gene expression was observed during coffee fruit ripening of both cultivars, and similar results were found for *CcETR1* in *Coffea canephora* fruits (Bustamante-Porras et al., 2007). On the other hand, *CaETR4-like* was induced during coffee ripening of both cultivars, suggesting that this gene may be up-regulated by ethylene, as observed in other species such as peaches (Rasori et al., 2002), tomato (Kevany et al., 2007), plum (El-Sharkawy et a., 2007) and kiwi (Yin et al., 2008). Six ethylene receptors have been identified in tomato and three of them (*SlETR3*, *SlETR4* and *SlETR6*) are up-regulated during ripening (Kevany et al., 2007). Since fruit ripening is dependent upon ethylene action and ethylene receptors act as negative regulators of the signaling pathway, an increase in receptor content during ripening leads to lower ethylene sensitivity, what would seem counter-intuitive. However, an important post-transcriptional mechanism has been shown to control receptor protein levels (Keavany et al., 2007). Although expression of ethylene receptors, such as *SlETR3*, *SlETR4* and *SlETR6*, are ethylene-inducible, protein analysis, throughout fruit development, revealed that receptor levels were highest during immature fruit development and significantly declined at the onset of ripening, despite increased RNA content, due to an enhanced receptor degradation following ethylene biding. Thus, according to this model, ethylene receptor content is a major determinant of when fruits initiate their ripening program (Kevany et al., 2007). The expression analysis of the ethylene biosynthesis genes *CaACO1-like* and *CaACO4-like* and the ethylene receptor *CaETR4-like*, suggest that the higher ethylene production levels in Catucaí 785-15 fruits may induce an enhanced *CaETR4-like* degradation, leading to an increase in ethylene sensitivity and consequently an earliness in the ripening process of this cultivar. Ethylene

production in Acauã fruits may not be sufficient to inactivate the *CaETR4*-like levels and thus ripening changes happen in a slower pace.

The expression profile of the signaling molecule *CaEIN2-like* did not change significantly during coffee fruit ripening of both cultivars (Figure 4), as observed in other species such as tomato (Wang et al., 2007). *EIN2* is a positive regulator of the ethylene signaling pathway and loss-of-function mutations result in complete loss of ethylene responsiveness**.** It encodes an integral membrane protein with 12 membrane-spanning regions at the N-terminal portion, which shows similarity to the Nramp metal-ion transport proteins, and a C-terminal region that does not show homology to any know protein. *EIN2* mRNA levels are not altered in response to ethylene (Alonso et al., 1999; Wang et al., 2007) and EIN2 protein accumulation is positively regulated by ethylene (Qiau et al., 2009). As the tomato *EIN2* gene (Wang et al., 2007), *CaEIN2-like* expression reached its highest levels in green fruits, was not induced during fruit ripening (Figure 2), and was shown to display a higher expression in leaf tissues (Figure 5).

The EIN3 transcriptional factors are represented by a small multigenic family in plants, whose members positively regulate the expression of ethylene responsive genes, such as including other transcriptional factors such as ERF1, a member of the ERF family of transcriptional factors (Chao et al., 1997; Solano et al., 1998). *EIN3* genes have been cloned and characterized in different climateric fruit species, such as tomato (Tieman et al., 2001, Yokotani et al., 2003), kiwi (Yin et al., 2008; Yin et al., 2010), and banana (Mbeguie-A-Mbeguie et al., 2008), and usually they are not differentially regulated at the transcriptional level by ripening and ethylene. The expression profile of *CaEIN3-like* shows that this gene is not up-regulated during fruit ripening (Figure 3), showing similar expression levels in leaf tissues (Figure 5), suggesting that it may be regulated at the protein level, with ethylene positively

regulating CaEIN3-like protein levels, as observed for AtEIN3 in Arabidopsis (Guo; Ecker, 2003).

At the last step of the ethylene signaling pathway, the ERFs also controls additional ethylene-responsive genes, acting as activators and repressor of gene expression (Ohmetakagi; Shinshi, 1995. ERFs are uniquely present in plant kingdom and belong to the AP2/ERF superfamily of transcriptional factors (Nakano et al., 2006). All members of this superfamily are characterized by the AP2/ERF domain, and according to the number and similarity within it, three families can be distinguished: AP2 (APETALA2), RAV (Related to ABI3/VP1) and ERF (Ethylene Response Factors) (Riechman et al. 2000). ERFs from different species have been shown to play an important role in modulating ethylene-induced fruit ripening, regulating genes directly associated to the ripening process, such as ethylene biosynthesis genes (Zhang et al., 2009; Bapat et al., 2010; Yin et al., 2010; Sharma et al., 2010). CaERF1 showed high expression levels in coffee fruits, especially in green fruits, and its expression pattern was partially similar to the tomato ERFs *SlERF35* and *SlERF78* (Sharma et al., 2010). SlERF35 showed a high expression levels during the initial stages of fruit development (immature green fruit), which decreased during the breaker stage, and subsequently increased during the final stages of ripening (red fruits), as observed for *CaERF1* (Figure 4). However *SlERF35* displayed high expression levels in leaf tissues, unlike *CaERF1* (Figure 5). *SlERF78* showed a different expression pattern from that observed for CaERF1, being up-regulated during the initial stages of fruit development (immature green fruit) and remained activated until the last stages of fruit ripening (red fruit), but showed similar expression levels to *CaERF1* in leaf tissues (Sharma et al., 2010), corroborating with the phylogenetic and in silico expression analysis (Figure 1 and 2).

This study shows that the ethylene biosynthesis and signaling members identified in this study, and by Lima et al. (2011), display great similarity levels to the tomato ones, indicating a high conservation level between these different species. Based on these results, a model of ethylene biosynthesis and signaling pathways in coffee fruits is proposed in figure 6.

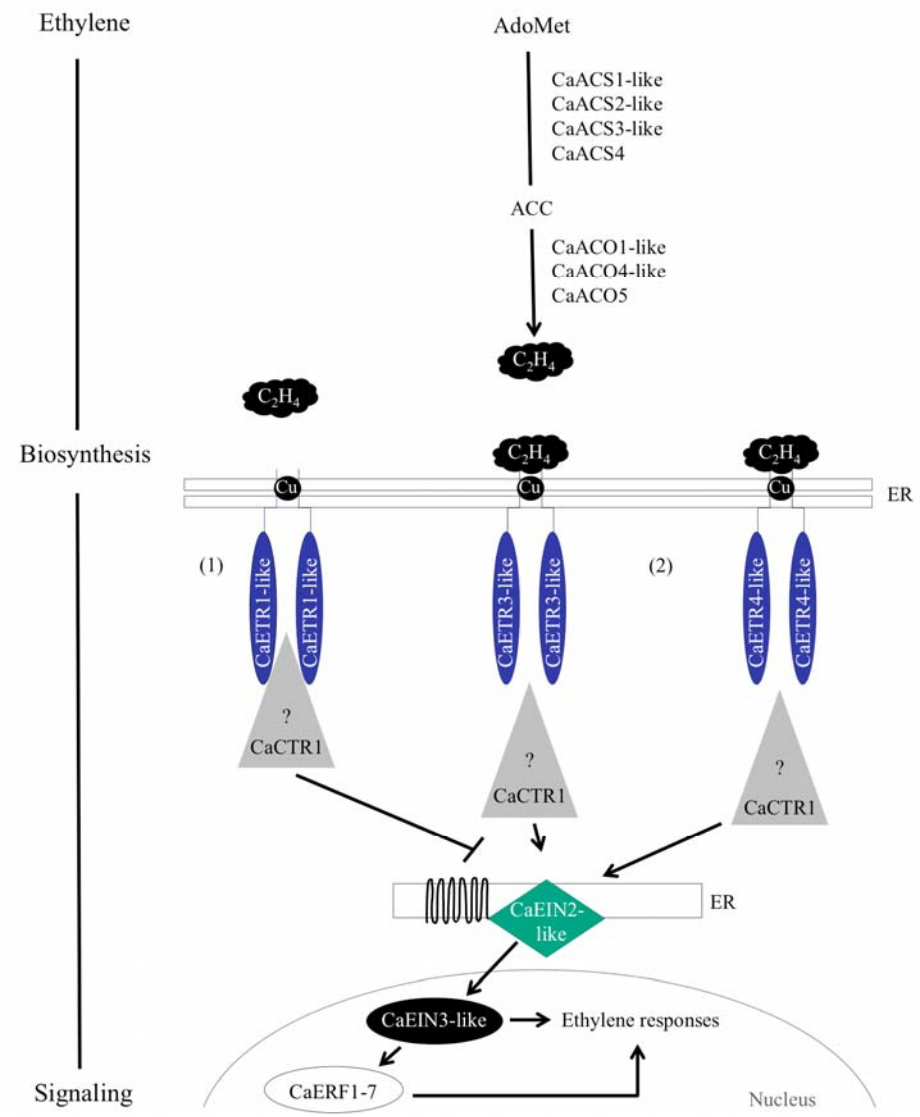

Figura 6 – Schematic representation of the two committed steps in ethylene biosynthesis, ACC generation and its conversion to ethylene, and the ethylene signal transduction pathway in coffee fruits. AdoMet (S-adenosilmetionina), an intermediary of the Yang cycle, is converted to ACC by ACS (CaACS1-like, CaACS2-like, CaACS3-like and CaACS4). ACC is oxidized to ethylene in a reaction catalyzed by ACO (CaACO1-like, CaACO4like and CaACO5-like). In absence of ethylene binding, the receptors (CaETR1-like, CaETR3-like and

CaETR4-like), located in the endoplasmatic reticulum (ER), are in an active state (1) and repress the ethylene responses by signaling through CTR1 (not identified in this study), a Raf-like MAPKK kinase that negatively regulates responses. Upon ethylene biding, which is mediated by a single copper ion (Cu) (Rodriguez et al. 1999), ethylene receptors are deactivated and are no longer able to recruit CTR1 proteins. As a result, CaEIN2-like is activated and a transcriptional cascade involving CaEIN3-like and CaERF1-7 is initiated, culminating in ethylene responses. Cu, copper; ER, endoplasmatic reticulum.

Thus, the expression analysis of the ethylene biosynthesis and signaling genes suggest that ethylene is directly involved on the determination of the ripening time of coffee fruits and *CaACO4-like* and *CaERF1* may display essential roles during coffee fruit ripening, given their high expression levels at different ripening stages and low expression levels in other tissues, such as leaves and flowers. The lower expression levels of *CaACO1-like* and *CaACO5-like* of Acauã fruits may have result in a reduced response to ethylene and ripening, due to lower ethylene receptor degradation levels in these fruits. the higher ethylene production levels of Catucaí 785-15 fruits may have lead to higher, and more even, ethylene receptor degradation levels, allowing that an increased number of fruits reach the cherry stage at the same time.

Supplementary material	
Lima et al 2011	**This study**
CaC20	CaETR1-like
-	CaETR3-like
-	CaETR4-like
CaC3	CaEIN2-like
CaC4	CaEIN3-like
CaC8	CaERF1
CaC5	CaERF2
CaC13	CaERF3
CaC23	CaERF4
CaC11	CaERF5
CaC18	CaERF6
CaC37	CaERF7
CaC7	CaERF8
CaC22	CaERF9
CaC2	CaERF10
CaC32	CaERF11
CaC33	CaERF12
CaC10	CaERF13
CaS1	CaERF14
CaS2	CaERF15

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ARTIGO 3 Estratégia molecular para o entendimento da fisiologia do etileno em frutos de café usando o tomateiro como espécie heteróloga

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NORMAS DA REVISTA CIENTIFICA

Resumo

O cafeeiro é uma cultura bastante estudada, mas ainda existem problemas quanto a produtividade, reflexo principalmente da maturação desuniforme dos frutos em razão do florescimento sequencial encontrado nesta espécie, dificultando a colheita e causando perdas na produção. Sincronizar a maturação dos frutos é um dos principais objetivos da pesquisa do cafeeiro, cujos resultados beneficiaria diretamente a renda do produtor, gerando redução nos custos e uma bebida de melhor qualidade. A maturação dos frutos é um processo dependente de etileno e para que se tenha um bom entendimento da ação do etileno durante a maturação de frutos de café é necessária a compreensão dos principais fatores genéticos que controlam sua ação, como os genes envolvidos na biossíntese e sinalização. Desta forma, este trabalho tem como objetivo o estudo molecular do genes da rota de biossíntese (*CaACO4-like*) e sinalização (*CaERF1-like*) do etileno utilizando o tomateiro (cv. Micro-Tom) como espécie heteróloga, visando o entendimento da fisiologia do etileno em frutos de café (*Coffea arabica*), pois o compreensão desse processo pode levar ao desenvolvimento de cultivares com maturação de frutos uniforme.

Palavras-chave *Coffea arabica*· Etileno· ACC oxidase · Sinalização · Mutantes

1 INTRODUÇÃO

O café é uma das mais importantes commodities naturais do mundo, sendo o Brasil o maior produtor e exportador dessa cultura, com 50,450 mil sacas de 60 kg/ano e 36,28% respectivamente, com área plantada de 2.346.48 ha e produtividade média de 25,80 sacas/ha (CONAB 2012; USDA 2012). Com relação à produção, mesmo o cafeeiro sendo uma cultura bastante estudada, ainda existem problemas com a produtividade, reflexo principalmente da maturação desuniforme dos frutos em razão do florescimento sequencial encontrado nesta espécie, dificultando a colheita e causando perdas na produção.

Sincronizar a maturação dos frutos pode contribuir na redução dos custos de produção e aumento da produtividade, bem como da qualidade final da bebida. A maturação dos frutos é um processo dependente de etileno, sendo altamente coordenado, geneticamente programado e irreversível, o qual envolve uma série de mudanças fisiológicas, bioquímicas e organolépticas que leva ao desenvolvimento de frutos dispondo de atributo de qualidade desejáveis e aptos para o consumo (Prasanna et al. 2007).

O etileno é formado a partir do aminoácido metionina via S-Adenosilmetionina (AdoMet), e o precursor imediato do etileno, denominado de Ácido-1-aminociclopropano-1-carboxílico (ACC) (Adams; Yang, 1979). AdoMet é sintetizada a partir da metionina por ação da enzima AdoMet sintetase

e a conversão de AdoMet em ACC é catalisada pela enzima ACC sintase (ACS) (Kende, 1993). A ação da ACS produz, além do ACC, a 5-Metiltioadenosina a qual é utilizada para a síntese de uma nova metionina através do ciclo modificado da metionina ou ciclo de Yang (Miyazaki; Yang, 1987). Um aumento na taxa respiratória fornece o ATP necessário para o ciclo de Yang e pode permitir que elevados níveis de etileno sejam produzidos mesmo na ausência de altos níveis intracelulares de metionina. O ACC gerado nessa etapa, é então convertido a etileno, essa conversão é catalisada pela enzima ACC oxidase (ACO), gerando além do etileno, CO_2 e ácido cianídrico (HCN) (Yang; Hoffman, 1984).

Existem dois sistemas que regulam a biossíntese de etileno nas plantas, um deles (Sistema 1) é operante tanto em frutos climatéricos como em não-climatéricos, assim como em tecidos vegetativos, e é responsável pela produção basal de etileno, enquanto o outro (Sistema 2) é operante durante a maturação de frutos climatéricos e é responsável pela produção autocatalítica de etileno nesse processo (McMurchie et al. 1972). Alguns autores sugerem o cafeeiro como climatérico, demostrando o envolvimento do etileno durante o amadurecimento dos frutos (Pereira et al., 2005; Salmona et al., 2008). Além disso, outros estudos relatam um efeito positivo na antecipação e sincronização da maturação de frutos de café pela aplicação exógena de Ethephon (Carvalho et al., 2003; Scudeler et al., 2004).

Para que se tenha um bom entendimento da ação do etileno durante a maturação de frutos de café é necessária a compreensão dos principais fatores genéticos que controlam sua ação, como os genes envolvidos na biossíntese e sinalização. Estudos genéticos relacionados com a formação e desenvolvimento de frutos foram realizados em Arabidopsis (Pinyopich et al., 2003), enquanto a maturação de frutos tem sido usado o tomateiro como modelo (Giovannoni, 2004, 2007; Hong; Lee, 1993), pois está espécie apresenta frutos carnosos e climatérico.

Além disso, o tomateiro tem promotores (E8 e E4) de resposta a genes do etileno, que foram extensivamente utilizados como promotores específicos de fruto (Cordes et al, 1989;. Coupe e Deikman 1997; Deikman et al 1992, 1998;. Deikman e Fischer, 1988; Kneissl e Deikman 1996; Lincoln et al, 1987;. Montgomery et ai, 1993a; Xu et al, 1996) e um grande número de mutantes bem caracterizados.

Já foram descritos mutantes relacionados com as principais classes de hormônios, tais como etileno, giberelinas, citocinina e ácido abscísico (Fujino et al., 1988; Bensen; Zeevaart, 1990; Pino-Nunes, 2005; Burbidge et al., 1999), bem como, brassinoesteróides e ácido jasmônico (MONTOYA et al., 2002; LI; LI; HOWE, 2001). Esse tipo de estudo tem possibilitado a compreensão dos mecanismos que regulam a maturação de frutos, através do estudo do mutantes *ripening-inhibitor* (*rin*), *nonripening (nor), colorless nonripening (Cnr), green-*

ripe (Gr), green flesh (gf), high pigment1 (hp1), high pigment2 (hp2), and never-ripe (Nr) (Lanahan et al., 1994; Mustilli et al., 1999; Vrebalov et al., 2002; Liu et al., 2004; Barry and Giovannoni, 2006; Manning et al., 2006; Barry et al., 2008).

Os locos *rin* e *Cnr* codificam fatores de transcrição MADS box e um SPBP, respectivamente, e são reguladores da maturação (Vrebalov et al, 2002; Manning et al, 2006). O gene *Gr* interage com componentes de resposta ao etileno em frutos (Barry e Giovannoni, 2006), enquanto que a mutação *Nr* tem sido caracterizado como um receptor de etileno ERS-like, com uma baixa capacidade para se ligar ao etileno (Lanahan et ai., 1994).

A cultivar Micro-Tom (MT) tem sido muito utilizado como modelo genético (Meissner et al. 1997) para o estudo de mutantes, por possuir porte pequeno, de 10 a 20 cm (Emmanuel; Levy, 2002), frutos e sementes viáveis, ciclo de apenas 70-90 dias, facilmente cultivada em laboratório e adequada para a utilização das técnicas de cultura de tecidos. Existem vários mutantes já introgredidos em MT, como o alelo *Rg1* de *S. peruvianum* que foi transferido para a cv MT (Lima et al., 2004) o que possibilitou melhorias no processo de transformação genética, por aumentar a capacidade de regeneração (Pino et al., 2010).

Desta forma, este trabalho teve como objetivo o estudo molecular dos genes da rota de biossíntese (CaACO4-like) e sinalização (CaERF1-like) do

etileno utilizando o tomateiro (cv. Micro-Tom) como espécie heteróloga, visando o entendimento da fisiologia do etileno em frutos de café (Coffea arábica), pois o compreensão desse processo pode levar ao desenvolvimento de cultivares com maturação de frutos uniforme.

2 MATERIAL E MÉTODOS

2.1 Estratégia das Construções

Neste trabalho foram utilizadas as sequências dos transcritos de C*aACO4-like* e *CaERF1* identificadas por Ságio et al 2012 (dados não publicados) para o cafeeiro e para o tomate foram utilizadas as sequencias depositadas no NCBI, que apresentaram a maior similaridade: SlACO4 (NM_001246938.1) e SlERF2 (AY192368.1).

Para cada transcrito, foi feita uma combinação com dois vetores de destino: pK7WG2.0 dirigido pelo promotor 35S do vírus do mosaico da couve-flor e pK7WG2.0 - dirigido pelo promotor E8 de tomate, que é tecido específico de fruto, além disso o promotor E8 foi inserido dentro do vetor pKGWFS7,que possui como marcador GFP (green fluorescent protein) e GUS (β-glucuronidase protein). O promotor 35S é amplamente utilizado para o estudo da expressão de genes em plantas, no entanto, em alguns casos o promotor 35S não é adequado por ser constitutivo, assim promove a expressão do gene durante o crescimento e

desenvolvimento da planta, por isso utilizamos também o promotor E8.As construções com os transcritos de *SlACO4* e *SlERF2* foram usadas como controle para o estudo dos mutantes.

2.2 Análise In Silico

As sequências dos transcritos *CaACO4-like* e *CaERF1* foram comparadas com ACO e ERF de outras espécies que estão depositadas no banco de genes do NCBI (National Center for Biotechnology: http://www.ncbi.nlm.nih.gov/), através do programa ClustalW (Thompson et al. 1994) com os parâmetros padrões (default), utilizando-se as sequências de nucleotídeos traduzidas em aminoácidos, os resultados foram visualizados com GeneDoc

2.3 Extração de RNA e síntese de cDNA de café

Para isolamento dos genes, foram separados frutos de café arábica e de tomate (cv Micro-Tom) no estádio cereja. Os frutos foram coletados e imediatamente congelados em nitrogênio líquido até o momento da extração do RNA. A extração do RNA total foi feita através do kit RNeasy Plant (QIAGEN), e a integridade das amostras foi verificada em gel de agarose 1,0% (m/v) e posteriormente quantificadas em espectrofotômetro (Nanodrop®

Espectrophotometer ND-1000) a A_{260}. As amostras que apresentaram alto grau de integridade e pureza foram usadas para a síntese de cDNA, através do Kit High-Capacity cDNA Reverse Transcription (Applied Biosystems). Após a síntese de cDNA as amostras foram armazenadas em freezer a -20° C até o uso.

2.4 Desenho de primer, amplificação dos fragmentos e eluição das bandas

Os primers para a clonagem dos genes foram desenhados utilizando o programa "OligoPerfect™ Designer" (Invitrogen). Quanto a amplificação, os genes foram amplificados a partir de amostras de cDNA de frutos, utilizando uma enzima de alta fidelidade que possui atividade de exonuclease 3'- 5', e *primer* específicos ("forward" e "reverse") para todos os genes estudados.

Os produtos das amplificações foram submetidos à eletroforese em gel de agarose 1,0% (m/v) corado com GelRed, sob corrente elétrica de 110 V em Tampão SB (acido bórico) por 40 min. Os fragmentos obtidos foram eluídos do gel de agarose por meio do Kit Qiaquick Gel Extraction.

2.5 Clonagem e transformaçãobacteriana

Para a clonagem foi utilizado o vetor *pENTR*™/D-TOPO®Cloning Kit da Invitrogen. Os produtos da eluição foram adicionados ao vetor, na proporção de 1:1 acrescentado de 1 μL de solução salina e água para um volume final de 6

μL. A reação foi incubada por 30 minutos à 22°C e colocadas no gelo para a utilização na transformação bacteriana.

Para a transformação foram utilizadas as células da bactéria quimicamente competente *Escherichia coli* DH5α™-T1^{R}. A essas células foram adicionados 2 μL da reação de clonagem com o vetor *pENTR*™/D-TOPO®, e incubados no gelo por 30 min. Para a introdução do vetor na bactéria foi utilizado o processo de choque térmico que consistiu em colocar a 42 °C por 30 segundos e imediatamente transferir os tubos para o gelo. Foram acrescentados 250 μL do meio S.O.C e deixados por 1 h a 37 °C em constante agitação a 200 rpm. Logo após a incubação, o plaqueamento foi realizado utilizando 50 μL da solução de transformação em placa de Petri, contendo 25 mL de meio de cultura Luria-Bertani (LB) ágar e 50 mg L^{-1} de canamicina.

As placas foram mantidas a 37 °C por aproximadamente 24 horas, para permitir o crescimento das colônias bacterianas. Após esse período, 5 colônias foram selecionadas aleatoriamente, transferidas a outra placa para subcultivo com o mesmo meio, e submetidas à PCR de colônia, para comprovar a presença do fragmento de interesse. As colônias que apresentaram o fragmento de interesse foram selecionadas para o processo de extração de DNA plasmidial.

A partir da confirmação da inserção do fragmento pela PCR, as colônias foram transferidas para meio líquido, possibilitando o crescimento das células, que foram utilizadas para a extração de DNA plasmidial e posterior

sequenciamento. Com o auxilio de palitos esterilizados, cada colônia foi transferida para 3 mL de meio LB suplementados com 50 mg L^{-1}de canamicina. Esse procedimento foi realizado em tubos Falcon, que foram mantidos a 37 °C, sob agitação, durante aproximadamente 16 horas, em agitador orbital ajustado para 250 rpm.

2.6 Miniprep e sequenciamento

O isolamento do DNA plasmidial foi feito através do protocolo de Alkaline Lysis Mini-Prep. Os plasmídeos contendo os insertos dos fragmentos correspondentes aos *genes* de interesse, foram sequenciados no Departamento de Biologia da West Virginia University. As sequências obtidas no sequenciamento foram comparadas e alinhadas com as sequencias previamente obtidas *in silico*, utilizando *ClustalW*,e com sequências de bancos públicos pelo *GenBank*. Essa comparação com o banco de dados foi realizada utilizando-se o programa *BlastX* (Altschul et al., 1997). As sequências confirmadas foram utilizadas para a transformação em *Agrobacterium tumefaciens*.

2.7 Preparo de células competentes e transformação

O preparo das células competentes da *Agrobacterium tumefaciens* EHA105, foi feita seguindo o protocolo *Freeze-Thaw,* adaptado de Höfgen and Willmitzer (1998).

Foi inoculado 200 ml de meio LB liquido, com 1 mL de cultura de Agrobacterim EHA105, previamente crescida em LB liquido em agitação, por 24 horas a 28°C. Após a inoculação, foi mantido em agitação a 28 °C até atingir a concentração de OD_{550nm}= 0.5-0.8.

Quando atingiram essa fase, as amostras foram centrifugadas a 500 rpm por 10 minutos a temperatura ambiente. O pellet foi lavado com tampão TE 1X, e as células foram ressuspendidas em 0,1X do volume original de LB. As amostras (células competentes), foram separadas em alíquotas de 250 µL em microtubos de 2,0 mL. Os microtubos foram imediatamente congelados em nitrogênio líquido e armazenados em freezer -80°C.

As células competentes foram descongeladas em gelo e em seguida foi adicionado 10 µL DNA em 250 µL de célula competente. A mistura foi mantida em gelo por 5 minutos e depois transferida para o nitrogênio liquido, por mais 5 minutos. Após esse período as amostras foram incubadas por mais 5 minutos em banho-maria a 37 °C. Foram adicionados 1 mL de meio LB em cada tubo e mantido em agitação por 4 horas a temperatura ambiente.

O sobrenadante foi coletado, após rápida centrifugação, e espalhado em placas de Petri (100x15) contendo meio LB, 50 mg L^{-1}de rifampicina e 50 mg L^{-1}de espectinomicina. As placas foram incubadas por 48horas a 28 °C.

Foram selecionadas colônias, e checadas com PCR. Um única colônia foi transferida para 3 mL de meio LB líquido suplementado com 50 mg L^{-1}de rifampicina e 50 mg L^{-1}de espectinomicinae cultivada a 28ºC por 48 h com agitação 120 rpm. Foi retirado 500 µL da suspensão e adicionadas a 50 ml de meio LB fresco em um frasco de 250 mL e cultivada a 28ºC overnight a 120 rpm. A suspensão bacteriana foi centrifugada a 3000 rpm por 15 min e o pellet dissolvido em meio MS líquido basal com vitaminas B5 suplementado com 30 g L^{-1} a uma concentração de OD_{600nm}=0,2-0,3. Dez minutos antes da inoculação dos explantes, foi acrescentado a suspensão bacteriana 100µM de acetoseringona.

2.8 Transformação de plantas de tomateiro cv. Micro-Tom

Primeiramente, as sementes de Micro-Tom foram esterilizadas por agitação em 100 ml de hipoclorito de sódio a (2,7%), com duas gotas de Tween 20, por 15 min, seguido de três lavagens com água destilada autoclavada, foram germinadas em meio MS meia força suplementado com vitamina B5, 15 g L^{-1} sacarose e 6 g L^{-1} de ágar. O pH foi ajustado com KOH 1 M para 5,8 antes da autoclavagem. Cerca de 30 sementes foram colocadas em frascos, contendo 30

mL desse meio. Os frascos foram vedados com PVC e mantidos a 25 ± 1ºC no escuro por quatro dias, após esse período foram transferidos para um regime de luz com 16 horas de fotoperíodo, mantendo a temperatura de 25 ± 1ºC.

Para a inoculação com *Agrobacterium*, os cotilédones foram isolados a partir de 8 dias após a semeadura e divididos transversalmente em dois pedaços, colocados com lado abaxial em placas de Petri (100 x 15mm), contendo meio MS sólido com vitaminas B5, suplementado com 30 g L^{-1} sacarose, 6 g L^{-1} ágar, 0.4 μM ANA e 100 μM AS. Foram utilizados um total de 120 explantes (4 placas de Petri com 20 explantes cada) por tratamento.

A suspensão de Agrobacterium em meio MS líquido foi gotejada sobre as placas contendo o explante, e incubadas à temperatura ambiente por 10 min, após esse período o excesso de suspensão bacteriana foi removido com uma pipeta esterilizada e os explantes foram secos em papel filtro estéril. As placas foram mantidas em condições de escuro a 28 º C por 2 dias para o co-cultivo. Os explantes foram então, transferidos para meio MS sólido com vitaminas B5, suplementado com 6 g L^{-1} ágar, 30 g L^{-1} sacarose, 5 μM zeatina, 100 mg L^{-1} canamicina e 25 mg L^{-1} Timetin e cultivadas por 3 semanas a 25 º C ± 1 e 16 horas de fotoperíodo. Durante este período foi realizado um subcultivo e os brotos bem desenvolvidos foram separados dos explantes e transferidos para frascos contendo 50 ml de meio MS suplementado com 30 g L^{-1} sacarose, 6 g L^{-1}

ágar, 0.4 μM ANA antibiótico e 100 mg L^{-1} canamicina,para o enraizamento por duas semanas.

Quando as raízes já estavam bem formadas, as plantas foram transferidas para vasos de 100 mL contendo substrato, e mantidas sob controle de umidade (70%) por uma semana, após a aclimatação, foram levadas para 16horas de fotoperíodo a 25 º C ± 1º.

3 RESULTADOS

A análise do alinhamento entre as sequências dos transcritos dos genes *ACO4-like* e *ERF1* de cafeeiro e as sequências dos transcritos de *ACO4* e *ERF2* de tomateiro, respectivamente, mostraram uma alta similaridade, com uma identidade de 82% entre as sequências de ACO e 60% para ERF (Figura 1). Sendo o tomate comprovadamente um bom modelo, para estudos genéticos em café. O café como um membro da família Rubiaceae, está distantemente relacionado com a espécie modelo Arabidopsis (Brassicaceae, Rosids). Estudos comparativos entre Arabidopsis e solanáceas (por exemplo, tomate, pimenta), indicaram que estes são melhores modelos genômicos para o café que Arabidopsis (Lin et al., 2005). Estes resultados são consistentes pois ambas, Rubiaceae e Solanaceae, estão evolutivamente mais próximas e pertencem ao grupo das Asterids, além de terem numero básico de cromossomos semelhantes (Chase et al.; 1993, Lin et al., 2005, Benedito, 2007).

Na figura 2 podemos acompanhar o desenvolvimento dos trabalhos utilizando o tomateiro como espécie heteróloga. O padrão de banda para os genes escolhidos, pode ser visualizado através da digestão com as enzimas Not I e Asc I (*CaACO* - 1429pb ; *SlACO*- 1174pb; *CaERF* - 888pb; *SlERF* - 939pb). Após a confirmação através do sequenciamento, todos os genes foram inseridos dentro do vetor de destino (pK7WG2). Para as construções 35S:SlERF2, 35S:CaERF, 35S:CaACO, seguimos o esquema de transformação descrito na Figura 4. Já para as análises usando o promotor E8 temos as construções em andamento.

Através do resultado da PCR para as plantas transformadas (T0) com 35: SlERF2 (figura 4), podemos observar que obtivemos a regeneração de vinte e quatro explantes, dentre esses dez apresentaram padrão de expressão positivo, com o tamanho de banda esperado.

O próximo passo, para a análise dos fenótipos dos mutantes, será a coleta de sementes para a geração T1 aplicando a seleção com spray de canamicina nas plântulas com 14 dias. Assim só as resistentes seguiram para as próximas gerações até homozigose.

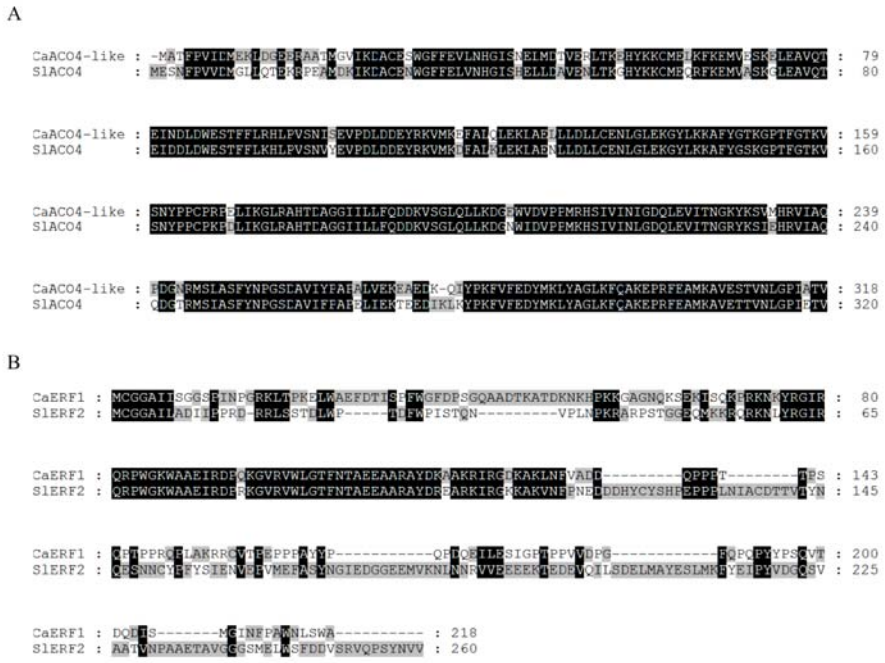

Figura 1 Comparação das prováveissequências de aminoácidos dos genes CaACO4-like e CaERF1 com o ACO4 e ERF2 de tomateiro. A – Comparação dos aminoácidos para o gene ACO. B - Comparação dos aminoácidos para o gene ERF.O alinhamento foigerado pelo programaClustalWe exibidoscomGeneDoc. Resíduos de aminoácidosidênticossão sombreadas empreto eos resíduos nãoconservadosem cinza.Barrasinvertidasindicam espaçosinseridospara a otimizaçãodo alinhamento.Posiçõesde aminoácidos são apresentadasno lado direito.

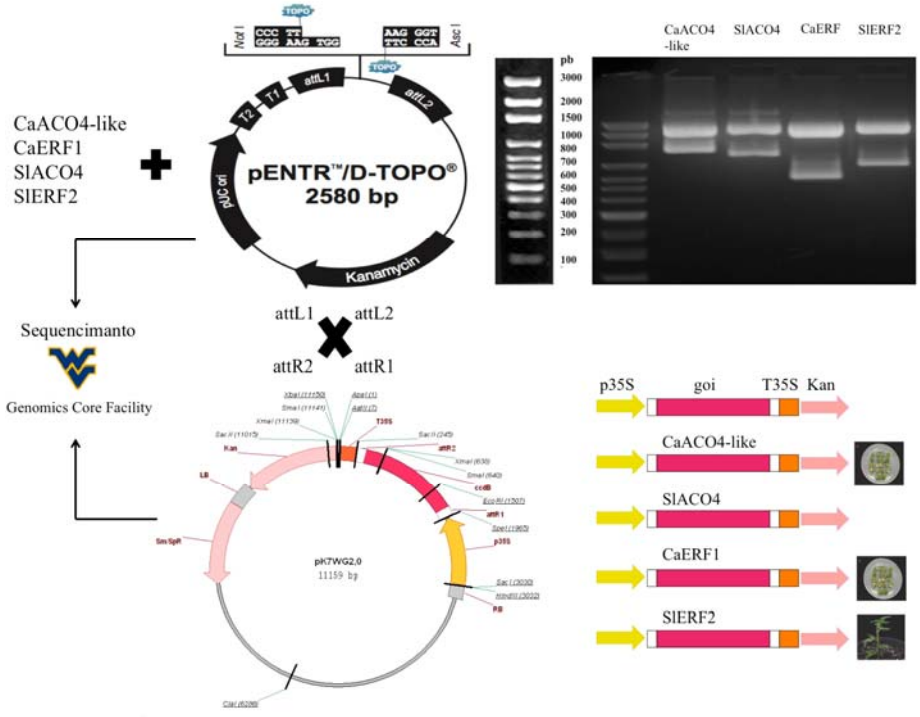

Figura 2 Esquema representativo das construções para os genes ACO4-like e ERF1 de cafeeiro e dos genes ACO4 e ERF2 de tomateiro, usando *pENTR*™/D-TOPO® como vetor de entrada e o pK7WG2.0 como vetor de destino. Cada etapa foi verificada com enzimas de restrição e sequenciamento.

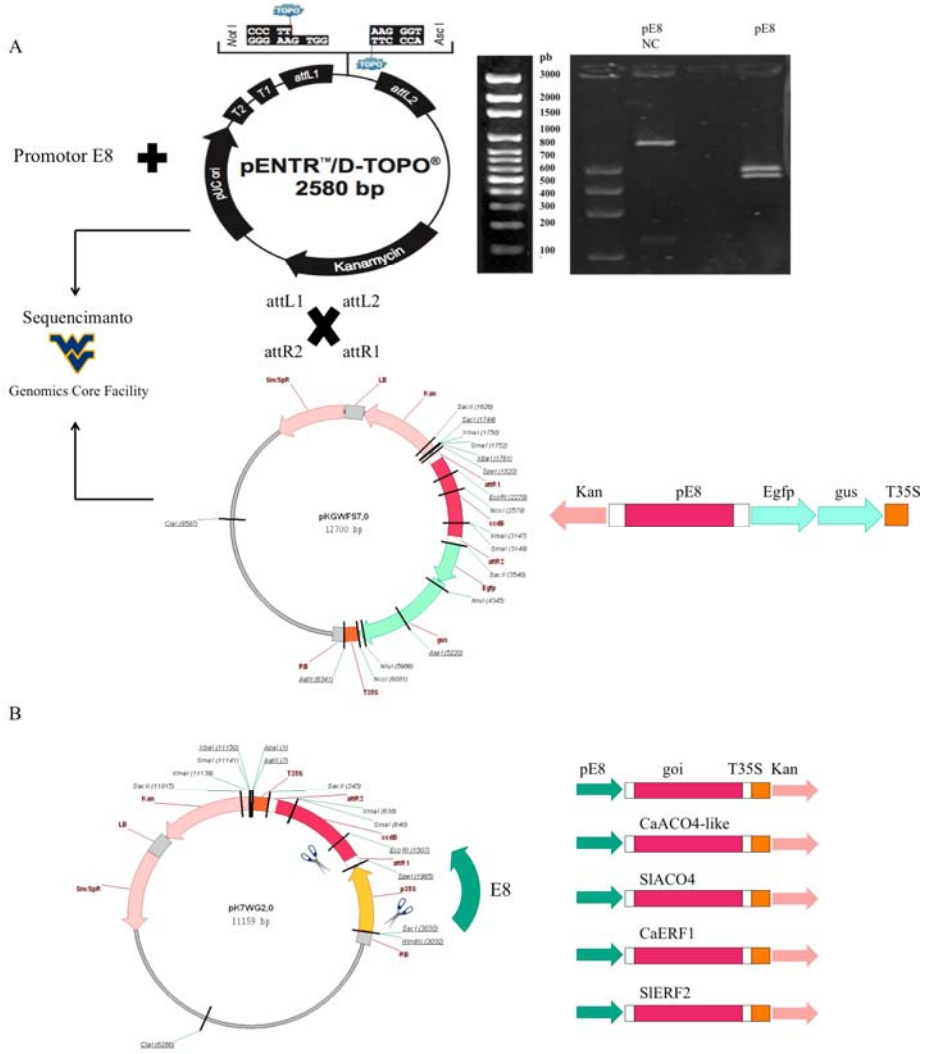

Figura 3 Esquema representativo das construções com os genes ACO4-like e ERF1 de cafeeiro e do promotor E8 e genes ACO4 e ERF2 de tomateiro, usando *pENTR*™/D-TOPO® como vetor de entrada e o pKGWFS7.0 e pK7WG2.0 como vetor de destino. A – Construção do vetor de destino para estudo do promotor E8 de tomateiro. B – Construção do vetores de destino para os genes CaACO4-like, CaERF1, SlACO4 e SlERF2.

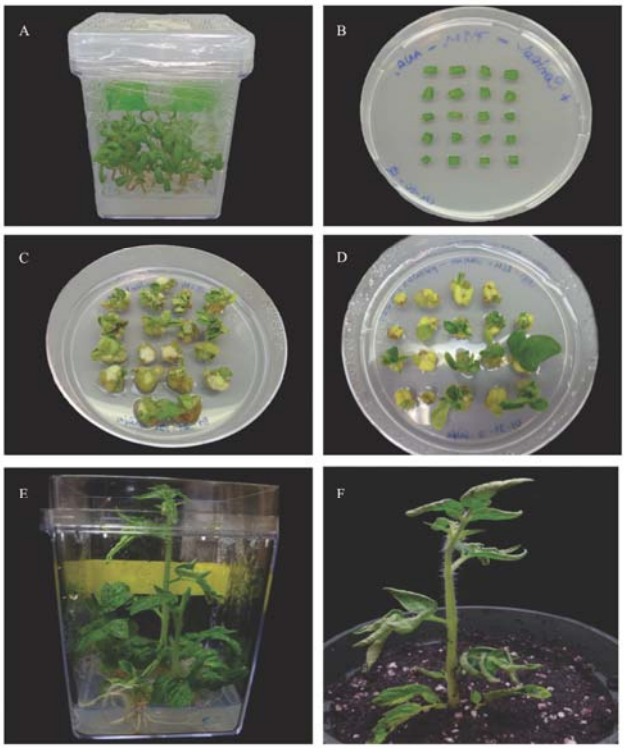

Figura 4 Esquema de transformação - (A,B) cotilédones de plântulas com 8 dias foram preparados para a inoculação com *Agrobacterium tumefaciens*. (C,D,E) Após 3 semanas observou-se o inicio da formação do sistema aéreo, sendo necessários mais 4 semanas para a formação das raízes e completo desenvolvimento das plantas. (F) Após esse período as plantas T0foram aclimatadas.O tomateiro (*Solanum lycopersicum* L.) cv Micro-Tom (MT) utilizado como espécie heteróloga para os trabalhos de transformaçãofoi proveniente da Escola Superior de Agricultura "Luiz de Queiroz" (ESALQ) da Universidade de São Paulo (USP).

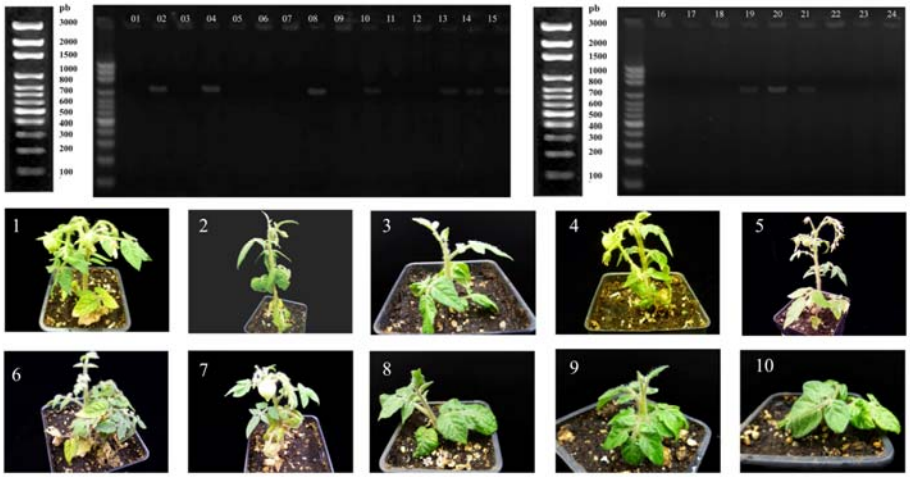

Figura 5 Gel da PCR de folhas de possíveis mutantes de tomate Micro-Tom transformado com construção para superexpressão constitutiva do transcrito ERF2 de tomateiro, utilizando promotor 35S e as plantas que apresentaram o padrão de banda esperado no gel (1100pb).

4 PERSPECTIVAS FUTURAS

A manipulação de genes envolvidos no processo de amadurecimento em frutos de café é o primeiro passo para a obtenção de cultivares com maior uniformidade quanto à maturação de seus frutos, para isso o uso de espécies modelos é fundamental.

Espera-se no final desse trabalho, a caracterização funcional do gene de biossíntese (ACO) e sinalização (ERF) de etileno, dando suporte para novos estudos, como a geração de duplos mutantes, a caracterização funcional dos demais genes que compõe a rota do etileno em café e finalmente a identificação da melhor estratégia para a transformação de plantas de café, visando solucionar de maneira eficiente a desuniformidade na maturação dos frutos.

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