Expression of candidates genes for drought tolerance related to ABA signaling in roots of *C. Canephora*

T. S. COSTA¹, J. A. T. MELO², F. A. CARNEIRO², N. G. VIEIRA¹, E. C. S. RÊGO², E. A. LIMA², M. G. COTTA¹, P. MARRACCINI^{2,3}, A. C. ANDRADE²

¹ University of Lavras, Lavras, MG, BR
² Embrapa Genetic Resources and Biotechnology, Brasilia, DF, BR
³ CIRAD UMR AGAP, Montpellier, FR

Background

Coffee is a major agricultural commodity in the world and Brazil is the largest producer and second largest consumer. Drought is one of the main limiting factors to the national coffee production, so the selection of tolerant cultivars is of great importance, especially given the expansion of Brazilian coffee to areas subject to water stress [1]. Of the two species of higher marketing, Coffea canephora is best suited to the drier regions. Plant growth under drought conditions is influenced by changes in photosynthesis, respiration, translocation, ion absorption, metabolism of nutrients and hormones [2]. The ABA is a very important hormone in response to abiotic stresses, being synthetized in the roots and leaves. The aim of this study was to evaluate the expression of genes directly related to ABA biosynthesis. The results presented here concerned expression studies in roots of C. canephora Conilon submitted to drought of genes CcNCED3, CcPYL7 and CcPP2C-1, known to encode for key proteins implicated in biosynthesis, perception and transduction of ABA signaling pathway. Our study aims to evaluate the expression of candidate genes tolerance to drought and coding elements responsible for ABA biosynthesis, related to the perception and transduction pathway such as CcNCED3, CcPYL7 and CcPP2C-1 in roots of clones tolerant (n. 14, 73 and 120) and susceptible (n. 22) to drought of C. canephora Conilon grown under controlled conditions (greenhouse) with or without irrigation. For each clone and every condition, total RNA was extracted from roots and the transcriptome profiles was evaluated using 454 sequencing. For these three genes, expression analyses also performed by real-time qPCR to validate the gene expression levels obtained by RNA-Seq in silico. In roots of all clones analyzed, we showed that the expression of genes CcNCED3, CcPP2C-1 and CcPYL7 increased with water suspension, particularly to the drought-tolerant clone14.

Methods

Plant material

Roots were collected from drought susceptible (n° 22) and drought-tolerante (n° 14, 73 and 120) clones of *C. canephora* Conilon grown under controlled conditions

(greenhouse) with (I) and without (NI) irrigation as previously described [3]. Under NI condition, predawn leaf water potential (Ψ PD) corresponded to -3.0 MPa for all clones.

RNA isolation, sequencing and in silico analysis

For all clones and irrigation conditions, total RNAs were extracted using TRIzol® reagent Reagent (Life Technologies) according to manufacturer's instructions and treated with RQ1 RNase-Free Kit DNase (Promega) to remove traces of contaminating genomic DNA. cDNAs were synthesized (ImProm II, Promega) and the sequencing was performed by pirosequencing-454 (Roche). In silico gene expression analysis was performed using the Q-software package SeqDNAStar (Lasergene), based on the quantification of reads. For both, the cDNA sequences 25.574 resulting from the Structural Genome Project of *Coffea canephora* were used as a reference in the analysis. The data were normalized by RPKM [4].

Expression analysis by RT-qPCR

Os genes candidatos foram selecionados de acordo com o fator de indução (FI), calculado pelo quociente da expressão do gene, em cada clone, na condição NI e pela expressão do mesmo gene no respectivo clone na condição I [5]. Foram sintetizados pares de primers, que possibilitaram a validação da expressão gênica por qPCR.

The candidate genes were selected according to the induction factor (FI), calculated by the ratio of gene expression in each clone, NI and the expression of the same gene in its clone in the condition I have been synthesized pairs of primers, which enabled the validation by qPCR gene expression. The qPCR was performed with SYBR green fluorochrom (SYBRGreen qPCR Mix-UDG/ROX, Invitrogen) according to the manufacturer and using a FAST7500 apparatus (Applied Biosystems). For each sample, gene expression levels were standardized with the expression of CcUBQ10 gene (endogenous control of constitutive expression) coding for the ubiquitin protein [6]. Data were treated by SDS 2.0.1 program (Applied Biosystems). Expression levels were calculated with the Δ CT (CT [gene] - CT [CcUBQ10]) method and expression levels were expressed in relative quantification by calculating the values of 2- $\Delta\Delta$ CT. The primer pairs used were:

CcNCED3gene	CcNCED3-F CcNCED3-R	5'- GCCTGGGAAGAGCCTGAAAC -3' 5'- CCCCTCGTCACATTCATTGAA -3'
CcPP2C-1gene	CcPP2C-1-F CcPP2C-1-R	5'- ATGGCTTGTGGGATGTCATGA -3' 5'- CGTTCTTCTTGTGCCAAAGCA -3'
CcPYL7gene	CcPYL7-F CcPYL7-R	5'-GAGAAGCACATTCTTGGGGATCAA-3' 5'- GGATGCACGGTAAGGATGGA -3'

Results and conclusions

• FI of candidate genes

For genes analyzed in this work, the calculation of FI proves that these genes exhibit differential expression and more expressive in tolerant clones (Figure 1G).

• Study of CcNCED3 gene expression

In roots of all clones analyzed, in silico and qPCR experiments showed that CcNCED gene expression increased under drought compared to control (irrigation) condition. It is worth noting that this increase was particularly high for the drought-tolerant clone 14.

• Study of CcPP2C-1 gene expression

Once again, a relatively good correlation was observed for this gene between in silico (Figure 1C) and qPCR results (Figure 1D), demonstrating an increase of CcPP2C-1 gene expression under drought condition in roots of all clones analyzed. However, this increase under NI condition was higher for the clone 14 than for the others.

• Study of CcPYL7 gene expression

For CcPYL7 gene coding for the ABA receptor PYL 7, no great differences of expression were observed in silico between clones and irrigation conditions. However, qPCR experiments clearly revealed higher expression in clone 14 than in others, as well as an over-expression of this gene under NI condition (Figures 1E and 1F).

Altogether, our results showed that:

- it exits a relatively good correlation between in silico analyses and results of qPCR experiments for the three genes presented,

- the drought-tolerant clone 14 was the only one presenting highest levels of gene induction under water deficit (NI), highlighting the fact that abscisic acid signaling system is of great importance for the mechanisms of drought tolerance in coffee,

- CcNCED3 gene expression increase in roots of *C. canephora* Conilon under drought stress. This observation, also reported before in other plants [7, 8].



Figure 1. Expression profiles of transcripts that encode 9-cis-epoxycarotenoid dioxygenase 3 (A and B), Phosphatase 2 c (C and D) and receiver ABA PYL (E and F) for in silico (A, C and E) and qPCR analyses (B, D and F) in roots of clones of *C. canephora* 14, 73 and 120 (drought tolerant) and 22 (sensitive to drought) subjected to water stress (NI: no irrigated) and without stress (I:

Irrigated). For qPCR the relative expression (ER evaluated in arbitrary units) was compared to reference gene expression (constitutive expression) CcUBQ10. The calibrator used for normalization of qPCR data was the sample 14I (ER = 1).

For ABA Signaling in cells, recent studies indicate direct interaction between intracellular receptors of ABA (PYR/Pyl/RCARS) and type 2C protein phosphatases, inducing a cascade of signals that ensure plant tolerance to water stress [9]. From the results presented here for CcNCED3, CcPYL7 and CcPP2C-1genes analyzed, it is worth noting that highest induction of expression were observed in roots of 14 clone under drought stress. This suggested the participation of ABA-dependent pathways in controlling drought tolerance in this clone of *C. canephora* and also highlighted the fact different molecular mechanisms account for drought tolerance in *C. canephora* Conilon [10].

Acknowledgments

Federal University of Lavras – UFLA, Brazilian Ministry of Education, CAPES, Coffee Research Consortium, EMBRAPA genetic resources and biotechnology.

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