# Study of Drought-Tolerance Mechanisms in Coffee Plants by an Integrated Analysis

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## SUMMARY

The principal aim of this study was to investigate the molecular mechanisms underlying the response to drought stress in coffee plants by different approaches. In order to indentify candidate genes involved in controlling drought tolerance in coffee plants, different strategies were followed in our laboratories. The first used the nucleic data generated by the Brazilian Coffee EST project to identify candidate genes (CG) by *in silico* analysis (electronic Northern-blot). Differential expression of these CG was verified in leaves and roots from drought-tolerant and susceptible clones of *C. canephora* var. Conilon by Northern-blot and quantitative PCR experiments. The second was based on the screening of macroarray membranes spotted with coffee ESTs which were hybridized separately with leaf cDNA probes of the same clones. Finally, 2D gel electrophoresis was also performed to selected proteins presenting differential accumulation in leaves of the same clones. These proteins were analyzed by MALDI-TOF-MS/MS leading to the identification of a new set of CG. Results concerning the identification of CG by these different approaches are presented and discussed.

## INTRODUCTION

It is well known that drought periods affect coffee plant development and productivity (DaMatta and Ramalho, 2006). In case of severe drought, this could led to plant death and abortion of developing fruits but also affect flowering, bean development and consequently coffee bean quality, in case of moderate stress. As a consequence of elevation of temperature due to global warming, coffee growing geographical regions could suffer delocalization (Assad et al., 2004), leading environmental, economical and social problems. In such a context, the generation for drought-tolerant coffee varieties now turns one of the priorities of the coffee research mainly in Brazilian research institutions. The development of molecular tools generated by the recent advances in coffee genomic (Lin et al., 2005; Poncet et al., 2006 ; Vieira et al., 2006)] now opens the way to study the genetic determinism of drought-tolerance and the identification of molecular markers that could be used to speed up breeding programs (Lashermes et al., 2008).

We initiated the search of such markers by the *in silico* analysis of the Brazilian Coffee Genome project. The identification of CG was also performed using cDNA macroarrays and looking for CG underlying leaf expression profiles varying with water stress condition applied to coffee plants. Finally, protein profiles by 2-DE coupled with tryptic peptide identification by MALDI-TOF-MS/MS was also tested.

## MATERIAL AND METHODS

### Plant material

Clones of *C. canephora* var. Conillon tolerant (14, 73 and 120) and sensitive (22) to drought were selected by the INCAPER (Ferrão et al., 2000) and grown in greenhouse with (unstressed condition) or without (stress, $\Psi_{H2O}$ leaves = -3.0 MPa) water. Some of them were already subjected to several physiological and biochemical analyses (DaMatta et al., 2003; Lima et al., 2002; Pinheiro et al., 2004; 2005; Praxedes et al., 2006). Leaves were collected, frozen in liquid nitrogen and further used for expression analyses.

### In silico identification of candidate genes (CG)

cDNA libraries from leaves of drought-stressed plants of *C. arabica* cv. Rubi MG 1189 (drought sensitive) and *C. canephora* clone 14 (drought tolerant) of the coffee genome datadase (Vieira et al., 2006; Vinecky et al., 2005) were tested by a statistical test of Fisher (1922) to select ESTs over- or under-expressed in both libraries. Sequence homologies were made by screening the GenBank database using TBLASTX program (Altschul et al., 1997).

### Northern-blot experiment

Total RNAs were extracted from collected tissues and tested by Northern-blot experiments as described before Geromel et al. (2006) using EST probes of CG labeled by random-priming with $\alpha$-$^{32}$P-dCTP.

### Quantitative PCR (qPCR)

Total RNAs were digested with DNaseI-RNase-free (Promega) and 1µg was reverse-transcribed with the ImPromII enzyme according to the recommendations of the furnisher (Promega). Synthesized single-strand cDNA were diluted (1/25 to 1/100) and tested by qPCR using CG primer pairs preliminary tested for their specificity and efficiency against cloned EST (data not shown). The qPCR was performed with 1µl of ss-cDNA in a final volume of 10µl with SYBR green fluorochrom (SYBRGreen qPCR Mix-UDG/ROX, Invitrogen) according to the manufacturer and using a Fast 7500 apparatus (Applied Biosystems). For each sample, GC expression levels were standardized to the expression of ubiquitine gene used as an internal control. Data were treated by SDS 1.3.1. program (Applied Biosystems). Expression levels were expressed in absolute quantification by comparison to standard curves of PCR realized with known concentrations of corresponding genes.

### Screening of macroarrays

The inserts of 3,388 clones of an unigene set designed based on clustering ESTs from drought stressed cDNA libraries (SH1 and SH3) (Vieira et al., 2006) were PCR amplified with universal M13 reverse and forward primers. Concentration and quality of the PCR products were checked on agarose gels. Aliquots of 50 µl each (± 250 ng.µl$^{-1}$) were transferred to 384 plates. An equal volume of DMSO was added to each well. Spotting of the PCR products on nylon Hybond-N+ membranes (GE Healthcare) was performed using a Q-Bot (Genetix Inc.). Each PCR product was spotted in duplicate, in a 3x2 format, totalizing 6,912 spots including 68 controls. After spotting, nylon membranes were treated with denaturing and neutralizing solutions, according to the manufacturer. DNA was fixed onto membranes by UV-cross-linking. Membranes were further hybridized with probes corresponding to ss-cDNAs obtained

by RT of 30 μg of total RNA extracted from leaves of plants of *C. canephora* clones 22 and 14 grown with or without water stress conditions. Labeling was performed by random-priming with $\alpha$-$^{33}$P-dCTP. After overnight hybridization, nylon membranes were washed and exposed for 3 days, before analysis on a Phosphoimager FLA3000 (Fuji). Differentially expressed genes were identified using the ArrayGauge software (Fuji).

**Proteome Analysis by 2D gel electrophoresis**

Proteins were extracted of leaves by a modified phenol/SDS method (Ramos et al., 2007) followed by two-dimensional electrophoresis. The first dimension (isoelectric focusing) was carried out using 13 cm IPG strips (pH 3-10 or pH 4-7) in an IPGphor system (GE Healthcare) and the samples (500-1000 micrograms of proteins) were loaded in during reswelling process at 20 °C for 12 h. The second dimension was in an SDS-PAGE (11%) using the Hoefer SE 600 Ruby system (GE Healthcare) under 15 mA/gel for 45 min and 30 mA/gel for 180 min at 12 °C. Gels were stained with Coomassie Blue G-250 and R-350, digitalized using an UMAX Image Scanner and analyzed with ImageMaster 2D Platinum 6.0. Protein spots differentially expressed were removed manually from gels and analyzed by mass spectrometry using a Maldi-Tof/Tof (Auto-flex, Bruker) mass spectrometer.

**Protein identification by MS**

Proteins were identified by PMF (Peptide Mass Fingerprinting) using PiumsGUI2.2 and MS/MS Ion Search using the X!Tandem software, against the translated HarvEST and Coffee Project Genone EST-based databases (Vieira et al., 2006). Trans-Proteomic Pipeline (TPP) and Scafold packages were used for analyzing, validating, and storing protein identification data. Additionally, the identification results were verified by visually inspection and by *de novo* sequencing using PepSeq software.

**RESULTS**

**Selection of CG by electronic-northern**

The *in silico* analysis ("electronic Northern") allowed us to identify several (n ≈ 20) CGs, some of them coding for putative proteins of known functions like the *rbcS* subunit (Marraccini et al., 2003) of the Rubisco [E-value 7e-$^{84}$], the cystatine [cystein protease inhibitor, E-value 7e-$^{100}$) and the mannose 6-P reductase [E-value 7e-$^{120}$]. However, some others (called "no hit") did not presented significant homologies after searches in public databases.

**Expression of CG selected by electronic-northern**

Firstly, CGs expression profiles were analyzed in leaves of *C. canephora* clones by Northern-blot and were classified in four types (Fig. 1). Independently of clones analyzed, some CG (i.e. *rbcS*) showed high expression under irrigation (I) but low expression under water limitation (NI) (Figure 1A). Inversely, other CG (i.e. mannose 6-P reductase) presented higher expression under water-stress independently of clones analyzed (Figure 1B). In that case, expression was higher in the clone 73 than in others. Interestingly, other CGs presented differential gene expression between the tolerant (14, 73 and 120) and sensible (22) clones of *C. canephora*. This was the case for CG10 that showed specific expression in the clone 22 that also increased under water-limitation (Figure 1C). Finally, CG with greater expression in tolerant than in sensible clones were also obtained like for the gene encoding cystatin-like protein (Figure 1D).

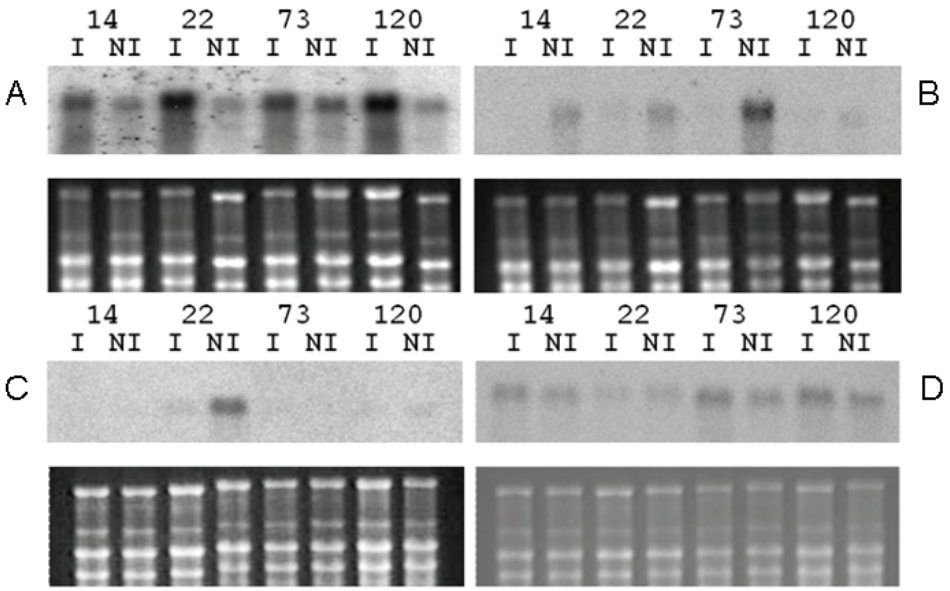


**Figure 1. Total RNAs from leaves of *C. canephora* clones grown under irrigated conditions (I) and water deficit (NI) were separated on agarose gel, transferred to nylon membranes and hybridized with probes corresponding to CG labeled with $^{32}P\alpha$-dCTP (upper parts). Total RNAs colored with BET (lower parts) indicates equal loading of the samples.**

Expression profiles of theses CGs were also checked by qPCR experiments that confirmed differential expressions detected by northern-blotting (Figure 2). In some cases, gene expression profiles obtained in roots were identical to those in leaves (data not shown).

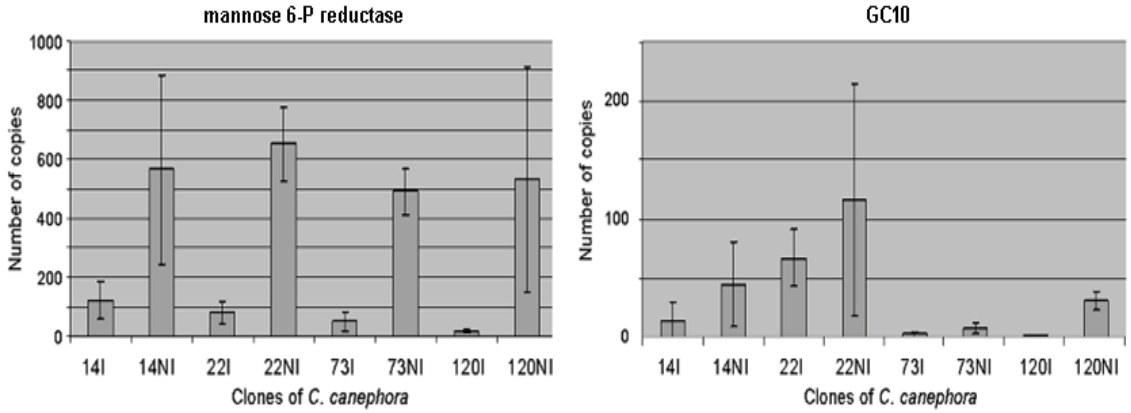


**Figure 2. Expression of mannose 6-P reductase and CG10 genes in leaves of *C. canephora* clones grown under irrigated (1) and water stress (NI) conditions. Results are expressed in absolute quantification (Number of copies).**

**Identification of CG genes by of macroarray screening**

After membrane hybridizations, several unigenes showing differential expression were observed. Hybridization profiles of 14I vs. 22I in the figure 3 are presented as an example. qPCR experiments are on-going to verify the differential expression of these unigenes.

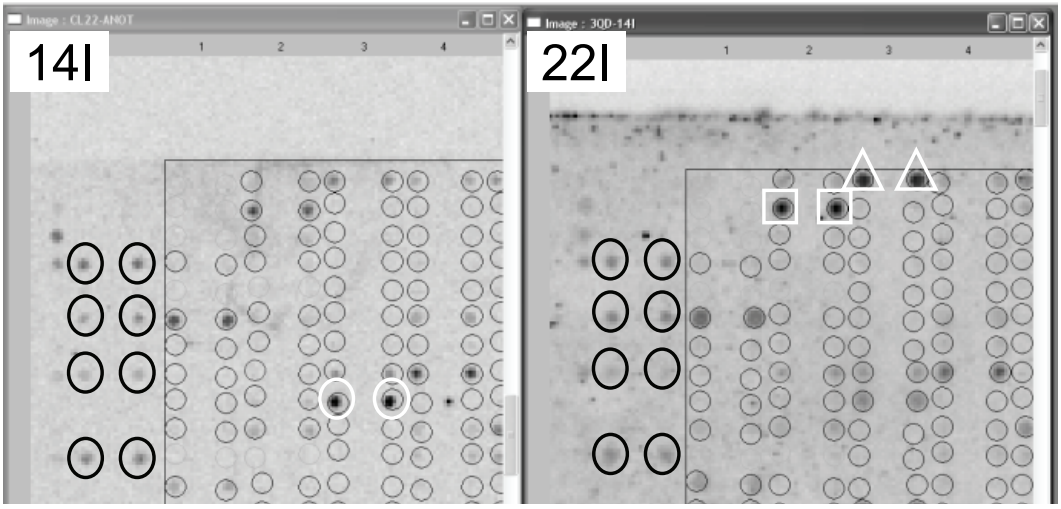


**Figure 3. Nylon membranes with unigenes (spotted in horizontal duplicate) were hybridized with *a*-$^{33}$P-dCTP ss-cDNA probes coming from RNA extracted from leaves of the clones 14 (left) and 22 (right) of *C. canephora* var. Conillon grown under irrigation (I). Unigenes showing identical expression and used to standardize expression levels (control) in both membranes, are in black circles. Unigenes highly expressed in the clones 22I are in white triangles and boxes. A unigene highly expressed in the clone 14I is identified by a white circle.**

**Protein expression pattern in response to drought**

In general, 700-1000 well resolved protein spots of each treatment were used comparative analyses and 40 more intense protein spots differentially expressed have been processed for protein identification by Maldi-Tof MS/MS.

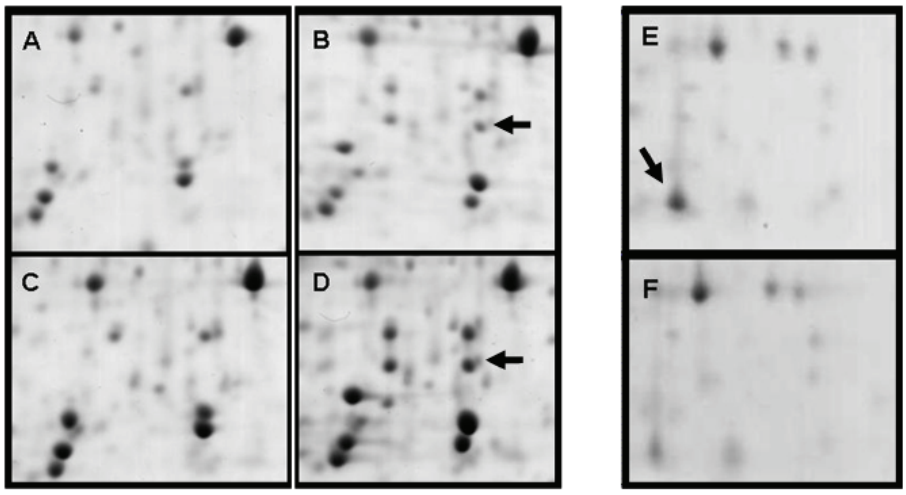


**Figure 4. Regions of the 2D gels showing differential accumulation of the HSP26 protein (A to D) and of chloroplast carbonic anhydrase (E and F) are presented. In both cases, proteins are marked by black arrows. 2D gels correspond to leaf proteins of the clone 14 grown under irrigated conditions (A and E) and water deficit (C) and of the clone 22 grown under irrigated conditions (B and F) and water deficit (D).**

The gel analysis permitted to identify several proteins that showed differential accumulation between the clones and with the water-stress conditions applied to the plants. This was the case of the coffee protein homologous to the *N. tabacum* HSP26 which was not detected in leaves of the drought-resistant clone 14 (Figure 4A and C) but was present in those of the drought-susceptible clone 22 (Figure 4B and D).

Another example concerns the chloroplast carbonic anhydrase which accumulate to higher amount in the clone 14 than in the clone 22 under unstressed conditions (Figure 4E-F). These 2D gel comparisons also permitted the identification of other proteins differentially expressed like those homologous to Oxygen-evolving enhancer (OEE) protein 2 that always accumulate to higher levels in the clone 22 (data not shown). In some cases, modifications of pI with water-stress conditions were also observed for several proteins particularly for the Rubisco small subunit (RbcS) (data not shown).

## CONCLUSIONS AND DISCUSSIONS

This integrated analysis led us to identify several CG and proteins that showed differential expression and accumulation under water stress condition. Interestingly, a large number of them encode for proteins implicated in the photosynthesis suggesting that the drought-tolerance of coffee clones could be directly linked to this biological function. Work is now under way to validate expression of CG identified by macroarray screenings (see poster PB640) or coming from the comparisons of 2D gels, using drought-tolerant clones of *C. canephora* like those forming the clonal variety Conilon Vitória-Incaper 8142 (Fonseca et al., 2004) but also in field-grown plants of cultivars Rubi and Iapar59 of *C. arabica* submitted to different irrigation conditions (Embrapa Cerrados, Planaltina-DF). Expression of CG will be also checked in clones of *C. canephora* representing the genetic diversity of this species where a great variability for drought tolerance was yet reported (Montagnon and Leroy, 1993). In addition to the differences of CG expression profiles observed, it is also worth noting that post-translational modifications should occur under drought stress condition. In order to increase our knowledge about metabolic changes occurring in drought stressed coffee plants, the consequences of such modifications and their implications on cell metabolism are now under investigations. CGs showing differential expression profiles between drought-tolerant and susceptible clones of *C. canephora*, but also with water limitation, could now be used for the search of nucleic markers (i.e. SNP) and promoter regions (see poster PB620) that could be further used as genomic tools either to speed-up conventional breeding programs or for coffee genetic transformation.

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## REFERENCES

Altschul S.F., Madden T.L., Schäffer A.A., Zhang J., Zhang Z., Miller W., Lipman D.J. (1997). “Gapped BLAST and PSI-BLAST: a newgeneration of protein database search programs”, Nucl. Acids Res. 25: 3389-3402.

Assad E.D., Pinto H.S., Zullo Jr J., Ávila A.M.H. (2004). Impacto das mudanças climáticas no zoneamento agroclimático do café no Brasil. Pesq. Agropec. Bras. 39: 1057-1064.

DaMatta F.M., Ramalho J.C. (2006). Impact of drought and temperature stress on coffee physiology and production: a review. Braz. J. Plant Physiol. 18: 55-81.

DaMatta F.M., Chaves A.R.M., Pinheiro H.A., Ducatti C., Loureiro M.E. (2003). Drought tolerance of two field-grown clones of *Coffea canephora*. Plant Sci. 164: 111-117.

Ferrão R.G., Fonseca A.F.A., Silveira J.S.M., Ferrão M.A.G., Bragança S.M. (2000). EMCAPA 8141 - Robustão Capixaba, variedade clonal de café conilon tolerante à seca, desenvolvida para o estado do Espírito Santo. Rev. Ceres 273: 555-560.

Fisher R.A. (1922). On the interpretation of $\chi^2$ from contingency tables, and the calculation of P. J. Royal Stat. Soc. 85: 87-94.

Fonseca A.F.A., Ferrão M.A.G., Ferrão R.G., Verdin Filho A.C., Volpi P.S., Zucateli F. (2004). Conilon Vitória - Incaper 8142: improved Coffea canephora var. Kouillou clone cultivar for the State Espírito Santo. Crop Breed. Appl. Biotech. 4: 1-3.

Geromel C., Ferreira L.P., Cavalari A.A., Pereira L.F.P., Guerreiro S.M.C., Vieira L.G.E., Leroy T., Pot D., Mazzafera P., Marraccini P. (2006). Biochemical and genomic analysis of sucrose metabolism during coffee (*Coffea arabica*) fruit development. J. Exp. Bot. 57: 3243-3258.

Lashermes P., Andrade A.C., Etienne H. (2008). Genomics of coffee, one of the world's largest traded commodities. *In*: "Genomics of Tropical Crop Plants". H Moore & R Ming eds. Springer, pp. 203-226.

Lima A.L.S., DaMatta F.M., Pinheiro H.A., Totola M.R., Loureiro M.E. (2002). Photochemical responses and oxidative stress in two clones of *Coffea canephora* under water stress. Env. Exp. Bot. 47: 239-247.

Lin C., Mueller L.A., McCarthy J., Crouzillat D., Pétiard V., Tanksley S.D. (2005). Coffee and tomato share common gene repertoires as revealed by deep sequencing of seed and cherry transcripts. Theor. Appl. Genet. 112: 114-130.

Marraccini P., Courjault C., Caillet V., Lausanne F., Lepage B., Rogers W.J, Tessereau S., Deshayes A. (2003). Rubisco small subunit of *Coffea arabica*: cDNA sequence, gene cloning and promoter analysis in transgenic tobacco plants. Plant Physiol. Biochem. 41: 17-25.

Montagnon C., Leroy T. (1993). Réaction à la sécheresse de jeunes caféiers *Coffea canephora* de Côte-d'Ivoire appartenant à différents groupes génétiques. Café Cacao Thé 37: 179-190.

Pinheiro H.A., DaMatta F.M., Chaves A.R.M., Fontes E.P.B., Loureiro M.E. (2004). Drought tolerance in relation to protection against oxidative stress in clones of *Coffea canephora* subjected to long-term drought. Plant Sci. 167: 1307-1314.

Pinheiro H.A., DaMatta F.M., Chaves A.R.M., Loureiro M.E., Ducatti C. (2005). Drought tolerance is associated with rooting depth and stomatal control of water use in clones of *Coffea canephora*. Ann. Bot. 96: 1001-108.

Poncet V., Rondeau M., Tranchand C., Cayrel A., Hamon S., de Kochko A., Hamon P. (2006). SSR mining in coffee tree EST databases: potential use of EST-SSRs AS marker across *Coffea* genus. Mol. Gen. Genet. 276: 436-449.

Praxedes S.C., DaMatta F.M., Loureiro M.E., Ferrão M.A.G., Cordeiro A.T. (2006). Effects of long-term soil drought on photosynthesis and carbohydrate metabolism in mature robusta coffee (*Coffea canephora* Pierre var. *kouillou*) leaves. Environ. Exp. Bot. 56: 263-273.

Ramos H.J.O., Seixas D.F., Pereira L.F.P., Cruz L. M., Fungaro M. H., Hungria M., Osaku C., Petzl-Erler M.L., Ayub R.A., Peralta M., Marur C.J., Souza E.M., Vieira L.G.E.,

Pedrosa F.O. (2007). Análise proteômica do estresse hídrico em cafeeiro. V Simpósio de Pesquisa dos Cafés do Brasil, Águas de Lindóia, CD-rom.

Vieira L.G.E., Andrade A.C., Colombo C.A., Moraes A.A.H., Metha A., Oliveira A.C., Labate C.A., Marino C.L., Monteiro-Vitorello C.B., Monte D.C., Giglioti E., Kimura E.T., Romano E., Kuramae E.E., Lemos E.G.M., Almeida E.R.P., Jorge E.C., Barros E.V.S.A., da Silva F.R., Vinecky F., Sawazaki H.E., Dorry H.F.A., Carrer H., Abreu I.N., Batista J.A.N., Teixeira J.B., Kitajima J.P., Xavier K.G., Lima L.M., Camargo L.E.A., Pereira L.F.P., Coutinho L.L., Lemos M.V.F., Romano M.R., Machado M.A., Costa M.M.C., Grossi de Sá M.F., Goldman M.H.S., Ferro M.I.T., Tinoco M.L.P., Oliveira M.C., Sluys M.A.V., Shimizu M.S., Maluf M.P., Eira M.T.S., Guerreiro Filho O., Arruda P., Mazzafera P., Mariani P.D.S.C., Oliveira R.L., Harakava R., Balbao S.F., Tsai S.M., Mauro S.M.Z., Santos S.N., Siqueira W.J., Costa G.G.L., Formighieri E.F., Carazzolle M.F., Pereira G.A.G. (2006). Brazilian coffee genome project: an EST-based genomic resource. Braz. J. Plant Physiol. 18: 95-108.

Vinecky F, de Brito KM, Da Silva FR, Andrade AC (2005) Análise *in silico* de genes potencialmente envolvidos na resposta aos estresses abióticos, presentes na base de dados do projeto Genoma café. IV Simpósio de Pesquisa dos Cafés do Brasil, Londrina-PR, Embrapa Café, CD-rom