KENIA DE CARVALHO

EXPRESSÃO DE GENES ENVOLVIDOS NA BIOSÍNTESE DE MANITOL EM *Coffea arabica* SUBMETIDO A ESTRESSES ABIÓTICOS E CONTRIBUIÇÃO DE HOMEÓLOGOS DE *Coffea canephora*.

Tese apresentada ao Curso de Pós-Graduação em Agronomia, área de concentração em Produção Vegetal, Departamento de Fitotecnia e Fitossanitarismo, Setor de Ciências Agrárias, Universidade Federal do Paraná, como parte das exigências para obtenção do título de Doutor.

Orientador: Prof. Dr. João Carlos Bespalhok Filho

Coorientador: Dr. Douglas Silva Domingues

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‘A ciência não pode prever o que vai acontecer.

Só pode prever a probabilidade de algo acontecer’

César Lattes

À mamãe

DEDICO

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À família LBI. Sempre ao meu lado. Obrigada pela ajuda e conforto nos momentos difíceis.

Ao Douglas, Filipe, Santista, Bespalhok e Fermino mais do que chefes e orientadores, grandes amigos.

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RESUMO GERAL

O Brasil se destaca como principal produtor e exportador mundial de café. Em 2013, o país deverá colher mais de 50 milhões de sacas de café beneficiado. Embora a produtividade e o consumo aumentem a cada ano, os cafeeiros ainda são sujeitos a oscilações de produção, o que pode ser explicado pelo ciclo bienal da planta e também por condições ambientais, como estresses abióticos. Dentre estes destacam-se seca, salinidade e altas temperaturas. Quando submetidas a estresses, as plantas podem sintetizar osmoprotetores, como o álcool açúcar manitol, um composto ainda pouco estudado em espécies perenes, como o café. Além disso, enquanto alotetraploide, as respostas transcricionais de *Coffea arabica* são um conjunto da expressão de dois subgenomas. Assim, o presente trabalho teve como objetivo investigar a influência da seca, salinidade e alta temperatura no perfil transcricional dos genes envolvidos na síntese e catabolismo de manitol, bem como a concentração de carboidratos sob esses estresses; além da contribuição do homeólogo de *C. canephora* na regulação transcricional destes genes. Para estudar a transcrição gênica, normalizadores foram previamente avaliados, onde, verificou-se que em condições de alta salinidade os normalizadores mais adequados são *EF1*, *EF1α* e *UBQ10*, e em condições de alta temperatura são os genes *MDH*, *GAPDH* e *EF1α*. Para o déficit hídrico, utilizou-se dois normalizadores da literatura, *GAPDH* e *UBQ10.* Esta avaliação possibilitou a inclusão de dois novos genes para normalização de dados, *MDH* e *EF1*. Este estudo também demonstrou que a síntese de manitol é alterada em condições de estresses abióticos; que há modulação transcricional dos genes *CaM6PR*, *CaPMI* e *CaMTD,* principalmente sob condição de seca; e que o subgenoma *Coffea canephora é* o principal responsável pelo perfil transcricional destes genes.

Palavras-chave: Café, qPCR, alta temperatura, salinidade, deficit hídrico, manitol, fosfomanose-isomerase, manose-6-fosfato redutase, manitol desidrogenase, subgenoma.

‘EXPRESSION OF GENES INVOLVED IN MANITOL BIOSYNTHESIS IN *Coffea arabica* SUBMITTED TO ABIOTIC STRESSES AND CONTRIBUTION OF *Coffea canephora* HOMEOLOGS’

ABSTRACT

Brazil stands out as the main world coffee producer and exporter. In 2013 Brazil’s production is estimated in more than 50 million bags of coffee. Although production and consumption increase every year, the coffee plants are still subjected to production variations, which could be explained by two year cycle of the plant and also by environmental conditions such as abiotic stresses. Among them, we can outline drought, salinity and high temperatures. When subjected to stress, plants can synthesize osmoprotectors, like the sugar alcohol mannitol, a compound poorly studied, especially in coffee. Moreover, being an allotetraploid, transcriptional responses of *Coffea arabica* are an expression set of two subgenomes. Thus, the present study aimed to investigate the influence of drought, salinity and high temperature in the transcriptional profile of genes involved in synthesis and catabolism of mannitol, as well as the contribution of *Coffea canephora* homeolog in the transcriptional pattern of these genes and the carbohydrates concentration under these stresses. To assess gene transcriptional pattern, potential reference genes were previously evaluated, where it was found that under conditions of high salinity the most suitable reference genes were *EF1*, *EF1α* and *UBQ10* and under high temperature were *MDH*, *EF1α* and *GAPDH*. In water deficit, we used two reference genes from literature, *GAPDH* and *UBQ10*. This study allowed the inclusion of two new genes for data normalization, *MDH* and *EF1*. This study also showed that the mannitol synthesis is regulated in abiotic stress conditions; that there is transcriptional modulation of *CaM6PR*, *CaPMI* and *CaMTD,* mainly under drought; and that *Coffea canephora* is the main responsible for the transcriptional profile of these genes.

Keywords: Coffee, qPCR, heat shock, salinity, water deficit, mannitol, phosphomannose-isomerase, mannose-6-phosphate reductase, mannitol dehydrogenase, subgenome.

1 INTRODUÇÃO GERAL

O café é um dos mais valiosos produtos primários no comércio mundial e seu cultivo, processamento, transporte e comercialização emprega cerca de 25 milhões de pessoas (WATSON; ACHINELLI, 2008). Além disso, essa cultura é crucial para a economia de diversos países em desenvolvimento, e seu comércio internacional chega a atingir 70 bilhões de dólares por ano (FAOSTAT, 2010). A produção mundial de café é de cerca de 144,5 milhões de sacas de 60 kg com uma renda de aproximadamente 23 bilhões de dólares em 2010/11. Atualmente, o Brasil é o principal produtor mundial de café (MISHRA; SLATER, 2012; MAPA, 2013).

Os estresses abióticos são os principais responsáveis por perda de produtividade agrícola, pois alteram as condições vegetais ótimas de modo a acarretar distúrbios funcionais na célula (JASPERS; KANGASJARVI, 2010). Entre eles, destacam-se o estresse térmico, o estresse salino e o déficit hídrico, que são desafios globais a serem compreendidos para garantir a sobrevivência das culturas agrícolas e produção sustentável de alimentos (JALEEL *et al.*, 2009). Levando-se em conta que atualmente os recursos hídricos para populações humanas já estão em risco, a agricultura poderá levar a uma sobreexploração dos mesmos, aumentando assim as restrições para o crescimento e sobrevivência das plantas e, portanto, para o desenvolvimento de culturas com potencial produtivo (PASSIOURA, 2007). A expansão da agricultura para regiões áridas e semi-áridas com o uso de irrigação intensiva agravará o processo de salinização secundária, resultado de mudanças no balanço hidrológico. Além disso, as previsões atuais de mudanças climáticas global e os cenários decorrentes dessas transformações sugerem um aumento da aridez no mundo. No Brasil, regiões que atualmente são as maiores produtoras de grãos poderão não estar mais aptas ao plantio mesmo com irrigação. Além disso, o aquecimento global poderá levar a perdas de até R$ 7,4 bilhões em 2020 (IPCC, 2009).

Durante os períodos de estresse, as plantas podem sintetizar osmoprotetores – compostos orgânicos como aminoácidos (prolina e ácido glutâmico), compostos quaternários e outras aminas (glicinabetaína e poliaminas) e uma variedade de açúcares e açúcares-alcoois, como o manitol, galactinol, trealose e rafinose (VINOCUR; ALTMAN, 2005). Estes compostos podem minimizar os

efeitos adversos da alta osmolaridade pelo aumento do conteúdo de água livre no citoplasma, estabilizando macromoléculas (IGNATOVA; GIERASCH, 2006) e estruturas de membrana (ZHIFANG; LOESCHER, 2003) e através de remoção de espécies reativas de oxigênio (BOHNERT; JENSEN, 1996).

O álcool de açúcar manitol pode agir como soluto compatível prevenindo perda de água ou balanceando o acúmulo de sal. A síntese de manitol é realizada através da isomerização da frutose-6-fosfato pela ação da enzima fosfomanose isomerase (PMI), formando manose-6-fosfato que, pela atuação da enzima chave manose-6-fosfato redutase NADPH dependente (M6PR), forma o manitol-1-fosfato (ZHIFANG; LOESCHER, 2003). O manitol é formado pela desfosforilação inespecífica do manitol-1-fosfato (SONG *et al*., 2010).

Em algumas espécies vegetais, o manitol é normalmente sintetizado como produto fotossintético primário e pode ser metabolizado (STOOP *et al*., 1996; NOIRAUD *et al*., 2001a; CONDE *et al*., 2007). Entretanto, culturas como trigo, tabaco e a planta-modelo *Arabidopsis thaliana* não produzem manitol. Desta forma, uma alternativa de aumento de tolerância a estresse nestas plantas é a introdução do mecanismo de síntese de manitol. Estudos recentes têm demonstrado aumento de tolerância a estresse hídrico e salino por produção constitutiva de manitol em plantas transgênicas, sem impactos relevantes no fenótipo em condições não-estressantes (WILLIAMSON *et al*., 2002; ABEBE *et al*., 2003; SICKLER *et al*., 2007). Entretanto, quase não há estudos avaliando a síntese estresse-induzida de manitol em plantas que não sejam transgênicas.

A análise *in silico* de seqüências do banco de dados do projeto brasileiro de transcriptoma do café identificou em *Coffea arabica* um ortólogo do gene *M6PR*, que codifica para a enzima chave na produção de manitol, e análises iniciais por Northern eletrônico indicam que este gene possui expressão diferencial em situações de estresse hídrico. Poucos estudos moleculares têm enfocado o gene *M6PR* em plantas e ainda não há relatos referentes à expressão deste gene em *Coffea arabica* em condições de estresses abióticos.

Apesar da importância do café, até agora, esforços limitados têm sido dedicados à compreensão de seus aspectos genéticos e genômicos (CENCI *et al*., 2012) quando comparado a outras culturas. Por ser alotetraploide, as respostas transcricionais de *C. arabica* são um conjunto da expressão de dois subgenomas (*C.*

canephora - Cc e *C. eugenioides* - *Ce*). A união de dois ou mais genomas de plantas têm se tornado foco de pesquisas e os recentes avanços em técnicas genômicas tem permitido elucidar diversos efeitos de poliploidia. Uma parte dos estudos analisa o impacto deste fenômeno em cópias gênicas de homeólogos parentais. A combinação de genomas diferentes pode conduzir a uma série de efeitos na expressão gênica, que vão desde quase a supressão completa de transcritos de um genoma (dominância nucleolar), a uma regulação diferencial (‘up and down’) (HEGARTY, 2011). Assim, uma consequência direta e observável da poliploidia é que genes homéologos são expressos em diferentes níveis e respondem diferentemente à alopoliploidia em vários órgãos, plantas e estresses (ADAMS, 2007; WANG *et al*., 2011).

Desta forma, este trabalho teve por objetivo avaliar respostas moleculares a estresses abióticos por meio de duas frentes: 1) investigar o papel do manitol em *C. arabica* submetido aos estresses hídrico, salino e térmico pela quantificação desse açúcar-álcool e seus precursores, e pela avaliação dos níveis transcricionais dos principais genes envolvidos em sua rota metabólica (*CaM6PR*, *CaPMI* e *CaMTD*). Para isso, avaliou-se a estabilidade de dez potenciais genes de referência para normalização de dados de qPCR envolvendo experimentos de estresses abióticos em café; 2) avaliar a modulação do homeólogo *Coffea canephora* (Cc), subgenoma de *C. canephora* dentro de *C. arabica*, em cada condição experimental e para cada um dos genes analisados ($CaM6PR_{Cc}$, $CaPMI_{Cc}$ e $CaMTD_{Cc}$).

2 REVISÃO DE LITERATURA

2.1 ASPECTOS ECONÔMICOS DA CAFEICULTURA

O café é uma das commodities mais comercializadas no mundo. Para muitos países, as exportações de café são responsáveis por uma proporção significativa de tributos e produto interno bruto. O comércio global do café no ano de 2012 totalizou cerca de 144.5 milhões sacas de 60 Kg (MAPA, 2013) e movimentou cerca de 15,4 bilhões de dólares (ICO, 2012). O Brasil se destaca como principal produtor e exportador mundial de café, seguidos por Vietnã, Indonésia, Colômbia, Etiópia, Honduras, Índia, México, Peru Guatemala, Costa do Marfim, Nicarágua e El Salvador.

De toda a produção mundial de café, o Brasil colaborou na última safra com 50.8 milhões de sacas de 60Kg respondendo com 35.17% de toda produção. Deste total, cerca de 28.7 milhões de sacas (25.24%) foram exportadas e 20 milhões (47.62%) foram utilizadas para consumo interno (MAPA, 2013). Os consumidores brasileiros tem cada vez mais elevado o consumo desta bebida. No período de um ano houve um acréscimo de cerca de 3,11% no consumo comparado ao período anterior (ABIC, 2012) . Este fator é proveniente da oferta no mercado de produtos diferenciados com melhor qualidade. Além disso, os brasileiros estão consumindo uma quantidade cada vez maior de café diariamente e diversificando as formas de consumo da bebida (ABIC, 2012).

A previsão atual para a produção nacional de café beneficiado indica que o país deverá colher 50.16 milhões de sacas de 60 quilos do produto beneficiado (MAPA, 2013). O resultado desta produção deverá ser superior ao da safra passada, acréscimo obtido principalmente pelo ano de alta bienalidade. Considerando-se as últimas quatro safras de bienalidade positiva, a produção mantém um crescimento constante, demonstrando que a maior utilização da mecanização, aliada às inovações tecnológicas, às exigências do mercado, à qualidade do produto e à boa gestão da atividade são fatores extremamente importantes e necessários para o avanço e modernização da cafeicultura (CONAB, 2012).

Dentre os estados brasileiros, Minas Gerais, Espírito Santo, São Paulo, Paraná, Bahia, Rondônia, Mato Grosso e Goiás são os principais responsáveis pela

produção nacional. Em Minas Gerais está concentrada a maior área com 1.040.966 hectares, predominando a produção de *Coffea arabica* (MAPA, 2013).

2.2 O CAFEEIRO

O cafeeiro é uma dicotiledônea lenhosa perene que pertence à família botânica Rubiaceae e que apresenta porte arbustivo ou arbóreo. Apresenta um ramo principal vertical (ortotropia) e ramos horizontais primários, secundários e terciários (plagiotrópicos) (COFFEE RESEARCH INSTITUTE, 2010). A família Rubiaceae possui cerca de 500 gêneros e mais de 6.000 espécies, entre os quais o gênero *Coffea* é o membro mais importante em termos econômicos (ICO, 2010). Embora o gênero *Coffea* contenha mais de 124 espécies (DAVIS *et al*., 2011), apenas duas têm significado econômico de destaque: *Coffea arabica* e *Coffea canephora*. Cerca de 70% do café produzido é proveniente da primeira, e 30% da segunda espécie (CONAB, 2009). *Coffea arabica*, apesar do seu exigente sistema de produção, é responsável pela maior parte da produção mundial, produz uma bebida de melhor qualidade, com baixos teores de cafeína, (BARRETO *et al*., 2012). Já *C. canephora* é matéria-prima para o café solúvel, produzindo grãos considerados de menor qualidade, porém com maior teor de cafeína e ácidos clorogênicos (LEROY *et al*., 2006).

O cafeeiro é uma planta tropical cujo cultivo comercial requer condições ambientais muito específicas. Fatores como temperatura, chuva, sol, vento e solos são importantes, mas as exigências variam conforme as variedades cultivadas. As temperaturas médias ideais vão de 15 a 24°C para *C. arabica* e de 22 a 26°C para *C. canephora*. A faixa ótima de precipitação anual para o café arábica é de 1200-1800 mm. Uma variação semelhante também é requerida pelo robusta, contudo este possui uma melhor adaptação sob condições de chuvas intensas superiores a 2000 mm (DA MATTA, 2003). A distribuição dos períodos de chuva e estiagem é importante para o crescimento, a formação dos botões e a floração (ICO, 2010). Um curto período de seca, com duração de 2-4 meses, o que corresponde à fase de crescimento quiescente, é importante para provocar a floração.

Coffea arabica é a única espécie alotetraplóide (2n=4x=44 cromossomos) do gênero, híbrido natural entre os diplóides *C. eugenioides* e *C. canephora*, e

predominantemente autofértil em aproximadamente 90% das flores, enquanto *C. canephora* e as outras espécies conhecidas do gênero *Coffea* são diplóides (2n=2x=22 cromossomos) e autoincompatíveis, multiplicando-se em sua grande maioria por fecundação cruzada (FAZUOLI *et al*., 1999). Esta espécie é caracterizada por apresentar baixa variabilidade genética (LASHERMES *et al*., 1996), o que é relacionado a sua biologia reprodutiva (planta autógama) (PRAKASH *et al*., 2002), ao seu centro de origem limitado e ao fato de terem sido utilizados poucos genótipos durante o processo de dispersão dos cafeeiros (ANTHONY *et al*., 2002; PINTO, 2006; VIDAL *et al*., 2010). Inclusive, por esta razão, outras espécies de *Coffea* não cultivadas comercialmente são consideradas fonte de variabilidade genética para o uso potencial em programas de melhoramento já que possuem características interessantes ao melhoramento de cafeeiros, como tolerância a estresses bióticos e abióticos (GEROMEL *et al*., 2008).

2.3 SUBGENOMAS DE *Coffea arabica*

Todas as espécies de *Coffea* são diplóides, exceto *C. arabica*, que é um alotetraplóide derivado de uma hibridização, seguida por duplicação genômica (LASHERMES *et al*., 1999). Assim, o transcriptoma de *C. arabica* é uma mistura da expressão de genes homéologos provenientes de dois sub-genomas, *C. canephora* e *C. eugenoides*, o provável progenitor feminino, baseado na análise molecular de cloroplastos (LASHERMES *et al*., 1999; VIDAL *et al*., 2010).

Os processos no qual dois genomas se adaptam para coexistir dentro de um mesmo núcleo é complexo. Estudos têm demonstrado mudanças dinâmicas na expressão gênica de plantas poliplóides, o que reflete na genômica e plasticidade funcional de genes duplicados (JACKSON; CHEN, 2009). Para investigar os efeitos da fusão genômica e duplicação, a divergência transcriptômica entre parentais e alopoliplóides naturais tem sido avaliada em diferentes estudos utilizando abordagens genômicas para medir o desvio de aditividade. De fato, no modelo aditivo, seria esperado que a expressão no alopoliplóide fosse equivalente à media de expressão das espécies parentais. Contudo, estudos em diversos alopoliplóides, como *Gossypium hirsutum* (CHAUDHARY *et al*., 2009) e *Triticum aestivum* (PUMPHREY *et al*., 2009), têm revelado uma tendência não aditiva na expressão

gênica. Bardil e colaboradores (2011) revelaram divergências no perfil transcricional entre o *C. arabica* e seus parentais, em condições ambientais de temperatura mais alta, ampliando assim os horizontes para o fenômeno de plasticidade em alopoliplóides. Também, o sequenciamento genômico têm demonstrado que as duas espécies parentais são intimamente relacionadas, e que os dois sub-genomas exibem baixa divergência de sequencias (YU et al., 2011; CENCI *et al.*, 2012).

Em alopoliplóides, localmente ou em todo o genoma, genes homeólogos podem exibir padrões de expressão desiguais, que podem variar de acordo com o tecido, desenvolvimento ou respostas a estresses. Podem ser observados tanto o silenciamento completo de uma cópia como o silenciamento recíproco (silenciamento de um gene homeólogo em um órgão e silenciamento de outros genes homeólogos em outros órgãos). Genética complexa e mecanismos regulatórios epigenéticos governam a expressão tendenciosa genoma-específicas em espécies de plantas alopoliplóides. Os níveis de expressão de homeólogos podem influir nas funções evolutivas de genes duplicados e, por consequência, na evolução de plantas alopoliplóides depois da poliploidização (BUGGS *et al*., 2010). Todas estas alterações na expressão de genes homeólogos atestam a capacidade de poliplóides de diversificação funcional e adaptativa (COMBES *et al*., 2012).

Um estudo baseado em uma análise em larga escala de ESTs demonstrou expressão diferencial de homeólogos em *C. arabica*, indicando que este fenômeno é comum em poliplóides (VIDAL *et al*., 2010). Em estudos recentes de expressão gênica por microarray, foi demonstrada uma expressão preferencial de homeólogos de *C. canephora* em plantas submetidas a altas temperaturas e de *C. eugenioides* em temperaturas reduzidas (BARDIL *et al*., 2011; PRIVAT *et al*., 2011).

Apesar de sua importância, até agora, esforços limitados têm sido dedicados à compreensão genética e genômica de *C. canephora* e *C. eugenioides* utilizadas para fins comerciais de produção de café (CENCI *et al.*, 2012). Alterações na expressão de homeólogos têm sido exploradas em diferentes órgãos (HOVAV *et al*., 2008; CHAUDHARY *et al*., 2009; FLAGEL *et al*., 2009; BUGGS *et al*., 2010), entretanto, poucos estudos tem explorado o efeito de diferentes tipos de estresse (LIU; ADAMS, 2007; STAMATI *et al*., 2009; DONG; ADAMS, 2011).

2.4 ESTRESSES ABIÓTICOS

O crescimento e produtividade das plantas são afetados em grande parte por estresses ambientais, de modo que estas desenvolveram, em diferentes níveis, um complexo mecanismo de tolerância (DUQUE *et al*., 2013), e muitas vezes a forma como cada planta percebe e responde às adversidades ambientais pode ser sobreposta (SREENIVASULU *et al*., 2007). Uma importante frente de resposta pode ocorrer através da expressão gênica, cuja regulação ocorre de maneira distinta em resposta a diferentes estresses.

Quando uma planta é submetida a um estresse, ocorre uma sinalização que leva a modulação transcricional de uma série de genes, que resulta em uma alteração no nível de diversos metabólitos e proteínas, algumas das quais podem ser responsáveis por conferir um certo grau de proteção e tolerância (BHATNAGAR-MATHUR *et al*., 2008). A rapidez na percepção do sinal e as conseqüentes alterações transcricionais que levam a uma adaptação e eventual tolerância ao estresse variam entre espécies e entre genótipos de uma mesma espécie (SREENIVASULU *et al*., 2007).

Na regulação gênica em nível transcricional há a ação de três elementos principais: remodelamento e modificação da cromatina, elementos cis-regulatórios que frequentemente são sítios de ligação como ‘enhancers’ e promotores, localizados up ou downstream à região codante; e elementos trans-regulatórios, como fatores de transcrição. Além da transcrição, outros dois níveis de regulação também podem ocorrer, pós-transcricional e pós traducional. A modulação pós-transcricional pode ocorrer das seguintes maneiras: processamento de pré mRNA (splicing, poliadenilação); tráfego núcleo-citoplasmático de mRNA; turnover e estabilidade de mRNA; e tradução do mRNA. Em nível pós-traducional a planta pode realizar processos de fosforilação, sumoilação e ubiquitinação de proteínas (DUQUE *et al*., 2013).

Embora grande parte dos esforços de caracterização de genes envolvam análises transcriptômicas, é relevante colocar que estas apenas refletem diferenças no acúmulo de RNAm, o que não significa necessariamante que o gene será convertido em um produto. Por isso, além do perfil transcricional, faz-se importante a avaliação de outros produtos que estejam relacionados ao processo de efetiva

tradução, armazenamento e/ou degradação destes RNAm durante o tratamento de estresse (URANO *et al*., 2010) .

Prevê-se uma maior incidência de estresse em razão das alterações climáticas globais de acordo com relatórios do Painel Intergovernamental de Mudanças Climáticas (http://www.ipcc.ch). Assim, a resposta a estresses abióticos é atualmente um dos tópicos mais importantes no estudo de plantas (HIRAYAMA; SHINOZAKI, 2010), e uma das estratégias biotecnológicas para enfrentar essa situação é o desenvolvimento de plantas geneticamente modificadas. Contudo, culturas transgênicas transformadas com um ou vários genes que confiram tolerância ao estresse podem apresentar problemas inesperados de metabolismo. Para tentar contornar algumas das dificuldades, é importante o conhecimento do sistema vegetal de resposta ao estresse, o que inclui regulação de energia, processos metabólicos e de desenvolvimento (Figura 1).

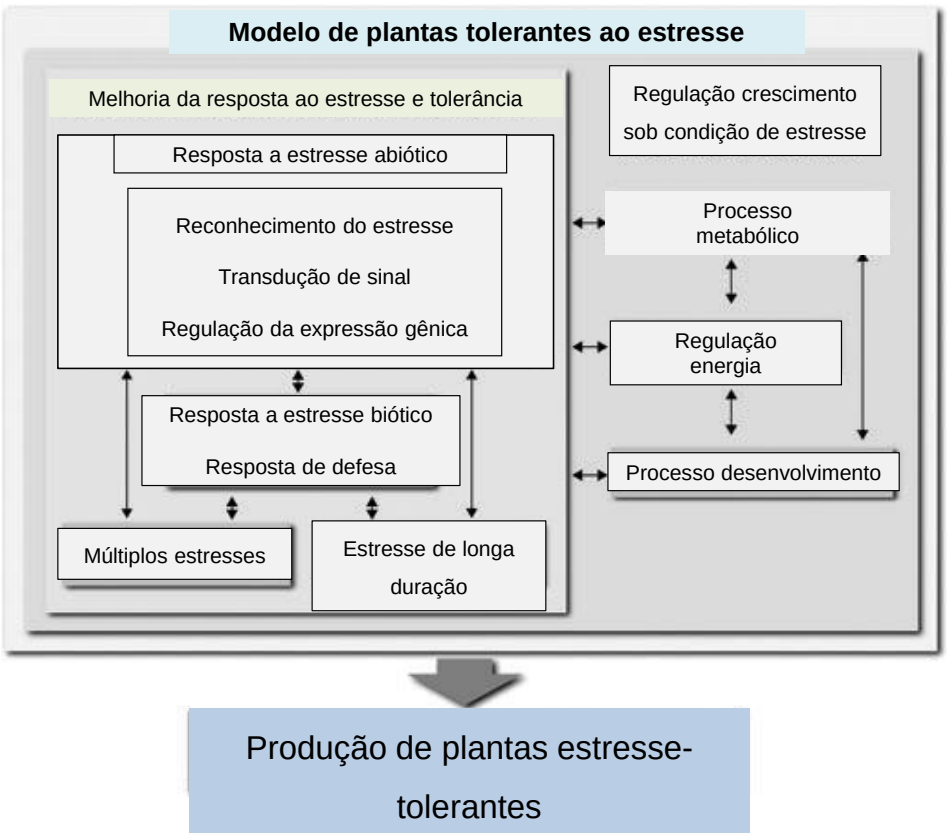

FIGURA 01 – Mecanismos envolvidos na resposta ao estresse (HIRAYAMA & SHINOZAKI, 2010).

Algumas redes regulatórias envolvidas na resposta da planta a estresses já foram propostas (VIJ; TYAGI, 2007). Entretanto, a integração das funções gênicas ainda permanece um desafio na compreensão do processo global de resposta da planta ao estresse (VALLIYODAN; NGUYEN, 2006). De maneira geral, os esforços de produção de plantas mais tolerantes a estresses abióticos por meio da transgenia geralmente envolvem a ação de um único gene, que confere modificação de um único metabólito, normalmente resultando-se em um aumento de tolerância devido a uma alteração em cascata de uma complexa rede. Entre esses genes alvo, estudos recentes têm priorizado o estudo de proteínas regulatórias na tentativa de melhorar a tolerância ao estresse, mas também pode-se destacar proteínas de canais de água, enzimas de detoxificação, enzimas de transporte e enzimas chave para biossíntese de osmólitos (como a prolina e a betaína; e açúcares como manitol, trealose e poliaminas) (BHATNAGAR-MATHUR *et al*., 2008).

Considerando-se o presente estudo, é importante destacar o papel dos açúcares durante condições de estresses abióticos. Como o aparato fotossintético é altamente sensível à condições de estresses abióticos, a assimilação de carbono e o metabolismo primário são afetados nestas condições. Assim, açúcares, álcool açúcares e aminoácidos são importantes metabólitos cuja concentração é alterada durante períodos de estresse (KRASENSKY; JONAK, 2012; VALERIO *et al.* 2011). Além de estarem envolvidos no controle transcricional, pós-transcricional e pós-traducional, os açúcares também atuam como moléculas sinalizadoras (ROLLAND *et al.* 2006; MULLER *et al*. 2011) e ocupam assim posição de destaque no balaço redox celular influenciando a produção de espécies reativas de oxigênio (ROS) (COUÉE *et al*. 2006). Álcool açucares, como o manitol, já foram associados a aumento de tolerância durante estresses abióticos por agirem como removedores de ROS.

2.4.1 ALTAS TEMPERATURAS

As altas temperaturas têm grande impacto em diversos processos fisiológicos e metabólicos (WAHID *et al*., 2007). O estresse térmico é prejudicial em termos de crescimento e produtividade vegetal, especialmente nos meses de verão e em regiões quentes (HALL, 2001). Além de ser uma preocupação para o cultivo de

culturas importantes agronomicamente em regiões quentes, o aquecimento global associado às emissões de gases do efeito estufa decorrentes da ação antrópica, podem aumentar a média de temperatura ambiente no mundo, agravando o problema do estresse térmico. Compreender os mecanismos de tolerância de calor é fundamental para atenuar os efeitos do estresse térmico na produtividade e auxiliar no desenvolvimento de plantas mais tolerantes (HUANG; XU, 2008).

Em temperaturas muito altas, danos celulares severos e até morte celular podem ocorrer em minutos, o que pode ser atribuído a um colapso na organização celular (SCHÖFFL *et al*., 1999). Entre os danos diretos decorrentes das altas temperaturas destacam-se a desnaturação e agregação de proteínas. Há também uma série de danos indiretos, como a inativação de enzimas em cloroplastos e mitocôndrias, inibição de síntese protéica, degradação de proteínas e perda de integridade de membrana (HOWARTH, 2005). O estresse térmico também afeta a organização de microtúbulos (SMERTENKO *et al*., 1997) e induz a produção de metabólitos secundários e compostos fenólicos, como flavonóides e fenilpropanóides (BHARTI; KHURANA, 1997; RIVERO *et al*., 2001). Estes danos eventualmente levam à inibição de crescimento, redução no fluxo iônico, produção de compostos tóxicos e espécies reativas de oxigênio (SCHÖFFL *et al*., 1999; HOWARTH, 2005).

O aumento da temperatura leva à redução das taxas fotossintéticas por favorecer a fotorrespiração (SCHUSTER; MONSON, 1990), ou por causar danos no aparelho fotossintético (SHARKEY, 2005). A atividade fotossintética dos cloroplastos está entre as funções celulares mais afetadas pelo calor (YORDANOV *et al*., 1986). Existem, no mínimo, três sítios estresse sensíveis na maquinaria fotossintética: os fotossistemas, principalmente o II (PSII), a geração de ATP e o processo de assimilação do carbono (MURATA *et al*., 2007; MOHANTY *et al*., 2007; ALLAKHVERDIEV *et al*., 2008).

Imediatamente após a exposição às altas temperaturas e percepção de sinais, mudanças moleculares alteram a expressão gênica e acúmulo de transcritos, levando assim à uma síntese de proteínas relacionadas a estresses como estratégia de tolerância (IBA, 2002). Uma resposta amplamente conhecida é a expressão de proteínas de choque térmico (HSPs), uma importante estratégia adaptativa de tolerância ao calor (FEDER; HOFFMAN, 1999). As HSPs apresentam massa molecular entre 10 e 200kDa e estão envolvidas na transdução de sinal durante o

estresse e com função deduzida como chaperonas, dobrando e desdobrando proteínas celulares e protegendo seus sítios funcionais (LINDQUIST, 1986; VIERLING, 1991). Chaperonas moleculares são proteínas que se ligam e estabilizam a conformação de outras proteínas, e pela ligação e liberação controlada, facilitam o seu destino correto *in vivo*, seja por dobramento, montagem oligomérica, transporte para um compartimento subcelular específico ou eliminação por degradação (HENDRICK; HARTL, 1995). Existem diversas famílias caracterizadas de HSPs/chaperonas: HSP100/Clp, HSP90, HSP70/DnaK, chaperoninas (GroEL e HSP60), e HSPs pequenas (small HSP) (WANG et *al*., 2004). Além das HSPs/chaperonas mencionadas, que constituem o mecanismo mais estudado para este tipo de estresse, outras proteínas, como as proteínas LEA (late embryogenesis abundant protein), também exercem papel fundamental durante os períodos de estresse (WAHID *et al*., 2007). Durante o estresse, pode também ocorrer um influxo induzido de Ca^{+2} e reorganização citoesquelética, resultando na regulação de MAP quinases (MAPK) e proteínas quinase cálcio dependentes (CDPK). A sinalização destas cascatas em nível nuclear levam à produção de antioxidantes e osmólitos compatíveis para o equilíbrio do balanço de água e ajustamento osmótico. A produção de espécies reativas de oxigênio nas organelas (cloroplasto e mitocôndrias) é de grande importância para a sinalização, bem como a produção de antioxidantes (BOHNERT *et al*., 2006).

A figura 2 apresenta uma síntese das diversas alterações e mecanismos moleculares que permitem que a planta tenha uma maior tolerância ao estresse. Os efeitos do estresse térmico podem ser observados em diversos níveis, incluindo membrana plasmática e vias bioquímicas operativas no citosol ou em organelas citoplasmáticas (SUNG *et al*., 2003).

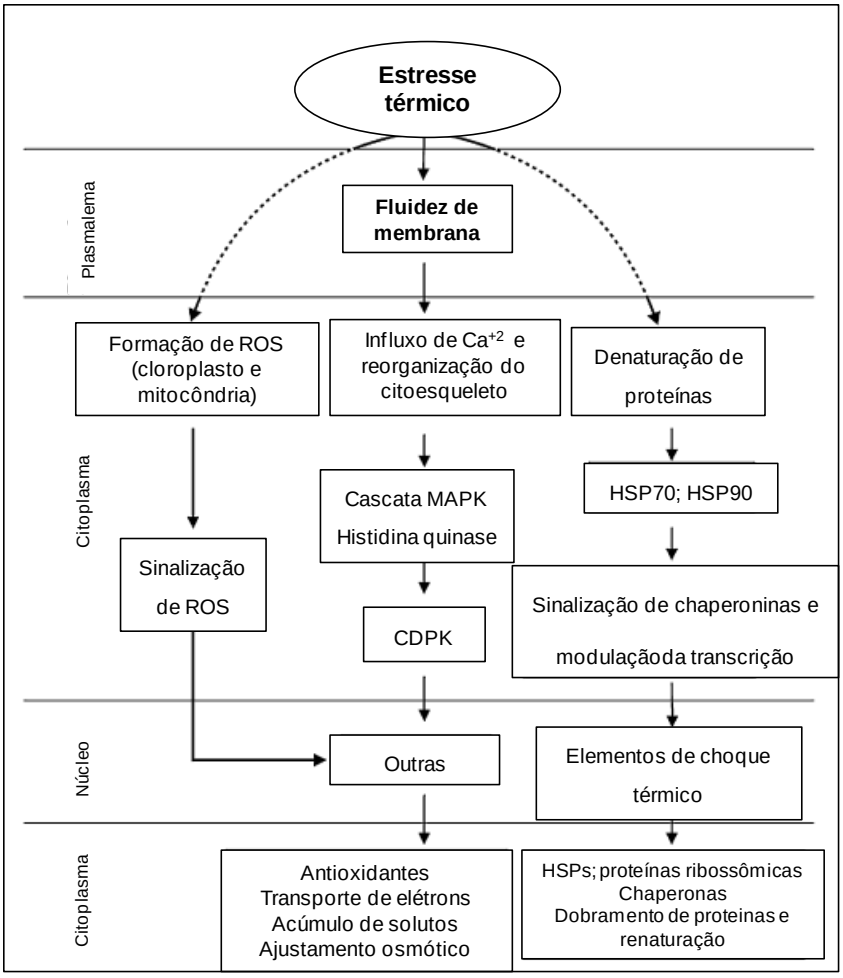

FIGURA 02 - Proposta de mecanismos de tolerância ao estresse térmico em plantas (WAHID *et al*., 2007).

Embora os mecanismos fisiológicos de tolerância ao calor sejam relativamente bem conhecidos, mais estudos são essenciais para determinar as bases fisiológicas do fracionamento de assimilados de tecidos fonte para os drenos, a flexibilidade fenotípica de plantas que leva à tolerância ao calor, e os fatores que modulam respostas a este estresse na planta. Nesse sentido, as abordagens genômica, proteômica e trascriptômica são essenciais para uma melhor compreensão das bases moleculares da resposta da planta ao estresse térmico, bem como a tolerância ao calor.

Como no caso da maioria dos outros estresses abióticos, as folhas são mais diretamente prejudicadas por altas temperaturas que as raízes. O conhecimento de mecanismos moleculares de resposta e tolerância abre caminho para a biotecnologia de plantas, o que pode ser a base para a produção de culturas de rendimento econômico em condições de estresse térmico (WAHID *et al*., 2007).

2.4.2 ESTRESSE OSMÓTICO

As plantas são freqüentemente submetidas a períodos de déficit hídrico durante seu ciclo de vida, assim como a altas concentrações salinas no solo em diversas áreas do globo. É estimado que mais de 6% do solo mundial e 30% das áreas irrigadas apresentem problemas de alta salinidade (http://www.unesco.org/water/water). A intensidade, duração e taxa de progressão do estresse influencia a resposta da planta à escassez de água e alta salinidade, uma vez que estes fatores irão ditar se o processo de mitigação associado à aclimatação vai ocorrer ou não (CHAVES *et al*., 2009). Respostas moleculares que são associadas à aclimatação ao estresse hídrico e salino são multigênicas e freqüentemente inter-relacionadas, portanto dificultando a identificação precisa de quais processos de sinalização são determinantes para tolerância específica a um estresse (PARDO, 2010).

Além de alterações fotossintéticas e no crescimento celular, o estresse salino e o déficit hídrico quando vagarosamente impostos, frequentemente induzem ajustamento osmótico, o que é considerado um importante mecanismo para manter a absorção de água e turgor celular sob condições de estresse. Os efeitos destes dois tipos de estresse na fotossíntese variam desde restrição na difusão do CO_2 para os cloroplastos a alterações na fotoquímica da folha e metabolismo de carbono. Estes efeitos variam de acordo com a intensidade e duração do estresse assim como com a espécie e a idade da folha - folhas mais velhas são mais afetadas por déficit hídrico e acumulam maiores quantidades de sal (FLEXAS *et al*., 2004; GALMÉS *et al*., 2007).

Outra consequência do estresse salino e do déficit hídrico é a produção de espécies reativas de oxigênio (ROS) que podem afetar estruturas celulares e o metabolismo. As espécies reativas de oxigênio (ROS) produzidas durante o estresse incluem oxigênio singleto, peróxido de hidrogênio, radicais ânion superóxido e radicais hidroxil (BARTELS, 2001; APEL; HIRT, 2004). As ROS são geradas predominantemente no cloroplasto por transferência direta da energia de excitação da clorofila para produzir oxigênio singleto, ou pela redução univalente do oxigênio no fotossistema I (FOYER *et al*., 1994; ALLEN, 1995) e, em certa medida, na mitocôndria. Os cloroplastos são os primeiros alvos em células de plantas uma vez

que este é o principal local de produção de ROS. O aumento da concentração de ROS inibe a capacidade de reparar os danos ao fotossistema II e inibe a síntese da proteína D1, uma das duas proteínas que constituem o centro de reação do FSII. Fotorrespiração e a atividade da NADPH também contribuem para o acúmulo de H_2O_2, que pode inativar enzimas por oxidação seus grupos tiol. A toxicidade do H_2O_2 não é devida a sua reatividade em si, mas na presença de um metal redutor forma radical hidroxil altamente reativo, que potencialmente reage com todas as moléculas biológicas (BARTELS; SUNKAR, 2005).

A adaptação ao estresse é um processo complexo que envolve muitas mudanças, entre elas o crescimento atenuado, a ativação/expressão aumentada ou indução de genes, aumentos transitórios nos níveis de ABA, o acúmulo de solutos compatíveis e proteínas protetoras, aumento nos níveis de antioxidantes e supressão de vias que consomem energia. No entanto, não há um consenso na definição dos processos-chave de determinação da tolerância e processos secundários posteriores (BARTELS; SUNKAR, 2005).

Durante a incidência de déficit hídrico/salino as plantas também podem acumular osmoprotetores em grandes quantidades, o que não interfere no metabolismo celular, a fim de se manter o turgor celular (YANCEY *et al*., 1982). Aminoácidos como prolina, compostos quaternários e outras aminas, como glicinabetaína e poliaminas, e uma variedade de açúcares e álcoois de açúcar, como manitol, trealose, galactinol e rafinose, estão entre os principais solutos compatíveis acumulados durante o estresse (MAHAJAN; TUJETA, 2005).

2.4.2.1 ESTRESSE SALINO

Mais de 800 milhões de hectares de terras são afetadas pela salinidade (FAO, 2008). Solos salinos estressam plantas de duas maneiras: altas concentrações de sal presente no solo podem dificultar a absorção de água pelas raízes, ou quando presente na planta pode ser tóxico. O ambiente salino fora das raízes tem efeito imediato no crescimento celular e metabolismo associado. Concentrações tóxicas intracelulares levam um certo tempo para afetar as funções da planta (MUNNS; TESTER, 2008).

A ação do intemperismo sobre as rochas provoca a liberação de sais solúveis de diversos tipos, principalmente cloretos de sódio, cálcio e magnésio, e em menor grau, sulfatos e carbonatos (SZABOLCS, 1989). Dentre os sais liberados, o cloreto de sódio é o mais solúvel e abundante. Uma outra causa do acúmulo de sais é relacionada a deposição de sais oceânicos transportados pelo vento e chuva. Mais impactante que a salinidade natural, uma significativa proporção de terras agrícolas cultivadas recentemente tornou-se salina devido ao desmatamento e/ou irrigação. As principais causas da salinização nas áreas irrigadas são os sais provenientes de água de irrigação e/ou do lençol freático, quando este se eleva até próximo à superfície do solo, aumentando assim a concentração de sais na zona da raiz (MUNNS; TESTER, 2008).

A deposição elevada de sal, particularmente do sódio (Na^+), pode alterar a textura de base do solo, resultando em diminuição da porosidade e, conseqüentemente, reduzir a aeração do solo e a condutância da água. O excesso de sal gera uma zona de baixo potencial de água dificultando o processo de absorção de água e nutrientes. O estresse salino, por conseguinte, acarreta condição de déficit hídrico nas plantas adquirindo forma de seca fisiológica (MAHAJAN; TUJETA, 2005).

A diversidade genética para tolerância ao sal é altamente variável entre os diversos gêneros vegetais. A maioria das culturas são sensíveis ou hipersensíveis ao sal (glicófitas), em oposição às halófitas, plantas nativas de ambientes salinos. Algumas halófitas possuem a capacidade de tolerar condições salinas extremas devido às suas adaptações anatômicas, morfológicas e fisiológicas (FLOWERS *et al*., 2007). Entretanto, estas características peculiares são reguladas por genes que não são facilmente incorporados por introgressão em cultivares.

De maneira geral, os mecanismos moleculares que mediam a homeostase celular e sinalização de estresse salino são similares em todas as plantas (HASEGAWA *et al*., 2000) e baseiam-se na compartimentalização em vacúolos dos íons citotóxicos de ambientes salinos, tipicamente Na^+ e Cl^-, que então são utilizados como solutos osmóticos (NIU *et al*., 2005). Este mecanismo geral proporciona a utilização de sistemas genéticos modelo para estudos de resposta à salinidade.

Com isso, a planta-modelo *Arabidopsis thaliana* tem proporcionado um grande aumento de informações a respeito de como os mecanismos de tolerância são integrados e coordenados, e também como estão relacionados com adaptações fenológicas. Como Arabidopsis é uma glicófita, uma planta hipersensível ao sal, esta pode ser utilizada como um modelo genético de resposta ao sal comparável a maioria das culturas, o que torna possível delinear se a tolerância é afetada em sua maioria por formas ou funções gênicas ou por diferenças na expressão de genes, comuns devido à presença de controles transcricionais ou pós-transcricionais (ZHU, 2007).

A regulação da homeostase iônica celular durante o estresse salino é crítica para a tolerância da planta a este estresse, uma vez que altas concentrações de sal podem influenciar o balanço de íons na célula e, por conseguinte, influenciam diretamente o gradiente de prótons utilizado no transporte de íons através de membranas (BARTELS; SUNKAR, 2005). Uma das respostas das células da planta é a geração de Ca^{+2} citosólico transiente (KNIGHT *et al*., 1997) e a subseqüente ativação da expressão e/ou atividade da proteína sensora de Ca^{+2}. Até recentemente, pouco era conhecido sobre os alvos *in vivo* e ação das vias de sinalização do estresse salino, entretanto, a identificação da via SOS (Salt-Overly-Sensitive) (Figura 3) em plantas mutantes de Arabidopsis ajudou a revelar componentes e mecanismos envolvidos na resposta ao estresse iônico (CHINNUSAMY *et al*., 2004). A análise molecular de mutantes *sos* de Arabidopsis levaram à identificação de uma cascata de três componentes (SOS1, SOS2 e SOS3) que tenta reestabelecer a homeostase iônica celular baseada em uma sinalização de Ca^{+2} induzida pelo estresse salino. Nesta cascata, SOS1 é um canal iônico, SOS2 é uma proteína quinase essencial para transdução de sinal induzida por estresse salino e fosforila o sinalizador SOS3, uma proteína que se liga a Ca^{+2}.

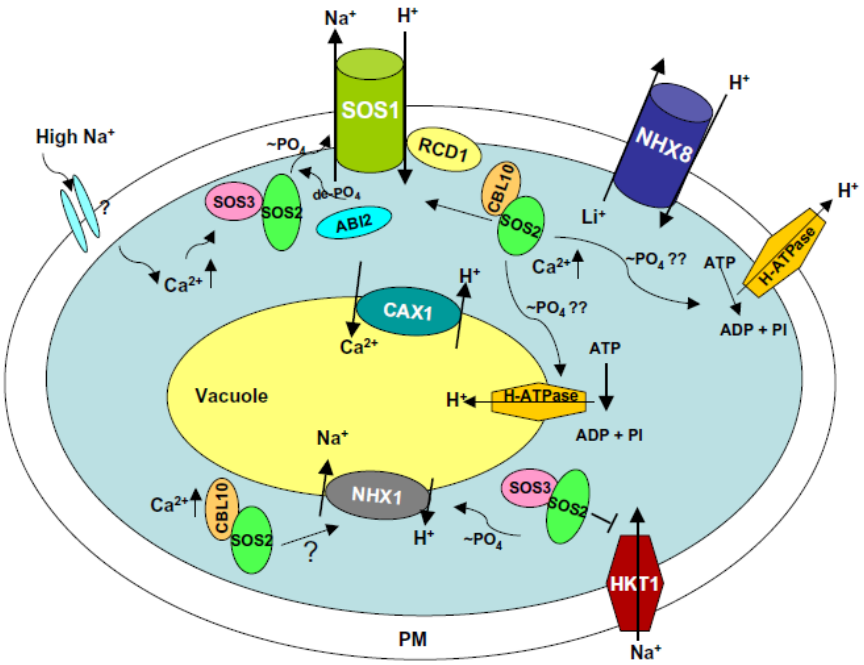

FIGURA 03- Principais componentes da via SOS envolvida no estresse salino. Sob condições de altas concentrações salinas, o aumento citosólico nos níveis de Na^+ causam danos em diversos processos celulares. Um dos principais mecanismos detoxificadores de sal é o complexo protéico cálcio ativado SOS2/SOS3, o qual ativa SOS1, um canal iônico Na^+/H^+- antiporte de membrana plasmática responsável pela extrusão de Na^+ para fora da célula. Ao mesmo tempo, o complexo SOS2/SOS3 também esta envolvido na inibição de HKT1, um transportador de potássio de baixa afinidade, que transporta Na^+ durante o estresse salino. Um outro membro da família SOS3 recentemete descrito, CBL10, também forma complexos com SOS2. Este complexo parece regular a extrusão de Na^+ (por regular SOS1) e seqüestro/compartimentalização de Na^+ nos vacúolos (Ativando transportadores tipo NHX que bombeiam o íon Na^+ no vacúolo). SOS1 também confere proteção contra ROS e estresse oxidativo. SOS2 também ativa H^+-ATPases sob estresse salino e ajuda no restabelecimento da homeostase iônica (MAHAJAN *et al*., 2008).

O excesso de sal na solução do solo apresenta um desafio adicional para a planta além de impedir a absorção de água. Na^{+2} e outros íons absorvidos pelas raízes são transportados para a parte aérea, onde eles acumulam ao longo do tempo, durante a transpiração. Elevadas concentrações de sal são armazenadas no apoplasto, e eventualmente dentro das células, ao evaporar a água. O acúmulo de íons nos tecidos das plantas acarreta danos progressivos (MUNNS; TESTER, 2008).

Em condições de alta salinidade é vital que a célula mantenha as concentrações de íons tóxicos abaixo de um limiar e acumule íons essenciais. A homeostase intracelular de K^+ e Na^+ é importante para o funcionamento de diversas enzimas citosólicas. O desequilíbio de Na^+ decorrente de estresse salino levam a uma série de anormalidades e patologias, afetando assim a sobrevivência,

crescimento e divisão celular (MAHAJAN *et al.*, 2008). Além disso, altas concentrações deste íon podem reduzir a fotossíntese e levar à produção de espécies reativas de oxigênio.

O potássio desempenha importante papel em diversos processos como metabolismo, crescimento e adaptação ao estresse. Na célula, o potássio complexa diretamente com proteínas resultando em ativação enzimática, estabilização de síntese protéica e neutralização de cargas negativas em proteínas. A alteração de sua concentração devido ao estresse salino também perturba o balanço osmótico, funcionamento de estômatos e enzimas (MAATHUIS; SANDERS, 1996; MAHAJAN; TUJETA, 2005).

Para contornar o acumulo de sódio no citoplasma, as plantas se utilizam de transportadores, como os do tipo Na^+/H^+, para realizar o efluxo de Na^+ para o apoplasto ou para seqüestra-los para dentro do vacúolo (APSE *et al*., 1999; QIU *et al*., 2002). O sódio tem um forte efeito inibitório na absorção de K^+ pelas células, provavelmente por interferir em transportadores de alta e baixa afinidade de K^+ presentes no plasmalema e tonoplasto (QI; SPALDING, 2004; RODRIGUEZ-NAVARRO; RUBIO, 2006).

O entendimento dos mecanismos envolvidos na tolerância ao estresse salino provavelmente passa pela identificação minunciosa dos caminhos convergentes e divergentes entre salinidade e outras respostas a estresses abióticos e as interfaces de convergência de sinalização. Entre as plantas halófitas, que podem habitar solos de altas concentrações salinas, existem uma série de adaptações que conferem tolerância o sal e resistência a déficit hídrico. A maioria destas espécies pode acumular solutos compatíveis. Os exemplos mais comuns deste tipo de soluto incluem betainas e polióis como o manitol (BOHNERT *et al*. 1995; SECKIN *et al*., 2009).

2.4.2.2 DÉFICIT HÍDRICO

A seca é uma das principais ameaças à produção agrícola. Mesmo nas áreas mais produtivas, períodos de deficiência hídrica levam a uma redução considerável de biomassa. A tolerância ao déficit hídrico é um fenômeno complexo que envolve diversas vias metebólicas e genes. A existência de diferenças na

tolerância ao estresse entre genótipos indica que existe uma base genética para os mecanismos de tolerância à seca (BARTELS; PHILLIPS, 2010).

Em cafeeiro, estudos na área de melhoramento têm visado a introdução de novos caracteres para obtenção de híbridos mais tolerantes a estresses abióticos, mas se conhece relativamente pouco sobre como os genótipos de café respondem nos níveis morfológicos, fisiológicos, e moleculares ao estresse hídrico (DA MATTA; RAMALHO, 2006).

Um dos principais alvos do melhoramento em áreas tropicais é a obtenção de plantas com um alto potencial de rendimento sob estresse hídrico. Em muitos casos, o alto potencial produtivo pode contribuir para o rendimento em ambiente de estresse moderado (BLUM, 1996). O déficit hídrico é considerado uma perda moderada de água, o que leva ao fechamento estomático, limitação de troca gasosa e diminuição do alongamento e crescimento celular (JALEEL *et al*., 2009). A deficiência hídrica inibe mais o alongamento do que a divisão celular e por afetar o crescimento afeta processos como fotossíntese, respiração, translocação, absorção iônica, carboidratos, metabolismo de nutrientes e promotores de crescimento (FAROOQ *et al*., 2008).

A condutância estomática em *C. arabica* parece constituir um indicador fisiológico precoce da falta de água, mostrando reduções imediatas tão logo um terço do aporte de água no solo decaia. Já *C. canephora* parece exibir um baixo controle estomático da transpiração (DA MATTA *et al*., 2003). O sistema antioxidante também pode estar envolvido na tolerância a seca em cafeeiros. Lima *et al.* (2002) propuseram que a tolerância à seca em *C. canephora* poderia estar parcialmente associada ao aumento da atividade de enzimas antioxidantes.

Condições de seca também desencadeiam a produção do ácido abscísico (ABA), que é um importante sinalizador na promoção do fechamento dos estômatos, bem como na modulação da expressão de genes responsivos ao estresse. Uma alternativa utilizada para modulação de genes influenciados por estresses é a aplicação de ABA exógeno. No entanto, nem todos os genes sofrem modificações em seu padrão transcricional, o que sugere a existência de vias de sinalização de estresse dependentes de ABA e outras indenpendentes deste hormônio.

Segundo Shinozaki e Yamaguchi-Shinozaki (2007), uma hipótese é que devam existir, a grosso modo, seis vias de transdução de sinal para a ativação dos

genes induzidos por seca, alta salinidade e frio, sendo três delas dependentes de ABA e três independentes. Uma destas vias independente de ABA é controlada por seca e salinidade, mas não por frio. Nesse processo, desempenham um papel importante os fatores de transcrição da família DREB (“Dehydration-responsive element binding protein”) que possuem um domínio altamente conservado chamado ERF/APE2 (LIU *et al.*, 2007). Por exemplo, DREB1 está relacionado com baixas temperaturas e o DREB2 com a tolerância à falta de água (Figura 4). Além disso, a elevação de ABA em condições de estresses abióticos também levam à expressão de genes com elementos conservados em sua região promotora, G-box e cis-atuantes, designados como ABREs (PyACGTGG/TC) (MARUYAMA *et al*., 2012).

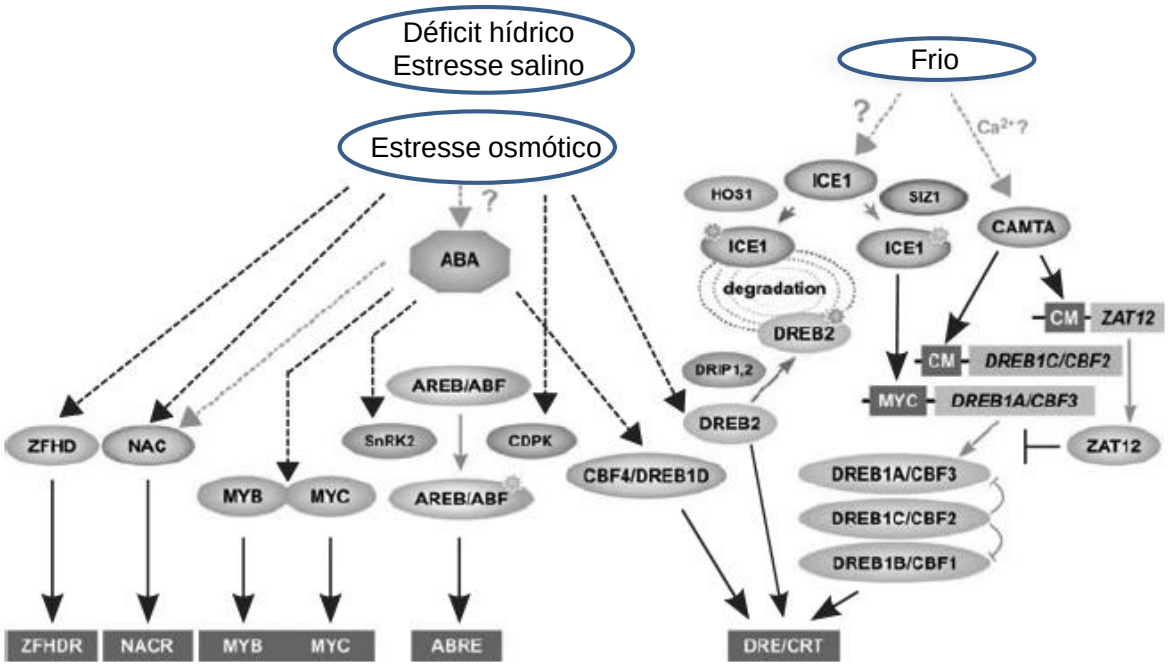

FIGURA 04 – Rede regulatória transcricional ativa em condição de déficit hídrico, estresse salino e baixas temperaturas. Objetos elípticos e retangulares indicam proteínas funcionais e cis-elementos, respectivamente. As linhas sólidas e linhas pontilhadas são links diretos ou indiretos, respectivamente. (HIRAYAMA; SHINOZAKI, 2010).

Para ampliar sua capacidade de obtenção de água no ambiente, as células acionam um mecanismo denominado de ajuste osmótico, que é o processo pelo qual o potencial hídrico pode ser diminuído sem que haja decréscimo no turgor ou no volume celular através da acumulação de solutos compatíveis, comumente

sintetizados pela célula e que não interferem nas funções das enzimas (TAIZ; ZEIGER, 2004).

2.4.3 ESTRESSES ABIÓTICOS EM CAFEEIROS

No cafeeiro, a influencia do ambiente, principalmente na produção, é bastante elevada. Assim, em muitas regiões de cultivo de café, os estresses abióticos limitam de forma preocupante a produtividade dos grãos (DA MATTA; RAMALHO, 2006). Devido a isso, mudanças em padrões climáticos decorrentes de alterações climáticas globais são consideradas cada vez mais importantes para o cultivo de café. Elevações de temperatura induzem condição de seca, o que pode gerar um reduzido crescimento vegetativo e vigor dos cafeeiros levando a anormalidades florais e frutos de baixa qualidade. Além disso, pode ocorrer um aumento na incidência de pragas e doenças nas plantas (MISHRA; SLATER, 2012).

As variações ambientais acabam por induzir uma bebida de baixa qualidade em função da alteração da composição bioquímica dos grãos (açúcares redutores, proteínas e cafeína) (MAZZAFERA, 2007). Por exemplo, o conteúdo de cafeína do grão foi reduzido quando as plantas de café foram submetidas à irrigação e altas temperaturas. Também, altas atividades de proteases e polifenol oxidase foram observadas em condições de temperatura elevada e baixa precipitação (SILVA *et al*., 2005).

Considerando-se a tolerância à seca, sabe-se que o gênero *Coffea* possui grande variabilidade genética. Estudos em *Coffea canephora* demonstraram que o grupo Kouillou possui maior tolerância a este estresse do que o Robusta (MONTAGNON, 2000). Essa maior tolerância atribuída ao grupo Kouillou pode ser devido ao processo de abertura estomatal e consequente ativação fotossintética. Além disso, as plantas Kouillou também apresentam um eficiente sistema de absorção de água pelas raízes. Dentre as estratégias exibidas por cafeeiros para lidar com a deficiência hídrica, o dobramento foliar e inclinação para reduzir a superfície foliar e perda de água por transpiração também são características observadas (MONTAGNON; LEROY, 1993).

O melhoramento de *C. arabica* inicialmente envolvia aumento de produtividade e adaptação local. Para isso, plantas elite em uma população eram

selecionadas, propagadas e cruzadas com cultivares existentes. Esse método acabou na identificação e desenvolvimento de cultivares produtivos e vigorosos que possuem um maior grau de variabilidade genética do que a população base. Dentre estas cultivares estão Mundo Novo, Caturra, Catuaí, Kents e Blue Mountain.

Embora o melhoramento convencional seja o mais utilizado para o *C. arabica*, este método é um processo longo que envolve diferentes técnicas como seleção, hibridização e avaliação de descendência, o que requer geralmente cerca de 30 anos para o desenvolvimento de uma nova cultivar. Mais ainda, o longo tempo exigido no desenvolvimento das plantas de café, os altos custos de trabalho de campo, a falta de acurácia, as diferenças de ploidia entre *C. arabica* e outras espécies diploides e a incompatibilidade são grandes limitantes associados a este tipo de melhoramento. Ainda, sobre o melhoramento convencional, a tolerância a estresses abióticos e resistência a herbicidas são características que não estão facilmente disponíveis no pool genético do cafeeiro ou são difíceis de serem incorporadas através deste método (MISHRA; SLATER, 2012).

C. canephora é muitas vezes utilizado como fonte de resistência a doenças e pragas (SRINIVASAN; NARASIMHASWAMY, 1975) e a identificação e seleção de clones combinando tolerância à seca com outras características agronômicas de interesse é de particular interesse para a geração de novas variedades mais adaptadas a mudanças climáticas. Durante a década passada, diversos clones tolerantes à seca de *C. canephora* var. Conilon foram caracterizadas como vigorosas e produtivas nestas condições (FERRÃO *et al*., 2000; FONSECA *et al*., 2004).

Considerando estes fatores, a transformação genética surgiu como uma ferramenta em potencial para o desenvolvimento de cafés elite que possuam caracteres agronômicos de interesse. Durante os últimos 20 anos, um progresso científico significativo tem sido alcançado na biotecnologia cafeeira. Estes avanços incluem projetos de transformação genética (RIBAS *et al*., 2005; ALPIZAR *et al*., 2008; RIBAS *et al*., 2011), onde Leroy e colaboradores (2000) foram os responsáveis pela obtenção das primeiras plantas geneticamente transformadas de café; do sequenciamento de sequencias expressas (ESTs) (LIN *et al*., 2005; PONCET *et al*., 2006; VIEIRA *et al*., 2006; VIDAL *et al*., 2010; MONDEGO *et al*., 2011); a construção de bibliotecas BACs (NOIR *et al*., 2004; LEROY *et al*., 2005);

mapas genéticos (CROUZILLAT *et al*., 2004; LEFEBVRE-PAUTIGNY *et al*., 2010; LEROY *et al*., 2011) e marcadores moleculares (LASHERMES *et al*., 2008; DE KOCHKO *et al*., 2010).

A abordagem mais promissora da engenharia genética em busca de tolerância a estresses abióticos em cafeeiros inclui a manipulação de genes regulatórios ou funcionais, e diversas pesquisas têm sido realizadas em condições de salinidade, altas temperaturas e déficit hídrico. Geralmente, o objeto de estudo destas pesquisas de tolerância são genes envolvidos na detoxificação ou respostas osmóticas, enzimas ativas em sinalizações, proteínas envolvidas no transporte de metabólitos e regulação do status de energia da planta (CHAVES *et al*., 2003; CHAVES; OLIVEIRA, 2004; CORAGGIO; TUBEROSA, 2004; DOS SANTOS *et al*., 2011). Além disso, genes relacionados com a qualidade da bebida do café também são alvos de estudo, como genes que codificam constituintes químicos como ácidos clorogênicos, carboidratos, proteínas, glicosídeos, lipídeos e compostos voláteis responsáveis pelo sabor. Um outro composto associado à qualidade dos grãos e bebida é a sacarose, que costuma ser mais alta em *C. arabica* do que *C. canephora*. Assim, a manipulação de genes envolvidos com a síntese de sacarose pode ser de grande valia para o melhoramento do cafeeiro (CLIFFORD, 1985; DE MARIA *et al*., 1996; LEROY *et al*., 2005). Um exemplo importante para o trabalho em questão é a síntese do álcool açúcar manitol, um osmoprotetor que pode direta ou indiretamente influenciar o cafeeiro.

2.5 OSMOPROTETORES

Quase todos os organismos sintetizam solutos compatíveis em resposta a estresses osmóticos (BURG *et al*., 1996). Estes osmoprotetores são moléculas não tóxicas, que consistem em açúcares simples (principalmente frutose e glicose), álcool açúcares (manitol, glicerol, inositóis metilados), combinações de uréia e metilaminas, açúcares complexos (trealose e rafinose), aminoácidos quaternários (prolina, glicina betaína, β-alanina betaína) e aminas terciárias (1,4,5,6-tetrahidro-2-metil-4-carboxil pirimidina) (BOHNERT; JENSEN, 1996; NUCCIO *et al.*, 1999; BARTELS; SUNKAR, 2005).

A vantagem evolutiva do sistema de osmólitos orgânicos é a compatibilidade com estruturas macromoleculares, o que permite a manutenção de funções proteicas celulares mesmo quando os osmoprotetores estão em concentração alta e variável. Esta compatibilidade é resultado da não perturbação ou efeitos favoráveis dos osmólitos em interações solvente-macromolécula (BURG; FERRARIS, 2008).

Altas concentrações de osmólitos orgânicos protetores estabilizam estruturas protéicas através da manutenção da forma compacta nativa. A configuração da estrutura proteica é o determinante mais importante para estabilização ou denaturação. Estes solutos compatíveis, por conseguinte, proporcionam a capacidade de manter a atividade de enzimas presentes em soluções salinas (HUSSAIN *et al*., 2010). Por exemplo, os açúcares podem proteger as células durante a dessecação atraves da formação de cristais (BLACK; PRITCHARD, 2002). Eles também podem interagir com a parte polar dos fosfolipídios em membranas de modo que a fusão da membrana é impedida (TAJI *et al*., 2002).

Sob condições adversas, o acúmulo de carboidratos não estruturais ocorre em diferentes níveis, variando de acordo com a espécie (BARTELS; SUNKAR, 2005). Os álcool açúcares estão intimamente relacionados aos açúcares uma vez que eles representam a forma quimicamente reduzida de um açúcar aldose ou cetose. Sugere-se que os álcool açúcares, em função de seus grupos hidroxil, possam imitar a estrutura da água e manter uma esfera artificial de hidratação em torno das moléculas (SCHOBERT, 1977; STOOP *et al*., 1996).

Acreditava-se inicialmente que solutos compatíveis tinham como principal função o ajustamento osmótico, contudo atualmente existem também discussões envolvendo outras possíveis funções (SERRAJ; SINCLAIR, 2002). De acordo com Hasegawa e colaboradores (2000), o acúmulo de solutos *per se* pode não ser importante para a tolerância ao estresse osmótico, porém as vias metabólicas podem ter valor adaptativo. Uma outra hipótese seria o envolvimento destes solutos na remoção de espécies reativas de oxigênio (HONG *et al.*, 2000; AKASHI *et al.*, 2001; CHEN; MURATA, 2002).

O acúmulo de osmoprotetores sob condições de estresses abióticos é uma resposta comum observada no reino vegetal (LUOA *et al*., 2008), contudo,

algumas espécies não possuem a habilidade de sintetizar altos níveis de osmoprotetores. Assim, os osmoprotetores se tornaram uma estratégia da engenharia metabólica para melhorar a tolerância das plantas a estresses abióticos (BHATNAGAR-MATHUR *et al.*, 2008). A superexpressão de álcool açúcares (manitol, trealose, mio-inositol e sorbitol) tem sido utilizada como uma abordagem em potencial para alcançar esta tolerância (WANG *et al.*, 2003).

Arabidopsis thaliana, uma planta não produtora de manitol, apresentou uma maior tolerância a salinidade quando transformada com o gene que possibilita a síntese e conseqüente acúmulo deste álcool açúcar (ZHIFANG; LOESCHER, 2003). Em um outro estudo, Sickler e colaboradores (2007) observaram que sob déficit hídrico, plantas de *Arabidopsis* com acúmulo de manitol apresentaram proteção do aparato fotossintético, mas não reduziram seu potencial osmótico de forma a proporcionar ajustamento osmótico. Plantas de tabaco e trigo com acúmulo de manitol apresentaram um maior acúmulo de biomassa e aumento de tolerância a estresse salino, oxidativo e osmótico (SHEN *et al.*, 1997; ABEBE *et al.*, 2003). Também, o acúmulo de trealose em plantas de tabaco e arroz levou a uma maior manutenção da capacidade fotossintética, da capacidade de retenção de água e menor dano oxidativo (PILON-SMITS *et al.*, 1998; GARG *et al.*, 2002).

Oligossacarídeos da família rafinose (OFR), como rafinose e estaquiose, podem ser acumulados em folhas de plantas submetidas a estresses ambientais (KEMPA *et al*., 2008; USADEL *et al*., 2008) e estão ligadas à remoção de ROS e proteção de membranas (HINCHA, 2003; NISHIZAWA *et al*., 2008). A biossíntese de OFRs se inicia com a formação de galactinol a partir de *myo*-inositol e UDP-galactose, através da ação da galactinol sintase (*GolS*). A adição sequencial de unidades de galactose, fornecidos pelo galactinol, à sacarose levam à formação de rafinose (PETERBAUER; RICHTER, 2001). Plantas de arabidopsis superexpressando arabidopsis *GolS1* ou *GolS2* acumularam altos níveis de galactinol e rafinose apresentando maior tolerância à seca e salinidade (TAJI *et al*., 2002; NISHIZAWA *et al*., 2008). O papel destes oligossacarídeos na aquisição de tolerância ao estresse ainda não está bem elucidada. Em café poucos estudos foram realizados envolvendo estes OFRs (CHABRILLANGE *et al*. 2000; JOET *et al*., 2009). Ainda assim, estes estudos não consideraram estresses ambientais em suas análises. Dos Santos *et al*. (2011) sugeriram que o acúmulo de rafinose e estaquiose

em folhas de *C. arabica* poderiam ter uma importante função na proteção da planta contra estresse osmótico. Mais ainda, análises transcricionais de *CaGolS* indicaram que os genes foram diferencialmente regulados de acordo com o estresse abiótico aplicado.

2.5.1 MANITOL

Diversas espécies de plantas superiores sintetizam sacarose e amido como produtos primários da assimilação fotossintética do carbono. A sacarose é a principal forma de carboidrato translocado através da planta pelo floema e o amido é uma reserva estável e insolúvel de carboidratos. Ambos são sintetizados a partir da triose fosfato gerada pelo ciclo de Calvin. Entretanto existem espécies, incluindo muitas de considerável importância econômica, que podem fracionar entre 15 e 60% do seu carbono assimilado em álcool açúcares acíclicos, como o sorbitol e manitol. Álcool açúcares raramente são produtos primários da assimilação do carbono, uma vez que as espécies de plantas que os sintetizam também sintetizam sacarose e amido. O fracionamento fotossintético do carbono entre outros produtos depende da espécie (LOESCHER; EVERARD, 1996), estádio de desenvolvimento do órgão, em especial as folhas (DAVIS *et al*., 1988) e fatores ambientais (EVERARD *et al*., 1994; LOESCHER *et al*., 1995). Estas evidências sugerem que a produção de álcool açúcar acontece sob controle metabólico restrito (EVERARD *et al*., 1997).

O manitol é o àlcool açúcar mais amplamente distribuído na natureza e tem sido relatado em mais de 100 espécies de plantas vasculares de diversas famílias, incluindo *Rubiaceae*, *Oleaceae* e *Apiaceae* (CONDE *et al*., 2007). Nas plantas vasculares produtoras de manitol, ele é sintetizado a partir de manose-6-fosfato através da ação da NADPH manose-6-fosfato redutase (M6PR), que catalisa a conversão de manose-6-fosfato à manitol-1-fosfato, seguido por desfosforilação por uma fosfatase ainda desconhecida (Figura 5). A síntese de álcool açúcar ocorre principalmente em folhas maduras, onde a M6PR é localizada predominantemente no citosol. Em aipo, o manitol é diretamente oxidado à manose pela atividade catalítica de uma manitol desidrogenase NAD-dependente (MTD). O catabolismo de manitol é espacialmente separado de sua síntese: a atividade de MTD é detectada em tecidos dreno e não em folhas maduras. A MTD promove os passos iniciais pela

qual a translocação de manitol entra no ciclo dos ácidos tricarboxílicos para ser utilizado como fonte de carbono e energia. A atividade da enzima M6PR não é detectada em tecidos dreno, como raízes e folhas imaturas (RUMPHO *et al*., 1983; FLORA; MADORE, 1993; LOESCHER *et al*., 1995; PHARR *et al*., 1995).

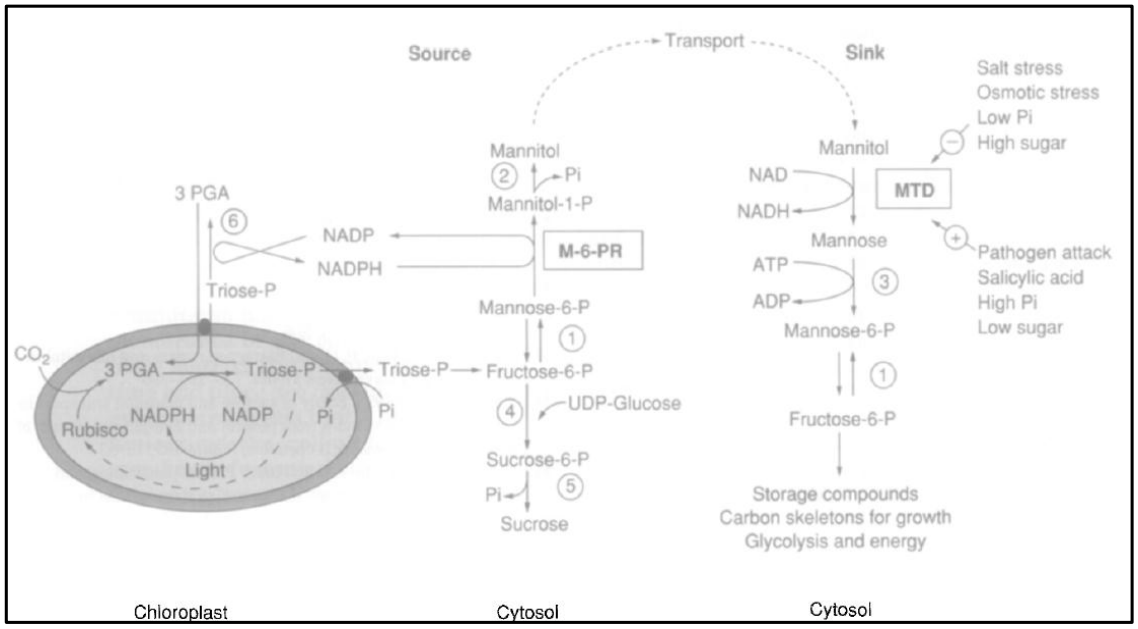

FIGURA 05 - Biossíntese, transporte e catabolismo de manitol em plantas. À esquerda, a via biossintética via M6PR (Tecidos fonte). A membrana externa do cloroplasto está representada. À direita, a via catabólica via MTD (Tecidos dreno). Outras enzimas envolvidas são: (1) fosfomanose isomerase (PMI); (2) manose-1-fosfato fosfatase; (3) hexoquinase; (4) sucrose-6-fosfato sintase; (5) sucrose-6-fosfato fosfatase; e (6) triose-fosfato desidrogenase não reversível. Fatores down-regulado (-) ou up-regulado (+). A expressão da MTD é indicada. Abreviações: PGA, ácido fosfoglicérico; P, fosfato; P_i, fosfato inorgânico (STOOP *et al.*, 1996).

O papel do manitol como osmoprotetor, removedor de espécies reativas de oxigênio, estabilizador de proteínas e estruturas de membrana e protetor fotossintético sob condições de estresse abiótico é bem documentado em diversas espécies incluindo o aipo, onde o manitol representa cerca de 50% do total de carboidratos translocados. O balanço destes papéis ocorre devido à uma coordenação precisa da síntese e catabolismo do manitol (STOOP *et al*., 1996; SECKIN *et al*., 2009, CHENG *et al*., 2009). A produção de manitol pode também conferir diversas outras vantagens que incluem uma maior eficiência na utilização do carbono (STOOP *et al.,* 1996), maior tolerância ao estresse oxidativo (SMIRNOFF; CUMBERS, 1989; WILLIAMSON *et al*., 1995; JENNINGS *et al*., 1998), e estresse

salino (SICKLER *et al*., 2007; ZHIFANG; LOESCHER, 2003). Por outro lado, este álcool açúcar também já foi relatado tendo efeitos em condições de estresse biótico, facilitando a infecção e sobrevivência do patógeno (DELAVAULT *et al*., 2002; VÉLËZ *et al*., 2008).

Uma vez que a síntese de osmoprotetores geralmente ocorre em folhas maduras, um aumento na concentração em órgãos dreno certamente está relacionado com elevação na taxa de transporte destas moléculas. No caso da prolina, outro importante osmoprotetor, plantas de *Arabidopsis* submetidas a estresse hídrico e salino apresentaram aumento na expressão de um transportador de prolina, contudo, a expressão de transportadores de outros aminoácidos foi reduzida, indicando que o transporte de prolina foi favorecido sob condição de estresse (RENTSCH et al., 1996). Desta forma, o transporte de osmólitos parece representar um evento relevante quando as plantas são submetidas a estresses (NOIRAUD *et al*., 2001b).

Embora diversos estudos indiquem que, após a síntese de álcool açúcares em folhas maduras, há um aumento na sua concentração em órgãos dreno em resposta a estresse hídrico e salino, e a salinidade altera atividade enzimática relacionada ao metabolismo destes açúcares (WILLIAMSON *et al*., 1995), pouco se conhece sobre a regulação da expressão dos transportadores dos alcoóis de açúcar em condição de estresse. Estudos realizados em *Olea europaea* submetidas a altas concentrações salinas demonstraram que houve uma elevação na expressão de um gene para o transporte de manitol (*OeMaT1*) assim como a sua atividade, juntamente com uma drástica redução na oxidação do manitol pela MTD. Isto permite o acúmulo intracelular de manitol para compensar a redução externa da atividade de água (Figura 6) (CONDE *et al.*, 2007).

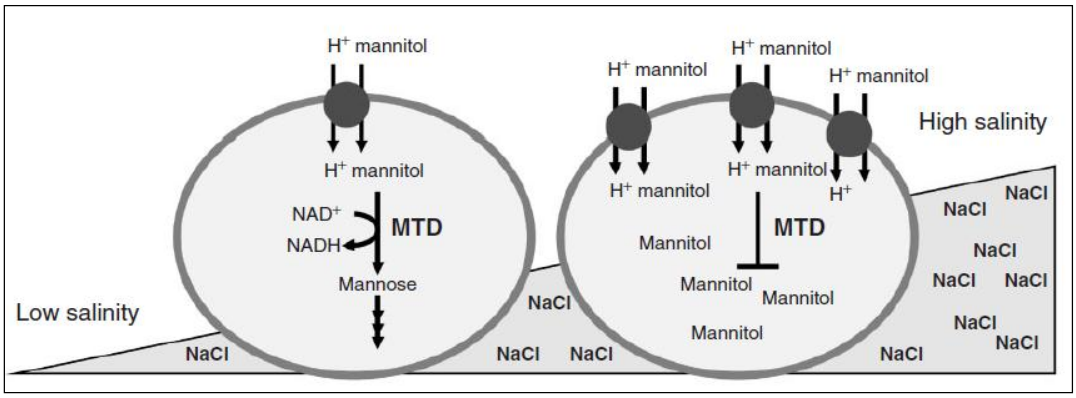

FIGURA 06 - Regulação do transporte e metabolismo do manitol como um mecanismo de tolerância à salinidade. Em condições de estresse salino a oxidação de manitol pela MTD é inibida enquanto que os tranportadores de manitol são ativados para permitir um maior acúmulo intracelular do mesmo (CONDE *et al*., 2007).

A translocação do manitol pelo floema, que ocorre de maneira muito similar à translocação de sacarose, de tecidos fonte para tecidos dreno já está clara, contudo, existem algumas controvérsias sobre as vias envolvidas no carregamento no floema e descarregamento. No caso do manitol, vias de descarregamento foram pouco estudadas, embora os carreadores mediando a captação do manitol tenha sido demonstrado em discos de tecido e vacúolos de armazenamento no parênquima de pecíolos de aipo (NOIRAUD *et al*., 2001a).

2.6 NORMALIZAÇÃO DE DADOS PARA PCR EM TEMPO REAL

A quantificação de níveis de expressão gênica é uma ferramenta importante que tem sido cada vez mais utilizada no meio científico para elucidar os mecanismos que abrangem redes gênicas regulatórias. O método quantitativo de PCR em tempo real (qPCR) passou recentemente a ser amplamente utilizado para estes fins devido sua sensibilidade, acurácia, precisão, reprodutibilidade, rapidez e facilidade (PFAFFL, 2004a). Além disso, o qPCR é de grande utilidade principalmente quando quantidades limitadas de RNA estão disponíveis (amostras reduzidas) (GACHON *et al*., 2004).

Para que a quantificação da expressão gênica seja feita com todas estas características, diversas variações experimentais devem ser consideradas, como a qualidade da amostra a ser analisada, presença de inibidores, desenho de primers, extração de RNA, eficiência de transcrição e manipulação na montagem de placas

(GINZINGER, 2002). A normalização de dados é um procedimento fundamental para que os resultados de expressão obtidos por qPCR sejam confiáveis, e para isto, o uso de genes de referência têm sido cada vez mais utilizado (VANDESOMPELE *et al.*, 2009).

Um gene de referência ideal deve possuir expressão estável entre amostras, incluindo diferentes tecidos e tipos celulares, estádios de desenvolvimento e condição de tratamento (HONG *et al*., 2008; WAN *et al*., 2010). Geralmente, genes relacionados a processos básicos e estruturais na célula (tubulina, actina, GAPDH, subunidades ribossomais, ubiquitina e outros) são utilizados diretamente, sem qualquer validação, como genes de referência para normalização (TRICARICO *et al*., 2002). Contudo, estudos demonstram que estes genes podem apresentar instabilidade de expressão sob diferentes condições e tecidos (CZECHOWSKI *et al*., 2005; CORDOBA *et al*., 2011). Com isso, existe a necessidade de selecionar genes caracterizados por baixa variação em sua expressão para que estes sejam confiáveis como controles internos (ANDERSEN *et al*., 2004; TUNBRIDGE *et al*., 2011).

Até alguns anos atrás estudos com genes de referência tinham grande foco na área médica (BUSTIN, 2002). Recentemente, um grande número de validações tem sido realizado em plantas, embora ainda não seja suficiente, uma vez que poucos estudos de expressão tem aplicado a utilização de genes de referência de forma adequada. Dentre os estudos já publicados, grande parte deles envolvem plantas modelo, plantas ornamentais e culturas de valor econômico como arroz (JAIN *et al*., 2006), arabidopsis (DEKKERS *et al*., 2012), tabaco (SCHMIDT; DELANEY, 2010), cana-de-açúcar (ISKANDAR *et al*., 2004), batata (NICOT *et al*., 2005), soja (LI *et al*., 2012), tomate (DEKKERS *et al*. 2012), *Urochloa* sp. (SILVEIRA *et al*., 2009), café (BARSALOBRES-CAVALLARI *et al*., 2009), citros (CARVALHO *et al*., 2010; MAFRA *et al*., 2012), entre outros.

Até o momento, a identificação do gene de referencia mais adequado para uma condição experimental específica tem sido realizado pela utilização dos softwares geNorm (VANDESOMPELE *et al*., 2002), NormFinder (ANDERSEN *et al*., 2004) e Bestkeeper (PFAFFL *et al*., 2004b).

3 REFERÊNCIAS

ABEBE, T.; GUENZI, A.; MARTIN, B.; CUSHMAN, J. C. Tolerance of mannitol-accumulating transgenic wheat to water stress and salinity. **Plant Physiology**, v. 131, p. 1748–1755, 2003.

ABIC. Produção e exportação mundial de café (http://abic.com.br/estat_exporta_ppaises.html.), 2012.

ADAMS, K. L. Evolution of duplicate gene expression in polyploid and hybrid plants. **Journal of Heredity**, v. 98, p. 136–141, 2007.

AKASHI, K.; MIYAKE, C.; YOKOTA, A. Citrulline, a novel compatible solute in drought-tolerant wild watermelon leaves, is an efficient hydroxyl radical scavenger. **FEBS Letters**, v. 508, p. 438–442, 2001.

ALLAKHVERDIEV, S. I.; KRESLAVSKI, V. D.; KLIMOV, V. V.; LOS, D. A.; CARPENTIER, R.; MOHANTY, P. Heat stress: an overview of molecular responses in photosynthesis. **Photosynthesis Research**, v. 98, p. 541–550, 2008.

ALLEN, R. Dissection of oxidative stress tolerance using transgenic plants. **Plant Physiology**, v. 107, p. 1049–1054, 1995.

ALPIZAR, E.; DECHAMP, E.; LAPEYRE-MONTES, F.; GUILHAUMON, C.; BERTRAND, B.; JOURDAN, C.; LASHERMES, P.; ETIENNE, H. *Agrobacterium rhizogenes*-transformed roots of coffee (*Coffea arabica*): conditions for long-term proliferation, and morphological and molecular characterization. **Annals of Botany**, v. 101, p. 929–940, 2008.

ANDERSEN, C. L.; JENSEN, J. L.; ORNTOFT, T. F. Normalization of realtime quantitative reverse transcription-PCR data: a model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets. **Cancer Research**, v. 64, p. 5245–5250, 2004.

ANTHONY, F.; COMBES, M. C.; ASTORGA, C.; BERTRAND, B.; GRAZIOSI, G.; CHARRIER, A.; LASHERMES, P. The origin of cultivated *Coffea arabica* L. varieties

revealed by AFLP and SSR markers. **Theoretical Applied Genetics**, v. 104, p. 894–900, 2002.

APEL, K.; HIRT, H. Reactive oxygen species: Metabolism, oxidative stress, and signal transduction. **Annual Review of Plant Biology**, v. 55, p. 373–399, 2004.

APSE, M. P.; AHARON, G. S.; SNEDDEN, W. A.; BLUMWALD, E. Salt Tolerance Conferred by Overexpression of a Vacuolar Na^+/H^+ Antiport in Arabidopsis. **Science**, v. 285, p.1256–1258, 1999.

BARDIL, A.; ALMEIDA, J. D.; COMBES, M. C.; LASHERMES, P.; BERTRAND, B. Genomic expression dominance in the natural allopolyploid Coffea arabica is massively affected by growth temperature. **New Phytologist**, v. 192, p. 760–774, 2011.

BARRETO, H. G.; LAZZARI, F.; SÁGIO, S. A.; CHALFUN-JUNIOR, A.; PAIVA, L. V.; BENEDITO, V. A. In Silico and Quantitative Analyses of the Putative FLC-like homologue in coffee (Coffea arabica L.). **Plant Molecular Biology Reports**, v. 30, p. 29–35, 2012.

BARSALOBRES-CAVALLARI, C. F.; SEVERINO, F. E.; MALUF, M. P.; MAIA, I.G. Identification of suitable internal control genes for expression studies in Coffea arabica under different experimental conditions. **BMC Molecular Biology**, v. 10:1, 2009.

BARTELS, D,; SUNKAR, R. Drought and Salt Tolerance in Plants. **Critical Reviews in Plant Sciences**, v. 24, p. 23–58, 2005.

BARTELS, D. Targeting detoxification pathways: An efficient approach to obtain plants with multiple stress tolerance? **Trends Plant Science**, v. 6, p. 284–286, 2001.

BARTELS, D.; PHILLIPS, J. Drought stress tolerance. **Genetic Modification of Plants Biotechnology in Agriculture and Forestry**, v. 64, p. 139-157, 2010.

BHARTI, A. K.; KHURANA, J. P. Mutant of Arabidopsis as tools to understand the regulation of phenylpropanoids pathway and UVB protection mechanism. **Photochemistry and photobiology**, v. 65, p. 765–776, 1997.

BHATNAGAR-MATHUR, P.; VADEZ, V.; SHARMA, K. K. Transgenic approaches for abiotic stress tolerance in plants: retrospect and prospects. **Plant Cell Reports**, v. 27, p. 411–424, 2008.

BLACK, M; PRITCHARD, H. W. **Desiccation and survival in plants: drying without dying**, 2002.

BLUM, A. **Constitutive traits affecting plant performance under stress**. In: Edmeades, G.O., M. Banziger, H.R. Mickelson and C.B. Pena- Valdivia (eds.), Developing Drought and Low N Tolerant Maize, p. 131–35. Proceedings of the Symposium, Mexico D.F.: Cimmyt, 1996.

BOHNERT, H. J.; GONG, Q.; LI, P.; MA, S. Unraveling abiotic stress tolerance mechanisms – getting genomics going. **Current Opinion in Plant Biology**, v. 9, p. 180–188, 2006.

BOHNERT, H. J.; JENSEN, R. G. Strategies for engineering water stress tolerance in plants. **Trends in Biotechnology**, v. 14, p. 89-97, 1996.

BOHNERT, H. J.; NELSON, D. E.; JENSEN, R. G. Adaptations to environmental stresses. **Plant Cell**, v. 7, p. 1099–1111, 1995.

BUGGS, R. J. A.; ELLIOTT, N. M.; ZHANG, L.; KOH, J.; VICCINI, L. F.; SOLTIS, D. E.; SOLTIS, P. S. Tissue-specific silencing of homoeologs in natural populations of the recent allopolyploid Tragopogon mirus. **New Phytologist**, v. 186(1), p. 175–183, 2010.

BURG, M. B.; FERRARIS J. D. Intracellular Organic Osmolytes: Function and Regulation. **The Journal of Biological Chemistry**, v. 283, n. 12, p. 7309–7313, 2008.

BURG, M. B.; KWON, E. D.; KULTZ, D. Osmotic regulation of gene expression. **FASEB Journal**, v. 10, p. 1598–1606, 1996.

BUSTIN, S. A. Quantification of mRNA using real-time reverse transcription PCR (RT-PCR): trends and problems. **Journal of Molecular Endocrinology**, v. 29, p. 23–39, 2002.

CARVALHO, K.; CAMPOS, M. K. F.; PEREIRA, L. F. P.; VIEIRA, L. G. E. Reference gene selection for real-time quantitative polymerase chain reaction normalization in ‘‘Swingle” citrumelo under drought stress. **Analytical Biochemistry**, v. 402, p. 197-199, 2010.

CENCI, A.; COMBES, M-C; LASHERMES, P. Genome evolution in diploid and tetraploid Coffea species as revealed by comparative analysis of orthologous genome segments. **Plant Molecular Biology**, v. 78, p. 135–145, 2012.

CHABRILLANGE, N.; DUSSERT, S.; ENGELMANN, F.; DOULBEAU, S.; HAMON, S. Desiccation tolerance in relation to soluble sugar contents in seeds of ten coffee (*Coffea* L.) species. **Seed Science Research**, v.10, p. 393-396, 2000.

CHAUDHARY, B.; FLAGEL, L.; STUPAR, R. M.; UDALL, J. A.; VERMA, N.; SPRINGER, N. M.; WENDEL, J. F. Reciprocal silencing, transcriptional bias and functional divergence of homeologs in polyploid cotton (Gossypium). **Genetics**, v. 182(2), p. 503–517, 2009.

CHAVES, M. M.; MAROCO, J. P.; PEREIRA, J. S. Understanding plant responses to drought—from genes to the whole plant. **Functional Plant Biology**, v. 30, n. 3, p. 239–264, 2003.

CHAVES, M. M.; OLIVEIRA, M. M. Mechanisms underlying plant resilience to water deficits: prospects for water-saving agriculture. **Journal of Experimental Botany**, v. 55, n. 407, p. 2365–2384, 2004.

CHAVES, M.M.; FLEXAS, J.; PINHEIRO, C. Photosynthesis under drought and salt stress: regulation mechanisms from whole plant to cell. **Annals of Botany**, v. 103, p. 551–560, 2009.

CHEN, T. H. H.; MURATA, N. Enhancement of tolerance of abiotic stress by metabolic engineering of betaines and other compatible solutes. **Current Opinion in Plant Biology**, v. 5, p. 250–257, 2002.

CHENG, F-Y; ZAMSKI, E.; GUO, W-W; PHARR, D. M.; WILLIAMSON, J. D. Salicylic acid stimulates secretion of the normally symplastic enzyme mannitol dehydrogenase: a possible defense against mannitol-secreting fungal pathogens. **Planta**, v. 230, p.1093–1103, 2009.

CHINNUSAMY, V.; SCHUMAKER, K.; ZHU, J-K. Molecular genetic perspectives on cross-talk and specificity in abiotic stress signalling in plants. **Journal of Experimental Botany**, v. 55, n. 395, p. 225-236, 2004.

CLIFFORD, M. N. **Chemical and physical aspects of green coffee and coffee products**. In: Coffee: Botany Biochemistry and Production of Beans and Beverages. Clifford, M. N.; Williamson, K. C. Eds., p. 304–374, AVI, Westport, Conn, USA, 1985.

Coffee Research Institute (http://www.coffeeresearch.org/>).

COKER, J. S.; DAVIES, E. Selection of candidate housekeeping controls in tomato plants using EST data. **BioTechniques**, v. 35, p. 740–742, p. 744-746, 2003.

COMBES, M-C; CENCI, A.; BARAILLE, H.; BERTRAND, B.; LASHERMES, P. Homeologous Gene Expression in Response to Growing Temperature in a Recent Allopolyploid (Coffea arabica L.). **Journal of Heredity**, v. 103(1), p. 36–46, 2012.

CONAB – Companhia Nacional de Abastecimento (http://www.conab.gov.br/conabweb/download/safra/2cafe_10.pdf) (2009, 2010, 2012)

CONDE, C.; SILVA, P.; AGASSE, A.; LEMOINE, R.; DELROT, S.; TAVARES, R.; GERÓS, H. Utilization and Transport of Mannitol in Olea europaea and Implications for Salt Stress Tolerance. **Plant Cell Physiology**, v. 48(1), p. 42–53, 2007.

CORAGGIO, I.; TUBEROSA, R. **Molecular bases of plant adaptation to abiotic stress and approaches to enhance tolerance to hostile environment**. In: Hand Book of Plant Biotechnology. Christou, P.; Klee, H. Eds., p. 413–466, 2004.

CORDOBA, E. M.; DIE, J. V.; GONZÁLEZ-VERDEJO, C. I.; NADAL, S.; ROMÁN, B. Selection of reference genes in Hedysarum coronarium under various stresses and stages of development. **Analytical Biochemistry**, v. 409, p. 236–243, 2011.

COUÉE, I.; SULMON, C.; GOUESBET, G.; EL AMRANI, A. Involvement of soluble sugars in reactive oxygen species balance and responses to oxidative stress in plants. **Journal of Experimental Botany**, v. 57, p. 449–459, 2006.

CROUZILLAT, D.; RIGOREAU, M.; BELLANGER, L.; PRIYONO, P.; MAWARDI, S.; SYAHRUDI; MCCARTHY, J.; TANKSLEY, S.; ZAENUDIN, I.; PÉTIARD, V. **A Robusta consensus genetic map using RFLP and microsatellite markers for the detection of QTL**. In: 20th International Conference on Coffee Science, Bangalore-India, CDrom, B202, 2004.

CZECHOWSKI, T.; STITT, M.; ALTMANN, T.; UDVARDI, M. K.; SCHEIBLE, W. R. Genome-wide identification and testing of superior reference genes for transcript normalization in Arabidopsis. **Plant Physiology**, v. 139, p. 5–17, 2005.

DA MATTA, F. M.; CHAVES, A. R. M.; PINHEIRO, H. A.; DUCATTI, C.; LOUREIRO, M. E. Drought tolerance of two field-grown clones of Coffea canephora. **Plant Science**, v.164, p. 111-117, 2003.

DA MATTA, F. M.; RAMALHO, J. D. C. Impacts of drought and temperature stress on coffee physiology and production: a review. **Brazilian Journal of Plant Physiology**, v.18, n.1, p.55-81, 2006.

DAVIS, J.M.; FELLMAN, J. K.; LOESCHER, W. H. Biosynthesis of sucrose and mannitol as a function of leaf age in celery (Apium graveolens L.). **Plant Physiology**, v. 86, p. 129-133, 1988.

DAVIS, A. P. *et al.* Growing coffee: *Psilanthus* (Rubiaceae) subsumed on the basis of molecular and morphological data; implications for the size, morphology, distribution and evolutionary history of *Coffea.* **Botanical Journal of the Linnean Society,** v. 167, n. 4, p. 357–377, 2011.

DE KOCHKO, A.; AKAFFOU, S.; ANDRADE, A. C. ET AL. Advances in Coffea Genomics. **Advances in Botanical Research**, v. 53, p. 23‑63, 2010.

DE MARIA, C. A. B.; TRUGO, L. C.; AQUINO NETO, F. R.; MOREIRA, R. F. A.; ALVIANO, C. S. Composition of green coffee water-soluble fractions and identification of volatiles formed during roasting. **Food Chemistry**, v. 55, n. 3, p. 203–207, 1996.

DEKKERS, B. J. W.; WILLEMS, L.; BASSEL, G. W.; VAN BOLDEREN-VELDKAMP, R. P.; LIGTERINK, W.; HILHORST, H. W. M. et al. Identification of reference genes for RT–qPCR expression analysis in Arabidopsis and tomato seeds. **Plant Cell Physiology**, v. 53(1), p. 28–37, 2012.

DELAVAULT, P.; SIMIER, P.; THOIRON, S.; VÉRONÉSI, C.; FER, A.; THALOUARN, P. Isolation of mannose 6—phosphate reductase cDNA, changes in enzyme activity and mannitol content in broomrape (Orabanche ramosa) parasitic on tomato roots. **Physiologia plantarum**, v. 115, 48-55, 2002.

DONG, S.; ADAMS, K. L. Differential contributions to the transcriptome of duplicated genes in response to abiotic stresses in natural and synthetic polyploids. **New Phytologist**, v. 190(4), p. 1045–1057, 2011.

DOS SANTOS, T. B.; BUDZINSKI, I. G. F.; MARUR, C. J., et al. Expression of three galactinol synthase isoforms in *Coffea arabica* L. and accumulation of raffinose and stachyose in response to abiotic stresses. **Plant Physiology and Biochemistry**, v. 49, p. 441-448, 2011.

DUQUE, A. S. *et al.* Abiotic Stress Responses in Plants: Unraveling the Complexity of Genes and Networks to Survive. In: Abiotic Stress - **Plant Responses and Applications in Agriculture**, 2013.

EVERARD, J. D.; CANTINI, C.; CRUMET, R.; PLUMMER, J.; LOESCHER, W. H. Molecular Cloning of Mannose-6-Phosphate Reductase and Its Developmental Expression in Celery. **Plant Physiology**, v. 113, p. 1427-1435, 1997.

EVERARD, J. D.; GUCCI, R.; KANN, S. C.; FLORE, J. A.; LOESCHER, W. H. Gas exchange and carbon partitioning in the leaves of celery (Apium graveolens L.) at Various Levels of Root Zone Salinity. **Plant Physiology**, v. 106, n. 1, p. 281-292, 1994.

FAO. FAO Land and Plant Nutrition Management Service. (http://www.fao.org/ag/agl/agll/spush), 2008.

FAOSTAT (2010) - Food Aand Agricultures Organization of the United Nations.

FAROOQ, M.; BASRA, S. M. A.; WAHID, A.; CHEEMA, Z. A.; CHEEMA, M. A.; KHALIQ, A. Physiological role of exogenously applied glycinebetaine in improving drought tolerance of fine grain aromatic rice (Oryza sativa L.). **Journal of Agronomy and Crop Science**, v. 194, p. 325–333, 2008.

FAZUOLI, L. C. Cultivares do IAC. O Agronômico, Campinas, v. 51, n. 1, p. 27-24, 1999.

FEDER, M. E.; HOFFMAN, G. E. Heat-shock proteins, molecular chaperones, and the stress response: evolutionary and ecological physiology. **Annual Review of Physiology**, v. 61, p. 243–282, 1999.

FERRÃO, R. G.; FONSECA, A. F. A.; SILVEIRA, J. S. M.; FERRÃO, M. A. G.; BRAGANCXA, S. M. EMCAPA 8141—Robustão Capixaba, variedade clonal de café conilon tolerante à seca, desenvolvida para o estado do Espírito Santo. **Revista Ceres**, v. 273, p. 555–560, 2000.

FLAGEL, L. E.; CHEN, L.; CHAUDHARY, B.; WENDEL, J. F. Coordinated and finescale control of homoeologous gene expression in allotetraploid cotton. **Journal of Heredity**, v. 100(4), p. 487–490, 2009.

FLEXAS, J.; BOTA, J.; LORETO, F.; CORNIC, G.; SHARKEY, T. D. Diffusive and metabolic limitations to photosynthesis under drought and salinity in C3 plants. **Plant Biology**, v. 6, p. 269–279, 2004.

FLORA, L. L.; MADORE, M. A. Stachyose and mannitol transport in olive (Olea europaea L.). **Planta**, v. 189, p. 484-490, 1993.

FLOWERS, T. J.; HAJIBAGHERI, M. A.; CLIPSON, N. J. W. Halophytes. **The Quarterly Review of Biology**, v. 61, p. 313-337, 2007.

FONSECA, A. F. A.; FERRÃO, M. A. G.; FERRÃO, R. G.; VERDIN FILHO, A. C.; VOLPI, P. S.; ZUCATELI, F. Conilon Vitória—Incaper 8142: improved Coffea canephora var. Kouillou clone cultivar for the State Espírito Santo. **Crop Breeding and Applied Biotechnology**, v. 4, p. 1–3, 2004.

FOYER, C. H.; Descourvieres, P.; Kunert, K. J. Protection against oxygen radicals, an important defense mechanism studied in transgenic plants. **Plant Cell Environment**, v. 17, p. 507–523, 1994.

GACHON, C.; MINGAM, A.; CHARRIER, B. Real-time PCR: what relevance to plant studies? **Journal of Experimental Botany**, v. 55, p. 1445–1454, 2004.

GALMÉS, J.; MEDRANO, H.; FLEXAS, J. Photosynthetic limitations in response to water stress and recovery in Mediterranean plants with different growth forms. **New Phytologist**, v. 175, p. 81–93, 2007.

GARG, A. K.; KIM, J-K.; OWENS, T. G.; RANWALA, A. P.; CHOI, Y. D.; KOCHIAN, L. V.; WU, R. J. Trehalose accumulation in rice plants confers high tolerance levels to different abiotic stresses. **PNAS**, v. 99, p. 15898–15903, 2002.

GEROMEL, C.; FERREIRA, L.P.; BOTTCHER, A.; POT, D.; PEREIRA, L.F.P.; LEROY, T.; VIEIRA, L.G.E.; MAZZAFERA, P.; MARRACINI. Sucrose metabolism during fruit development in Coffea racemosa. **Annals of Applied Biology**, v. 152, n. 2, p. 179-187, 2008.

GINZINGER, D. G. Gene quantification using real-time quantitative PCR: An emerging technology hits the mainstream. **Experimental Hematology**, v. 30, p. 503–512, 2002.

GONZÁLEZ-VERDEJO, C. I.; DIE, J. V.; NADAL, S.; JIMÉNEZ-MARÍN, A.; MORENO, M. T.; ROMÁN, B. Selection of housekeeping genes for normalization by real-time RT-PCR: analysis of Or-MYB1 gene expression in Orobanche ramosa development. **Analytical Biochemistry**, v. 379, p. 176–181, 2008.

HALL, A. E. **Crop Responses to Environment**. CRC Press LLC, Boca Raton, Florida, 2001.

HASEGAWA, P. M.; BRESSAN, R.; ZHU, J-K.; BOHNERT, H-J. Plant cellular and molecular responses to high salinity. **Annual Review of Plant Physiology and Plant Molecular Biology**, v. 51, p. 463–499, 2000.

HEGARTY, M. Hybridization: ExpressingYourself in aCrowd. **Current Biology**, v. 21(7), p. R254–R255, 2011.

HENDRICK, J. P.; HARTL, F. U. The role of molecular chaperones in protein folding. **FASEB Journal**, v. 9, p. 1559–1569, 1995.

HINCHA, D. K. Effects of calcium-induced aggregation on the physical stability of liposomes containing plant glycolipids. **Biochimica et Biophysica Acta**, v. 1611, p. 180–186, 2003.

HIRAYAMA, T.; SHINOZAKI, K. Research on plant abiotic stress responses in the post-genome era: past, present and future. **The Plant Journal**, v. 61, 1041-1052, 2010.

HONG, S-Y; SEO, P. J.; YANG, M-S; XIANG, F.; PARK, C-M. Exploring valid reference genes for gene expression studies in Brachypodium distachyon by real-time PCR. **BMC Plant Biology**, v. 8, p. 112, 2008.

HONG, Z.; LAKKINENI, K.; ZHANG, Z.; VERMA, D. P. S. Removal of feedback inhibition of 1-pyrroline-5-carboxylate synthetase results in increased proline

accumulation and protection of plants from osmotic stress. **Plant Physiology**, v. 122, p. 1129–1136, 2000.

HOVAV, R.; UDALL, J.; CHAUDHARY, B.; RAPP, R.; FLAGEL, L.;WENDEL, J. F. Partitioned expression of duplicated genes during development and evolution of a single cell in a polyploid plant. **PNAS**, v.105(16), p.6191–6195, 2008.

HOWARTH, C.J. GENETIC IMPROVEMENTS OF TOLERANCE TO HIGH TEMPERATURE. IN: ASHRAF, M., HARRIS, P.J.C. (Eds.), **Abiotic Stresses: Plant Resistance Through Breeding and Molecular Approaches**. Howarth Press Inc., New York, 2005.

HUANG, B.; XU, C. Identification and Characterization of Proteins Associated with Plant Tolerance to Heat Stress. **Journal of Integrative Plant Biology**, v. 50, n. 10, p. 1230–1237, 2008.

HUSSAIN, K.; NISAR, M. F.; MAJEED, A.; NAWAZ, K.; BHATTI, K. H.; AFGHAN, S.; SHAHAZAD, A.; ZIA-UL-HUSSNIAN, S. What molecular mechanism is adapted by plants during salt stress tolerance? **African Journal of Biotechnology**, v. 9, n. 4, p. 416-422, 2010.

IBA, K. Acclimative response to temperature stress in higher plants: approaches of gene engineering for temperature tolerance. **Annual Review of Plant Biology**, v. 53, p. 225–245, 2002.

ICO - International Coffee Organization. As estatísticas do comercio do Café. Site: www.ico.org., 2009, 2010, 2012.

IGNATOVA, Z.; GIERASCH, L. M. Inhibition of protein aggregation in vitro and in vivo by a natural osmoprotectant. **PNAS**, v. 103, p. 13357–13361, 2006.

IPCC - Painel Intergovernamental de Mudanças Climáticas (www.ipcc.ch), 2009.

ISKANDAR, H. M.; SIMPSON, R. S.; CASU, R. E.; BONNETT, G. D.; MACLEAN, D. J.; MANNERS, J. M. Comparison of reference genes for quantitative real-time

polymerase chain reaction analysis of gene expression in sugarcane. **Plant Molecular Biology Reports**, v. 22, p. 325–337, 2004.

JACKSON, S.; CHEN, Z. J. Genomic and expression plasticity of polyploidy. **Current Opinion in Plant Biology**, v. 13, p. 1–7, 2009.

JAIN, M.; NIJHAWAN, A.; TYAGI, A. K.; KHURANA, J. P. Validation of housekeeping genes as internal control for studying gene expression in rice by quantitative real-time PCR. **Biochemical and Biophysical Research Communications**, v. 345, p. 646–651, 2006.

JALEEL, C. A.; MANIVANNAN, P.; WAHID, A.; FAROOQ, M.; SOMASUNDARAM, R.; PANNEERSELVAM, R. Drought stress in plants: a review on morphological characteristics and pigments composition. **International journal of agriculture & biology**, v. 11, p. 100–105, 2009.

JASPERS, P.; KANGASJARVI, J. Reactive oxygen species in abiotic stress signaling. **Physiologia Plantarum**, v. 138, p. 405-413, 2010.

JENNINGS, D. B.; EHRENSHAFT, M.; PHARR, D. M.; WILLIAMSON, J. D. Roles for mannitol and mannitol dehydrogenase in active oxygen-mediated plant defense. **PNAS**, v. 95, p. 15129–15133, 1998.

JOET, T.; LAFFARGUE, A.; SALMONA, J.; DOULBEAU, S.; DESCROIX, F.; BERTRAND, B. et al. Metabolic pathways in tropical dicot albuminous seeds: *Coffea arabica* as a case study. **New Phytologist**, v.182, p. 146-162, 2009.

KEMPA, S.; KRASENSKY, J.; DAL SANTO, S.; KOPKA, J.; JONAK, C. A central role of abscisic acid in stress-regulated carbohydrate metabolism. **PLoS One**, v. 3, e3935, 2008.

KNIGHT, H.; TREWAVAS, A. J.; KNIGHT, M. R. Calcium signalling in Arabidopsis thaliana responding to drought and salinity. **The Plant Journal**, v. 12, p. 1067-1078, 1997.

KRASENSKY, J.; JONAK, C. Drought, salt, and temperature stress-induced metabolic rearrangements and regulatory networks. **Journal of Experimental Botany**, v. 63, p. 1593–1608, 2012.

LASHERMES, P.; ANDRADE, A. C.; ETIENNE, H. **Genomics of Coffee One of the World's Largest Traded Commodities**. In: genomics of tropical crop plants: Plant Genetics and Genomics: Crops and Models, v. 1, p. 203-226, 2008.

LASHERMES, P.; COMBES, M-C; ROBERT, J.; TROUSLOT, P.; D'HONT, A.; ANTHONY, F.; CHARRIER, A. Molecular characterisation and origin of the *Coffea arabica* L. genome. **Molecular and General Genetics**, v. 261, p. 259–266, 1999.

LASHERMES, P.; TROUSLOT, P., ANTHONY, E.; COMBES, M.C.; CHARRIER, A. Genetic diversity for RAPD markers between cultivated and wild accessions of Coffea arabica. **Euphytica**, v. 87, p. 59–64, 1996.

LEFEBVRE-PAUTIGNY, F.; WU, F. N. et al. High resolution synteny maps allowing direct comparisons between the coffee and tomato genomes. **Tree Genetics and Genomes**, v. 6, p. 565–577, 2010.

LEROY, T.; DE BELLIS, F.; LEGNATE, H. et al. Improving quality of African robustas: QTLs for yield- and quality-related traits in *Coffea canephora*. **Tree Genetics and Genomes**, v. 7, p. 781–798, 2011.

LEROY, T.; HENRY, A. M.; ROYER, M. et al. Genetically modified coffee plants expressing the *Bacillus thuringiensis* cry1Ac gene for resistance to leaf miner. **Plant Cell Reports**, v. 19, n. 4, p. 382-385, 2000.

LEROY, T.; MARRACCINI, P.; DUFOUR M. et al. Construction and characterization of a Coffea canephora BAC library to study the organization of sucrose biosynthesis genes. **Theoretical and Applied Genetics**, v. 111, n. 6, p. 1032–1041, 2005.

LEROY, T.; RIBEYRE, F.; BERTRAND, B.; CHARMETANT, P.; DUFOUR, M.; MONTAGNON, C.; MARRACCINI, P.; POT, D. Genetics of coffee quality. **Brazilian Journal of Plant Physiology**, v.18, p. 229-242, 2006.

LI, Q.; FAN, C-M; ZHANG, X-M; FU, Y-F. Validation of reference genes for real-time quantitative PCR normalization in soybean developmental and germinating seeds. **Plant Cell Reports**, v. 31, p.1789–1798, 2012.

LIMA, A. L. S.; DAMATTA, F. M.; PINHEIRO, H. A.; TOTOLA, M. R.; LOUREIRO, M. E. Photochemical responses and oxidative stress in two clones of Coffea canephora under water stress. **Environmental and Experimental Botany**, v.47, p. 239-247, 2002.

LIN, C.; MUELLER, L. A.; MCCARTHY, J.; CROUZILLAT, D.; PÉTIARD, V.;TANKSLEY, S. D. Coffee and tomato share common gene repertoires as revealed by deep sequencing of seed and cherry transcripts. **Theoretical and Applied Genetics**, v. 112, p. 114–130, 2005.

LINDQUIST, S. The heat-shock response. **Annual Review of Biochemistry**, v. 55, p. 1151–1191, 1986.

LIU, N.; ZHONG, N. Q.; WANG, G. L.; LI, L. J.; LIU, X. L.; HE, Y. K.; XIA, G. X. Cloning and functional characterization of Ppdbf1 gene enconding a DRE-binding transcription factor from Physcomitrella patens. **Planta**, v.226, p.827-838, 2007.

LIU, Z; ADAMS, K. L. Expression partitioning between genes duplicated by polyploidy under abiotic stress and during organ development. **Current Biology**, v. 17(19), p.1669–1674, 2007.

LOESCHER, W. H. et al. Sugar alcohol metabolism in source leaves, in Carbon Partitioning and Source-Sink Interactions in Plants (CmTent Topics in Plant Physiology, v.. 13) (Madora, M.A. and Lewis, W.J., eds), p. 170-179, **American Society of Plant Physiologists**, 1995.

LOESCHER, W. H.; EVERARD, J. D. **Sugar alcohol metabolism in sinks and sources**. In E Zamski, AA Schaffer, eds, Photoassimilate Distribution in Plants and Crops: Source-Sink Relationships. Marcel Dekker, New York, p. 185-207, 1996.

LOESCHER, W. H.; EVERARD, J. D.; CANTINI, C.; GRUMET, R. **Sugar alcohol metabolism in source leaves**. In: MA Madore, WJ Lucas, eds, Carbon Partitioning

and Source-Sink Interactions in Plants, v. 13. American Society of Plant Physiologists, Rockville, MD, p. 170-179, 1995.

LUOA, Y.; LI, W-M; WANG, W. Trehalose: Protector of antioxidant enzymes or reactive oxygen species scavenger under heat stress? **Environmental and Experimental Botany**, v. 63, p. 378–384, 2008.

MAATHUIS, F. J. M.; SANDERS, D. Mechanisms of potassium absorption by higher plant roots. **Physiologia Plantarum**, v. 96, p. 158–168, 1996.

MAFRA, V.; KUBO, K. S.; ALVES-FERREIRA, M.; RIBEIRO-ALVES, M.; STUART, R. M. et al. Reference Genes for Accurate Transcript Normalization in Citrus Genotypes under Different Experimental Conditions. **PLoS ONE**, v. 7(2), p. e31263, 2012.

MAHAJAN, S. et al. Calcium- and salt-stress signaling in plants: Shedding light on SOS pathway. **Archives of Biochemistry and Biophysics**, v. 471, p. 146–158, 2008.

MAHAJAN, S.; TUJETA, N. Cold, salinity and drought stresses: an overview. **Archives of Biochemistry and Biophysics**, v. 444, p. 139-58, 2005.

MAPA - Ministério Da Agricultura, Pecuária E Abastecimento, 2013.

MAROUFI, A.; VAN BOCKSTAELE, E.; DE LOOSE, M. Validation of reference genes for gene expression analysis in chicory (Cichorium intybus) using quantitative real-time PCR. **BMC Molecular Biology**, v. 11, p. 15, 2010.

MARRACCINI, P.; VINECKY, F.; ALVES, G. S. C.; RAMOS, H. J. O.; ELBELT, S.; VIEIRA, N. G.; CARNEIRO, F. A.; SUJII, P. S.; ALEKCEVETCH, J. C.; SILVA, V. A.; DAMATTA, F. M.; FERRÃO, M. A. G.; LEROY, T.; POT, D.; VIEIRA, L. G. E.; DA SILVA, F. R.; ANDRADE. A. C. Differenttialy expressed genes and proteins upon drought acclimation in tolerant and sensitive genotypes of Coffea canephora. **Journal of Experimental Botany**, v. 63, n. 11, p. 4191–4212, 2012.

MAZZAFERA, P. **Estresse hídrico e consequencias na composição das sementes de café e qualidade da bebida**. In: Garcia Salva, T. J.; Guerreiro Filho, O.; Thomaziello, R. A.; Fazuoli, L. C. Eds. Cafés de qualidade: aspectos tecnológicos, científicos e comerciais. IAC: Campinas, SP, Brazil, p. 73–90, 2007.

MISHRA, M. K.; SLATER, A. Recent Advances in the Genetic Transformation of Coffee. **Biotechnology Research International**, p.17, 2012.

MOHANTY, P.; ALLAKHVERDIEV, S. I.; MURATA, N. Application of low temperature during photoinhibition allows characterization of individual steps in photodamage and repair of photosystem II. **Photosynthesis Research**, v. 94, v. 217–234, 2007.

MONDEGO, J. M. C; VIDAL, R. O.; CARAZZOLLE, M. F. et al. An EST-based analysis identifies new genes and reveals distinctive gene expression features of *Coffea arabica* and *Coffea canephora*. **BMC Plant Biology**, v. 11, p. 30, 2011.

MONTAGNON, C. 2000. **Optimisation des gains génétiques dans le schéma de sélection récurrente réciproque de Coffea canephora Pierre**. PhD Thesis. Montpellier University, France, 2000.

MONTAGNON, C.; LEROY, T. Réaction ála sé cheresse de jeunes caféiers Coffea canephora de Côte-d'Ivoire appartenant a` différents groupes génétiques. **Cafe´ Cacao The´**, v. 37, p. 179–190, 1993.

MULLER, B.; PANTIN, F.; GÉNARD, M.; TURC, O. et al. Water deficits uncouple growth from photosynthesis, increase C content, and modify the relationships between C and growth in sink organs. **Journal of Experimental Botany**, v. 62, p. 1715–1729, 2011.

MUNNS, R.; TESTER, M. Mechanisms of Salinity Tolerance. **Annual Review in Plant Biology**, v. 59, p. 651-681, 2008.

MURATA, N.; TAKAHASHI, S.; NISHIYAMA, Y.; ALLAKHVERDIEV, S. I. Photoinhibition of photosystem II under environmental stress. **Biochimica et Biophysica Acta**, v. 1767, p. 414–421, 2007.

NICOT, N.; HAUSMAN, J. F.; HOFFMANN, L.; EVERS, D. Housekeeping gene selection for real-time RT–PCR normalization in potato during biotic and abiotic stress. **Journal of Experimental Botany**, v. 56, p. 2907–2914, 2005.

NISHIZAWA, A.; YABUTA, Y.; SHIGEOKA S. Galactinol and raffinose constitute a novel function to protect plants from oxidative damage. **Plant Physiology**, v. 147, p.1251–1263, 2008.

NIU, X.; BRESSAN, R. A.; HASEGAWA, P. M.; PARDO, J. M. Ion homeostasis in NaCl stress environments. **Plant Physiology**, v. 109, p. 735-742, 2005.

NOIR, S.; PATHEYRON, S.; COMBES, M. C.; LASHERMES, P.; CHALHOUB, B. Construction and characterization of a BAC library for genome analysis of the allotetraploid coffee species (Coffea arabica L.). **Theoretical and Applied Genetics**, v. 109, p. 225–230, 2004.

NOIRAUD, N.; MAUROUSSET, L.; LEMOINE, R. Identification of a mannitol transporter, AgMaT1, in celery phloem. **Plant Cell**, v. 13, p. 695–705, 2001b.

NOIRAUD, N.; MAUROUSSET, L.; LEMOINE, R. Transport of polyols in higher plants. **Plant Physiology and Biochemistry**, v. 39, p. 717−728, 2001a.

NUCCIO, M. L.; RHODES, D.; MCNEIL, S. D.; HANSON, A. D. Metabolic engineering of plants for osmotic stress resistance. **Current Opinion in Plant Biology**, v. 2, p. 128-134, 1999.

PARDO, J. M. Biotechnology of water and salinity stress tolerance. **Current Opinion in Biotechnology**, v. 21, p. 185-196, 2010.

PASSIOURA, J. The drought environment: physical, biological and agricultural perspectives. **Journal of Experimental Botany**, v. 58, p. 113–117, 2007.

PETERBAUER, T.; RICHTER, A. Biochemistry and physiology of raffinose family oligosaccharides and galactosyl cyclitols in seeds. **Seed Scence Research**, v. 11, p. 185–197, 2001.

PFAFFL, M. W.; TICHOPAD, A.; PRGOMET, C.; NEUVIANS, T. P. Determination of stable housekeeping genes, differentially regulated target genes and sample integrity: BestKeeper—Excel-based tool using pair-wise correlations. **Biotechnology Letters**, v. 26, p. 509–515, 2004b.

PFAFFL, M.W. **Quantification strategies in real-time PCR**. In A–Z of quantitative PCR. Edited by Bustin, S.A. p. 87–112. International University Line (IUL), La Jolla, CA, 2004a.

PHARR, D. M. et al. The dual role of mannitol as osmoprotectant and photoassimilate in celery. **HortScience**, v. 30, p. 1182-1188, 1995.

PILON-SMITS, E. A. H.; TERRY, N.; SEARS TOBIN, K. H. et al. Trehalose producing transgenic tobacco plants show improved growth performance under drought stress. **Jounal of Plant Physiology**, v. 25, p. 525–532, 1998.

PINTO, F.O. **Associação de marcadores EST-SSR à resistência ao bicho-mineiro em cafeeiros**. Campinas. 71f. Dissertação (Mestrado em Agricultura Tropical e Subtripical) – Curso de Pós Graduação em Agricultura Tropical e Subtropical, Instituto Agronômico de Campinas, 2006.

PONCET, V.; RONDEAU, M.; TRANCHAND, C.; CAYREL, A.; HAMON, S.; DE KOCHKO, A.; HAMON, P. SSR mining in coffee tree EST databases: potential use of EST-SSRs as marker across Coffea genus. **Molecular and General Genetics**, v. 276, p. 436–449, 2006.

PRAKASH, N.S.; COMBES, M.C.; SOMANNA, N.; LASHERMES, P. AFLP analysis of introgression in coffee cultivars (Coffea arabica L.) derived from a natural interspecific hybrid. **Euphytica**, n. 124, p. 265–271, 2002.

PRIVAT, I.; BARDIL, A.; BOMBARELY GOMEZ A. et al. The ‘PUCE CAFE’ project: the first 15K coffee microarray, a new tool for discovering candidate genes correlated to agronomic and quality traits. **BMC Genomics**, v. 12, p. 5, 2011.

PUMPHREY, M.; BAI, M.; LAUDENCIA-CHINGCUANCO, D.; ANDERSON, O.; GILL, B. S. Nonadditive expression of homeologous genes is established upon polyploidisation in hexaploid wheat. **Genetics**, v. 181, p. 1147–1157, 2009.

QI, Z.; SPALDING, E. P. Protection of plasma membraneK+ transport by the salt overly sensitive1 Na+–H+ antiporter during salinity stress. **Plant Physiology**, v. 136, p. 2548-2555, 2004.

QIU, Q. S; GUO, Y.; DIETRICH, M. A.; SCHUMAKER, K. S.; ZHU, J.K. Regulation of SOS1, a plasma membrane Na^+/H^+ exchanger in *Arabidopsisthaliana*, by SOS2 and SOS3. **PNAS**, v. 99, p. 8436–8441, 2002.

RENTSCH, D.; HIRNER, B.; SCHMELZER, E.; FROMMER, W. B. Salt stress-induced proline transporters and salt stressrepressed broad specificity amino acid permeases identified by suppression of a yeast amino acid permeasetargeting mutant. **Plant Cell**, v. 8, p. 1437–1446, 1996.

RIBAS, A. F.; DECHAMP, E.; CHAMPION, A.; BERTRAND, B.; COMBES, M. C.; VERDEIL, J. L.; LAPEYRE, F.; LASHERMES, P.; ETIENNE, H. Agrobacterium-mediated genetic transformation of Coffea arabica (L.) is greatly enhanced by using established embryogenic callus cultures. **BMC Plant Biology**, v. 11, p. 92, 2011.

RIBAS, A. F.; KOBAYASHI, A. K.; PEREIRA, L. F. P.; VIEIRA, L. G. E. Genetic transformation of Coffea canephora by particle bombardment. **Biologia Plantarum**, v. 49, n. 4, p. 493–497, 2005.

RIVERO, R. M.; RUIZ, J. M.; GARCÍA, P. C.; LÓPEZ-LEFEBRE, L. R.; SÁNCHEZ, E.; ROMERO, L. Resistance to cold and heat stress: accumulation of phenolic compounds in tomato and watermelon plants. **Plant Science**, v. 160, p. 315–321, 2001.

RODRIGUEZ-NAVARRO, A.; RUBIO, F. High-affinity potassium and sodium transport systems in plants. **Jornal of Experimental Botany**, v. 57, p. 1149-1160, 2006.

ROLLAND, F.; BAENA-GONZALEZ, E.; SHEEN J. Sugar sensing and signaling in plants: conserved and novel mechanisms. **Annual Review of Plant Biology**, v. 57, p. 675–709, 2006.

RUMPHO, M. E.; EDWARDS, G. E.; LOESCHER, W. H. A pathway for photosynthetic carbon flow to mannitol in celery leaves: activity and localization of key enzymes. **Plant Physiology**, v. 73, p. 869-873, 1983.

SCHMIDT, G. W.; DELANEY, S. K. Stable internal reference genes for normalization of real-time RT–PCR in tobacco (Nicotiana tabacum) during development and abiotic stress. **Molecular Genetics and Genomics**, v. 283, p. 233–241, 2010.

SCHOBERT, B. Is there an osmotic regulatory mechanism in algae and higher plants? **Journal of theoretical biology**, v. 68, p. 17-26, 1977.

SCHÖFFL, F.; PRANDL, R.; REINDL, A. MOLECULAR RESPONSES TO HEAT STRESS. IN: SHINOZAKI, K., YAMAGUCHI-SHINOZAKI, K. (Eds.), **Molecular Responses to Cold, Drought, Heat and Salt Stress in Higher Plants**. R.G. Landes Co., Austin, Texas, pp. 81–98, 1999.

SCHUSTER, W. S.; MONSON, R. K. An examination of the advantages of C3-C4 intermediate photosynthesis in warm environments. **Plant, Cell and Environment**, v. 13, p. 903–912, 1990.

SECKIN, B.; SEKMEN, A.H.; TÜRKAN, I. An Enhancing Effect of Exogenous Mannitol on the Antioxidant Enzyme Activities in Roots of Wheat Under Salt Stress. **Journal of Plant Growth Regulation**, v. 28, p. 12–20, 2009.

SERRAJ, R.; SINCLAIR, T. R. Osmolyte accumulation: Can it really help increase in crop yield under drought conditions? **Plant Cell Environment**, v. 25, p. 333– 341, 2002.

SHARKEY, T. D. Effects of moderate heat stress on photosynthesis: importance of thylakoid reactions, rubisco deactivation, reactive oxygen species, and thermotolerance provided by isoprene. **Plant, Cell and Environment**, v. 28 , p. 269–277, 2005.

SHEN, B.; JENSEN, R. G.; BOHNERT, H. J. Mannitol protects against oxidation by hyderoxyl radicals. **Plant Physiology**, v. 115, p. 527–532, 1997.

SHINOZAKI, K.; YAMAGUCHI-SHINOZAKI, K. Gene networks involved in drought stress response and Tolerance. **Journal of Experimental Botany**, v.58, n.2, p.221–227, 2007.

SICKLER, C. M.; EDWARDS, G. E.; KIIRATS, O.; GAO, Z.; LOESCHER, W. Response of mannitol-producing Arabidopsis thaliana to abiotic stress. **Functional Plant Biology**, v. 34, p. 382–391, 2007.

SILVA, E. A.; MAZZAFERA, P.; BRUNINI, O.; SAKAI, E.; ARRUDA, F. B.; MATTOSO, L. H. C.; CARVALHO, C. R. L.; PIRES, R. C. M. The influence of water management and environmental conditions on the chemical composition and beverage quality of coffee beans. **Brazilian Journal of Plant Physiology**, v. 17, p. 229–238, 2005.

SILVEIRA, E. D.; ALVES-FERREIRA, M.; GUIMARÃES, L. A.; SILVA, F. R.; CARNEIRO, V. T. D. C. Selection of reference genes for quantitative real-time PCR expression studies in the apomictic and sexual grass Brachiaria brizantha. **BMC Plant Biology**, v. 9, p. 84, 2009.

SMERTENKO, A.; DRABER, P.; VIKLICKY, V.; OPATRNY, Z. Heat stress affects the organization of microtubules and cell division in Nicotiana tabacum cells. **Plant Cell Environment**, v. 20, p. 1534–1542, 1997.

SMIRNOFF, N.; CUMBERS, Q. Hydroxyl radical scavenging activity of compatible solutes. **Phytochemistry**, v. 28, p. 1057–1060, 1989.

SONG, G-Q; SINK, K. C.; MA, Y.; HERLACHE, T.; HANCOCK, J. F.; LOESCHER, W. H. A novel manose-based selection system for plant transformation using celery mannos-6-phosphate reductase gene. **Plant Cell Report**, v. 29, p. 163-172, 2010.

SREENIVASULU, N.; SOROPY, S.K.; KISHOR, P.B.K. Deciphering the regulatory mechanisms of abiotic stress tolerance in plants by genomic approaches. Gene, v. 388, p. 1-13, 2007.

SRINIVASAN, K. H.; NARASIMHASWAMY, R. L. A review of coffee breeding work done at the Government coffee Experiments Station. **Indian Coffee**, v. 39, n. 10-11, p. 311-321, 1975.

STAMATI, K.; MACKAY, I.; POWELL, W. A quantitative genomic imbalance gene expression assay in a hexaploid species: wheat (Triticum aestivum). **Genome**, v. 52(1), p.89–94, 2009.

STEPHEN, T. S.; JAMES, T. The RNA polymerase II Core promoter. **Annual Review of Biochemistry**, v. 72, p. 449-479, 2003.

STOOP, J. M. H.; WILLIAMSON, J. D.; PHARR, D. M. Mannitol metabolism in plants: a method for coping with stress. **Trends in Plant Science**, v. 5, p. 139–144, 1996.

SUNG, D-Y; KAPLAN, F.; LEE, K-J; GUY, C. L. Acquired tolerance to temperature extremes. **Trends in Plant Science**, v. 8, p. 179–187, 2003.

SZABOLCS I. 1989. **Salt-Affected Soils**. Boca Raton, FL: CRC Press, 2009.

TAIZ, L.; ZEIGER, E. **Fisiologia Vegetal**. 3º edição, Ed. Artmed, Porto Alegre-RS, 2004.

TAJI, T.; OHSUMI, C.; IUCHI, S.; SEKI, M.; KASUGA, M.; KOBAYASHI, M.;YAMAGUCHI - SHINOZAKI, K.; SHINOZAKI, K. Important roles of drought- and cold inducible genes for galactinol synthase in stress tolerance in Arabidopsis thaliana. **Plant Journal**, v. 29, p. 417–426, 2002.

TRICARICO, C.; PINZANI, P.; BIANCHI, S.; PAGLIERANI, M.; DISTANTE, V.; PAZZAGLI, M.; BUSTIN, S. A.; ORLANDO, C. Quantitative real-time reverse transcription polymerase chain reaction: normalization to rRNA or single housekeeping genes is inappropriate for human tissue biopsies. **Analytical Biochemistry**, v. 309, p. 293–300, 2002.

TUNBRIDGE, E. M.; EASTWOOD, S. L.; HARRISON, P. J. Changed relative to what? housekeeping genes and normalization strategies in human brain gene expression studies. **Biological psychiatry**, v. 69, p.173–179, 2011.

URANO, K.; KURIHARA, Y.; SEKI, M.; SHINOZAKI, K. Omics analyses of regulatory networks in plant abiotic stress responses. **Current Opinion in Plant Biology**, v. 13, p. 132-138, 2010.

USADEL, B.; BLASING, O. E.; GIBON, Y.; POREE, F.; HOHNE, M.; GUNTER, M.; TRETHEWEY, R.; KAMLAGE, B.; POORTER, H.; STITT, M. Multilevel genomic analysis of the response of transcripts, enzyme activities and metabolites in Arabidopsis rosettes to a progressive decrease of temperature in the non-freezing range. **Plant, Cell and Environment**, v. 31, p. 518–547, 2008.

VALERIO, C.; COSTA, A.; MARRI, L.; ISSAKIDIS-BOURGUET, E.; PUPILLO, P.; TROST, P.; SPARLA, F. Thioredoxin-regulated beta-amylase (BAM1) triggers diurnal starch degradation in guard cells, and in mesophyll cells under osmotic stress. **Journal of Experimental Botany,** v. 62, p. 545–555, 2011.

VALLIYODAN, B.; NGUYEN, H.T. Understanding regulatory networks and engineering for enhanced drought tolerance in plants. **Current Opinion in Plant Biology**, v. 9, p. 189–195, 2006.

VANDESOMPELE, J.; DE PRETER, K.; PATTYN, F.; POPPE, B.; VAN ROY, N.; DE PAEPE, A.; SPELEMAN, F. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. **Genome Biology**, v. 3, 2002.

VANDESOMPELE, J.; KUBISTA, M.; PFAFFL, M. W. **Reference gene validation software for improved normalization**. Real-time PCR: Current Technology and Applications, p. 47–64, 2009.

VÉLËZ, H.; GLASSBROOK, N.J.; DAUB, M.E. Mannitol biosynthesis is required for plant pathogenicity by Alternaria alternate. **FEMS Microbiol Letters**, v. 285, 122-129, 2008.

VIDAL, R. O.; MONDEGO, J. M. C.; POT, D.; AMBRÓSIO, A. B.; ANDRADE, A. C.; PEREIRA, L. F. P.; COLOMBO, C. A.; VIEIRA, L. G. E.; CARAZZOLLE, M. F.; PEREIRA, G. A. G. A high-throughput data mining of single nucleotide

polymorphisms in Coffea species expressed sequence tags suggests differential homeologous gene expression in the allotetraploid Coffea arabica. **Plant Physiology**, v. 154, p. 1053–1066, 2010.

VIEIRA, L. G. E.; ANDRADE, A. C. et al. Brazilian coffee genome project: an EST-based genomic resource. **Brazilian Journal of Plant Physiology**, v. 18, p. 95–108, 2006.

VIERLING, E. The role of heat shock proteins in plants. **Annual Review of Plant Physiology and Plant Molecular Biology**, v. 42, p. 579–620, 1991.

VIJ, S.; TYAGI, A. K. Emerging trends in the functional genomics of the abiotic stress response in crop plants. **Plant Biotechnology Journal**, v. 5, n. 3, p 361–380, 2007.

VINOCUR, B.; ALTMAN, A. Recent advances in engineering plant tolerance to abiotic stress: achievements and limitations. **Current Opinion in Biotechnology**, v. 16, p. 123–132, 2005.

WAHID, A.; GELANI, S.; ASHRAF, M.; FOOLAD, M. R. Heat tolerance in plants: an overview. **Environmental and Experimental Botany**, v. 61, p. 199–223, 2007.

WAN, H.; ZHAO, Z.; QIAN, C.; SUI, Y.; MALIK, A. A.; CHEN, J. Selection of appropriate reference genes for gene expression studies by quantitative real-time polymerase chain reaction in cucumber. **Analytical Biochemistry**, v. 399, p. 257–261, 2010.

WANG, J.; LIU, D.; GUO, X.; YANG, W.; WANG, X.; ZHAN, K.; ZHANG, A. Variability of Gene Expression After Polyhaploidization in Wheat (*Triticum aestivum* L.). **G3: Genes, Genomes and Genetics**, v. 1, n. 1, p. 27-33, 2011.

WANG, W.; VINOCUR, B.; ALTMAN, A. Plant responses to drought, salinity and extreme temperatures: towards genetic engineering for stress tolerance. **Planta**, v. 218, p. 1–14, 2003.

WANG, W.; VINOCUR, B.; SHOSEYOV, O.; ALTMAN, A. Role of plant heat-shock proteins and molecular chaperones in the abiotic stress response. **Trends in Plant Science**, v. 9, p. 244–252, 2004.

WATSON, K.; ACHINELLI, M. L. Context and contingency: the coffee crisis for conventional small-scale coffee farmers in Brazil. **The Geographical Journal**, v. 174, n. 3, p. 223–234, 2008.

WILLIAMSON, J. D.; JENNINGS, D. B.; GUO, W-E.; PHARR, D. M.; EHRENSHAFT, M. Sugar alcohols, salt stress and fungal resistance: polyolsmultifunctional plant protection? **Journal of the American Society for Horticultural Science**, v. 127, p. 467– 473, 2002.

WILLIAMSON, J. D.; STOOP, J. M. H.; MASSEL, M. O.; CONKLING, M. A.; PHARR, D. M. Sequence analysis of a mannitol dehydrogenase cDNA from plants reveals a function for the pathogenesis-related protein ELI3. **PNAS**, v. 92., p. 7148–7152, 1995.

YORDANOV, I.; DILOVA, S; PETKOVA, R.; PANGELOVA, T.; GOLTSEV, V.; SUSS, K.-H. Mechanisms of the temperature damage and acclimation of the phitosynthetic apparatus. **Photochemistry and photobiology**, v. 12, p. 147–155, 1986.

YU, Q.; GUYOT, R.; DE KOCHKO, A.; BYERS, A.; NAVAJAS-PÉREZ, R.; LANGSTON, B. J.; DUBREUIL-TRANCHANT, C.; PATERSON, A. H.; PONCET, V.; NAGAI, C.; MING, R. Micro-collinearity and genome evolution in the vicinity of an ethylene receptor gene of cultivated diploid and allotetraploid coffee species (*Coffea*). **Plant Journal**, v. 67, n. 2, p. 305-17, 2011.

ZHIFANG, G.; LOESCHER, W. H. Expression of a celery mannose 6-phosphate reductase in Arabidopsis thaliana enhances salt tolerance and indices biosynthesis of both mannitol and a glucosyl-mannitol dimer. **Plant, Cell and Environment**, v. 26, p. 275-283, 2003.

ZHU, J. K. Plant salt tolerance. **Trends in Plant Science**, v. 6, p. 66-71, 2007.

4 ARTIGO

DEFICIÊNCIA DE NITROGÊNIO, ESTRESSE SALINO E TÉRMICO EM CAFÉ (*Coffea arabica* L.): IDENTIFICAÇÃO E VALIDAÇÃO DE NOVOS GENES PARA NORMALIZAÇÃO DE qPCR.

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(ANEXO I)

Deficiência de nitrogênio, estresse salino e térmico em café (*Coffea arabica* L.): identificação e validação de novos genes para normalização de qPCR.

RESUMO

Os estresses abióticos estão entre os principais fatores que alteram a produtividade de culturas. Estes desafios ambientais podem ser enfrentados através de modulação transcricional. O PCR quantitativo em tempo real (qPCR) é um método sensível, rápido e confiável na detecção de mRNA e tem se tornado uma poderosa ferramenta em estudos de tolerância a estresses em plantas; no entanto, genes de referencia adequados são necessários para se realizar a normalização de dados. Genes de referência em plantas de café submetidas à deficiência de nitrogênio, estresses salino e térmico ainda não foram relatados. Este trabalho avaliou a estabilidade de expressão de dez potencias genes de referencia utilizando os softwares geNorm PLUS, NormFinder e BestKeeper em plantas submetidas à deficiência de nitrogênio, estresses salino e térmico. *EF1*, *EF1α*, *GAPDH*, *MDH* e *UBQ10* foram os genes mais estáveis em todos os tratamentos e softwares analisados, enquanto que *RPL39* e *RPII* foram classificados como os genes de referencia menos confiáveis. Para a validação, o padrão transcricional de uma hemoglobina não-simbiótica de *Coffea* (*CaHb1*) foi analisado utilizando os dois novos genes de referencia recomendados e o gene de referencia mais instável para normalização. O gene mais instável pode levar a interpretações equivocadas ao se analisar o nível transcricional de *CaHb1*. Assim, neste trabalho nós recomendamos dois novos genes de referencia em *Coffea* para o uso de normalização de dados em experimentos de estresses abióticos: *MDH* e *EF1*.

Palavras-chave: nutrição mineral, alta temperatura, salinidade, limitação de nitrogênio, café, normalização de qPCR, folhas e raízes.

Nitrogen starvation, salt and heat stress in coffee (*Coffea arabica* L.): identification and validation of new genes for qPCR normalization

ABSTRACT

Abiotic stresses are among the most important factors that affect food production. One important step to face these environmental challenges is the transcriptional modulation. Quantitative real-time PCR (qPCR) is a rapid, sensitive and reliable method for the detection of mRNAs and it has become a powerful tool to mitigate plant stress tolerance; however, suitable reference genes are required for data normalization. Reference genes for coffee plants during nitrogen starvation, salinity and heat stress have not yet been reported. We evaluated the expression stability of ten candidate reference genes using geNorm PLUS, NormFinder and BestKeeper softwares, in plants submitted to nitrogen starvation, salt and heat stress. *EF1*, *EF1α*, *GAPDH, MDH* and *UBQ10* were ranked as the most stable genes in all stresses and software analyses, while *RPL39* and *RPII* were classified as the less reliable references. For reference gene validation, the transcriptional pattern of a *Coffea* non-symbiotic hemoglobin (*CaHb1*) was analyzed using the two new recommended and the most unstable gene references for normalization. The most unstable gene may lead to incorrect interpretation of *CaHb1* transcriptional analysis. Here, we recommend two new reference genes in *Coffea* for use in data normalization in abiotic stresses: *MDH* and *EF1*.

Keywords: mineral nutrition, high temperature, salinity, nitrogen limitation, coffee, qPCR normalization, leaves, roots.

4.1 INTRODUCTION

Coffee is an important crop and is crucial to the economy of many developing countries, generating around US$70 billion per year. This way, coffee is one of the most valuable international exchange commodities in agricultural trade and coffee farmings are crucial to the economy of many developing countries where its cultivation, processing, transportation and marketing provide employment for millions of people worldwide (WATSON. ACHINELLI, 2008). Abiotic stress is one of the major factors that affect food production worldwide and in tropical countries, drought, salinity, extreme temperatures, nutrient deficiencies and mineral toxicities are among the most important limitants in crop yield (ROY *et al,.* 2011). It is important to note that, in a scenario of climate changes, these stresses will be increasingly important, associated with land degradation and declining water quality (TESTER; LANGRIDGE, 2010).

In this way, several studies that depict the impact of abiotic stresses uses reverse transcription quantitative real-time PCR (qPCR) as a tool to understand the mechanism of plant stress tolerance (KODAIRA *et al.,* 2011; TILLETT *et al.,* 2011). qPCR is a valuable technique to study rare transcripts, to work with members of a multigene family or with small sample sizes and offers advantages in sensitivity and specificity. However, this method is only valid if proper internal controls are included. Extensive transcriptomic data mining and experimental validation in different plants has shown that the reliability of these endogenous controls can be influenced by plant species, growth conditions and organs/tissues examined, and there are no universally applicable reference genes with an invariant expression and that commonly used reference genes exhibit a large expression fluctuation (CHEN *et al.,* 2011). Diverse statistical algorithms have been developed to help validate reference genes that led to recommendations for refining qPCR standards in the plant research community. These debates provide guidance for refining qPCR standards in a defined set of RNA samples in a single species.

The continued use of inappropriate reference genes may have already resulted in the misinterpretation of some published results (GUÉNIN *et al,.* 2009), although studies involving the selection of appropriate reference genes have

increased (ARTICO *et al,.* 2010; FERNANDEZ *et al.,* 2010; GU *et al.,* 2011;). In recent years, coffee (*Coffea ssp*.) has become the subject of increasing research in gene expression analysis (SALMONA *et al.,* 2008; NOBILE *et al,.* 2010). However, few works have focused on selecting reliable reference genes for data normalization in studies involving coffee plants under different stress conditions (CRUZ *et al.,* 2009; BARSALOBRES-CAVALLARI *et al.,* 2009). Moreover, few studies explore the suitability of reference genes for nitrogen (N), salt and heat stress.

Considering that, in the current study we evaluated the transcriptional profile of a *Coffea arabica* hemoglobin and the stability of ten *Coffea arabica* genes to normalize the results of gene expression studies in N starvation, salt stress and high temperature conditions using three algotithms: geNorm PLUS (VANDESOMPELE *et al,.* 2002), NormFinder (ANDERSEN *et al.,* 2004) and BestKeeper (PFAFFL *et al.,* 2004).

4.2 MATERIALS AND METHODS

4.2.1 PLANT MATERIAL AND ABIOTIC STRESS TREATMENTS

For all experiments, we used *C. arabica* cv IAPAR 59. In the N starvation treatment, coffee seeds were allowed to germinate in vermiculite in a greenhouse at 25 ºC on a 12h/12h day/night cycle. The seeds were irrigated twice a week with distilled water. After 4 weeks, seedlings were irrigated with Clark (CLARK, 1975) nutritive solution, with pH adjusted to 5.5-6.0. Prior to the experiments, 6-month-old plants (5-6 leaf pairs) with a single shoot were selected for size uniformity, and transferred to a growth chamber under the following conditions: 12/12 h light/dark, 25 ºC/23 ºC day/night temperature, 45% humidity and photosynthetic photon flux density of approximately 250 µmol m^{-2} s^{-1}. Plants were maintained in a aerated solution modified from Clark (1975) with the following components: 5330 µM NH_4NO_3, 260 µM KH_2PO_4, 330 µM $MgSO_4$, 1060 µM K_2SO_4, 660 µM $CaCl_2$, 200 µM Na-Fe-EDTA, 7 µM H_3BO_3, 3 µM $MnSO_4$, 2.5 µM $ZnSO_4$, 0.1 µM $CuSO_4$, e 0.7 µM $NaMoO_4$. Nutrient solutions were renewed weekly. After 4 weeks on hydroponic devices, plants were harvested for time point 0 and then transferred to the modified Clark (1975) N-free solution, where leaves (5th and 6th pair) and lateral roots were harvested at 1 day and 10 days after transfer into the N-free solution. Experiments

were conducted twice with a minimum of three biological replicates per experiment. Biological replicates were represented by pools of coffee leaves or lateral roots of at least nine plants at the same developmental stage. After harvesting, all samples were frozen immediately in liquid N_2 and stored at -80ºC until RNA extraction.

Basic procedures of the salinity and heat stress treatments followed Dos Santos *et al.* (2011). The salt stress experiment was carried out using leaves of nine 18-month coffee plants with uniform growth conditions. In order to avoid osmotic shock, on the first day plants were irrigated with 50 mM NaCl and the second day with 100 mM NaCl. From the third day up to the end of the experiment, plants were daily irrigated with 150 mM NaCl. Leaves were harvested as following: day 0 (control without addition of NaCl), day 4, 6 and 12 (150 mM NaCl). Sampling was carried out in three pools of leaves (each pool contained a mixture of two pairs of leaves from three plants). Each pool represented a biological repetition.

In the heat stress treatment, we used leaves from nine coffee plants. Firstly, plants were maintained in the chamber for seven days at 24 °C and controlled photoperiod (12h:12h) for acclimatization. In sequence, the chamber temperature was raised to 37°C for five days. To avoid water deficit, plants were irrigated during all the heat stress period. Samples were collected at four evaluation stages: non-stressed control (plants maintained 7 days in growth chamber at 24°C), day 1, 3 and 5 (plants maintained at 37°C). Leaf sampling was also held in pools, where each of the three pools contained a mixture of two leaf pairs from three plants under the same stress conditions. Again, each pool represented a biological repetition. For both salt and heat stress, only the second pair of leaves at the same developmental stage, of each plagiotropic branch was collected and were immediately immersed in liquid N_2 and stored at -80°C until the assays were performed.

4.2.2 RNA ISOLATION, PURIFICATION AND cDNA SYNTHESIS

Total RNA from *C. arabica* cv. IAPAR-59 leaves and roots (in the case of N starvation) was isolated as described by Chang and coworkers (1993). RNA samples were purified using the Pure Link Micro to Midi Total RNA Purification System (Invitrogen) and their integrity was examined by 1% agarose gel electrophoresis and treated with DNase (RNase-free). RNA concentration and purity

were determined using a NanoDrop® ND-100 spectrophotometer and the absence of genomic DNA contamination was confirmed by PCR using *GAPDH* primers and about100ng of RNA (data not shown). Complementary DNA (cDNA) was synthesized by a SuperScript III Reverse Transcriptase (Invitrogen) following the manufacturer's instructions in a final volume of 20µl using 5 µg of total RNA. The final cDNA products were diluted 10-fold prior to use in qPCR.

4.2.3 REFERENCE GENES SELECTION, PRIMER DESIGN AND AMPLIFICATION EFFICIENCY

A set of ten potential reference genes, including large ribosomal subunit 39 (*RPL39*), polyubiquitin 10 (*UBQ10*), ribosomal protein S24 (*S24*), glyceraldehyde-3-phosphate-dehydrogenase (*GAPDH*), β-tubulin (*β-TUB*), tonoplast intrinsic protein (*TIP41*), elongation factor 1 (*EF1*), elongation factor 1-alpha (*EF1α*), RNA polymerase-II transcription factor (*RPII*) and malate dehydrogenase (*MDH*), were chosen to assess their stability as reliable reference genes. For *RPL39*, *UBQ10*, *S24* and *GAPDH* we used primers pairs already reported in previous studies in *Coffea* (SIMKIN *et al.,* 2006; CRUZ *et al.,* 2009; BOTTCHER *et al.,* 2011), having Tm of 60 °C ± 1 °C and amplicon length between 69 and 100 bp. We associated previously reported primers based on private database sequences to public data from the Brazilian Coffee Genome Project Consortium (MONDEGO *et al.,* 2011) (Table 1).

In the case of *β-TUB*, *TIP41*, *EF1*, *EF1α*, *RPII* and *MDH*, genes were selected from *Coffea arabica* EST sequences developed by the Brazilian Coffee Genome Project Consortium (MONDEGO *et al,.* 2011) (Table 1). For these genes, primers were designed using Primer Express software (version 3.0) according to parameters established to obtain amplicons of 100 bp with a Tm of 60 °C ± 1 °C (Table 1). Specific products and amplicon length of all primer pairs were verified in 1% agarose gel electrophoresis with SB buffer (0.5 M NaOH, pH adjusted to 8.5 with boric acid) (Supplementary material 1).

The efficiency of each primer pair was calculated according to a previous work from our group (CARVALHO *et al.,* 2010), using a standard curve generated from a serial dilution of cDNA using $E = [10^{(-1/slope)} - 1]$. Four serial dilutions were set up to determine cycle threshold (Ct) values, designated here as quantification cycle

(C_q), and the reaction efficiencies for all primers pairs. Standard curves were generated for each primer pair using the Ct value versus the logarithm of each cDNA dilution factor.

Table 01 - Description of genes and primer sequences used for qPCR

Gene	Abbreviation	Primer forward	Primer reverse	Efficiency	NCBI accession	Arabidopsis ortholog
Large ribosomal subunit 39	*RPL39*	GAACAGGCCCATCCCTTATTG	CGGCGCTTGGCATTGTA	104%	GT720707.1	AT4G31985
Polyubiquitin 10	*UBQ10*	CAGACCAGCAGAGGCTGATT	AGAACCAAGTGAAGGGTGGA	92%	GT697658.1	AT4G05320
Ribosomal protein S24	*S24*	GCCCAAATATCGGCTTATCA	TCTTCTTGGCCCTGTTCTTC	101%	GT730897.1	AT3G04920
GAPDH	*GAPDH*	AGGCTGTTGGGAAAGTTCTTC	ACTGTTGGAACTCGGAATGC	99%	GW488886.1	AT1G13440
β-tubulin	*β-TUB*	GTGCCGGTAATAACTGGGCTAA	CAGTCACAATTTTCGGCTTCTTT	99%	GT707405.1	AT1G75780
Tonoplast intrinsic protein	*TIP41*	CTTCCAAAGCTTCCATCCTCAA	AAGGAGCTATGTCCGAAAATCATCT	102%	GW454965.1	AT4G34270
Elongation factor 1	*EF1*	CTGTCCTTGATTGCCACACTTCT	CTTGGGCTCCTTCTCAAGCTC	95%	GW484749.1	AT1G07920
Elongation factor 1-alpha	*EF1α*	AAGGGAGCTTCCAGCTTTACCT	TGTGAGAGGTGTGGCAGTCAA	96%	GT708303.1	AT1G07940
RNA pol-II transcription factor	*RPII*	AATGGAAGGCAGATCGTACCAG	TATGCTGGCATCAGTGTCGC	110%	GT730193.1	AT2G15430
Malate dehydrogenase	*MDH*	CCTGATGTCAACCACGCAACT	GTGGTTATGAACTCTCCATTCAACC	98%	GW464198.1	AT1G04410
Hemoglobin	*CaHb1*	GAACGCTCCATTGGAACAGAAC	CCAGCTTTCCGGAGTTGAAC	102%	GW457930.1	AT2G16060

4.2.4 qPCR AND DATA ANALYSIS

The transcript abundance for each of the ten genes was analyzed by qPCR (7500 Fast Real-Time PCR System, Applied Biosystems) using the SYBR Green PCR Master Mix (Applied Biosystems). The reaction mixture contained 12.5 µl of 2x SYBR Green master mix, 1µl of each primer (10 µM), 1 µl of cDNA 1:10 diluted and Milli-Q water to a total volume of 25 µl. Thermal conditions were 95 °C for 10 min, followed by 40 cycles of 95 °C for 30s and 60 °C for 60s. Melting curves were analyzed to verify the presence of a single product including a negative control. All reactions were performed in triplicate for each of the three biological replicates in all treatments studied. Relative expression levels of the candidates reference genes were imported to geNorm PLUS (VANDESOMPELE *et al.,* 2002) Biogazelle, Ghent), NormFinder (ANDERSEN *et al.,* 2004) and BestKeeper (PFAFFL *et al.,* 2004) analysis tools which were used as described in their respective manuals. In this work, we have followed the minimum information for publication of quantitative real-time PCR experiments (MIQE) according to Bustin and coworkers (BUSTIN *et al.,* 2009). Additional information concerning the qPCR experiment can be found in the MIQE document (Supplementary material 2), that followed the recommendations of Privat *et al.* (2011) in a large-scale transcriptional study in coffee.

4.2.5 REFERENCE GENE VALIDATION

In order to validate the reference gene, the relative expression level of *C. arabica* Hemoglobin1 (*CaHb1*) (Table 1), an ortholog of *Arabidopsis* Class I hemoglobin (DORDAS *et al.,* 2004), was performed in all RNA samples using two stable reference genes and the most unstable, from each experimental condition, to demonstrate how the adoption of different reference genes can affect the normalization of the expression data for a gene of interest. The transcriptional activity of *CaHb1* was calculated using the $2^{-\Delta\Delta Ct}$ method (LIVAK; SCHMITTGEN, 2001).

4.3. RESULTS

4.3.1 RNA INTEGRITY AND AMPLIFICATION SPECIFICITY

The integrity and quality of RNA samples were evaluated by electrophoretic analysis agarose gels stained with ethidium bromide. All samples analyzed showed clear 28S and 18S rRNA bands (data not shown), confirming that the RNA samples were appropriate for transcript level analysis. The average yield of RNA extractions were 25.5µg g^{-1} of fresh tissue (ft) for salt stressed leaves, 10.5µg g^{1} ft for heat stressed leaves, 27.8µg g^{-1}ft for N-starved leaves and 13.5µg g^{-1} ft for N-starved lateral roots. As expected, RNA yield of lateral roots was lower than the obtained for leaves. Among leaves, the heat stress resulted in the lowest yield. The purity (ratio A_{260}/A_{280}) of the RNA samples used for the qPCR analysis was evaluated by spectrophotometry, and ranged from 1.7 to 2.1. Also, prior to carrying out qPCR reactions, the viability of all RNA samples was examined by PCR with the primer pair selected for each reference gene (Table 1). All the PCR reactions produced a single specific fragment with the predicted molecular size on agarose gel visualized with ethidium bromide staining. Also, using each primer pair, gene-specific amplification was confirmed by the appearance of a single peak in melting curve analyses. The amplification efficiencies ranged from 92 to 110% (Table 1).

4.3.2 EXPRESSION PROFILES OF REFERENCE GENES

To reveal the differences in transcript expression levels between the studied genes, the quantification cycle (C_q) values were determined for each gene across all of the experimental samples and scatter plots were constructed (Figure 7). We observed different C_q values from qPCR amplifications, with the lowest mean C_q value (20.13) in *RPL39* and the highest (36.05) in *RPII*. Considering all the stresses, C_q values for many reference genes showed rather large standard deviation (SD), which was due to the large number of biological samples and in accordance with similar studies in other plant species (TILLETT *et al.,* 2011; CHEN *et al.,* 2011). The smallest variation in gene expression is observed for *RPL39* and *S24* (lower than 2 cycles), while *β-TUB* and *TIP41* were the genes with the most variable levels inter-run C_q variation (over 2.9 cycles).

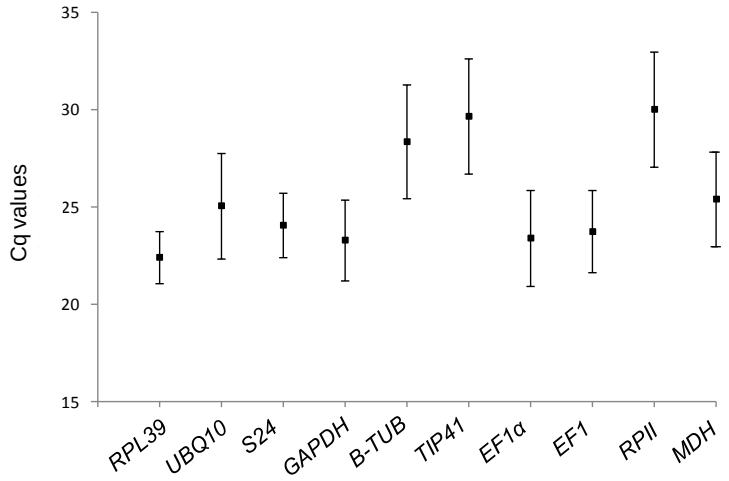


FIGURE 07 - Expression levels of candidate reference genes in different experimental samples. Values are given as quantification cycle (Cq) in 42 samples. Bars indicate standard deviations.

4.3.3 EVALUATION OF EXPRESSION STABILITY

The expression stability of ten potential reference genes was evaluated in a set of 42 cDNA samples that included leaves of plants under three abiotic stresses (N starvation, salt stress and heat shock) and roots of N starved plants. Altogether, our results showed that geNorm PLUS, NormFinder and BestKeeper algorithms obtained, in most cases, to the same results concerning the best candidate reference gene to use for expression normalization for at least one reference gene.

4.3.3.1 GENORM PLUS ANALYSIS

Gene expression stability (M) of the ten studied reference genes was calculated using geNorm PLUS software (VANDESOMPELE *et al.,* 2002) (Figure 8). This program recommends using an M value below the threshold of 1.5 to identify reference genes with stable expression. *MDH* (0.104) and *GAPDH* (0.113) had the highest expression stability in leaf during N starvation (Figure 9A) while in roots the best reference genes were *GAPDH* (0.179) and *EF1* (0.180) (Figure 8B). In salt stress, *EF1* (0.469) and *EF1α* (0.493) appeared among the best candidates (Figure 8C), while during the heat stress the best reference genes were *MDH* (0.216) and *EF1α* (0.241) (Figure 8D). Considering the three first reference genes (more stable)

and the three last (more unstable) from each condition, GeNorm classified *EF1* and *EF1α* as the most stable reference genes and *RPL39* and *RPII* as the least stable.

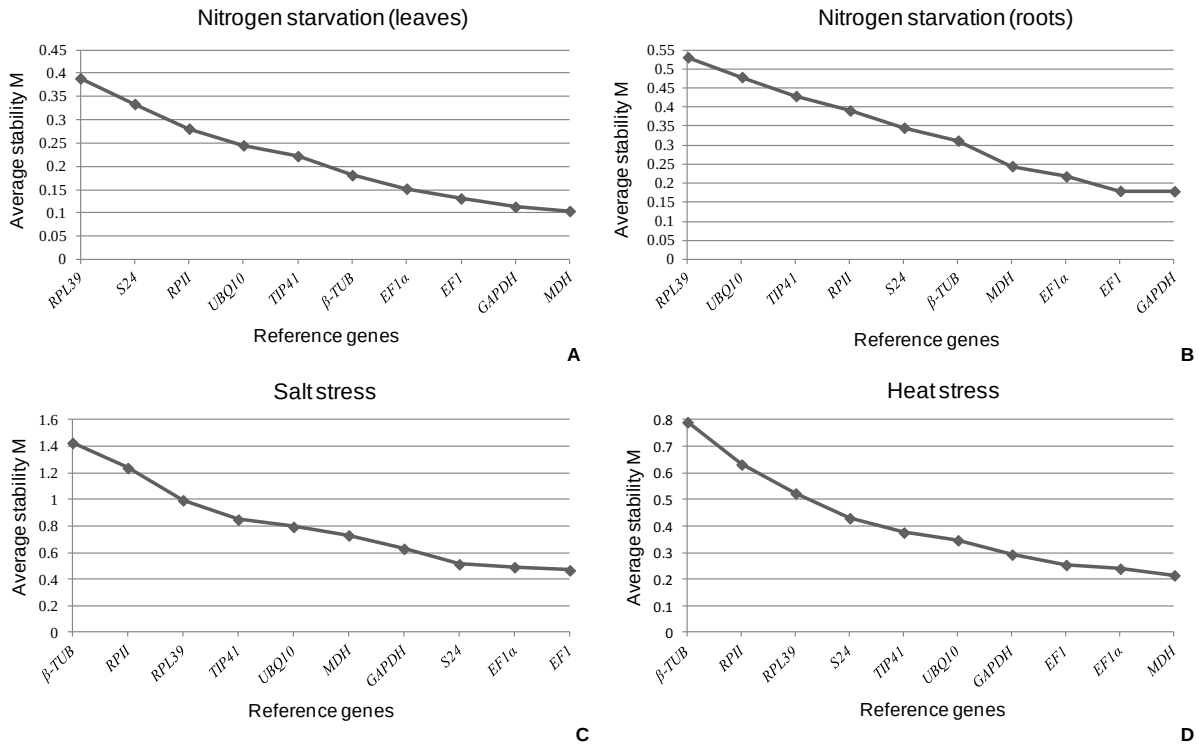


FIGURE 08 - Gene expression stability and ranking of the ten reference genes as calculated by geNorm PLUS (M values). A lower M value indicates more stable expression. Nitrogen starvation (leaves) – A; Nitrogen starvation (roots) – B; Salt stress – C; Heat stress – D.

Several works use one single gene as an internal control for normalization, even in *C. arabica* gene expression studies (SALMONA *et al.,* 2008; OLIVEIRA *et al.,* 2010), but it has been suggested that the use of two or more reference genes for normalization might produce more reliable results (LIN; LAI, 2010). To determine the optimal number of reference genes, geNorm calculates the pairwise variation V_n/V_{n+1} between two sequential normalization factors N_{Fn} and N_{Fn+1} that contain an increasing number of reference genes. Vandesompele *et al.* (2002) recommended 0.15 as a cutoff value for the variation below which the inclusion of an additional reference gene is not required. However, this threshold should not be an absolute cutoff value, because it will depend on the data (WAN *et al.,* 2010). In three of the four experimental conditions (N starved leaves – Figure 9A; N starved roots – Figure

9B and Heat stress – Figure 9D), $V_{2/3}$ was already bellow the cutoff value, implying that the use of the two best reference genes is sufficient to reliable gene expression normalization of target genes. On the other hand, salt stress displayed a recommended variation cutoff value only at $V_{5/6}$, indicating that five genes are the ideal set for data normalization (Figure 9C).

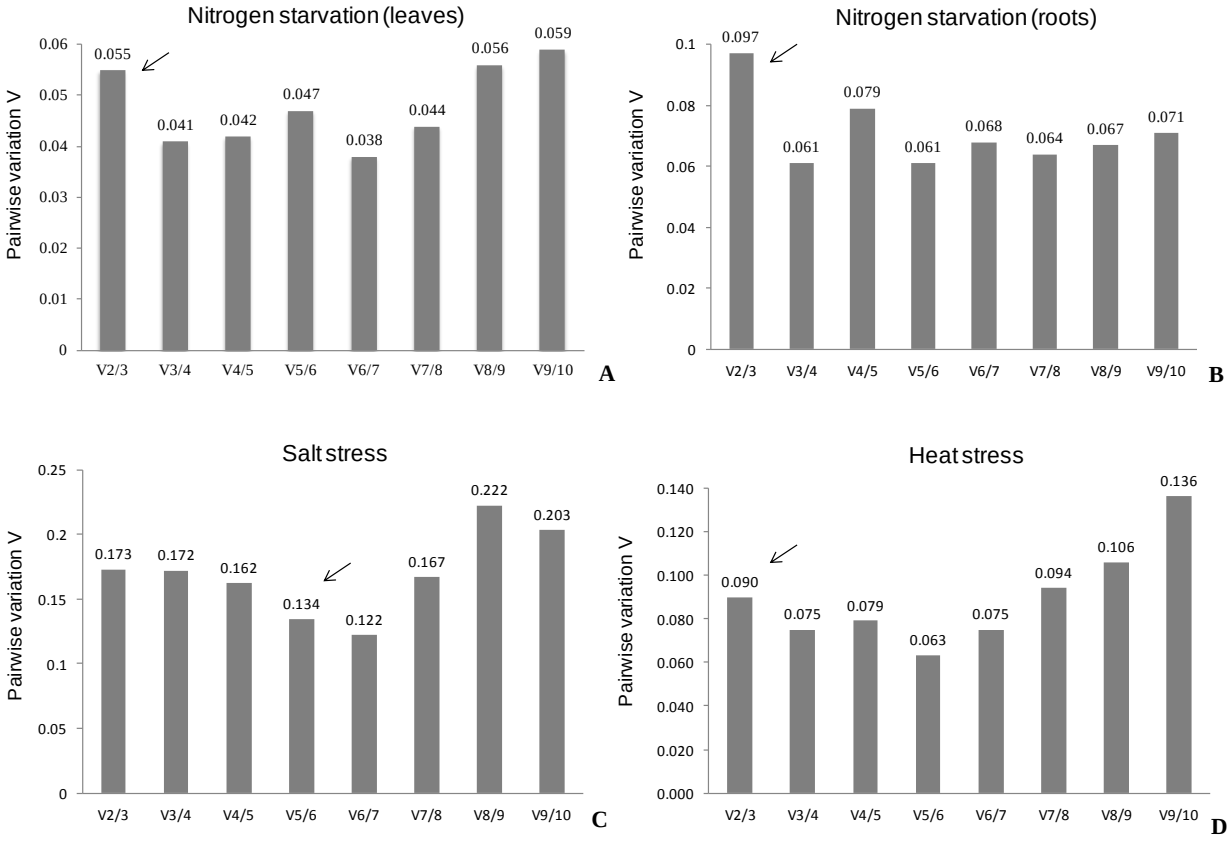


FIGURE 9 - Pairwise variation (V) analysis of the candidate reference genes. The pairwise variation (V_n/V_{n+1}) was analyzed between the normalization factors N_{Fn} and N_{Fn+1} using geNorm PLUS to determine the optimal number of reference genes required for qPCR data normalization in each condition. Nitrogen starvation (leaf) – A; Nitrogen starvation (roots) – B; Salt stress – C; Heat stress – D. Arrow indicates the optimal number of genes for normalization in each sample sets.

4.3.3.2 NORMFINDER ANALYSIS

NormFinder ranks the set of candidate reference genes on a model-based approach and generates a stability measure of which a lower value indicates increased stability in gene expression (ANDERSEN *et al.,* 2004). In the N starvation experiment, the best stabilities assigned by the applet for reference genes in leaves were *EF1* and *EF1α* (0.046 and 0.056, respectively), and the best combination of two genes was *EF1α* and *MDH*, which lead the stability value to 0.025, indicating a more reliable normalization than that based on single genes (Table 2). During N starvation using root tissues, *GAPDH* and *EF1* were the most stable reference genes, with stability values of 0.102 and 0.106, respectively. The best combination was *GAPDH* and *β-TUB* (0.061).

Table 02 - Expression stability values in four different conditions calculated by NormFinder software

Rank	N starvation (leaves)		N starvation (roots)		Salt stress		Heat stress	
	Gene	Stability	Gene	Stability	Gene	Stability	Gene	Stability
1	*EF1*	0.046	*GAPDH*	0.102	*UBQ10*	0.074	*GAPDH*	0.118
2	*EF1α*	0.056	*EF1*	0.106	*EF1*	0.164	*EF1α*	0.237
3	*MDH*	0.084	*β-TUB*	0.116	*EF1α*	0.199	*UBQ10*	0.239
4	*GAPDH*	0.093	*EF1α*	0.187	*S24*	0.238	*MDH*	0.256
5	*β-TUB*	0.117	*S24*	0.193	*GAPDH*	0.275	*S24*	0.318
6	*UBQ10*	0.161	*RPII*	0.230	*MDH*	0.285	*TIP41*	0.328
7	*TIP41*	0.223	*MDH*	0.239	*TIP41*	0.303	*EF1*	0.337
8	*S24*	0.302	*TIP41*	0.242	*RPL39*	0.488	*RPL39*	0.468
9	*RPII*	0.328	*UBQ10*	0.344	*β-TUB*	0.684	*RPII*	0.531
10	*RPL39*	0.343	*RPL39*	0.347	*RPII*	0.725	*β-TUB*	0.784
BCTG*	*EF1α* and *MDH*	0.025	*GAPDH* and *β-TUB*	0.061	*UBQ10* and *EF1*	0.092	*GAPDH* and *EF1α*	0.149

*Best combination of two genes

Normfinder ranked for salt stress *UBQ10* (0.074) and *EF1* (0.164) as the most stable reference genes, which were also the best combination (0.092). Heat stress showed *GAPDH* (0.118) and *EF1α* (0.237) with the most constant expression; these genes were also the best combination (0.149). Considering all treatments, *EF1* appeared more frequently among the most stable genes and *RPL39* and *RPII* were always among the least suitable reference genes.

4.3.3.3 BESTKEEPER ANALYSIS

This tool estimates gene expression stability for all individual reference genes based on coefficient of correlation (r), standard deviation (SD) and percentage covariance (CV). It is important to emphasize that BestKeeper uses Ct values as input data instead of the raw data (relative quantities) used by geNorm and NormFinder and consequently may lead to misinterpretation related to the lack of correction for intersample/intercondition variation, especially when transcriptional variation among reference genes is greater than two-fold (SD >1.0) (PFAFFL *et al.,* 2004). Here, the criteria analyzed for gene expression stability were r values over 0.95 and SD < 1.0. Additional descriptive statistics of the BestKeeper analyses are provided in Supplementary Material 3.

In N-starved leaves, *EF1* (r = 0.998; SD = 0.91) and *EF1α* (r = 0.996; SD = 0.96) were the most stable reference genes based on higher r values and lower CV values. Also, in this condition all of the ten candidates presented r values above 0.97 and most of them displayed low SD (only *UBQ10*, *TIP41* and *RPII* had SD > 1). In N-starved roots, *GAPDH* (r = 0.996; SD = 0.96) and *EF1* (r = 0.994; SD = 0.98) were the most stable genes considering the criteria described above (Supplementary material 3). During salt stress, *UBQ10* and *EF1α* had the most constant transcription levels (r > 0.97) and in heat stressed leaves, *MDH* and *GAPDH* were the most stable reference genes (r > 0.98), but in both cases standard deviation was above 1 (Supplementary material 3).

4.3.4 REFERENCE GENE VALIDATION

To assess the validity of the candidate reference genes, the transcriptional pattern of a target gene, non-symbiotic hemoglobin *CaHb1* (DORDAS *et al.,* 2004), under different experimental conditions, including N starvation in leaves and roots, salt and heat stress, were analyzed using the two new recommended reference genes (*EF1* and *MDH*), and the most unstable gene for normalization (*RPL39*). *CaHb1* transcriptional profile showed that mRNA levels of this gene was upregulated in short-term in N-starved leaves (day 1) while in N-starved roots *CaHb1*was

downregulated. During salt stress transcriptional levels of *CaHb1*was short-term upregulated (day 4) and in heat stress mRNA levels showed little variation (Figure 10). Unlike *RPL39*, similar expression patterns were observed when *EF1* and *MDH* were used for normalization of *CaHb1* (Figure 10), showing their expression stability and capability to generate reliable results of relative expression levels.

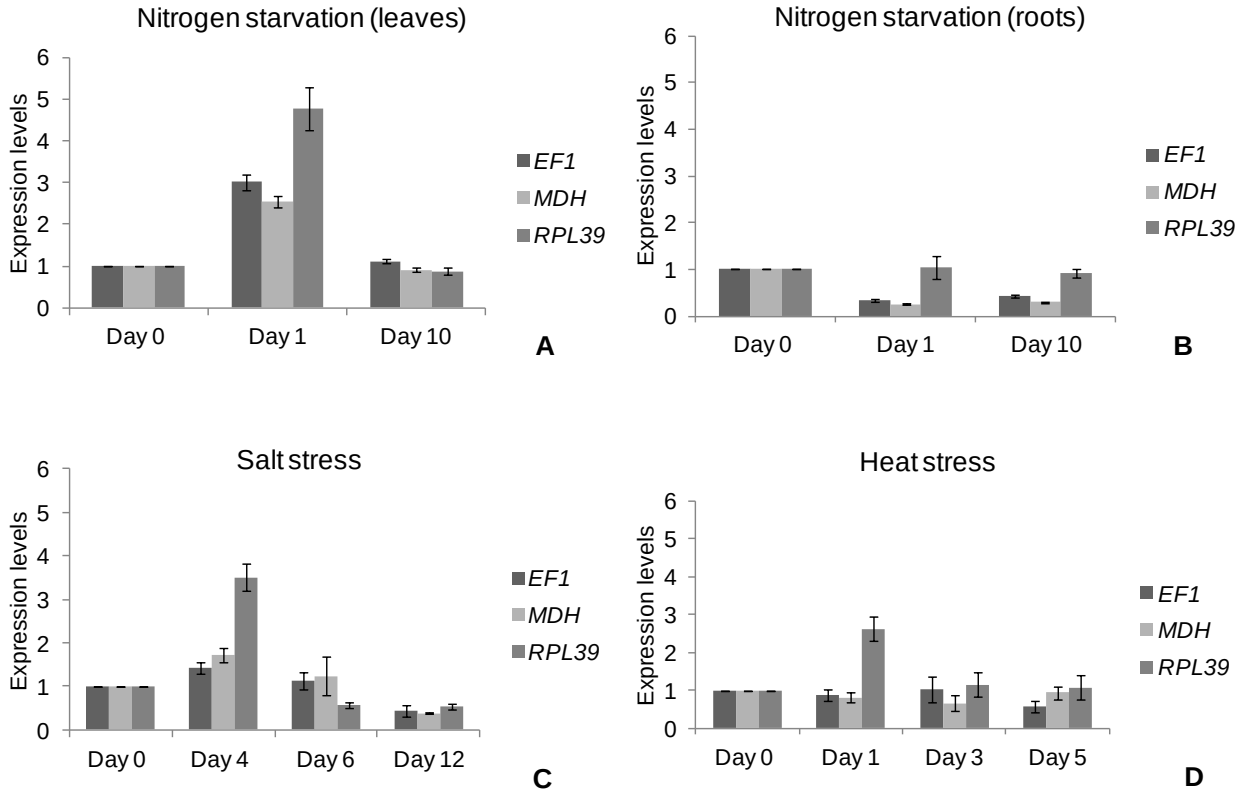


FIGURE 10 - Hemoglobin (*CaHb1*) expression profile during nitrogen starved leaves (A), nitrogen starved roots (B), salt stress (C) and heat stress (D) normalized with two stable reference genes (*EF1* and *MDH*) and the most unstable reference gene (*RPL39*). Values are represented by means ± SE (n = 3)

4.4. DISCUSSION

Coffee (*Coffea ssp*.) is among the main commodities commercialized in the world and it has been target for many studies involving gene expression by qPCR (SALMONA *et al.,* 2008; NOBILE *et al.,* 2010; OLIVEIRA *et al.,* 2010). This technique is widely applied for accurate and sensitive quantification of gene transcript levels,

even for those genes for which transcript levels are low (ZHONG *et al.,* 2011). The accuracy of qPCR is strongly influenced by stability of the internal reference genes used for data normalization. It is advisable to validate the expression stability of candidate reference genes under specific experimental conditions rather than using reference genes published elsewhere aiming to avoid erroneous analysis (LEE *et al.,* 2010). Several studies have shown that the expression of the commonly used reference genes is not always stably expressed when tested in other species or in a wider range of experimental treatments (SILVEIRA *et al.,* 2009; CHEN *et al.,* 2011). A few studies identified candidate reference genes in *C. arabica* for some experimental conditions (CRUZ *et al.,* 2009; BARSALOBRES-CAVALLARI *et al.,* 2009) but, to our knowledge, this work represents the first effort in qPCR studies of experiments involving N starvation, salt and heat stress conditions in *Coffea.*

In order to select suitable reference genes we used geNorm PLUS, BestKeeper and NormFinder algorithms to analyze the expression stability of ten potential reference genes, six of them evaluated for the first time in coffee. Results obtained here indicate that none of the ten genes analyzed had a constant expression level in all conditions, which is common in plant studies (BUSTIN *et al.,* 2009; MONDEGO *et al.,* 2011). This may be partially explained by the fact that reference genes are not only implicated in the basal cell metabolism but also participate in other cellular functions (SINGH; GREEN, 1993). The discrepancy in the expression of the ten potential reference genes in different samples reinforces the importance in selecting an appropriate reference gene to normalize gene expression under specific experimental conditions. Even the most stable reference gene(s) reported in a given species should be validated in a new experimental set-up or when using other genotype/species (CHEN *et al.,* 2011).

4.4.1. REFERENCE GENE STABILITY

In general, the most stable and unstable genes were almost the same for all used programs. *EF1*, *EF1α*, *GAPDH*, *MDH* and *UBQ10* were usually ranked as the most stable genes regardless the software used or the treatment applied, while

RPL39 and *RPII* were usually classified as the most unstable. The differences observed in ranking position are expected because the programs are based on distinct statistical algorithms: geNorm calculates gene expression stability based on its average pairwise expression ratio, whereas Normfinder estimate the overall expression variation of the candidate reference genes to provide a stability value for each gene and BestKeeper considers the least variation of a single reference gene (WAN *et al.,* 2010). According to Andersen *et al.* (2004) coregulated reference genes should be avoided when using multiple references because they may bias the results and geNorm may be insensitive to these cases. Once we observed that *EF1* and *EF1α* were commonly ranked together as the most suitable reference genes, we checked eventual coregulation between these genes removing *EF1* from our analysis, in a strategy analog to Lovdal and Lillo (2009) in tomato. There was not any significant difference in the ranking in the four analysed conditions. In N-starved leaves, no changes in ranking position were observed, while in roots the three most stable reference genes changed only their relative position (*EF1α*, *MDH* and *GAPDH*, in decreasing order from the most stable). In salinity *EF1α* and *S24* changed positions with each other and in heat stress treatment *EF1α* and *MDH* also changed positions. This result shows that the potential coregulation of *EF1* and *EF1α* did not significantly affect the ranking of reference genes.

The best combination of reference genes to reach the appropriate normalization factor in Normfinder is not necessarily the one containing the most stable genes according to a gene-by-gene comparison (CRUZ *et al.,* 2009), which can explain the fact that, in some cases, using the best ranked gene alone is better than the combination of two best genes (stability value is lower).

Although all three softwares produced slightly diverse rankings of stability, the set of most reliable genes was similar, with the main differences being observed in the intermediate ranking positions. This inconsistency among softwares results has already been observed and shows that the choice of the program represents a crucial but difficult task (JIAN *et al.,* 2008; MIGOCKA; PAPIERNIAK, 2011). Despite the differences observed in ranking position from one algorithm to another, geNorm presented values below 1.5 in all of the tested reference genes, showing the potential of the genes to maintain a constancy in their expression profile (VANDESOMPELE *et*

al., 2002). In N starvation and heat stress, we observed high stability (average geNorm 0.2 ≤ M ≤ 0.5), which is typically seen in a homogeneous set of samples, while intermediate reference gene stability (average geNorm M ≤ 1.0) was observed in salt stress. The results through pairwise variation (V values) analysis showed that only salt stress condition required five genes for a better normalization data, whereas only two genes were sufficient for the other three experimental conditions.

The same stability patterns were observed in all analyzed conditions using geNorm PLUS and NormFinder, where salt stress also showed the most inconsistent values with higher variation among samples. BestKeeper results might be considered only for N starvation, where r was over 0.95 and SD was below 1 for most primer pairs. The two most suitable reference genes selected by geNorm PLUS, Normfinder and BestKeeper (only for N stress) are summarized in table 3.

Table 03 - The most suitable reference genes selected by geNorm PLUS, NormFinder and BestKeeper in all analyzed conditions

Conditions	geNorm PLUS	Normfinder	Bestkeeper *
N starvation (leaves)	*MDH*	*EF1*	*EF1*
	GAPDH	*EF1 α*	*EF1 α*
N starvation (roots)	*GAPDH*	*GAPDH*	*GAPDH*
	EF1	*EF1*	*EF1*
Salt stress	*EF1*	*UBQ10*	**
	EF1 α	*EF1*	**
Heat stress	*MDH*	*GAPDH*	**
	EF1 α	*EF1 α*	**

*Primers with r > 0.95 and SD < 1.0

**No primer pair was suitable with r > 0.95 and SD < 1.0

We found that *RPII* was among the least stable reference genes in all conditions used in this work. Similar findings were reported in tomato (EXPOSITO-RODRIGUES *et al.,* 2008), *Arabidopsis* (GUTIERREZ *et al.,* 2008), and cucumber (WAN *et al.,* 2010). However, Zhong *et al.* (2011) and Tong *et al*. (2009) observed that *RPII* showed better expression stability in different tissues in litchi and peach.

TIP41 and *S24* were also poorly ranked, but they were among the best reference genes in studies realized by Cruz *et al.* (2009) and Migocka and Papierniak (2009) in coffee and cucumber, respectively. Considering several plant species, *β-TUB* yielded contrasting result, with high relative expression variability in litchi (ZHONG *et al.,* 2011), *Arabidopsis* (GUTIERREZ *et al.,* 2008), in tomato (LOVDAL, LILO, 2009) and in many other different organisms and experimental contexts (REID *et al.,* 2006; ARTICO *et al.,* 2010). However, in studies with wheat (PAOLACCI *et al.,* 2009) and pea (DIE *et al.,* 2010) *β-TUB* was identified as the most stable reference gene. In our case, the fact that the ranking of this gene varied from intermediate to the least stable reference gene depending to experimental condition indicates that *β-TUB* is not suitable for normalization.

EF1α, *UBQ10* and *GAPDH* are among the most commonly used reference genes. These three genes were also indicated as appropriate reference genes in this study. *EF1α*, often described as a stable gene used as a reference in many species (REID *et al.,* 2006; HONG *et al.,* 2008; WAN *et al.,* 2010; ZHONG *et al.,* 2011), was ranked as a suitable reference genes in N starvation conditions. Similar result was observed by Lovdal and Lillo (2009) in tomato, while in *Arabidopsis EF1α* was never represented among the 100 most stably expressed genes (CZECHOWSKI *et al.,* 2005). Studies carried out in coffee by Cruz *et al.* (2009) considered *GAPDH* and *UBQ10* the most appropriate reference genes. Even though these two genes are constantly described as reliable internal control in several studies (ISKANDAR *et al.,* 2004; EXPOSITO-RODRIGUEZ *et al.,* 2008; ZHONG *et al.,* 2011), including coffee (BARSALOBRES-CAVALLARI *et al.,* 2009), in some conditions their expression profiles can vary (JAIN *et al.,* 2006; DIE *et al.,* 2010). The reasons that may lead to fluctuations in reference gene expression may be found not only in the activation their respective activities but also in their active roles in other processes as well (DIE *et al.,* 2010).

Two potential reference genes (*MDH* and *EF1*) that are not commonly used in expression normalization were classified as highly stable in almost every condition by all methods. *MDH* was better ranked by geNorm PLUS in N starved leaf tissue and heat stressed plants. This reference gene had been only analyzed by Reid *et al.* (2006) in grapevine fruits, where *MDH* did not present a good performance.

This might be attributed to the fact that coffee species exhibit all the physiological and biochemical characteristics of plants with predominantly C3 photosynthetic pathways (CARELLI *et al.,* 2003), where *MDH* transcriptional activity is low and stable (FRESCHI *et al.,* 2010). Our results also showed that some recommended reference genes for *Coffea* expression studies were not suitable in the specific conditions used in this work. For instance, *RPL39* has been extensively used as an internal control when comparing gene expression profiles of target genes among different coffee organs (SIMKIN *et al.,* 2008; PRÉ *et al.,* 2008), in coffee cell cultures (BOTTCHER *et al.,* 2011) and also when comparing control and drought-stressed leaves (SIMKIN *et al.,* 2008). Our work revealed that *RPL39* is not the most accurate reference gene since it was poorly ranked compared to other genes.

4.4.2. REFERENCE GENE VALIDATION

To demonstrate the need for accurate relative quantification using suitable reference genes, the expression of the *C. arabica* Hemoglobin1 (*CaHb1*) gene was studied. *CaHb1* is an ortholog of *Arabidopsis* Class I hemoglobin. Hemoglobins (Hbs) are heme containing proteins found in most organisms related to oxygen sequestration, and they are one of many different strategies that plants have evolved to overcome stress conditions and survive (DORDAS *et al.,* 2004). In *Arabidopsis*, hemoglobin 1 RNA blot analysis demonstrated that this gene is upregulated by cold and drought stress in leaves, but it was not possible to detect transcriptional activity in control and heat-stressed plants (TREVASKIS *et al.,* 1997). In *Alnus firma*, Hb1 is lowly expressed in leaves and is also transcriptionally active under different N sources SASAKURA *et al.,* 2006).

Using the two new recommended reference genes (*EF1* and *MDH*), we obtained similar expression patterns in all experimental conditions. On the other hand, the normalized expression levels illustrate that *CaHb1* is significantly up regulated when using *RPL39* in comparison to *EF1* and *MDH*. Misleading results obtained by the utilization of a non suitable reference gene have been also observed in other studies (ZHONG *et al.,* 2011; LI *et al.,* 2011). Our results reinforces the

importance of validating reference genes to ensure that low precision or misleading results did not occur, especially with genes with low transcript abundance in normal physiological conditions, like hemoglobins.

In conclusion, the use of three different statistical algorithms allowed the identification of different combinations of reference genes for qPCR expression data normalization in *C. arabica* plants subjected to abiotic stresses. The profiling of the gene expression pattern of 10 putative reference genes showed that *MDH*, *GAPDH*, *EF1* and *EF1α* reference genes can be used in N starvation experiments using leaves as the analyzed tissue, while *GAPDH* and *EF1* can be used in N-starved roots. For salt stress treatments *EF1*, *EF1α* and *UBQ10* were selected as the most suitable reference genes and *MDH*, *EF1α* and *GAPDH* are the best choice for heat stress conditions.

To our knowledge, this is the first work regarding the selection of reliable reference genes for transcriptional studies in coffee plants under N starvation, salt and heat stress. Additionally, we recommended two additional potential reference genes that are not commonly used as internal control for normalization of qPCR data (*MDH* and *EF1*).

4.5 REFERENCES

ANDERSEN, C. L.; JENSEN, J. L.; ORNTOFT, T. F. Normalization of real-time quantitative reverse transcription-PCR data: A model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets. **Cancer Research**, v. 64, p. 5245-5250, 2004.

ARTICO, S.; NARDELI, S. M.; BRILHANTE, O. et al. Identification and evaluation of new reference genes in *Gossypium hirsutum* for accurate normalization of real-time quantitative RT-PCR data. **BMC Plant Biology**, v.10, p. 49, 2010.

BARSALOBRES-CAVALLARI, C. F.; SEVERINO, F. E.; MALUF, M. P.; MAIA, I. G. Identification of suitable internal control genes for expression studies in *Coffea*

arabica under different experimental conditions. **BMC Molecular Biology**, v. 10, n. 1, 2009.

BOTTCHER, A.; NOBILE, P. M.; MARTINS, P. F.; CONTE, F. F.; AZEVEDO, R. A.; MAZZAFERA, P. A role for ferritin in the antioxidant system in coffee cell cultures. **Biometals**, v. 24, n. 2, p. 225-37, 2011.

BUSTIN, S. A., BENES, V., GARSON, J. A., et al. (2009) The MIQE guidelines: minimum information for publication of quantitative real time PCR experiments. **Clinical Chemistry**, v. 55, p. 611-622, 2009.

CARELLI, M. L. C.; QUEIROZ-VOLTAN, R. B.; FAHL, J. I.; TRIVELIN, P. C. O. Leaf anatomy and carbon isotope composition in *Coffea* species related to photosynthetic pathway. **Brazilian Journal of Plant Physiology**, v. 15, n. 1, 19-24, 2003.

CARVALHO, K.; CAMPOS, M. K. F.; PEREIRA, L. F. P.; VIEIRA, L. G. E. Reference gene selection for real-time quantitative polymerase chain reaction normalization in ‘‘Swingle” citrumelo under drought stress. **Analytical Biochemistry**, v. 402, p. 197-199, 2010.

CHANG, S.; PURYEAR, J.; CAIRNEY, J. A simple and efficient method for isolating RNA from pine trees. **Plant Molecular Biology Reporter**, v. 11, p. 113-116, 1993.

CHEN, L.; ZHONG, H.; KUANG, J. et al. Validation of reference genes for RT-qPCR studies of gene expression in banana fruit under different experimental conditions. **Planta**, v. 234, p. 377-390, 2011.

CLARK, R. B. Characterization of phosphatase of intact maize roots. **Journal of Agricultural and Food Chemistry**, v. 23, p. 458-460, 1975.

CRUZ, F.; KALAOUN, S.; NOBILE, P. et al. Evaluation of coffee reference genes for relative expression studies by quantitative real-time RT-PCR. **Molecular Breeding**, v. 23, n. 4, p. 607-616, 2009.

CZECHOWSKI, T.; STITT, M.; ALTMANN, T. et al. Genome-wide identification and testing of superior reference genes for transcript normalization in Arabidopsis. **Plant Physiology**, v. 139, n. 1, p. 5-17, 2005.

DIE, J. V.; ROMÁN, B.; NADAL, S.; GONZÁLEZ-VERDEJO, C. I. Evaluation of candidate reference genes for expression studies in *Pisum sativum* under different experimental conditions. **Planta**, v. 232, p. 145-153, 2010.

DORDAS, C.; HASINOFF, B. B.; RIVOAL, J.; HILL, R. D. Class-1 hemoglobins, nitrate and NO levels in anoxic maize cell-suspension cultures. **Planta**, v. 219, p. 66-72, 2004.

DOS SANTOS, T. B.; BUDZINSKI, I. G. F.; MARUR, C. J., et al. Expression of three *galactinol synthase* isoforms in *Coffea arabica* L. and accumulation of raffinose and stachyose in response to abiotic stresses. **Plant Physiology and Biochemistry**, v. 49, p. 441-448, 2011.

EXPOSITO-RODRIGUEZ, M.; BORGES, A. A.; BORGES-PEREZ, A.; PEREZ, J. A. Selection of internal control genes for quantitative real-time RT-PCR studies during tomato development process. **BMC Plant Biology**, v. 8, p. 131, 2008.

FERNANDEZ, P.; RIENZO, J.A.; MOSCHEN, S. et al. Comparison of predictive methods and biological validation for qPCR reference genes in sunflower leaf senescence transcript analysis. **Plant Cell Reports**, v. 30, n. 1, p. 63-74, 2010.

FRESCHI, L.; RODRIGUES, M. A.; DOMINGUES, D. S. et al. Nitric oxide mediates the hormonal control of Crassulacean acid metabolism expression in young pineapple plants. **Plant Physiology**, v. 152, n. 4, p. 1971-85, 2010.

GU, C., CHEN, S., LIU, Z., et al. Reference gene selection for quantitative real-time PCR in Chrysanthemum Subjected to Biotic and Abiotic Stress. **Molecular Biotechnology**, v. 49, p. 192–197, 2011.

GUÉNIN, S.; MAURIAT, M.; PELLOUX, J. et al. Normalization of qRT-PCR data: the necessity of adopting a systematic, experimental conditions-specific, validation of references. **Journal of Experimental Botany**, 60, 487-493, 2009.

GUTIERREZ, L.; MAURIAT, M.; GUÉNIN, S. et al. The lack of a systematic validation of reference genes: a serious pitfall undervalued in reverse transcription polymerase chain reaction (RT-PCR) analysis in plants. **Plant Biotechnology Journal**, v. 6, p. 609-618, 2008.

HONG, S. Y.; SEO, P. J.; YANG, M. S., et al. Exploring valid reference genes for gene expression studies in *Brachypodium distachyon* by real-time PCR. **BMC Plant Biology**, v. 8, p. 112, 2008.

ISKANDAR, H. M.; SIMPSON, R. S.; CASU, R. E. et al. Comparison of reference genes for quantitative real time-PCR analysis of gene expression in sugarcane. **Plant Molecular Biology Reporter**, v. 22, p. 325-337, 2004.

JAIN, M.; NIJHAWAN, A.; TYAGI, A. K.; KHURANA, J. P. Validation of housekeeping genes as internal control for studying gene expression in rice by quantitative real-time PCR**. Biochemical and Biophysical Research Communications**, v. 345, p. 646-651, 2006.

JIAN, B.; LIU, B.; BI, Y. et al. Validation of internal control for gene expression study in soybean by quantitative real-time PCR. **BMC Molecular Biology**, v. 9, p. 59, 2008.

KODAIRA, K. S.; QIN, F.; TRAN, L. S. P. et al. Arabidopsis C2H2 Zinc-Finger Proteins AZF1 and AZF2 Negatively Regulate ABA-Repressive and Auxin-Inducible Genes under Abiotic Stress Conditions. **Plant Physiology**, v. 157, n. 2, 742-56, 2011.

LEE, J. M.; ROCHE, J. R.; DONAGHY, D. J. et al. Validation of reference genes for quantitative RT-PCR studies of gene expression in perennial ryegrass (*Lolium perenne* L.). **BMC Molecular Biology**, v. 11, p. 8, 2010.

LI, H.; QINA, Y.; XIAO, X.; TANGA, C. Screening of valid reference genes for real time RT-PCR data normalization in Hevea brasiliensis and expression validation of a sucrose transporter gene HbSUT3. **Plant Science**, v. 181, p. 132-139, 2011.

LIN, Y. L.; LAI, Z. X. Reference gene selection for qPCR analysis during somatic embryogenesis in longan tree. **Plant Science**, v. 178, p. 359-365, 2010.

LIVAK, J. K.; SCHMITTGEN, T. D. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta Ct}$ method. **Methods**, v. 25, p. 402-408, 2001.

LOVDAL, T.; LILLO, C. Reference gene selection for quantitative real-time PCR normalization in tomato subjected to nitrogen, cold, and light stress. **Analytical Biochemistry**, v. 387, p. 238-242, 2009.

MIGOCKA, M.; PAPIERNIAK, A. Identification of suitable reference genes for studying gene expression in cucumber plants subjected to abiotic stress and growth regulators. **Molecular Breeding**, v. 28*,* p. 343-357, 2011.

MONDEGO, J. M. C.; VIDAL, R. O.; CARAZZOLLE, M. F. et al. An EST-based analysis identifies new genes and reveals distinctive gene expression features of *Coffea arabica* and *Coffea canephora*. **BMC Plant Biology**, v. 11, p. 30, 2011.

NOBILE, P. M.; QUECINI, V.; BAZZO, B. et al. Transcriptional profile of genes involved in the biosynthesis of phytate and ferritin in *Coffea*. **Journal of Agricultural and Food Chemistry**, v. 58, n. 6, p. 3479-3487, 2010.

OLIVEIRA, R. R.; CHALFUN-JUNIOR, A.; PAIVA, L. V.; ANDRADE, A. C. *In silico* and quantitative analyses of MADS-Box genes in *Coffea arabica.* **Plant Molecular Biology Reporter**, v. 28, p. 460-472, 2010.

PAOLACCI, A.; TANZARELLA, O.; PORCEDDU, E.; CIAFFI, M. Identification and validation of reference genes for quantitative RT-PCR normalization in wheat. **BMC Molecular Biology**, v. 10, n. 1, p. 11, 2009.

PFAFFL, M. W.; TICHOPAD, A.; PRGOMET, C.; NEUVIANS, T. P. Determination of stable housekeeping genes, differentially regulated target genes, and sample integrity: BestKeeper–Excel-based tool using pair-wise correlations. **Biotechnology Letters**, v. 26, p. 509-515, 2004.

PRÉ, M.; CAILLET, V.; SOBILO, J.; MCCARTHY, J. Characterization and expression analysis of genes directing galactomannan synthesis in coffee. **Annals of Botany**, v. 102, p. 207-220, 2008.

PRIVAT, I.; BARDI, A.; GOMEZ, A. B.; SEVERAC, D.; DANTEC, C.; FUENTES, I.; MUELLER, L. et al. The 'PUCE CAFE' Project: the First 15K Coffee Microarray, a New Tool for Discovering Candidate Genes correlated to Agronomic and Quality Traits. **BMC Genomics**, v.12, p. 5, 2011.

REID, K. E,; OLSSON, N.; SCHLOSSER, J. et al. An optimized grapevine RNA isolation procedure and statistical determination of reference genes for real-time RT-PCR during berry development. **BMC Plant Biology**, v. 6, p. 27-37, 2006.

ROY, S. J.; TUCKER, E. J.; TESTER, M. Genetic analysis of abiotic stress tolerance in crops. **Current Opinion in Plant Biology**, v. 14, p. 232-239, 2011.

SALMONA, J.; DUSSERT, S.; DESCROIX, F., et al. Deciphering transcriptional networks that govern *Coffea arabica* seed development using combined cDNA array and real-time RT-PCR approaches. **Plant Molecular Biology**, v. 66, p. 105-124, 2008.

SASAKURA, F.; UCHIUMI, T.; SHIMODA, Y., et al. A class 1 hemoglobin gene from *Alnus firma* functions in symbiotic and non-symbiotic tissues to detoxify nitric oxide. **Molecular Plant-Microbe Interactions**, v. 19, p. 441-450, 2006.

SILVEIRA, E. D.; ALVES-FERREIRA, M.; GUIMARAES, L. A. et al. Selection of reference genes for quantitative realtime PCR expression studies in the apomictic and sexual grass *Brachiaria brizantha*. **BMC Plant Biology**, v. 9, p. 84, 2009.

SIMKIN, A. J.; MOREAU, H.; KUNTZ, M. et al. An investigation of carotenoid biosynthesis in *Coffea canephora* and *Coffea arabica.* **Journal of Plant Physiology**, v. 165, p. 1087-1106, 2008.

SIMKIN, A. J.; QIAN, T. Z.; CAILLET, V.; MICHOUX, F.; BEN AMOR, M.; LIN, C.W.; TANKSLEY, S.; MCCARTHY, J. Oleosin gene family of *Coffea canephora*:

quantitative expression analysis of five oleosin genes in developing and germinating coffee grain. **Journal of Plant Physiology**, v. 163, p. 691–708, 2006.

SINGH, R.; GREEN, M. R. Sequence-specific binding of transfer RNA by glyceraldehyde-3-phosphate dehydrogenase. **Science**, 259(5093), 365-368, 1993.

TESTER, M.; LANGRIDGE, P. Breeding technologies to increase crop production in a changing world. **Science**, v. 327, p. 818-822, 2010.

TILLETT, R. L.; ERGÜ, A.; ALBION, R. L. et al. Identification of tissue-specific, abiotic stress responsive gene expression patterns in wine grape (*Vitis vinifera* L.) based on curation and mining of large-scale EST data sets. **BMC Plant Biology**, v. 11, p. 86, 2011.

TONG, Z.; GAO, Z.; WANG, F. et al. Selection of reliable reference genes for gene expression studies in peach using real-time PCR. **BMC Molecular Biology**, v. 10, p. 71, 2009.

TREVASKIS, B.; WATTS, R. A; ANDERSSON, C. R. et al. Two hemoglobin genes in *Arabidopsis thaliana*: the evolutionary origins of leghemoglobins. **PNAS**, v. 94, p. 12230-12234, 1997.

VANDESOMPELE, J.; DE PRETER, K.; PATTYN, F. et al. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. **Genome Biology**, v. 3, p. 34, 2002.

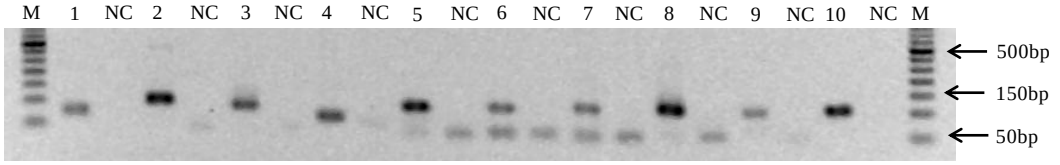
WAN, H.; ZHAO, Z.; QIAN, C. et al. Selection of appropriate reference genes for gene expression studies by quantitative real-time polymerase chain reaction in cucumber. **Analytical Biochemistry**, v. 399, p. 257-261, 2010.

WATSON, K.; ACHINELLI, M. L. Context and contingency: the coffee crisis for conventional small-scale coffee farmers in Brazil. **The Geographical Journal**, v. 174, n. 3, p. 223-234, 2008.

ZHONG, H. Y.; CHEN, J. W.; LI, C.Q. et al. Selection of reliable reference genes for expression studies by reverse transcription quantitative real-time PCR in litchi under different experimental conditions. **Plant Cell Reports**, v. 30, p. 641-653, 2011.

SUPPLEMENTARY MATERIAL I

Supplementary material 1 Specificity and amplicon length of all primer pairs



Supplementary material 1 1- *RPL39*, 2- *UBQ10*, 3- *S24*, 4-*GAPDH*, 5- *βTUB*, 6- *TIP41*, 7- *EF1α*, 8- *EF1*, 9- *RPII*, 10- *MDH*. M- molecular marker (Invitrogen – 50bp), NC- Negative control.

Supplementary material 2

EXPERIMENTAL DESIGN
Definition of experimental and control groups
Number within each group
Assay carried out by core lab or investigator's lab?
Acknowledgement of authors' contributions
SAMPLE
Description
Volume/mass of sample processed
Microdissection or macrodissection
Processing procedure
If frozen - how and how quickly?
If fixed - with what, how quickly?
Sample storage conditions and duration (especially for FFPE samples)

Experimental design is provided in the material and methods section. Tissues, also described in material and methods section, were harvested in the greenhouse and immediately frozen in liquid nitrogen. Tissues were stored in -80°C freezer until RNA extraction would be performed. Assays (qPCR experiment) were carried out by the main author in Plant Biotechnology Laboratory of Instituto Agronômico do Paraná.

NUCLEIC ACID EXTRACTION
Procedure and/or instrumentation
Name of kit and details of any modifications
Source of additional reagents used
Details of DNase or RNAse treatment
Contamination assessment (DNA or RNA)
Nucleic acid quantification
Instrument and method
Purity (A_{260}/A_{280})
Yield
RNA integrity method/instrument
RIN/RQI or Cq of 3' and 5' transcripts
Electrophoresis traces
Inhibition testing (Cq dilutions, spike or other)

Tissues were ground into a powder in liquid nitrogen and total RNA was extracted as described by Chang and coworkers [20], then treated with and treated with DNase (RNase-free – Invitrogen) following the manufacturer's instructions. RNA samples were purified using the Pure Link Micro to Midi Total RNA Purification System (Invitrogen). All RNA samples were analyzed by formaldehyde agarose gel electrophoresis to assess their integrity. To test for contamination by polyphenols, carbohydrates and proteins, a a NanoDrop® ND-100 spectrophotometer was used. Only RNA samples with purity (ratio A_{260}/A_{280}) ranging from 1.7 to 2.1 were used for further analysis. Exact values for each RNA can be provided upon request. The average yield of RNA extractions were 25.5µg.g^{-1} of fresh tissue (ft) for salt stressed leaves, 10.5µg.g^{-1} ft for heat stressed leaves, 27.8µg.g^{-1}ft for nitrogen-starved leaves and 13.5µg.g^{-1} ft for nitrogen-starved lateral roots. As expected, RNA yield of lateral roots was lower than the obtained for leaves. Among leaves, the heat stress resulted in the lowest yield.

REVERSE TRANSCRIPTION
Complete reaction conditions
Amount of RNA and reaction volume
Priming oligonucleotide (if using GSP) and concentration
Reverse transcriptase and concentration
Temperature and time
Manufacturer of reagents and catalogue numbers
Cqs with and without RT
Storage conditions of cDNA

Complementary DNA (cDNA) was synthesized by a SuperScript III Reverse Transcriptase (Invitrogen) following the manufacturer's instructions. 5µg of RNA, 1µL of oligo (dT)20 (50µM) and 1µl of dNTP mix (10µM) were mixed, incubated at 65°C for 5min, and quickly chilled on ice. 4µL of 5x Buffer, 1µL of DTT 100mM, 1µL of RNAse OUT (40U/uL) and 1µL of SuperScript III RTwere added. The reaction mix was incubated at 50°C for 60min, and then at 70°C for 15min. cDNA was stored at -20°C.

qPCR TARGET INFORMATION
If multiplex, efficiency and LOD of each assay.
Sequence accession number
Location of amplicon
Amplicon length
In silico specificity screen (BLAST, etc) **Pseudogenes, retropseudogenes or other homologs?** **Sequence alignment**
Secondary structure analysis of amplicon
Location of each primer by exon or intron (if applicable)
What splice variants are targeted?

Multiplex qPCR was not performed. Information about genes, primers and amplicons, including NCBI accession number, Arabidopsis ortholog and primer efficiency, can be found in Table 1 or in the material and methods section.

qPCR OLIGONUCLEOTIDES
Primer sequences
RTPrimerDB Identification Number
Probe sequences
Location and identity of any modifications
Manufacturer of oligonucleotides
Purification method

Primer sequences are included in Table 1, including those extracted from literature as mentioned in the material and methods section. This section also describes primers concentration used in the qPCR reactions.

qPCR PROTOCOL
Complete reaction conditions
Reaction volume and amount of cDNA/DNA
Primer, (probe), Mg^{++} and dNTP concentrations
Polymerase identity and concentration
Buffer/kit identity and manufacturer
Exact chemical constitution of the buffer
Additives (SYBR Green I, DMSO, etc.)
Manufacturer of plates/tubes and catalog number
Complete thermocycling parameters
Reaction setup (manual/robotic)
Manufacturer of qPCR instrument

The PCR reactions were performed using a reaction mixture containing 12.5 µl of 2x SYBR Green master mix, 1µl of each primer (10 µM), 1 µl of cDNA 1:10 diluted and Milli-Q water to a total volume of 25 µl. Thermal conditions were 95 °C for 10 min, followed by 40 cycles of 95 °C for 30s and 60 °C for 60s. Melting curves were analyzed to verify the presence of a single product including a negative control. All reactions were performed in triplicate for each of the three biological replicates in all treatments studied.

qPCR VALIDATION
Evidence of optimisation (from gradients)
Specificity (gel, sequence, melt, or digest)
For SYBR Green I, Cq of the NTC
Standard curves with slope and y-intercept
PCR efficiency calculated from slope
r2 of standard curve
Linear dynamic range
Cq variation at lower limit
Evidence for limit of detection
If multiplex, efficiency and LOD of each assay.

The specificity of the amplification products have been confirmed by analyzing their melting curves, and also by size estimations on a 1% agarose gel.

DATA ANALYSIS
qPCR analysis program (source, version)
Cq method determination
Outlier identification and disposition
Results of NTCs
Justification of number and choice of reference genes
Description of normalisation method

Number and concordance of biological replicates
Number and stage (RT or qPCR) of technical replicates
Repeatability (intra-assay variation)
Reproducibility (inter-assay variation, %CV)
Power analysis
Statistical methods for result significance
Software (source, version)
Cq or raw data submission using RDML

qPCR analysis program (source, version): Applied Biosystems
No data have been exclude from the calculations
Results of NTCs: no amplification products present thus no Cqs
Justification of number and choice of reference genes: genes have been selected based on the literature, some commonly used and some do not.
Three algorithms were used to evaluate stability
Number and concordance of biological replicates: 3
Number and stage (RT or qPCR) of technical replicates: 3 at qPCR level, 1 for RT
Repeatability (intra-assay variation): was below one Cq

Supplementary material 3 BestKeeper results for ten selected reference genes in four experimental conditions

N starvation (leaves)	(A)									
Factor	Reference genes									
	RPL39	*UBQ10*	*S24*	*GAPDH*	*β-TUB*	*TIP41*	*EF1 α*	*EF1*	*RPII*	*MDH*
n	9	9	9	9	9	9	9	9	9	9
GM[C_q]	21.3459	23.6106	23.0523	21.7178	27.4529	28.5414	21.8501	22.5311	29.4464	23.6049
AM[C_q]	21.3531	23.6459	23.0628	21.7423	27.4753	28.5708	21.8767	22.5546	29.4768	23.6286
Min[C_q]	20.5106	21.5808	22.1373	20.2029	25.8047	26.6436	20.2764	21.0379	27.4310	22.0566
Max[C_q]	22.2363	25.6341	24.1210	23.4790	28.9445	30.2568	23.3709	24.0974	31.3814	25.3631
SD[±C_q]	0.46	1.08	0.62	0.87	0.98	1.16	0.96	0.91	1.13	0.89
CV [%C_q]	2.17	4.55	2.69	3.98	3.57	4.04	4.40	4.05	3.84	3.78
r	0.987	0.993	0.97	0.991	0.991	0.995	0.996	0.998	0.98	0.995
Ranking stability	8	5	10	7	6	4	**2**	**1**	9	3
N starvation (roots)	(B)									
Factor	Reference genes									
	RPL39	*UBQ10*	*S24*	*GAPDH*	*β-TUB*	*TIP41*	*EF1 α*	*EF1*	*RPII*	*MDH*
n	9	9	9	9	9	9	9	9	9	9
GM[C_q]	21.8012	24.1104	23.7078	22.7273	26.5095	28.2300	22.1820	23.8886	29.0459	24.4392
AM[C_q]	21.8130	24.1644	23.7307	22.7604	26.5354	28.2405	22.2261	23.9202	29.0646	24.4831
Min[C_q]	20.1266	21.1949	21.7868	20.6692	24.1833	27.1532	19.7514	21.8550	26.8571	22.0965
Max[C_q]	22.9793	26.5612	25.9153	25.2904	28.5756	29.6843	25.2676	26.4936	30.7034	27.6674
SD[±C_q]	0.45	1.41	0.70	0.96	0.90	0.64	0.99	0.98	0.78	1.13
CV [%C_q]	2.08	5.82	2.96	4.23	3.40	2.25	4.45	4.10	2.68	4.62
r	0.942	0.957	0.980	0.996	0.989	0.964	0.990	0.994	0.967	0.991
Ranking stability	10	9	6	**1**	5	8	4	**2**	7	3
Salt stress	(C)									
Factor	Reference genes									
	RPL39	*UBQ10*	*S24*	*GAPDH*	*β-TUB*	*TIP41*	*EF1 α*	*EF1*	*RPII*	*MDH*
n	12	12	12	12	12	12	12	12	12	12
GM[C_q]	22.2255	23.5514	23.5912	22.4979	26.9271	29.1000	22.6972	22.4235	27.8822	24.8981
AM[C_q]	22.2476	23.6558	23.6416	22.5503	27.0919	29.1696	22.7876	22.5020	28.0503	24.9908
Min[C_q]	20.9209	20.0681	21.5764	19.6882	21.4213	26.2292	19.6884	20.0823	22.1385	22.3236
Max[C_q]	24.1978	27.8791	27.6338	25.4228	31.1571	33.6653	27.6587	27.0981	32.7067	30.6584
SD[±C_q]	0.86	1.84	1.12	1.25	2.34	1.49	1.57	1.43	2.47	1.68
CV [%C_q]	3.86	7.76	4.74	5.54	8.62	5.10	6.90	6.35	8.80	6.72
r	0.857	0.990	0.954	0.935	0.838	0.890	0.970	0.957	0.916	0.944
Ranking stability	9	**1**	4	6	10	8	**2**	3	7	5
Heat stress	(D)									
Factor	Reference genes									
	RPL39	*UBQ10*	*S24*	*GAPDH*	*β-TUB*	*TIP41*	*EF1 α*	*EF1*	*RPII*	*MDH*
n	12	12	12	12	12	12	12	12	12	12
GM[C_q]	23.7830	28.1266	25.4203	25.5486	31.5662	31.6883	25.9618	25.6368	33.0094	27.7614
AM[C_q]	23.8121	28.1921	25.4720	25.6051	31.6166	31.9981	26.0317	25.7005	33.0722	27.8263
Min[C_q]	21.4575	24.3891	22.2372	22.5066	28.4105	19.7738	22.3744	21.8284	29.7962	23.9657
Max[C_q]	25.9175	30.7286	28.2900	27.9905	34.0550	35.5194	28.5767	28.0110	36.0459	30.4181
SD[±C_q]	0.90	1.53	1.27	1.37	1.56	2.46	1.53	1.43	1.79	1.50
CV [%C_q]	3.77	5.42	5.00	5.35	4.94	7.70	5.88	5.57	5.41	5.40
r	0.954	0.985	0.963	0.985	0.708	0.864	0.985	0.984	0.92	0.993
Ranking stability	7	3	6	**2**	10	9	4	5	8	**1**

Note: n, number of coffee samples; GM[C_q], geometric mean of C_q value; SD[±C_q]:, standard deviation of C_q value; CV[%C_q], coefficient of variance expressed as percentage of C_q value; r, coefficient of correlation

5 ARTIGO

EXPRESSÃO DE GENES ENVOLVIDOS NA BIOSÍNTESE DE MANITOL EM *Coffea arabica* SUBMETIDO A ESTRESSES ABIÓTICOS E CONTRIBUIÇÃO DE HOMEÓLOGOS DE *Coffea canephora*.

***Planta**

‘Expressão de genes envolvidos na biosíntese de manitol em *Coffea arabica* submetido a estresses abióticos e contribuição de homeólogos de *Coffea canephora*.’

RESUMO

O objetivo deste estudo foi investigar a influência do estresse hídrico, salinidade e alta temperatura na concentração do manitol e seu precursor, frutose, assim como analisar as respostas transcricionais dos genes envolvidos na biossíntese e catabolismo de manitol em folhas de *C. arabica*. Esta é uma espécie alotetraplóide originada a partir da hibridização natural entre *C. canephora* e *C. eugenioides* e responsável por três quartos da produção mundial de café. Estresses abióticos, como altas temperaturas, salinidade e seca representam a principal causa de perda de produtividade mundial. O acúmulo de solutos compatíveis, como o manitol, em resposta a estes estresses podem proteger a planta em diversas espécies. Nosso estudo demonstrou que a concentração de manitol aumentou durante o estresse hídrico e salinidade, mas foi reduzida com estresse térmico. Os níveis de frutose seguiram o de manitol somente em condição de alta temperatura. Os níveis transcricionais de enzimas chave envolvendo a síntese de manitol, *CaM6PR, CaPMI* e *CaMTD* foram modulados de maneira distinta dependendo do estresse aplicado. Além disso, o subgenoma de *Coffea canephora* não seguiu o mesmo padrão transcricional entre os genes, sugerindo regulação diferencial entre homeólogos. Os resultados também demonstraram que o acúmulo de manitol durante seca e alta salinidade é devido, pelo menos em parte, ao aumento da expressão gênica dos genes chave envolvidos na biossíntese de manitol. Desta forma, este estudo adiciona novas informações sobre a ação do álcool-açúcar manitol durante a tolerância a estresse abióticos e expressão diferencial dos genes homeólogos de *C. arabica* sob condições ambientais adversas..

Palavras-chave: manitol, fosfomanose-isomerase, manose-6-fosfato redutase, manitol desidrogenase, café, déficit hídrico, estresse salino, alta temperatura, genes homeólogos

‘Expression of genes involved in manitol biosynthesis in *Coffea arabica* submitted to abiotic stress and contribution of *Coffea canephora* homeologs.’

ABSTRACT

The aim of this study was to investigate the influence of drought, salinity and high temperature in the concentration of mannitol and its precursor, fructose, as well as to investigate transcriptional responses of genes involved in mannitol biosynthesis and catabolism in *Coffea arabica* leaves. *C. arabica* is an allotetraploid originated from a natural hybridization between *C. canephora* and *C. eugenioides* and it is responsible to three quarters of the world coffee production. Abiotic stresses, like extreme temperatures, salinity and drought represent the principal cause of crop losses worldwide. The accumulation of compatible solutes, such as mannitol, in response to this stresses may protect plants in several species. Our study revealed that mannitol concentration is increased under drought and salinity, but reduced in heat stress. Fructose levels follow mannitol only in heat stress, suggesting the partion of this sugar in other metabolites during drought and salt stress. Transcripts of key enzymes involved in mannitol biosynthesis, *CaM6PR*, *CaPMI* and *CaMTD* were modulated in distinct ways depending on the abiotic stress. Besides, *Coffea canephora* subgenome expression do not follow the same pattern of overall gene transcriptional response, suggesting differential regulation among homeologs. The resultas showed that mannitol accumulation during drought and salt stress is due, at least in part, to an increase in gene expression of the key genes involved in mannitol biosynthesis. Thus, this study adds new information concerning the sugar alcohol mannitol during abiotic stress tolerance and the differential expression of *C. arabica* homeologous genes under adverse environmental condition.

Keywords: mannitol, phosphomannose-isomerase, mannose-6-phosphate reductase, mannitol dehydrogenase, coffee, water deficit, salt stress, high temperature, homeologous genes.

5.1 INTRODUCTION

Coffee is one of the most valuable agricultural international commodities and its cultivation, processing, transportation and marketing provide employment for millions of people worldwide. Coffee exports in 2009/10 were estimated in US$ 15.4 billion, when 93.4 million bags were traded (www.ico.org). Brazil is the largest producer and exporter contributing with a significant portion of these values.

A FAO report stated that only 3.5% of the global land area is not affected by some environmental constraint (CRAMER *et al.*, 2011). Environmental factors exert influence on nearly every aspect of plant function throughout its life cycle. In response to changing and often unfavorable conditions, a complex and overlapping network of molecular machinery must regulate plant responses to these conditions. A stress perception in plants initiates a signal transduction events that lead to expression of specific genes and generation of “protecting” metabolites (BARKLA, PANTOJA 2011). Stressful abiotic environmental conditions such as drought, salinity and high temperatures are among the major stresses that adversely affect plant growth and productivity, including coffee plants.

Drought, salinity and extreme temperatures are often interconnected, and may induce similar cellular damage affecting coffee plant development and production. For instance, drought and/or salinization are manifested primarily as osmotic stress, resulting in the homeostasis disruption, ion distribution in the cell (SERRANO *et al*., 1999; ZHU, 2001) and impairment of photosynthetic capacity (BRINI *et al*., 2007). The photosynthetic activity of chloroplasts is considered among the most heat sensitive cell function, where the primary targets of thermal damage in plants are the oxygen evolving complex along with the associated cofactors in photosystem II (PSII) (ALLAKHVERDIEV *et al*., 2008).

Plants can overcome abiotic stresses through intracellular accumulation of organic compatible solutes that acts as osmoprotectants, such as polyols, proline and quaternary ammonium compounds (RATHINASABAPATHI, 2000). Polyols are the reduced form of aldoses and ketoses, and can be found in all living forms (NOIRAUD *et al*., 2001). Mannitol is the most common polyol in nature and has been observed in more than 100 vascular plant species of several families including the Rubiaceae (ROGERS *et al*., 1999). In plants, mannitol is synthesized through

fructose-6-phosphate isomerization by the action of the phosphomannose-isomerase enzyme (PMI), forming mannose-6-phosphate that, through the activity of the key enzyme NADPH-dependent mannose-6-phosphate reductase (M6PR), generates mannitol-1-phosphate. This sugar alcohol can be translocated through the phloem to heterotrophic sink tissues, where it may be either stored or oxidized to mannose through an NAD^+-dependent mannitol dehydrogenase (MTD) and used as a carbon and energy source (CONDE *et al*., 2011).

Coffea arabica is an allotetraploid (2n = 4x = 44) derived from a recent interespecific hybridization (approximately one million years) between two diploid species: *C. eugenioides* and *C. canephora* (VIDAL *et al.*, 2010; Yu *et al.*, 2011). Several allopolyploid species has been used to investigate variations in gene expression by comparing the ratio of transcripts derived from each homeolog; however, few studies focus on different types of stress (LIU, ADAMS 2007; STAMATI *et al*., 2009; DONG, ADAMS, 2011). The homeologous expression can change in response to abiotic stresses and no single unifying factor has been reported, suggesting that each allopolyploid plant has its own regulatory mechanism (CHAUDHARY *et al.,* 2009). In *C. arabica*, previous studies confirmed the existence of genes with homeologous differential expression (VIDAL *et al*., 2010; MARRACCINI *et al*., 2011), but this characteristic is not a general feature to all *C. arabica* genes (VIDAL *et al.* 2010; COMBES *et al*., 2012).

The transcriptome of *C. arabica* is a mixture of homeologous genes expressed from two sub-genomes and thus, is important to identify the colaboration of each homeolog in different abiotic stress conditions in order to add new information concerning coffee evolution and stress tolerance (LIMA *et al*., 2002; BARDIL *et al*., 2011). Breeders usually atribute abiotic and biotic stress tolerance to the “expression” of *C. canephora* traits contained in *C. arabica*. Moreover, the transcriptional contribution of each homeolog may be affected by abiotic stress, as previously observed for RBCS in *C. arabica* (MARRACCINI *et al*., 2011). Considering that mannitol is directly involved in photosynthesis for being a photosynthetic product (CHAN *et al*., 2011) and that drought, salinity and extreme temperatures damages the photosynthetic apparatus, the elucidation of the role of mannitol in protecting against abiotic stress might be important for the enhancement of yield of coffee plants and other species.

Here we investigate the role of mannitol in *C. arabica* leaves submitted to drought, salinity and high temperature using three strategies: measuring fructose and mannitol levels during these stresses, analyzing the transcriptional pattern of the key genes involved in mannitol metabolism (*CaM6PR*, *CaPMI* and *CaMTD*), and the contribution of *C. canephora* homeolog transcripts in each abiotic stress. This analysis helps to elucidate the role of mannitol in plants and stress tolerance mechanism in coffee plants.

5.2 MATERIAL AND METHODS

5.2.1 IDENTIFICATION OF CANDIDATE GENES INVOLVED IN MANNITOL BIOSYNTHESIS

From the database of the Brazilian Coffee Genome Project (http://bioinfo04.ibi.unicamp.br/coffea/; VIEIRA *et al*., 2006; MONDEGO *et al*., 2011) we performed a gene search for *M6PR* using the keyword 'NADPH dependent mannose 6 phosphate reductase' (M6PR). All sequences obtained for *M6PR,* designated here as *CaM6PR*, were grouped by similarity in order to form contigs using CLC (Main Workbench 4.1.1) software. Each contig was analyzed and compared with individual protein sequences deposited in the NCBI database (National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/). The sequences were individually analyzed in the database of the Brazilian Coffee Genome Project Consortium to verify similarity, and identity.

For *PMI*, named here as *CaPMI,* we used the sequence from the *Arabidopsis thaliana* (At1g67070) ortholog obtained through TAIR database (http://www.arabidopsis.org/) described by MARUTA *et al*. (2008) as a starting point. In the case of *MTD* (*CaMTD*), we used the sequence of *Apium graveolens* ortholog, previously mentioned by MUSTORP *et al*. (2008) (GenBank Acession AF067082.1). These protein sequences of *PMI* and *MTD* were used as queries in a TBLASTN against *Coffea* spp ESTs from dbEST (NCBI) database. These sequences were also used as queries in the Brazilian Coffee Genome Project (VIEIRA *et al*., 2006; MONDEGO *et al*., 2011) database.

5.2.2 PHYLOGENETIC ANALYSIS

For phylogenetic analyses, sequences for *M6PR*, *PMI* and *MTD* were obtained from several complete plant genomes in Phytozome database (http://www.phytozome.net/) and used as queries for a TBLASTN search.

For *PMI* and *MTD* phylogenetic analysis, we also used sequences from Maruta *et al.* (2008), and Barakat *et al.* (2009) and Conde *et al.* (2011), respectively.

All protein sequences selected were aligned using MUSCLE (Edgar, 2004), and a phylogenetic tree was constructed with the aligned protein sequences by the neighbor-joining method. In order to test the analysis reliability, bootstrap analysis was carried out (500 trials) to assess the support for individual nodes. All analyses were developed in MEGA version 5 (TAMURA *et al.,* 2011). In the case of *MTD*, subclasses were classified according to Barakat *et al.* (2009).

5.2.3 PLANT MATERIAL AND ABIOTIC STRESS TREATMENTS

For all experiments, we used used leaves of 18-month old plants of *C. arabica* cv IAPAR 59 with uniform growth. Basic procedures of abiotic stress treatments followed Dos Santos *et al.* (2011) and Carvalho *et al.* (2013a). In all experiments we used the same pair of leaves (second plagiotropic pair) and sampling consisted of three pools of leaves with each pool containing two pairs of leaves from three different plants. Each pool represented a biological repetition. The leaves were immediately immersed in liquid N_2 and stored at -80°C until the assays were performed.

For drought stress treatment, in order to monitor plant water status, leaf discs of approximately 2 cm^2 were collected and placed in thermocouple psychrometer chambers (model C-30, Inc., Wescor) assembled with a datalogger (model CR-7, Campbell Scientific, Inc.) for determination of leaf water potential (Ψ_w). The treatments were established as: irrigated (IR; Ψ_w = -1.34 MPa), moderate stress (MS; Ψ_w = -2.4), severe stress (SE; Ψ_w = -4.5 MPa) and recovery (REC, 72 h after re-irrigation).

The salt stress experiment was carried out in the same conditions used for the drought stress experiment. In order to avoid osmotic shock, plants were irrigated

with 50 mM NaCl in the first day and then with 100 mM NaCl on the second day of the experiment. From the third day up to the end of the experiment, the plants were daily irrigated with 150 mM NaCl. Leaves were harvested as following: day 0 (control without addition of NaCl), day 4, 6 and 12 after adding 150 mM NaCl.

In the heat stress experiment, the coffee plants were maintained in a growth chamber for 7 days at 24°C and controlled photoperiod (12h:12h) for acclimatization. In sequence, the chamber temperature was raised to 37°C for five days. To avoid water deficit, plants were irrigated daily during all the heat stress period. Samples were collected at four evaluation stages: non-stressed control (plants maintained 7 days in growth chamber at 24°C), day 1, 3 and 5 (at 37°C).

5.2.4 QUANTIFICATION OF FRUCTOSE, MANNOSE AND MANNITOL

Leaves collected from coffee plants under stress treatments were lyophilized and submitted to an extraction process for obtaining low molecular weight oligosaccharides according to Albini *et al*. (1994). Fructose, mannose and mannitol were determined by high performance liquid chromatography (HPLC) using a Shimadzu system (Japan) equipped with CBM-10A interface module, CTO-10A column oven, LC-10AD pump and with a RID-10A refractive index detector. A Supelcogel Ca column (Supelco _ USA), 30 cm x 7.8 mm and Supelcogel Ca pre-column, 5 cm x 4.6 mm were used. The HPLC-column was eluted with water at a flow rate of 0.5 mL min^{-1} at 80^0C.

Differences in carbohydrates concentration inside each abiotic stress were analyzed by one-way ANOVA, taking $P < 0.05$ as significant according to Student t test.

5.2.5 RNA ISOLATION, PURIFICATION AND cDNA SYNTHESIS

Total RNA were isolated from leaves in a protocol modified from Chang *et al.* (1993). RNA samples were purified using the Pure Link Micro to Midi Total RNA Purification System (Invitrogen), their integrity was examined by 1% agarose gel electrophoresis and treated with DNase (RNase-free, Invitrogen). RNA concentration and purity were determined using a NanoDrop® ND-100 spectrophotometer (Thermo

Scientific) and the absence of genomic DNA contamination was confirmed by PCR using *GAPDH* primers (data not shown). Complementary DNA (cDNA) was synthesized by a SuperScript III Reverse Transcriptase (Invitrogen) following the manufacturer's instructions using 5 µg of total RNA in a final volume of 20 µl. The final cDNA products were diluted 10-fold prior to use in qPCR.

5.2.6 PRIMER DESIGN AND AMPLIFICATION EFFICIENCY

The reads for *C. arabica* of each gene were obtained for primers design. Then, the amplicons were analyzed through BLAST to verify specificity. Primers were designed using Primer Express v. 3.0 (Applied Biosystems) in order to obtain amplicons around 100 base pairs with a Tm of 60°C ± 2°C. Primers for total gene expression were named here as *CaM6PR*, *CaPMI*, *CaMTD* and primers *C. canephora* subgenome specific were named as $CaM6PR_{Cc}$, *CaPMICc* and $CaMTD_{Cc}$ (Supplementary material 4). The three allelic specific primer pairs were designed to amplify sequences in the same gene region of the primers used to assess total gene expression.

The efficiency of each primer pair was calculated using a standard curve generated from a serial dilution of cDNA using $E = [10^{(-1/slope)} - 1]$. The cDNA used to this analysis was from leaves of non-treated plants. Four serial dilutions were set up to determine quantification cycle (C_q) and reaction efficiencies for all primers pairs. Standard curves were generated for each primer pair using the Cq value versus the logarithm of each cDNA dilution factor.

For qPCR data normalization we used reference genes according to Carvalho *et al.* (2013a).

5.2.7 *Coffea canephora* HOMEOLOG IDENTIFICATION

In order to study the contribution of *C. canephora* homeolog expression (within *C. arabica*), we used the same rational of Vidal et al., (2010) and Marraccini et al. (2011), based in Taq-MAMA method (LI *et al*., 2004). This approach relies on differential amplification based on Single Nucleotide Polymorphisms detected between *C. arabica* and *C. canephora* ESTs. To this purpose, the sequences for

M6PR, *PMI* and *MTD* obtained from the database of the Brazilian Coffee Genome Project (mentioned above) were used to obtain *C. arabica* and *C. canephora* contigs. These contigs were submitted to Blast2sequences in the NCBI database to detect SNPs.

5.2.8 qPCR AND DATA ANALYSIS

The transcripts levels for each gene were detected by qPCR (7500 Fast Real-Time PCR System, Applied Biosystems) using SYBR Green PCR Master Mix (Applied Biosystems). The reaction mixture contained 12.5 µl of 2x SYBR Green master mix, 1 µl of each primer (10 µM), 1 µl of cDNA 1:10 diluted and Milli-Q water to a total volume of 25 µl. Thermal conditions were 95°C for 10 min, followed by 40 cycles of 95°C for 30 s and 60°C for 60 s. Melting curves were analyzed to verify the presence of a single product including a negative control. All reactions were performed in triplicate for each of the three biological replicates in all treatments studied. Fragments obtained in qPCR were sequenced in order to confirm their identity with the target genes.

Relative expression levels of the genes were analyzed by GenEX software (MultiD Analyses AB, Göteborg, Sweden) with normalization perfomed using two reference genes specific to each abiotic stress according to Carvalho *et al.* (2013a) (Supplementary material 4). This software compares control and treatment Cq values to obtain the concentration of expression (C), where $C = E^{meanCq(control)-meanCq(treatment)}$; then, it calculates the relative expression (RE) ratio, where RE= $C_{target\ gene}$/geometric average $C_{reference\ gene}$; and performs a pairwise fixed reallocation randomization test (bootstrap = 2,000 permutation) to obtain *p* values. *CaGAPDH* and *CaUBQ10* were used to normalize relative expression of drought stress treatment (CRUZ *et al.* 2009), *CaEF1* and *CaUBQ10* were used to normalize relative expression of salt stress treatment and *CaGAPDH* and *CaEF1α* were used to normalize relative expression of heat stress treatment. In all cases the value 1 was attributed to the lowest expression.

We also evaluated gene expression and the *C. canephora* contribution through the ratio of Ca/Ca_{Cc}, where Ca represents total gene expression and Ca_{Cc} represents the *C. canephora* specific amplification within *C. arabica*. Our inference

was based in a previous report on *C. arabica* subgenomes expression (MARRACCINI *et al*., 2011). Ca/Ca_{Cc} ratio corresponded to $(1+E)^{-\Delta Ct}$, where $\Delta Ct = Ct_{mean}Ca - Ct_{mean}\ Ca_{Cc}$ with E as the efficiency of gene amplification.

5.3 RESULTS

5.3.1 CARBOHYDRATES QUANTIFICATION

During the abiotic stresses applied, mannitol and fructose levels in the coffee leaves showed variations depending on the stress intensity (Figure 11). In all cases, mannose was not detected in the samples. According to Stein *et al.* (1999), mannose is a toxic intermediate to plants.

5.3.1.1 *Drought stress*

Mannitol and fructose concentration in coffee leaves were altered during drought stress (Figure 11A). Mannitol content increased in moderate and severe stress at similar levels when compared to normal water supply condition. After re-irrigation, mannitol concentration reached lower levels than those observed in Day 0. Fructose content was detected at lower levels compared to mannitol with increased content during moderate stress and reduction during severe stress and recovery (Figure 11A).

5.3.1.2 *Salt stress*

Similarly to drought stress, there was a substantial increase in mannitol content in leaves of coffee plants submitted to salt stress (Figure 11B) while fructose showed significant reduction until Day 6 with a small but significant increase at Day 12. With the exception of Day 0, mannitol content presented higher levels compared to fructose.

5.3.1.3 *Heat stress*

Differently from drought and salt stress, mannitol and fructose presented substantial reduction during the five days of heat stress. No significant differences between Day 3 and Day 5 were observed (Figure 11C).

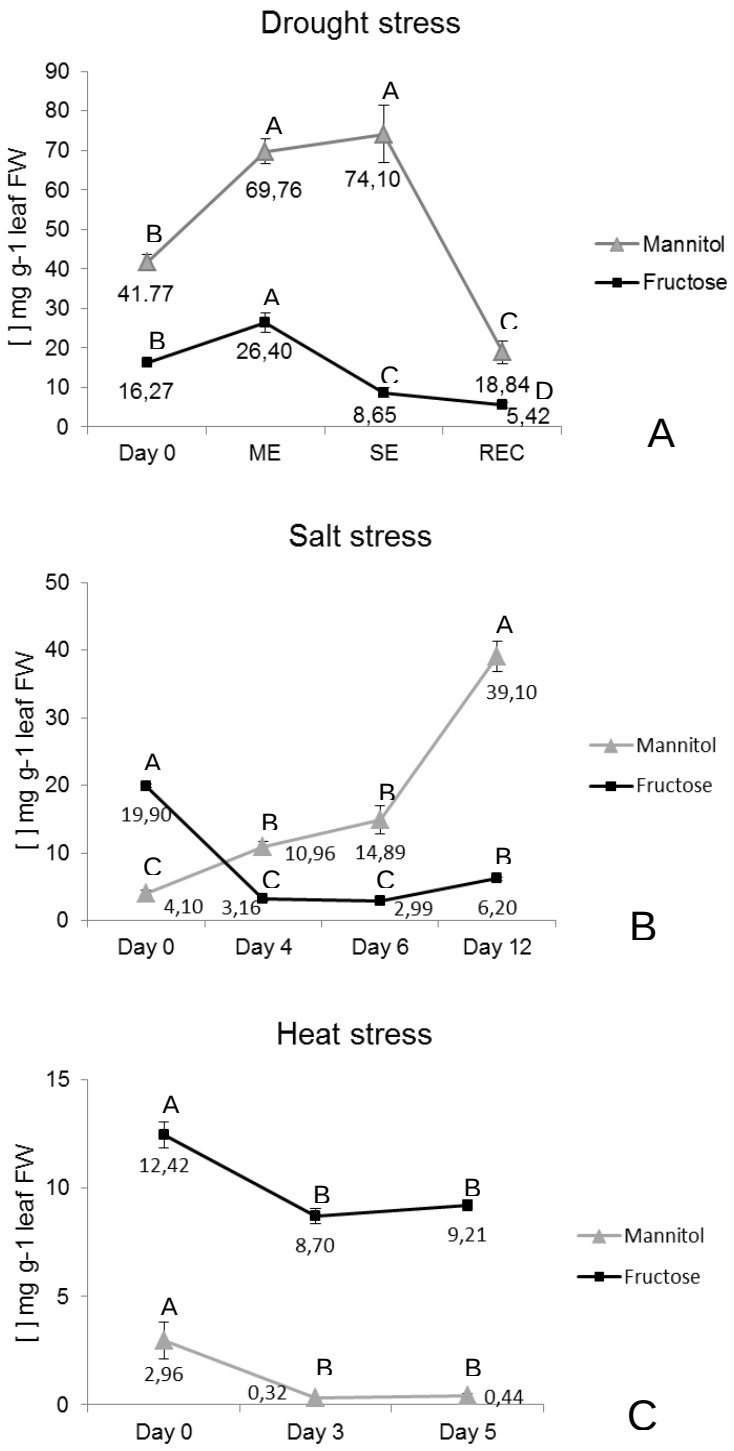


FIGURE 11 - Mannitol and fructose concentrations in leaves of *Coffea arabica* cv. IAPAR-59 submitted to drought stress conditions (A) - Non-stressed (Day 0), moderate stress (ME), severe stress (SE) and recovery (REC). Plants under salt stress conditions (B) - Non-stressed (Day 0) and 4, 6 and 12 days after the beginning of salt stress. Plants under heat stress conditions (C) - Non-stressed (Day 0), moderate stress (Day 3) and severe stress (Day 5). Values are means±SD (n = 3). Different letters represent significant difference between means at $P < 0.05$ level determined by Student t test.

5.3.2 PHYLOGENETIC ANALYSIS

The deduced amino acid sequences of *C. arabica M6PR*, *PMI* and *MTD* were aligned with homologous sequences from several plant genomes and Neighbor joining (NJ) trees were drawn for each gene. In *M6PR* and *PMI* tree, a separation between monocot and eudicot sequences was observed (Figure 12A and B).

The classification obtained with *MTD* tree showed that *CaMTD* was allocated in the subclass Class II (Figure 12C), a subclass previously reported by Barakat *et al.* (2009). This class, together with Class III, represents genes that are related to mannitol degradation under stress conditions. These authors identified a *C. canephora* sequence in Class I (Figure 12C). It is important to note that Class I is related to cinnamyl alcohol dehydrogenases involved in lignin biosynthesis, and they are not involved in responses to stressful conditions, such as mannitol dehydrogenase is.

5.3.3 TRANSCRIPTIONAL ANALYSIS OF *CaM6PR*, *CaPMI* AND *CaMTD*

5.3.3.1 RNA QUALITY AND AMPLIFICATION SPECIFICITY

The quality of RNA samples were confirmed by electrophoretic analysis in agarose gels stained with ethidium bromide and the purity, evaluated by spectrophotometry, ranged from 1.7 to 2.1 (A_{260}/A_{280}). Each primer pair, described in Supplementary material 4, had their specificity confirmed by the identification of a single specific fragment with the predicted molecular size on agarose gel and by the observation of a single peak in the melting curve analyses of qPCR. Primers for amplification of *C. canephora* subgenome were tested using *C. canephora* and *C. eugeniodes* DNA and cDNA to confirm the allelic-specific amplification (Figure 13 and Supplementary material 5). The amplification efficiencies for all primer pairs ranged from 89 to 108% (Supplementary material 4).

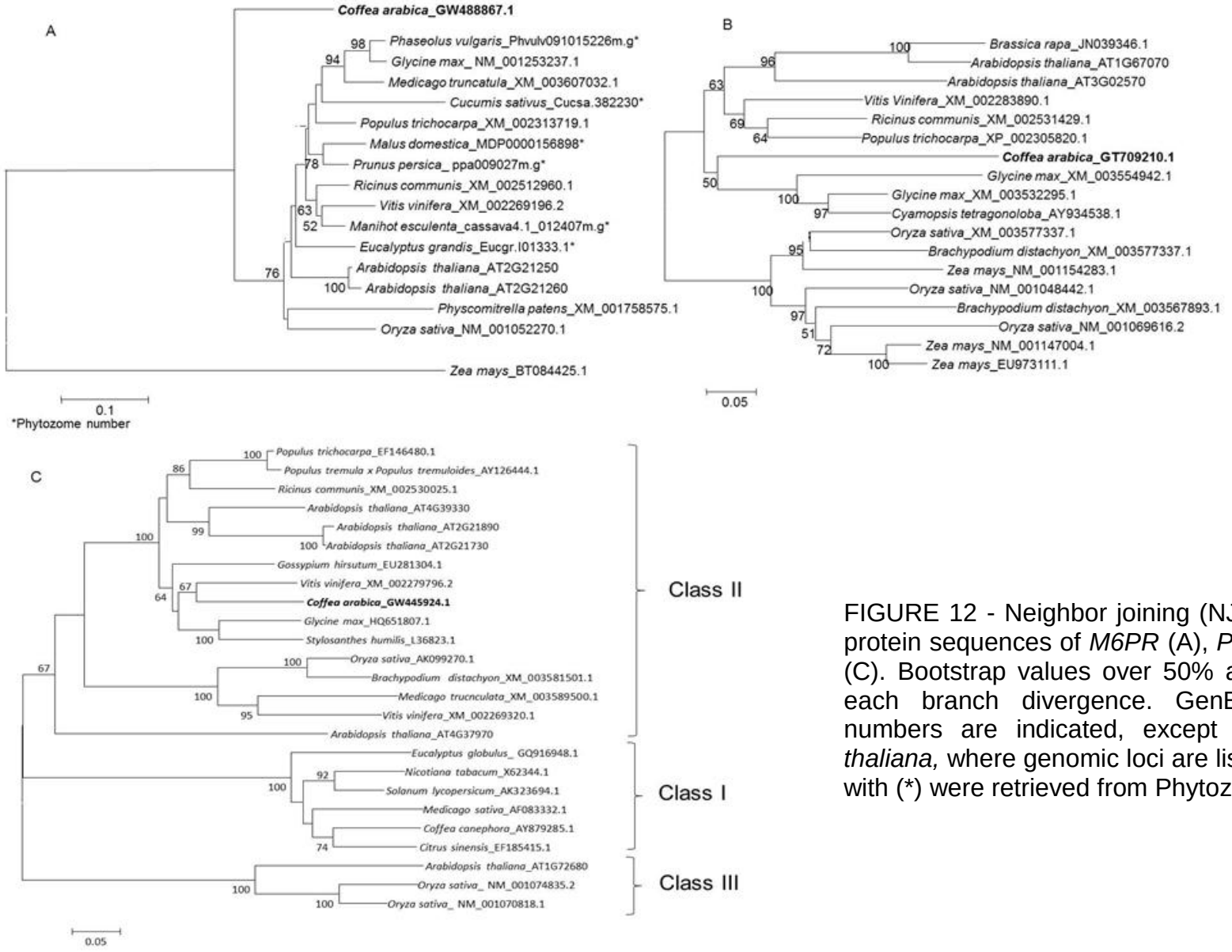


FIGURE 12 - Neighbor joining (NJ) tree based on protein sequences of *M6PR* (A), *PMI* (B) and *MTD* (C). Bootstrap values over 50% are indicated for each branch divergence. GenBank accession numbers are indicated, except for *Arabidopsis thaliana,* where genomic loci are listed. Sequences with (*) were retrieved from Phytozome.

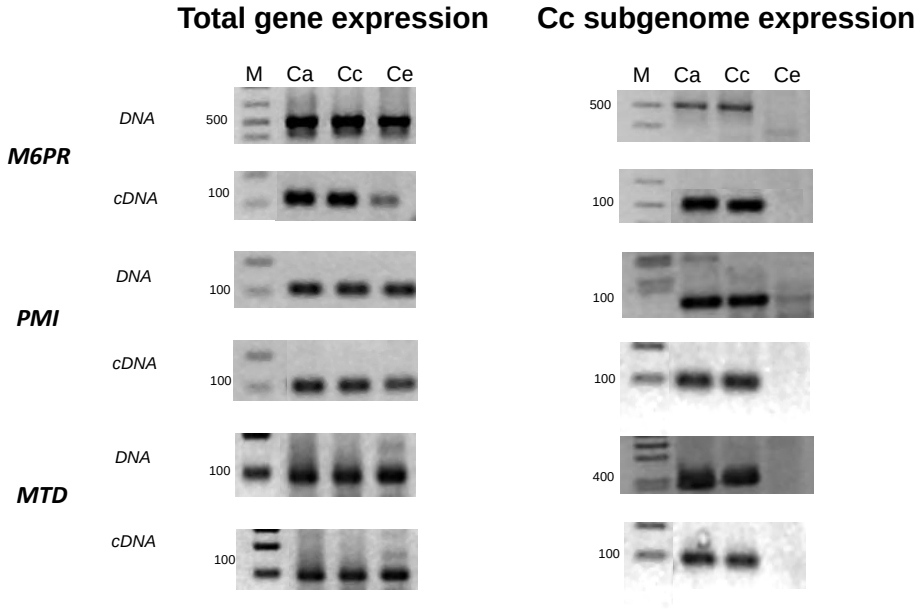


FIGURE 13 - Amplification products in Coffea species using primers for total gene expression and *C. canephora* subgenome expression using DNA and cDNA of non-stressed plants. Primers were specified in Supplementary material 4. (M) Molecular weight standard, (Ca) *C. arabica*, (Cc) *C. canephora*, (Ce) *C. eugenioides*.

5.3.3.2 *Drought stress*

The expression pattern of *CaM6PR*, *CaPMI* and *CaMTD* were assayed by qPCR, revealing differential regulation during drought stress imposition (Figure 14). *CaM6PR* and *CaPMI* presented the same transcriptional pattern, with up-regulation under moderate and severe stress. This modulation is more pronunciated for *CaM6PR,* where it was about 20-fold higher than *CaPMI* (Figure 14A). *CaMTD* was down-regulated with stress imposition but during moderate and severe stress the expression was maintained with posterior increased after recovery. Interestingly, after recovery *CaM6PR* and *CaPMI* were down-regulated (Figure 14A).

5.3.3.3 *Salt stress*

During salt stress, *CaM6PR* expression presented lower values when compared to drought stress. With salt stress imposition *CaM6PR* was up-regulated mainly after 12 days. *CaPMI* expression presented the highest level at day 6 of the experiment with a great reduction in the latest day. On contrary, *CaMTD* showed an increase in mRNA levels in the first days of the stress imposition being down-regulated at Day 12 with increase in the latest days of the salt stress treatment (Figure 14B).

5.3.3.4 *Heat stress*

The expression profile of *CaM6PR*, *CaPMI* and *CaMTD* were distinct during heat stress. *CaM6PR* was down regulated with stress imposition. On the other hand, *CaPMI* and *CaMTD* were first up regulated followed by a down regulation, with *CaMTD* being five-fold more expressed than *CaPMI* at day 1 and ten-fold than *CaM6PR* (Figure 14C).

5.3.4 *Coffea canephora* – SPECIFIC TRANSCRIPTIONAL PATTERN CONTRIBUTION

qPCR analysis specific to the *C. canephora* subgenome were performed for the same three abiotic stresses (Figure 15). The ratio between Ca/Ca_{Cc} was also evaluated. Low values indicates high contribution of *C. canephora* homeolog (Table 4).

5.3.4.1 *Drought stress*

Although different expression values were observed, $CaM6PR_{Cc}$ presented the same pattern during this treatment when compared to *CaM6PR*, with increases in transcriptional levels during moderate and severe stress with posterior

reduction after plant were recovered (Figure 15A). *CaPMI*$_{Cc}$ *also* had an increase with water stress imposition and *CaMTD*$_{Cc}$ displayed a pronunciated upregulation under drought (Figure 15A). Table 4 confirms the expression profile of *M6PR* and *MTD* showing the high contribution of *C. canephora* homeolog during stress imposition.

5.3.4.2 *Salt stress*

Allelic-specific transcriptional level of *CaM6PR*$_{Cc}$ when plants were submitted to salinity showed a great increase in the latest day of the experiment (day 12), while the highest expression of *CaPMI*$_{Cc}$ occurred at day 6. This result is consistent to those observed in table 4, where it is possible to suggest the great contribution of *C. canephora* homeolog. The highest transcript levels of *CaMTD*$_{Cc}$ was also observed at day 6 (Figure 15B).

5.3.4.3 *Heat stress*

When plants were submitted to heat stress, homeolog transcriptional analysis displayed a reduction with stress imposition for *CaM6PR*$_{Cc}$ (Figure 15C, Table 4) showing that *C. canephora* contribution was lower in this condition. On the other hand, *CaMTD*$_{Cc}$ presented a hugh expression in final day of the experiment evidencing that in this case *C. canephora* is probably the main responsible for the gene expression (Figure 15C, Table 4).

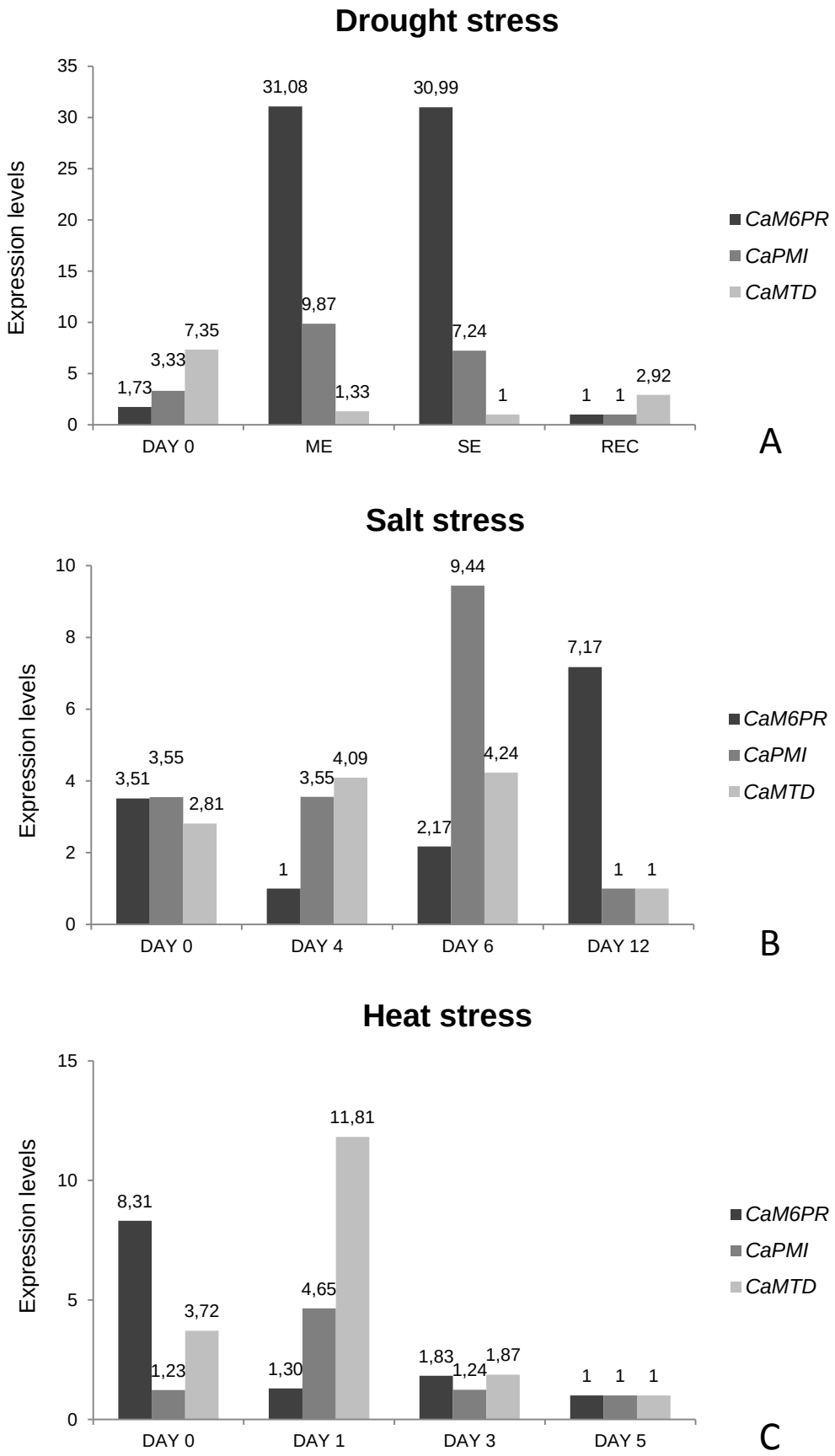


FIGURE 14 – qPCR of total gene expression of mannose-6-phosphate reductase (*CaM6PR*), phosphomannose-isomerase (*CaPMI*) and mannitol dehydrogenase (*CaMTD*) during drought (A), salt (B) and heat (C) stress imposition. Non-stressed (Day 0), moderate stress (ME), severe stress (SE) and recovery (REC) (A). The value 1 was attributed to the lowest expression

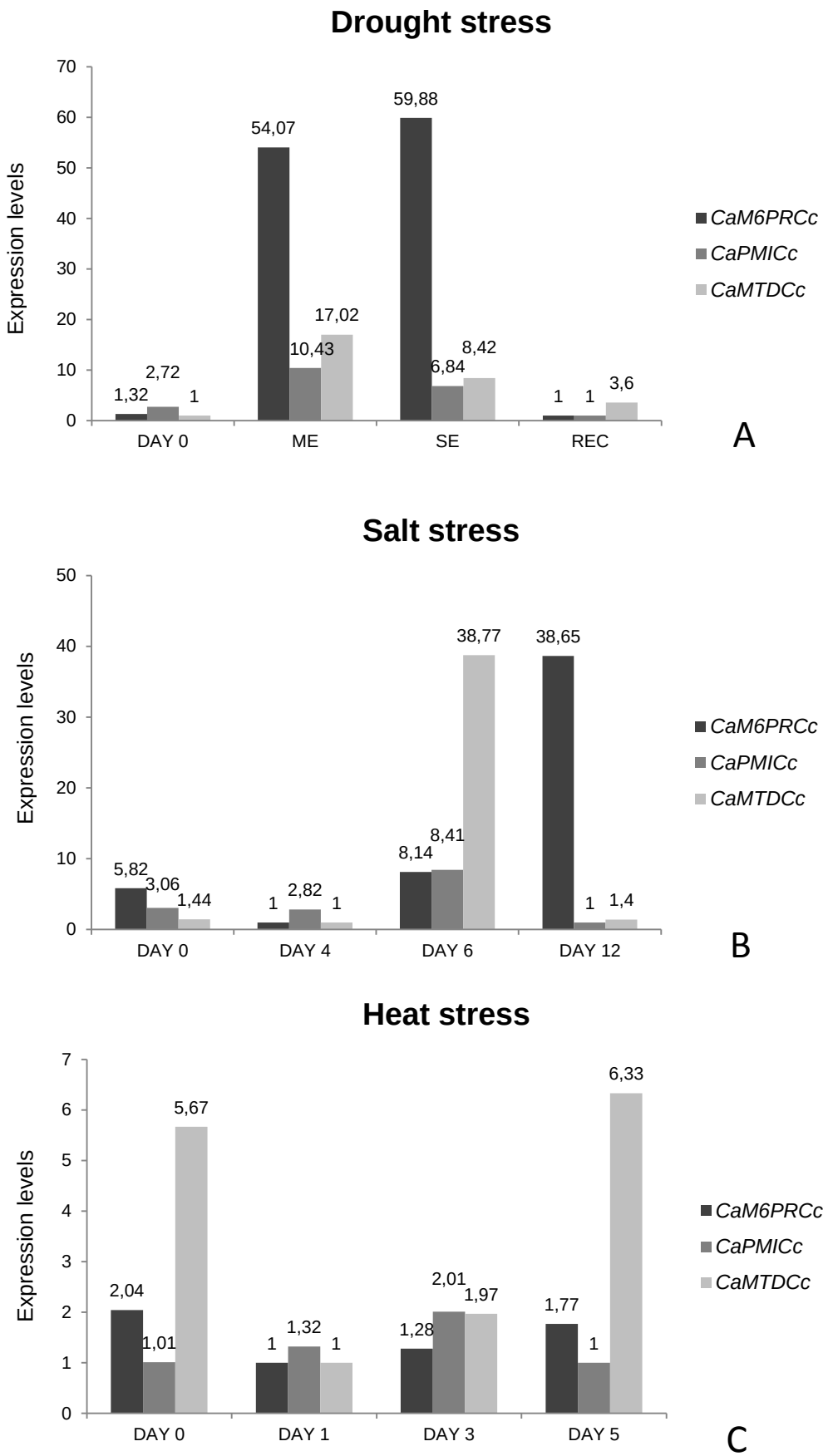


FIGURE 15 - qPCR of *C. canephora* subgenome of mannose-6-phosphate reductase (*CaM6PR*$_{Cc}$), phosphomannose-isomerase (*CaPMI*$_{Cc}$) and mannitol dehydrogenase (*CaMTD*$_{Cc}$) during drought (A), salt (B) and heat (C) stress imposition. Non-stressed (Day 0), moderate stress (ME), severe stress (SE) and recovery (REC) (A). The value 1 was attributed to the lowest expression.

Table 04 – Expression of *M6PR*, *PMI* and *MTD* measured by the ratio of Ca/Ca_{Cc}

Treatments	**Ca/Ca_{Cc}**		
Drought stress	*M6PR*	*PMI*	*MTD*
DAY 0	37,08	1,42	1771,69
ME	16,42	1,64	28,96
SE	14,65	1,72	42,09
REC	28,57	1,87	265,88
Salt stress	*M6PR*	*PMI*	*MTD*
DAY 0	120,10	2,46	838,59
DAY 4	199,51	2,28	1499,27
DAY 6	53,17	2,13	38,85
DAY 12	36,83	2,51	333,66
Heat stress	*M6PR*	*PMI*	*MTD*
DAY 0	130,99	1,79	22,32
DAY 1	41,53	4,08	361,50
DAY 3	45,60	0,84	33,77
DAY 5	18,02	1,51	5,83

5.4 DISCUSSION

Coffee production is subjected to regular oscillations that can be explained by the natural biennial cycle, but also by the adverse effects of climatic conditions. Among them, drought, salt stress and high temperatures are key factors affecting coffee plant development and production (DAMATTA; RAMALHO, 2006; DOS SANTOS *et al*., 2011). Also, as a result of global climate change, periods of drought may become more pronounced, and the sustainability of total production, productivity and coffee quality may become more difficult to maintain (ASSAD *et al*., 2004).

Many abiotic stresses directly or indirectly affect the synthesis, concentration, metabolism, transport and storage of sugars (CRAMER *et al*., 2011). Soluble sugars act as potential signals interacting with light, nitrogen and abiotic stress to regulate plant growth and development (OBERTELLO *et al*., 2010; KANG *et al*., 2010).Growth is limited by the plant ability to osmotically adjust or conduct water. The epidermal cells can increase the water potential gradient by osmotic adjustment, which may be largely supplied by solutes from the phloem. Such solutes are supplied

by photosynthesis that is also supplying energy for growth and other metabolic functions in the plant. With long- term stress, photosynthesis declines due to stomatal limitations for CO_2 uptake and increased photoinhibition from difficulties in dissipating excess light energy (PINHEIRO, CHAVES, 2011; CRAMER *et al*., 2011).

There is great interest in dissecting the processes that are involved in the sugar sensing and response pathways allowing plants to adapt to the constantly changing environment, but the sugars' dual function as nutrients and signalling molecules significantly complicates the analyses of the mechanisms involved (ROLLAND *et al*., 2001). Sugar alcohols, such as mannitol, are of interest as sweeteners and industrial precursors. Achieving higher yields of endogenous sugars in highly selected crops will require the integration of activity across multiple enzymes and possibly multiple pathways; but more knowledge of endogenous regulatory mechanisms is required to select likely targets (PATRICK *et al*., 2012).

Mannitol, an osmoprotectant sugar alcohol, is synthesized during stressful conditions and is by far the most abundant polyol (LOESHER *et al*., 1987). Plant cells can convert mannose by a non-specific hexokinase to mannose-6-phosphate, which can lead to cell growth inhibition by blocking glycolysis through phosphate sequestration (GOLDSWORTHY; STREET, 1965). However, in some higher plants endogenous PMI and/or M6PR can catalyze the conversion of mannose-6-phosphate to fructose-6-phosphate and mannitol-1-phosphate, respectively (PRIVALLE *et al*., 2002, GAO; LOESHER 2003). Mannitol is also involved in translocation and storage of metabolites and helps in the tolerance of plants against salt and osmotic stress (YAMAMOTO *et al*., 1997), and also against pathogens (STOOP *et al*., 1996). In order to assess the relationships between the concentrations of mannitol under conditions of abiotic stresses in *C. arabica* plants, the levels of fructose and mannitol were measured by HPLC and the relative transcriptional levels of the three most important genes in mannitol biosynthesis were evaluated by qPCR.

Studies involving the synthesis and accumulation of mannitol in coffee have only been reported during fruits development of *C. canephora* and *C. arabica* (ROGERS *et al*., 1999) which revealed maximum concentration peaks of 0.18 μg g^{-1} for *C. canephora* and 0.70 μg g^{-1} for *C. arabica*, both in the early stages of fruit development. In this study, the high mannitol levels observed in the *C. arabica* leaves presented are probably due to the higher photosynthetic activity in this tissue

compared to immature green fruits, which may also explain the non-detection of mannitol in ripe fruits (ROGERS *et al*., 1999). In tobacco, a non-mannitol producing plant, overexpressing the mannitol-1-phosphate dehydrogenase gene (*mt1D*), accumulated about to 3.8 mg g^{-1} of the sugar alcohol mannitol in leaves (KARACAS *et al*., 1997).

Studies have shown that an increased accumulation of mannitol under conditions of drought and salinity stress improved plant tolerance to stresses (WILLIAMSON *et al*., 2002; ABEBE *et al*., 2003; SICKLER *et al*., 2007) but there is no physiological evidence on the impact of this accumulation in tolerance to these stresses.

Our data showed that after plants were recovered from drought stress, mannitol rates were drastically reduced (Figure 11A). The fact that mannitol production is an alternative pathway of the carbohydrates production by photosynthesis and the energy used in the synthesis can be diverted to other routes involved in plants recovery, such as synthesis of proteins like Rubisco, a limiting enzyme for high photosynthetic efficiency, may explain this decrease. According to Sakamoto and Murata (2002), the abiotic stress conditions imposed to the plants leaded to a multiple sites of damage in the chloroplasts through PSII and Rubisco inactivation by over-reduction on the acceptor side of PSII together with other enzymes of carbon assimilation (SICKLER *et al*., 2007).

In our study, most of the time mannitol was present at higher levels compared to fructose, probably by the fact that fructose, besides being a mannitol precursor, it is also a sugar highly used in other pathways for carbohydrates production. The high mannitol content in coffee leaves contrasts with results observed by Karacas *et al*. (1997), that verified that in adverse conditions mannitol had the lowest accumulation among osmolytes and the improvement in plant tolerance to salt and osmotic adjustment occurred indirectly and not by the presence of mannitol *per se* in transgenic tobacco. However, other studies have shown that mannitol provides protection by acting as compatible solute by protecting the hydration shell around membranes and proteins or by scavenging ROS even at very low levels of accumulation (GALINSKI *et al*., 1993; SHEN *et al*., 1997).

Another important point from our study is that, as a general pattern, during drought and salt stress, mannitol increased their levels while fructose presented

reduction with stress imposition. The heat stress treatment showed a distinct profile with both sugars reducing their concentration with stress imposition with considering lower concentrations since before the stress treatment. Several factors may have contributed to the differences in carbohydrates quantification, before and after the stress imposition. According to Goldschmidt and Huber (1992) when there is a photosynthesis reduction due to stress, the accumulation of carbohydrates is very variable and can be regulated by the levels of photosynthetic metabolites, and not necessarily by the action the final products themselves (GEIGER 1987; PLAUT *et al*., 1987; BAGNALL *et al*., 1988; FOYER, 1988). Another important observation is that plants used in heat stress were exposed to a lower radiance when compared to natural light conditions and, consequently, the photosynthetic activity of these plants were probably lower than in drought and salt stress, explaining the lowest sugar content. Also, a more detailed study of physiological characterization of hexoses synthesis during stress might help to elucidate these variables. According to Lawlor and Cornic (2002) an increase in hexoses content probably could also be related to the osmoregulation.

Altogether, our results show that mannitol accumulation in *C. arabica* leaves is highly responsive to drought and salt but not to heat stress. According to Chen and Murata (2002), the presence of mannitol in the chloroplasts resulted in enhance resistance to oxidative stress due to an increased capacity to scavenge hydroxyl radicals which can help to explain why during heat stress only a few days is necessary to lead the plant to a collapse. Considering mannitol accumulation, since less mannitol is synthesized, reduced protection in photosynthesis machinery is expected and together with the damage of the photosynthetic apparatus and enzymes inactivation by heat, this abiotic stress seems to be more aggressive for the plant in a short period of time. Also, the heat stress imposition was applied in a short period of time, compared to the other two abiotic stresses, and the temperature seems to be too high for coffee plants. Acording to DaMatta (2004) when coffee is submitted to high temperatures (above 30^{o}C) the coffee production decreased showing the importance of shading for coffee plantation. Also, the same author suggested that the benefits of shading increase as the environment becomes less favorable for coffee cultivation.

The protective effects of soluble sugars against oxidative stress have been mostly attributed to indirect signalling effects, triggering the production of specific ROS scavengers (COUÉE *et al*., 2006; RAMEL *et al*., 2009). It has been recently proposed that soluble sugars, especially when they are present at higher concentrations, might act as ROS scavengers themselves (VAN DEN ENDE, VALLURU, 2009). Moreover, Bolouri-Moghaddam *et al*. (2010) hypothesized that the synergistic interaction of sugars (or sugar-like compounds) and phenolic compounds forms part of an integrated redox system, quenching ROS and contributing to stress tolerance, especially in tissues or organelles with high soluble sugar concentrations. According to Bartels (2001) and Zhifang and Loescher (2003) the difference of NADH and/or NADPH in relation to stress tolerance is not known; however, lowering NADH and/or NADPH has been suggested to have beneficial effects on mitochondrial respiration, energy charge, and possibly reducing ROS. Since mannitol synthesis involves an aldo-keto reductase and use of reductant, such a process may, at least in part, contribute to enhanced abiotic stress tolerance.

The presence of mannose was not detected in the samples studied detected. Literature shows that this sugar can be toxic to plant cells (SONG *et al*., 2010). The mannose is an unusable carbon source for several plant species; this sugar is phosphorylated by a hexokinase to mannose-6-phosphate, which leads to a glycolysis blocking by the inhibition of the phosphoglucoisomerase resulting in a severe inhibition of growth (DEGENHARDT *et al*., 2006). In this way, an alternative to the plant not accumulate this sugar is through the isomerization of mannose-6-phosphate to fructose-6-phosphate by the action of the fosfomanose isomerase (PMI) enzyme which catalyzes this conversion. Fructose-6-phosphate is then used in the metabolic pathway of glycolysis (MARUTA *et al*., 2008). For mannose tolerant species, levels of PMI and/or M6PR are high (SONG *et al*., 2010).

The phylogenetic analysis performed with the three genes focused in this study revealed that for *MTD* tree, monocots and dicots were mixed (Figure 12), while *M6PR* and *PMI* showed a clear division between monocots and dicots (Figure 12). These results suggests that these genes suffered distinct impacts of selection, and *PMI* and *M6PR* as the most conserved of them, perhaps for suffering less substitution (LYNCH, CONERY 2003). *CaMTD* was classified, according to Barakat

et al. (2009), in a group that was previously related to genes that may function under stress conditions.

As a first observation of our results is that a relation between mannitol content and transcript level could be observed, even though complex network regulations is involved among this two processes. According to Everard *et al*. (1997) there is a tight link among M6PR activity, carbon fixation and mannitol synthesis, because fast changes in flux is required to maintain balance in partitioning. It seems more plausible that mannitol biosynthetic pathway is subjected to other regulation controls besides gene expression. Also, metabolic effects may result from changes in enzyme activities in the absence of transcriptional changes. Although previous studies in mannitol production in plants suggested that their effects were primarily as compatible solutes, osmolytes or osmoprotectors, one single gene can have far-ranging effects on other genes expression where many can be involved in stress tolerance (CHAN *et al*., 2011). This observation agrees with the transcriptional regulation observed in our study, where *CaM6PR*, *CaPMI* and *CaMTD* were regulated in distinct ways depending on the abiotic stress. It is also important to notice that previous studies involving mannitol in tolerance to abiotic stresses were focused on salt stress tolerance. Here we showed that drought and heat stress can also regulate genes involved in mannitol biosynthesis in different levels.

CaM6PR was drastically up-regulated by drought stress and high levels of mannitol was present in cells, a result different from those observed by Sickler *et al*. (2007), where M6PR transformants show no increased protection against water deficit, suggesting that the plants do not adequately lower the osmotic potential for protection against dehydration by osmotic adjustment, while increased tolerance to salinity was observed. This same study also suggested that the mannitol synthesis in these plants may be limited by low PMI activity. In our case, *CaM6PR* transcript levels during salt stress presented lower levels compared to drought stress together with lower mannitol content, suggesting that coffee plants might have different responses to water deprivation in comparison to salinity. Differently from non-mannitol producing species, in coffee, mannitol may have functions in protection against water stress. Interestingly, *CaPMI* followed the same profile pattern of *CaM6PR*, confirming the tight regulation of these two genes during drought stress.

However, when plants were submitted to heat stress mannitol was present in lower levels compared to fructose although, except for day 1, *CaPMI* was expressed in lower levels than *CaM6PR*.

Studies with compatible solutes and osmoprotectants during abiotic stress have revealed a crosstalk between them, maybe because much of the genetic variation in gene expression is hidden by non-linearity in response functions, a system that could prevent disfunction of the organism (FU *et al*., 2009). A good example of this system can be seen between proline, an osmoprotectant which also occupies a prominent position under conditions of abiotic stress (CARVALHO *et al*., 2013b), and mannitol. Although studies concerning the functions of mannitol as an osmoprotector are still insufficient, it is possible to establish an analogy with proline. The *M6PR* enzyme plays a similar role to *P5CS*, since both acts in mannitol and proline synthesis, respectively, while *MTD* is involved in the mannitol catabolism just like P5CR is involved in proline degradation. However, proline seems to play other functions in increasing stress tolerance different from those played by mannitol. One possible hypothesis would be high proline accumulation compared to mannitol due to the energy that plant spent in the degradation of these osmoprotectors.

According to Freire *et al.* (2013) in *C. canephora CcM6PR* was up-regulated by water stress in genotypes sensitive and tolerant to drought. But to our knowledge, this is the first work that investigates the relationship between mannitol production and gene expression in coffee. In other plant species, transcriptional profile of this gene was previous studied in two cases, by Northern blot analysis (ZHIFANG, LOESCHER 2003, DELAVAULT *et al*., 2002), and none of them considered abiotic stresses or subgenome contribution to gene expression.

Besides total gene expression of *M6PR*, *PMI* and *MTD*, the present study also evaluated the contribution of *C. canephora* homeolog based on the fact that in an allopolyploid, two diverged genomes merge in a common nucleus can contribute either equaly or disproportionately to the transcriptome (CHAUDHARY *et al*., 2009; COMBES *et al*., 2012). Also, the divergence between subgenomes may indicate that there is a mechanism to prevent *C. arabica* genome homogenization by avoiding the recombination between *CaCc* and *CaCe* (VIDAL *et al*., 2010). The phenomenon of differential expression of homeologous genes has been reported and increasingly studied in alloplyploids species such as wheat (MOCHIDA *et al*., 2003), cotton

(UDAL *et al*., 2006; HOVAV *et al*., 2008b) and coffee (VIDAL *et al*., 2010, MARRACCINI *et al*., 2011; BARDIL *et al*., 2011, COMBES *et al*., 2012). The results in our study is in agreement with the fact that the relative contribution of homeologues to the transcriptome varied according to the abiotic stress applied, since *C. canephora* evidenced to be highly participative during water stress imposition (Table 4). In salt and heat stress homeologues of *C. canephora* demonstrated to be much more involved in gene expression than *C. eugeniodes* since the values of expression level were similar to those observed in total gene expression (Table 4).

During drought, salt and heat stress, one homeologue gene was more recruited to be expressed whereas the other is recruited little or not at all. This were also observed by Bardil *et al.* (2011) and Marraccini *et al.* (2011) in studies with coffee submitted to heat and drought stress, respectively. Also, a similar observation was reported by Vidal *et al*. (2010), where *C. canephora* sub-genome within *C. arabica* transcriptome seems to be responsible for expressing genes coding for regulatory proteins and *C. eugenioides* sub-genome expression appears to be more closely associated with basal processes. A different result was reported by Petitot *et al.* (2008) for genes involved in coffee defense response to biotic stress. These authors reported that *CaWRKY1a* and *CaWRKY1b,* genes that code for transcription factors, were concomitantly expressed, and both homeologous genes contributed to the transcriptional expression. Therefore, our allelic-specific transcriptional expression of *CaM6PRCc*, *CaPMICc* and *CaMTDCc* showed a great participation in total gene expression of *C. Arabica*. This is also consistent with the fact that, in general, as observed in the studies mentioned before, *C. canephora* sub-genome is frequently reported to be more involved in expression of genes related to abiotic stresses.

5.4.1 CONTRIBUTIONS AND PERSPECTIVES

Considering that it is still not completely clear if the abiotic stress-induced accumulation of mannitol may itself directly allow an increased tolerance, or if it is just participating in a much more complex mechanism of stress tolerance by acting synergistically with other key intervenients, our transcriptional evaluation in coffee

plants considering three different abiotic stresses together with other results using transgenic M6PR plants might give an important new information about mannitol regulation.

At the present time, there are no data in the literature about mannitol quantification in coffee leaves, so this is the first work on this sugar alcohol that shows that besides the synthesis, there is a response induced by drought and salt stress in *C. arabica*. Besides, we showed that *C. arabica* sub-genomes do not contribute at the same proportion. Thus, although the action of the sugar alcohol mannitol is unclear, our data integrated with ongoing studies add new information and should help to understand the role of mannitol during abiotic stresses and the transcriptome regulation of homeologous.

5.5 REFERENCES

ABEBE, T.; GUENZI, A.; MARTIN, B.; CUSHMAN, J. C. Tolerance of mannitol-accumulating transgenic wheat to water stress and salinity. **Plant Physiology**, v. 131, p. 1748–1755, 2003.

ADAMS, K. L.; CRONN, R.; PERCIFIELD, R.; WENDEL, J. F. Genes duplicated by polyploidy show unequal contributions to the transcriptome and organspecific reciprocal silencing. **Proceedings of the National Academy of Sciences**, v. 100, n. 8, p. 4649–4654, 2003.

ALBINI, F. M.; MURELLI, C.; PATRITTI, G. et al. Molecular weight substances from the resurrection plant *Sporobolus stapfianus*, **Phytochemistry**, v. 37, p. 137-142, 1994.

ALLAKHVERDIEV, S. I.; KRESLAVSKI, V. D.; KLIMOV, V. V. et al. Heat stress: an overview of molecular responses in photosynthesis. **Photosynthesis Research**, v. 98, p. 541–550, 2008.

BAGNALL, D. J.; KING, R. W.; FARQUHAR, G. D. Temperature-dependent feedback inhibition of photosynthesis in peanut. **Planta**, v. 175, p. 348-354, 1988.

BARAKAT, A.; BAGNIEWSKA-ZADWORNA, A.; CHOI, A. et al. The cinnamyl alcohol dehydrogenase gene family in *Populus*: phylogeny, organization, and expression. **BMC Plant Biology**, v. 9, p. 26, 2009.

BARDIL, A.; ALMEIDA, J. D.; COMBES, M. C.; LASHERMES, P.; BERTRAND, B. Genomic expression dominance in the natural allopolyploid *Coffea arabica* is massively affected by growth temperature. **New Phytologist**, v. 192, p. 760–774, 2011.

BARKLA, B. J.; PANTOJA, O. Plasma Membrane and Abiotic Stress. In: The Plant Plasma Membrane. **Plant Cell Monographs**, v. 19, p. 457-470, 2011.

BARTELS, D. Targeting detoxification pathways. An efficient approach to obtain plants with multiple stress tolerance ? **Trends in Plant Science**, v. 6, p. 284-286, 2001.

BOLOURI-MOGHADDAM, M. R.; LE ROY, K.; XIANG, L. et al. Sugar signalling and antioxidant network connections in plant cells. **FEBS Journal**, v. 277,p. 2022–2037, 2010.

BRINI, F.; HANIN, M.; M EZGHANI, I. et al. Overexpression of wheat Na+/H+ antiporter TNHX1 and H+-pyrophosphatase TVP1 improve salt- and drought-stress tolerance in *Arabidopsis thaliana* plants. **Journal of Experimental Botany**, v. 58, n. 2, p. 301–308, 2007.

CARVALHO, K., CAMPOS, M. K. F., PEREIRA, L. F. P., VIEIRA, L. G. E. Reference gene selection for real-time quantitative polymerase chain reaction normalization in ‘‘Swingle” citrumelo under drought stress. **Analytical Biochemistry**, v. 402, p. 197-199, 2010.

CARVALHO, K.; BESPALHOK FILHO, J. C.; DOS SANTOS, T. B.; et al. Nitrogen Starvation, Salt and Heat Stress in Coffee (*Coffea arabica* L.): Identification and Validation of New Genes for qPCR Normalization. **Molecular Biotechnology**, v. 53, n. 3, p. 315-325, 2013a.

CARVALHO, K.; DE CAMPOS, M. K. F.; DOMINGUES, D. S.; PEREIRA, L. F. P.; VIEIRA, L. G. E. The accumulation of endogenous proline induces changes in gene expression of several antioxidant enzymes in leaves of transgenic Swingle citrumelo. **Molecular Biology Reports**, v 40, n. 4, p 3269-3279, 2013b.

CHAN, Z.; GRUMET, R.; LOESCHER, W. Global gene expression analysis of transgenic, mannitol-producing, and salt-tolerant *Arabidopsis thaliana* indicates widespread changes in abiotic and biotic stress-related genes. **Journal of experimental botany**, v. 62, n. 14, p. 4787-4803, 2011.

CHANG, S., PURYEAR, J., CAIRNEY, J. A simple and efficient method for isolating RNA from pine trees. **Plant Molecular Biology Reporter**, v. 11, p. 113-116, 1993.

CHAUDHARY, B.; FLAGEL, L.; STUPAR, R. M.; UDALL, J. A. et al. Reciprocal silencing, transcriptional bias and functional divergence of homeologs in polyploid cotton (*Gossypium*). **Genetics**, v. 182, n. 2, p. 503–517, 2009.

CHEN, T. H. H.; MURATA, N. Enhancement of tolerance of abiotic stress by metabolic engineering of betaines and other compatible solutes. **Current Opinion in Plant Biology**, v. 5, p. 250–257, 2002.

COMBES, M-C.; CENCI, A.; BARAILLE, H. et al. Homeologous Gene Expression in Response to Growing Temperature in a Recent Allopolyploid (*Coffea arabica* L.). **Journal of Heredity**, v. 103, n. 1, p. 36–46, 2012.

CONDE, A.; SILVA, P.; AGASSE, A. et al. Mannitol Transport and Mannitol Dehydrogenase Activities are Coordinated in *Olea europaea* Under Salt and Osmotic Stresses. **Plant Cell Physiology**, v. 52, n. 10, p. 1766–1775, 2011.

COUÉE, I.; SULMON, C.; GOUESBET, G.; EL AMRANI, A. An involvement of soluble sugars in reactive oxygen species balance and responses to oxidative stress in plants. **Journal of Experimental Botany**, v. 57, p. 449–459, 2006.

CRAMER, G. R.; URANO, K.; DELROT, S. et al. Effects of abiotic stress on plants: a systems biology perspective. **BMC Plant Biology**, v. 11, p. 163, 2011.

CRUZ, F.; KALAOUN, S.; NOBILE, P. et al. Evaluation of coffee reference genes for relative expression studies by quantitative real-time RT-PCR. **Molecular Breeding**, v. 23, n. 4, p. 607-616, 2009.

DAMATTA, F. M. Ecophysiological constraints on the production of shaded and unshaded coffee: a review. **Field Crops Research**, v. 86, p. 99–114, 2004.

DAMATTA, F. M.; RAMALHO, J. C. Impact of drought and temperature stress on coffee physiology and production: a review. **Brazilian Journal of Plant Physiology**, v. 18, n. 1, 55-81, 2006.

DEGENHARDT, J.; POPPE, A.; MONTAG, J.; SZANKOWSKI, I. The use of the phosphomannose-isomerase/mannose selection system to recover transgenic apple plants. **Plant Cell Reports**, v. 25, p. 1149–1156, 2006.

DELAVAULT, P.; SIMIER, P.; THOIRON, S.; VÉRONÉSI, C.; FER, A.; THALOUARN, P. Isolation of mannose 6—phosphate reductase cDNA, changes in enzyme activity and mannitol content in broomrape (*Orabanche ramosa*) parasitic on tomato roots. **Physiologia plantarum**, v. 115, p. 48-55, 2002.

DONG, S.; ADAMS, K. L. Differential contributions to the transcriptome of duplicated genes in response to abiotic stresses in natural and synthetic polyploids. **New Phytologist**, v. 190, n. 4, p. 1045–1057, 2011.

DOS SANTOS, T. B., BUDZINSKI, I. G. F., MARUR, C. J. et al. Expression of three galactinol synthase isoforms in Coffea arabica L. and accumulation of raffinose and stachyose in response to abiotic stresses. **Plant Physiology and Biochemistry**, v. 49, p. 441-448, 2011.

EVERARD, J. D.; CANTINI, C.; GRUMET, R. et al Molecular cloning of mannose-6-phosphate reductase and its developmental expression in celery. **Plant Physiology**, v. 113, p. 1427-1435, 1997.

FOYER, C. H. Feedback inhibition of photosynthesis through source-sink regulation in leaves. **Plant Physiology and Biochemistry**, v. 26, p. 483-492, 1988.

FREIRE, L. P.; MARRACCINI, P. R.; RODRIGUES, G. C.; ANDRADE, A. C. Analise da expressão do gene manose 6 fosfato redutase em cafeeiros submetidos ao déficit hídrico. **Coffee Science**, v. 8, n. 1, 2013.

GALINSKI, E. A. Compatible solutes of halophytic eubacteria: molecular principles, water-solute interaction, stress protection. **Experientia**, v. 49, p. 487-496, 1993.

GAO, Z.; LOESCHER, W. H. Expression of celery mannose-6-phosphate reductase in *Arabidopsis thaliana* enhances salt tolerance and induces biosynthesis of both mannitol and a mannitol dimer. **Plant Cell Environment**, v. 26, p. 275-283, 2003.

GEIGER, D. R. Understanding interactions of source and sink regions in plants. **Plant Physiology and Biochemistry**, v. 25, p. 659-666, 1987.

GOLDSCHMIDT, E. E.; HUBER, S. Regulation of photosynthesis by end-product accumulation in leaves of plants storing starch, sucrose, and hexose sugars. **Plant Physiology,** v. 99, p. 1443-1448, 1992.

GOLDSWORTHY, A.; STREET, H. E. The carbohydrate nutrition of tomato roots VIII. The mechanism of the inhibition by D-mannose of the respiration of excised roots. **Annals of Botany**, v. 29, p. 45-58, 1965.

HOVAV, R.; UDALL, J. A.; CHAUDHARY, B.; RAPP, R. et al. Partitioned expression of duplicated genes during development and evolution of a single cell in a polyploid plant. **PNAS USA**, v. 105, p. 6191–6195, 2008a.

HOVAV, R.; CHAUDHARY, B.; UDALL, J. A.; et al. Parallel domestication, convergent evolution and duplicated gene recruitment in allopolyploid cotton. **Genetics**, v. 179, p. 1725–1733, 2008b.

KANG, S. G.; PRICE, J.; LIN, P. C. et al. The Arabidopsis bZIP1 transcription factor is involved in sugar signaling, protein networking, and DNA binding. **Molecular Plant**, v. 3, n. 2, p. 361-373, 2010.

KARACAS, B.; OZIAS-AKINS, P.; STUSHNOFF, C.; SUEFFERHELD, M.; RIEGER, M. Salinity and drought tolerance of mantiol-accumulating transgenic tobacco. **Plant Cell and Environment**, v. 20, p. 609-616, 1997.

LASHERMES, P.; COMBES, M. C.; ROBERT, J. et al. Molecular characterisation and origin of the Coffea arabica L. genome. **Molecular and General Genetics MGG**, v. 261, n. 2, p. 259–266, 1999.

LAWLOR, D. W.; CORNIC, G. Photossynthetic carbon assimilation and associated metabolism in relation to water déficits in higher plants. **Plant, Cell and Environment**, v. 25, p. 275-294, 2002.

LIMA, A. L. S.; DAMATTA, F. M.; PINHEIRO, H. A. et al. Photochemical responses and oxidative stress in two clones of *Coffea canephora* under water deficit conditions. **Environmental and Experimental Botany**, v. 47, n. 3, p. 239-247, 2002.

LI, B.; KADURA, I.; FU, D-J; WATSON, D. E. Genotyping with TaqMAMA. **Genomics**, v. 83, p. 311– 320, 2004.

LIU, Z.; ADAMS, K. L. Expression partitioning between genes duplicated by polyploidy under abiotic stress and during organ development. **Current Biology**, v. 17, n. 19, p. 1669–1674, 2007.

LOESCHER, W. H. Physiology and metabolism of sugar alcohols in higher plants. **Physiologia Platarum**, v. 70, p. 553-557, 1987.

LYNCH, M.; CONERY, J. S. The evolutionary demography of duplicate genes. **Genome Evolution**, v. 3, p. 35–44, 2003.

MARRACCINI, P.; FREIRE, L. P.; ALVES, G. S. C. et al. RBCS1 expression in coffee: Coffea orthologs, *Coffea arabica* homeologs, and expression variability between genotypes and under drought stress. **BMC Plant Biology**, v. 11, p. 85, 2011.

MARUTA, T.; YONEMITSU, M.; YABUTA, Y. et al. *Arabidopsis* phosphomannose isomerase 1, but not phosphomannose isomerase 2, is essential for ascorbic acid

biosynthesis. **Journal of Biological Chemistry**, v. 283, n. 43, p. 28842-28851, 2008.

MOCHIDA, K.; YAMAZAKI, Y.; OGIHARA, Y. Discrimination of homoeologous gene expression in hexaploid wheat by SNP analysis of contigs grouped from a large number of expressed sequence tags. **Molecular Genetics and Genomics**, v. 270, p. 371–377, 2003.

MONDEGO, J. M. C.; VIDAL, R. O.; CARAZZOLLE, M. F. et al. An EST-based analysis identifies new genes and reveals distinctive gene expression features of *Coffea arabica* and *Coffea canephora*. **BMC Plant Biology**, v. 11, n. 30, 2011.

MUSTORP, S.; ENGDAHL-AXELSSON, C.; SVENSSON, U.; HOLCK, A. Detection of celery (*Apium graveolens*), mustard (*Sinapis alba, Brassica juncea, Brassica nigra*) and sesame (*Sesamum indicum*) in food by real-time PCR. **European Food Research and Technology**, v. 226, p.771-778, 2008.

NOIRAUD, N.; MAUROUSSET, L.; LEMOINE, R. Identification of a mannitol transporter, AgMaT1, in celery phloem. **Plant Cell**, v. 13, n. 695–705, 2001.

OBERTELLO, M.; KROUK, G.; KATARI, M. S.; RUNKO, S. J.; CORUZZI, G. M. Modeling the global effect of the basic-leucine zipper transcription factor 1 (bZIP1) on nitrogen and light regulation in Arabidopsis. **BMC Systems Biology**, v. 4, p. 111, 2010.

OSBORN, T. C.; PIRES, J. C.; BIRCHLER, J. A.; AUGER, D. L.; CHEN, Z. J. et al. Understanding mechanisms of novel gene expression in polyploids. **Trends in Genetics**, v. 19, p. 141–147, 2003.

PETITOT, A. S.; LECOULS, A. C.; FERNANDEZ, D. Sub-genomic origin and regulation patterns of a duplicated WRKY gene in the allotetraploid species *Coffea arabica*. **Tree Genetics and Genomes**, v. 3, n. 4, p. 379-390, 2008.

PIGNATTA D, COMAI L. Parental squabbles and genome expression: lessons from the polyploids. **Journal of Biology**, v. 8, n. 4, p. 43, 2009.

PINHEIRO, C.; CHAVES, M. M. Photosynthesis and drought: can we make metabolic connections from available data? **Journal of Experimental Botany**, v. 62, n. 3,869-882, 2011.

PRIVALLE, L. S. Phosphomannose isomerase, a novel plant selection system. Potential allergenicity assessment. **Annals of the New York Academy of Sciences**, v. 964, p.129-138, 2002.

RAMEL, F.; SULMON, C.; BOGARD, M.; COUÉE, I.; GOUESBET, G. Differential patterns of reactive oxygen species and antioxidative mechanisms during atrazine injury and sucrose-induced tolerance in *Arabidopsis thaliana* plantlets. **BMC Plant Biology**, v. 9, p. 28, 2009.

RATHINASABAPATHI, B. Metabolic engineering for stress tolerance: installing osmoprotectant synthesis pathways. **Annals of Botany**, v. 86, p. 709–716, 2000.

ROGERS, W. J.; MICHAUX, S.; BASTIN, M.; BUCHELI, P. Changes to the content of sugars, sugar alcohols, myo-inositol, carboxylic acids and inorganic anions in developing grains from different varieties of Robusta (*Coffea canephora*) and Arabica (*C. arabica*) coffees. **Plant Science**, v. 149, p. 115–123, 1999.

ROLLAND, F.; WINDERICKX, J.; THEVELEIN, J. M. Glucose sensing mechanisms in eukaryotic cells. **Trends in Biochemical Sciences**, v. 26, p. 310–317, 2001.

SERRANO, R.; MULET, J. M.; RIOS, G. et al. A glimpse of the mechanisms of ion homeostasis during salt stress. **Journal of Experimental Botany**, v. 50, p. 1023–1036, 1999.

SHEN, B.; JENSEN, R. G.; BOHNERT, H. J. Increased resistance to oxidative stress in transgenic plants by targeting mannitol biosynthesis to chloroplasts. **Plant Physiology**, v. 113, p. 1177-1183, 1997.

SICKLER, C. M.; EDWARDS, G. E.; KIIRATS, O. et al. Response of mannitol-producing *Arabidopsis thaliana* to abiotic stress. **Functional Plant Biology**, v. 34, p. 382–391, 2007.

STAMATI, K.; MACKAY, I.; POWELL, W. A quantitative genomic imbalance gene expression assay in a hexaploid species: wheat (*Triticum aestivum*). **Genome**, v. 52, n. 1, p. 89–94, 2009.

STOOP, J. M. H.; WILLIAMSON, J. D.; PHARR, D. M. Mannitol metabolism in plants: a method for coping with stress. **Trends of Plant Science Review**, v. 1, n. 5 139-144, 1996.

TAMURA, K.; PETERSON, D.; PETERSON, N. et al. MEGA5: Molecular Evolutionary Genetics Analysis using Maximum Likelihood, Evolutionary Distance, and Maximum Parsimony Methods. **Molecular Biology and Evolution**, v. 28, n.10, p. 2731-2739, 2011.

UDALL, J. A.; SWANSON, J. M.; HALLER, K. et al. A global assembly of cotton ESTs. **Genome Research**, v. 16, p. 441–450, 2006.

VAN DEN ENDE, W.; VALLURU, R. Sucrose, sucrosyl oligosaccharides, and oxidative stress: scavenging and salvaging? **Journal of Experimental Botany**, v. 60, p. 9–18, 2009.

VIDAL, R. O.; MONDEGO, J. M. C.; POT, D. et al. A high-throughput data mining of SNPs in Coffea spp Suggests differential ESTs homeologous gene expression in the allotetraploid *Coffea arabica*. **Plant Physiology,** v. 154, p. 1053-1066, 2010.

WILLIAMSON, J. D.; JENNINGS, D. B.; GUO, W-E. et al. Sugar alcohols, salt stress and fungal resistance: polyolsmultifunctional plant protection? **Journal of the American Society for Horticultural Science**, v. 127, p. 467–473, 2002.

YAMAMOTO, Y. T.; ZAMSKI, E.; WILLIAMSON, J. D. et al. Subcellular localization of celery mannitol dehydrogenase: a cytosolic metabolic enzyme in nuclei. **Plant Physiology**, v. 155, n. 4, p. 1397-1403, 1997.

ZHIFANG, G.; LOESCHER, W. H. Expression of a celery mannose 6-phosphate reductase in *Arabidopsis thaliana* enhances salt tolerance and indices biosynthesis of both mannitol and a glucosyl-mannitol dimer. **Plant, Cell and Environment** , v. 26, p. 275-283, 2003.

ZHU, J. K. Plant salt tolerance. **Trends in Plant Science**, v. 6, p. 66–71, 2001.

SUPPLEMENTARY MATERIAL II

Supplementary material 4

Supplementary material 4. Description of genes and primers sequences used for qPCR

Gene	Abbreviation	Primer forward	Primer reverse	Efficiency	NCBI accession	Arabidopsis ortholog
Mannose-6-phosphate reductase	*CaM6PR*	GAAGAACTTGGGGAAGCCCT	AGAACGTGGTCATGGTCTGAATTC	95%	GW488867.1	AT2G21250**
	* *CaM6PR* $_{Cc}$	GAAGAACTTGGGGAAGCCCT	GCTGTCTTTGCAAGCCTGG	95%	GT649509.1	_____
Phosphomannose-isomerase	*CaPMI*	ACTCCGGACCCTCCTATGTTG	GCCCTGGGATTTTTCTCGAT	108%	GT709210.1	AT1G67070
	**CaPMI* $_{Cc}$	ACTCCGGACCCTCCTATGTTG	CCCTGGGATTTTTCTCGACC	93%	DV688525.1	_____
Mannitol dehydrogenase	*CaMTD*	TGTCCAGAAATTCCAAGTTGGTG	GGACAGTAATTCTCCAAGTCTTGTTG	102%	GW445924.1	AT2G21730
	**CaMTD* $_{Cc}$	CCAGAAATTCCAAGTTGGTGATTT	GGACAGTAATTCTCCAAGTCTTGTTG	89%	GT652950.1	_____
Glyceraldehyde-3-phosphate-dehydrogenase	*CaGAPDH*	AGGCTGTTGGGAAAGTTCTTC	ACTGTTGGAACTCGGAATGC	99%	GW451281.1	AT1G13440
Elongation factor 1	*CaEF1*	CTGTCCTTGATTGCCACACTTCT	CTTGGGCTCCTTCTCAAGCTC	95%	GW484749.1	AT1G07920
Elongation factor 1-alpha	*CaEF1α*	AAGGGAGCTTCCAGCTTTACCT	TGTGAGAGGTGTGGCAGTCAA	96%	GT708303.1	AT1G07940
Polyubiquitin 10	*CaUBQ10*	CAGACCAGCAGAGGCTGATT	AGAACCAAGTGAAGGGTGGA	92%	GT697658.1	AT4G05320
**CaM6PRCc, CaPMICc* and *CaMTDCc* are primers *C. canephora* subgenome specific						
***Arabidopsis thaliana* has a ortholog for M6PR even though is not a mannitol producer						

Supplementary material 5

Genes	Species	Contig*	
M6PR	Ca	Contig12129	
	Cc	Contig6031	
PMI	Ca	Contig11388	
	Cc	Contig5789	
MTD	Ca	Contig1070	
	Cc	Contig4109	
*Contig from assembly made by Mondego et al (2011)			

ANEXO I

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RESEARCH

Nitrogen Starvation, Salt and Heat Stress in Coffee (*Coffea arabica* L.): Identification and Validation of New Genes for qPCR Normalization

Kenia de Carvalho · João Carlos Bespalhok Filho · Tiago Benedito dos Santos · Silvia Graciele Hülse de Souza · Luiz Gonzaga Esteves Vieira · Luis Filipe Protasio Pereira · Douglas Silva Domingues

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Abstract Abiotic stresses are among the most important factors that affect food production. One important step to face these environmental challenges is the transcriptional modulation. Quantitative real-time PCR is a rapid, sensitive, and reliable method for the detection of mRNAs and it has become a powerful tool to mitigate plant stress tolerance; however, suitable reference genes are required for data normalization. Reference genes for coffee plants during nitrogen starvation, salinity and heat stress have not yet been reported. We evaluated the expression stability of ten candidate reference genes using geNorm PLUS, NormFinder, and BestKeeper softwares, in plants submitted to nitrogen starvation, salt and heat stress. *EF1*, *EF1α*, *GAPDH*, *MDH*, and *UBQ10* were ranked as the most stable genes in all stresses and software analyses, while *RPL39* and *RPII* were classified as the less reliable references. For reference gene validation, the transcriptional pattern of a *Coffea* non-symbiotic hemoglobin (*CaHb1*) was analyzed using the two new recommended and the most unstable gene references for normalization. The most unstable gene may lead to incorrect interpretation of *CaHb1* transcriptional analysis. Here, we recommend two new reference genes in *Coffea* for use in data normalization in abiotic stresses: *MDH* and *EF1*.

Keywords Mineral nutrition · High temperature · Salinity · Nitrogen limitation · Coffee · qPCR normalization · Leaves · Roots

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K. de Carvalho · T. B. dos Santos · S. G. H. de Souza · L. G. E. Vieira · L. F. P. Pereira · D. S. Domingues (✉)
Laboratório de Biotecnologia Vegetal, Instituto Agronômico do Paraná, CP 481, Londrina, PR CEP 86047-902, Brazil
e-mail: doug@iapar.br

K. de Carvalho
e-mail: keniadecarvalho@gmail.com

T. B. dos Santos
e-mail: tiagobio02@yahoo.com.br

S. G. H. de Souza
e-mail: silviahulse@yahoo.com.br

L. G. E. Vieira
e-mail: lvieira@iapar.br

L. F. P. Pereira
e-mail: filipe.pereira@embrapa.br

K. de Carvalho · J. C. Bespalhok Filho
Programa de Pós-Graduação em Agronomia (Produção Vegetal), Departamento de Fitotecnia e Fitossanitarismo, Universidade Federal do Paraná, Curitiba, PR CEP 80035-050, Brazil

J. C. Bespalhok Filho
e-mail: bespa@ufpr.br

L. F. P. Pereira
Embrapa Café, Brasília, DF CEP 70770-901, Brazil

Introduction

Coffee is an important crop and is crucial to the economy of many developing countries, generating around US$70 billion per year. This way, coffee is one of the most valuable international exchange commodities in agricultural trade and coffee farmings are crucial to the economy of many developing countries where its cultivation, processing, transportation and marketing provide employment for millions of people worldwide [1]. Abiotic stress is one of the major factors that affect food production worldwide and in tropical countries, drought, salinity, extreme temperatures, nutrient deficiencies and mineral toxicities are

among the most important limitants in crop yield [2]. It is important to note that, in a scenario of climate changes, these stresses will be increasingly important, associated with land degradation and declining water quality [3].

In this way, several studies that depict the impact of abiotic stresses uses reverse transcription quantitative real-time PCR (qPCR) as a tool to understand the mechanism of plant stress tolerance [4, 5]. qPCR is a valuable technique to study rare transcripts, to work with members of a multigene family or with small sample sizes and offers advantages in sensitivity and specificity. However, this method is only valid if proper internal controls are included. Extensive transcriptomic data mining and experimental validation in different plants has shown that the reliability of these endogenous controls can be influenced by plant species, growth conditions and organs/tissues examined, and there are no universally applicable reference genes with an invariant expression and that commonly used reference genes exhibit a large expression fluctuation [6]. Diverse statistical algorithms have been developed to help validate reference genes that led to recommendations for refining qPCR standards in the plant research community. These debates provide guidance for refining qPCR standards in a defined set of RNA samples in a single species.

The continued use of inappropriate reference genes may have already resulted in the misinterpretation of some published results [7], although studies involving the selection of appropriate reference genes have increased [8–10]. In recent years, coffee (*Coffea* ssp.) has become the subject of increasing research in gene expression analysis [11, 12]. However, few works have focused on selecting reliable reference genes for data normalization in studies involving coffee plants under different stress conditions [13, 14]. Moreover, few studies explore the suitability of reference genes for nitrogen (N), salt and heat stress.

Considering that, in this study, we evaluated the transcriptional profile of a *Coffea arabica* hemoglobin and the stability of ten *C. arabica* genes to normalize the results of gene expression studies in N starvation, salt stress and high temperature conditions using three algorithms: geNorm PLUS [15], NormFinder [16], and BestKeeper [17].

Materials and Methods

Plant Material and Abiotic Stress Treatments

For all experiments, we used *C. arabica* cv IAPAR 59. In the N starvation treatment, coffee seeds were allowed to germinate in vermiculite in a greenhouse at 25 °C on a 12 h/12 h day/night cycle. The seeds were irrigated twice a week with distilled water. After 4 weeks, seedlings were irrigated with Clark [18] nutritive solution, with pH adjusted to 5.5–6.0. Prior to the experiments, 6-month-old plants (5–6 leaf pairs) with a single shoot were selected for size uniformity, and transferred to a growth chamber under the following conditions: 12 h/12 h light/dark, 25 °C/23 °C day/night temperature, 45 % humidity, and photosynthetic photon flux density of ~250 μmol/m^2/s. Plants were maintained in a aerated solution modified from Clark [18] with the following components: 5330 μM NH_4NO_3, 260 μM KH_2PO_4, 330 μM $MgSO_4$, 1060 μM K_2SO_4, 660 μM $CaCl_2$, 200 μM Na–Fe–EDTA, 7 μM H_3BO_3, 3 μM $MnSO_4$, 2.5 μM $ZnSO_4$, 0.1 μM $CuSO_4$, and 0.7 μM $NaMoO_4$. Nutrient solutions were renewed weekly. After 4 weeks on hydroponic devices, plants were harvested for time point 0 and then transferred to the modified Clark [18] N-free solution, where leaves (5th and 6th pair) and lateral roots were harvested at 1 day and 10 days after transfer into the N-free solution. Experiments were conducted twice with a minimum of three biological replicates per experiment. Biological replicates were represented by pools of coffee leaves or lateral roots of at least nine plants at the same developmental stage. After harvesting, all samples were frozen immediately in liquid N_2 and stored at −80 °C until RNA extraction.

Basic procedures of the salinity and heat stress treatments followed Dos Santos et al. [19]. The salt stress experiment was carried out using leaves of nine 18-month coffee plants with uniform growth conditions. In order to avoid osmotic shock, on the first day plants were irrigated with 50 mM NaCl and the second day with 100 mM NaCl. From the third day up to the end of the experiment, plants were daily irrigated with 150 mM NaCl. Leaves were harvested as following: day 0 (control without addition of NaCl), day 4, 6, and 12 (150 mM NaCl). Sampling was carried out in three pools of leaves (each pool contained a mixture of two pairs of leaves from three plants). Each pool represented a biological repetition.

In the heat stress treatment, we used leaves from nine coffee plants. Firstly, plants were maintained in the chamber for 7 days at 24 °C and controlled photoperiod (12 h:12 h) for acclimatization. In sequence, the chamber temperature was raised to 37 °C for 5 days. To avoid water deficit, plants were irrigated during all the heat stress period. Samples were collected at four evaluation stages: non-stressed control (plants maintained 7 days in growth chamber at 24 °C), day 1, 3, and 5 (plants maintained at 37 °C). Leaf sampling was also held in pools, where each of the three pools contained a mixture of two leaf pairs from three plants under the same stress conditions. Again, each pool represented a biological repetition. For both salt and heat stress, only the second pair of leaves at the same developmental stage, of each plagiotropic branch was collected and were immediately immersed in liquid N_2 and stored at −80 °C until the assays were performed.

RNA Isolation, Purification and cDNA Synthesis

Total RNA from *C. arabica* cv. IAPAR-59 leaves and roots (in the case of N starvation) was isolated as described by Chang et al. [20]. RNA samples were purified using the Pure Link Micro to Midi Total RNA Purification System (Invitrogen) and their integrity was examined by 1 % agarose gel electrophoresis and treated with DNase (RNase-free). RNA concentration and purity were determined using a NanoDrop® ND-100 spectrophotometer and the absence of genomic DNA contamination was confirmed by PCR using *GAPDH* primers and about 100 ng of RNA (data not shown). Complementary DNA (cDNA) was synthesized by a SuperScript III Reverse Transcriptase (Invitrogen) following the manufacturer's instructions in a final volume of 20 µl using 5 µg of total RNA. The final cDNA products were diluted tenfold prior to use in qPCR.

Reference Genes Selection, Primer Design and Amplification Efficiency

A set of ten potential reference genes, including large ribosomal subunit 39 (*RPL39*), polyubiquitin 10 (*UBQ10*), ribosomal protein S24 (*S24*), glyceraldehyde-3-phosphate-dehydrogenase (*GAPDH*), *β*-tubulin (*β-TUB*), tonoplast intrinsic protein (*TIP41*), elongation factor 1 (*EF1*), elongation factor 1-alpha (*EF1α*), RNA polymerase-II transcription factor (*RPII*) and malate dehydrogenase (*MDH*), were chosen to assess their stability as reliable reference genes. For *RPL39*, *UBQ10*, *S24*, and *GAPDH*, we used primers pairs already reported in previous studies in *Coffea* [13, 21, 22], having Tm of 60 °C ± 1 °C and amplicon length between 69 and 100 bp. We associated previously reported primers based on private database sequences to public data from the Brazilian Coffee Genome Project Consortium [23] (Table 1).

In the case of *β-TUB*, *TIP41*, *EF1*, *EF1α*, *RPII*, and *MDH*, genes were selected from *C.arabica* EST sequences developed by the Brazilian Coffee Genome Project Consortium [23] (Table 1). For these genes, primers were designed using Primer Express software (version 3.0) according to parameters established to obtain amplicons of 100 bp with a Tm of 60 °C ± 1 °C (Table 1). Specific products and amplicon length of all primer pairs were verified in 1 % agarose gel electrophoresis with sodium boric acid (SB) buffer (0.5 M NaOH, pH adjusted to 8.5 with boric acid) (Supplementary Material 1).

The efficiency of each primer pair was calculated according to a previous work from our group [24], using a standard curve generated from a serial dilution of cDNA using $E = [10^{(-1/\text{slope})} - 1]$. Four serial dilutions were set up to determine cycle threshold (C_t) values, designated here as quantification cycle (C_q), and the reaction

Table 1 Description of genes and primer sequences used for qPCR

Gene	Abbreviation	Primer forward	Primer reverse	Efficiency (%)	NCBI accession	Arabidopsis ortholog
Large ribosomal subunit 39	*RPL39*	GAACAGGCCCATCCCTTATTG	CGGCGCTTGGCATTGTA	104	GT720707.1	AT4G31985
Polyubiquitin 10	*UBQ10*	CAGACCAGCAGAGGCTGATT	AGAACCAAGTGAAGGGTGGA	92	GT697658.1	AT4G05320
Ribosomal protein S24	*S24*	GCCCAAATATCGGCTTATCA	TCTTCTTGGCCCTGTTCTTC	101	GT730897.1	AT3G04920
GAPDH	*GAPDH*	AGGCTGTTGGGAAAGTTCTTC	ACTGTTGGAACTCGGAATGC	99	GW488886.1	AT1G13440
β-tubulin	*β-TUB*	GTGCCGGTAATAACTGGGCTAA	CAGTCACAATTTTCGGCTTCTTT	99	GT707405.1	AT1G75780
Tonoplast intrinsic protein	*TP41*	CTTCCAAAGCTTCCATCCTCAA	AAGGAGCTATGTCCGAAAATCATCT	102	GW454965.1	AT4G34270
Elongation factor 1	*EF1*	CTGTCCTTGATTGCCACACTTCT	CTTGGGCTCCTTCTCAAGCTC	95	GW484749.1	AT1G07920
Elongation factor 1-alpha	*EF1α*	AAGGGAGCTTCCAGCTTTACCT	TGTGAGAGGTGTGGCAGTCAA	96	GT708303.1	AT1G07940
RNA pol-II trans cription factor	*RPII*	AATGGAAGGCAGATCGTACCAG	TATGCTGGCATCAGTGTCGC	110	GT730193.1	AT2G15430
Malate dehydrogenase	*MDH*	CCTGATGTCAACCACGCAACT	GTGGTTATGAACTCTCCATTCAACC	98	GW464198.1	AT1G04410
Hemoglobin	*CaHb1*	GAACGCTCCATTGGAACAGAAC	CCAGCTTTCCGGAGTTGAAC	102	GW457930.1	AT2G16060

efficiencies for all primers pairs. Standard curves were generated for each primer pair using the C_t value versus the logarithm of each cDNA dilution factor.

qPCR and Data Analysis

The transcript abundance for each of the ten genes was analyzed by qPCR (7500 Fast Real-Time PCR System, Applied Biosystems) using the SYBR Green PCR Master Mix (Applied Biosystems). The reaction mixture contained 12.5 µl of 2× SYBR Green master mix, 1 µl of each primer (10 µM), 1 µl of cDNA 1:10 diluted, and Milli-Q water to a total volume of 25 µl. Thermal conditions were 95 °C for 10 min, followed by 40 cycles of 95 °C for 30 s and 60 °C for 60 s. Melting curves were analyzed to verify the presence of a single product including a negative control. All reactions were performed in triplicate for each of the three biological replicates in all treatments studied. Relative expression levels of the candidates reference genes were imported to geNorm PLUS [15] (Biogazelle, Ghent), NormFinder [16], and BestKeeper [17] analysis tools which were used as described in their respective manuals. In this work, we have followed the minimum information for publication of qPCR experiments (MIQE) according to Bustin et al. [25]. Additional information concerning the qPCR experiment can be found in the MIQE document (Supplementary material 2), that followed the recommendations of Privat et al. [26] in a large-scale transcriptional study in coffee.

Reference Gene Validation

In order to validate the reference gene, the relative expression level of *C. arabica* Hemoglobin1 (*CaHb1*) (Table 1), an ortholog of *Arabidopsis* Class I hemoglobin [27], was performed in all RNA samples using two stable reference genes and the most unstable, from each experimental condition, to demonstrate how the adoption of different reference genes can affect the normalization of the expression data for a gene of interest. The transcriptional activity of *CaHb1* was calculated using the $2^{-\Delta\Delta Ct}$ method [28].

Results

RNA Integrity and Amplification Specificity

The integrity and quality of RNA samples were evaluated by electrophoretic analysis agarose gels stained with ethidium bromide. All samples analyzed showed clear 28S and 18S rRNA bands (data not shown), confirming that the RNA samples were appropriate for transcript level analysis. The average yield of RNA extractions were 25.5 µg/g of fresh tissue (ft) for salt stressed leaves, 10.5 µg/g ft for heat-stressed leaves, 27.8 µg/g ft for N-starved leaves and 13.5 µg g/ft for N-starved lateral roots. As expected, RNA yield of lateral roots was lower than the obtained for leaves. Among leaves, the heat stress resulted in the lowest yield. The purity (ratio A_{260}/A_{280}) of the RNA samples used for the qPCR analysis was evaluated by spectrophotometry, and ranged from 1.7 to 2.1. Also, prior to carrying out qPCR reactions, the viability of all RNA samples was examined by PCR with the primer pair selected for each reference gene (Table 1). All the PCR reactions produced a single specific fragment with the predicted molecular size on agarose gel visualized with ethidium bromide staining. Also, using each primer pair, gene-specific amplification was confirmed by the appearance of a single peak in melting curve analyses. The amplification efficiencies ranged from 92 to 110 % (Table 1).

Expression Profiles of Reference Genes

To reveal the differences in transcript expression levels between the studied genes, the quantification cycle (C_q) values were determined for each gene across all of the experimental samples and scatter plots were constructed (Fig. 1). We observed different C_q values from qPCR amplifications, with the lowest mean C_q value (20.13) in *RPL39* and the highest (36.05) in *RPII*. Considering all the stresses, C_q values for many reference genes showed rather large standard deviation (SD), which was due to the large number of biological samples and in accordance with similar studies in other plant species [5, 6]. The smallest variation in gene expression is observed for *RPL39* and *S24*

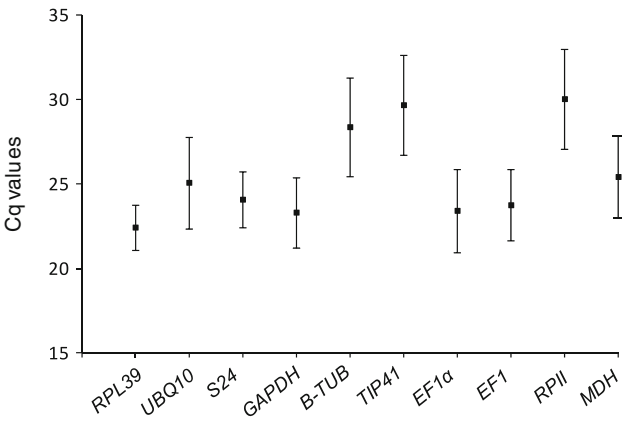

Fig. 1 Transcriptional levels of *Coffea* candidate reference genes in nitrogen starvation, heat, and salt stress. Expression levels of candidate reference genes in different experimental samples. Values are given as quantification cycle (C_q) in 42 samples. *Bars* indicate standard deviations

(lower than 2 cycles), while *β-TUB* and *TIP41* were the genes with the most variable levels inter-run C_q variation (over 2.9 cycles).

Evaluation of Expression Stability

The expression stability of 10 potential reference genes was evaluated in a set of 42 cDNA samples that included leaves of plants under three abiotic stresses (N starvation, salt stress and heat shock) and roots of N-starved plants. Altogether, our results showed that geNorm PLUS, NormFinder, and BestKeeper algorithms obtained, in most cases, to the same results concerning the best candidate reference gene to use for expression normalization for at least one reference gene.

geNorm PLUS Analysis

Gene expression stability (M) of the ten studied reference genes was calculated using geNorm PLUS software [15] (Fig. 2). This program recommends using an M value below the threshold of 1.5 to identify reference genes with stable expression. *MDH* (0.104) and *GAPDH* (0.113) had the highest expression stability in leaves during N starvation (Fig. 2a) while in roots the best reference genes were *GAPDH* (0.179) and *EF1* (0.180) (Fig. 2b). In salt stress, *EF1* (0.469) and *EF1α* (0.493) appeared among the best candidates (Fig. 2c), while during the heat stress the best reference genes were *MDH* (0.216) and *EF1α* (0.241) (Fig. 2d). Considering the three first reference genes (more stable) and the three last (more unstable) from each condition, GeNorm classified *EF1* and *EF1α* as the most stable reference genes and *RPL39* and *RPII* as the least stable.

Several works use one single gene as an internal control for normalization, even in *C. arabica* gene expression studies [11, 29], but it has been suggested that the use of two or more reference genes for normalization might produce more reliable results [30]. To determine the optimal number of reference genes, geNorm calculates the pairwise variation V_n/V_{n+1} between two sequential normalization factors N_{Fn} and N_{Fn+1} that contain an increasing number of reference genes. Vandesompele et al. [15] recommended 0.15 as a cutoff value for the variation below which the inclusion of an additional reference gene is not required. However, this threshold should not be an absolute cutoff value, because it will depend on the data [31]. In three of the four experimental conditions (N-starved leaves—Fig. 3a; N-starved roots—Fig. 3b, and heat stress—Fig. 3d), $V_{2/3}$ was already bellow the cutoff value, implying that the use of the two best reference genes is sufficient to

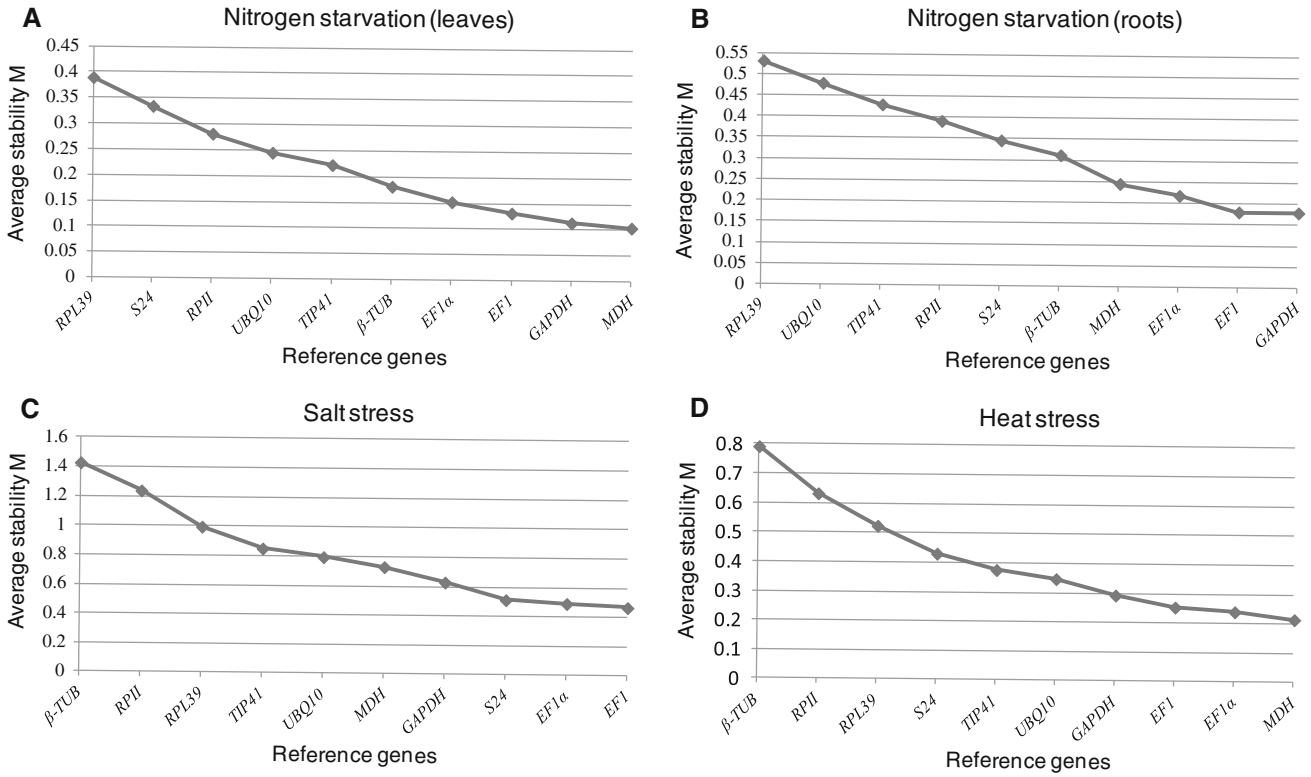

Fig. 2 Gene expression stability and ranking of the ten reference genes as calculated by geNorm PLUS (*M* values). A lower *M* value indicates more stable expression. Nitrogen starvation (leaves) (**a**), nitrogen starvation (roots) (**b**), salt stress (**c**), and heat stress (**d**)

reliable gene expression normalization of target genes. On the other hand, salt stress displayed a recommended variation cutoff value only at $V_{5/6,}$ indicating that five genes are the ideal set for data normalization (Fig. 3c).

NormFinder Analysis

NormFinder ranks the set of candidate reference genes on a model-based approach and generates a stability measure of which a lower value indicates increased stability in gene expression [16]. In the N starvation experiment, the best stabilities assigned by the applet for reference genes in leaves were *EF1* and *EF1α* (0.046 and 0.056, respectively), and the best combination of two genes was *EF1α* and *MDH*, which lead the stability value to 0.025, indicating a more reliable normalization than that based on single genes (Table 2). During N starvation using root tissues, *GAPDH* and *EF1* were the most stable reference genes, with stability values of 0.102 and 0.106, respectively. The best combination was *GAPDH* and *β-TUB* (0.061).

Normfinder ranked for salt stress *UBQ10* (0.074) and *EF1* (0.164) as the most stable reference genes, which were also the best combination (0.092). Heat stress showed *GAPDH* (0.118) and *EF1α* (0.237) with the most constant expression; these genes were also the best combination (0.149). Considering all treatments, *EF1* appeared more frequently among the most stable genes and *RPL39* and *RPII* were always among the least suitable reference genes.

BestKeeper Analysis

This tool estimates gene expression stability for all individual reference genes based on coefficient of correlation (r), standard deviation (SD), and percentage covariance (CV). It is important to emphasize that BestKeeper uses C_t values as input data instead of the raw data (relative quantities) used by geNorm and NormFinder and consequently may lead to misinterpretation related to the lack of correction for intersample/intercondition variation, especially when transcriptional variation among reference genes is greater than twofold (SD > 1.0) [17]. Here, the criteria analyzed for gene expression stability were r values over 0.95 and SD < 1.0. Additional descriptive statistics of

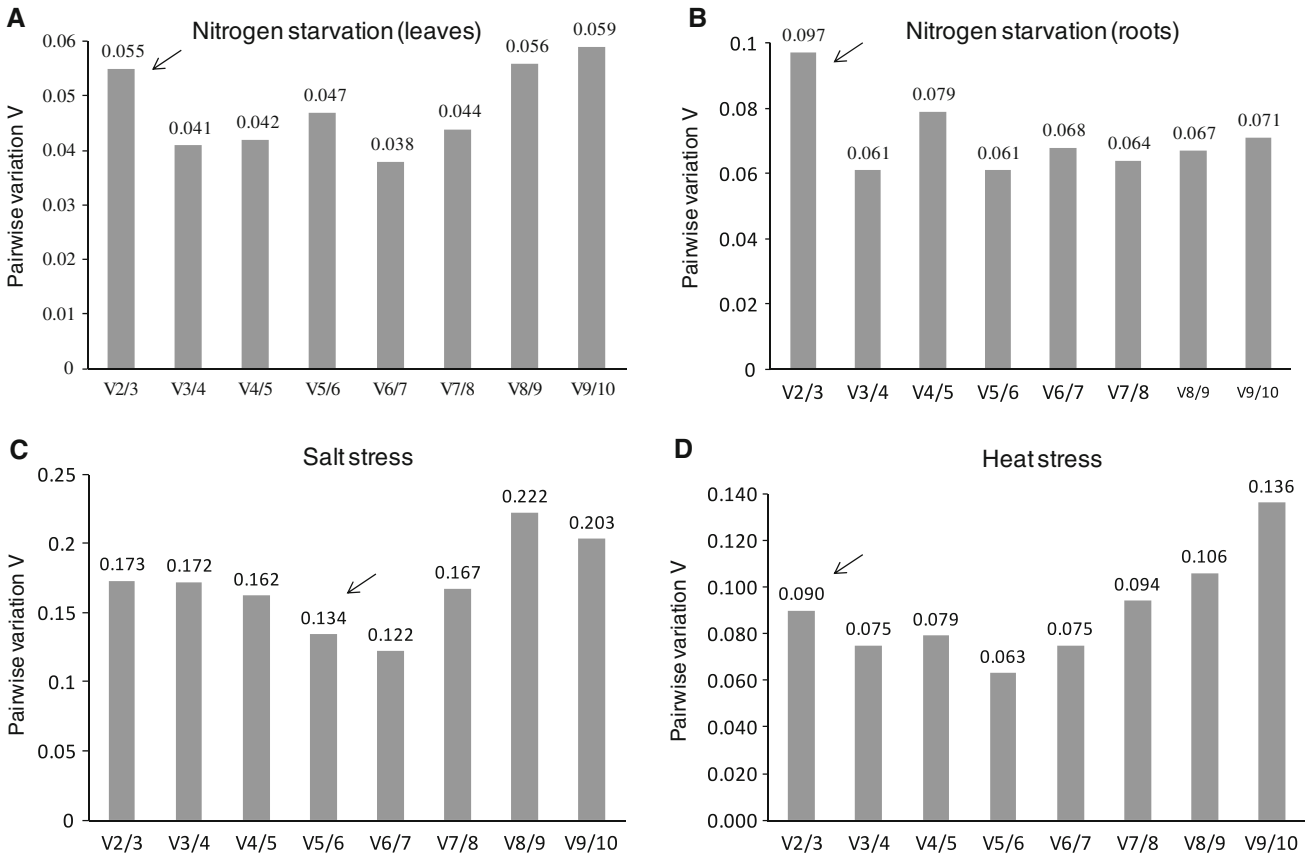

Fig. 3 Pairwise variation (*V*) analysis of the candidate reference genes. The pairwise variation (V_n/V_{n+1}) was analyzed between the normalization factors N_{Fn} and N_{Fn+1} using geNorm PLUS to determine the optimal number of reference genes required for qPCR data normalization in each condition. Nitrogen starvation (leaves) (**a**), nitrogen starvation (roots) (**b**), salt stress (**c**), and heat stress (**d**). *Arrow* indicates the optimal number of genes for normalization in each sample sets

Table 2 Expression stability values in four different conditions calculated by NormFinder software

Rank	N starvation (leaves)		N starvation (roots)		Salt stress		Heat stress	
	Gene	Stability	Gene	Stability	Gene	Stability	Gene	Stability
1	*EF1*	0.046	*GAPDH*	0.102	*UBQ10*	0.074	*GAPDH*	0.118
2	*EF1α*	0.056	*EF1*	0.106	*EF1*	0.164	*EF1α*	0.237
3	*MDH*	0.084	*β-TUB*	0.116	*EF1α*	0.199	*UBQ10*	0.239
4	*GAPDH*	0.093	*EF1α*	0.187	*S24*	0.238	*MDH*	0.256
5	*β-TUB*	0.117	*S24*	0.193	*GAPDH*	0.275	*S24*	0.318
6	*UBQ10*	0.161	*RPII*	0.230	*MDH*	0.285	*TIP41*	0.328
7	*TIP41*	0.223	*MDH*	0.239	*TIP41*	0.303	*EF1*	0.337
8	*S24*	0.302	*TIP41*	0.242	*RPL39*	0.488	*RPL39*	0.468
9	*RPII*	0.328	*UBQ10*	0.344	*β-TUB*	0.684	*RPII*	0.531
10	*RPL39*	0.343	*RPL39*	0.347	*RPII*	0.725	*β-TUB*	0.784
BCTG[a]	EF1α and MDH	0.025	GAPDH and β-TUB	0.061	UBQ10 and EF1	0.092	GAPDH and EF1α	0.149

[a] Best combination of two genes

the BestKeeper analyses are provided in Supplementary Material 3.

In N-starved leaves, *EF1* ($r = 0.998$; SD = 0.91) and *EF1α* ($r = 0.996$; SD = 0.96) were the most stable reference genes based on higher *r* values and lower CV values. Also, in this condition all of the ten candidates presented r values above 0.97 and most of them displayed low SD (only *UBQ10*, *TIP41* and *RPII* had SD > 1). In N-starved roots, *GAPDH* ($r = 0.996$; SD = 0.96) and *EF1* ($r = 0.994$; SD = 0.98) were the most stable genes considering the criteria described above (Supplementary material 3). During salt stress, *UBQ10* and *EF1α* had the most constant transcription levels ($r > 0.97$) and in heat stressed leaves, *MDH* and *GAPDH* were the most stable reference genes ($r > 0.98$), but in both cases standard deviation was above 1 (Supplementary Material 3).

Reference Gene Validation

To assess the validity of the candidate reference genes, the transcriptional pattern of a target gene, non-symbiotic hemoglobin *CaHb1* [27], under different experimental conditions, including N starvation in leaves and roots, salt and heat stress, were analyzed using the two new recommended reference genes (*EF1* and *MDH*), and the most unstable gene for normalization (*RPL39*). *CaHb1* transcriptional profile showed that mRNA levels of this gene was upregulated in short-term in N-starved leaves (day 1) while in N-starved roots *CaHb1*was downregulated. During salt stress transcriptional levels of *CaHb1*was short-term upregulated (day 4) and in heat stress mRNA levels showed little variation (Fig. 4). Unlike *RPL39*, similar expression patterns were observed when *EF1* and *MDH* were used for normalization of *CaHb1* (Fig. 4), showing their expression stability and capability to generate reliable results of relative expression levels.

Discussion

Coffee (*Coffea* ssp.) is among the main commodities commercialized in the world and it has been target for many studies involving gene expression by qPCR [11, 12, 29]. This technique is widely applied for accurate and sensitive quantification of gene transcript levels, even for those genes for which transcript levels are low [32]. The accuracy of qPCR is strongly influenced by stability of the internal reference genes used for data normalization. It is advisable to validate the expression stability of candidate reference genes under specific experimental conditions rather than using reference genes published elsewhere aiming to avoid erroneous analysis [33]. Several studies have shown that the expression of the commonly used reference genes is not always stably expressed when tested in other species or in a wider range of experimental treatments [6, 34]. A few studies identified candidate reference genes in *C. arabica* for some experimental conditions [13, 14] but, to our knowledge, this work represents the first effort in qPCR studies of experiments involving N starvation, salt and heat stress conditions in *Coffea.*

In order to select suitable reference genes, we used geNorm PLUS, BestKeeper, and NormFinder algorithms to analyze the expression stability of ten potential reference genes, six of them evaluated for the first time in coffee. Results obtained here indicate that none of the ten genes analyzed had a constant expression level in all conditions, which is common in plant studies [23, 35]. This may be partially explained by the fact that reference genes are not

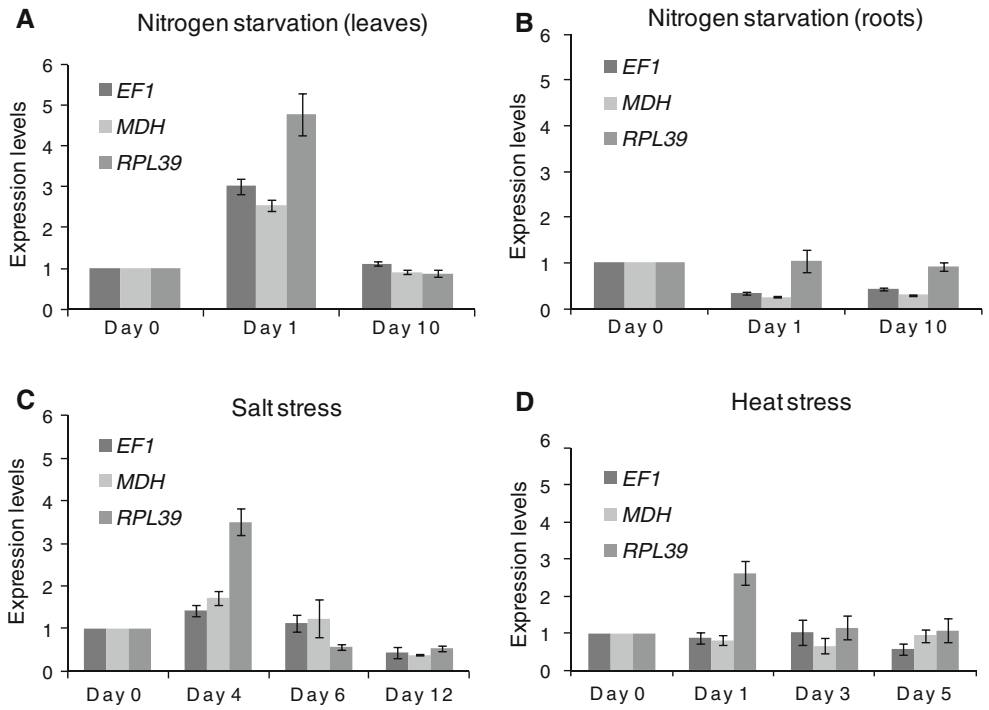

Fig. 4 Hemoglobin transcriptional pattern. Hemoglobin (*CaHb1*) expression profile in nitrogen starved leaves (**a**), nitrogen starved roots (**b**), salt stress (**c**), and heat stress (**d**) normalized with two stable reference genes (*EF1* and *MDH*) and the most unstable reference gene (*RPL39*). Values are represented by means ± SE ($n = 3$)

only implicated in the basal cell metabolism but also participate in other cellular functions [36]. The discrepancy in the expression of the ten potential reference genes in different samples reinforces the importance in selecting an appropriate reference gene to normalize gene expression under specific experimental conditions. Even the most stable reference gene(s) reported in a given species should be validated in a new experimental set-up or when using other genotype/species [6].

Reference Gene Stability

In general, the most stable and unstable genes were almost the same for all used programs. *EF1*, *EF1α*, *GAPDH*, *MDH*, and *UBQ10* were usually ranked as the most stable genes regardless the software used or the treatment applied, while *RPL39* and *RPII* were usually classified as the most unstable. The differences observed in ranking position are expected because the programs are based on distinct statistical algorithms: geNorm calculates gene expression stability based on its average pairwise expression ratio, whereas Normfinder estimate the overall expression variation of the candidate reference genes to provide a stability value for each gene and BestKeeper considers the least variation of a single reference gene [31]. According to Andersen et al. [16] coregulated reference genes should be avoided when using multiple references because they may bias the results and geNorm may be insensitive to these cases. Once we observed that *EF1* and *EF1α* were commonly ranked together as the most suitable reference genes, we checked eventual coregulation between these genes removing *EF1* from our analysis, in a strategy analog to Lovdal and Lillo [37] in tomato. There was not any significant difference in the ranking in the four analyzed conditions. In N-starved leaves, no changes in ranking position were observed, while in roots the three most stable reference genes changed only their relative position (*EF1α*, *MDH* and *GAPDH*, in decreasing order from the most stable). In salinity *EF1α* and *S24* changed positions with each other and in heat stress treatment *EF1α* and *MDH* also changed positions. This result shows that the potential coregulation of *EF1* and *EF1α* did not significantly affect the ranking of reference genes.

The best combination of reference genes to reach the appropriate normalization factor in Normfinder is not necessarily the one containing the most stable genes according to a gene-by-gene comparison [13], which can explain the fact that, in some cases, using the best ranked gene alone is better than the combination of two best genes (stability value is lower).

Although all three softwares produced slightly diverse rankings of stability, the set of most reliable genes was similar, with the main differences being observed in the intermediate ranking positions. This inconsistency among

softwares results has already been observed and shows that the choice of the program represents a crucial but difficult task [38, 39]. Despite the differences observed in ranking position from one algorithm to another, geNorm presented values below 1.5 in all of the tested reference genes, showing the potential of the genes to maintain a constancy in their expression profile [15]. In N starvation and heat stress, we observed high stability (average geNorm $0.2 \leq M \leq 0.5$), which is typically seen in a homogeneous set of samples, while intermediate reference gene stability (average geNorm $M \leq 1.0$) was observed in salt stress. The results through pairwise variation (V values) analysis showed that only salt stress condition required five genes for a better normalization data, whereas only two genes were sufficient for the other three experimental conditions.

The same stability patterns were observed in all analyzed conditions using geNorm PLUS and NormFinder, where salt stress also showed the most inconsistent values with higher variation among samples. BestKeeper results might be considered only for N starvation, where r was over 0.95 and SD was below 1 for most primer pairs. The two most suitable reference genes selected by geNorm PLUS, Normfinder and BestKeeper (only for N stress) are summarized in Table 3.

We found that *RPII* was among the least stable reference genes in all conditions used in this work. Similar findings were reported in tomato [40], *Arabidopsis* [35], and cucumber [31]. However, Zhong et al. [32] and Tong et al. [41] observed that *RPII* showed better expression stability in different tissues in litchi and peach. *TIP41* and *S24* were also poorly ranked, but they were among the best reference genes in studies realized by Cruz et al. [13] and Migocka and Papierniak [37] in coffee and cucumber, respectively. Considering several plant species, *β-TUB* yielded contrasting results, with high relative expression variability in litchi [32], *Arabidopsis* [35], in tomato [37] and in many other different organisms and experimental contexts [8, 42]. However, in studies with wheat [43] and pea [44] *β-TUB* was identified as the most stable reference gene. In our case, the fact that the ranking of this gene varied from intermediate to the least stable reference gene depending to experimental condition indicates that *β-TUB* is not suitable for normalization.

Table 3 The most suitable reference genes selected by geNorm PLUS, NormFinder, and BestKeeper in all analyzed conditions

Conditions	geNorm PLUS	Normfinder	Bestkeeper[a]
N starvation (leaves)	*MDH*	*EF1*	*EF1*
	GAPDH	*EF1α*	*EF1α*
N starvation (roots)	*GAPDH*	*GAPDH*	*GAPDH*
	EF1	*EF1*	*EF1*
Salt stress	*EF1*	*UBQ10*	[b]
	EF1α	*EF1*	[b]
Heat stress	*MDH*	*GAPDH*	[b]
	EF1α	*EF1α*	[b]

[a] Primers with $r > 0.95$ and SD < 1.0

[b] No primer pair was suitable with $r > 0.95$ and SD < 1.0

EF1α, *UBQ10*, and *GAPDH* are among the most commonly used reference genes. These three genes were also indicated as appropriate reference genes in this study. *EF1α*, often described as a stable gene used as a reference in many species [31, 32, 42, 45], was ranked as a suitable reference genes in N starvation conditions. Similar result was observed by Lovdal and Lillo [37] in tomato, while in *Arabidopsis EF1α* was never represented among the 100 most stably expressed genes [46]. Studies carried out in coffee by Cruz et al. [13] considered *GAPDH* and *UBQ10* the most appropriate reference genes. Even though these two genes are constantly described as reliable internal control in several studies [32, 40, 47], including coffee [14], in some conditions their expression profiles can vary [44, 48]. The reasons that may lead to fluctuations in reference gene expression may be found not only in the activation their respective activities but also in their active roles in other processes as well [44].

Two potential reference genes (*MDH* and *EF1*) that are not commonly used in expression normalization were classified as highly stable in almost every condition by all methods. *MDH* was better ranked by geNorm PLUS in N starved leaves and heat stressed plants. This reference gene had been only analyzed by Reid et al. [42] in grapevine fruits, where *MDH* did not present a good performance. This might be attributed to the fact that coffee species exhibit all the physiological and biochemical characteristics of plants with predominantly C3 photosynthetic pathways [49], where *MDH* transcriptional activity is low and stable [50]. Our results also showed that some recommended reference genes for *Coffea* expression studies were not suitable in the specific conditions used in this work. For instance, *RPL39* has been extensively used as an internal control when comparing gene expression profiles of target genes among different coffee organs [51, 52], in coffee cell cultures [21] and also when comparing control and drought-stressed leaves [51]. Our work revealed that *RPL39* is not the most accurate reference gene since it was poorly ranked compared to other genes.

Reference Gene Validation

To demonstrate the need for accurate relative quantification using suitable reference genes, the expression of the *C. arabica* Hemoglobin1 (*CaHb1*) gene was studied. *CaHb1* is an ortholog of *Arabidopsis* Class I hemoglobin. Hemoglobins (Hbs) are heme containing proteins found in

most organisms related to oxygen sequestration, and they are one of many different strategies that plants have evolved to overcome stress conditions and survive [27]. In *Arabidopsis*, hemoglobin 1 RNA blot analysis demonstrated that this gene is upregulated by cold and drought stress in leaves, but it was not possible to detect transcriptional activity in control and heat-stressed plants [53]. In *Alnus firma*, Hb1 is lowly expressed in leaves and is also transcriptionally active under different N sources [54].

Using the two new recommended reference genes (*EF1* and *MDH*), we obtained similar expression patterns in all experimental conditions. On the other hand, the normalized expression levels illustrate that *CaHb1* is significantly up regulated when using *RPL39* in comparison to *EF1* and *MDH*. Misleading results obtained by the utilization of a non suitable reference gene have been also observed in other studies [32, 55]. Our results reinforces the importance of validating reference genes to ensure that low precision or misleading results did not occur, especially with genes with low transcript abundance in normal physiological conditions, like Hbs.

In conclusion, the use of three different statistical algorithms allowed the identification of different combinations of reference genes for qPCR expression data normalization in *C. arabica* plants subjected to abiotic stresses. The profiling of the gene expression pattern of 10 putative reference genes showed that *MDH*, *GAPDH*, *EF1* and *EF1α* reference genes can be used in N starvation experiments using leaves as the analyzed tissue, while *GAPDH* and *EF1* can be used in N-starved roots. For salt stress treatments *EF1*, *EF1α*, and *UBQ10* were selected as the most suitable reference genes and *MDH*, *EF1α*, and *GAPDH* are the best choice for heat stress conditions.

To our knowledge, this is the first work regarding the selection of reliable reference genes for transcriptional studies in coffee plants under N starvation, salt and heat stress. In addition, we recommended two additional potential reference genes that are not commonly used as internal control for normalization of qPCR data (*MDH* and *EF1*).

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References

1. Watson, K., & Achinelli, M. L. (2008). Context and contingency: The coffee crisis for conventional small-scale coffee farmers in Brazil. *The Geographical Journal, 174*(3), 223–234.
2. Roy, S. J., Tucker, E. J., & Tester, M. (2011). Genetic analysis of abiotic stress tolerance in crops. *Current Opinion in Plant Biology, 14*, 232–239.
3. Tester, M., & Langridge, P. (2010). Breeding technologies to increase crop production in a changing world. *Science, 327*, 818–822.
4. Kodaira, K. S., Qin, F., Tran, L. S. P., et al. (2011). Arabidopsis C2H2 zinc-finger proteins AZF1 and AZF2 negatively regulate ABA-repressive and auxin-inducible genes under abiotic stress conditions. *Plant Physiology, 157*(2), 742–756.
5. Tillett, R. L., Ergü, A., Albion, R. L., et al. (2011). Identification of tissue-specific, abiotic stress responsive gene expression patterns in wine grape (*Vitis vinifera* L.) based on curation and mining of large-scale EST data sets. *BMC Plant Biology, 11*, 86.
6. Chen, L., Zhong, H., Kuang, J., et al. (2011). Validation of reference genes for RT-qPCR studies of gene expression in banana fruit under different experimental conditions. *Planta, 234*, 377–390.
7. Guénin, S., Mauriat, M., Pelloux, J., et al. (2009). Normalization of qRT-PCR data: The necessity of adopting a systematic, experimental conditions-specific, validation of references. *Journal of Experimental Botany, 60*, 487–493.
8. Artico, S., Nardeli, S. M., Brilhante, O., et al. (2010). Identification and evaluation of new reference genes in *Gossypium hirsutum* for accurate normalization of real-time quantitative RT-PCR data. *BMC Plant Biology, 10*, 49.
9. Gu, C., Chen, S., Liu, Z., et al. (2011). Reference gene selection for quantitative real-time PCR in chrysanthemum subjected to biotic and abiotic stress. *Molecular Biotechnology, 49*, 192–197.
10. Fernandez, P., Rienzo, J. A., Moschen, S., et al. (2010). Comparison of predictive methods and biological validation for qPCR reference genes in sunflower leaf senescence transcript analysis. *Plant Cell Reports, 30*(1), 63–74.
11. Salmona, J., Dussert, S., Descroix, F., et al. (2008). Deciphering transcriptional networks that govern *Coffea arabica* seed development using combined cDNA array and real-time RT-PCR approaches. *Plant Molecular Biology, 66*, 105–124.
12. Nobile, P. M., Quecini, V., Bazzo, B., et al. (2010). Transcriptional profile of genes involved in the biosynthesis of phytate and ferritin in *Coffea*. *Journal of Agricultural and Food Chemistry, 58*(6), 3479–3487.
13. Cruz, F., Kalaoun, S., Nobile, P., et al. (2009). Evaluation of coffee reference genes for relative expression studies by quantitative real-time RT-PCR. *Molecular Breeding, 23*(4), 607–616.
14. Barsalobres-Cavallari, C. F., Severino, F. E., Maluf, M. P., & Maia, I. G. (2009). Identification of suitable internal control genes for expression studies in *Coffea arabica* under different experimental conditions. *BMC Molecular Biology, 10*(1), 1.
15. Vandesompele, J., De Preter, K., Pattyn, F., et al. (2002). Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biology, 3*, 34.
16. Andersen, C. L., Jensen, J. L., & Orntoft, T. F. (2004). Normalization of real-time quantitative reverse transcription-PCR data: A model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets. *Cancer Research, 64*, 5245–5250.
17. Pfaffl, M. W., Tichopad, A., Prgomet, C., & Neuvians, T. P. (2004). Determination of stable housekeeping genes, differentially regulated target genes, and sample integrity: BestKeeper-Excel-based tool using pair-wise correlations. *Biotechnology Letters, 26*, 509–515.
18. Clark, R. B. (1975). Characterization of phosphatase of intact maize roots. *Journal of Agricultural and Food Chemistry, 23*, 458–460.
19. Dos Santos, T. B., Budzinski, I. G. F., Marur, C. J., et al. (2011). Expression of three *galactinol synthase* isoforms in *Coffea arabica* L. and accumulation of raffinose and stachyose in response to abiotic stresses. *Plant Physiology and Biochemistry, 49*, 441–448.

20. Chang, S., Puryear, J., & Cairney, J. (1993). A simple and efficient method for isolating RNA from pine trees. *Plant Molecular Biology Reporter, 11*, 113–116.
21. Bottcher, A., Nobile, P. M., Martins, P. F., Conte, F. F., Azevedo, R. A., & Mazzafera, P. (2011). A role for ferritin in the antioxidant system in coffee cell cultures. *BioMetals, 24*(2), 225–237.
22. Simkin, A. J., Qian, T. Z., Caillet, V., Michoux, F., Ben Amor, M., Lin, C. W., et al. (2006). Oleosin gene family of *Coffea canephora*: Quantitative expression analysis of five oleosin genes in developing and germinating coffee grain. *Journal of Plant Physiology, 163*, 691–708.
23. Mondego, J. M. C., Vidal, R. O., Carazzolle, M. F., et al. (2011). An EST-based analysis identifies new genes and reveals distinctive gene expression features of *Coffea arabica* and *Coffea canephora*. *BMC Plant Biology, 11*, 30.
24. Carvalho, K., Campos, M. K. F., Pereira, L. F. P., & Vieira, L. G. E. (2010). Reference gene selection for real-time quantitative polymerase chain reaction normalization in "Swingle" citrumelo under drought stress. *Analytical Biochemistry, 402*, 197–199.
25. Bustin, S. A., Benes, V., Garson, J. A., et al. (2009). The MIQE guidelines: minimum information for publication of quantitative real time PCR experiments. *Clinical Chemistry, 55*, 611–622.
26. Privat, I., Bardi, A., Gomez, A. B., Severac, D., Dantec, C., Fuentes, I., et al. (2011). The 'PUCE CAFE' project: The first 15 K coffee microarray, a new tool for discovering candidate genes correlated to agronomic and quality traits. *BMC Genomics, 12*, 5.
27. Dordas, C., Hasinoff, B. B., Rivoal, J., & Hill, R. D. (2004). Class-1 hemoglobins, nitrate and NO levels in anoxic maize cell-suspension cultures. *Planta, 219*, 66–72.
28. Livak, J. K., & Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta Ct}$ method. *Methods, 25*, 402–408.
29. Oliveira, R. R., Chalfun-Junior, A., Paiva, L. V., & Andrade, A. C. (2010). In silico and quantitative analyses of MADS-Box genes in *Coffea arabica*. *Plant Molecular Biology Reporter, 28*, 460–472.
30. Lin, Y. L., & Lai, Z. X. (2010). Reference gene selection for qPCR analysis during somatic embryogenesis in longan tree. *Plant Science, 178*, 359–365.
31. Wan, H., Zhao, Z., Qian, C., et al. (2010). Selection of appropriate reference genes for gene expression studies by quantitative real-time polymerase chain reaction in cucumber. *Analytical Biochemistry, 399*, 257–261.
32. Zhong, H. Y., Chen, J. W., Li, C. Q., et al. (2011). Selection of reliable reference genes for expression studies by reverse transcription quantitative real-time PCR in litchi under different experimental conditions. *Plant Cell Reports, 30*, 641–653.
33. Lee, J. M., Roche, J. R., Donaghy, D. J., et al. (2010). Validation of reference genes for quantitative RT-PCR studies of gene expression in perennial ryegrass (*Lolium perenne* L.). *BMC Molecular Biology, 11*, 8.
34. Silveira, E. D., Alves-Ferreira, M., Guimaraes, L. A., et al. (2009). Selection of reference genes for quantitative realtime PCR expression studies in the apomictic and sexual grass *Brachiaria brizantha*. *BMC Plant Biology, 9*, 84.
35. Gutierrez, L., Mauriat, M., Guénin, S., et al. (2008). The lack of a systematic validation of reference genes: a serious pitfall undervalued in reverse transcription polymerase chain reaction (RT-PCR) analysis in plants. *Plant Biotechnology Journal, 6*, 609–618.
36. Singh, R., & Green, M. R. (1993). Sequence-specific binding of transfer RNA by glyceraldehyde-3-phosphate dehydrogenase. *Science, 259*(5093), 365–368.
37. Lovdal, T., & Lillo, C. (2009). Reference gene selection for quantitative real-time PCR normalization in tomato subjected to nitrogen, cold, and light stress. *Analytical Biochemistry, 387*, 238–242.
38. Migocka, M., & Papierniak, A. (2011). Identification of suitable reference genes for studying gene expression in cucumber plants subjected to abiotic stress and growth regulators. *Molecular Breeding, 28*, 343–357.
39. Jian, B., Liu, B., Bi, Y., et al. (2008). Validation of internal control for gene expression study in soybean by quantitative real-time PCR. *BMC Molecular Biology, 9*, 59.
40. Exposito-Rodriguez, M., Borges, A. A., Borges-Perez, A., & Perez, J. A. (2008). Selection of internal control genes for quantitative real-time RT-PCR studies during tomato development process. *BMC Plant Biology, 8*, 131.
41. Tong, Z., Gao, Z., Wang, F., et al. (2009). Selection of reliable reference genes for gene expression studies in peach using real-time PCR. *BMC Molecular Biology, 10*, 71.
42. Reid, K. E., Olsson, N., Schlosser, J., et al. (2006). An optimized grapevine RNA isolation procedure and statistical determination of reference genes for real-time RT-PCR during berry development. *BMC Plant Biology, 6*, 27–37.
43. Paolacci, A., Tanzarella, O., Porceddu, E., & Ciaffi, M. (2009). Identification and validation of reference genes for quantitative RT-PCR normalization in wheat. *BMC Molecular Biology, 10*(1), 11.
44. Die, J. V., Román, B., Nadal, S., & González-Verdejo, C. I. (2010). Evaluation of candidate reference genes for expression studies in *Pisum sativum* under different experimental conditions. *Planta, 232*, 145–153.
45. Hong, S. Y., Seo, P. J., Yang, M. S., et al. (2008). Exploring valid reference genes for gene expression studies in *Brachypodium distachyon* by real-time PCR. *BMC Plant Biology, 8*, 112.
46. Czechowski, T., Stitt, M., Altmann, T., et al. (2005). Genome-wide identification and testing of superior reference genes for transcript normalization in Arabidopsis. *Plant Physiology, 139*(1), 5–17.
47. Iskandar, H. M., Simpson, R. S., Casu, R. E., et al. (2004). Comparison of reference genes for quantitative real time-PCR analysis of gene expression in sugarcane. *Plant Molecular Biology Reporter, 22*, 325–337.
48. Jain, M., Nijhawan, A., Tyagi, A. K., & Khurana, J. P. (2006). Validation of housekeeping genes as internal control for studying gene expression in rice by quantitative real-time PCR. *Biochemical and Biophysical Research Communications, 345*, 646–651.
49. Carelli, M. L. C., Queiroz-Voltan, R. B., Fahl, J. I., & Trivelin, P. C. O. (2003). Leaf anatomy and carbon isotope composition in *Coffea* species related to photosynthetic pathway. *Brazilian Journal of Plant Physiology, 15*(1), 19–24.
50. Freschi, L., Rodrigues, M. A., Domingues, D. S., et al. (2010). Nitric oxide mediates the hormonal control of Crassulacean acid metabolism expression in young pineapple plants. *Plant Physiology, 152*(4), 1971–1985.
51. Simkin, A. J., Moreau, H., Kuntz, M., et al. (2008). An investigation of carotenoid biosynthesis in *Coffea canephora* and *Coffea arabica*. *Journal of Plant Physiology, 165*, 1087–1106.
52. Pré, M., Caillet, V., Sobilo, J., & McCarthy, J. (2008). Characterization and expression analysis of genes directing galactomannan synthesis in coffee. *Annals of Botany, 102*, 207–220.
53. Trevaskis, B., Watts, R. A., Andersson, C. R., et al. (1997). Two hemoglobin genes in *Arabidopsis thaliana*: The evolutionary origins of leghemoglobins. *PNAS, 94*, 12230–12234.
54. Sasakura, F., Uchiumi, T., Shimoda, Y., et al. (2006). A class 1 hemoglobin gene from *Alnus firma* functions in symbiotic and non-symbiotic tissues to detoxify nitric oxide. *Molecular Plant-Microbe Interactions, 19*, 441–450.
55. Li, H., Qina, Y., Xiao, X., & Tanga, C. (2011). Screening of valid reference genes for real time RT-PCR data normalization in *Hevea brasiliensis* and expression validation of a sucrose transporter gene HbSUT3. *Plant Science, 181*, 132–139.