LUANA FERREIRA TORRES

EXPRESSION OF THE *CcDREB1D* PROMOTER IN *Coffea arabica*: FUNCTIONAL GENOMICS AND TRANSCRIPTOME ANALYSIS

LAVRAS – MG
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Tese apresentada à Universidade Federal de Lavras como parte das exigências do Programa de Pós-Graduação em Biotecnologia Vegetal, área de concentração em Biologia Molecular, para a obtenção do título de Doutor.

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APROVADA em 20 de Abril de 2017.
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"Aqui, no entanto nós não olhamos para trás por muito tempo. Nós continuamos seguindo em frente, abrindo novas portas e fazendo coisas novas, porque somos curiosos...e a curiosidade continua nos conduzindo por novos caminhos. Siga em frente! – Walt Disney"

RESUMO GERAL

A mudança climática está colocando um grande desafio para a produção mundial de café e uma das estratégias para superar esses problemas consiste na geração de culturas com maior tolerância à seca e outros estresses abióticos, através de abordagens biotecnológicas e avançadas técnicas de melhoramento molecular. Ao longo de sua evolução, as plantas desenvolveram uma série de mecanismos de tolerância ao estresse abiótico. No entanto, a ativação desses mecanismos varia dependendo da espécie da planta, que determina o seu nível de tolerância ao estresse. A elucidação da função dos genes de tolerância ao estresse e também do processo de defesa vegetal nestas condições é de fundamental importância para a obtenção de variedades agrícolas mais resistentes, o que consequentemente pode contribuir para a redução de perdas nas culturas em condições climáticas adversas. Estudos recentes resultaram na identificação de muitos genes candidatos em *Coffea* para tolerância à seca apresentando perfis de expressão diferencial contrastantes entre genótipos. Entre estes genes, os *DREBs* estão envolvidos na via de resposta ao estresse hídrico como os fatores de transcrição vegetais que regulam a expressão de muitos genes induzíveis por estresse e desempenham um papel crítico na melhoria da tolerância ao estresse abiótico das plantas através da interação com um cis –elemento (DRE/CRT) presente na região promotora de vários genes responsivos ao estresse abiótico. Entre outros *DREBs*, o gene *CcDREB1D* mostrou um aumento nos níveis de mRNA durante a aclimatação por estresse de seca. Isto indica uma regulação distinta para a expressão de genes homólogos nos dois genótipos e sugere que este gene pode contribuir para a diversidade observada entre genótipos. Atualmente, as estratégias de sequenciamento de alto desempenho do transcriptoma (RNA-Seq) permitem gerar um grande volume de dados, a baixo custo e em um curto período de tempo. Esta informação permite a realização de estudos de perfis transcripcionais e redes genéticas numa compreensão aprofundada de vários perfis de genes. Diante deste contexto, o capítulo 1 apresenta uma revisão de literatura englobando desde as características de origem e comerciais do café até o estudo de genes e seu transcriptoma. O Capítulo 2 compreendeu o estudo de três haplótipos do promotor *CcDREB1D* isolados de clones tolerantes e sensíveis à seca de *C. canephora* avaliando a capacidade do promotor para controlar a expressão do gene repórter *uidA* em resposta ao déficit hídrico. O Capítulo 3 compreendeu o estudo do haplótipo pHP16L isolado de *C. canephora* e sua participação na expressão do gene repórter *uidA* em resposta aos estresses de seca, altas e baixas temperaturas, alta intensidade luminosa e aplicação exógena de ABA, acompanhados de dados de RT-qPCR. O capítulo 4 compreendeu analisar as condições de expressão do haplótipo pHP16L do promotor *DREB1D* sob uma faixa representativa dos estresses abióticos mais comuns, utilizando a técnica de RNA-seq como ferramenta para entender o mecanismo de tolerância ao estresse das plantas e a expressão dos genes estresse induzido bem como dos genes *DREBs*, acompanhados de validação dos dados pela técnica RT-qPCR.

Palavras-chave: *Coffea arabica*. *DREB*. Expressão gênica. Transcriptômica.

GENERAL ABSTRACT

Climate change is posing a major challenge to coffee worldwide production and one of the strategies to overcome these problems consists in the generation of crops with increased tolerance to drought and other abiotic stresses, through biotechnological approaches and advanced molecular breeding techniques. Throughout its evolution, plants have developed a series of mechanisms of tolerance to abiotic stresses. However, the activation of these mechanisms varies depending on the plant species, which determines your level of tolerance to stress. The elucidation of the function of the genes of tolerance to stress and also of the process of vegetal defense in these conditions is of fundamental importance for the obtaining of more resistant agricultural varieties, which consequently can contribute to the reduction of losses in the crops in adverse climatic conditions. Recent studies resulted in the identification of many candidate genes in *Coffea* for drought tolerance presenting contrasted differential expression profiles between genotypes. Among those are found the genes involved in the water stress response pathway, such as the *DREB* transcription factors. *DREBs (Dehydration Responsive Element Binding)* are important plant transcription factors that regulate the expression of many stress-inducible genes and play a critical role in improving the abiotic stress tolerance of plants by interacting with a DRE/CRT cis-element present in the promoter region of various abiotic stress-responsive genes. Among others *DREBs*, the *CcDREB1D* gene displayed an increase in mRNA levels during drought stress acclimation. This indicates a distinct regulation for homologous gene expression in the two genotypes and suggests that this gene might contribute to the diversity observed between genotypes. Currently, high-performance sequencing strategies of the transcriptome (RNA-Seq) allow to generate a large volume of data, at low cost and in a short period of time. This information allows the realization of studies of transcriptional profiles and gene networks in an in-depth understanding of several gene profiles. Facing this context, the Chapter 1 presents a review of the literature encompassing from the origin and commercial characteristics of coffee, to the study of genes and their transcriptome. The Chapter 2 evaluated the activity of three *CcDREB1D* promoter haplotypes isolated from drought-tolerant and drought-susceptible clones of *Coffea canephora* by evaluating their ability to control the expression of the *uidA* reporter gene in response to water deficit. The Chapter 3 comprised the study of the haplotype pHP16L isolated from *C. canephora* and its participation in the expression of the *uidA* reporter gene in response to drought, high and low temperatures, high light intensity and exogenous ABA application stresses, accompanied by RT-qPCR data. The Chapter 4 comprised analyzing the expression conditions of the pHP16L haplotype of the *DREB1D* promoter under a representative range of the most common abiotic stresses using the RNA-Seq technique as a tool to understand the mechanism of plant stress tolerance and induced stress genes expression as well as *DREBs* gene, accompanied by data validation by the RT-qPCR technique.

Keywords: *Coffea arabica*. *DREB.* Gene expression. Transcriptome.

SUMMARY

FIRST PART

1 INTRODUCTION

The coffee (*Coffea* sp.) is one of the most important crops in the world economy with cultivation in more than 70 countries, handling around 91 billion dollars a year. World production has estimated increase of 1,4% in production in 2015/16 compared to 2014/15, wherein 70.17 million bags of Arabica were exported in the twelve months ending December 2015, compared to 68.96 million bags last year (INTERNATIONAL COFFEE ORGANIZATION – ICO, 2016).

Climate change - rising temperatures, longer droughts, excessive rainfall - appears to threaten the sustainability of arabica coffee production. Brazil has been facing reduced production due to severe droughts in 2014, but is likely to bounce back with its combination of strong public coffee research centres, effective extension services and a resilient private sector (BRANDO, 2014).

The plants be exposed to various adverse environmental conditions during their life cycle. These environmental factors limit the growth and reproduction of plants and consequently, agricultural productivity in case of intensive cultivation of plants. The plants respond to these stresses through various physiological and molecular responses (SHINOZAKI; YAMAGUCHI-SHINOZAKI, 2000).

Low and high temperature, drought, and high salinity are common stress conditions that adversely affect plant growth and crop production. Understanding the mechanisms by which plants perceive environmental signals and transmit the signals to cellular machinery to activate adaptive responses is of fundamental importance to biology. Knowledge about stress signal transduction is also vital for continued development of rational breeding and transgenic strategies to improve stress tolerance in crops (XIONG et al., 2002).

Hundreds of studies investigating the effect of individual and combinatorial stresses have allowed us to piece together the complex network of molecular interactions controlling plant stress responses. Plants activate both specific and nonspecific stress responses as a reaction to adverse environmental conditions, allowing them to maximize efficiency in responding to the exact set of conditions encountered, at the same time as conserving resources for growth. Signal specificity is achieved through the precise interplay between components of each pathway, particularly the hormones ABA (abscisic acid), SA (salicylic acid) and JA (jasmonic acid), TFs (transcription

factors), HSFs (heat shock factors), ROS (reactive oxygen species) and small RNAs (ATKINSON; URWIN, 2012).

Transcription factors are of key importance in generating specificity in stress responses. Their manipulation provides one of the greatest opportunities for conferring multiple stress tolerance transgenically, as they control a wide range of downstream events (XU et al., 2011). The TFs interact with cis-elements in the promoter regions of various stress-related genes to up-regulate the expression of many downstream genes, thus imparting stress tolerance (AGARWAL; JHAa, 2010).

A family of genes known as C-repeat binding factors (CBFs) or dehydration-responsive element binding factors (DREBs) are key transcription factors implicated in drought, salt and cold adaptation and regulate many essential stress-responsive genes which modulate physiological adaptation of plants to abiotic stress. *DREB/CBF* compose the abscisic acid (ABA) -dependent and -independent pathways of signal transduction in abiotic stress response, and regulate the expression of several stress-related genes. Indeed, the overexpression of *DREB* genes in several genetic engineered plants lead to up regulation of cold-regulated genes (CORE) and osmotic-stress responsive genes (OR), resulting in increased abiotic stress tolerance (CHEN et al., 2009).

In an attempt to better understand the tissular localization and regulation of *CcDREB1D* promoter haplotypes (pHP15L, pHP16L and pHP17L), our study set out to analyze their ability to regulate the expression of the *uidA* reporter gene in *C. arabica* transgenic plants submitted to different abiotic stresses such as low and high temperatures, water stress mimicked by low relative humidity (RH), application of exogenous ABA and photo-oxidative stress mimicked by high irradiance. The expression of pHP16L haplotype was evaluated by RT-qPCR and RNA-seq to confirm his participation in plant response to stress.

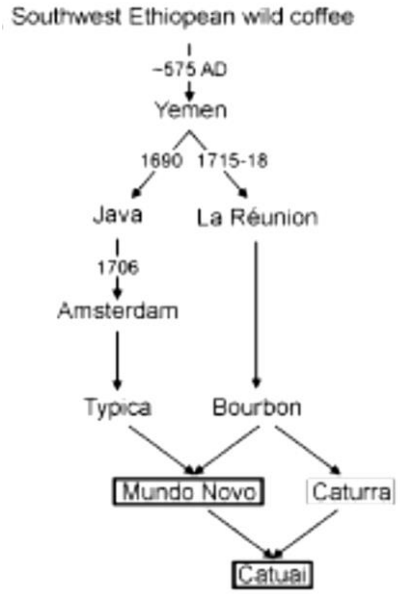
2 LITERATURE REVIEW

2.1 General characteristics of coffee origin and production

The coffee belongs to the Gentianales order, Rubiaceae family and Ixoreideae subfamily, and is divided into two genus, *Coffea* L. and *Coffea* Hook f., which comprise more than 100 species which differ in morphology, size and ecological adaptation (DAVIS et al., 2011). The genus *Coffea* L., belonging to the group of dicotyledons, is characterized by persistent leaves, shrub size, woody stem and hermaphrodite flowers (FAZUOLI et al., 1986).

Arabica coffee is native to the tropical forests of Ethiopia, Kenya, and Sudan, at altitudes of 1500-2800 m, between the latitudes of 4°N and 9°N (ANTHONY et al., 1987). Historically, the genetic inheritance of modern *Coffea arabica* cultivars arise from two main populations, known as Typica and Bourbon, that were spread worldwide in the 18th century (ANTHONY et al., 2002). This is the case of the most commercial *C. arabica* cultivars, Mundo Novo, Catuai and Caturra. The Caturra cultivar is a dwarf mutant of the Bourbon group. Mundo Novo is a hybrid between Bourbon and Typica, while Catuai cultivar derived from a cross between Mundo Novo and Caturra. Each cultivar displays distinctive plant architecture and physiological properties (Figure 1).

Figure 1- Origin of cultivated commercial cultivars of *C. arabica.*



Source: Vidal et al., (2010).

C. arabica is an species autogamous and alotetraploid (2n = 4X = 44 chromosomes) (CLARINDO; CARVALHO, 2011). It originated in Ethiopia Central highlands to about one million years as a result of a natural cross between outcrossing diploid species *C. canephora* and *C. eugenoides* (LASHERMES et al., 1999).

As a consequence of its evolutionary history and autogamy, the genetic diversity of *C. arabica* is low and unrestricted access to its genetic resources (and also other Coffea spp.), being crucial to advanced variety development (VAN DER VOSSEN; BERTRAND, 2015).

The species *C. canephora* is allogamous and diploid (2n = 2X = 22 chromosomes), with approximately 568.6 Mb in its genome (DENOEUD et al., 2014). It is native to the forest lowlands of the Congo River basin, where it extends to Lake Victoria, Uganda. It presents high genetic variability and great adaptation to the most varied climatic conditions, which made it possible to gather this species in two distinct groups, denominated Congolese and Guinean, established according to their geographic origin (MONTAGNON; CUBRY; LEROY, 2012).

The Congolese group has several subgroups of which two are of major importance: the SG1 (drought tolerant) subgroup, typical of an arid climate region, located in a more continental area of the continent, and the subgroup SG2 (drought-sensitive), characteristic of a region of tropical climate with high rainfall levels and well distributed throughout the year, which gives it susceptibility to drought (FAZUOLI, 2007).

Commercial coffee production is mainly based on two species: *C. arabica* and *C. canephora*, which account for about 65% and 35% of world production, respectively. In 2016, the largest producer were Brazil, followed by Vietnam and Colombia, with approximately 55.000; 25.500 and 14,5 thousand 60kg bags, respectively produced. Monthly data for February/2017, indicate how the main exporters Brazil, Vietnam and Colombia, with 14.883; 10.175 and 6.328 thousand 60kg bags exported, respectively (INTERNATIONAL COFFEE ORGANIZATION - ICO, 2017). Regarding the quality of the drink, *C. arabica* is considered of better cup quality because it has a low caffeine content, and the drink from it is the most appreciated in the world market (LEROY et al., 2006).

In january of 2017, the coffee market recovered from the fall in prices which was recorded towards the end of last year. While a price increase could be observed across all groups, it was most notable for Robusta. Exports for the first quarter of coffee year 2016/17 were 8.3% higher than last year at 29.8 million bags (INTERNATIONAL COFFEE ORGANIZATION - ICO, 2017).

The production of the 2017 harvest is estimated between 43,650.1 and 47,509.8 thousand bags benefited from coffee. The total area used for cultivation should be 2.228,2 thousand hectares (331,8 thousand hectares in formation and 1.896,4 thousand hectares in production). The production of arabica should be between 35.013,1 and 37.881,7 thousand bags. This year is of negative bienniality in most producing states, which consequently results in an average productivity lower than the previous year and a larger area to be managed (COMPANHIA NACIONAL DE ABASTECIMENTO - CONAB, 2017).

Climatic variability has always been the main factor responsible for the fluctuation of coffee yields worldwide, and the climate change, as a result of global warming, is expected to presents a major challenge to the coffee industry (VAN HILTEN, 2011).

2.2 Environmental stresses and plant defense mechanisms

Environmental stress is exerted on the plant by biotic or abiotic factors. The biotic factors involve the interaction between living organisms such as pathogens, algae or weeds, while abiotic stress is caused by high or low temperatures, excess or lack of water, high salt concentration and also by chemical components (QURESHI et al., 2007). These environmental factors severely limit the growth and reproduction of plants and, consequently, the agricultural productivity in case of plants of intense cultivation. Among all types of abiotic stresses, dehydration and extremes of temperature are the ones that most affect plant development. The plants respond to the water deficit, as well as to the high and low temperatures, with diverse physiological and molecular responses (SHINOZAKI; YAMAGUCHI-SHINOZAKI, 2000).

A given environmental stress that affects one plant may not be stressful for another because some plants have developed adaptive characteristics against these stresses by activating defense mechanisms. The plant's response to stress is the most crucial function of the plant cell and occurs through the alteration in the gene expression model (QURESHI et al., 2007), whose products (proteins) may be involved in several adaptive functions (THOMASHOW, 1999). Genes induced during abiotic stresses encode proteins that function in the control of gene expression and signal transduction, as well as the proteins involved, for example, in the protection and detoxification of cells (SHINOZAKI; YAMAGUCHI-SHINOZAKI, 2000).

Multiple signaling pathways regulate plant stress responses (KNIGHT; KNIGHT, 2001) and there is an overlap between patterns of gene expression that are induced in response to different stressors (CHEN et al., 2002).

Molecular responses to abiotic stresses include stress perception, signal transduction to cellular components, gene expression, and, finally, metabolic changes imparting stress tolerance. The genes thus induced by stress not only function in protecting cells from stress by the production of important metabolic proteins but also in regulating the downstream genes for signal transduction (AGARWAL et al., 2006).

Plant responses to abiotic stresses are highly complex and involve expression of a large number of genes encoding stress related proteins and enzymes working in biosynthetic pathways of osmoprotectants and other stress-related metabolites (VINOCUR; ALTMAN, 2005).

Functional proteins are characterized by protecting the cell against dehydration. In this group are aquaporins (water movement through the plasma membrane), the osmoprotective enzymes (related to the accumulation of solutes in the cytosol, with the purpose of promoting the maintenance of cellular turgor), the protective proteins of macromolecules and cell membranes, as Late Embyogenesis Abundant proteins (LEA), molecular chaperones such as HSP 70 BiP (70 kDa Heat Shock Binding Protein) and antifreeze proteins, detoxifying enzymes (neutralizing free radicals from oxidative stress) and proteases (CUSHMAN; BOHNERT, 2000).

Regulatory proteins are characterized by the regulation of signal transduction and gene expression. In the group of regulatory proteins, protein kinases and transcription factors are present (SHINOZAKI; YAMAGUCHI-SHINOZAKI, 1997). They include various transcription factors (TFs) such as myelocytomatosis oncogene (MYC), myeloblastosis oncogene (MYB), basic leucine zipper (bZIP), NAM, ATAF, and CUC (NAC), dehydration responsive element binding (DREB), etc. suggesting the role of various transcriptional regulatory mechanisms in the stress signal transduction pathways (AGARWAL et al., 2006).

2.3 Transcription factors

Transcription factors (TFs) bind to the cis-acting elements present in the promoter region of several genes that are regulated by signaling pathways that trigger their activation. *Cis*-acting elements are specific binding sites for proteins involved in the initiation and regulation of

transcription (HERNANDEZ-GARCIA; FINER, 2014). These regulatory elements participate in the control of various biological processes, including abiotic stress responses, hormone responses and developmental processes (YAMAGUCHI-SHINOZAKI; SHINOZAKI, 2006).

Transcription factors fall in the category of early genes and are induced within minutes of stress (HUANG et al., 2012). They are of key importance in generating specificity in stress responses. Their manipulation provides one of the greatest opportunities for conferring multiple stress tolerance transgenically, as they control a wide range of downstream events (XU et al., 2011). They interact with cis-elements in the promoter regions of various stress-related genes to up-regulate the expression of many downstream genes, thus imparting stress tolerance (AGARWAL; JHA, 2010). A list of TFs that may be crucial in controlling the response to biotic and abiotic stresses is given in Atkinson and Urwin (2012).

TFs do not bind indiscriminately to every gene with a matching response element, as there may be thousands of such sites across the genome. Instead, specificity can be generated by TFs forming into homo- or heterodimers, which then bind to a pair of response elements at an appropriate distance from each other, or by the the co-operation of bridging or scaffold proteins which direct TFs to the correct response elements (VAAHTERA; BROSCHE, 2011).

Plant genomes assign approximately 7% of their coding sequence to TFs, which proves the complexity of transcriptional regulation (AGARWAL et al., 2006b). Transcriptome data in *Arabidopsis* and in numerous other plants suggest that there are several pathways that independently respond to environmental stresses (in both ABA dependent- and independent-manner), suggesting that stress tolerance or susceptibility is controlled at the transcriptional level by an extremely intricate gene regulatory network (AGARWAL; JHA, 2010).

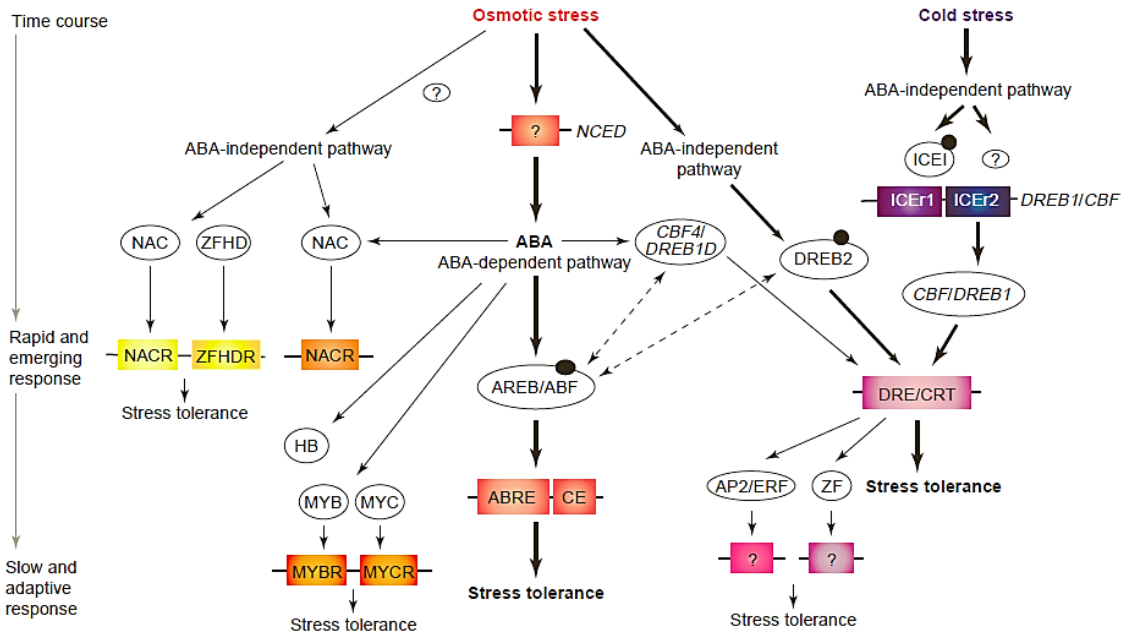
2.4 Signal transduction pathways involved in plant stress response

Four signal transduction pathways involved in the plant response to stress were described: pathways I and II ABA dependent and pathway III and IV ABA non-dependent (SHINOZAKI; YAMAGUCHI-SHINOZAKI, 2000). ABA-dependent signalling systems have been described as pathways that mediate adaptation to stress by the activation of at least two different TFs can be identified: (I) the AREB/ABF (ABA-responsive element-binding protein/ABA-binding factor) regulon; and (II) the MYC/MYB regulon. On the other hand, ABA-independent TFs are: (III) the

CBF/DREB (cold-binding factor/dehydration responsive element binding) regulon; and (IV) the NAC and ZF-HD (zinc-finger homeodomain) regulon (SAIBO et al., 2009).

Many other transcriptional regulation systems are involved in the expression of genes responsive to stress. In parallel, these four main pathways relate and converge to the activation of genes involved in the stress response. Figure 2 shows a model of signal transduction pathways of biotic and abiotic stresses from perception to gene expression. There are cross-talks between these signaling pathways, implying common transcription factors for biotic and abiotic stresses (YAMAGUCHI-SHINOZAKI; SHINOZAKI, 2005; CIARMIELLO et al., 2011).

Figure 2 - A schematic representation of stress signal perception and gene expression via ABA-dependent and independent pathways at cellular level in plants.



Legend: Transcription factors controlling stress-inducible gene expression are depicted as ellipses. *Cis*-acting elements involved in stress responsive transcription are depicted as colored boxes. Small, black, filled circles reveal modification of transcription factors in response to stress signals for their activation, such as phosphorylation. Regulatory cascade of stress-responsive gene expression is shown from top to bottom. Early and emergency responses of gene expression are shown in the upper part, and late and adaptive responses in the lower part. Thick black arrows indicate the major signaling pathways; these pathways regulate many downstream genes. Broken arrows indicate protein–protein interactions. Abbreviations: ABA, abscisic acid; AREB, ABRE-binding proteins; ABRE, ABA-responsive element; CBF, C-repeat-binding factor; DRE/CRT, dehydration- responsive element/C-repeat; DREB, DRE-binding protein; ICE, Inducer of CBF Expression; MYBR, MYB recognition site; MYCR, MYC recognition site; NACR, NAC recognition site; ZFHDR, zinc-finger homeodomain recognition site.
Source: Yamaguchi-Shinozaki and Shinozaki (2005).

2.5 DREB/CBF (Dehydration-Response-Element-Binding/C-repeat-binding factor)

The DREB are important AP2 (APETALA2)/ERF (ethylene responsive factor) plant TFs that induce a set of abiotic stress-related genes. The 145 members of this large AP2/ERF family of TFs in *Arabidopsis* were classified into four subfamilies namely, DREB/CBF, ERF, AP2, RAV (SAKUMA et al., 2002; LATA; PRASAD, 2011), based on the similarities of their sequences and on the numbers of AP2/ERF binding domains (NAKANO et al., 2006).

The first work about this family of transcription factors was performed by Yamaguchi-Shinozaki et al. (1993; 1994), through the identification of Responsive to Desiccation (rd) cDNAs of *A. thaliana*, which were expressed not only in response to water deficit, but also to saline stress, low temperature and abcisic acid.

Members of the AP2/ERF subfamily contain a highly conserved domain consisting of a sequence of 60 to 70 amino acids. AP2/ERF proteins play important roles in regulating gene transcription in a variety of biological processes related to growth and development, as well as various responses to environmental stimuli that regulate the expression of plant genes responsive to biotic and abiotic stresses (AGARWAL et al., 2006b). AP2/ERF TFs have long been associated with stress signalling, and recent discoveries suggest that they may make excellent targets for improving broad-spectrum tolerance in crops through genetic engineering (XU et al., 2011).

The *DREB* transcription factors are divided into two classes, *DREB1* and *DREB2* (CHINI et al., 2004). Three *DREB1/CBF* genes are involved in responses and acclimation to cold stress (NOVILLO et al., 2007; FANG et al., 2015), whereas *DREB2* genes are induced by dehydration, high salinity and heat (SAKUMA et al., 2006; MIZOI et al., 2012). The subclasse *DREB1/CBF* has the following variations: *DREB1B/CBF1*, *DREB1C/CBF2* and *DREB1A/CBF3* (STOCKINGER et al., 1997; LIU et al., 1998; MEDINA et al., 1999).

The *DREB* plays important roles in regulating the expression of genes (*rd29A, kin1* and *erd10*) in response to a variety of abiotic and biotic stresses (YAMAGUCHI-SHINOZAKI; SHINOZAKI, 20056; AGARWAL et al., 2006).

It has been established that they are major transcription factors involved in plant abiotic stress responses by regulating gene expression via the cis-acting *DRE/CRT* (dehydration-responsive element/C-repeat) (YAMAGUCHI-SHINOZAKI; SHINOZAKI, 2005). These TFs were isolated from *Arabidopsis* and designated as *DREB1A, DREB1B, DREB1C*, *DREB2A* and

DREB2B. Since *DREB1A*, *DREB1B, DREB1C* are induced by cold stress, and *DREB2A* and *DREB2B* by water stress conditions and high salt concentration (DUBOUZET et al., 2003).

The *DREB* genes have been studied and characterized in several plant species, such as *Arabidopsis*, wheat, rye, tomato, maize, rice, barley, buckwheat, grape and rapeseed and under various adverse environmental conditions. DRE/CRT was identified as an essential cis-acting element in the *Arabidopsis thaliana RD29A* promoter for expression in response to dehydration and cold, regardless of the presence of ABA. The *RD29A* (Responsive to Dessication) gene has been very characterized and consists of a sequence of nine base pairs - TACCGACAT - that regulates its induction under conditions of water deficit, low temperatures and salt stress (YAMAGUCHI-SHINOZAKI; SHINOZAKI, 1994; SAKUMA et al., 2006; FANG et al., 2015; ZANDKARIMI et al., 2015).

DREB genes play an important role in the ABA-independent stress-tolerance pathways that induce the expression of various stress-responsive genes in plants. The first isolated cDNAs encoding DRE binding proteins, CBF1 (CRT binding factor1), *DREB1A* and *DREB2A* were first isolated by using yeast one hybrid screening (STOCKINGER et al., 1997; LIU et al., 1998) from *Arabidopsis*. Since then, numerous *DREB* genes have been isolated from a number of plants (LATA; PRASAD, 2011; FANG et al., 2015; ZANDKARIMI et al., 2015).

A 125 bp region of the *DREB1C* promoter was demonstrated to be sufficient to drive cold-induced transcription, and two segments within the promoter, designated *ICEr1* and *ICEr2* (inducer of *CBF* expression region 1 and 2), contribute to cold responses (ZARKA et al., 2003). ICE1 and MYB15 have also been identified as transcription factors upstream of the induction of *DREB* genes in response to cold stress (AGARWAL et al., 2006). However, little is known regarding the upregulation of *DREB2* genes (CHEN et al., 2012).

These observations suggest that the *DREB* proteins are important TFs in regulating abiotic stress-related genes and play a crucial role in imparting stress tolerance to plants. The *DREB1* and *DREB2* regulons can thus be used to improve the tolerance of various kinds of agriculturally important crop plants to drought, high-salinity, and freezing stresses by gene transfer (LATA; PRASAD, 2011).

A diversity of *DREBs* genes has been functionally characterized in model plants by homologous transformation. In *Arabidopsis* and rice, as an example, members of subfamilies groups *DREB1* and *DREB2* have shown promising results in inducing cold, salt and drought

tolerance (SAKUMA et al., 2002; DUBOUZET et al., 2003; ITO et al., 2006; LIM et al., 2007; TAKASAKI et al., 2010; KIM et al., 2011; CHEN et al., 2009; CHEN et al., 2012).

DREBs genes have been less extensively characterized in non-model plants in parts due to inefficient protocols for genetic transformation or quite laborious *in vitro* regeneration selection. To cope with this limitation, heterologous transformation in model plants has been used to functional characterization of crop *DREBs* genes and results have been demonstrating a certain degree of functional conservation across plants species (BIHANI et al., 2010).

Despite numerous physiological investigations to discover how *DREB* transcription factors regulate target genes, transcriptional regulation of the *DREB* genes themselves has not yet been fully characterized. Little is known about its spatial expression during the development of plant organs submitted to environmental stress. Therefore, there is a need to find out how these transcription factors function during the regulation of a specific gene in a temporal and spatial manner.

2.5.1 *DREBs* genes in *Coffea canephora*

To identify the candidate genes involved in drought tolerance in coffee plants different strategies were used, among them the identification of these candidate genes through the Coffee Genome Project, which has already unmasked more than 200 thousand cDNA sequences and identified about 30 thousand genes of coffee tree (MONDEGO et al., 2011).

Most of the sequences obtained were deposited in the international biotechnology information database, the National Center for Biotechnology Information (NCBI). They are available the sequences of the genes that were expressed - EST (Expressed Sequence Tags) - removed the coffee tissues in their stages of development or at the time that these tissues respond to biotic or abiotic stresses. It is possible to reassemble the RNA molecule, ie, the copy of the DNA (cDNA) of the plant that expresses itself at the time of the stresses (VIEIRA et al., 2006).

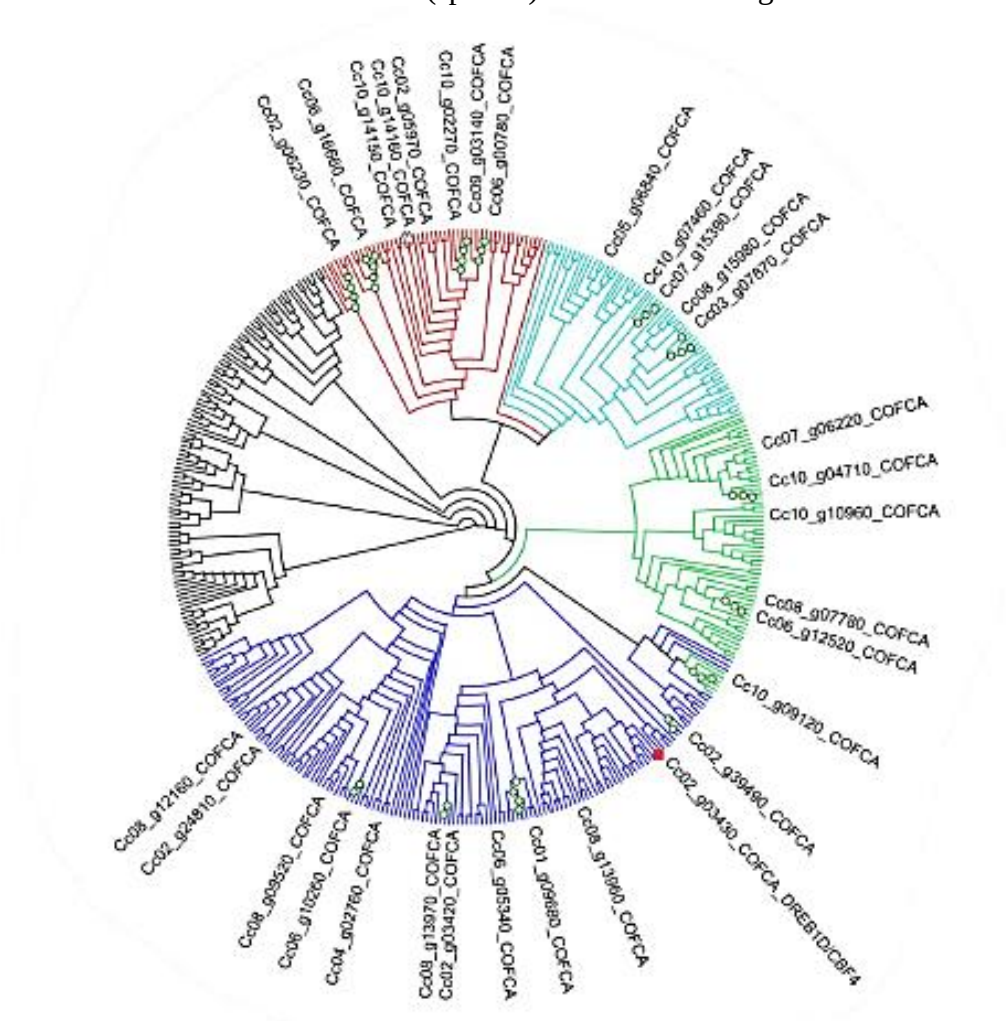
Among the candidate genes that showed differential expression in leaves of *C. canephora* conilon tolerant (14 and 120) and sensitive (clone 22) to drought clones grown in greenhouse with irrigation (I) or without (NI), were identified several genes, particularly the *CcDREB1D* gene (MARRACCINI et al., 2012).The transcriptional activity of allelic and homolog forms of

CcDREB1D promoter from *C. canephora* were studied and isolated from the clone 14 (tolerant) and clone 22 (sensible) by genetic transformation of *C. arabica* (ALVES et al.,, 2015).

The *CcDREB1D* gene and other members of the *DREB* subfamily of *C. canephora* were identified and divided into four SGs: I, II, III and IV (Figure 3) by Alves (2015). This identification was possible due to the complete sequencing of the genome of *C. canephora* (DENOEUD et al., 2014), whose information was made available in an integrative system of genome information, the Coffee Genome Database Hub (DEREEPER et al., 2015).

Based on this information and the identification of the members of the *DREB* subfamily of *C. canephora*, it became easier to analyze the expression of *DREB* genes and their respective subgroups, in order to elucidate their mechanisms of action in other stresses, in addition to drought.

Figure 3 - The reconciled taxonomic (species) tree with *DREB* gene tree.



Legend: *DREB* tree harboring homologs sequences from *A. thaliana, Coffea canephora, S. lycopersicon, S. tuberosum, O. sativa .j, V. vinifera* and *Z. mays* was rooted with viridiplantae phylogenetic tree. *DREB* subgroups I, II, III and IV are highlighted in light blue, green, dark blue, and red, respectively. *CcDREB* members are in bold, green circle nodes indicate speciation with high bootstrap confidence, blue triangle marks the *CcDREB* genes undergone duplication, and the red square shows *CcDREB1D*.
Source: Alves (2015).

2.6 Coffee physiology and the main abiotic stresses that affect plants

Under field conditions, plant performance in terms of growth, development, biomass accumulation and yield depends on acclimation ability to the environmental changes and stresses, exercising specific tolerance mechanisms that involve a complex network of biochemical and molecular processes (WANG et al., 2003). Overall, drought and unfavourable temperatures are the major climatic limitations for coffee production. These limitations are expected to become increasingly important in several coffee growing regions due to the recognized changes in global climate, and also because coffee cultivation has spread towards marginal lands, where water shortage and unfavourable temperatures constitute major constraints to coffee yield (DAMATTA; RAMALHO, 2006)

Primary effects of stresses, such as cold and drought, are often related, causing cellular damage and secondary stresses, e.g., osmotic and oxidative stress, resulting in disruption of osmotic and ionic homeostasis and damage to proteins and membranes. These imbalances will trigger downstream signalling and transcription control, which activate mechanisms, such as antifreezing and chaperone functions (e.g., chitinases, Heat shock proteins, late embriogenesis abundant proteins, Cold-related genes), detoxification [e.g., superoxide dismutase (SOD), ascorbate peroxidase (APX)], osmoprotection (e.g., proline, glycine betaine, sugar polyols), etc., that re-establish cellular homeostasis (Wang et al., 2003).

With regard to arabica coffee plants, peroxidation of membrane lipids plays a decisive role in the high cold sensitivity of roots, probably linked to a limitation in the functioning of antioxidative systems provoked by a decreased respiratory activity. The latter brings about a low availability of reducing power that in turn leads to the failure of antioxidative enzymes to protect lipid membranes, causing a higher root tissue damage and membrane rigidity (QUEIROZ et al., 1998).

An set of studies with potted plants of several coffee genotypes, which were submitted to low positive temperatures (CAMPOS et al., 2003; RAMALHO et al., 2003) showed that Icatu (a hybrid of *C. arabica* x *C. canephora*) exhibited better cold acclimation ability than the other genotypes studied, namely due to the small membrane selectivity loss and peroxidative degradation. This would be related to the maintenance (or increase) in the fatty acid saturation of membrane lipids and the reinforcement of the antioxidative system (observed also in cv. Catuaí). Such changes allow the coffee plants to maintain higher photosynthetic activity due to an organized and functional membrane structure (still, more rigid) less sensitive to ROS. Such qualitative changes in membrane lipids seem also to play an importante role in acclimation of the coffee tree to other conditions that promote oxidative stress, such as high-irradiance exposure (RAMALHO et al., 1998), becoming important in a quite short (8 h) time (GASCÓ et al., 2004).

Species or cultivars more tolerant to drought generally differ morphologically and/or physiologically, with mechanisms allowing greater production under restricted water supply. Understanding such mechanisms in genotypes naturally adapted to drought could help to improve their agronomic performance. In coffee, some physiological traits have been shown to potentially contribute to yield under drought conditions (DAMATTA, 2004b), but the development of an eficiente breeding method for drought tolerance is still a long-standing objective. A major component of differential adaptation to drought among arabica and robusta coffee genotypes appears to be behavioural, and may be governed by rates of water use and/or efficiency of extraction of soil water (PINHEIRO et al., 2005).

2.6.1 Abscisic acid – ABA, hormone of stress

ABA is an important plant hormone that plays a regulatory role in many physiological processes in plants, such as embryo maturation, seed germination and development, seed and bud dormancy, root growth, fruit ripening, regulation of stomatal aperture and the activation of stress responsive genes (AGARWAL; JHA, 2010). Increased levels of ABA are triggered by a variety of environmental stresses such as drought, salinity, cold, desiccation, heat and wounding. Further, it is also proved that ABA is a major physiological signal that induces drought and high salinity responses (FAROOQ et al., 2009).

ABA is synthesized in response to a reduction in water potential (CHRISTMANN et al., 2007) and the biosynthesis is partitioned between plastids and the cytosol. The oxidative cleavage of the precursor carotenoid 99-cis-neoxanthin to xanthoxin, catalyzed by the plastidial enzyme 99-cis-epoxycarotenoid dioxygenase (NCED), is the committed step for ABA biosynthesis (NAMBARA; MARION-POLL, 2005).

Abiotic stress responses are largely controlled by the hormone ABA, while defence against different biotic assailants is specified by antagonism between the salicylic acid (SA) and jasmonic acid (JA)/ethylene signalling pathways. However, recent findings suggest that ABA acts both synergistically and antagonistically with biotic stress signalling, creating a complex network of interacting pathways with cross-talk at different levels (FUJITA et al., 2006; ASSELBERGH et al., 2008b).

Plants under water stress usually have a higher accumulation of abscisic acid, which exerts various physiological effects on development of plants and has been identified as a messenger in perceptual response pathways of water stress and other environmental stresses, such as low temperatures and high salinity (WAN; LI, 2006; CLEMENT et al., 2008). Some studies have shown that the application of ABA in intact plants can increase your tolerance to stress (YIN et al, 2004).

Increased content of ABA during water stress has been found in all photosynthetic organisms. The biosynthesis of ABA has previously been thought to occur only in the roots, but more recent studies show that ABA is also synthesized in mesophyll cells, vascular tissue and stomata. It has been found that genes regulating at least the last steps in the ABA biosynthesis (NCED and AAO) are the most important and are strongly up regulated during water stress, showing the important role of ABA as a rapid stress response (SEO et al., 2000).

Many stress-responsive genes are upregulated by ABA. The first studies of ABA in osmotic stress signal transduction was the stress induction of several of these genes in the *Arabidopsis*. A general conclusion from these studies was that whereas low-temperature–regulated gene expression is relatively independent of ABA, osmotic stress–regulated genes can be activated through both ABA-dependent and ABA-independent pathways (SHINOZAKI; YAMAGUCHI-SHINOZAKI, 2000).

2.6.2 Low temperature

Occurrence of frosts, even if sporadic, may strongly limit the economic success of the crop. In that case, low temperatures cause the discoloration of leaves, exposure to temperatures below 1°C can result in serious lesions on both the leaves and coffee cherries and below -2°C for durations of 6 hours or more can cause plant death (DESCROIX; SNOECK, 2004).

Arabica coffee trees are severely damaged by frost, and are therefore not suited to regions that experience sub-zero temperatures, even for short periods of time (GAY et al., 2006). Large changes in diurnal temperatures can affect yield and quality, and the maximum tolerance is a range of 19°C (DESCROIX; SNOECK, 2004).

Cold stress, which includes chilling (<20°C) and/or freezing (<0°C) temperatures, adversely affects the growth and development of plants. Chilling and freezing are stresses that show different effects on plants: the first leads to slow biochemical reactions, such as enzyme and membrane transport activities; the second leads to ice crystal formation that can cause the disruption of cell membrane system (CHINNUSAMY et al., 2007).

Low temperature is one of the most important abiotic factors limiting growth, productivity and geographical distribution of agricultural crops. Many plants increase in freezing tolerance in response to low temperature, a phenomenon known as cold acclimation. Cold acclimation is the process that allows hardy plants to develop essential tolerance for cold stress survival through multiple levels of biochemical and cell biological changes. These responses are due to reprogramming of gene expression which results in the adjusted metabolic alterations. The first step in switching on such molecular responses is to perceive the stress as it occurs and to relay information about it through a signal transduction pathway (HEIDARVAND; MAALI AMIRI, 2010).

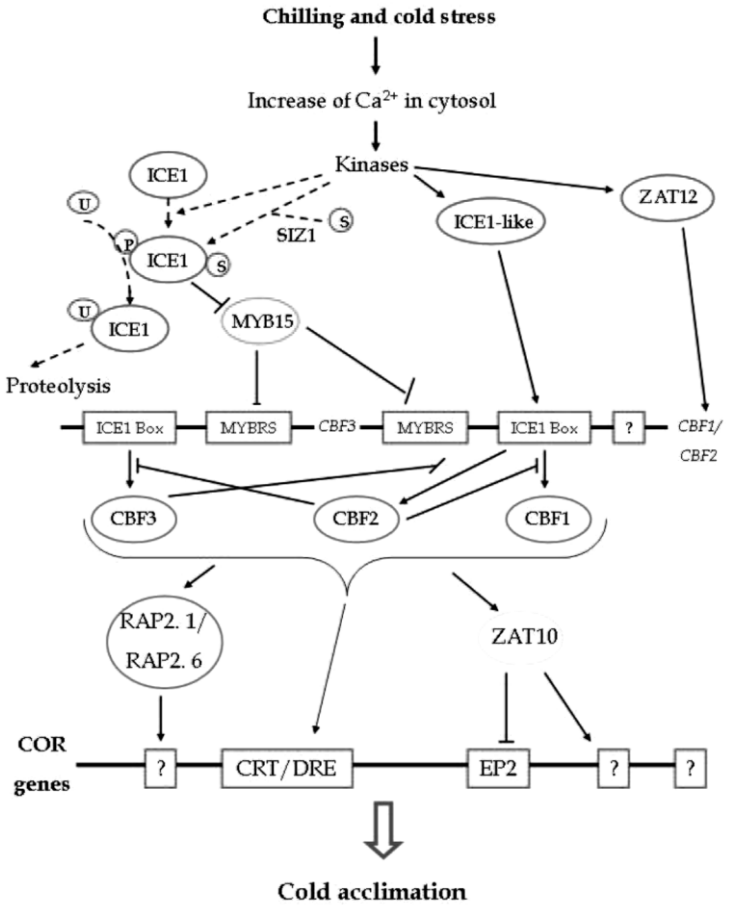
Little is known about cold sensors in plants and details of the early low temperature – signaling pathway are missing. Some data have suggested a role of membrane physical state on cold transduction (HUMPHREY et al., 2007).

Cold stress induces the expression of AP2/ERF family TFs, that is, CBFs, which can bind to *cis*-elements in the promoters of COR genes and activate their expression (CIARMIELLO et al., 2011) (Figure 4). CBFs regulate the expression of genes involved in phosphoinositide metabolism, transcription, osmolyte biosynthesis, ROS detoxification, membrane transport, hormone

metabolism and signalling and many others with known or presumed cellular protective functions (LEE et al., 2005).

A large number of studies have used a transcriptional profiling approach to identify genes in *Arabidopsis* that respond to cold (4°C) and chilling (13°C) temperatures. Results have shown that plants respond to low temperatures by altering mRNA levels of a large number of genes belonging to different and independent pathways. The cold induction of genes involved in calcium signalling, lipid signalling or encoding receptor-like protein kinases are also affected by the ice1 mutation gene (LEE et al., 2005).

Figure 4 - Cold-responsive transcriptional network in *Arabidopsis*.



Legend: CBFs regulate the expression of COR genes that confer cold tolerance. CBFs might cross-regulate each other's transcription. CBFs induce the expression of ZAT10 which might downregulate the expression of COR genes. Costitutive expressed ICE1 is actived throught sumoylation and phosphorylation induced by cold stress. ICE1 actived induce the transcription of *CBFs* and reprime *MYB15*. The expression of *CBFs* is negatively regulated by MYB15 and ZAT12. HOS1 mediates the ubiquitination and proteolysis of ICE1,

thus negatively regulates CBF regulons. Lines ending with bar indicate negative regulation; question mark (?) indicate unknown *cis*-elements; broken arrows indicate post-translational regulation; solid arrows indicate activation; lines ending with bar indicate negative regulation.
Source: Ciarmiello et al, (2011).

In *Arabidopsis*, ICE1 (Inducer of CBF Expression1), a MYC-type bHLH TF, can bind to MYC recognition elements in the CBF3 promoter and is important for the expression of CBF3 during cold acclimation. ICE1 is constitutively expressed and localized in the nucleus, but it induces expression of CBFs only under cold stress. This suggests that cold stress induced post-translational modification is necessary for ICE1 to activate downstream genes in plants (CHINNUSAMY et al., 2003).

The expression of cold-regulated genes is regulated by both ABA independent and ABA-dependent pathways (CHINNUSAMY et al., 2004). Promoter analysis of the cold-regulated genes has shown that they contain sequence elements that mediate the stress induction of the genes. There are some transcription factors which identify these elements and bind it (HEIDARVAND; MAALI AMIRI, 2010).

2.6.3 High temperature

For *C. arabica* the optimums mean annual air temperature range from 18 to 23°C. Above 23°C, fruit development and ripening are accelerated, often leading to loss of quality (CAMARGO, 1985). Continuous exposure to daily temperatures as high as 30°C could result in not only depressed growth but also in abnormalities such as yellowing of leaves (DAMATTA; RAMALHO, 2006). The optimum day/night temperature is 18/22°C, with tolerated extremes for Arabica coffee extending to a 15°C minimum during the night, and between 25°C - 30°C during the day (DESCROIX; SNOECK, 2004).

High temperatures can significantly damage the productivity of coffee plants in several ways. Continuous exposure to temperatures as high as 30°C leads to depressed growth and anormalities, such as the yellowing of leaves, growth of tumors on the stem and during blossoming may cause abortion of flowers, reducing coffee yields. Besides, favor the development of Coffee Leaf Rust (*Hemileia vastatrix*) and fruit blight, while Coffee Berry Disease is more prominent in cool regions (VAN DER VOSSEN; BERTRAND, 2015).

High temperature is now a major concern for crop production and approaches for sustaining high yields of crop plants under this stress are important agricultural goals (HASANUZZAMAN et al., 2013a).

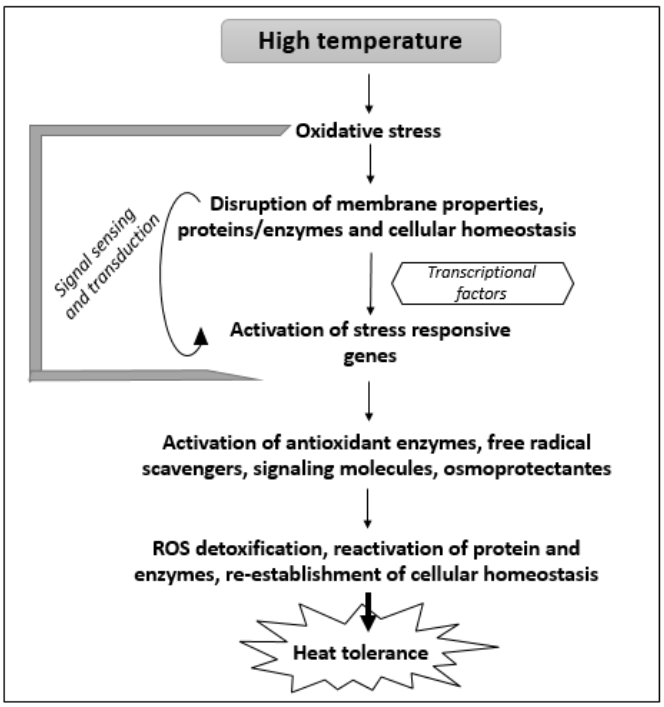
Conditions that exceed the optimal temperature for growth are recognized as heat stress (HS) in living organisms. Plant cells respond to HS by temperature perception mechanisms, signaling transduction pathways and accumulation of conserved heat shock transcription factors (Hsfs), as well as HS-related proteins such as molecular chaperones or heat shock proteins (Hsps) (KOTAK et al., 2007). One of the major consequences of heat stress is the excess generation of reactive oxygen species (ROS), which leads to oxidative stress (HASANUZZAMAN et al, 2013a).

Several papers in the literature cited either in structural level as at the molecular level, the processes occurring in the plant cells during heat stress. Plants alter their metabolism in various ways in response to heat temperature, particularly by producing compatible solutes that are able to organize proteins and cellular structures, maintain cell turgor by osmotic adjustment, and modify the antioxidant system to re-establish the cellular redox balance and homeostasis. At the molecular level, heat stress causes alterations in expression of genes involved in direct protection from heat temperature stress. These include genes responsible for the expression of osmoprotectants, detoxifying enzymes, transporters and regulatory proteins (KRASENSKY; JONAK, 2012).

Transcriptional regulation of HS-responsive nuclear gene expression is one of the main targets of HS-related signal transduction cascades and requires sets of *trans*-acting factors and *cis*-regulatory elements (CREs) in the promoter regions of HS-inducible genes (CHEN et al., 2012). HSFs serve as the terminal components of signal transduction, mediating the expression of HSPs and other HS-induced transcripts. Plants possess multiple HSF-encoding genes, with 21 members defined in *Arabidopsis* (KOTAK et al., 2007).

Upregulation of many genes has been reported to help the plant to withstand the stress conditions which leads to plant adaptation (TUTEJA, 2009). Upon stress plants perceive the external and internal signals through different independent or interlinked pathways which are used to regulate various responses for its tolerance development. Plant adaptation to heat stress includes avoidance and tolerance mechanisms which employ a number of strategies (HASANUZZAMAN et al., 2013a) (Figure 5).

Figure 5 - Schematic illustration of heat induced signal transduction mechanism and development of heat tolerance in plants.



Source: Adapted of Hasanuzzaman et al, (2013a).

2.6.4 Salinity

Salt stress is the exposure of plants to salinity, the main component of which is NaCl. Salt stress can be in one of two forms (1) gradual exposure to increasing levels of NaCl or (2) exposure of plants to low levels of salinity – or it may be a combination of both. Salt shock is an extreme form of salt stress, where plants are exposed suddenly to a high level of salinity. Salt shock rarely occurs in either agricultural practice or in natural ecosystems because increases in NaCl concentration in soils occur gradually, via rising water tables, deep penetration of roots, or slow, seasonal drying of the soil profile and removal of soil water via evaporation and transpiration (SHAVRUKOV, 2013).

Osmotic stress (or the osmotic component of salt stress) occurs immediately when roots come into contact with solutions containing unfavourably high concentrations of salts in hydroponic systems or in soil. But osmotic stress is also a component of the initial stages of drought stress, which similarly involves increasing cellular concentrations of osmolytes and regulation of stomatal conductance (JAMES et al., 2008).

Na^+ exclusion and tissue tolerance are two common mechanisms of tolerance to the ionic component of salt stress, where one or both of these mechanisms are employed by tolerant plant types (MUNNS; TESTER, 2008).

In contrast, salt shock occurs when plants are suddenly transferred from normal growth solution (without NaCl) into solution containing high concentrations of NaCl. The main component of salt shock is osmotic shock or plasmolysis, especially in root cells (MUNNS, 2002).

Apoplastic solutes, containing high concentrations of Na+, can freely flow through the open spaces in root cells and be transported to the shoot with minimal control by the plant. There is consequently rapid activation of many genes, in response to osmotic shock and damaged plasma membrane in root cells and to ionic stress in shoot cells. The mechanism of osmotic shock is universal for all plant species because it results from the physical–chemical effects of loss of cell turgor. Plants from different species, regardless of their level of salt tolerance, will only differ in the degree of damage to the plasma membrane during plasmolysis and in how quickly normal structure and function of affected cells is restored (SHAVRUKOV, 2013).

Highly upregulated expression of some genes in response to osmotic shock can be registered within minutes after sudden exposure of plants to salinity. Many genes identified in experiments involving salt shock are directly related to osmotic shock responses. Cell turgor maintenance, accumulation of soluble sugars, other osmolytes, and water balance are the most important processes of osmotic adjustment and they are controlled by genes with osmotic function (MUNNS; TESTER, 2008).

Large numbers of genes are expressed within a few minutes to several hours following the imposition of a salt shock treatment. These genes are mainly associated with the initial defence of plant cells against plasmolysis/salt shock, including processes such as signal transduction pathways, osmoregulation, and water loss. In contrast, following incremental application of salt, plants respond more smoothly to the osmotic phase of salt stress, with fewer genes associated with this phase. Genes showing altered expression are primarily responsible for osmotic adjustment and osmolyte production (SHAVRUKOV, 2013).

Changes in gene expression using model legume plant (*Louts japonicus*) and gradual salt stress treatment (150 mM NaCl) were analysed over a longer time (16 days) (SANCHEZ et al., 2008). The authors found that the most responsive genes were either related to transcription and signaling pathways (17% of the total number of genes responsive to NaCl), including members of

the transcription factor families AP2/ERF and MYB, membrane and cytoplasmatic receptor-like kinases and other classes of kinases, or were transport-related genes (8% of genes). These genes are not likely to be involved in the reaction of plants to osmoregulation but are clearly expected to be responsive to ionic changes.

2.6.5 Photo-oxidative stress

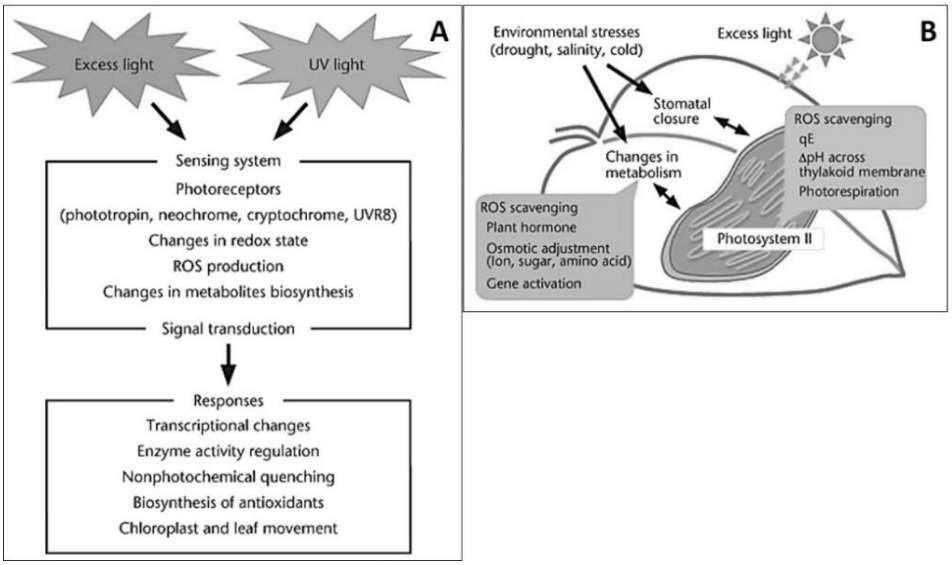
For plants, light is arguably the single most important environmental parameter, as it provides the ultimate source of biological energy. Plays a critical role in regulating plant growth and development through the modulation of expression levels of light-responsive genes that regulate developmental and metabolic processes (QUAIL, 2002b).

Although light is essential for photosynthesis and, thus, crucial for the survival of plants, it can also cause oxidative stress. Exposure of a plant to light exceeding what is utilized in photochemistry leads to inactivation of photosynthetic functions and the production of reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2), superoxide (O_2^-), hydroxyl radicals and singlet oxygen ($^1O_2$) (NIYOGI, 1999).

The effects of these ROS cause disturbances in the normal redox state of cells can cause toxic effects through the production of peroxides and free radicals that damage all components of the cell, including proteins, lipids, and DNA. Oxidative stress from oxidative metabolism causes base damage, as well as strand breaks in DNA (KALA et al, 2015). Besides excess light, a range of abiotic environmental conditions such as O3, salt, toxic metals, and temperature can induce increased production of ROS by limiting the ability of a plant to utilize light energy through photosynthesis (SHINOZAKI; YAMAGUCHI-SHINOZAKI, 2000).

Oxidative stress is described as an imbalance between reactive oxygen species (ROS) and antioxidants in biological systems. It can be triggered by increased ROS formation and / or reduced antioxidant defenses, and a variety of stressful conditions, both biotic and abiotic, triggers an increase in ROS in animal and plant cells (MAHALINGAM; FEDOROFF, 2003) (Figura 6).

Figura 6 – Perception light stress by ROS.



Legend: (A) Scheme of the sensing and responses to excess light. Light stresses are perceived by photoreceptors and cause changes in metabolite biosynthesis, production of reactive oxygen species (ROS) and redox state. Downstream pathways are activated and control the plant responses such as gene activation, enzyme activity, nonphotochemical quenching, biosynthesis of antioxidants and chloroplast and leaf movements. (B) Plant photoprotection in excess light stress and the environmental stress responses in plants. Photodamaged PSII is repaired by ROS scavenging, ΔpH-dependent qE, and photorespiration in chloroplasts. Stomatal closure, ROS production and metabolic changes under environmental stress affect these repair mechanisms.
Source: Osakabe et al. (2012).

Although toxic at high concentrations, reactive oxygen species act as signaling molecules during plant development, as well as signaling for stress-responsive pathways (MØLLER; SWEETLOVE, 2010).

To avoid the overproduction of ROS inside the cells, the plants have mechanisms that aim to decrease the production of these molecules in stress situations (MITTLER, 2002). The mechanisms used may be via anatomical adaptations, physiological, molecular (JOHNSON et al., 2011) and via alternative oxidases enzymes, which take electrons from the photosynthetic chain to use them in the reduction of intracellular molecular oxygen, thus reducing the flow of electrons and molecular oxygen concentration, both leading to a decrease in ROS production (MITTLER, 2002).

In addition to the mechanisms that aim to decrease ROS production in the plant cell, they also have a complex antioxidant system. This system constitutes a complex range of enzymatic and non-enzymatic antioxidants that regulate ROS, protecting cells from oxidative damage

(VRANOVÁ et al., 2002). Enzymes, including catalase (CAT), superoxide dismutase (SOD), peroxidase (POX), ascorbate peroxidase (APX) and glutathione reductase (GR) and non-enzymatic antioxidants such as tocopherols, ascorbic acid, flavonoids and glutathione work together to regulate amounts of ROS (HUNG et al., 2005).

Among these, the enzymes SoD, Cat and APx are highlighted in the detoxification of hydrogen peroxide and the superoxide ion. The process of detoxification of H_2O_2 catalyzed by the enzyme ascorbate-peroxidase is accompanied by a series of reactions that together form one of the most important antioxidant pathways present in plants, the ascorbate-glutathione cycle (GILL; TUTEJA, 2010).

Classical genetic and molecular approaches have identified various regulators downstream of photoreceptors. Many of these encode TFs, as well as kinases, phosphatases and degradation-pathway proteins. Although some of these regulators are specific for light quality, others regulate signal transduction networks in response to various light signals, representing potential signal integration points. Several basic posttranslational mechanisms are involved in regulating TF activities and the subcellular localization in response to light. The phosphorylation of TFs is a common modification that can influence their ability to bind to promoters (KLIMCZAK et al., 1995).

The HY5, a bZIP transcription factor that is one of the key regulators of cryptochrome and phytochrome controlled photomorphogenesis, is an important component of the UVB-induced signalling network. UVB promotes rapid transcriptional activation of HY5 independently of all known photoreceptors, and loss of HY5 results in the impairment of the transcriptional induction of a subset of UVB-responsive genes (ULM et al., 2004). Given that HY5 appears to regulate the expression of several *Arabidopsis* genes known to respond to abiotic stress conditions (e.g. *CBF1, DREB2A, RD20* and *MYB59*). It is inferred that HY5 could also be involved in the regulation of photosynthesis by adverse environmental conditions (LEE et al., 2007).

Arabidopsis thaliana leaves exposed to high light accumulate hydrogen peroxide (H_2O_2) in bundle sheath cell chloroplasts as part of a retrograde signaling network that induced ascorbate peroxidase 2 (APX2) (GALVEZ-VALDIVIESO et al., 2009).

Changes in mRNA level of *Arabidopsis* genes after exposure to high light conditions were related by Rossel et al. (2002). The authors identified an increase in expression of known antioxidant genes such as ascorbate peroxidase (APX1) and dehydroascorbate reductase (DHAR),

as well as unknown genes with homologies to regulatory genes and metabolic enzymes. Furthermore, they found that several Heat Shock Protein genes were up-regulated, implicating them in the antioxidant response in addition to their chaperones function.

In response to high light, wild-type *Arabidopsis* plants showed much enhanced expression of CSD1 and CSD2 and higher enzyme activity of MKK5 (mitogen-activated protein kinase kinase 5), which mediated a signal of the high light-induced expression of the genes CSD1 and CSD2. Manipulating MKK5 and thereby up-regulating the levels of CSD1 and CSD2 transcripts can improve plant tolerance to high light stress (XING et al., 2013).

2.6.6 Drought

Several definitions for water deficit are available in the literature. Taiz and Zeiger (2004) define as one of the main stresses that restrict the capacity and efficiency of the plant to perform important biochemical processes, caused by the water of deficit. This can be defined as "any water content of a tissue or cell that is below the highest water content exhibited in the state of greatest hydration."

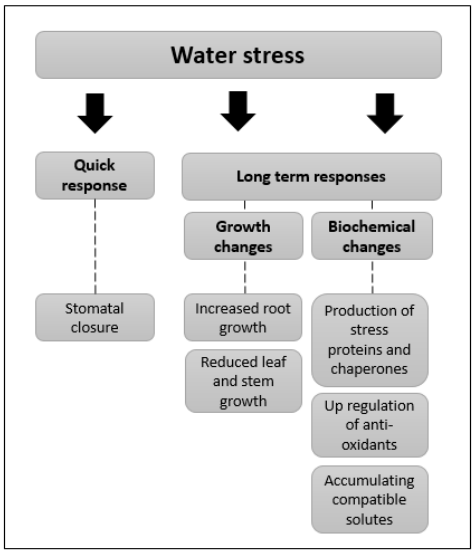
Another definition would be reduced water availability; either by water scarcity (drought) or osmotic stress (high salt concentrations) or water logging; too much water. In nature water stress is common either for long or short periods of time, depending on the local climate. Most plants therefore have some adaptation or response to enhance the growth and survival rate during water stress and subsequent recovery (ARVE et al., 2011).

Water stress may reduce photosynthesis, respiration and ion uptake, change the metabolic and growth patterns in the plant and in severe cases result in plant death. The environments that provide this water deficit are drought, high salinity and low temperature conditions (SHINOZAKI; YAMAGUCHI-SHINOZAKI, 1997; SEKI et al., 2003).

In agriculture drought stress is one of the major problems, causing major crop losses every year as well as loss of aesthetic value in ornamentals. With the global human population rapidly increasing, simultaneously as water scarcity increases, the loss of crop will be even more serious than before. The discovery and development of stress tolerant crops to avoid yield loss during water stress is therefore very important. In response to water stress plants have developed several

different mechanisms that increase the desiccation tolerance and water retention. These responses can be divided into short term and long term responses (Figure 7) (ARVE et al., 2011).

Figure 7 – Plant responses to water stress.



Source: Adapted of ARVE et al. (2011).

2.6.6.1 Long term responses

During prolonged water stress plants must be able to survive with low water content and maintain a minimum amount of water, through water uptake and retention. To cope with prolonged drought stress plants respond with energy demanding processes that alter the growth pattern, chemical content of the plants and the up or down regulation of genes (ARVE et al., 2011).

When the water availability is reduced, plants change the biochemistry to be able to retain as much water as possible and take up whatever water they can. During water stress plants produce and accumulate compatible solutes such as sugars, polyols and amino acid to lower the osmotic potential in the cells to facilitate water absorption and retention. Some of the compatible solutes also contribute to maintaining the conformation of macromolecules by preventing misfolding or denaturation (XIONG; ZHU, 2002). Plants also produce higher levels of the plant stress hormone ABA during water stress and this affects their growth pattern and stress tolerance (ARVE et al., 2011).

A group of proteins called late embryogenesis abundant like (LEA) proteins are also produced during water stress. These LEA-like proteins are highly hydrophilic, glycine-rich and highly soluble and have been found to be regulated by ABA (XIONG; ZHU, 2002). The LEA-like proteins are thought to act as chaperones, protecting enzymatic activities and preventing misfolding and denaturation of important proteins (XIONG; ZHU, 2002).

Some studies show that ABA can function as a signal to reduce leaf growth rate, both when ABA is applied exogenously or generated by water stress. By reducing the leaf expansion, the leaves become smaller and therefore transpire less. In some cases, water stress can even lead to leaf abscission. The reduction of cell volume also concentrates the solutes in the cells and compresses the plasma membranes causing them to increases in thickness (WILKINSON; DAVIES, 2010).

2.6.6.2 Short term response

When plants suddenly encounter drought it is important to respond as quickly as possible. A faster drought response means that less water is lost and the survival rate of the plants is increased. The most important quick response is stomatal closure (FAROOQ et al., 2009).

Development of stomata if often considered one of the most important developments in plant evolution. Much of the water loss occurs via stoma through perspiration, due to foliar exposure to sunlight, and consequent acquisition of sunlight and carbon dioxide (CO_2), which are fundamental to the photosynthetic process (BRODRIBB; McADAM, 2011).

Stomata are small pores present on the epidermis of green tissues that mediate exchanges between the plant and the atmosphere: CO_2 enters through stomatal pore as the carbon source for photosynthesis, while water vapor is released by transpiration. Stomata are surrounded by a pair of highly specialized cells, called guard cells, whose changes of turgor pressure control opening and closure of stomata (SIRICHANDRA et al., 2009). Interestingly, the guard cell transcriptome is particularly rich in TF-encoding genes (BATES et al., 2012).

During water stress the stomatal pore can be closed to reduce water loss. By closing the stomatal pore the water use efficiency is increased (FAROOQ et al., 2009), reducing the amount of water lost per CO_2 molecule assimilated. Several mechanisms work together to close the stomata, such as hydro passive closure and chemical signals from the plant stress hormone ABA. Increased levels of ABA also cause increased hydraulic conductivity in the roots and xylem,

enabling the plants to transport more water and thereby recover more rapidly after water stress (KUDOYAROVA et al., 2011).

2.7 Transcriptome

The transcriptome comprises the complete set of transcripts of a cell, as well as their amount, for a particular specific stage of the development or physiological condition of an organism. Their understanding is essential for the interpretation of the functional elements of the genome, for the discovery of the molecular components of cells and tissues, and for the understanding of stages of development and disease. The main objectives of transcriptomy are to catalog all species of transcripts (mRNAs, non-coding RNAs, small RNAs, etc.); to determine the transcriptional structure of the gene (its initiation sites; 5 'and 3' extremities; splice patterns and other post-transcriptional modification) and quantifying changes in gene expression levels of each transcript during development of the organism and in other different conditions (WANG; GERSTEIN; SNYDER, 2009).

The investigation of plant molecular responses to multiple stresses has often focused on overlapping transcriptional patterns. To this effect, several studies have been carried out in which different groups of plants are exposed to either one stress or another in parallel, and their gene expression patterns compared. Overlapping sets of genes that are regulated by both stresses are then identified and proposed to represent a generalized stress response or points of cross-talk between signalling pathways (HUANG et al., 2008).

A complete genome sequence and high-density genetic map of *Coffea canephora* was published recently (DENOEUD et al., 2014). This will offer opportunities for enhancing breeding progress to increase crop quality and yield, as well as to protect the coffee crop from major losses caused by diseases, insect pests and abiotic stresses related to climatic changes (GONGORA et al., 2015).

Further transcriptome studies in various species have identified genes, hormones, and processes important in controlling plant response to multiple abiotic or multiple biotic stresses and provided targets for the improvement of stress tolerance (WANG et al., 2010; GRIGOROVA et al., 2011).

Most coffee transcriptome sequencing data relies on two major cultivated species (*C. arabica* and *C. canephora*). Sanger EST sequencing projects were developed for *C. arabica*

(VIEIRA et al., 2006; VIDAL et al., 2010; MONDEGO et al., 2011) and *C. canephora* (LIN et al., 2005; PONCET et al., 2006). Transcriptome analysis using next-generation sequence—NGS has been performed in studies investigating biotic and abiotic interactions (FERNANDEZ et al., 2004; COMBES et al., 2013). The two subgenomes contained in the *C. arabica* allotetraploid genome (subgenome *C. canephora*—CaCc and subgenome *C. eugenioides*—CaCe) do not contribute equally to the transcriptome (VIDAL et al., 2010; COTTA et al., 2014).

In *Coffea* sp., EST resources have been developed for various species and tissues including roots, leaves, and fruits (LIN et al., 2005). However, no genomic resources are available for shoot apices, which are considered as key organs for plant development by integrating several signals, such as environmental stimuli as well as hormones (abscisic acid [ABA], auxins, cytokinins) and transcription (TRAAS; VERNOUX, 2002). On the other hand, next-generation sequencing (NGS) provides new opportunities to study transcriptomic responses and to combine high-throughput sequencing with the functional annotation capacity of generated ESTs (TORRES et al., 2008).

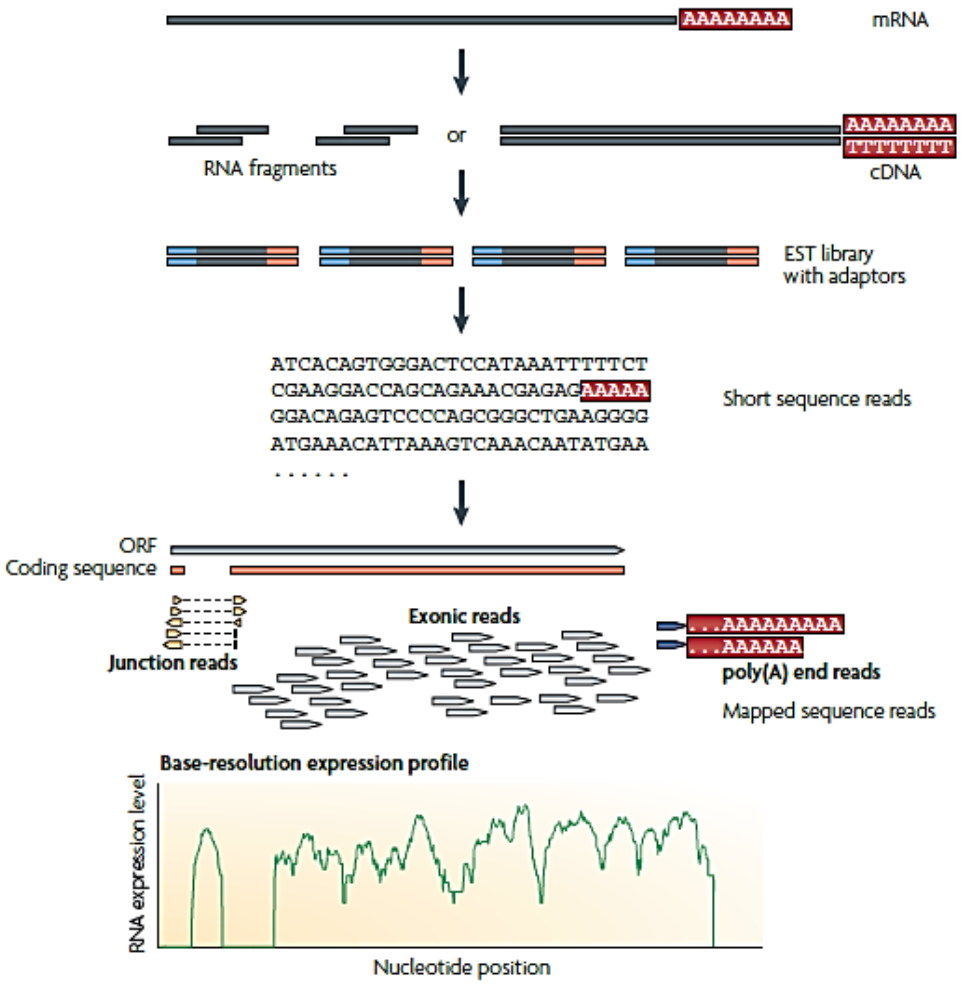
2.8 Resources for genome analysis using RNA-seq data

Contrary to the genome, the cell transcriptome is dynamic and specific for a given developmental stage and physiological condition. The understanding of the transcriptome is essential in the interpretation of the functional elements of the genome and in the development of cellular molecular constituents. The recent development of new high-throughput methods for DNA sequencing has led to the emergence of a new methodology that allows the sequencing of RNA molecules at unprecedented resolutions. This method is called RNA-Seq and has emerged as the preferred technology in the characterization and quantification of cellular transcripts (CRUZ, 2013).

RNA-Seq is the first method based on sequencing that allows a complete transcriptome is searched in large-scale and quantitatively, offering resolution up to a single base for annotation and gene expression levels "digital" in genomic scale, usually at a much lower cost when compared to high density microarray techniques or EST sequencing. It stands out as a valuable tool for understanding transcriptional dynamics, not only during the normal physiological changes associated with the development of the organism, but also in the comparison of normal and diseased tissues (WANG; GERSTEIN; SNYDER, 2009).

Over the years, a wide number of methodologies have been developed with the purpose of profile the transcriptome. One of these methods revolutionized expression profiling by enabling the measurement of thousands of genes simultaneously is the modern high-throughput sequencing-based RNA-Seq technology (Figure 8) (WANG; GERSTEIN; SNYDER, 2009).

Figure 8 - A typical RNA-seq experiment**.** Briefly, long RNAs are first converted into a library of cDNA fragments through either RNA fragmentation or DNA fragmentation.



Legend: Sequencing adaptors (blue) are subsequently added to each cDNA fragment and a short sequence is obtained from each cDNA using high-throughput sequencing technology. The resulting sequence reads are aligned with the reference genome or transcriptome, and classified as three types: exonic reads, junction reads and poly(A) end-reads. These three types are used to generate a base-resolution expression profile for each gene, as illustrated at the bottom; a yeast ORF with one intron is shown.
Source: Wang, Gerstein and Snyder, (2009).

A further advantage is that RNA-Seq technology can be used not only for gene expression profiling but also for detection of gene fusion events (MAHER et al., 2009), discovery of single nucleotide polymorfisms (LALONDE et al., 2011), investigation of post transcriptional RNA mutations (GARCION et al., 2004), study of alternative splicing events (PAN et al., 2008), discovery of novel transcripts (GUTTMAN et al., 2010) or investigation of allele specific expression (DEGNER et al., 2009).

There are several technologies available to perform high-throughput sequencing of DNA molecules. Currently, the ones that dominate the field are: 454 GS-FLX from Roche Applied Science, Genome Analyzer II from Illumina, Inc. and AB SOLiD from Applied Biosystems. Nevertheless, other technologies are being developed with potentially even higher quality throughputs (e.g. Pacific Biosciences, Helicos). The different technologies require distinct experimental protocols. Although, the essence of these systems is the same: to miniaturize individual sequencing reactions (BENTLEY et al., 2008).

2.9 Expression analysis of RNA - seq data using tools Bioinformatics and Statistics

In this context, bioinformatics and computational biology are interdisciplinary fields that combine computer technology, mathematics and molecular biology to answer fundamental questions in life sciences (HOGEWEG, 2011). Particularly, they present powerful tools that are suitable to analyse data generated from high-throughput sequencing platforms. Consequently, the success of next-generation sequencing (NGS) technologies is tightly related with the creation of efficient computational tools that are able to process the data. In fact, without the means provided by computational tools, the processing of NGS data would be almost impossible (POP; SALZBERG, 2008).

The RNA-seq platform addresses a multitude of applications, including relative expression analyses, alternative splicing, discovery of novel transcripts and isoforms, RNA editing, allele-specific expression and the exploration of non-model-organism transcriptomes (WANG; GERSTEIN; SNYDER, 2009).

One of the primary goals of RNA-sequencing analysis software is to reconstruct the full set of transcripts (isoforms) of genes that were present in the original cells. In addition to the transcript structures, experimenters need to estimate the expression levels for all transcripts. The first step in

the analysis process is to map the RNAseq reads against the reference genome, which provides the location from which the reads originated (MORTAZAVI et al., 2008).

In contrast to DNA-sequence alignment, RNA-seq mapping algorithms have two additional challenges. First, because genes in eukaryotic genomes contain introns, and because reads sequenced from mature mRNA transcripts do not include these introns, any RNA-seq alignment program must be able to handle gapped (or spliced) alignment with very large gaps. Second, the presence of processed pseudogenes, from which some or all introns have been removed, may cause many exon-spanning reads to map incorrectly (PEI et al., 2012).

In order to handle this problem, the software TopHat2 uses a two-step procedure. TopHat is a popular spliced aligner for RNA-seq experiments can align reads of various lengths produced by the latest sequencing technologies, while allowing for variable-length indels with respect to the reference genome (KIM et al., 2013).

Of particular interest is the discovery of differentially expressed genes across different conditions (e.g., tissues, perturbations) while optionally adjusting for other systematic factors that affect the data-collection process. There are a number of subtle yet crucial aspects of these analyses, such as read counting, appropriate treatment of biological variability, quality control checks and appropriate setup of statistical modeling. Several variations have been presented in the literature, and there is a need for guidance on current best practices (YU et al., 2013; CHU, 2014).

A crucial component of such an analysis is the statistical procedure used to call differentially expressed genes. There are two protocols widely used for this task: DESeq (ANDERS; HUBER, 2010) and edgeR (ROBINSON; McCARTHY; SMYTH, 2010), both of which are available as packages of the Bioconductor software development project. For RNA-seq data, the strategy taken is to count the number of reads that fall into annotated genes and to perform statistical analysis on the table of counts to discover quantitative changes in expression levels between experimental groups (ANDERS; HUBER, 2010).

OVERVIEW

One of the possible strategies to better prepare coffee producers to this future challenge is the development of new varieties that could cope with higher temperatures and water limitations, and remain highly productive contributing to the long-term sustainability of coffee cultivation in lands potentially affected by climate change.

In several plant species, the application of *DREB* genes in crop improvement has achieved promising results to desiccation tolerance engineering, however knowledge on natural diversity of these genes and its association to phenotypic variability in coffee is little known.

Unlike the genome, the cell transcriptome is dynamic and specific for a given cell developmental stage or physiological condition. Understanding the transcriptome is essential for interpreting the functional elements of the genome and revealing the molecular constituents of cells, Enabling the discovery and study of promising new genes that will participate in the improvement of many economically important plant species that are being affected by climate change.

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SECOND PART

ARTICLES

ARTICLE 1

Differential fine tune regulation of gene expression in coffee leaves by *CcDREB1D* promoter haplotypes under water deficit

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Running title: Fine tuning of coffee *DREB1D* expression under water deficit

Highlight: Fine tuning of *DREB1D* expression in stomata guard cells under water deficit is mediated differentially by promoter haplotypes from sensitive and tolerant coffee genotypes.

Abstract

Despite the importance of the *DREB1D* gene (also known as *CBF4*) in plant responses to water deficit and cold stress, studies analyzing its regulation by transgenic approaches are lacking. In the current work, a functional study of three *CcDREB1D* promoter haplotypes (called HP15, HP16 and HP17) isolated from drought-tolerant and drought-sensitive clones of *Coffea canephora* was carried out on plants of *C. arabica* stably transformed by *Agrobacterium tumefaciens* by analyzing their ability to regulate the expression of the *uidA* reporter gene in response to water deficit mimicked by PEG (-2.0 MPa) and low relative humidity (RH) treatments. A deletion analysis of their corresponding 5′-upstream regions revealed the increased specificity of GUS activities in the PEG and low RH treatments, with high expression in leaf mesophyll and guard cells in full-length constructions. RT-qPCR assays also revealed that the HP16 haplotype (specific to the clone tolerant to water deficit) had stronger and earlier activity compared to the HP15 and HP17 haplotypes. As most of the *cis*-regulatory elements involved in ABA-dependent and -independent networks, tissue-specificity and light regulation, are common to these haplotypes, we propose that their organization, as well as the nucleic polymorphisms present outside these boxes, may play a role in modulating activities in guard cells.

Key words: Coffee, *DREB1D* gene, genetic transformation, promoter haplotypes, *uidA* reporter gene, water deficit.

Abbreviations:

ABA: abscisic acid; ABRE: ABA-responsive element; CBF: cold-binding factor; CREs: *cis*-regulatory elements; DOF: DNA-binding one zinc finger; DRE: dehydration-responsive element; DREB: dehydration responsive element binding transcription factor; GUS: ß-glucuronidase; ICE: inducer of CBF expression; PEG: polyethylene glycol; RH: relative humidity; SNP: single-nucleotide polymorphism; VPD: vapor pressure deficit.

Introduction

Climate change leads to increasingly extreme temperatures and drought periods which are major abiotic factors affecting coffee production (DaMatta and Ramalho, 2006). Recent modelling studies delivered warnings on the threat of the occurrence of severe droughts and high temperatures on coffee production by increasing attacks by pest and pathogens (Avelino *et al.*, 2004; Jaramillo *et al.*, 2011; Magrach and Ghazoul, 2015). These climatic events that already affect coffee production are planned to modify areas suitable for future coffee cultivation crops (Davis *et al*., 2012; Bunn *et al*., 2015; Craparo *et al*., 2015). Drought is a limiting factor that affects flowering and yield of coffee (DaMatta and Ramalho, 2006), as well as bean development and biochemical composition, hence the final quality of the beverage (Silva *et al.*, 2005; Vinecky *et al.*, 2016). Increased [CO_2] in air is also a key factor for coffee plant acclimation to high temperature, strengthening photosynthetic pathway, metabolism and antioxidant protection, modifying gene transcription and mineral balance (Ramalho *et al.*, 2013; Martins *et al.*, 2014, 2016; Ghini *et al.*, 2015; Rodrigues *et al.*, 2016). In such a context, understanding the genetic determinism of coffee adaptation to climate change became essential for creating new varieties (Cheserek and Gichimu, 2012; van der Vossen *et al.*, 2015).

Within the *Coffea* genus, substantial genetic variability exists in relation to drought tolerance, particularly in the cultivated species *C. canephora* (Montagnon, 2000). This species, also commercially known as 'Robusta', is diploid (2n = 2x = 22), autogamous and phylogenetically separated into distinct groups according to their geographical origin in the inter-tropical region of Africa (Cubry *et al*., 2013). For instance, the Congolese group contains sub-group 1 (SG1), originating from the coastal lowlands and relatively tolerant to water deficit, while plants belonging to sub-group 2 (SG2) are more sensitive to water deficit. In Brazil, breeding programs implemented in the last decade on *C. canephora* Conilon (related to SG1, Montagnon *et al*., 2012) identified several drought-tolerant (D^T) and drought-sensitive (D^S) clones that were intensively characterized (Lima *et al*., 2002; Pinheiro *et al*., 2004; Praxedes *et al.* 2006). Among their anatomical and physiological traits, better root development and greater water use efficiency were highlighted as important factors of tolerance to water deficit in D^T clones (Pinheiro *et al*., 2005).

In order to investigate the molecular determinism of tolerance to water deficit in coffee, several of these *C. canephora* clones (such as D^T clone 14 and D^S clone 22) were used to identify candidate genes whose expression was induced by drought stress (Marraccini *et al.*, 2011, 2012; Vieira *et al.*,

2013). Of those genes, *CcDREB1D* (encoding the Dehydration Responsive Element Binding transcription factor 1D) was of outstanding interest since its expression was up-regulated under conditions of water deficit in leaves of D^T clone 14 but not in those of D^S clone 22 (Marraccini *et al*., 2012; Vieira *et al*., 2013).

The *DREB* gene family includes key transcription factors involved in responses to various abiotic stresses, such as water deficit, cold or salt stress, regulating the expression of essential responsive genes (Lata and Prasad, 2011; Sakuma *et al*., 2012). DREB factors act downstream of ABA-dependent and -independent signal transduction pathways in abiotic stress responses. Indeed, the overexpression of *DREB* genes in several genetically engineered plants leads to up-regulation of cold-regulated genes and osmotic-stress responsive genes, resulting in increased abiotic stress tolerance (Agarwal *et al*., 2006a). Several DREB transcription factors have also been functionally characterized in model plants. In *Arabidopsis thaliana* and rice, for instance, overexpression of *DREB1* and *DREB2* have shown promising results in inducing cold, salt and tolerance to water deficit (Novillo *et al*., 2004; Ito *et al*., 2006; Lim *et al*., 2007; Kim *et al*., 2011). However, *DREB* genes have been less extensively characterized in non-model (e.g. perennial) plants, particularly due to laborious genetic transformation protocols often limiting plant regeneration.

Even though *DREB1*/*CBF* (cold-binding factor) gene expression is mainly induced by cold, it was also reported to be involved in the control of plant development and response to other stress stimuli (Lata and Prasad, 2011). The expression of *AtDREB1D* (also known as *AtCBF4*) is rapidly induced by water deficit but not by cold in *A. thaliana* (Haake *et al*., 2002). Expression of *CBF4* genes was also reported to be up-regulated in response to low temperatures, water deficit, and salinity in different *Vitis* species (Xiao *et al*., 2008; Zandkarimi *et al*., 2015) and in *Medicago truncatula* (Li *et al*., 2011). The fact that overexpression of the *DREB1D/CBF4* gene increased tolerance to water deficit in transgenic plants of *Arabidopsis* (Haake *et al*., 2002) and *Glycine max* (Guttikonda *et al*., 2014), suggests that it plays an important role in plant responses to abiotic stress.

Despite the importance of *DREB* genes in plant response pathways to abiotic stress, limited number of studies analyzed the regulation of their corresponding promoters. Promoter regions of *AtDREB1C* (Zarka *et al.*, 2003), *OsDREB1B* (Gutha and Reddy, 2008), *GmDREB3* (Chen *et al.*, 2009) and *AtDREB2C* (Chen *et al.*, 2012) were shown to be sufficient for regulating the transcription of *uidA* reporter gene by abiotic stresses in transgenic *A. thaliana*. More recently, Fang *et al.* (2015) also demonstrated that a 1278-bp of the *FeDREB1* promoter from the common

buckwheat enhanced GUS activities by drought in leaves of transgenic tobacco. However, functional characterization of *DREB1D* promoter has neither been reported. In an attempt to better understand the tissular and environmental regulation of *CcDREB1D* promoter activity, as well as the regulation of allele-specific expression depending on the haplotype sequence, our study set out to (i) isolate and compare the promoter haplotypes of *CcDREB1D* genes from D^T clone 14 and D^S clone 22 of *C. canephora*, (ii) evaluate their ability to regulate expression of the *uidA* reporter gene by monitoring GUS histochemical activity in transgenic plants of *C. arabica* subjected to different abiotic stresses, and (iii) analyze the expression of *uidA* and endogenous *CcDREB1D* genes in leaves of these coffee plants.

Materials and methods

Plant Material and Growing Conditions

The D^T clone 14 and D^S clone 22 of *C. canephora* Conilon were previously described (Marraccini *et al*., 2011, 2012). For *Agrobacterium tumefaciens*-mediated transformation experiments, *C. arabica* var. Caturra plants were grown in *in vitro* conditions with a 12 h photoperiod (20 µmol.m^{-2}.s^{-1} light intensity) at 26°C and 80% RH.

DNA extraction and isolation of CcDREB1D promoter haplotypes

Leaves of D^T clone 14 and D^S clone 22 of *C. canephora* Conilon were used to extract genomic DNA and isolate *CcDREB1D* promoters. Genomic DNA was extracted from 20 mg of ground leaves as previously described by Cotta *et al*. (2014). For each coffee clone, *CcDREB1D* promoter haplotypes were amplified independently by PCR with high fidelity *Taq* Platinum® DNA polymerase according to the supplier's instructions (Invitrogen) (initial denaturation: 94°C-2 min followed by 30 cycles: 94°C-30 s, 68°C-30 s, 72°C-3 min and a final extension step: 72°C-10 min) using 100 ng of genomic DNA and 0.2 µM of the DREB-F1/DREB-R1 primers (Supplementary Table 1). Amplified products were cloned into the pCR2.1-TOPO vector (Invitrogen) and propagated in the *E. coli* DH5α strain. As *C. canephora* is a diploid species, recombinant plasmids were extracted from 12 independent *E. coli* colonies in order to identify the two haplotypes of

CcDREB1D promoter regions for each clone that were further double-strand sequenced using M13F and M13R universal primers and internal DREB-F2 and DREB-R2 primers (Table 2).

Bioinformatics analyses

The sequence of the unique gene *Cc02_g03430* (*CcDREB1D*: GSCOCP00020227001) from *C. canephora* (Denoeud *et al*., 2014), available at Coffee Genome Hub (http://coffee-genome.org/), was analyzed using the PlantPAN (http://plantpan.mbc.nctu.edu.tw, Chang *et al*., 2008) and the PlantProm DB (http://www.softberry.com, Shahmuradov *et al*., 2005) web interfaces. The DREB-F1/DREB-R1 primers (Supplementary Table 1) were designed to amplify the region (-1,308/+153 in Fig. 1) of *CcDREB1D* haplotypes from D^T clone 14 and D^S clone 22 that contained most of *cis*-regulatory elements (CREs) supposed to be essential for their regulation by abiotic stress. The same web interfaces were used to predict the presence of CREs in the HP15, HP16 and HP17 haplotypes of the *CcDREB1D* promoters (Table 1). The significance of the putative CREs was evaluated through a maximum threshold of core similarity (equal to 1.0) and matrix similarity of 0.75.

Construction of recombinant vectors

Binary vectors were generated by PCR using high fidelity *Taq* Platinum DNA polymerase amplification and recombinant plasmids harboring the HP15, HP16, and HP17 haplotypes of *CcDREB1D* promoters, as templates. The PCR reactions used the forward primers DREB-L, DREB-M and DREB-S carrying the *Hind*III restriction site and the reverse primer DREB-R carrying the *Bgl*II restriction site (Table 2). The position of the DREB-R primer made it possible to include the entire 5'-UTR region of the *CcDREB1D* gene in the final constructions. PCR reactions were carried out using 10 ng of plasmids, *Taq* Platinum DNA polymerase and primers as previously described, except that the first 10 amplification cycles were carried out at an annealing temperature of 60°C to ensure primer mismatch (calculated with the PrimerQuest^{SM}® software, Integrated DNA Technologies - IDT). The resulting PCR products were sub-cloned into the pGEM-T Easy vector (Promega, Madison, WI, USA), digested with *Hind*III and *Bgl*II and cloned in the pBI121 vector (Clontech, Palo Alto, CA, USA) double-digested by the same enzymes. Following ligation and transformation in the *E. coli*, the recombinant constructions were named according to their haplotype (HP) of the *CcDREB1D* promoter and length (S: short, M: medium and L: long), namely as pHP16S and pHP17S (+182/-608, relative to ATG of the *uidA* reporter gene), pHP16M

(+182/-966) and pHP15L, pHP16L and pHP17L (+182/-1,308) (Fig. 2B). Each recombinant vector was then transferred independently by electroporation into competent cells of the disarmed *A. tumefaciens* strain LBA1119. After each cloning step, recombinant vectors were systematically extracted with the Wizard® Plus SV Minipreps DNA Purification System and *CcDREB1D* promoter fragments were verified by double-strand sequencing (Genome Express, France).

Agrobacterium tumefaciens-mediated transformation and regeneration of C. arabica

A. tumefaciens strain LBA1119 harboring the recombinant vectors was grown at 28°C for 24 h in YMB (Yeast Extract Mannitol Agar) selective medium with rifampicin (25 $mg.l^{-1}$) and kanamycin (50 $mg.l^{-1}$). Transgenic lines of *C. arabica* were generated by *A. tumefaciens*-mediated transformation using twelve-month-old embryogenic calli competent for transformation as previously described (Etienne, 2005; Ribas *et al*., 2011). At least 8 independent putatively transformed cell lines were obtained for the six pHP constructions, as well as for coffee events transformed by the pBI121 (*CaMV35S:uidA*) and pBI101 (*uidA*-promoterless) vectors used as positive and negative controls, respectively. Each kanamycin resistant callus regenerated cotyledonary embryos giving plantlets after 4-5 months which were cultivated on MS medium with active charcoal (1 $g.l^{-1}$) under sterile conditions prior to testing for fast dehydration experiments. Once regenerated, DNA was extracted from leaf explants and successively tested by i) PCR using the *nptII*- (kanamycin) and *uidA*-specific primers to confirm T-DNA integration in genomic DNA of *C. arabica*-transformed plantlets, and ii) RT-QPCR to select transgenic lines harboring a single insertion of T-DNA (data not shown) representing around 50 % of T1 transgenic coffee lines (Ribas *et al*. 2011).

Abiotic assays used to study transformed plants of C. arabica

Regulation of the *CcDREB1D* promoter haplotypes was studied in primary transformants (T1) of *C. arabica* grown *in vitro* and subjected to the abiotic treatments described below. For each construction, 64 plantlets from 8 independent transformation events were arranged in eight batches of 8 plants. In each assay, each plant batch was subjected independently to water deficit for 0, 3, 6, 12 or 24 h, with 0 h (10 am, i.e. after 2h of light exposure) corresponding to 'no stress conditions'.

- Fast dehydration

Genetically transformed *in vitro* plantlets with two to four pairs of leaves were placed in a laminar flow cabinet under a 0.49 $m.s^{-1}$ air flow for 1 h until reaching a visible dehydrated state. Each pHP construction was composed of five plantlets corresponding to independent transformation events.

- Low RH assay (vapor pressure deficit)

A vapor pressure deficit (*D*) was induced by 9% RH at 27°C under controlled conditions. A theoretical *D* was calculated applying the formula $D = \left(1 - \left(\frac{RH}{100}\right)\right) * P_{WS}$ (kPa), where P_{WS} (saturated vapor pressure) = 3779 Pa at 28°C. To create a 9% RH, 400 mL of KOH supersaturate solution was poured into the lower compartment of a 5L- bioreactor (Matis®, CID Plastiques, France) which remained sealed throughout the experiment. Plantlets were placed in the upper compartment of the bioreactor in 55 mm Petri dishes having their upper part exposed to the outside environment and radicles immersed in MS (Murashige and Skoog, 1962) medium through a small hole in the Petri dish cover. Seven plants were placed inside the bioreactor in the resulting 9% RH.

- PEG assay

Osmotic potential was fixed at -2.0 MPa by adding polyethylene glycol (PEG) (molecular weight 6000; Sigma) to the MS medium, at 25°C, using the empirical equations defined by Michel and Kaufmann (1973). Batches of 7 plantlets were placed over 60 mg of vermiculite fully imbibed with 40 mL of PEG diluted in MS solution in sterile plastic Magenta® boxes.

GUS staining

Histochemical β-glucuronidase (GUS) assays were performed with different tissues (root, stem, apical meristem and leaf) derived from the same plant as those immersed in GUS staining solution (100 mM sodium phosphate buffer, pH 7.2, 10 mM sodium EDTA, 0.1% Triton X-100, and 1 $mg.ml^{-1}$ 5-bromo-4-chloro-3-indolyl-D-glucuronic acid (Sigma) and 2.5 mM potassium ferrocyanidine). After infiltrating (vacuum for 2 x 10 min) the staining solution in plant tissues, the samples were incubated at 37°C for 24 h, and then rinsed with water before image acquisition taken with a Retiga 2000R camera (G-Imaging Co., Wetzlar, Germany).

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Histology to reveal β-glucuronidase in organs of transgenic coffee plants

Prior to observation, the GUS-stained samples were fixed in fixative (50% methanol and 10% acetic acid) at 4°C for 24 h, rinsed with water and then dehydrated (10 min in 50% ethanol, 10 min in 70% ethanol and 10 min in 90% ethanol). After observation with a Nikon binocular SMZ 1500 loupe, samples were embedded in 6% agarose for subsequent sections in a Microm HM650V vibratome. For bright field microscopy observation, 50 µm-thick leaf sections were examined using a DM600 Leica microscope (Leica Microsystems GmbH, Wetzlar, Germany) and photographed. GUS expression patterns appeared highly conserved for the 8 lines transformed with the same promoter construction.

Proportion of GUS-stained guard cells in PEG and low RH assays

For bright field microscopy observation, GUS-stained leaves were fixed (50% methanol and 10% acetic acid) at 4°C for 24h, rinsed with water and incubated for at least 3 days in clearing solution (chloral hydrate: glycerol: water solution (4:1:2, v/v/v) to remove all leaf pigments. Prior to observation, tissues were rinsed with 70% ethanol and assembled in microscope slides for photography as described before. The proportion of GUS-stained guard cells on the abaxial epidermis of coffee leaves was calculated to estimate *CcDREB1D* promoter activity. For each pHP-construction, the same three independent transgenic lines were studied along the entire kinetics. The proportion of GUS-stained guard cells (p) was obtained by $p = x/n$, where x is the number of stained guard cells and n the total number of guard cells (= 150) per leaf. These values were assessed in 24 x 36 mm areas distributed in six pre-delimited leaf zones. For each pHP construction, three leaves from plants of 3 independent transformation events were sampled for each time (0, 3, 6, 12 and 24 h) in the PEG and low RH assays, and the respective proportion means (from 450 guard cells) and standard deviations for each time point were calculated.

RNA isolation

Total RNA was extracted from leaves frozen in liquid nitrogen which were further ground and treated as described previously (Breitler *et al.*, 2016). RNA quantification was performed using a NanoDrop™ 1000 Spectrophotometer (Waltham, MA, USA).

Real-time RT-PCR assays

PCR experiments were performed as previously described by Marraccini *et al*. (2012). Primers (Supplementary Table S1) were designed using Primer Express software (Applied Biosystems). Data were analyzed using SDS 2.1 software (Applied Biosystems) to determine cycle threshold (Ct) values. The specificity of the PCR products generated for each set of primers was verified by analyzing the Tm (dissociation) of amplified products. PCR efficiency (E) was estimated using absolute fluorescence data captured during the exponential phase of amplification of each reaction with the equation $(1+E) = 10(-1/^{slope})$ (Ramakers *et al*., 2003). Expression levels were calculated by applying the formula $(1+E)^{-\Delta\Delta Ct}$ where $\Delta Ct_{\text{target}} = Ct_{\text{target gene}} - Ct_{CaGAPDH}$ and $\Delta\Delta Ct = \Delta Ct_{\text{target}} - \Delta Ct_{\text{reference sample}}$, with the T0 samples being used as references for each construction. Gene expression levels were normalized with the expression *CaGAPDH* (GB accession number GW445811) gene as the endogenous control (Cruz *et al*., 2009).

Accession Numbers

Sequence data for the HP15, HP16, and HP17 haplotypes of *CcDREB1D* promoters can be found in the GenBank database under accession numbers KM281308, KM281309 and KM281311, respectively.

Results

Comparison of DNA motifs and CREs between the different CcDREB1D promoter haplotypes isolated from drought-tolerant and drought-sensitive clones

The comparison of *CcDREB1D* promoter regions of *C. canephora* drought-tolerant (D^T) clone 14 and drought-sensitive (D^S) clone 22 revealed the existence of three haplotypes called HP15, HP16 and HP17, diverging from each other by several SNPs and INDELs (Fig. 1, Supplementary Table S1 and Fig. S1). The HP15 haplotype was common (without any SNPs) to both clones, while HP16 was specific to D^T clone 14 and HP17 specific to D^S clone 22 (Fig. 2A). Upstream of the first nucleotide (+1) of the *CcDREB1D* mRNA, a sliding window comparison of the haplotype sequences revealed two highly polymorphic domains (between base pairs -627/-806 and -932/-1,121) concentrating most of the nucleotide variability (Fig. 2B), while the -115/-188 region was

highly conserved between the three haplotypes, and the -188/-520 region was conserved between HP15 and HP16. Several CREs were also observed (Table 1) such as ABA-responsive (ABRE), dehydration-responsive (DRE), inducer of CBF expression region 2 (ICEr2), DOF (DNA-binding one zinc finger) binding sites (required for gene-specific expression in guard-cells), GT-1 binding sites (essential for light-inducible expression), the CG-1 element (also known as CAMTA: calmodulin binding transcription activator) and the conserved motif 6 (CM6), considered as a repressor of DREB1/CBF activation.

The comparison of *CcDREB1D* haplotypes revealed that the ICEr2 (+115/+120) and DOF (-1,017/-1,013) DNA boxes present in HP15 and HP16 were missing in HP17 (Table 1). However, HP17 displayed some elements, such as one MYBCORE (+9/+14) and one MYC (-340/-335) element, as well as two GT-1 binding sites (-489/-484 and -1,179/-1,174) not present in the HP15 and HP16 haplotypes (Table 1). Except for these differences, the HP15, HP16 and HP17 haplotypes had all CREs in common.

Tissular activities of dehydration-induced CcDREB1D promoters in C. arabica transgenic plants

The activities of *CcDREB1D* promoter haplotypes were observed by comparing the GUS staining detected in *C. arabica* plants transformed by the short (pHP16S and pHP17S, Fig. 3A, 3B), the medium (pHP16M, Fig. 3C) and the full-length (pHP15L, pHP16L and pHP17L, Figs. 3D to 3F) constructions and subjected to fast dehydration.

No staining was observed in dehydrated wild type plants and pBI101-transformed plants, whereas strong staining was seen in dehydrated plants transformed by pBI121 (Supplementary Fig. S2). In dehydrated pHP16S-transformed coffee plants, intense GUS staining was detected in root vascular tissues, while weak staining was observed in stem vascular tissues (Figs. 3B1, 3B2). In meristems, longitudinal-sections revealed weak GUS activity in leaf primordia and auxiliary buds, while intense staining was observed in apical meristems (Fig. 3B3). Strong GUS activities were also detected in leaf epidermis, as well as in spongy and palisade parenchyma (Fig. 3B4). GUS activities in pHP17S-transformed plants were similar (at spatial, tissue and cell-specific levels) to those observed in pHP16S-transformed plants (Fig. 3A2, 3A4), except in roots where no staining was seen (Fig. 3A1).

For the pHP16L, pHP15L and pHP17L constructions, no GUS staining was observed in roots, stems, and meristems (Figs. 3D1 to D3, 3E1 to E3, 3F1 to F3, respectively). Microscopic analyses of leaf transverse sections revealed GUS staining in palisade and spongy parenchyma of pHP15L-transformed plants (Fig. 3E4), and in several guard cells and stomata of pHP16L-transformed plants (Fig. 3D4).

The effects of promoter length were observed by comparing GUS activities in plants transformed by the pHP16S, pHP16M and pHP16L constructions (Figs. 3B, 3C, 3D, respectively). For example, GUS activities were detected in leaves in pHP16M plants, particularly in the epidermis, in spongy and palisade parenchyma, and in the guard cells (Fig. 3C4) whereas non-tissue specificity of staining was observed with pHP16S-transformed plants (Fig. 3B4). The greatest tissue-specificity of GUS staining was shown for the plants transformed with the pHP16L construction, with histochemical staining observed in guard cells only (Fig. 3D4). The comparison of pHP17S- and pHP17L-transformed plants showed drastic reduction of GUS staining with the longer promoter (Figs. 3A, 3F, respectively).

CcDREB1D promoters target uidA gene expression in guard cells of C. arabica under water deficit

In order to fine-tune the GUS staining results previously observed during the fast-drying assay, transformed coffee plants were subjected to controlled water deficit mimicked by low RH (9%) and further analyzed to investigate GUS coloration in abaxial leaf surfaces. As controls, no staining was detected in plants transformed with the pBI101 vector (Fig. 4A) whereas strong staining was detected in guard and epidermis cells of leaves from pBI121- transformed plants (Fig. 4B). Under unstressed conditions, both epidermal cells and stomata were completely unstained in plants transformed by pHP17S, pHP17L, pHP16S and pHP16L (Figs. 4C, E, G and I). After 24 hours of reduced RH, GUS activities were clearly detected in guard cells of the same transgenic plants (Figs. 4D, F, H and J). GUS staining also appeared to be more intense in the colored stomata of pHP16L-transformed plants (Fig. 4J) than in those of pHP16S-transformed plants (Fig. 4H). For the three constructions, our results also clearly showed that in the same abaxial leaf area, stomata with stained guard cells coexisted with completely unstained stomata under a low RH, as observed for the HP16 haplotype (Figs. 4G, 4I). Longitudinal and cross-sections of the pHP16L constructions were also made to immunolocalize GUS proteins at cellular level by using anti-β-glucuronidase

polyclonal antibody. This clearly detected GUS protein in guard cells with fluorescence signals that were highly concentrated in their thickened inner wall (Suppl. Fig. S3A). Fluorescence signals were not detected in all guard cells, but were observed in subsidiary cells, in spongy and palisade parenchyma cells, and in epidermis cells (Suppl. Fig. S3B).

The activity of the HP16L CcDREB1D promoter was up-regulated by the low RH and PEG assays in leaves of transgenic C. arabica

In order to see whether the nucleic polymorphisms existing in the three longest haplotypes (HP15L, HP16L and HP17L) of the *CcDREB1D* promoter influenced the regulation of these sequences, the activity of these promoters was evaluated by calculating the proportion of GUS-stained guard cells on leaf abaxial regions in a time-course response to water deficit assays simulated by PEG (equivalent to -2.0 MPa) and to low RH assays (Fig. 5). For the pHP16L- and pHP17L-transformed plants, the proportion of GUS-stained guard cells increased in the first hours of PEG treatment, reaching 20% and 10% maximum on average after 12 h and 6 h, respectively (Fig. 5). However, the proportion of GUS-stained guard cells remained relatively low and stable during the PEG assay for pHP15L-transformed plants. For the low RH assay, around 20% of guard cells were GUS-stained after 24 h of treatment in pHP16L-transformed plants. This percentage was reached after 6 h in pHP15L-transformed plants and gradually decreased over the time course to reach 5% after 24 h of stress. In pHP17L-transformed plants, the proportion of GUS-stained guard cells increased slowly to reach a mean of 10% after 12 h of low RH or after 5 h of PEG treatment and decreased thereafter.

uidA and CcDREB1D gene expression

The monitoring of the of the *uidA* gene expression in leaves of coffee plants transformed by the pHP15L, pHP16L and pHP17L and subjected to PEG treatment showed low variability for each construction at each time of the water deficit bioassay. Similar expression profiles were obtained for all the constructions, showing a gradual increase in *uidA* gene expression with PEG treatment, which reached maximum expression after 6 h (pHP16L and pHP17L) or after 12 h (pHP15L) and declined thereafter (Fig. 6A). The main differences observed between the constructions concerned relative expression values in the first hours of stress (3 h and 6 h), with *uidA* gene expression being higher in pHP16L-transformed plants than in pHP15L- and pHP17L-transformed plants. As

controls, expression of the *CaDREB1D* endogenous gene increased with PEG treatment, reaching a peak at 6 h and then decreased gradually afterwards (Fig. 6B).

Discussion

In our study, we investigated the responses of different haplotypes of the *CcDREB1D* (CBF4) promoters of *C. canephora* to PEG and low RH treatments by analyzing their ability to regulate the expression of the *uidA* reporter gene in stably transformed *C. arabica* plants.

Multiple CREs are involved in the regulation of CcDREB1D promoter haplotypes

The results presented here indicate that the HP15, HP16 and HP17 haplotypes of *CcDREB1D* promoters harbored several abiotic-stress responsive CREs in common, including those involved in tissue-specific and light regulation. They also contained ABRE and DRE elements, thereby suggesting possible regulation of these promoters by both ABA-dependent and -independent networks (Haake *et al*., 2002; Yamaguchi-Shinozaki and Shinozaki, 2005). Interestingly, the co-occurrence in parallel strands and opposite direction of ABRE) and DRE elements within 100 bp, identified herein for all haplotypes, is the most active structural organization observed for these CREs in drought-responsive genes (Mishra *et al*., 2014). If we consider that DRE elements share a common DNA motif with LTRE (low-temperature responsive element), a functional implication of this gene could be expected in coffee plant responses to cold stress, as observed for its homologous genes in several plants (Zhang *et al*., 2004; Ito *et al*., 2006; Mao and Chen, 2012). This is also reinforced by the fact that the three *CcDREB1D* haplotypes reported here also contain other CREs such as one ICEr2 box (except in the HP17 haplotype) and eight MYC-related binding sites (CANNTG), which are both motifs known to play a key role in the regulation of DREB1/CBF genes by cold-stress. Regarding MYC-related DNA motifs, two of them contained the preferable target sequence CAAATG (-842/-837 for all HPs and -340/-335 only for HP17) of the positive regulator ICE1 (inducer of CBF expression 1).

The three *CcDREB1D* haplotypes also contained several MYB-binding sites that can act as either an enhancer or repressor of promoter activity. One of them (MYB1AT: AAACCA) is a putative DNA binding site of the MYB15 trans-acting factor known to regulate negatively the expression of DREB1D/CBF genes (Agarwal *et al*., 2006b). The remaining MYB boxes are potential positive regulators of DREB1/CBF expression, especially MYB2AT (YAACTG) binding sites required for

activation of salt- and dehydration-responsive genes (Urao *et al*., 1993; Abe *et al*., 2003). *In silico* analyses of the *CcDREB1D* haplotypes also revealed the occurrence of several DOF-binding sites, already reported as key DNA motifs mediating guard cell-specific gene expression, and of several GT-1 binding sites essential for light-inducible expression, mainly located in the last 500 bp region (-921/-1,183) of the *CcDREB1D* promoters (Plesch *et al*., 2001; Galbiati *et al*., 2008; Yang *et al*., 2008; Gardner *et al*., 2009; Cominelli *et al*., 2011).

Modular organization of DOF and light-induced DNA elements may account for preferential expression of CcDREB1D promoter haplotypes in guard cells

Previous studies showed that *OsDREB1B, AtDREB1C, AtDREB2C* and *FeDREB1* promoters contained all CREs necessary for enhancing gene expression by several abiotic within the proximal regions (up to 1 kb) of these promoters (Zarka *et al.*, 2003; Gutha and Reddy, 2008; Chen *et al.*, 2009; Chen *et al.*, 2012; Fang *et al.*, 2015). The results of GUS activities presented here in transgenic coffee plants transformed by the short (S: +182/-608), medium (M: +182/-966) or full-length (L: +182/-1,308) sequences of *CcDREB1D* haplotypes, clearly revealed the importance of the CREs located in the -608/-1,308 region in regulating the expression of the *uidA* reporter gene in a tissue-specific manner. Indeed, while ubiquitous and strong GUS activities were observed in different tissues of coffee plants transformed by the shortest constructions (pHP16S and pHP17S), fine-tuned regulation of the *uidA* reporter gene was observed in coffee plants transformed by the full-length constructions (pHP15L, pHP16L and pHP17L), with GUS activities restricted to leaf mesophyll and guard cells under stressed conditions. In that case, the degree of *CcDREB1D* haplotype activity in guard cells also appeared to increase with the number of DOF-binding sites, from the short (6 sites) to the full-length (11/12 sites) promoter regions. One possible explanation could be an increase in the numbers of regulatory sites recognized by trans-acting factors known in the longest constructions of *CcDREB1D* haplotypes. For example, of the five MYB DNA boxes found within the -608/-1308 region, three MYBCORE and one MYB2AT are positive regulators and one (MYB1AT) is a putative binding site of MYB15 trans-acting factor known to repress *DREB1/CBF* genes (Agarwal *et al*., 2006b) but also to regulate guard cell stomatal closure under drought conditions (Ding *et al*., 2009). The presence of such DNA boxes could explain the low GUS activities observed in coffee plants transformed with the longest constructions (pHP15L, pHP16L and pHP17L) compared to those observed in plants transformed with the shortest

constructions (pHP16S and pHP17S). The increase in leaf-specific GUS activities observed from the short to the medium and full-length constructions, also tallied with the fact that cluster arrangements of DOF-binding sites are essential in determining guard cell-specific gene expression (Galbiati *et al*., 2008; Yang *et al*., 2008). However, this does not preclude the participation of other CREs functioning conjointly with DOF-binding sites to fine-tune the transcriptional regulation of gene expression in guard cells, as previously proposed in grape *VvMYB60*, potato *KST1* and cotton *GbSLSP* promoters, for example (Plesch *et al*., 2001; Gardner *et al*., 2009; Cominelli *et al*., 2011; Galbiati *et al*., 2011; Han *et al*., 2013). Of the CREs that could play such roles, it is worth noting the presence of several GT-1 DNA binding sites in the *CcDREB1D* promoter haplotypes, with three of them (one in the medium (-608/-966) and two in the distal (-966/-1,308) regions of the *CcDREB1D* promoters) closely located to DOF-binding elements. Therefore, GT-1 DNA binding sites could participate together with DOF elements in forming such clusters necessary for targeting guard cell-specific gene expression.

The CcDREB1D promoter haplotypes respond differentially to RH and PEG abiotic stress

Few studies already reported the existence of functional variation between haplotypes (or alleles) of plant promoters in regulating gene expression differentially (de Meaux *et al.*, 2005; Takeshima *et al.*, 2016). The results presented here regarding the proportion of GUS-stained guard cells measured in transgenic coffee lines subjected to low water potential (PEG treatment) clearly showed greater activity for the HP16 and HP17 full-length haplotypes compared to very low activity for the HP15L haplotype. However, the activity of all the full-length *CcDREB1D* haplotypes was up-regulated under low air relative humidity (RH 9%). In that case, a slight increase in GUS-stained guard cells was observed in pHP15L- and pHP17L-transformed plants, while this proportion increased slightly but regularly in pHP16L-transformed plants in the RH treatment. The different time courses of GUS-stained guard cells observed between the HP15, HP16 and HP17 haplotypes during the PEG and RH treatments clearly revealed differences in the fine-tuning of the regulation of these *CcDREB1D* promoters, which were undoubtedly related to the nucleic polymorphism identified between these sequences directly affecting CREs and/or altering their cluster organization (see discussion below). Our results also indicated that the PEG and RH treatments affected the time-course activity of the HP16 and HP17 haplotypes of *CcDREB1D*

promoters differently. For example, GUS-stained guard cells were detected earlier in pHP16L- and pHP17L-transformed plants in response to the PEG treatment compared to the RH treatment. PEG treatment is known to mediate low Ψ_w by reducing the osmotic potential of the nutrient solution, thereby mimicking low soil water potential which leads to a water deficit in the whole plant (Blum, 2014). Following the perception of water deficit by roots (major ABA producing organs), ABA would rapidly amplify the drought signal and activate the *CcDREB1D* promoter in guard cells, probably through the involvement of ABRE regulatory elements. The fact that exogenous ABA up-regulated the expression of the *uidA* and *CaDREB1D* gene mainly in stomata guard cells of pHP16L-transformed coffee plants but also of genes from the supports the idea that this phytohormone plays a key role in coffee responses to water deficit (Torres *et al.*, 2016). While ABA induces early responses of *CcDREB1D* promoters by root-sensing water deficit, an inverse drought stimulus (where water stress is "sensed" by aerial parts rather than roots) is expected to delay the response of the *CcDREB1D* promoter to water deficit. To simulate such conditions, our transgenic coffee plants were subjected to a water deficit of -2.0 MPa mimicked by the low RH treatment, which is known to increase the leaf-to-air water vapor pressure deficit (VPD), to generate evaporative demand and, consequently, to elevate the transpiration rate (Farooq *et al.*, 2009). The increase over time in GUS-stained guard cells in pHP16L- and pHP17L-transformed plants with the low RH (high VPD) treatment tends to support the existence of such a mechanism. However, while the frequency of GUS activity in guard cells increased gradually with a low RH in pHP16L-transformed plants, it increased in the first 12 h only and then decreased slowly in pHP17L-transformed plants, thereby demonstrating that these haplotypes differed in activity and/or regulation.

The nucleic polymorphisms existing in CcDREB1D haplotypes are probably involved in modulating promoter activities

The comparison of HP15, HP16 and HP17 haplotype sequences revealed that (1) they had most of the essential CREs in common, and that (2) HP15 and HP16 were more closely related than HP17 regarding INDEL-type polymorphisms. By comparing CREs between HP16 and HP15, very few differences were observed regarding the boxes known to recognize key transcription factors involved in abiotic stress. The fact that these haplotypes of *CcDREB1D* promoters are regulated in a different manner in controlling the expression of the *uidA* reporter gene suggests that the

nucleotide polymorphisms observed outside of the CREs might be responsible for these differences, as could be the case for the 2 bp insertion (AA: -1,033/-1,032) in HP16, with this INDEL being localized close to GT1 (-1,037/-1,027) and DOF (-1,017/-1,013) DNA binding sites. Several other polymorphisms, localized close to essential CREs, were also identified mainly in the polymorphic domains -627/-806 and -932/-1,121. Within the -627/-806 region, the presence of (1) MYB- and MYC-like regulatory elements, respectively known as activator and repressors of transcription by drought, and (2) a tandem (x3) repeat of the GAAWTT unidentified motif (-722/-739) only present in the HP16 haplotype is worth noting. Compared to the HP16 sequence, a G to A transition (in position -1,013) in the HP17 haplotype, leading to the loss of one DOF domain, is also worth noting. Since DOF transcription factors are involved in the regulation of various plant processes (Le Hir and Bellini, 2013), such as stomata functioning, for example (Gardner *et al*., 2009; Negi *et al*., 2013), this might explain the differences in activity observed between these two haplotypes under PEG and low RH assays.

Drought stress up-regulates the expression of CcDREB1D haplotypes in transgenic plants of C. arabica

The increase in GUS activities observed in the water deficit mimicking treatments in leaves of transgenic coffee plants transformed by the pHP15L, pHP16L and pHP17L constructions was confirmed by monitoring expression of the *uidA* reporter gene. For all the haplotypes, *uidA*-specific transcripts were accumulated after 3 h to 12 h of PEG treatment (maximum after 6-12h) and declined thereafter. Although similar in terms of profiles, differences in promoter strength were found, with the HP16L promoter being stronger and occurring sooner (significant expression after 3h) than the other two haplotypes. It is worth noting that the accumulation of *uidA* transcripts followed quite well the expression profile of the endogenous *CaDREB1D* gene which was also up-regulated in the PEG treatment. In that case, the quantity of *CaDREB1D* transcripts detected after 24 h of treatment was very weak and did not differ from that before water deficit treatment. *CaDREB1D* transcripts were almost barely detectable after 6h. Our results strongly suggest that the transient expression of the *CaDREB1D* gene under water deficit directly reflected the transitory activity of *DREB1D* promoters.

Even though the expression of *DREB1*/*CBF* genes has been commonly reported to be mainly induced by cold, our results clearly indicated that the expression of the *DREB1D* gene of *C. arabica*

555 was rapidly induced by water deficit mimicking treatments and that the up-regulated expression of
556 this gene was controlled at transcriptional level. These results also showed that the different
557 promoter haplotypes of the *CcDREB1D* gene of the diploid *C. canephora* were correctly
558 recognized by the transcriptional machinery of the allotetraploid species *C. arabica*.
559 Summarizing, the variations in intensities and staining patterns between the three haplotypes tested
560 during this study suggested that HP16 (isolated from D^T clone 14 of *C. canephora*) had greater
561 strength, as well as longer activity under low RH conditions compared to HP15 (found in both D^T
562 clone 14 and D^S clone 22) and HP17 (isolated from D^S clone 22) haplotypes. These characteristics
563 might explain the higher stomatal conductance (g_s), and consequently, the more efficient
564 mechanisms in controlling the transpiration rate observed under water deficit in D^T clone 14
565 compared to D^S clone 22 (Marraccini *et al*., 2012). Based on the results of this study, work is now
566 on-going to analyze the genetic diversity of *DREB1D* promoters in other D^T and D^S plants of the
567 *Coffea* species with the objective to identify molecular markers for breeding drought resistant
568 cultivars, and also to study the effects of other types of abiotic stress (e.g. high temperature and
569 light intensities, cold stress, etc.) on the regulation and activity of the *CcDREB1D* haplotypes.
570

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582

583 **Author contributions:** G.S.C.A. cloned and sequenced the *CcDREB1D* promoter haplotypes,
584 made the constructions and analyzed these sequences. G.S.C.A., L.F.T., H.E and E.D. carried out

585 the genetic transformation of *C. arabica*, applied abiotic stress, and carried out GUS staining and
586 microscopy analyses with the help of F.G. L.F.T. extracted RNA samples used by J-C.B. to perform
587 qPCR experiments and analyse the results together with T.J. H.E, B.B., A.C.A and P.M. designed
588 the study, drew up the experimental design and implemented it. G.S.C.A., A.C.A, T.J., P.M. and
589 H.E. wrote the article which was approved by all the authors.

590

Table 1. *Cis*-regulatory elements (CREs) present in the *CcDREB1D* promoter haplotypes.

	CREs	Sequence	TFs	Gene	Stress condition	Ref.	Sites	pHP15	pHP16	pHP17
ABRE	ABRELATERD1	ACGTG	bZIP	*erd1*	Water deficit, ABA	6	-1,272/-1,268 (+)	+	+	+
	ABRE-LIKE	BACGTGKM	bZIP	*rd29*	Water deficit, ABA	2	-557/-550 (-)	+	+	+
							-347/-340 (+)	+	+	+
DRE	DRE	CCGNC	ERF/AP2	*rd29A*	Water deficit, cold	1	-267/-263 (-)	+	+	+
	LTRECOREATCOR15	CCGAC	ERF/AP2	*cor15A*	Water deficit, cold	6				
MYB	MYBCORE	CNGTTR	bHLH	*rd22*	Water deficit, ABA	6	-1,228/-1,223 (-)	+	+	+
							-1,012/-1,007 (-)	+	+	+
							-695/-690 (-)	+	+	+
							+9/+14 (-)	-	-	+
	MYB1AT	WAACCA	bHLH (MYB15)	*rd22*	Water deficit, ABA	6	-1,248/-1,243 (+)	+	+	+
	MYB2AT	YAACTG	bHLH (MYB2)	*rd22*	Water deficit, ABA	6	-1,235/-1,230 (+)	+	+	+
							-575/-570 (-)	+	+	+
							+104/+109 (-)	+	+	+
MYC	MYCCONSENSUSAT	CANNTG	bHLH (ICE1)	*rd22*	Water deficit	6	-1,273/-1,268 (+)	+	+	+
							-842/-837 (+)	+	+	+
							-575/-570 (+)	+	+	+
							-387/-382 (+)	+	+	+
							-347/-342 (+)	+	+	+
							-340/-335 (+)	-	-	+
							-220/-215 (+)	+	+	+
							+104/+109 (+)	+	+	+
ICE	ICEr2	ACTCCG	bHLH (ICE)	*CBF2/ DREB1C*	Cold	7	+115/+120 (+)	+	+	-
CG-1	CG-1	VCGCGB	bHLH (CAMTA/SR1)	*PAL1*	CaM/Ca^{+2} - inducible	3	-1,263/-1,258 (+)	+	+	+
							-1,255/-1,250 (+)	+	+	+
							-148/-143 (+)	+	+	+
CM6	CM6	AAGATTGTCA	unknown	*unknown*	Water deficit, ABA	5	-626/-617 (+)	+	+	+

Table 1 (continued).

	CREs	Sequence	TFs	Gene	Stress condition	Ref.	Sites	pHP15	pHP16	pHP17
GT-1	GT-1	GRWAAW	bHLH	*RBCS*	Light-inducible	6	-1,286/-1,281 (-)	+	+	+
							-1,179/-1,174 (+)	-	-	+
							-1,137/-1,132 (+)	+	+	+
							-1,129/-1,124 (+)	+	+	+
							-1,037/-1,027 (+)	+	+	-
							-1,005/-999 (+)	+	+	+
							-938/-933 (-)	+	+	+
							-921/-916 (+)	+	+	+
							-912/-907 (-)	+	+	+
							-899/-894 (-)	+	+	+
							-564/-559 (-)	+	+	+
							-538/-533 (+)	+	+	+
							-489/-484 (+)	-	-	+
							+143/+148 (-)	+	+	+
DOF	DOF	WAAAG	ZF	*KST1*	Water deficit, ABA	4	-1,183/-1,179 (+)	+	+	+
							-1,017/-1,013 (+)	+	+	-
							-1,000/-996 (+)	+	+	+
							-968/-964 (+)	+	+	+
							-929/-925 (-)	+	+	+
							-925/-921 (+)	+	+	+
							-584/-580 (+)	+	+	+
							-529/-525 (+)	+	+	+
							-487/-483 (+)	+	+	+
							-236/-232 (+)	+	+	+
							+49/+53 (-)	+	+	+
							+94/+98 (-)	+	+	+
W-BOX	W-BOX	TTGAC	ZF	*NPR1*	Wounding	6	-1,230/-1,226 (-)	+	+	+
							-552/-548 (-)	+	+	+
							-506/-502 (+)	+	+	+
							-482/-478 (-)	+	+	+
							-403/-399 (-)	+	+	+
							-231/-227 (+)	+	+	+
							+2/+6 (-)	+	+	+

Table 2. List of primers used in this study.

Legend: ([a]): primers used to amplify HP15, HP16 and HP17 haplotype promoters from the *CcDREB1D* (*Cc02_g03430*) gene of *Coffea canephora* (Denoeud et al., 2014). ([b]): internal primers used for sequencing to verify the sequence of *CcDREB1D* promoters. ([c]), ([d]) and ([e]): forward primers used to amplify the short (S: +182/-608), medium (M: +182/-966) and long (L: +182/-1.308) fragments of the *CcDREB1D* promoter haplotypes, respectively. The *Hind*II restriction site of these primers is underlined. ([f]): reverse primer used with to introduce the *Bgl*II restriction site. Other primer pairs were used in RT-qPCR experiments to determine gene expression levels of *uidA* (GUS-F/R) and *CcDREB1D* (DREBA09-F/R). ([g]): the primer pair GAPDH-F/R was used to amplify the transcripts of *CaGAPDH* (glyceraldehyde-3-phosphate dehydrogenase) gene used as reference to standardize the results of RT-qPCR experiments.

Primers	Sequences
DREB-F1 ([a])	5’ ACTCCTAGTAAGCGGCACGTTGTT 3’
DREB-R1 ([a])	5’ TGGCTTTGCAGGCATTGACTACG 3’
DREB-F2 ([b])	5’ TCGTGCATTCAACAGCACCGTCA 3’
DREB-R2 ([b])	5’ CCTTTCGTGGTTGTCTCTTGACCT 3’
S-DREB1D ([c])	5’ TAATTCCAAGCTTTGTCTGAAGT 3’
M-DREB1D ([d])	5’ AAGAGAACAACAAGCTTCTTGT 3’
L-DREB1D ([e])	5’ TCCTAGTAAGCTTCACGTTGT 3’
R-DREB1D ([f])	5’ TGTTGAGAAATGGTTAGATCTTGAA 3’
GUS-F	5’ GCACTAGCGGGACTTTGCAA 3’
GUS-R	5’ CGCGAAGCGGGTAGATATCA 3’
DREBA09-F	5' CAATGCCTGCAAAGCCAATTA 3'
DREBA09-R	5' TTTTCCTGCCTGCACGTTTC 3'
GAPDH-F ([g])	5' TTGAAGGGCGGTGCAAA 3'
GAPDH-R ([g])	5' AACATGGGTGCATCCTTGCT 3'

Figure legends

Fig. 1. Consensus sequence of of *CcDREB1D* promoter haplotypes.

The consensus sequence contains all single nucleotide polymorphisms (SNPs, in gray) and Insertion/deletions (INDELs, lower case underlined with horizontal brackets) found in the HP16 and HP17 haplotypes of *CcDREB1D* promoters. The nucleotides are numbered (on the right) on each lane using the first nucleotide (+1) of the *CcDREB1D* mRNA sequence (based on RNAseq data evidence available in the Coffee Genome Hub) as the start of numbering. CREs are identified by boxes with their corresponding name below excepted DOF/guard-cell (underlined), W-BOX (underlined and italics) and ABRE-LIKE (bold). Horizontal arrows indicate the primers (Table 2) used to amplify the full-length and truncated versions of coffee *CcDREB1D* promoter sequences (Fig. 2C). The TCA microsatellite GAAWTT unidentified motif and the putative TATA box are also indicated (bold and brackets). The 19 bp in italics upstream the ATG (+174) of the *ß*-glucuronidase correspond to the multiple cloning site of the pBI121 vector.

Fig. 2. Haplotypes of *CcDREB1D* promoters of *C. canephora*.

(A): *CcDREB1D* haplotypes found in drought-tolerant (D^T) clone 14 (HP15/HP16) and drought-sensitive (D^S) clone 22 (HP15/HP17) of *C. canephora*. Color code for haplotypes: HP15 (white), HP16 (black) and HP17 (gray). (B): Graphic representation of nucleotide variability detected in *CcDREB1D* promoter haplotypes. The *x* axis corresponds to the bases of the HP15 sequence (Fig. 1). The y axis corresponds to the frequency of polymorphic sites (S) observed in the HP16 (continuous line) and HP17 (dotted line) haplotypes compared to the HP15 sequence used as a reference. (C): Schematic representation of the *CcDREB1D* haplotypes analyzed in transgenic plants of *C. arabica*. The schematic map of *CcDREB1D* (*Cc02_g03430*) is given in the upper part together with the DREB primers (F1, R1 in brackets, see Table 2) used to amplify the *CcDREB1D* promoter haplotypes. Plasmid names used for stable transformation of *C. arabica* are given for each construction, indicating the haplotype studied with its corresponding length (L, long; M, medium and S, short). The fragments were amplified using the forward primers (including the *Hind*III [AAGCTT] restriction site) corresponding to L-DREB1D (L, white square), M-DREB1D (M, white star) and S-DREB1D (S, white triangle) and the reverse primer R-DREB1D (including the *Bgl*II [AGATCT] restriction site) indicated by a black point and further cloned in front of the *uidA* reporter gene.

pBI121 (CaMV35S:*uidA* gene construct) and pBI101 (*uidA* promoterless gene) were used as positive and negative controls of GUS enzymatic activities, respectively.

Fig. 3. Histochemical localization of GUS activity in different tissues of transgenic *C. arabica* subjected to dehydration.

Columns 1: root; 2: stem; 3: meristem; 4: leaf. The tissues belong to plants regenerated from constructions pHP17S (A), pHP16S (B), pHP16M (C), pHP16L (D), pHP15L (E) and pHP17L (F). The histochemical localization of GUS activity in control plants (WT, pBI101 and pBI121) is given in Supplementary Fig. S2. The scales (in µm) are given for each image. Arrows indicate GUS-staining restricted to specific cells and tissues: leaf epidermis (le), guard cell (gc), parenchyma (p), vascular tissue (vt), apical meristem (am).

Fig. 4. Histochemical localization of GUS activity in guard cells of *C. arabica* subjected to low air relative humidity (RH 9%).

Guard cells visualized by bright field microscopy (20 x magnification) on the abaxial detached epidermis of coffee leaves. The explants were from coffee plants transformed by pBI101 (A, negative control), pBI121 (B, positive control), pHP17S (C, D), pHP17L (E, F), pHP16S (G, H) and pHP16L (I, J). Unstressed conditions for C, E, G and I. Stressed conditions (9 % RH) for A, B, D, E, H and J.

Fig. 5. Proportions of GUS-stained guard cells: water deficit-induced regulation of GUS activity in mature leaf stomata driven by *CcDREB1D* promoter haplotypes.

Evolution of GUS-stained guard cells' proportions following 0, 3, 6, 12 and 24 h of exposure to PEG (equivalent to -2.0 MPa) and low air relative humidity (RH 9%) treatments in pHP15L-, pHP16L- and pHP17L- transformed coffee plants. Box-and-whisker plot display variation in proportions of coffee plants for each time assessed. The lower and upper hinges represent the first and third quartile respectively, the bold line represents the median, and the whiskers represent the smallest and the greatest values.

Fig. 6. Expression profiles of the *uidA* and *CaDREB1D* genes in leaves of transgenic *C. arabica* during water deficit.

Expression of the *uidA* (A) and *CaDREB1D* (B) genes was tested in leaves of coffee plants transformed by the pHP15L (white isobars), pHP16L (light gray isobars) and pHP17L (dark gray isobars) constructions and subjected to 0, 3, 6, 12 and 24 h osmotic stress (PEG treatment)

765 by RT-qPCR experiments using the GUS-F/R and DREBA09-F/R primer pairs, respectively.
766 Expression levels are indicated in relative quantification using the expression of the *CaGAPDH*
767 gene as a reference. The results are expressed using T0 samples as internal calibrators for each
768 construction. The relative quantification values correspond to the mean of at least three
769 biological repetitions analyzed by 3 technical replicates ±SD. The significance of expression
770 level differences was evaluated using the pairwise Wilcoxon rank test (non-parametric test).
771 Treatments sharing the same letter are not significantly different.
772
773 **Table 1.** *Cis*-regulatory elements (CREs) present in the *CcDREB1D* promoter haplotypes.
774 The *cis*-regulatory elements present in the *CcDREB1D* promoter consensus sequence (Fig. 1),
775 involved in osmotic- and cold-stress responsive gene expression, were identified using
776 PlantPAN (http://plantpan.mbc.nctu.edu.tw, Chang *et al*., 2008) and the TSSP
777 (http://www.softberry.com, Shahmuradov *et al*., 2005) web interfaces. Experimental results
778 supporting the involvement of CREs in the regulation of gene expression by abiotic stresses are
779 also available in the literature (Ref.: 1, Sakuma *et al*., 2002; 2, Yamaguchi-Shinozaki and
780 Shinozaki, 2005; 3, Silva, 1994; 4, Plesch et *al*., 2001; 5, Doherty *et al*., 2009; 6, Higo *et al*.,
781 1999; 7, Chinnusamy *et al*., 2004). Base abbreviations (IUPAC notation) are as follows: B =
782 [not A], K = [G,T], M = [A,C], N = [any nucleotide], R = [A,G], V = [not T], W = [A,T], Y =
783 [C,T]. TFs: transcription factors. CREs were found either in sense (+) or antisense (-) DNA
784 strands.
785
786 **Table 2.** List of primers used in this study.
787
788 **Supplementary Data**

789 **Supplementary Fig. S1.** Nucleic alignments of *CcDREB1D* haplotypes (HP15, HP16 and
790 HP17) with the *CcDREB1D* consensus sequence (see Fig. 1).

791 **Supplementary Fig. S2.** Histochemical localization (root, stem, meristem, leaf) of GUS
792 activity in dehydrated control plants of *C. arabica*. No staining in untransformed plants (A:
793 wild type) and in pB101-transformed plants (B) while strong staining was observed in all tissues
794 of pB121-transformed coffee plants. The scales (in µm) are given for each image.

795 **Supplementary Fig. S3.** Confocal laser scanning microscopy (CLSM) micrographs from leaf
796 cross-sections with immunolocalization of GUS protein. Leaves of pHP16L-transformed plants

were sampled after 24 h of exposure to 9% RH. Fluorescence immunolabeling of GUS proteins was carried out with 5% bovine serum albumin (BSA) in PBS (blocking buffer, 3 h), anti-*β*-glucuronidase rabbit antibody (1:200 in blocking buffer, overnight at 4°C), PBS (3 × 15 min washing). Leaves were also treated with anti-GUS rabbit antibody and then with a secondary anti-rabbit IgGs mouse antibody coupled to an Alexa Fluor® 488 probe (4 $\mu g.ml^{-1}$ in 2% BSA in PBS, 1 h in the dark), and PBS (3 × 15 min washing). Sections were mounted in PBS and observed under an LSM510 META NLO Axiovert 200M Inverted Microscope (Carl Zeiss, Germany), equipped with a Chameleon Ultra II laser (Coherent, Glasgow, UK) with the following settings: laser 488 nm, BP 500-530 nm. Micrograph deconvolution of spectrum signals was achieved using the Linear Unmixing technique (A and B). Cells testing positive for GUS green fluorescence are indicated by white arrows. Epidermal cell (ec); guard cell (gc); parenchyma cell (pc).

Supplementary Table S1. List of nucleic polymorphisms found in the HP15, HP16, and HP17 haplotypes of *CcDREB1D* coffee promoters.

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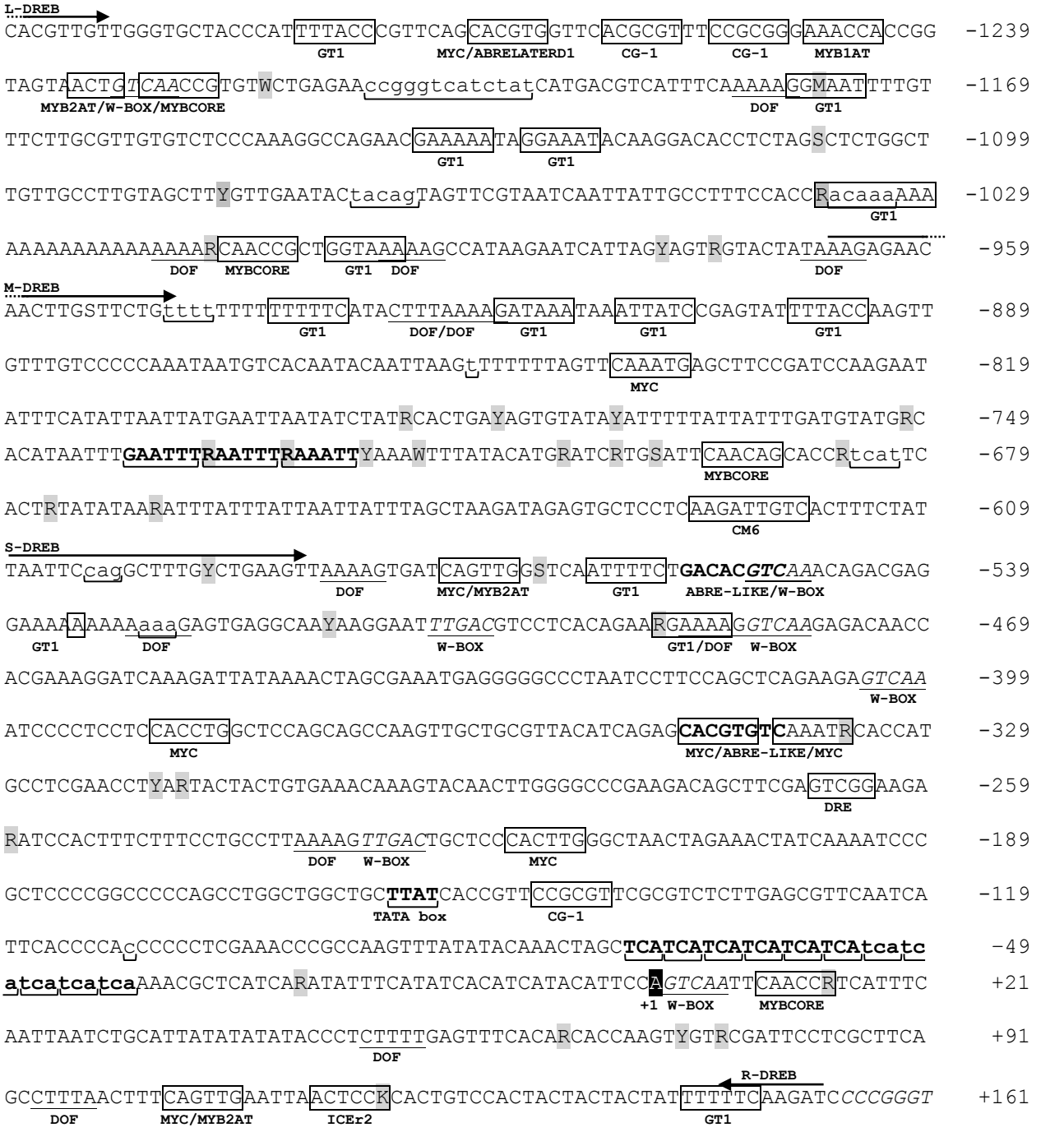
Figure 1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MYB2AT/W-BOX/MYBCORE DOF GT1
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GT1 GT1
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GT1
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DOF MYBCORE GT1 DOF DOF
M-DREB
AACTTGSTTCTGtttttTTTTTTTTTCATACTTTAAAAGATAAATAAATTATCCGAGTATTTTACCAAGTT -889
GT1 DOF/DOF GT1 GT1 GT1
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MYBCORE
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CM6
S-DREB
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DOF MYC/MYB2AT GT1 ABRE-LIKE/W-BOX
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GT1 DOF W-BOX GT1/DOF W-BOX
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W-BOX
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MYC MYC/ABRE-LIKE/MYC
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DRE
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DOF W-BOX MYC
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TATA box CG-1
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+1 W-BOX MYBCORE
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DOF
R-DREB
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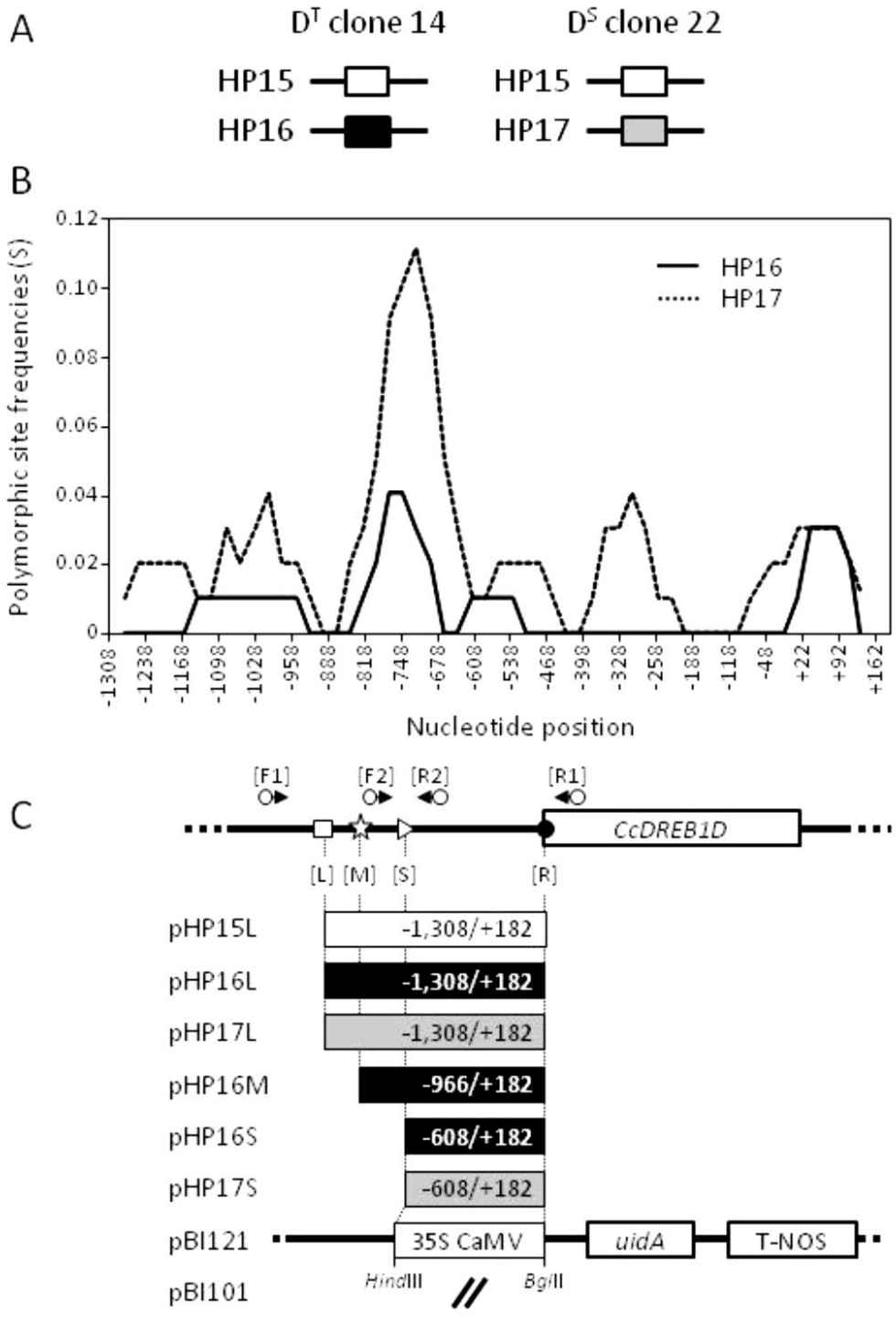
Figure 2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D^S clone 22
HP15
HP16
HP15
HP17
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Polymorphic site frequencies (S)
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0.06
0.04
0.02
0
HP16
HP17
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Nucleotide position
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[R]
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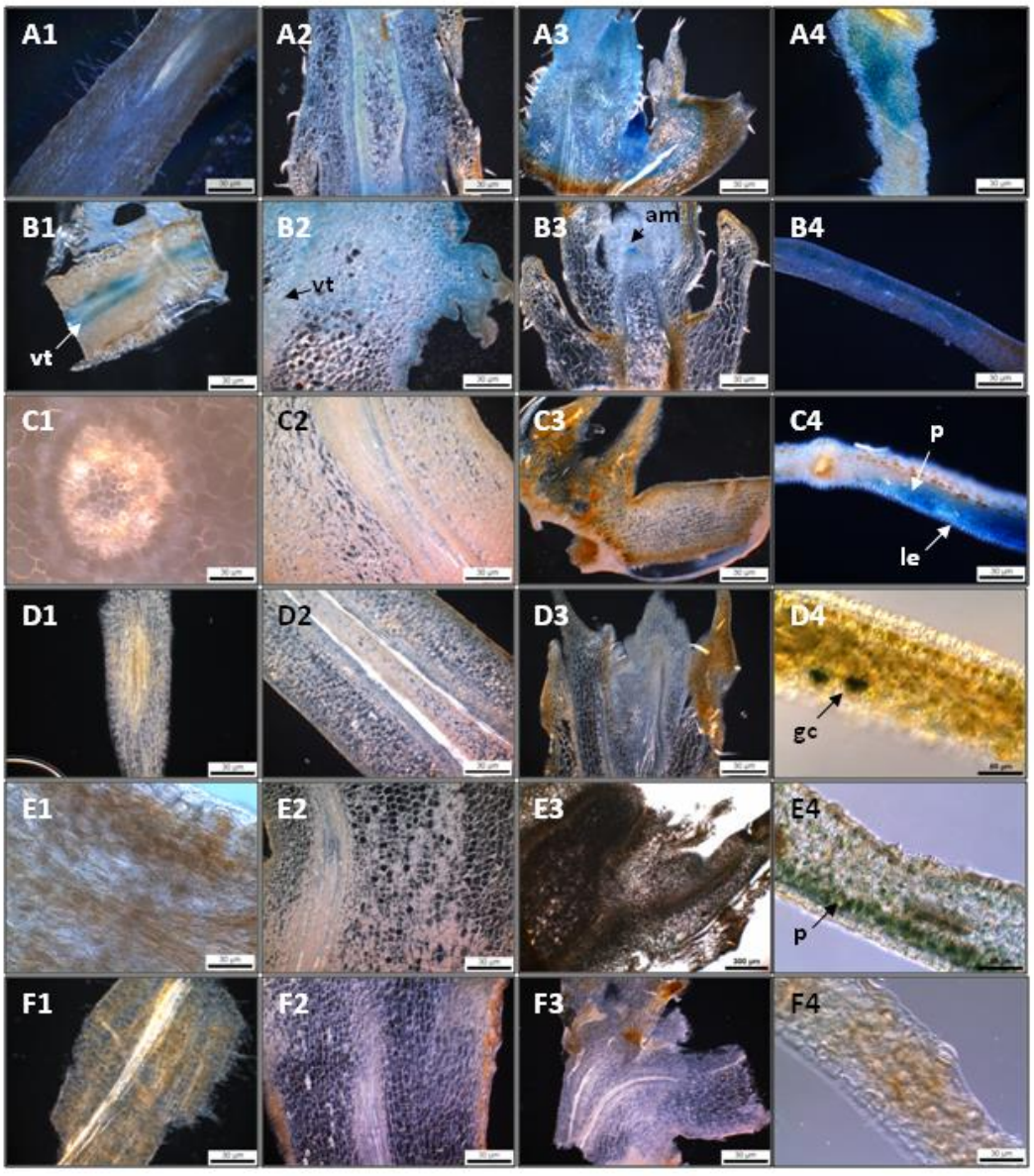
BglII

Figure 3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E4
p
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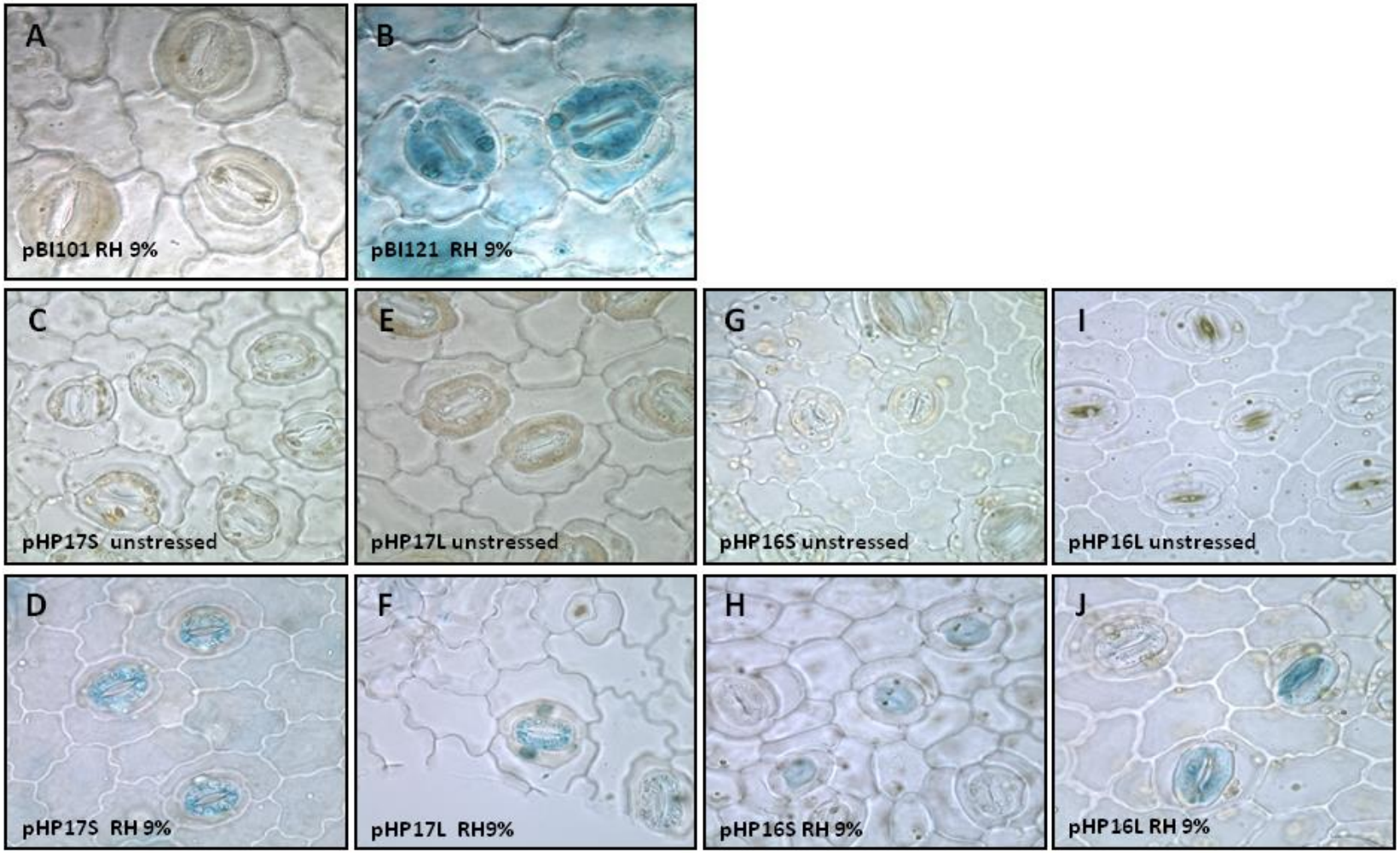
F3
F4

Figure 4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G
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I
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pHP16S RH 9%
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pHP16L RH 9%

Figure 5

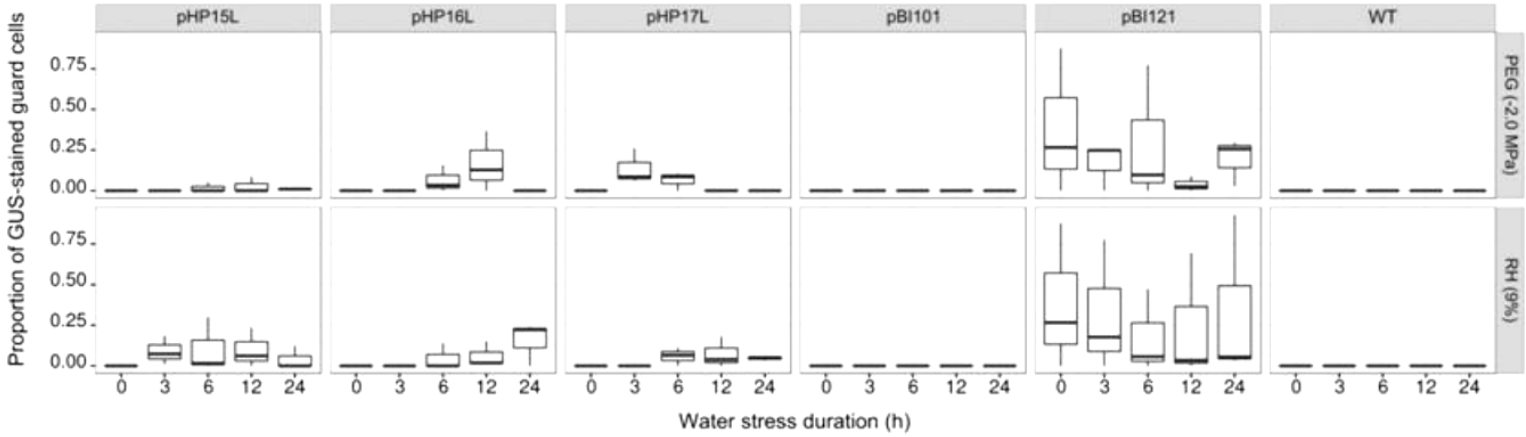
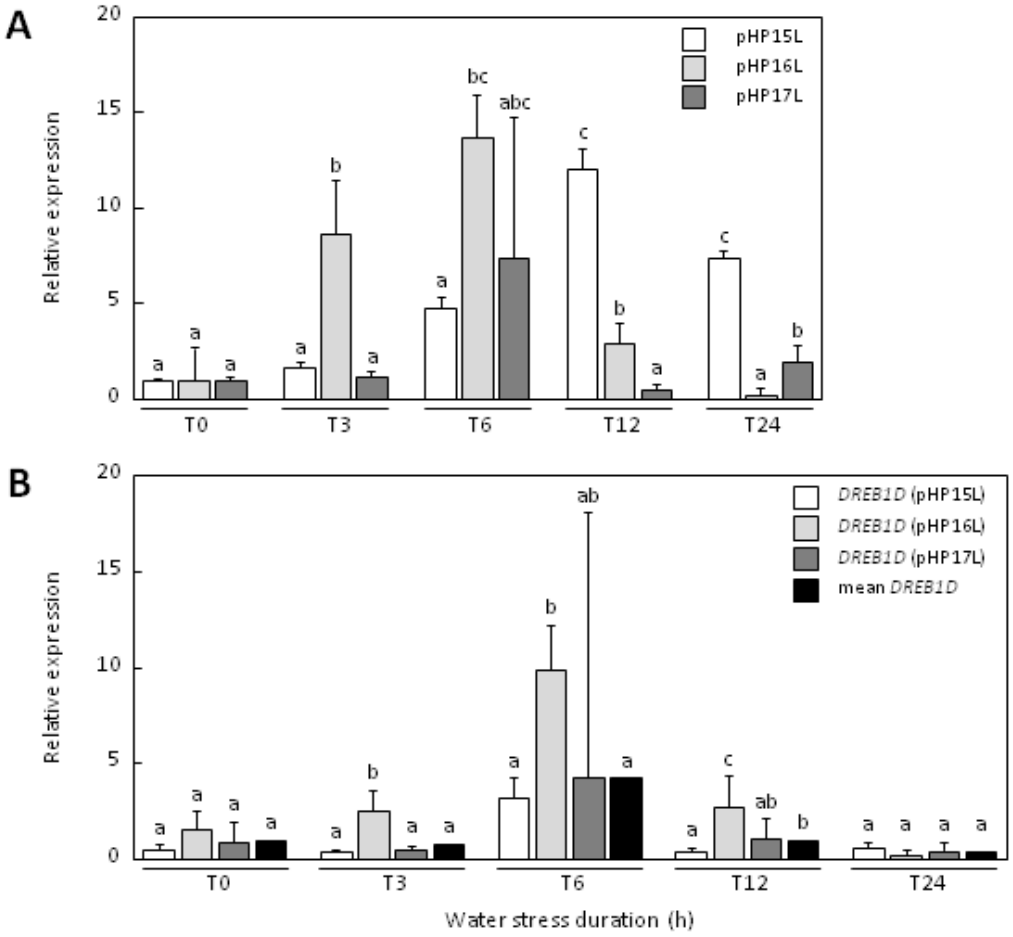
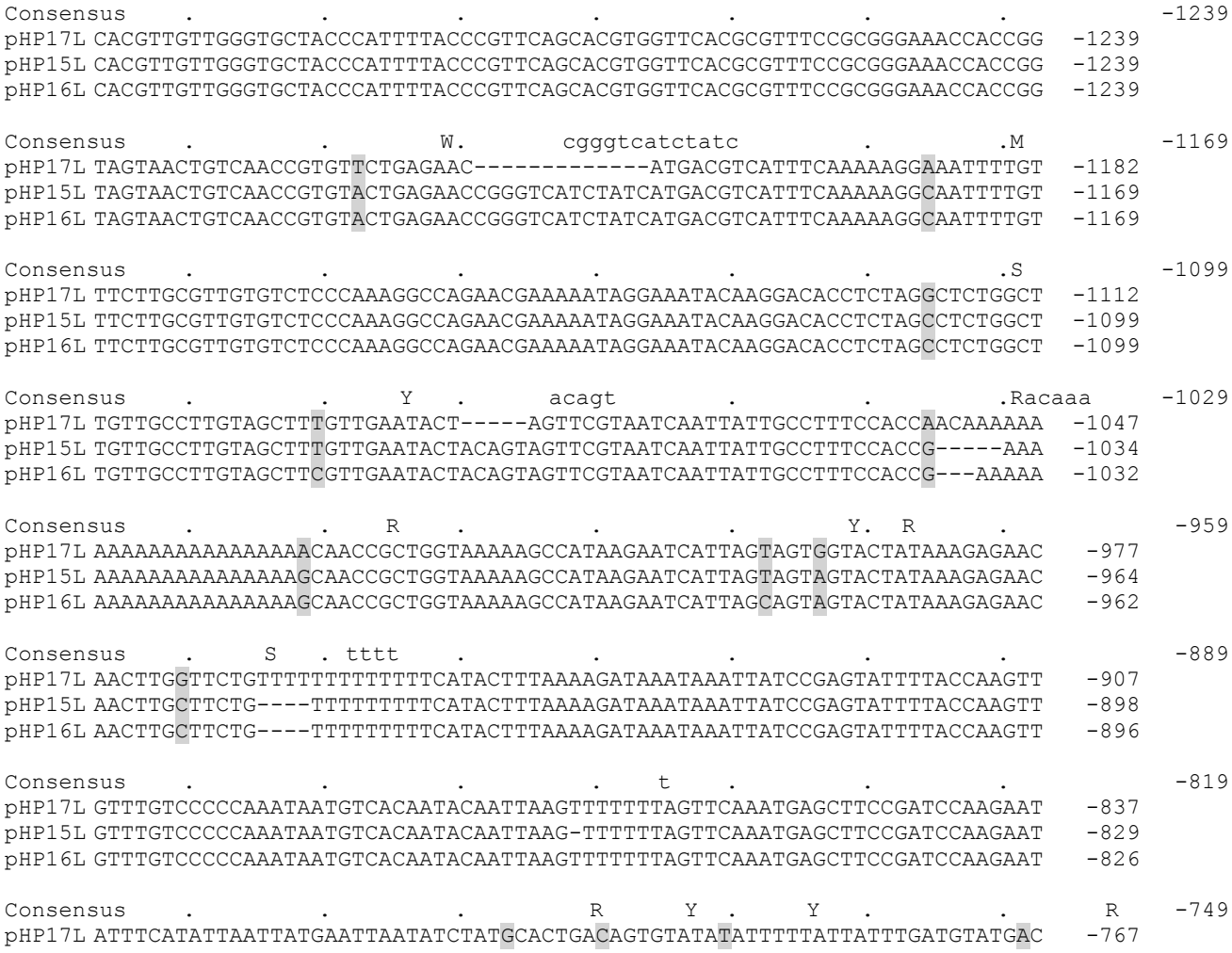

pHP15L
pHP16L
pHP17L
pBI101
pBI121
WT
PEG (-2.0 MPa)
RH (9%)
Proportion of GUS-stained guard cells
0.75
0.50
0.25
0.00
0
3
6
12
24
Water stress duration (h)

Figure 6


A
Relative expression
pHP15L
pHP16L
pHP17L
T0
T3
T6
T12
T24
B
Relative expression
DREB1D (pHP15L)
DREB1D (pHP16L)
DREB1D (pHP17L)
mean DREB1D
Water stress duration (h)

Supplementary Figure S1



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```

Supplementary Figure 1 (continued)

```
Consensus    .         .    R    .R     Y  .W        . R   R  S.         .  Rtcat     -679
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pHP16L ACATAATTTGAATTTGAATTTGAAATTCAAATTTTATACATGAATCGTGCATTCAACAGCACCG----TC   -690

Consensus    .  R      .R        .         .         .         .         .           -609
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pHP16L ACTATATATAAAATTTATTTATTAATTATTTAGCTAAGATAGAGTGCTCCTCAAGATTGTCACTTTCTAT   -620

Consensus    .     cag .    Y    .         .         S         .         .           -539
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Consensus    .aaa      .         .   Y     .         .        R.         .           -469
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pHP15L G---AAAAAAAAAGAGTGAGGCAATAAGGAATTTGACGTCCTCACAGAAAGAAAAGGTCAAGAGACAACC   -486
pHP16L G---AAAAAAAAAGAGTGAGGCAATAAGGAATTTGACGTCCTCACAGAAAGAAAAGGTCAAGAGACAACC   -486

Consensus    .         .         .         .         .         .         .           -399
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pHP15L ACGAAAGGATCAAAGATTATAAAACTAGCGAAATGAGGGGGCCCTAATCCTTCCAGCTCAGAAGAGTCAA   -416
pHP16L ACGAAAGGATCAAAGATTATAAAACTAGCGAAATGAGGGGGCCCTAATCCTTCCAGCTCAGAAGAGTCAA   -416

Consensus    .         .         .         .         .         .         .  R        -329
pHP17L ATCCCCTCCTCCACCTGGCTCCAGCAGCCAAGTTGCTGCGTTACATCAGAGCACGTGTCAAATGCACCAT   -347
pHP15L ATCCCCTCCTCCACCTGGCTCCAGCAGCCAAGTTGCTGCGTTACATCAGAGCACGTGTCAAATACACCAT   -346
pHP16L ATCCCCTCCTCCACCTGGCTCCAGCAGCCAAGTTGCTGCGTTACATCAGAGCACGTGTCAAATACACCAT   -346

Consensus    .         .Y R      .         .         .         .         .           -259
pHP17L GCCTCGAACCTCAGTACTACTGTGAAACAAAGTACAACTTGGGGCCCGAAGACAGCTTCGAGTCGGAAGA   -277
pHP15L GCCTCGAACCTTAATACTACTGTGAAACAAAGTACAACTTGGGGCCCGAAGACAGCTTCGAGTCGGAAGA   -276
pHP16L GCCTCGAACCTTAATACTACTGTGAAACAAAGTACAACTTGGGGCCCGAAGACAGCTTCGAGTCGGAAGA   -276
```

```
Consensus    R         .         .         .         .         .          -189
pHP17L GATCCACTTTCTTTCCTGCCTTAAAAGTTGACTGCTCCCACTTGGGCTAACTAGAAACTATCAAAATCCC   -207
pHP15L AATCCACTTTCTTTCCTGCCTTAAAAGTTGACTGCTCCCACTTGGGCTAACTAGAAACTATCAAAATCCC   -206
pHP16L AATCCACTTTCTTTCCTGCCTTAAAAGTTGACTGCTCCCACTTGGGCTAACTAGAAACTATCAAAATCCC   -206
```

Supplementary Figure 1 (continued)

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Consensus    .         .         .         .         .         .          -119
pHP17L GCTCCCCGGCCCCCAGCCTGGCTGGCTGCTTATCACCGTTCCGCGTTCGCGTCTCTTGAGCGTTCAATCA   -137
pHP15L GCTCCCCGGCCCCCAGCCTGGCTGGCTGCTTATCACCGTTCCGCGTTCGCGTCTCTTGAGCGTTCAATCA   -136
pHP16L GCTCCCCGGCCCCCAGCCTGGCTGGCTGCTTATCACCGTTCCGCGTTCGCGTCTCTTGAGCGTTCAATCA   -136

Consensus    .        C.         .         .         .         .        .   atcatc    -49
pHP17L TTCACCCCACCCCCCTCGAAACCCGCCAAGTTTATATACAAACTAGCTCATCATCATCATCATCATCATC    -67
pHP15L TTCACCCCA-CCCCCTCGAAACCCGCCAAGTTTATATACAAACTAGCTCATCATCATCATCATC------    -73
pHP16L TTCACCCCACCCCCCTCGAAACCCGCCAAGTTTATATACAAACTAGCTCATCATCATCATCATC------    -72

Consensus    atcatcatc .         . R       .         .         .        . R          +21
pHP17L ATCATCATCAAAACGCTCATCAGATATTTCATATCACATCATACATTCCAGTCAATTCAACCGTCATTTC     +3
pHP15L ---------AAAACGCTCATCAAATATTTCATATCACATCATACATTCCAGTCAATTCAACCATCATTTC    -12
pHP16L ---------AAAACGCTCATCAAATATTTCATATCACATCATACATTCCAGTCAATTCAACCATCATTTC    -11

Consensus    .         .         .         .        . R       .Y  R     .           +91
pHP17L AATTAATCTGCATTATATATATACCCTCTTTTGAGTTTCACAACACCAAGTCGTACGATTCCTCGCTTCA    +73
pHP15L AATTAATCTGCATTATATATATACCCTCTTTTGAGTTTCACAGCACCAAGTCGTGCGATTCCTCGCTTCA    +58
pHP16L AATTAATCTGCATTATATATATACCCTCTTTTGAGTTTCACAACACCAAGTTGTACGATTCCTCGCTTCA    +59

Consensus    .         .         .       K .         .         .         .          +161
pHP17L GCCTTTAACTTTCAGTTGAATTAACTCCTCACTGTCCACTACTACTACTATTTTTTCAAGATCCCCGGGT    +143
pHP15L GCCTTTAACTTTCAGTTGAATTAACTCCGCACTGTCCACTACTACTACTATTTTTTCAAGATCCCCGGGT    +128
pHP16L GCCTTTAACTTTCAGTTGAATTAACTCCGCACTGTCCACTACTACTACTATTTTTTCAAGATCCCCGGGT    +129

Consensus    .         .         .              +182
pHP17L GGTCAGTCCCTTATGTTACGT             +164
pHP15L GGTCAGTCCCTTATGTTACGT             +149
pHP16L GGTCAGTCCCTTATGTTACGT             +150
```
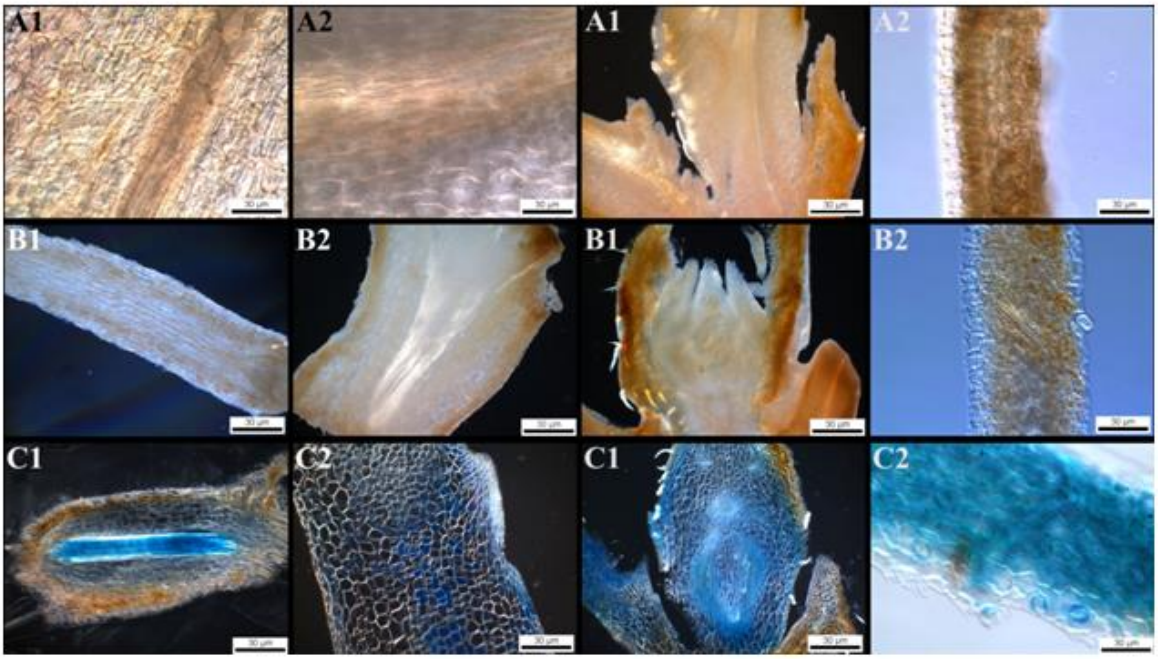
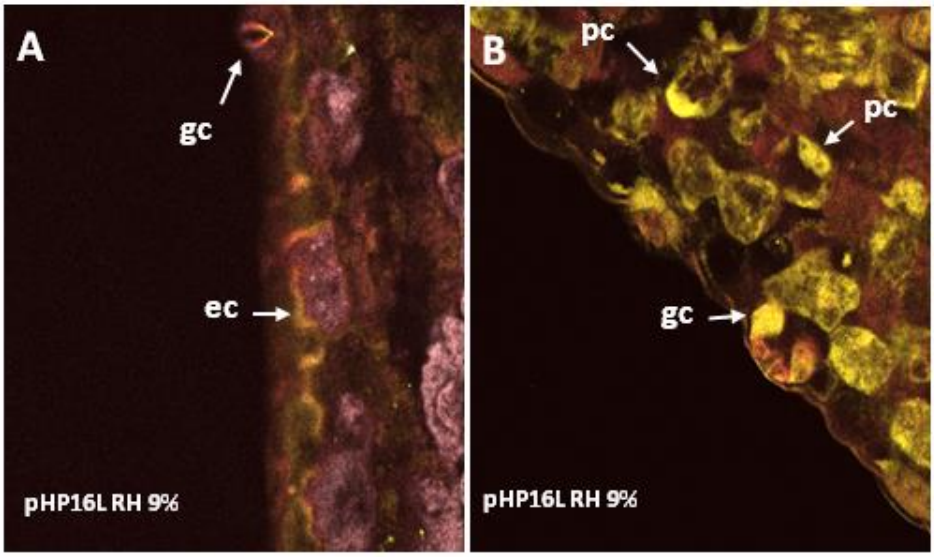

Supplementary Fig. 2



Suppl. Fig. 3



Supplementary Table 1. List of nucleic polymorphisms found in the HP15, HP16, and HP17 haplotypes of *CcDREB1D* coffee promoters.

Polymorphism	Consensus Sequence	Variant (s)	Polymorphism Type	Minimum	Maximum	Length	HP15	HP16	HP17
A -> T	W	T	SNP (transversion)	-1,219	-1,219	1	A	A	T
-CCGGGTCATCTAT	CCGGGTCATCTAT	----------	Deletion	-1,211	-1,199	13	CCGGGTCATCTAT	CCGGGTCATCTAT	----------
C -> A	M	A	SNP (transversion)	-1,177	-1,177	1	C	C	A
C -> G	S	G	SNP (transversion)	-1,107	-1,107	1	C	C	G
T -> C	Y	C	SNP (transition)	-1,082	-1,082	1	T	C	T
-TACAG	TACAG	-----	Deletion	-1,072	-1,068	5	TACAG	TACAG	-----
-ACAAA	---AA	ACAAA	Insertion	-1,036	-1,032	5	-----	---AA	ACAAA
G -> A	R	A	SNP (transition)	-1,013	-1,013	1	G	G	A
T -> C	Y	C	SNP (transition)	-984	-984	1	T	C	T
A -> G	R	G	SNP (transition)	-980	-980	1	A	A	G
C -> G	S	G	SNP (transversion)	-957	-957	1	C	C	G
(TT)4 -> (TT)6	----	TTTT	Insertion (tandem repeat)	-946	-943	4	----	----	TTTT
(T)6 -> (T)7	-	T	Insertion (tandem repeat)	-853	-853	1	-	T	T
A -> G	R	G	SNP (transition)	-788	-788	1	A	A	G
T -> C	Y	C	SNP (transition)	-781	-781	1	T	T	C
C -> T	Y	T	SNP (transition)	-772	-772	1	C	T	T
G -> A	R	A	SNP (transition)	-750	-750	1	G	G	A
A -> G	R	G	SNP (transition)	-733	-733	1	A	G	G
G -> A	R	A	SNP (transition)	-727	-727	1	G	G	A
C -> T	Y	T	SNP (transition)	-721	-721	1	C	C	T
A -> T	W	T	SNP (transversion)	-717	-717	1	A	T	T
G -> A	R	A	SNP (transition)	-706	-706	1	G	A	A
G -> A	R	A	SNP (transition)	-702	-702	1	G	G	A
C -> G	S	G	SNP (transversion)	-699	-699	1	C	C	G

Polymorphism	Consensus Sequence	Variant (s)	Polymorphism Type	Minimum	Maximum	Length	HP15	HP16	HP17
G -> ATCAT	R----	ATCAT	Insertion	-685	-681	5	G----	G----	ATCAT
A -> G	R	G	SNP (transition)	-675	-675	1	A	A	G
A -> G	R	G	SNP (transition)	-667	-667	1	A	A	G
-CAG	CAG	---	Deletion	-602	-600	3	CAG	---	CAG
T -> C	Y	C	SNP (transition)	-593	-593	1	T	T	C
G -> C	S	C	SNP (transversion)	-568	-568	1	G	C	G
(AAA)3 -> (AAA)4	---	AAA	Insertion (tandem repeat)	-528	-526	3	---	---	AAA
T -> C	Y	C	SNP (transition)	-514	-514	1	T	T	C
A -> G	R	G	SNP (transition)	-489	-489	1	A	A	G
A -> G	R	G	SNP (transition)	-335	-335	1	A	A	G
T -> C	Y	C	SNP (transition)	-317	-317	1	T	T	C
A -> G	R	G	SNP (transition)	-315	-315	1	A	A	G
A -> G	R	G	SNP (transition)	-258	-258	1	A	A	G
(C)5 -> (C)6	-	C	Insertion (tandem repeat)	-109	-110	1	-	C	C
(TCA)6 -> (TCA)11	----------	ATCATCATCATCATC	Insertion (tandem repeat)	-53	-39	15	----------	----------	ATCATCATCATCATC
A -> G	R	G	SNP (transition)	-26	-26	1	A	A	G
A -> G	R	G	SNP (transition)	+14	+14	1	A	A	G
G -> A	R	A	SNP (transition)	+64	+64	1	G	A	A
C -> T	Y	T	SNP (transition)	+73	+73	1	C	T	C
G -> A	R	A	SNP (transition)	+76	+76	1	G	A	A
G -> T	K	T	SNP (transversion)	+120	+120	1	G	G	T

Supplementary Table 1 (continued)

ARTICLE 2

Differential and specific expression patterns of three coffee *CcDREB1D* promoter haplotypes under different abiotic stresses

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Original Article

Full Title: Differential and specific expression patterns of three coffee *CcDREB1D* promoter haplotypes under different abiotic stresses

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Running title: Expression of *CcDREB1D* promoter haplotypes under different abiotic stresses

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ABSTRACT

Background and Aims *DREB* transcription factors play important roles in regulating the expression of genes in response to a variety of abiotic and biotic stresses, consisting of two subclasses, *DREB1* and *DREB2*, involved in signal transduction pathways under low temperature and dehydration, respectively. The aim of this work was to perform a functional analysis of three *CcDREB1D* promoter haplotypes isolated from drought-tolerant and susceptible clones of *C. canephora* in stably transformed plants of *C. arabica* subjected to different abiotic stresses.

Methods The activities and regulation of *CcDREB1D* promoter haplotypes (pHP15L, pHP16L, pHP17L), together with the expression of the *CaDREB1D* endogenous gene of *C. arabica*, were studied by performing GUS histochemical assays in different tissues of transformed coffee plants subjected to drought, cold, heat, photo-oxidative and exogenous application of abscisic acid (ABA) and monitoring the expression of *uidA* reporter gene by RT-qPCR experiments.

Key Results Staining results clearly highlighted the fact that specific expression of each haplotype occurred for the different stresses in plant tissues/organs (almost exclusively in leaves and apical meristems) of transformed plants of *C. arabica*. This enabled to fully characterize the specificity of the pHP16L haplotype, with expression levels ranging from low (cold, photo-oxidative) to the high levels (low RH, ABA treatment) – compared to other haplotypes - depending of abiotic stress applied. These results were confirmes by the amount of *uidA* transcripts in pHP16L transformed plants that clearly increased under cold and drought (low RH) conditions, as well as (but to a lower extend) under 24h of ABA treatment. These results also revealed the specific activity of the promoter *CcDREB1D* in guard cells of stomata when subjected to most of the abiotic stresses. The different proportions of GUS-stained guard cells observed between the HP15, HP16 and HP17 haplotypes during the different abiotic

treatments clearly revealed the differences of these *CcDREB1D* promoters in the fine-tuning of their regulation.

Conclusions The variations of GUS-stained guard cells and leaf *uidA* gene expression profiles observed between the three *CcDREB1D* promoter haplotypes in transgenic coffee plants subjected to cold and drought conditions, suggest that the nucleic polymorphisms previously identified among these sequences are probably responsible in fine-tuning of their regulation in response to abiotic stress.

Keywords: abiotic stress**,** *Coffea arabica* L., *DREB, β*-glucuronidase, gene expression, histochemical assay, promoter.

INTRODUCTION

Most of the traditional cultivars of *Coffea arabica* are high yielding and reputed to produce outstanding cup quality under optimal conditions of climate, soil and crop management. However, almost all of them are highly susceptible to the major coffee pests and diseases (Jaramillo *et al*., 2011) which makes them increasingly difficult to maintain - for economic and ecological reasons - in many regions particularly exposed to future climate changes (Bunn *et al*., 2015; Van der Vossen *et al*., 2015).

Plants have evolved to live in different environments by developing specific mechanisms that allow them to cope with abiotic (drought, high salt, and temperature change) and biotic stresses, while maintaining growth and production. Signal specificity is achieved through the precise interplay between components of each pathway, particularly the hormones (abscisic acid-ABA, salicylic acid-SA and jasmonic acid-JA), TFs (transcription factors), HSFs (heat shock factors), ROS (reactive oxygen species), and small RNAs (Atkinson and Urwin, 2012).

In higher plants, DREB (*Dehydration responsive element binding*) transcription factors are extensively studied because they play important roles in regulating the expression of genes in response to a variety of abiotic and biotic stresses (Shinozaki and Yamaguchi-Shinozaki, 2007; Khan, 2011). These genes belong to the AP2/ERF (Apetala2-ethylene responsive factor) family of transcription factors, consisting of two subclasses. *DREB* genes are commonly separated in *DREB1* and *DREB2* families, involved in signal transduction pathways under low temperature and dehydration, respectively (Agarwal *et al.*, 2006; Lata and Prasad, 2011).

Even if mainly induced by cold, expression of *DREB1D* (also known as *CBF4*) was also reported to be up-regulated by drought in *Arabidopsis* (Haake *et al*., 2002), *Medicago truncatula* (Li *et al*., 2011), as well as in different grape species and varieties (Xiao *et al*., 2008; Zandkarimi *et al*., 2015). Overexpression of the *DREB1D*/*CBF4* gene was shown to increase

tolerance to drought, cold and/or salt in transgenic plants (Haake *et al*., 2002; Li *et al*., 2011; Guttikonda *et al*., 2014), highlighting its key role in plant responses to abiotic stress.

Among the coffee candidate genes whose expression profiles were up-regulated by drought stress, the *CcDREB1D* was of outstanding interest because its expression was greatly enhanced under drought conditions in leaves of the drought-tolerant (D^T) clone 14 but not in those of the drought-susceptible (D^S) clone 22 of *C. canephora* cv. Conilon (Marraccini *et al*., 2012; Vieira *et al*., 2013). More recently, Thioune *et al.* (2017) also showed a rapid up-regulated expression of *CcDREB1* in leaves of *C. canephora* subjected to a sharp drop in relative humidity (RH). In order to understand the regulation of this gene, its promoter haplotypes from the D^T clone 14 and D^S clone 22 were characterized (Alves *et al*., 2017). This revealed the existence of three haplotypes (HP15, HP16 and HP16) diverging from each other by several SNPs and INDELs, with HP15 haplotype being common to both clones, while HP16 and HP17 were specific of the clones 14 and 22, respectively. The function and regulation of these promoter haplotypes were studied by analyzing their ability to regulate the expression of the *uidA* reporter gene in response to drought stress mimicked by low relative humidity (RH) and PEG osmotic treatments in transgenic plants of *C. arabica* var. Caturra (Alves *et al*., 2017). These results evidenced that HP16 was able to drive high expression of *uidA* gene in leaf mesophyll and guard cells in a stronger and earlier manner compared to the HP15 and HP17 haplotypes. The nucleic polymorphism detected in these sequences should explain the differences of expression and regulation observed between these haplotypes. In a recent study analyzing the genetic diversity of *CcDREB1D* promoter haplotypes, it was demonstrated that HP15 and HP16 haplotypes clustered in the sub-group 1 (SG1) of Congolese from *C. canephora,* while the HP17 haplotype was more phylogenetically related to the sub-group 2 (SG2) of Congolese (Torres *et al.*, 2016). Interestingly, plants of SG1 group of *C. canephora*, to which Brazilian Conilon cultivars are

phylogenetically linked (Montagnon *et al*., 2012), are known to be more drought-tolerant than those of SG2 usually considered as drought-susceptible (Montagnon, 2000).

In an attempt to better understand the tissular localization and regulation of *CcDREB1D* promoter haplotypes (pHP15L, pHP16L and pHP17L), our study set out to analyze their ability to regulate the expression of the *uidA* reporter gene in *C. arabica* transgenic plants subjected to different abiotic stresses such as low and high temperatures, water stress mimicked by low relative humidity (RH), application of exogenous ABA and photo-oxidative stress mimicked by high irradiance.

MATERIALS AND METHODS

Recombinant vectors

The pHP15L, pHP16L and pHP17L constructions used in this study (Fig. 1) were previously described by Alves *et al*. (2017). The pBI121 (CaMV35S::*uidA*) and pBI101 (*uidA*-promoterless) vectors (Clontech, Palo Alto, CA, USA) were used as positive and negative controls, respectively. These constructions were transferred independently by electroporation into competent cells of the disarmed *Agrobacterium tumefaciens* strain LBA1119. After each cloning step, recombinant vectors were systematically extracted with the Wizard® Plus SV Minipreps DNA Purification System and the sequences of *CcDREB1D* promoter fragments were verified by double-strand sequencing (Genome Express, France).

Regeneration of C. arabica transgenic plants

Embryogenic calli from *C. arabica* var. Caturra were obtained as described by Etienne (2005) and were placed in proliferation conditions during twelve months in view of selecting proembryogenic masses (PEMs) for *Agrobacterium*–mediated genetic transformation experiments (Ribas *et al*., 2011). Transgenic lines of *C. arabica* var. Caturra were generated by

A. tumefaciens-mediated transformation as previously described by Ribas *et al*. (2011). Briefly, *A. tumefaciens* strain LBA1119 harboring the recombinant vectors were grown at 28°C for 24 h in YMB selective medium (Yeast Extract Mannitol Agar) with rifampicin (25 mg L^{-1}) and kanamycin (50 mg L^{-1}). Three highly competent embryogenic callus cultures of 12 month-old were placed in baby food jars and immersed with 10 mL of *A. tumefaciens* suspension (OD_{600} = 0.6) for 10 min. Bacterial suspension was then removed and the inoculated calli were co-cultivated at 20°C for five days in the dark. For decontamination, the co-cultivated calli were rinsed twice with 20 mL sterile water and further placed on a rotary shaker at 30 rpm for 3 h with on half-strength MS (Murashige and Skoog, 1962) embryogenic callus production medium (ECP, Etienne, 2005) containing 1.2 g L^{-1} cefotaxime. The liquid was removed and the calli rinsed one last time with ECP medium for 15 min. Finally, calli were blotted on dry filter paper to remove excess bacterial solution and were subsequently placed in Petri dishes containing ECP medium with 500 mg L^{-1} cefotaxime. After decontamination, the embryogenic cultures were subcultured every four weeks twice on half-strength MS 'R' regeneration medium containing 17.76 µM 6-benzylaminopurine and 100 mg L^{-1} kanamycin and decreasing cefotaxime concentrations (250, 125 mg L^{-1}) and twice on 'M' maturation medium (Etienne, 2005) containing 1.35 µM 6-benzylaminopurine (6-BA), 100 mg L^{-1} hygromicin and 125 mg L^{-1} cefotaxime. The other subcultures were carried out on half-strength MS 'M' maturation medium containing 1.35 µM 6-BA devoid of cefotaxime and kanamycin until plantlets developed. Each kanamycin resistant callus regenerated multiple cotyledonary embryos. Plantlets with both leaf pairs and roots were cultivated on MS medium with active charcoal (1g L^{-1}) in baby food jars under sterile conditions until bioassays. During the entire regeneration process, the cultures were maintained under a 12 h photoperiod (20 µM m^{-2} s^{-1} light intensity) at 26°C and 80% RH. Once regenerated, DNA was extracted from small leaf explants and tested by PCR using *nptII*- (kanamycin) and *uidA*-specific primers to confirm T-DNA integration in

genomic DNA of *C. arabica*-transformed plantlets (data not shown). The numbers of T-DNA insertions in plants regenerated from each transgenic line were assessed through Southern blot analyzes. Only plants regenerated from lines showing a unique insertion were conserved for further bioassays on abiotic stresses. Transgenic plants were cultivated in Gerber flasks at 26°C on MS medium without growth regulators under a 12 h photoperiod (70 μM m^{-2} s^{-1}).

Abiotic stresses experiments

The objective was to understand the conditions of expression of the different haplotypes of the *DREB1D* promoter under a representative range of the most common abiotic stresses. Before applying the different stresses, transgenic plants were cultured in baby food jars in optimal environmental conditions under a 12 h photoperiod (70 μM m^{-2} s^{-1}) at 26°C under 80% RH. The drought, cold, heat, photo-oxidative and ABA stresses were performed as described below. To minimize possible effects of circadian clock (subset of biological rhythms with period), all experiments with the different stresses were started in the morning period at 10 a.m. after 2 h of light period. All the stresses were applied during 12 h except ABA that was applied for longer periods of 24 and 48 h. Phenotyping was performed at the end of these periods through histochemical characterization. All experiments were carried out with the plants transformed with the three different haplotypes and designed as follows: for each haplotype subjected to a particular abiotic stress, four plants derived from independent transformation events were studied, one plant per baby food jar representing an independent replicate.

Drought stress: Drought was mimicked by low (9%) relative humidity (RH). This atmosphere was created using 500 mL of KOH supersaturate solution that was poured in the lower compartment of a temporary immersion bioreactor (Matis®, CID Plastiques, France) (Supplementary data, Fig. S1). Coffee transformed plants were placed in the upper compartment over 55 mm Petri dishes having their upper part exposed to outside environment

and radicles immersed on MS medium with active charcoal (1g L^{-1}) through a small hand-made hole on the Petri dish cover (one plant per Petri dish). To avoid any water vapor exchange between MS medium and outside bioreactor atmosphere, the hand-made hole was further closed with high-vacuum silicone grease (Dow Corning®, Sigma) and Petri dishes were sealed with plastic film. Batches of 10 plants were then placed inside the bioreactor in a resulting atmosphere 9% RH for 12 h in the growth chamber with a 26°C temperature.

Cold stress: The baby food jars were directly transferred from the growth chamber with a 26°C temperature into a cold chamber at 5°C for 12 h.

Heat stress: The baby food jars were quickly transferred from the growth chamber with a 26°C temperature to a stove at 40°C for 12 h.

Photo-oxidative stress: The baby food jars were kept in the same culture chamber conditions, but light intensity was suddenly increased from 70 to 200 µM m^{-2} s^{-1} without any change in the spectrum. All the other culture parameters were unmodified. The plants were exposed for 12 h to this high light before histochemical characterization.

Abscisic acid: The plants were transferred from the 'M' maturation medium into baby food jars containing the same medium supplemented with 10^{-5} M ABA (Sigma Aldrich, St Louis, USA). The baby food jars were maintained under the same environmental conditions for 24 or 48 h.

GUS staining

In view of histochemical characterization of expression patterns of *CcDREB1D* haplotype promoters under abiotic stresses, GUS staining was performed after 12 h (drought, heat, cold and photo oxidative), 24 and 48 h (ABA) in leaves, apical buds and roots of transgenic (four biological repetitions for each of the three haplotypes) and control (pBI101- and pB121-transformed) coffee plants. The collected material was immersed in GUS staining solution (100 mM sodium phosphate buffer, pH 7.2, 10 mM sodium EDTA, 0.1% Triton X-100, and 1 mg

mL^{-1} 5-bromo-4-chloro-3-indolyl-D-glucuronic acid [Sigma] and 2.5 mM potassium ferrocyanidine), infiltrated by 10 min of vacuum, incubated at 37°C for 24 h, and then rinsed with ethanol 70%.

Prior to observation the GUS-stained samples were fixed in fixative (50% methanol and 10% acetic acid) at 4°C for 24 h. The tissues were rinsed with water and then dehydrated for 10 min in 50% ethanol, 10 min in 70% ethanol and 10 min in 90% ethanol. After observation with a Nikon binocular SMZ 1500 loupe, samples were embedded in 6% agarose for subsequent sections in a Microm HM650V vibratome. For bright field microscopy observation, 50 µm-thick leaf sections were examined using a DM600 Leica microscope (Leica Microsystems GmbH, Wetzlar, Germany). Pictures were taken with a Retiga 2000R camera (G-Imaging Co., Wetzlar, Germany).

Proportion of GUS-stained guard cells in abiotic stress assays

For bright field microscopy observation, GUS-stained leaves were fixed in fixative (50% methanol and 10% acetic acid) at 4°C for 24 h. The tissues were rinsed with water and incubated for at least 3 days in clearing solution (chloral hydrate: glycerol: water solution (4:1:2, v/v/v) to remove all leaf pigments. Prior to observation, tissues were rinsed with 70% ethanol and assembled in microscope slides. Whole leaves were examined using a DM600 Leica microscope (Leica Microsystems GmbH, Wetzlar, Germany). Pictures were taken with a Retiga 2000R camera (G-Imaging Co., Wetzlar, Germany). The proportion of GUS-stained guard cells on the abaxial epidermis of coffee leaves was calculated to estimate the activity of *CcDREB1D* promoter haplotypes. The proportion of GUS-stained guard cells (p) was obtained by $p = x/n$, where x is the number of stained guard cells and n the total number of guard cells (= 150) observed per leaf. These values were assessed in 24 x 36 mm areas distributed in six pre-

delimited leaf zones. For each pHP construction, four leaves from plants of 4 independent transformation events were sampled for each abiotic stress.

Gene expression assays

The expression of *uidA* and *CaDREB1D* genes was checked in leaves of pHP16L-transformed plants subjected to above described abiotic stresses. After 3 h of stress exposure, leaves were collected, transferred in liquid nitrogen and then kept at -80°C until RNA extraction. Total RNA was extracted from tissues ground in liquid nitrogen and treated as described by Breitler *et al*. (2016). RNA quantification was performed using a NanoDropTM 1000 Spectrophotometer (Waltham, MA, USA). RNA quality and integrity were verified by the Agilent 2100 Bioanalyzer (Agilent Technologies) ensuring RIN values equal to and greater than 7.0. For cDNA synthesis kit was used Taq® The One RT-qPCR (New England Biolabs, Ipswich, USA).

Real-time RT-PCR assays

Quantitative PCR was carried out with synthesized single-strand cDNA described above using the protocol recommended for the use of 7500 Fast Real-Time PCR Systems (Applied Biosystems, Foster City, CA, USA). cDNA preparations were diluted (1/20) and tested by qPCR. Primers were designed using Primer Express software (Applied Biosystems) and preliminarily tested for their specificity and efficiency against a mix of cDNA (data not shown). PCR reactions were performed with 1 µL of diluted ss-cDNA and 0.2 µM (final concentration) of each primer in a final volume of 10 µL with SYBR green fluorochrome (SYBRGreen qPCR Mix-UDG/ROX, Invitrogen). The reaction mixture was incubated for 2 min at 50°C (Uracil DNA-Glycosilase treatment), then 5 min at 95°C (inactivation of UDGase), followed by 40 amplification cycles of 3 s at 95°C and 30 s at 60°C (annealing and elongation). Data were analyzed using `7500` Fast `Software v2.0.6` (Applied Biosystems) to determine cycle

threshold (Ct) values. Specificity of the PCR products generated for each set of primers was verified by analysing the Tm (dissociation) of amplified products. Gene expression levels were normalized to expression level of *CaGAPDH* as a constitutive reference gene (de Carvalho *et al.*, 2013). Expression was expressed as relative quantification by applying the formula $(1+E)^{-\Delta\Delta Ct}$, where $\Delta Ct_{target} = Ct_{target\ gene} - Ct_{reference\ gene}$ and $\Delta\Delta Ct = \Delta Ct_{target} - \Delta Ct_{internal\ calibrator}$.

RESULTS

GUS enzymatic activity regulated by the three DREB1D promoter haplotypes under a variety of abiotic stresses

The regulation of HP15, HP16 and HP17 promoter haplotypes of the *CcDREB1D* gene was studied by analyzing *β*-glucuronidase (GUS) enzyme activity in leaves, apical buds and roots of *C. arabica* transgenic plants subjected to different abiotic stresses (Fig. 2, Table 2). Whatever the stress applied, strong GUS staining was observed in leaves and apical buds in the pBI121-transformed coffee plants used as a positive control (see Supplementary file Fig. S2, Table 2). In contrast, and whatever the abiotic stress conditions applied, no GUS activity was detected in pBI101-transformed coffee plants (negative control).

Drought stress

Faint GUS staining was observed mainly around the secondary veins in leaves of pHP15L- (Figs. 2 A1 and A7) and pHP16L-transformed coffee plants (Figs. 2 A3 and A9) subjected to low RH. Under higher magnification, several GUS-stained guard cells were also observed in leaves of these two transgenic lines. However, GUS activities were not detected in leaves of pHP17L-transformed coffee plants (Figs. 2 A5 and A11). In apical buds, GUS staining was stronger in pHP16L-transformed plants (Figs. 2 A4 and A10) than in those of plants transformed by pHP15L (Figs. 2 A2 and A8). On the other hand, no GUS staining was observed

in leaves (Figs. 2 A5 and A11) and apical buds (Figs. 2 A6 and A12) of pHP17L-transformed plants. Results are summarized in Table 2. For the three *CcDREB1D* promoter haplotypes, no staining was observed in the roots.

Cold stress

No GUS staining was observed in leaves of pHP15L- (Figs. 2 B1 and B7) and pHP16L-transformed plants (Figs. 2 B3 and B9) subjected to cold treatment. On the contrary, weak GUS activity was noticed in leaves of pHP17L-transformed coffee (Figs. 2 B5 and B11). In apical buds, faint GUS staining was observed in pHP15L- (Figs. 2 B2 and B8) and pHP17L-transformed plants (Figs. 2 B6 and B12), while moderate GUS staining was detected in pHP16L-transformed plants (Figs. 2 B4 and B10). For all *CcDREB1D* promoter haplotypes, no GUS activities were observed in the roots as summarized in Table 2.

Heat stress

Whatever the *CcDREB1D* promoter haplotype, no GUS expression was observed in leaves (Figs. 2 C1, C3, C5, C7, C9 and C11) and roots of transformed plants subjected heat stress (Table 2). Moderate GUS staining was observed in the apical buds of pHP15L- (Figs. 2 C2 and C8) and pHP16L- (Figs. 2 C4 and C10) transformed plants. Compared to these haplotype responses, higher GUS staining was detected in the apical buds of pHP17L-transformed plants (Figs. 2 C6 and C12).

ABA assay

After 24 h of ABA treatment, faint GUS staining was systematically observed in leaves of pHP15L- (Figs. 2 D1 and D3), pHP16L- (Figs. 2 D3 and D9) and pHP17L- (Figs. 2 D5 and D11) transformed coffee. GUS staining was considered as weak, medium and strong in apical buds of pHP15L- (Figs. 2 D2 and D8), pHP16L- (Figs. 2 D4 and D10) and pHP17L- (Figs. 2

D6 and D12) transformed plants, respectively (Table 2). Strong GUS staining was also observed in roots of pHP15L-transformed plants (Figs. 3 A and B), while no GUS activity was detected in those of plants transformed by pHP16L and pHP17L constructions (Table 2). After 48 h of ABA treatment, GUS staining was considered as weak in leaves of pHP16L-transformed plants (Figs. 2 E3 and E9) and moderate in those of pHP15L-transformed plants (Figs. 2 E1 and E7). In apical buds, moderate and strong GUS activities were observed in pHP15L- (Figs. 2 E2 and, E8), and pHP16L-transformed plants (Figs. 2 E4 and E10), respectively. However, and whatever the tissues tested, no GUS staining was detected in the pHP17L-transformed plants (Figs. 2 E5, E6, E11 and E12). For the three *CcDREB1D* promoter haplotypes, no GUS staining was detected in roots (Table 2).

Photo-oxidative stress

Under photo-oxidative stress, intense GUS staining was observed in apical buds, particularly in pHP16L- (Figs. 2 F4 and F10) and pHP17L- (Figs 2 F6 and F12) transformed plants and to a lesser extent, in those transformed by pHP15L (Figs. 2 F2 and F8). Weak GUS staining was also observed in leaves of pHP15L- (Figs. 2 F1 and F7) and pHP17L- (Figs. 2 F5 and F11) transformed plants while no GUS activities were observed in leaves of pHP16L-transformed plants (Figs. 2 F3 and F9). As summarized in Table 2, GUS activities were not detected in roots of all pHP-transformed plants.

Proportion of GUS stained guard cells in leaves subjected to abiotic stresses

In order to assess how HP15L, HP16L and HP17L *CcDREB1D* promoter haplotypes are regulated, the activity of these sequences was evaluated by analyzing the proportion of GUS-stained guard cells on leaf abaxial regions of pHP-transformed coffee plants subjected to different abiotic stress. GUS-stained guard cells were mainly observed under low RH and ABA-

48 h treatments in the leaves of pHP15L- (Figs. 4A and D) and pHP16L- (Figs. 4B and E) transformed coffee plants. In pHP17L-transformed plants, GUS-stained guard cells were observed under cold (Fig. 4C) and photo-oxidative (Fig. 4F) treatments. As expected, a high proportion of GUS-stained guard cells was observed in the leaves of pBI121-transformed coffee plants (positive control, Fig. 4G). Moreover, no GUS-stained guard cells were observed in pBI101-transformed plants and in untransformed (WT) coffee plants (negative controls, Figs. 4H and I), as well as in pHP-transformed plants under unstressed (control) conditions (Fig. 5). However, a high proportion of GUS-stained guard cells was observed in pHP16L-transformed plants, particularly under low RH (47%) and ABA (24 and 48 h, ± 20%) conditions, but also to a lesser extend under photo-oxidative and cold treatments (< 7%) (Fig. 5). In pHP17L-transformed plants, highest proportions of GUS-stained guard cells were detected under cold (13%) and photo-oxidative (8%) treatments. On the other hand, and whatever the stress conditions, the proportion of GUS stained cells remained always relatively low (< 6%) in pHP15L-transformed plants. As a positive control, the proportion of GUS-stained guard cells was always high (> 80%) in pBI121-tyransformed plants while no GUS-stained guard cells were detected in pBI101-transformed plants.

RT-qPCR expression

Since highest proportions of GUS-stained guard cells were observed for pHP16L-transformed plants, these plants were used to check the leaf expression of *uidA* and *CaDREB1D* endogenous genes under cold, heat, low RH, photo-oxidative and ABA (24 h) treatments by RT-qPCR experiments (Fig. 6). Under unstressed conditions, *uidA* and *CaDREB1D* transcripts were undetected. On the opposite, the transcripts of these two genes were highly accumulated under cold and RH treatments. Compared to these two stress conditions, the up-regulated expression

of *uidA* and *CaDREB1D* observed under heat, photo-oxidative and ABA treatments was considered as weaker.

DISCUSSION

Among the genes involved in the plant responses to stress conditions, the expression of many of them was shown to be under the control of the *DREB* factor pathway (Zhou *et al*., 2010; Lata and Prasad, 2011). Despite the importance of these genes, a limited number of *DREB* promoters were analyzed by transgenic approaches. Because functional characterization of *DREB1D* promoter has neither been reported elsewhere, the main purpose of this work was to study the regulation of three different promoter haplotypes of *CcDREB1D* coffee gene through their capacity to control the expression of the *uidA* reporter gene in transgenic plants of *C. arabica* subjected to most important abiotic stresses.

The results of GUS staining presented here indicated that the pHP15L, pHP16L and pHP17L promoter haplotypes did not function in leaves, apical buds and roots of unstressed coffee plants. However, blue-stained tissues were detected in transgenic coffee transformed by all *DREB* promoter haplotypes when these plants were subjected to drought (mimicked by low RH), cold, heat, photo-oxidative and ABA treatments. For example, strong GUS staining was observed in roots of pHP15L-transformed coffee plants under 24 h of ABA treatment but also in apical buds of pHP16L-transformed plants subjected to ABA (48 h) and drought, as well as in those of pHP17L-transformed plants subjected to ABA (24 h), heat and photo-oxidative stress treatments. These results are similar to those already reported for promoters *AtDREB1C* (Zarka *et al.,* 2003), *AtDREB2C* (Chen *et al.,* 2012), *OsDREB1B* (Gutha and Reddy, 2008), *GmDREB3* (Chen *et al.,* 2009), and *FeDREB1* (Fang *et al.*, 2015) showing that proximal regions (up to 1.3-kb) harbored all *cis*-regulatory elements (CREs) essential to correctly regulate the expression of *uidA* reporter gene in transgenic plants. Moreover, the fact that high

accumulation of the *uidA* transcripts in pHP16L-transformed plants under cold and drought was concomitant with the increase of *CaDREB1D* endogenous transcripts also indicated that the leaf transcriptional machinery of the allotetraploid *C. arabica* correctly recognized and regulated the *CcDREB1D* promoter of *C. canephora* diploid species, therefore suggesting that the molecular mechanisms for upstream regulation of *CcDREB1D* promoters and its ortholog are conserved between closely related species (Rusconi *et al.*, 2013).

GUS staining results presented here also clearly highlighted the fact that HP15, HP16 and HP17 haplotypes responded in different manners regarding the same abiotic treatments and the considered tissues. For example, GUS staining referred as medium and weak was observed in leaves of pHP15L- and pHP16L-transformed plants subjected to 48 h of ABA and drought conditions, respectively. On the other hand, GUS staining was noticed as medium and strong in apical buds of the same plants and conditions. However, in both tissue and stress treatments, GUS-stained guard cells were neither observed in pHP17L-transformed plants. For the three *DREB1D* haplotypes, no GUS staining was detected in guard-cells of transformed coffee plants under unstressed condition. However, the proportion of GUS-stained guard cells varies regarding the *DREB1D* promoter haplotypes and stress conditions applied to transgenic coffee plants. For example, the proportion of GUS-stained guard-cells was higher in pHP16L-transformed plants subjected to drought but also to exogenous ABA (after both 24 h and 48 h of treatment) than to that measured in pHP15L- and pHP17L-transformed plants. These results were similar to those previously reported from the same plants by Alves *et al.* (2017) that showed a higher proportion of GUS-stained guard cells in pHP16L- than in pHP15L- and pHP17L-transformed coffee lines subjected to low water potential mimicked by PEG (equivalent to -2.0 MPa). The different proportions of GUS-stained guard cells observed between the HP15, HP16 and HP17 haplotypes during the different abiotic treatments clearly revealed the differences of these *CcDREB1D* promoters in the fine-tuning of their regulation.

This increased proportion of GUS stained guard cells observed in pHP16L-transformed coffee plants subjected to abiotic stress was also confirmed by analyzing the expression of the *uidA* reporter gene. In that case, the amount of *uidA* transcripts clearly increased under cold and drought (low RH) conditions, as well as (but to a lower extend) under 24h of ABA treatment. Once again, these results are in accordance with those already reported with the same coffee lines by Alves *et al.* (2017), showing a peak of *uidA* reporter gene in pHP16L-transformed plants after 3 h of PEG treatment while *uidA* expression peaks were observed after 6 h and 12 h in pHP17L- and pHP15L-transformed plants, respectively. The results presented here in pHP16L-transformed plants also showed that the *uidA* transcripts were barely detected after 3 h of heat and photo-oxidative treatments in pHP16L-transformed plants, therefore confirming the low proportion (up to the absence) of GUS-stained guard cells under these treatments. However, the low *uidA* gene expression observed after 3 h of ABA treatment did not match with the relatively high proportion of GUS-stained stomata observed after 24 h and 48 h of ABA treatment. The up-regulated expression of the *uidA* reporter occurring after 3 h of ABA treatment could explain such a situation. In that sense, it is noteworthy that *Arabidopsis* genes containing the *DREB1A/CBF3* motif in their promoter were mainly upregulated in after 6 h of ABA treatment (Huang *et al.*, 2007).

Several guard cell-specific genes were already reported (Leonhardt *et al.*, 2004; Wang *et al.*, 2011; Virlouvet and Fromm, 2015). Interestingly, the guard cell transcriptome is particularly rich in transcription factor-encoding genes such as *DREB*, *WRKY*, *MYB* and *MYC*, for example (Hachez *et al.*, 2011; Bates *et al.*, 2012; Baldoni *et al.*, 2015). For a grand majority, functional analyses of their promoters showed that expression was not strictly restricted to guard cells, but guard cell-preferred, with expression often also observed in mesophyll cells, as well as in leaf veins and trichomes, for example (Han *et al.*, 2013). However, guard cell-exclusive expression was reported for *Arabidopsis* promoters of *GC1* (formally At1g22690 encoding a GASA9

gibberellin regulated cysteine rich protein), *CYP86A2* and *MYB60* genes (Galbiati *et al.*, 2008; Yang *et al.*, 2008; Francia *et al.*, 2008; Cominelli *et al.*, 2011). In addition to its guard cell specificity, the activity of *AtMYB60* promoter, but also of *VvMYB60* promoter from *Vitis vinifera*, was also shown to be rapidly down-regulated by ABA (Cominelli *et al.*, 2011; Galbiati *et al.*, 2011; Rusconi *et al.*, 2013). On the other hand, and even though its promoter was not functionally characterized, *CYP86A2* gene expression was reported to be up-regulated by ABA and dehydration treatments (Francia *et al.*, 2008). One of the characteristics of these guard-cell specific promoters is that they share in common several CREs, at least clusters of [T/A]AAAG DOF (DNA binding with One Finger)-binding site evidenced to drive guard cell-specific gene expression (Plesch *et al.*, 2001; Cominelli *et al.*, 2011). Computational analyses also showed that the classical G-box containing the classical ABA-regulated elements (ABRE) BACGTGKM (where B=C/G/T, K=G/T and M=A/C) was overrepresented in guard cell specific promoters ABA-upregulated but missed in guard cell specific promoters ABA-downregulated (Leonhardt *et al.*, 2004; Wang *et al.*, 2011). Interestingly, the HP15, HP16 and HP17 *CcDREB1D* promoter haplotypes all contained (in their long versions, L) three ABRE-like and several DOF motifs (Alves *et al.*, 2017), together with MYC, MYB and ICE2 DNA motifs all known in participating to drought-, cold- and abscisic acid-regulated gene expression (Abe *et al.*, 2003; Fursova *et al.*, 2009).

To our knowledge, the results presented here are the first demonstrating guard-cell up-regulated expression of a *DREB1D* promoter in response to ABA, cold and drought stresses, mainly in pHP16L-transformed coffee plants. However, heat shock and high-light treatments appeared less efficient in up-regulating the expression of this haplotype. The fact that *DREB* genes responded in different manners to abiotic stress was already reported in the literature. For example, the expression of *AtDREB1D* (also known as *AtCBF4*) was up-regulated under drought but not by cold in *Arabidopsis* (Haake *et al.*, 2002). However, cold and drought up-

regulated the expression of *DREB1D* in *Vitis* sp. (Xiao *et al.*, 2008; Zandkarimi *et al.*, 2015) and *Medicago truncatula* (Li *et al.*, 2011). The presence/absence in these promoters of CREs specifically involved for each abiotic stress probably explain such responses (Abe *et al.*, 2003; Fursova *et al.*, 2009).

The studies demonstrating haplotypes (or alleles) of a same promoter function differentially are also very restricted in plants (de Meaux *et al.*, 2005; Takeshima *et al.*, 2016). In the present work, the comparison of GUS staining in stomata guard cells, leaves, roots and apical buds together with the results of *uidA* gene expression in pHP-transgenic plants clearly demonstrated that the three *CcDREB1D* promoter haplotypes responded in different manners to abiotic stress. For example, *uidA* gene expression after 24 h of ABA treatment was higher in pHP16L-transformed plants than in pHP17L- and pHP15L-transformed plants. Similar observations were made using the same transgenic lines when they were subjected to drought stress mimicked by low RH (Alves *et al.*, 2017). Since the HP16 haplotype was isolated from D^T clone 14 of *C. canephora*, this could explain why *CcDREB1D* gene expression was highly up-regulated by drought in leaves of this clone but not in those of D^S clone 22 harboring HP15 and HP17 haplotypes (Marraccini *et al.*, 2012). The fact that HP16 haplotype clustered genetically to *CcDREB1D* haplotypes found in the drought-tolerant plants of Congolese sub-group 1 (SG1) from *C. canephora* is also worth noting (Torres *et al.*, 2016). Since the sequences of ABRE regulatory elements, as well as those of many other DNA boxes (e.g. dehydration-responsive elements-DRE) are very conserved in HP15, HP16 and HP17 *CcDREB1D* promoter haplotypes, it was proposed that single nucleotide polymorphisms (SNPs) and/or insertion/deletions (INDELs) detected in these haplotypes close or between essential CREs might explain the differences of regulation observed between these sequences.

In roots, water deficiency up-regulated the expression of *DREB1* genes in pine (Lorenz *et al.*, 2011), soybean (Ha *et al.*, 2015) and poplar (Cohen *et al.*, 2010). The fact that higher root-to-

shoot ratio or larger number and longer roots were observed in transgenic plants overexpressing *DREB* genes also demonstrated the key role of these genes in root system development (Janiak *et al.*, 2016). Interestingly, Pinheiro *et al.* (2005) showed that the D^T clones 14 of *C. canephora* had higher root depth than D^S clones, therefore suggesting that root system architecture contributed to drought tolerance in coffee. Even though no expression of *CcDREB1D* was detected in roots of drought-stressed D^S and D^T clones of *C. canephora* (Costa *et al.*, to be submitted), the results presented here clearly showed ABA-induced GUS staining in roots of pH15L-transformed plants. The fact that drought up-regulates *CcNCED3* expression in roots of *C. canephora* also suggests for a key role of ABA in these coffee tissues. Altogether, these results presented here clearly highlighted the function of *CcDREB1D* promoter haplotypes in regulating by different manners the expression of the *uidA* reporter gene in guard cells of transgenic coffee plants, thereby suggesting a key role for this gene in controlling the molecular responses to drought in coffee.

CONCLUSION

The specific and spatio-temporal expression of the *pCcDREB1D* occurred in plant tissues/organs of transformed plants of *C. arabica*, and the specific activity of the promoter *CcDREB1D* in guard cells of stomata during cold, heat, photo-oxidative, water stress and ABA exogenous application were detected. The expression of *uidA* gene explicitly observed in cold and drought conditions, and *CaDREB1D* gene also observed under the same conditions and additional to the exogenous ABA conditions suggests that nucleic polymorphisms identified among the different *DREB1D* promoter haplotypes interfere with the expression and perhaps regulation of these sequences. RNA-Seq data are underway to confirm these histochemical results.

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FIGURE LEGENDS

Figure 1 - Schematic representation of the *CcDREB1D* promoter constructions used to transform *C. arabica*. Plasmid names used for stable transformation of *C. arabica* are given for each construction, indicating the *CcDREB1D* promoter haplotype (HP) studied. The HP16L and HP17L haplotypes were cloned from D^T clone 14 and D^S clone 22 of *C. canephora*, respectively, while the HP15L was found in both clones. These promoters were amplified using the forward (L, white square), and reverse (R, black circle) primers and cloned in *Hind*III and *Bgl*II of digested pBI121 as previously described (Alves *et al.*, 2007). The pBI121 (CaMV35S:*uidA*) and pBI101 (*uidA* promoterless gene) vectors were used as positive and negative controls for GUS expression, respectively.

Figure 2 - Histochemical localization of GUS activity in *C. arabica* var. Caturra transgenic plants transformed independently by *CcDREB1D* promoter haplotype constructions, named pHP15L, pHP16L and pHP17L, and subjected to low relative humidity (RH 9%) (A), cold (B), heat (C), ABA (D, 24 h; E, 48 h), and photo-oxidative (E) treatments. For each construction and stress condition, GUS staining performed in leaves and apical buds was analyzed by

binocular loupe (1-6, with bar scales of 3.0 mm for leaves and 1.5 mm for apical buds) and by bright field microscopy of organs in longitudinal- or cross-sections (7-12, with bar scales of 80 µm for leaves and 300 µm for apical buds). These images correspond to the most representative patterns of GUS staining observed for each pHP construction.

Figure 3 - Histochemical localization of GUS activity in roots of *CcDREB1D* promoter haplotype HP15L (pHP15L)-transformed plants of *C. arabica* subjected ABA 10^{-5} M treatment during 24 h. A: Binocular loupe image (bar = 1.5 mm). B: bright field microscopy of root tissues in cross-section (bar = 40 µm). Tissue abbreviations: Rc: root cap; Ep: epidermis; Co: cortex; Xy: xylem; Ph: phloem; Cv: vascular cylinder.

Figure 4 - Histochemical detection of GUS activity in guard cells of *CcDREB1D* promoter haplotype (pHP)-transformed plants of *C. arabica* subjected to abiotic stresses. Stomata were visualized by bright field microscopy on abaxial region of coffee leaves from pHP15L- and pHP16L-transformed plants subjected to low relative humidity (RH 9%) (A, B) and ABA-48 h (D, E) treatments and from leaves of pHP17L-transformed plants subjected to cold (C) and photo-oxidative (F) treatments. Leaves of pBI121-transformed coffee plants subjected to low relative humidity (G) were used as a positive control while those of pBI101-transformed (H) and untransformed (I: WT, wild type) coffee plants subjected to low relative humidity were used as negative controls. For each construction and stress condition, images correspond to the most representative patterns of GUS staining. Bars represent 80 µm.

Figure 5 - Proportion of GUS stained guard cells in leaves of *CcDREB1D* promoter haplotype (pHP)-transformed plants of *C. arabica* subjected to different abiotic stresses. The colors used for each abiotic stress (cold, heat, RH 9%, photo-oxidative and ABA 24 h/48 h) are indicated in the figure. The proportion of GUS stained guard cells in pBI121- and pBI101-transformed coffee plants were used as positive and negative controls, respectively.

Figure 6 - Expression profiles of *uidA* and *CaDREB1D* genes in leaves of *CcDREB1D* promoter haplotype HP16L (pHP16L)-transformed plants of *C. arabica* subjected to cold, heat, low relative humidity (RH 9%), photo-oxidative and ABA (24 h) treatments. The expression was analyzed by RT-qPCR using the GUS-F/R and DREB-F/R primer pairs (see Table 1) for *uidA* (black isobars) and *CaDREB1D* (white isobars) genes, respectively. Expression values corresponding to the mean of three biological and technical repetitions (±SD) are expressed in arbitrary units (AU) using the expression of the *CaGAPDH* gene as control endogenous. Reference samples (relative expression = 1) corresponded to *uidA* gene expression under photo-oxidative and to *CaDREB1D* gene expression under unstressed condition.

Table 1 - List of primers used in this study. Primer pairs were used in RT-qPCR experiments to determine gene expression levels of *uidA* (GUS-F/R) and *CcDREB1D* (DREB-F/R). The primer pair GAPDH-F/R was used to amplify the transcripts of *CaGAPDH* (glyceraldehyde-3-phosphate dehydrogenase) gene used as reference to standardize the results of RT-qPCR experiments.

Table 2 - Specificity and intensity of GUS staining in T1 transgenic plants of *C. arabica* transformed by pHP-constructions and subjected to different abiotic stresses. The intensity of *β*-glucuronidase (GUS) enzymatic activity was checked in leaves (L), apical buds (A) and roots (R), and evaluated as follows: unstained (-), weak (+), medium (++), strong (+++) and intense (++++). Staining intensity was assessed from the observation of four biological repetitions for each of the three *CcDREB1D* promoter haplotypes-transformed plants and control coffee plants (pBI101- and pB121-transformed).

SUPPLEMENTARY DATA

Supplementary data are available online at www.aob.oxfordjournals.org and consist of the following figures:

Fig. S1: Transgenic plants of *C. arabica* var. Caturra subjected to a low relative humidity (RH = 9%) in Matis@ bioreactors.

Fig. S2: GUS staining in transgenic plants of *C. arabica* var. Caturra transformed by pBI101 (negative control) and pBI121 (positive control) constructions and subjected to abiotic stres (cold, heat, RH 9%, photo-oxidative and ABA 24 h/48 h).

Table 1

Primers	Sequences
GUS-F	5’ GCACTAGCGGGACTTTGCAA 3’
GUS-R	5’ CGCGAAGCGGGTAGATATCA 3’
DREB-F	5' CAATGCCTGCAAAGCCAATTA 3'
DREB-R	5' TTTTCCTGCCTGCACGTTTC 3'
GAPDH-F	5' TTGAAGGGCGGTGCAAA 3'
GAPDH-R	5' AACATGGGTGCATCCTTGCT 3'

Table 2.

	Control			Cold			Heat			RH 9%			Photo-oxidative			ABA 24 h			ABA 48 h		
	L	A	R	L	A	R	L	A	R	L	A	R	L	A	R	L	A	R	L	A	R
pBI101	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
pBI121	+++	++++	++	++++	++++	+++	+++	+++	+++	++++	++++	-	++++	++++	-	++++	++++	+++	++++	++++	+++
pHP15L	-	-	-	-	+	-	-	++	-	++	++	-	+	+++	-	+	+	+++	++	++	-
pHP16L	-	-	-	-	++	-	-	++	-	+	+++	-	-	+++	-	+	++	-	+	+++	-
pHP17L	-	-	-	+	+	-	-	++	-	-	-	-	+	+++	-	+	+++	-	-	-	-

Fig. 1

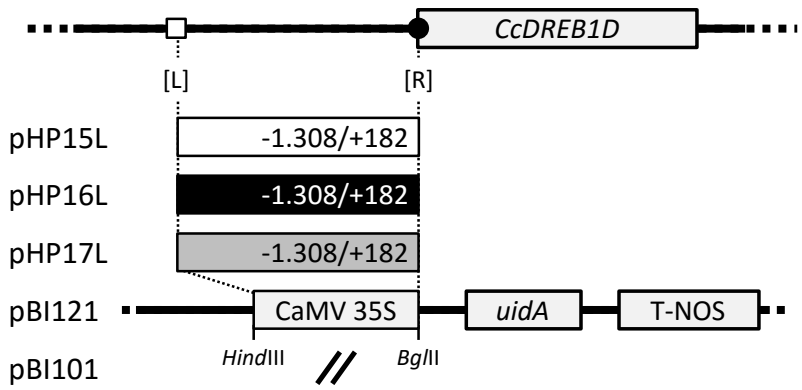

CcDREB1D
[L]
[R]
pHP15L
-1.308/+182
pHP16L
-1.308/+182
pHP17L
-1.308/+182
pBI121
CaMV 35S
uidA
T-NOS
pBI101
HindIII
BglII

Fig. 2

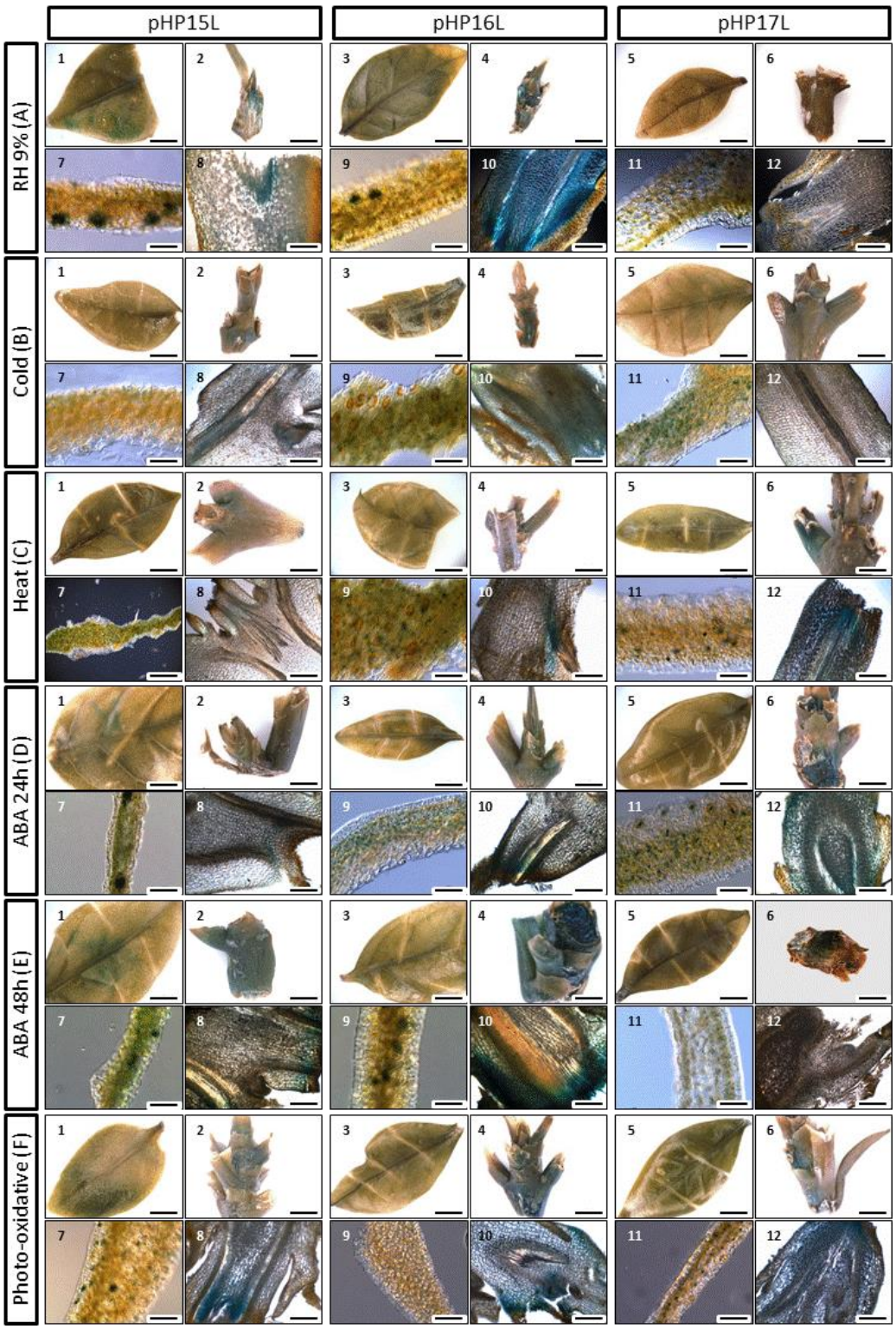

pHP15L
pHP16L
pHP17L
RH 9% (A)
Cold (B)
Heat (C)
ABA 24h (D)
ABA 48h (E)
Photo-oxidative (F)
1
2
3
4
5
6
7
8
9
10
11
12

Fig. 3

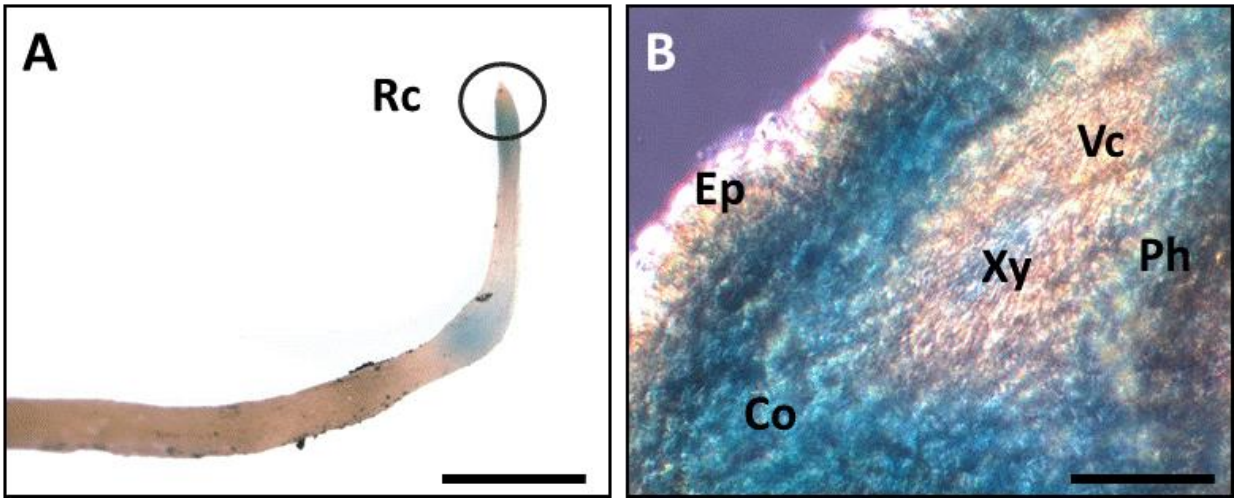


Fig. 4

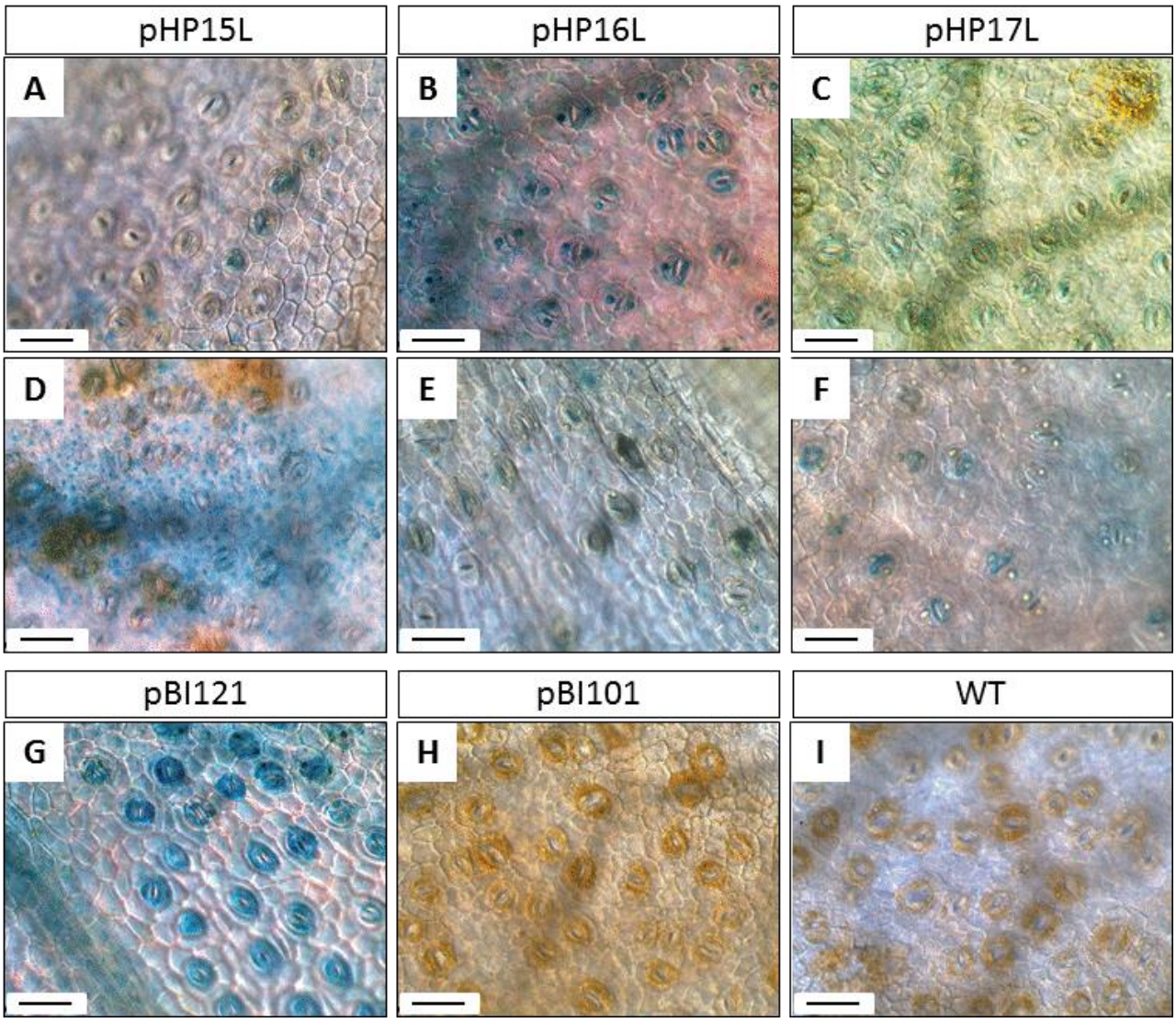


Fig. 5

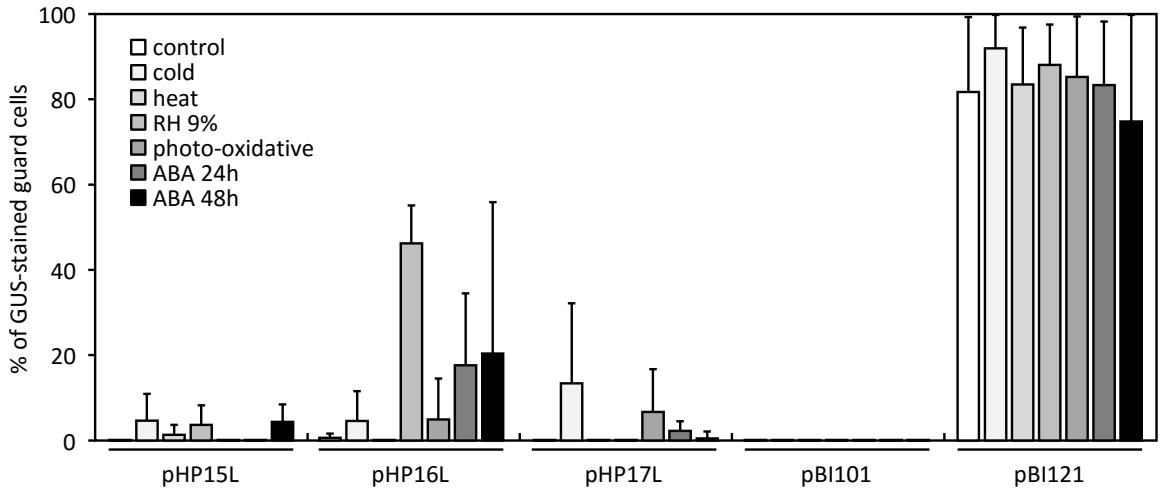
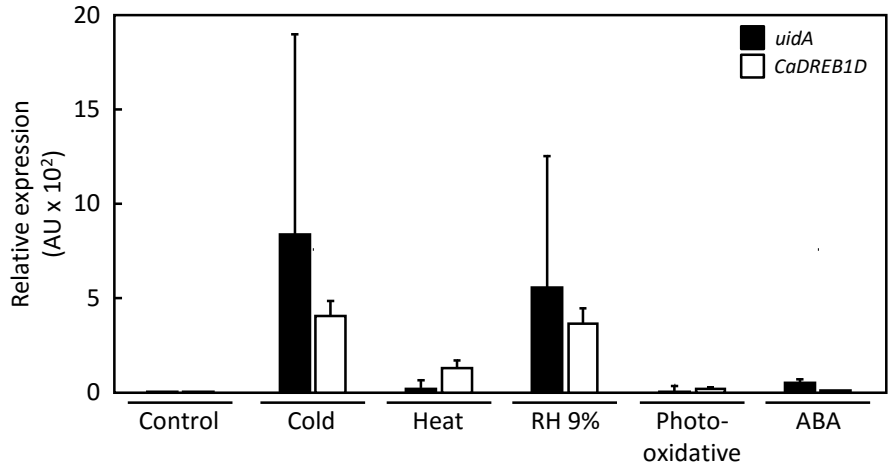
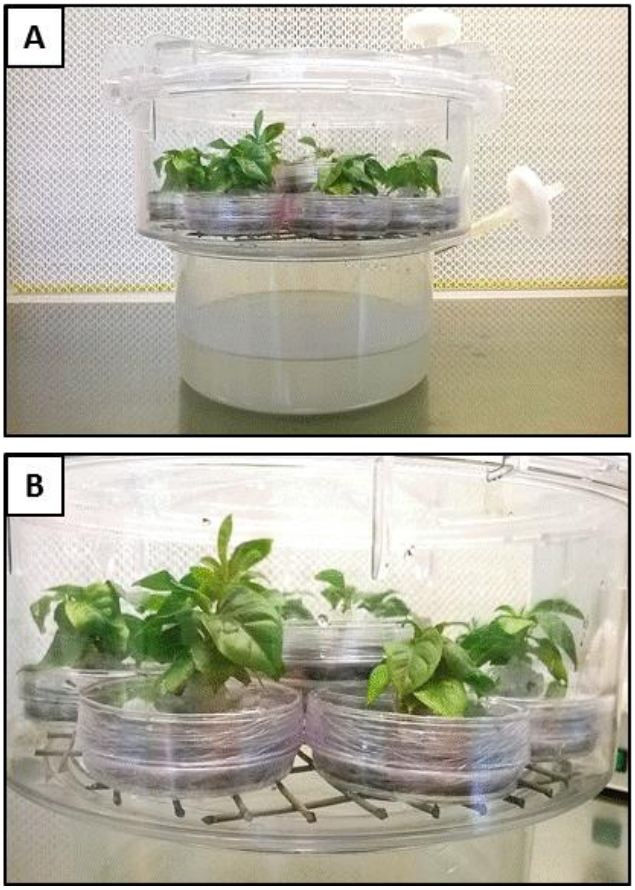

100
80
60
40
20
0
% of GUS-stained guard cells
control
cold
heat
RH 9%
photo-oxidative
ABA 24h
ABA 48h
pHP15L
pHP16L
pHP17L
pBI101
pBI121

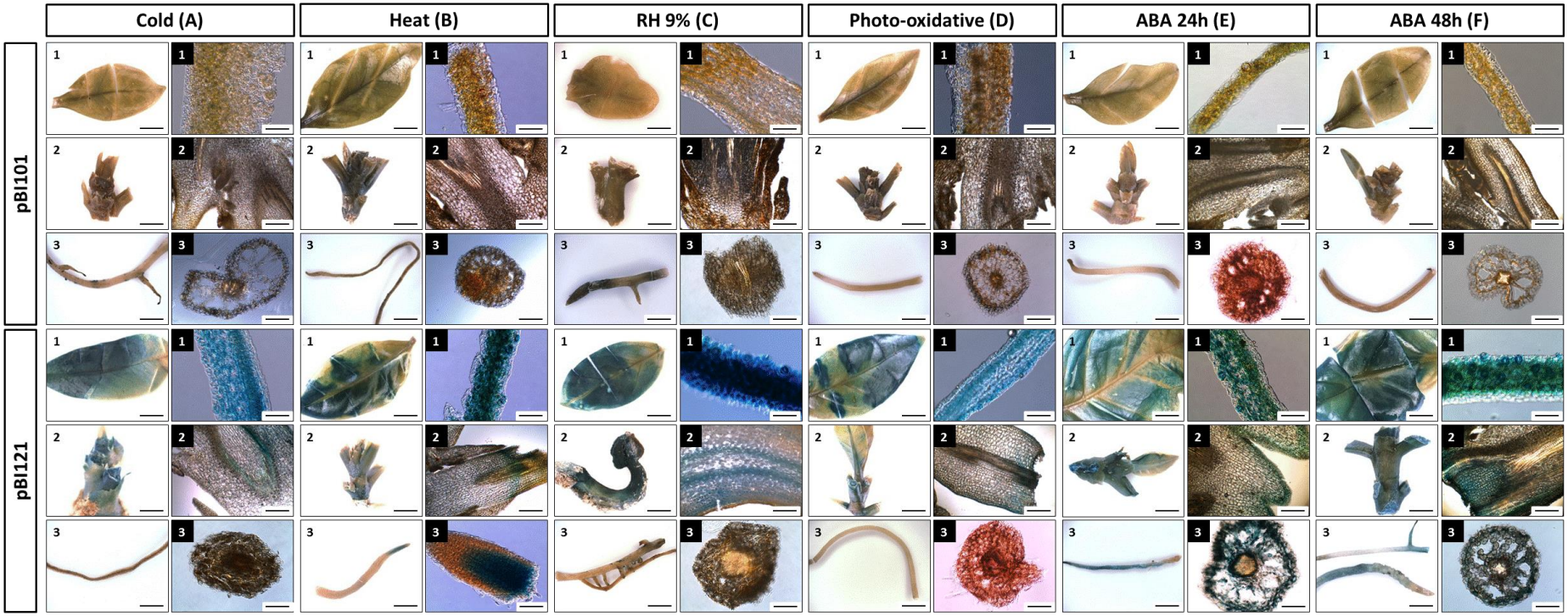
Fig. 6


20
15
10
5
0
Relative expression
(AU x 10²)
uidA
CaDREB1D
Control
Cold
Heat
RH 9%
Photo-
oxidative
ABA

Supp. Fig.1



Supp. Fig.2


Cold (A)
Heat (B)
RH 9% (C)
Photo-oxidative (D)
ABA 24h (E)
ABA 48h (F)
pBI101
pBI121

ARTICLE 3

Transcriptome analysis of *Coffea arabica* leaves reveals differentially expressed genes under several abiotic stresses

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Transcriptome analysis of *Coffea arabica* leaves reveals differentially expressed genes under several abiotic stresses

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ABSTRACT

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. *DREB* transcription factors play important roles in regulating the expression of genes in response to a variety of abiotic and biotic stresses. The objective of the present work was to molecularly characterize arabica coffee plants transformed with the *CcDREB1D* promoter in order to list the genes induced under a representative range of abiotic stresses. In this way, the RNA-seq Illumina technique was used to understand the molecular responses of coffee plants under different abiotic stresses. Special attention was focused on genes belonging to the four subgroups of the *DREB* family. Using these data, candidate genes were selected and their differential expression profiles were confirmed by RT-qPCR experiments in leaves of arabica coffee var. Caturra under stress conditions, such as low and high temperature, photo oxidative, application of exogenous ABA (acid abscisic) and drought stress. The identification of these genes should help advance our understanding of the genetic determinism of abiotic stress tolerance in coffee. Furthermore, this work will open new avenues for studies into specific genes and pathways in this species, especially related to abiotic stress, and our data have a potential value in assisted breeding applications.

Keywords: *Coffea*. *DREB*. RNA Seq. Differentially expressed genes. Abiotic stresses.

INTRODUCTION

The coffee (*Coffea* sp.) is one of the most important crops in the world economy with cultivation in more than 70 countries, handling around 91 billion dollars a year. World production has estimated increase of 1,4% in production in 2015/16 compared to 2014/15, wherein 70.17 million bags of Arabica were exported in the twelve months

ending December 2015, compared to 68.96 million bags last year (International Coffee Organization – ICO, 2016). The Brazilian crop production of 2017 is estimated at 43.650,1 and 47.509,8 million 60kg bags of coffee (Companhia Nacional de Abastecimento - CONAB, 2017).

However, growth constraints and stress due to environmental changes result in reduced productivity and significant crop losses (Lata and Prasad 2011). Climatic variability has always been the main factor responsible for the fluctuation of coffee yields worldwide, and the climate change, as a result of global warming, is expected to present a major challenge to the coffee industry (Van Hilten 2011).

In recent years, *Coffea* ssp. has become the subject of increasing research in gene expression analysis (Nobile et al. 2010). However, few works have focused on selecting reliable reference genes for data normalization in studies involving coffee plants under different abiotic stress conditions (Cruz et al. 2009).

In the quest to find genetic factors working in concert of abiotic signaling pathways, various transcription factors have been discovered. Some of these important transcription factors respond to drought, low temperature and high salinity stress (Agarwal et al. 2006). Among these, the *DREB* transcription factors have gained much attention due to their involvement in the regulation of many stress-related genes that play an important role in cascading a response to environmental stimuli (Lata and Prasad 2011). Transformation of plants with *DREBs* is one of the preferred strategies to develop multiple abiotic stress tolerance. In response to abiotic stresses, this transcription factor up-regulate the expression of many stress-related genes, the products of which work in various ways confer protection (Khan 2011).

Gene expression analysis has been widely used as a method to study the complex signaling and metabolic pathways underlying cellular and developmental processes in biological organisms, including plants. Growing number of studies of expression levels of several genes in plants have been carried out in order to understand the cellular and molecular mechanisms involved in plant development and growth, as well as, in plant responses to biotic (pathogen infection) and abiotic (environmental) stresses (Dechorgnat el al. 2012). The gene expression analysis has been performed by using different methods such as, northern blotting, ribonuclease protection assay, RT-qPCR, DNA microarrays and next generation sequencing (NGS) technologies. These last three technologies in particular have gained a wider appeal for the quantification of gene expression (Velada et al. 2014).

In order to identify candidate genes involved in several abiotic stress in coffee plants, leaves from transgenic *C. arabica* harboring *CcDREB1D* promoter under different stress conditions, such as temperature, photo-oxidative, application of exogenous ABA (acid abscisic) and drought stress, were used to generate libraries that were sequenced using the Illumina technique. A reference full transcriptome was annotated and compared to identify the transcription profiles of differentially genes expressed under several abiotic stress conditions. Furthermore, expression of these genes were analysed by RT-qPCR in the leaves of these plants.

Therefore, the main objectives were to obtain a global overview of transcriptionally active genes in this species using next generation sequencing and to analyze specific genes that were highly expressed in leaves with potential exploratory characteristics for breeding and understanding the evolutionary biology of coffee.

MATERIALS AND METHODS

Plant materials

Embryogenic calli from *C. arabica* var. Caturra were obtained as described by Etienne (2005) and were placed in proliferation conditions during twelve months in view of selecting proembryogenic masses (PEMs) for *Agrobacterium*–mediated genetic transformation experiments. Transgenic lines of *C. arabica* var. Caturra were generated by *A. tumefaciens*-mediated transformation as previously described by Ribas et al. (2011). The construction used in this study contains full length version of haplotype 16 of *CcDREB1D* promoters of *C. canephora* fused to the *uidA* reporter gene, described by Alves et al. 2017.

Abiotic stresses experiments

Before applying the different stresses, transgenic plants were cultured in baby food jars in optimal environmental conditions under a 12 h photoperiod (70 μM m^{-2} s^{-1}) at 26°C under 80% RH. The drought, cold, heat, photo-oxidative and ABA stresses were performed as described below. To minimize possible effects of circadian clock (subset of biological rhythms with period), all experiments with the different stresses were started in the morning period at 10 a.m. after 2 h of light period. All experiments were carried out with plants transformed with the hp16L haplotype and designed as follows: for each haplotype subjected to a particular abiotic stress, four plants derived from independent

transformation events were studied, one plant per baby food jar representing an independent replicate.

Drought stress: Drought was mimicked by low (9%) relative humidity (RH). This atmosphere was created using 500 mL of KOH supersaturate solution that was poured in the lower compartment of a temporary immersion bioreactor (Matis®, CID Plastiques, France). Transformed coffee plants were placed in the upper compartment over 55 mm Petri dishes having their upper part exposed to outside environment and radicles immersed on MS medium with active charcoal (1g L^{-1}) through a small hand-made hole on the Petri dish cover (one plant per Petri dish). To avoid any water vapor exchange between MS medium and outside bioreactor atmosphere, the hand-made hole was further closed with high-vacuum silicone grease (Dow Corning®, Sigma) and Petri dishes were sealed with plastic film. Batches of 10 plants were then placed inside the bioreactor in a resulting atmosphere 9% RH in the growth chamber with a 26°C temperature.

Temperature stress: The baby food jars were directly transferred from the growth chamber with a 26°C temperature into a cold chamber at 5°C and similarly, the baby food jars were quickly transferred from the growth chamber with a 26°C temperature to a stove at 40°C.

Photo-oxidative stress: The baby food jars were kept in the same culture chamber conditions, but light intensity was suddenly increased from 70 to 200 µM m^{-2} s^{-1} without any change in the spectrum. All the other culture parameters were unmodified.

Abscisic acid: The plants were transferred from the 'M' maturation medium into baby food jars containing the same medium supplemented with 10^{-5} M ABA (Sigma Aldrich, St Louis, USA).

In silico gene expression

The expression of the pHP16L haplotype of the Cc*DREB1D* promoter was analyzed though a transcriptomic approach under all the abiotic stresses described above. After a 3 h stress exposure period, leaves were collected, transferred to liquid nitrogen and then kept at -80°C until RNA extraction.

Total RNA was extracted from leaves frozen in liquid nitrogen which were further ground and treated as previously described (Breitler et al. 2016). RNA quantification was performed using a NanoDropTM 1000 Spectrophotometer (Waltham, MA, USA). RNA quality and integrity were verified by the Agilent 2100 Bioanalyzer (Agilent Technologies) ensuring RIN values equal to and greater than 7.0.

The preparation of the mRNA library was done via the TruSeq Stranded mRNA Sample Preparation Kit from Illumina. The validation of the mRNA banks was performed by the quantification (concentration and size of the fragments) of the cDNAs in the Fragment Analyzer and by qPCR (ROCHE Light Cycler 480).

Sequencing was performed on the HiSeq 2500 platform (Illumina) through the SBS (Synthesis By Sequencing) technique by MGX - Montpellier Genomix (Montpellier, France). Base calling was performed by RTA software provided by Illumina.

Description of the sequencing

For the RNA-Seq experiment, three samples were used in biological triplicate for each stress condition tested. Sequence quality control was performed by the FastQC software and contaminant search in the FastQ Screen software (Bowtie2 aligner), which allowed the testing of the alignment of a large set of data from different genomes representing potential sources of contamination. Mapping was performed by the TopHat2 algorithm v2.0.13 (with Bowtie 2.2.3). Cufflinks and Cuffdiff was also performed, following the Tuxedo protocol for RNAseq analysis (ref), followed by the cummeRbund packge of R, for the visualization of the results. The HTSeq-count (version 0.6.1p1) software was also used for counting aligned reads on the genes.

Statistical analyzes

The experiment consisted of six treatments (RH 9%, Cold 5°C, Heat 40°C, ABA 10^{-5} M, Photo-oxidative 200 $\mu mol.m^{-2}s^{-1}$ and Control - no stress) with three biological triplicates for each. Among the six treatments, 15 comparisons were performed (2 to 2 comparison of each condition). To identify the differentially expressed genes, three R statistical software packages were used: EdgeR (Robinson et al. 2010), DESeq and DESeq2 (Anders and Huber 2010). The versions used were EdgeR 3.6.7, DESeq 1.16.0, DESeq2 1.4.5 and R 3.1.0.

Before the statistical analyzes, the data were normalized in order to correct bias errors. The normalization factor was calculated for each sample in order to adjust the

count of the number of reads per gene between the different samples. The standardization method used was RLE - Relative Log Expression.

Differential expression analysis

Differential expression analysis was performed from the raw read counts using the DESeq2 package (Anders and Huber 2010). Genes identified as differentially expressed in each abiotic stress tested were required to have at least twofold change and p-value ≤ 0.05.

Additionally, GO functional enrichment analysis was performed using the identified differentially expressed genes and performing Fisher's exact test using AgriGO web resource (http://bioinfo.cau.edu.cn/agriGO/).

RT-qPCR assays

Based on DESeq2 results for differential expression, genes responding to drought stress and corresponding genes of each DREB subgroup in the arabica coffee leaves under the different abiotic stresses, besides the condition without stress, identified by the in silico analyses, were selected for expression analysis by RT-qPCR to validate the RNA-seq analysis. These genes were chosen based on a normalized log2 fold-change for each stress. The primers used are described in Table 1.

Quantitative PCR was carried out with synthesized single-strand cDNA described above using the protocol recommended for the use of 7500 Fast Real-Time PCR Systems (Applied Biosystems, Foster City, CA, USA). cDNA preparations were diluted (1/20) and tested by qPCR. Primers were designed using Primer Express software (Applied Biosystems) and preliminarily tested for their specificity and efficiency against a mix of cDNA (data not shown). qPCR was performed with 1 µL of diluted ss-cDNA and 0.2 µM (final concentration) of each primer in a final volume of 10 µL with 2,5 µL SYBR green fluorochrome (SYBRGreen qPCR Mix-UDG/ROX, Invitrogen). The reaction mixture was incubated for 2 min at 50°C (Uracil DNA-Glycosilase treatment), then 5 min at 95°C (inactivation of UDGase), followed by 40 amplification cycles of 3 s at 95°C and 30 s at 60°C (annealing and elongation). Data were analyzed using *7500* Fast *Software v2.0.6* (Applied Biosystems) to determine cycle threshold (Ct) values. Specificity of the PCR products generated for each set of primers was verified by analysing the Tm (dissociation) of amplified products. Gene expression levels were normalized to expression level of

GaPDH as a constitutive reference (de Carvalho et al. 2013). Expression was expressed as relative quantification by applying the formula $(1+E)^{-\Delta\Delta Ct}$, where $\Delta Ct_{target} = Ct_{target\ gene} - Ct_{reference\ gene}$ and $\Delta\Delta Ct = \Delta Ct_{target} - \Delta Ct_{internal\ calibrator}$.

Table 1 - Efficiency of primer pairs in *Coffea arabica* leaf tissue selected for *in vivo* gene expression (RT-qPCR) analyzes. As an optimal efficiency range, values equal to or greater than 80% were considered.

PRIMER	SEQUENCE 5' – 3'	EFFICIENCY
Cc10_g14150	F – GCACAGCTCAACCTCCCAAA	100%
	R - GCAAACTCTGCATGGGAAGTG	
Cc10_g14160	F – GATAAAAGTGGCCCCATGGA	100%
	R - CCCCTTGGGATTATGCATTCT	
Cc10_g04710	F- TTCAACAAACTGCGGTGCAT	100%
	R - CCAGGCGAGGAGGAGTAGAA	
Cc10_g07460	F – AGGGCTCACTTGGGCTCAAT	100%
	R - TCGTTCTGGAGGCTGAATCG	
Cc08_g09520	F – TCCCCATTATCGCCAAACAC	80%
	R - TTGGACTCTGCATCCTGATTGT	
Cc06_g12520	F – GGTGGTCCAGTTGGAGAGTGA	87%
	R - TGGGTGGCACAAAAAAATCA	
Cc10_g10960	F – TCACCGGACTCACCATCTCA	80%
	R - GGAGGATGATCAGGTGGTGAA	
Cc07_g06220	F – TGATGCCGTTCAAAGTTCCA	100%
	R - CTGAGTCGGTGTTGCATGGT	
Cc08_g07780	F – TGTCTTCGTGGCAAGAATGC	100%
	R - GGTTGGCGTATTCAGATGCA	
Cc02_g24810	F – GCGAAGATCAATGGCAATGA	100%
	R - CCCCTGTAGGTCGGATGCTT	
Cc03_g07870	F – TGCTCAAGGGCTATGATGCA	88%
	R - TCAACGGAATGTGGCTTCCT	
Cc04_g02760	F – CGGCAACAAGCATCCAGTTT	99%
	R - GAGCCATATGCGCGATTTTT	
Cc05_g06840	F – GGCGAGAATGCTAGGCTCAA	95%
	R - AGGGTCCGAGGCTGATGAAT	
Cc06_g00780	F – AGCAGCCACCAGAGAACCAA	86%
	R - GCACCAACAGGATCGTCGAT	
Cc02_g03420	F – ATCACGACGGCATCCTCATT	100%
	R - CCAGCCATATGCGCGTAGTT	
Cc02_g03430	F – ATGGATGAGGAGGCGGTTTT	92%
	R - TCATCCCCAACTGCACATTG	
Cc02_g05970	F – GGGCAAATGGGTAGCTGAAA	100%
	R - CCTCAACTGCAGTCCCAAATG	
Cc07_g15390	F – GTTCCAACCACAGCCCACAT	100%
	R - TTGAACCACCACCTGATTGCT	
DHN - Dehydrin	F – AAGGCGGGAGGAGGAAGAA	88%

	R - GTCCAGGTTGAGCCTCCTTGT	
GolS - galactinol synthase	F – CCCTTTGGTGGTTGCAGTTT R - AGGCTCGATCTCCCGGACTATA	83%

RESULTS

The results obtained with the HTSeq-count software for the percentage of reads aligned in the genome are shown in Table 2. A reference full transcriptome was then built using *Coffea canephora* as the reference genome. Access to these data was facilitated by an integrative genome information system, Coffee Genome Hub (Dereeper et al. 2015).

Table 2 - Results obtained with HTSeq-count software. Gene count: The number of reads (or pairs of reads) aligned in the exons of a gene unambiguously. CB: number of raw clusters. CAF: number of clusters after the passage of the filter. Ambiguous: number of reads aligned in the exons of several genes (if genes overlap). Unique aligned: proportion of reads aligned in a single position (belonging to the three cases above) in relation to the number of clusters filtered. GUS: number of reads aligned on the glucuronidase sequence.

sample	gene counts	CB	CAF	ambiguous	% unique aligned	GUS
ABA	85.144.084	185.909.314	166.883.308	58.017	225,39	145
Heat	53.797.638	107.618.747	102.186.703	158.487	233,65	139
Cold	40.848.106	132.364.802	100.354.058	22.476	200,7	2.238
Light	72.475.771	116.148.903	110.498.961	50.100	248,23	17
Drought	65.431.098	166.800.702	125.956.111	56.503	222,44	2.514
No stress	64.443.466	127.640.697	114.016.870	40.780	229,66	27

Statistical analyzes

The number of sequences anchored in each contig (read counts) was subjected to differential expression analysis between the libraries using DEseq, DEseq2 and EdgeR software in the R/Bioconductor package. A unigene was considered as differentially expressed when it was identified in at least one software considering fold-change ≥ 2 (or foldchange ≤ -2) and/or p-value ≤ 0.05. The Table 3 shows the numbers of differentially expressed genes reported (p-value <0.05) in arabica coffee leaves for each comparasion and statistically tested. The analysis performed in the DESeq2 software was shown to be the most efficient to detect the differentially expressed genes in each comparison. We emphasize that only for the COLD vs NO STRESS comparison the most efficient analysis was using the EdgeR software. The results of the Cuffdiff analisys is shown in figure 1 and shows that it was also very efficient in detecting differential expression, among the conditions tested.

Table 3 - Number of differentially expressed genes by comparison between the treatments and the statistical analyzes tested.

Comparison	EdgeR	DESeq	DESeq2
ABA vs NO STRESS	1.672	960	2.217
COLD vs NO STRESS	1.141	146	812
DROUGHT vs NO STRESS	4.090	2.550	4.263
HEAT vs NO STRESS	7.144	4.754	8.008
LIGHT vs NO STRESS	1.930	782	2.099
ABA vs HEAT	7.654	5.253	8.815
ABA vs COLD	2.111	415	2.932
ABA vs LIGHT	1.894	939	2.066
ABA vs DROUGHT	4.784	3.758	6.096
HEAT vs COLD	6.871	3.139	7.556
HEAT vs LIGHT	7.871	5.427	8.847
HEAT vs DROUGHT	7.502	5.407	8.070
COLD vs LIGHT	3.686	640	4.726
COLD vs DROUGHT	4.142	1.106	4.449
LIGHT vs DROUGHT	5.772	4.133	6.422

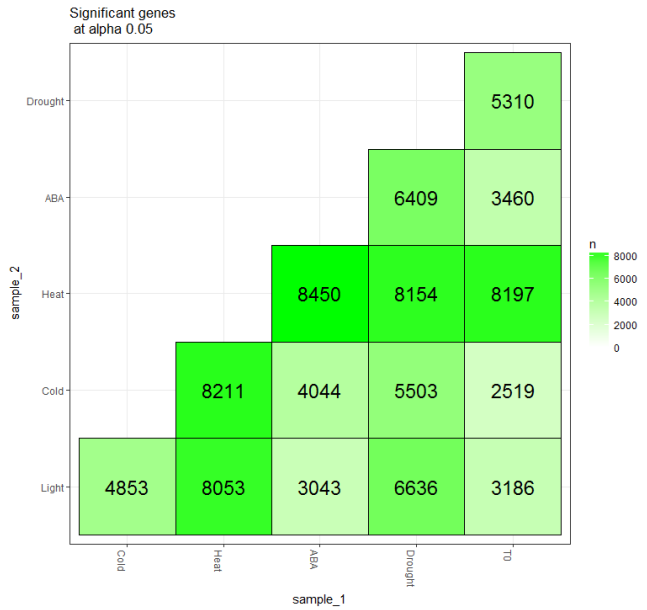

Figure 1 – Number of differentially expressed genes detected by the Cuffdiff analysis among the conditions tested.

The results of the *in silico* gene expression analysis (Table 3 and Figure 1) showed that more differentially expressed genes (DEG) in the comparisons with the control

condition, non stress (T0), was observed by the applied Heat and Drought stresses. ABA, Light and Cold had less DEG, repectively. The scatter-plot matrix of all comparisons, the MDS-Plot and a dendogram clustering produced on the cuffdiff results also corroborates with these observations (Figure 2). Futhermore, results presented on figure 2 shows that the molecular responses observed upon ABA and light stresses were more similar than the other stresses applied.

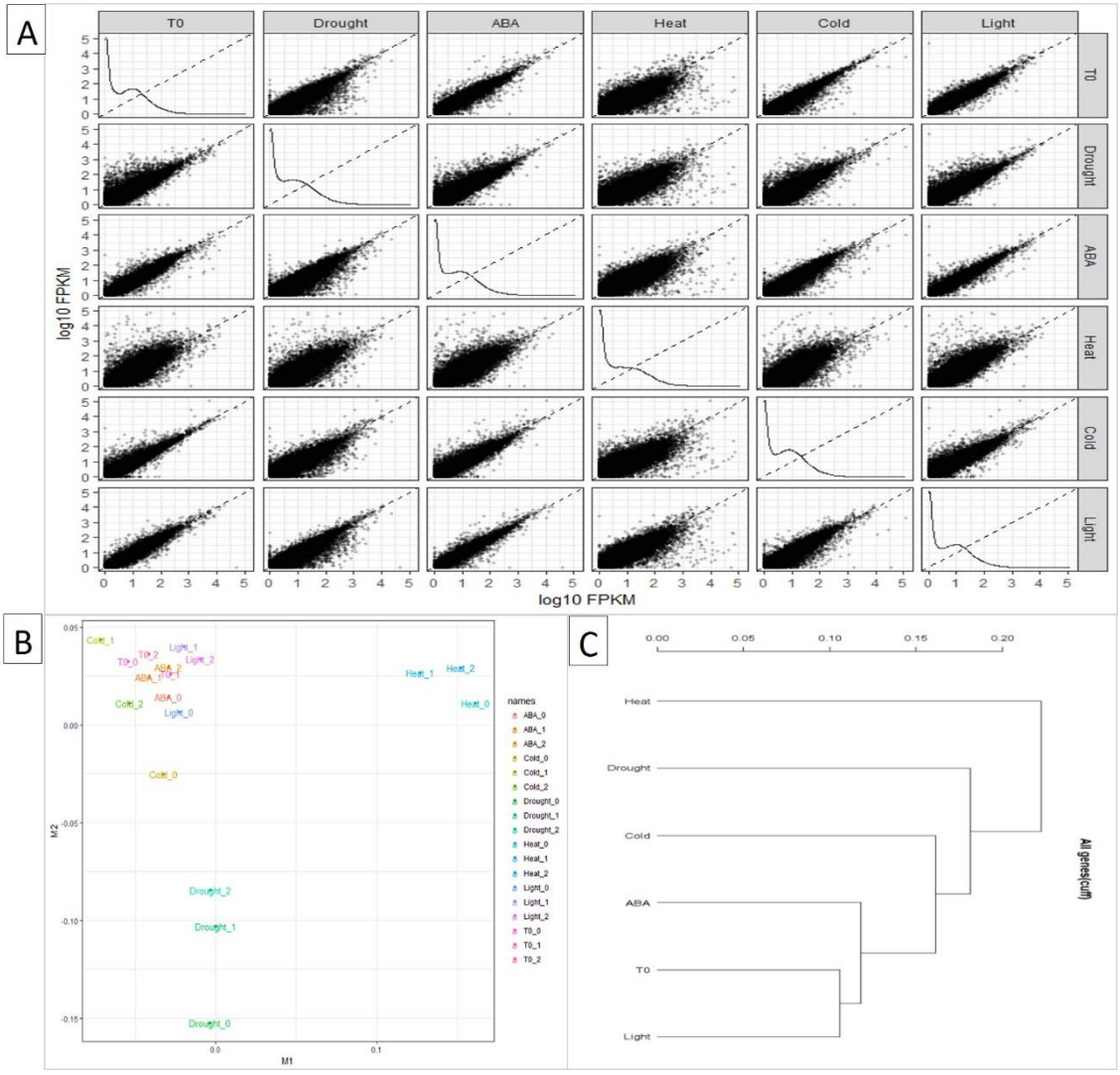

Figure 2 – Scatter-plot matrix (A), MDS-Plot (B) and a Dendogram (C) of all comparisons of differentially expressed genes obtained with the Cuffdiff analysis, produce by the cummeRbund package.

In general, the total values of differentially expressed genes (p-value <0.05) are shown in figure 3, for the three types of statistical analyzes analyzed, without taking into account the comparisons. According to these results, data obtained by DESeq2 displayed greater efficiency in detecting differentially expressed genes.

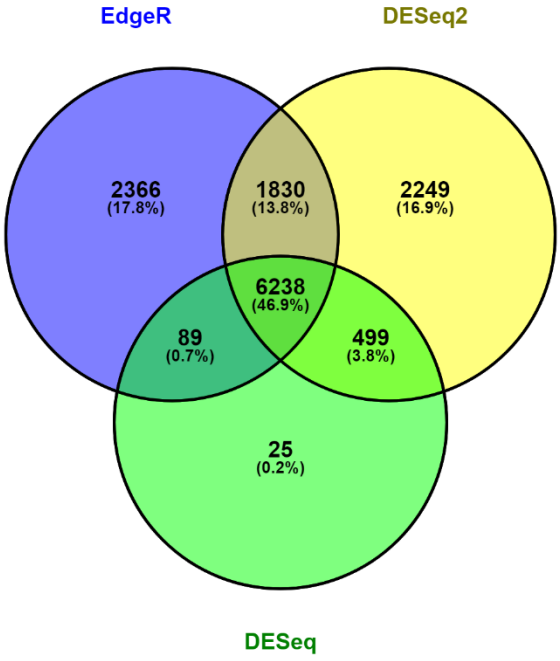

Figure 3 - Venn Diagram representing the number of differentially expressed genes in all abiotic stresses tested in the statistical analysis EdgeR, DESeq and DESeq2 in relation to the control treatment – No stress.

Clustering differential expression

The results obtained with Cuffdiff analysis was k-means clustered using the function "*cscluster*" of the cummeRbund package, which uses the Jensen-Shannon distance metric for the analysis. Figure 4 displays the results of this analysis using k=5. Interestingly, by using this tool, we have been able to identify differentialy expressed genes that display an up-regulation upon heat stress (Cluster 1), cold (Cluster 4) and drought (Cluster 5), repectively (Figure 4), althought with some cross-talks between some conditions could also be observed. In addition, Cluster 2, seems to be specifically down-regulated upon heat-stress. The Cluster 3 pattern displays a set of genes that seems to do not respond consistently to any of the abiotic stress condition applied in our work.

A heat-map of the expression levels of a 50 genes set from the three identified clusters (Clusters 1, 4 and 5) is shown on figure 5, 6 and 7, respectively. Results displayed on figure 5-7, corroborate with the almost specific up-regulation in function of the applied abiotic stress. The list of these genes is presented on Tables 4, 5 and 6.

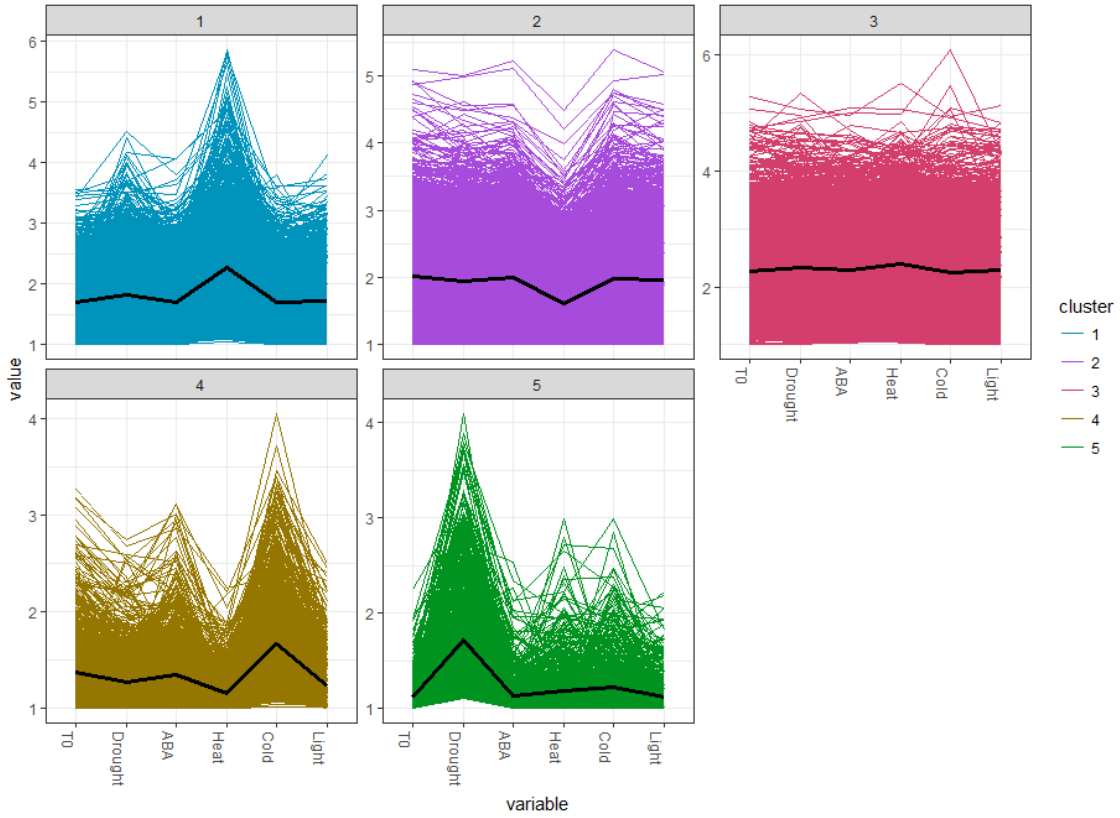

Figure 4 – Clustering results of the Cuffdiff data obtained with the *cscluster* function of the cummeRbund package which the Jensen-Shannon distance metric for the analysis.

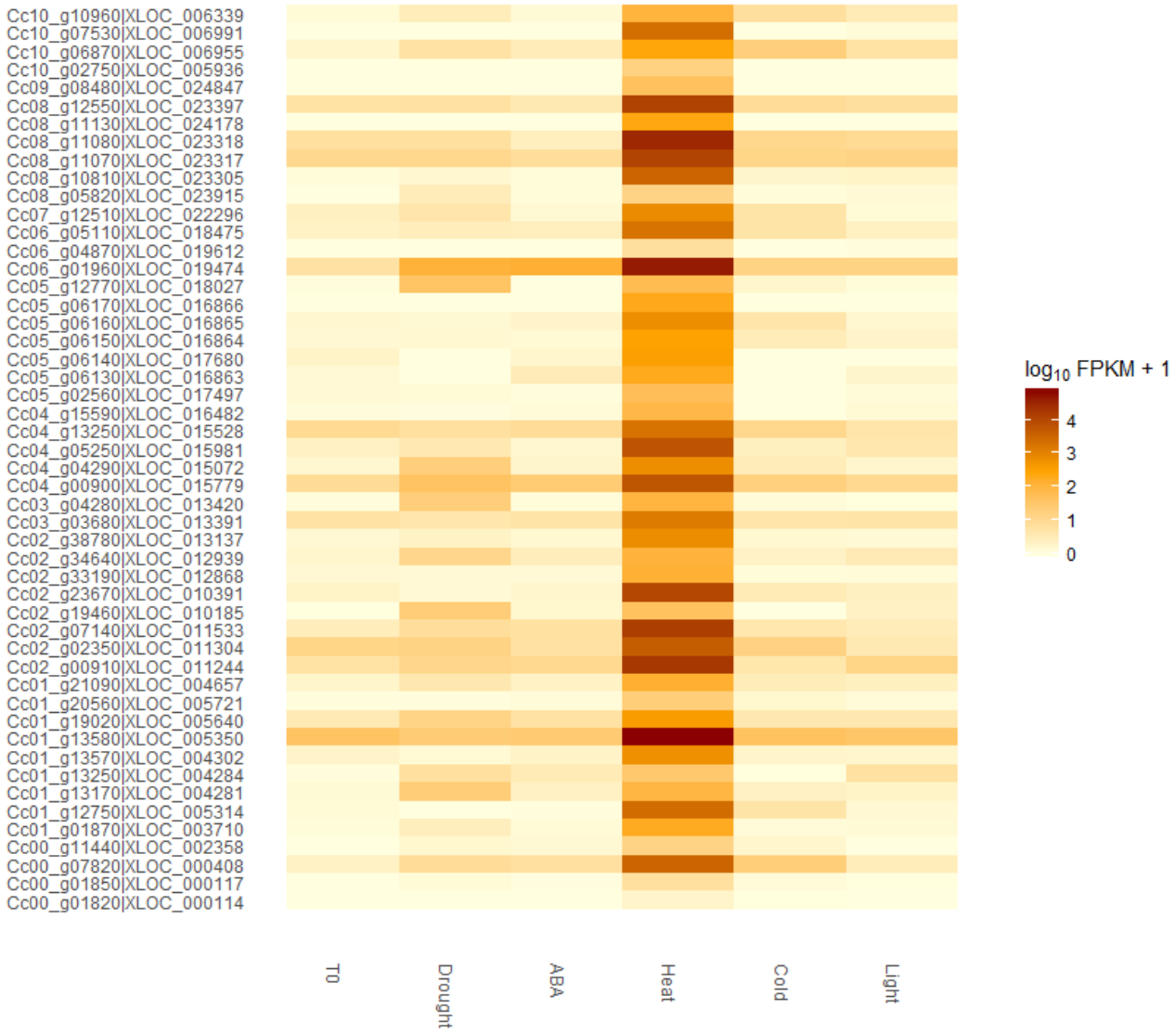

Figure 5 – Heat-map of the expression levels of a set of 50 genes from Cluster 1.

Table 4 – Genes up-regulated upon heat stress (Cluster 1).

	BLAST C. canephora genome	***C. arabica* gene**	**Annotation *C. canephora* genome**
XLOC_006991	*Cc10_g07530*	GSCOCP00034510001	gi|125620180|gb|ABN46982.1| small molecular heat shock protein 1... 120 7e-26
XLOC_023305	*Cc08_g10810*	GSCOCP00027060001	gi|406047594|gb|AFS33109.1| heat stress transcription factor A2 ... 397 e-108
XLOC_010391	*Cc02_g23670*	GSCOCP00015069001	gi|297739445|emb|CBI29627.3| unnamed protein product [Vitis vini... 228 6e-58
XLOC_019474	*Cc06_g01960*	GSCOCP00023436001	gi|356521398|ref|XP_003529343.1| PREDICTED: 17.5 kDa class I hea... 174 6e-42
XLOC_005314	*Cc01_g12750*	GSCOCP00028220001	gi|255585824|ref|XP_002533590.1| heat-shock protein, putative [R... 211 5e-53
XLOC_011533	*Cc02_g07140*	GSCOCP00009477001	gi|225449270|ref|XP_002280821.1| PREDICTED: 18.2 kDa class I hea... 242 2e-62
XLOC_023318	*Cc08_g11080*	GSCOCP00027100001	***** No hits found ******
XLOC_015981	*Cc04_g05250*	GSCOCP00022272001	gi|255543359|ref|XP_002512742.1| heat shock protein, putative [R... 1113 0.0
XLOC_011244	*Cc02_g00910*	GSCOCP00019892001	gi|255582541|ref|XP_002532054.1| heat shock protein, putative [R... 227 2e-57
XLOC_024847	*Cc09_g08480*	GSCOCP00020811001	gi|224072528|ref|XP_002303771.1| predicted protein [Populus tric... 148 2e-33
XLOC_000408	*Cc00_g07820*	GSCOCP00007023001	gi|297734525|emb|CBI15772.3| unnamed protein product [Vitis vini... 134 6e-30
XLOC_023397	*Cc08_g12550*	GSCOCP00030466001	gi|299891471|gb|ADJ57588.1| mitochondrial small heat shock prote... 199 5e-49
XLOC_005350	*Cc01_g13580*	GSCOCP00028328001	gi|351725131|ref|NP_001235546.1| uncharacterized protein LOC1005... 241 5e-62
XLOC_018475	*Cc06_g05110*	GSCOCP00043044001	gi|225441008|ref|XP_002283685.1| PREDICTED: uncharacterized prot... 132 2e-29
XLOC_005936	*Cc10_g02750*	GSCOCP00024783001	gi|224059052|ref|XP_002299693.1| predicted protein [Populus tric... 72 3e-10
XLOC_013137	*Cc02_g38780*	GSCOCP00027812001	gi|224064112|ref|XP_002301387.1| predicted protein [Populus tric... 290 2e-75
XLOC_002358	*Cc00_g11440*	GSCOCP00012461001	gi|377806997|ref|YP_005090412.1| orf2 gene product (mitochondrio... 335 9e-90
XLOC_023317	*Cc08_g11070*	GSCOCP00027098001	gi|359476259|ref|XP_002279425.2| PREDICTED: putative methyltrans... 627 e-177
XLOC_015072	*Cc04_g04290*	GSCOCP00022152001	gi|225442975|ref|XP_002267925.1| PREDICTED: 26.5 kDa heat shock ... 228 1e-57
XLOC_016865	*Cc05_g06160*	GSCOCP00042095001	gi|388506148|gb|AFK41140.1| unknown [Medicago truncatula] 110 1e-22
XLOC_003710	*Cc01_g01870*	GSCOCP00019259001	gi|359482368|ref|XP_003632762.1| PREDICTED: pre-mRNA-processing-... 4561 0.0
XLOC_015779	*Cc04_g00900*	GSCOCP00021718001	gi|356520108|ref|XP_003528707.1| PREDICTED: 17.4 kDa class III h... 187 5e-46
XLOC_013420	*Cc03_g04280*	GSCOCP00026138001	gi|255552051|ref|XP_002517070.1| Heat shock factor protein HSF30... 364 1e-98

XLOC_004302	*Cc01_g13570*	GSCOCP00028327001	gi|629669|pir||S39507 glucuronosyl transferase homolog, ripening... 517 e-144
XLOC_016864	*Cc05_g06150*	GSCOCP00042094001	gi|225447661|ref|XP_002275421.1| PREDICTED: uncharacterized prot... 86 3e-15
XLOC_016863	*Cc05_g06130*	GSCOCP00042092001	gi|225447661|ref|XP_002275421.1| PREDICTED: uncharacterized prot... 86 2e-15
XLOC_022296	*Cc07_g12510*	GSCOCP00036863001	gi|147852346|emb|CAN80115.1| hypothetical protein VITISV_032527 ... 1048 0.0
XLOC_010185	*Cc02_g19460*	GSCOCP00014514001	gi|296083098|emb|CBI22502.3| unnamed protein product [Vitis vini... 493 e-137
XLOC_016482	*Cc04_g15590*	GSCOCP00042332001	gi|297734029|emb|CBI15276.3| unnamed protein product [Vitis vini... 794 0.0
XLOC_018027	*Cc05_g12770*	GSCOCP00021147001	***** No hits found ******
XLOC_011304	*Cc02_g02350*	GSCOCP00020088001	gi|1708314|sp|P51819.1|HSP83_IPONI RecName: Full=Heat shock prot... 1160 0.0
XLOC_006339	*Cc10_g10960*	GSCOCP00016651001	gi|358248764|ref|NP_001239936.1| ethylene-responsive transcripti... 124 2e-26
XLOC_023915	*Cc08_g05820*	GSCOCP00024373001	gi|359475200|ref|XP_002282080.2| PREDICTED: B3 domain-containing... 268 1e-69
XLOC_006955	*Cc10_g06870*	GSCOCP00034602001	gi|3759184|dbj|BAA33810.1| phi-1 [Nicotiana tabacum] 494 e-137
XLOC_017680	*Cc05_g06140*	GSCOCP00042093001	gi|225447663|ref|XP_002275464.1| PREDICTED: uncharacterized prot... 80 2e-13
XLOC_004281	*Cc01_g13170*	GSCOCP00028280001	gi|42761378|dbj|BAD11646.1| FtsJ cell division protein-like [Ory... 63 3e-08
XLOC_013391	*Cc03_g03680*	GSCOCP00026065001	gi|224099789|ref|XP_002311619.1| predicted protein [Populus tric... 967 0.0
XLOC_019612	*Cc06_g04870*	GSCOCP00043012001	gi|297738679|emb|CBI27924.3| unnamed protein product [Vitis vini... 56 4e-06
XLOC_012868	*Cc02_g33190*	GSCOCP00015336001	gi|386685665|gb|AFJ20202.1| putative luminal-binding protein [Vi... 1055 0.0
XLOC_024178	*Cc08_g11130*	GSCOCP00027106001	gi|5257560|gb|AAD41409.1|AF159562_1 cytosolic class II low molec... 183 1e-44
XLOC_015528	*Cc04_g13250*	GSCOCP00018903001	gi|225457237|ref|XP_002284179.1| PREDICTED: chaperone protein Cl... 1604 0.0
XLOC_016866	*Cc05_g06170*	GSCOCP00042096001	gi|118485443|gb|ABK94578.1| unknown [Populus trichocarpa] 45 0.004
XLOC_004284	*Cc01_g13250*	GSCOCP00028289001	gi|225434873|ref|XP_002283159.1| PREDICTED: uncharacterized prot... 279 7e-73
XLOC_000117	*Cc00_g01850*	GSCOCP00002930001	***** No hits found ******
XLOC_004657	*Cc01_g21090*	GSCOCP00005023001	gi|41353548|gb|AAS01337.1| ERF-like transcription factor [Coffea... 629 e-178
XLOC_000114	*Cc00_g01820*	GSCOCP00002920001	***** No hits found ******
XLOC_005721	*Cc01_g20560*	GSCOCP00015918001	gi|359479814|ref|XP_002268578.2| PREDICTED: wee1-like protein ki... 644 0.0
XLOC_005640	*Cc01_g19020*	GSCOCP00016112001	gi|225437154|ref|XP_002280618.1| PREDICTED: heat stress transcri... 432 e-119
XLOC_017497	*Cc05_g02560*	GSCOCP00020936001	gi|297744323|emb|CBI37293.3| unnamed protein product [Vitis vini... 61 8e-08
XLOC_012939	*Cc02_g34640*	GSCOCP00000723001	gi|225450647|ref|XP_002278369.1| PREDICTED: GATA transcription f... 114 5e-24

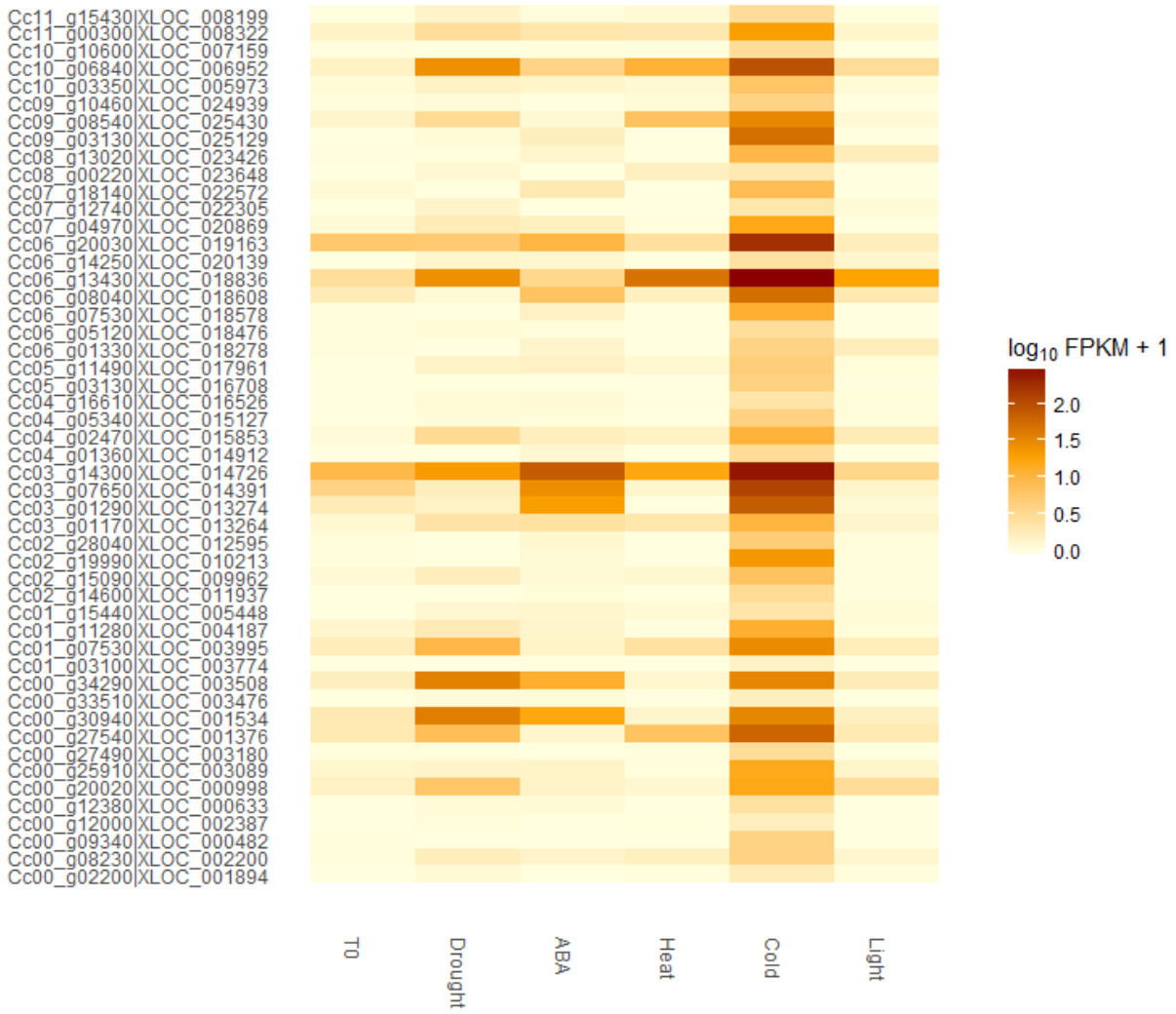

Figure 6 – Heat-map of the expression levels of a set of 50 genes from Cluster 4.

Table 5 – Genes up-regulated upon cold stress (Cluster 4).

	BLAST C. canephora genome	***C. arabica* gene**	**Annotation *C. canephora* genome**
XLOC_025129	*Cc09_g03130*	GSCOCP00017586001	gi|237899560|gb|ACR33100.1| putative tea geometrid larvae-induci... 584 e-164
XLOC_010213	*Cc02_g19990*	GSCOCP00014579001	gi|225429027|ref|XP_002267686.1| PREDICTED: uncharacterized prot... 66 2e-09
XLOC_006952	*Cc10_g06840*	GSCOCP00034605001	gi|225459247|ref|XP_002285762.1| PREDICTED: uncharacterized prot... 452 e-125
XLOC_018578	*Cc06_g07530*	GSCOCP00031250001	gi|356530607|ref|XP_003533872.1| PREDICTED: probable flavin-cont... 674 0.0
XLOC_017961	*Cc05_g11490*	GSCOCP00021309001	gi|147768650|emb|CAN71664.1| hypothetical protein VITISV_011991 ... 294 2e-77
XLOC_023426	*Cc08_g13020*	GSCOCP00030396001	gi|255555789|ref|XP_002518930.1| Glucan endo-1,3-beta-glucosidas... 164 7e-39
XLOC_018836	*Cc06_g13430*	GSCOCP00041399001	gi|401466646|gb|AFP93557.1| xyloglucan endotransglycosylase/hydr... 405 e-111
XLOC_011937	*Cc02_g14600*	GSCOCP00013876001	gi|255563090|ref|XP_002522549.1| conserved hypothetical protein ... 769 0.0
XLOC_016708	*Cc05_g03130*	GSCOCP00033505001	gi|357462217|ref|XP_003601390.1| Endoglucanase [Medicago truncat... 775 0.0
XLOC_015127	*Cc04_g05340*	GSCOCP00022284001	gi|356564882|ref|XP_003550676.1| PREDICTED: AP2-like ethylene-re... 257 1e-66
XLOC_020869	*Cc07_g04970*	GSCOCP00039758001	gi|359488343|ref|XP_002283020.2| PREDICTED: bifunctional dihydro... 439 e-121
XLOC_013274	*Cc03_g01290*	GSCOCP00025735001	gi|359477157|ref|XP_002273790.2| PREDICTED: major allergen Pru a... 207 8e-52
XLOC_020139	*Cc06_g14250*	GSCOCP00041282001	gi|255545130|ref|XP_002513626.1| conserved hypothetical protein ... 163 2e-38
XLOC_015853	*Cc04_g02470*	GSCOCP00021919001	gi|147790299|emb|CAN69978.1| hypothetical protein VITISV_011280 ... 164 8e-38
XLOC_025430	*Cc09_g08540*	GSCOCP00020824001	gi|224152016|ref|XP_002337181.1| predicted protein [Populus tric... 427 e-117
XLOC_022305	*Cc07_g12740*	GSCOCP00036837001	gi|296084381|emb|CBI24769.3| unnamed protein product [Vitis vini... 433 e-119
XLOC_008199	*Cc11_g15430*	GSCOCP00038054001	gi|224102455|ref|XP_002312684.1| predicted protein [Populus tric... 189 2e-46
XLOC_003774	*Cc01_g03100*	GSCOCP00019030001	gi|224119134|ref|XP_002331333.1| cytochrome P450 [Populus tricho... 533 e-149
XLOC_001376	*Cc00_g27540*	GSCOCP00000280001	gi|297802938|ref|XP_002869353.1| hypothetical protein ARALYDRAFT... 52 4e-05
XLOC_018608	*Cc06_g08040*	GSCOCP00031181001	gi|224087088|ref|XP_002308060.1| predicted protein [Populus tric... 142 8e-32
XLOC_014912	*Cc04_g01360*	GSCOCP00021784001	gi|14269343|gb|AAK58023.1|AF377310_1 recombinant myb-like transc... 241 9e-62
XLOC_003476	*Cc00_g33510*	GSCOCP00006255001	gi|297736139|emb|CBI24177.3| unnamed protein product [Vitis vini... 155 1e-35
XLOC_001894	*Cc00_g02200*	GSCOCP00007914001	***** No hits found ******

XLOC_024939	*Cc09_g10460*	GSCOCP00041041001	gi|225428253|ref|XP_002279474.1| PREDICTED: proline-rich recepto... 661 0.0
XLOC_023648	*Cc08_g00220*	GSCOCP00005578001	gi|359475860|ref|XP_003631764.1| PREDICTED: uncharacterized prot... 61 3e-07
XLOC_003508	*Cc00_g34290*	GSCOCP00008238001	gi|225453712|ref|XP_002271138.1| PREDICTED: BON1-associated prot... 171 8e-41
XLOC_012595	*Cc02_g28040*	GSCOCP00025164001	gi|357131100|ref|XP_003567180.1| PREDICTED: protein ULTRAPETALA ... 218 8e-55
XLOC_003089	*Cc00_g25910*	GSCOCP00006243001	gi|419789|pir||S31196 hypothetical protein - potato 315 9e-84
XLOC_000482	*Cc00_g09340*	GSCOCP00008208001	gi|147862409|emb|CAN81911.1| hypothetical protein VITISV_042289 ... 84 9e-15
XLOC_008322	*Cc11_g00300*	GSCOCP00019459001	gi|359489039|ref|XP_003633861.1| PREDICTED: putative vacuolar pr... 152 2e-35
XLOC_014391	*Cc03_g07650*	GSCOCP00026622001	***** No hits found ******
XLOC_002200	*Cc00_g08230*	GSCOCP00003334001	gi|356566878|ref|XP_003551653.1| PREDICTED: uncharacterized prot... 58 7e-07
XLOC_022572	*Cc07_g18140*	GSCOCP00018794001	gi|255552408|ref|XP_002517248.1| Pectinesterase inhibitor, putat... 214 8e-54
XLOC_013264	*Cc03_g01170*	GSCOCP00025722001	gi|225431873|ref|XP_002271623.1| PREDICTED: uncharacterized prot... 272 6e-71
XLOC_004187	*Cc01_g11280*	GSCOCP00024253001	***** No hits found ******
XLOC_016526	*Cc04_g16610*	GSCOCP00035767001	gi|388508792|gb|AFK42462.1| unknown [Lotus japonicus] 118 4e-25
XLOC_005973	*Cc10_g03350*	GSCOCP00024859001	***** No hits found ******
XLOC_003995	*Cc01_g07530*	GSCOCP00009386001	gi|225436011|ref|XP_002273485.1| PREDICTED: uncharacterized prot... 270 3e-70
XLOC_005448	*Cc01_g15440*	GSCOCP00016560001	gi|297745741|emb|CBI15797.3| unnamed protein product [Vitis vini... 262 7e-68
XLOC_019163	*Cc06_g20030*	GSCOCP00022898001	gi|159161160|ref|YP_001542447.1| photosystem I P700 apoprotein A... 159 2e-37
XLOC_000633	*Cc00_g12380*	GSCOCP00013523001	gi|393990627|dbj|BAM28984.1| UDP-glucose glucosyltransferase [Ga... 358 1e-96
XLOC_018278	*Cc06_g01330*	GSCOCP00023348001	gi|77416921|gb|ABA81856.1| unknown [Solanum tuberosum] 82 4e-14
XLOC_001534	*Cc00_g30940*	GSCOCP00000514001	gi|224067852|ref|XP_002302565.1| predicted protein [Populus tric... 123 1e-26
XLOC_007159	*Cc10_g10600*	GSCOCP00036012001	gi|356573167|ref|XP_003554735.1| PREDICTED: protein PIR-like [Gl... 243 6e-63
XLOC_009962	*Cc02_g15090*	GSCOCP00013943001	gi|255556938|ref|XP_002519502.1| Transitional endoplasmic reticu... 1117 0.0
XLOC_014726	*Cc03_g14300*	GSCOCP00031828001	***** No hits found ******
XLOC_018476	*Cc06_g05120*	GSCOCP00043045001	gi|225441006|ref|XP_002283672.1| PREDICTED: glucomannan 4-beta-m... 981 0.0
XLOC_000998	*Cc00_g20020*	GSCOCP00008215001	gi|255545130|ref|XP_002513626.1| conserved hypothetical protein ... 176 3e-42
XLOC_002387	*Cc00_g12000*	GSCOCP00000024001	gi|224145649|ref|XP_002325717.1| nbs-lrr resistance protein [Pop... 277 5e-72
XLOC_003180	*Cc00_g27490*	GSCOCP00007278001	gi|225444918|ref|XP_002279617.1| PREDICTED: uncharacterized prot... 385 e-104

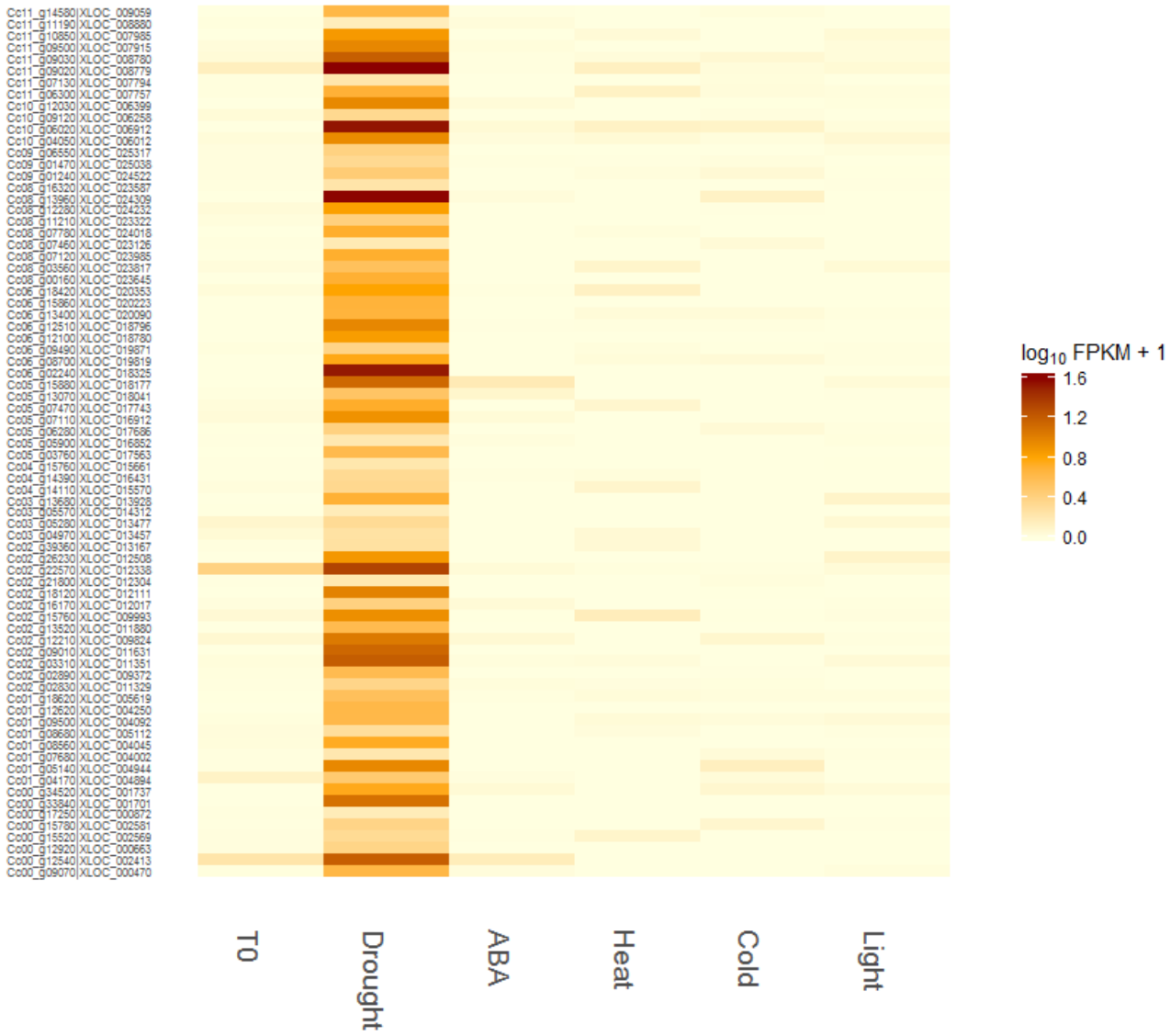

Figure 7 – Heat-map of the expression levels of a set of 50 genes from Cluster 5.

Table 6 – Genes up-regulated upon drought stress (Cluster 5).

	BLAST C. canephora genome	***C. arabica* gene**	**Annotation *C. canephora* genome**
XLOC_004944	*Cc01_g05140*	GSCOCP00011477001	gi|167018103|gb|ABZ05022.1| delta-12 fatty acid desaturase [Davi... 410 e-112
XLOC_005619	*Cc01_g18620*	GSCOCP00016181001	gi|18000070|gb|AAL54886.1|AF092915_1 cytochrome P450-dependent f... 743 0.0
XLOC_011351	*Cc02_g03310*	GSCOCP00020210001	gi|224134715|ref|XP_002321889.1| predicted protein [Populus tric... 83 2e-14
XLOC_017686	*Cc05_g06280*	GSCOCP00042111001	gi|307136044|gb|ADN33896.1| NADH dehydrogenase [Cucumis melo sub... 887 0.0
XLOC_016431	*Cc04_g14390*	GSCOCP00004344001	gi|359491313|ref|XP_002284678.2| PREDICTED: probable receptor-li... 1055 0.0
XLOC_008780	*Cc11_g09030*	GSCOCP00032797001	gi|342306018|dbj|BAK55745.1| UDP-glucose glucosyltransferase [Ga... 777 0.0
XLOC_007915	*Cc11_g09500*	GSCOCP00032712001	***** No hits found ******
XLOC_000470	*Cc00_g09070*	GSCOCP00012320001	gi|225462354|ref|XP_002269543.1| PREDICTED: protein SOMBRERO [Vi... 392 e-107
XLOC_007757	*Cc11_g06300*	GSCOCP00020638001	gi|297737596|emb|CBI26797.3| unnamed protein product [Vitis vini... 429 e-118
XLOC_006012	*Cc10_g04050*	GSCOCP00034984001	gi|255579535|ref|XP_002530610.1| Squalene monooxygenase, putativ... 727 0.0
XLOC_004092	*Cc01_g09500*	GSCOCP00024004001	gi|225436277|ref|XP_002264897.1| PREDICTED: cytochrome P450 94A1... 736 0.0
XLOC_018041	*Cc05_g13070*	GSCOCP00021108001	gi|380751740|gb|AFE56211.1| terpene synthase [Camellia sinensis] 633 e-179
XLOC_008779	*Cc11_g09020*	GSCOCP00032798001	gi|342306018|dbj|BAK55745.1| UDP-glucose glucosyltransferase [Ga... 798 0.0
XLOC_002581	*Cc00_g15780*	GSCOCP00000087001	gi|359488557|ref|XP_003633778.1| PREDICTED: LOW QUALITY PROTEIN:... 696 0.0
XLOC_009372	*Cc02_g02890*	GSCOCP00020158001	gi|22857914|gb|AAL91667.1| pollen specific actin-depolymerizing ... 254 4e-66
XLOC_004045	*Cc01_g08560*	GSCOCP00023884001	gi|225464826|ref|XP_002272164.1| PREDICTED: uncharacterized prot... 62 6e-08
XLOC_016912	*Cc05_g07110*	GSCOCP00042234001	gi|225446345|ref|XP_002272406.1| PREDICTED: GPI-anchored protein... 173 1e-41
XLOC_011329	*Cc02_g02830*	GSCOCP00020152001	gi|224134853|ref|XP_002321921.1| cytochrome P450 [Populus tricho... 729 0.0
XLOC_020353	*Cc06_g18420*	GSCOCP00040457001	gi|376008196|gb|AFB18311.1| F-box protein [Citrus clementina] 187 1e-45
XLOC_017743	*Cc05_g07470*	GSCOCP00042276001	gi|224095515|ref|XP_002310404.1| predicted protein [Populus tric... 484 e-134
XLOC_024232	*Cc08_g12280*	GSCOCP00030503001	gi|1518057|gb|AAB67881.1| membrane channel protein [Solanum tube... 326 3e-87
XLOC_009824	*Cc02_g12210*	GSCOCP00029313001	gi|224126823|ref|XP_002319935.1| predicted protein [Populus tric... 179 7e-43
XLOC_009993	*Cc02_g15760*	GSCOCP00014035001	gi|302142248|emb|CBI19451.3| unnamed protein product [Vitis vini... 328 2e-87

XLOC_015661	*Cc04_g15760*	GSCOCP00042355001	gi|225456647|ref|XP_002270812.1| PREDICTED: probable peptide/nit... 781 0.0
XLOC_016852	*Cc05_g05900*	GSCOCP00035177001	gi|224145550|ref|XP_002325682.1| predicted protein [Populus tric... 236 4e-60
XLOC_024522	*Cc09_g01240*	GSCOCP00017837001	gi|359494848|ref|XP_002267221.2| PREDICTED: subtilisin-like prot... 1156 0.0
XLOC_014312	*Cc03_g05570*	GSCOCP00026316001	gi|2979526|gb|AAC61881.1| 11S storage globulin [Coffea arabica] 689 0.0
XLOC_025317	*Cc09_g06550*	GSCOCP00005106001	gi|255554674|ref|XP_002518375.1| ring finger protein, putative [... 85 1e-14
XLOC_012017	*Cc02_g16170*	GSCOCP00014094001	gi|225462452|ref|XP_002266118.1| PREDICTED: GDSL esterase/lipase... 521 e-145
XLOC_023817	*Cc08_g03560*	GSCOCP00034140001	gi|224105209|ref|XP_002313728.1| predicted protein [Populus tric... 232 3e-59
XLOC_019871	*Cc06_g09490*	GSCOCP00041906001	gi|225445290|ref|XP_002281248.1| PREDICTED: probable sulfate tra... 845 0.0
XLOC_013167	*Cc02_g39360*	GSCOCP00027727001	gi|297745451|emb|CBI40531.3| unnamed protein product [Vitis vini... 244 8e-63
XLOC_012304	*Cc02_g21800*	GSCOCP00014827001	gi|225447860|ref|XP_002268766.1| PREDICTED: LL-diaminopimelate a... 648 0.0
XLOC_004002	*Cc01_g07680*	GSCOCP00015435001	gi|359479927|ref|XP_003632376.1| PREDICTED: subtilisin-like prot... 855 0.0
XLOC_000663	*Cc00_g12920*	GSCOCP00000111001	gi|255571897|ref|XP_002526891.1| serine-threonine protein kinase... 156 5e-36
XLOC_024309	*Cc08_g13960*	GSCOCP00030281001	gi|224122476|ref|XP_002318846.1| AP2/ERF domain-containing trans... 201 1e-49
XLOC_023587	*Cc08_g16320*	GSCOCP00035589001	gi|297742360|emb|CBI34509.3| unnamed protein product [Vitis vini... 852 0.0
XLOC_023322	*Cc08_g11210*	GSCOCP00027117001	gi|255566767|ref|XP_002524367.1| conserved hypothetical protein ... 147 7e-34
XLOC_025038	*Cc09_g01470*	GSCOCP00017805001	gi|359483418|ref|XP_003632954.1| PREDICTED: probable F-box prote... 394 e-107
XLOC_006912	*Cc10_g06020*	GSCOCP00034723001	gi|224093804|ref|XP_002309999.1| predicted protein [Populus tric... 102 4e-20
XLOC_018325	*Cc06_g02240*	GSCOCP00023474001	***** No hits found ******
XLOC_002569	*Cc00_g15520*	GSCOCP00000738001	gi|18324|emb|CAA49162.1| beta-fructofuranosidase [Daucus carota] 848 0.0
XLOC_000872	*Cc00_g17250*	GSCOCP00006075001	gi|85068628|gb|ABC69394.1| CYP71D47v1 [Nicotiana tabacum] 526 e-147
XLOC_015570	*Cc04_g14110*	GSCOCP00035938001	***** No hits found ******
XLOC_023126	*Cc08_g07460*	GSCOCP00025374001	gi|334305730|sp|A6YIH8.1|C7D55_HYOMU RecName: Full=Premnaspirodi... 547 e-153
XLOC_007794	*Cc11_g07130*	GSCOCP00018101001	gi|400750|sp|Q02200.1|PERX_NICSY RecName: Full=Lignin-forming an... 452 e-125
XLOC_002413	*Cc00_g12540*	GSCOCP00002073001	gi|224086034|ref|XP_002307787.1| predicted protein [Populus tric... 116 2e-24
XLOC_005112	*Cc01_g08680*	GSCOCP00023900001	gi|255565739|ref|XP_002523859.1| short chain alcohol dehydrogena... 348 5e-94
XLOC_008880	*Cc11_g11190*	GSCOCP00032476001	gi|224111802|ref|XP_002315983.1| predicted protein [Populus tric... 511 e-143
XLOC_011631	*Cc02_g09010*	GSCOCP00029714001	gi|167600644|gb|ABZ89186.1| putative protein [Coffea canephora] 421 e-116

GO-enrichment analysis

GO enrichment analysis on gene sets was performed with AgriGO. For example, given a set of genes that are up-regulated under certain conditions, an enrichment analysis will find which GO terms are over-represented (or under-represented) using annotations for that gene set.

We used GO annotations for *C. canephora* available at AgriGO to assign each gene to a set of the biological process categories. Cellular process, metabolic process, biological regulation, response to stimulus and establishment of localization were among the most highly represented groups under the biological process category. In addition to these, we cite catalytic activity, binding and transcription regulator activity particularly for the drought condition (Figure 8).

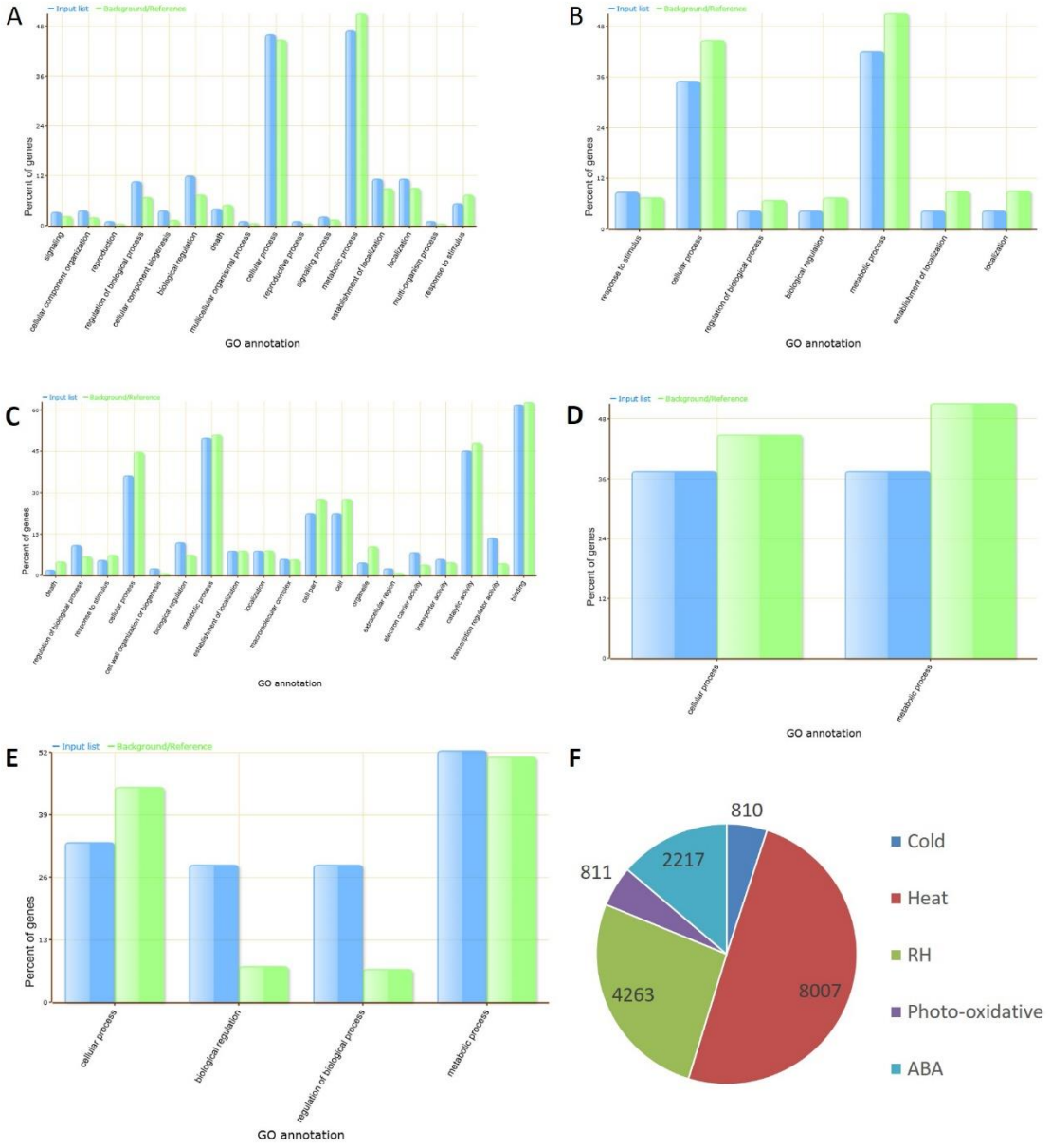

Figure 8 – GO enrichment analysis in *C. arabica* under several abiotic stresses. Genes were classified into different functional groups based on a set in the biological process categories. A) Cold stress B) Heat stress C) Drought stress D) Photo-oxidative stress E) ABA supply stress. F) Representation of the amount of differentially expressed genes (Foldchange ≥ 4) in each stress according to the DESeq2 for the GO enrichment analysis.

Abiotic Stress validation

For each stress, of all differentially expressed genes identified by DESeq2, we chose the four largest foldchange values to validate the transcriptional pattern in *C. arabica* leaves, to ascertain that the applied abiotic stress induced the expression of previously described genes known to respond to the corresponding stress. Results

presented at Figure 9, shows that *Cc00_g10160* and *Cc00_g02570* genes were mostly expressed under cold stress, *Cc02_g22170, Cc10_g00400* and *Cc00_g10880* under heat stress, *Cc00_g07510, Cc00_g34090* and *Cc00_g10160* under drought stress, *Cc00_g10880* and *Cc00_g19080* in response to the photo-oxidative stress, and *Cc00_g10980* and *Cc11_g15020* responded to the ABA supply stress (Figure 9). The functional anotation of each gene corresponding to the *C. canephora* genome is shown in table 7 and shows that known responsive genes are present in this selected set.

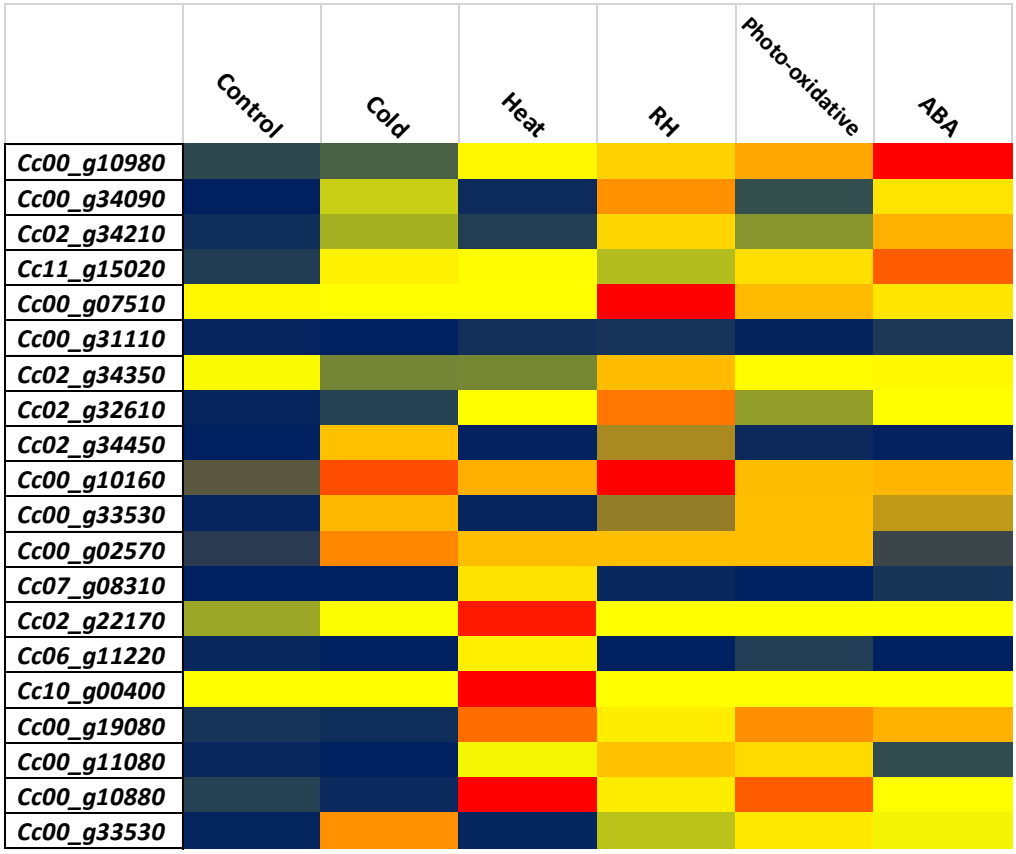

Figure 9 - Representation "heatmap" of the RPKM expression values of the genes stress induced in leaves of *C. arabica* transformed with *CcDREB1D* promoter haplotype 16L. The colors closer to the intense red indicate higher gene expression.

Table 7 - Candidate genes found in arabica coffee plants subject to drought stress, high and low temperatures, high luminous intensity (photo-oxidative stress) and exogenous application of ABA and their corresponding identifications and functions in the genome of *C. canephora.*

C. arabica gene	***BLAST*** ***C. canephora genome***	***Annotation*** ***C. canephora genome***
"GSCOCT00042866001"	*Cc00_g10980*	neutral/alkaline invertase 2
"GSCOCT00039580001"	*Cc00_g34090*	putative calcium-binding protein
"GSCOCT00039726001"	*Cc02_g34210*	No hits
"GSCOCT00041755001"	*Cc11_g15020*	uncharacterized protein
"GSCOCT00037166001"	*Cc00_g07510*	dehydrin DH1a
"GSCOCT00039597001"	*Cc00_g31110*	NAC domain
"GSCOCT00034982001"	*Cc02_g34350*	ABA responsive element-binding protein
"GSCOCT00042862001"	*Cc02_g32610*	galactinol synthase [*Coffea arabica*]
"GSCOCT00041508001"	*Cc02_g34450*	pectinesterase 3
"GSCOCT00034783001"	*Cc00_g10160*	zinc finger DNA-binding protein
"GSCOCT00037003001"	*Cc00_g33530*	hypothetical protein RCOM 00172
"GSCOCT00041399001"	*Cc00_g02570*	xyloglucan endotransglycosylase/hydrolase
"GSCOCT00027106001"	*Cc07_g08310*	cytosolic class II low molecular
"GSCOCT00027060001"	*Cc02_g22170*	heat stress transcription factor A2
"GSCOCT00034510001"	*Cc06_g11220*	small molecular heat shock protein 1
"GSCOCT00015069001"	*Cc10_g00400*	unnamed protein product
"GSCOCT00014441001"	*Cc00_g19080*	uncharacterized protein
"GSCOCT00020190001"	*Cc00_g11080*	uncharacterized protein
"GSCOCT00034975001"	*Cc00_g10880*	unnamed protein product
"GSCOCT00037003001"	*Cc00_g33530*	hypothetical protein RCOM_00172

Futhermore, of the four genes identified *in silico* for the drought condition, two were selected for validation by RT-qPCR: Dehydrin (*Cc00_g07510)* and Galactinol synthase (*Cc02_g32610*), both with fold change> 2. These genes were also analyzed *in vivo* by the RT-qPCR technique to prove the biological functionality of drought stress applied to coffee plants transformed with HP16L haplotype and presented (Figure 10). A very good correlation with the obtained *in silico* data Figure 9.

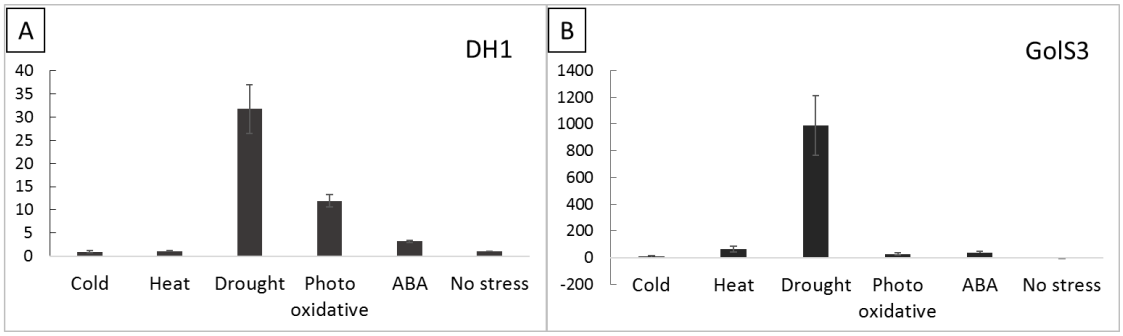

Figure 10 - Validation of *in silico* data by RT-qPCR of Dehydrin- *Cc00_g07510* (A) and Galactinol synthase- *Cc02_g32610* (B) genes. Relative expression values of genes up-regulated in leaves of *C. arabica* by RT-qPCR. Relative quantification of each transcript

in each stress was normalized against *GAPDH*. The condition with the lowest expression (No stress = Control) was used to calibrate the relative value between the stress. *Bars* represent the standard deviation values.

In silico expression of *DREB* subgroup genes

The *DREB1D* mutated promoter (containing haplotype 16) responded to the abiotic stresses applied by expression of the *uidA* gene, which is present in the constructions used to evaluate the response *CcDREB1D* promoter. It is possible to note its greater expression in the cold and drought condition, followed by a lower expression with the exogenous heat and ABA condition. Low expression was observed in the condition of photo oxidative and control, without stress (Figure 11).

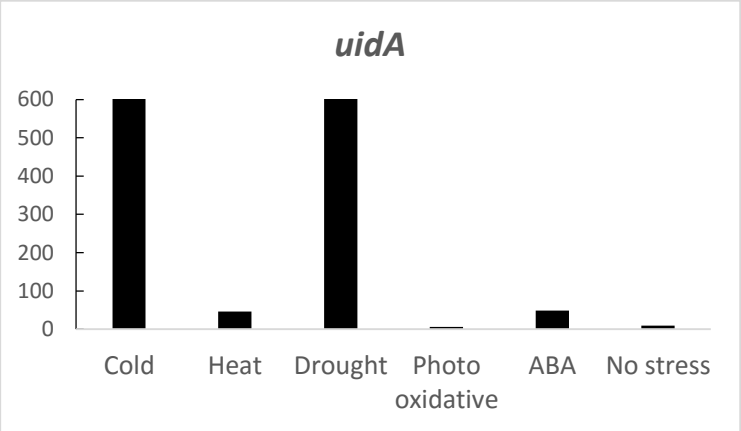

Figure 11 - *In silico* expression in RPKM of the *uidA* gene, present in the *CcDREB1D* promoter, under all stress conditions tested.

In addition to the induced stress genes, a special focus was dedicated to genes belonging to the four subgroups of the *DREB* family and their expression upon the various stresses applied to the plants transformed with the *DREB1D* promoter was analyzed, in order to increase the knowledge about the responses of this family to the abiotic stresses.

The *in silico* expression of the members of each *DREB* subgroup based on their fold change values in each tested stress condition is presented in figure 12.

For subgroup I, the genes *Cc03_g07870, Cc10_g07460* and *Cc05_g06840* were the most expressed in terms of response to all conditions tested, being these genes less related to heat. For cold and drought conditions, these genes had equivalent levels of expression. The other genes belonging to the subgroup, such as the genes *Cc07_g15390* and *Cc08_g15980* were not expressed under all conditions, however, *Cc07_g15390*

presented the highest level of expression, especially for heat, being the only gene with fold change> 2 in this subgroup. The *Cc08_g15980* gene showed expression only in the exogenous ABA condition.

In subgroup II, the genes *Cc10_g09120, Cc10_g04710, Cc08_g07780* and *Cc10_g10960* were the most expressed in terms of response to all conditions tested. The most heat responsive genes were *Cc10_g09120* and *Cc10_g04710*, and the coldest responsive genes, *Cc10_g10960* and *Cc08_g07780*. The *Cc07_g06220* gene did not show any expression, whereas the *Cc06_g12520* gene was not expressed under all conditions, but still had one of the highest expression values for heat. The genes *Cc10_g04710* and *Cc06_g12520* were those that presented fold change> 2 for this subgroup, both being induced under the heat condition.

For genes of subgroup III, the discrepancy of the *Cc08_g09520* gene expressed in the heat condition is remarkable when compared to the other genes. The others had an average equivalent expression for all conditions. The *Cc02_g39490* and *Cc08_g13970* genes showed higher expression under the exogenous ABA condition, while the *Cc06_g10260* and *Cc02_g03420* genes were expressed in the drought and cold condition, respectively. In addition, the *Cc08_g09520* and *Cc08_g13970* genes were also the most expressed in the photo oxidative condition. All of these genes have fold fold> 2 for this subgroup.

For subgroup IV, all genes showed expression in all conditions tested. The highest levels of expression occurred in the genes *Cc06_g16660, Cc06_00780* and *Cc10_g02270* for cold, heat and ABA respectively, and in the *Cc10_g14150* gene for heat, drought and photo oxidative. The genes *Cc06_g16660, Cc06_00780* and *Cc10_g14150* were those that presented fold change ≥2 for this subgroup, being for the cold conditions and the last two genes for heat, respectively.

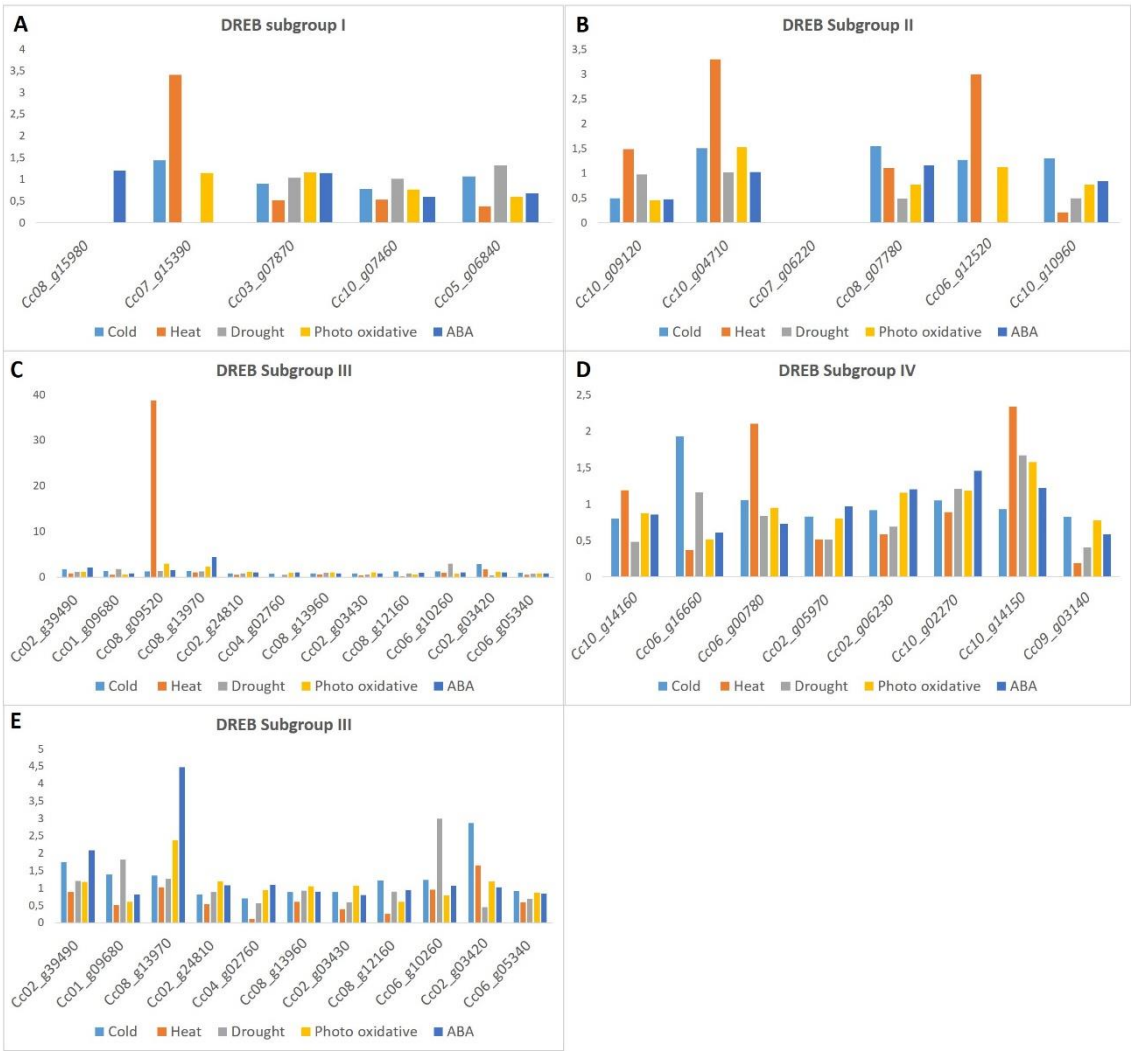

Figure 12 – *In silico* expression of the members of each subgroup *DREB* based on their fold change values in each stress condition tested. (A) Subgroup I (B) Subgroup II (C) Subgroup III (D) Subgroup IV (E) Expression of subgroup III genes without the presence of the gene with the highest expression value of this subgroup (*Cc08_g09520*).

RT-qPCR expression of *DREB* subgroup genes

Subgroup I

Of the five genes of this subgroup, it was possible to analyze the expression of four genes. The gene *Cc07_g15390* is up-regulated by drougt, high luminosity and the exougenous application of ABA. These three stresses also induced the expression of *Cc03_g07870* gene but in this case, high luminosity had the highest effect. For the *Cc10_g07460* gene, the water deficit was the main influencing stress, since it presented the highest expression values for this condition in the RT-qPCR analysis. At the end of

this group, the *Cc05_g06840* gene was highly induced by the drought condition, but also presented some induction for photo oxidative and exogenous application of ABA (Figure 13).

Subgroup II

Of the six genes of this subgroup, it was possible to analyze the expression of five genes. The genes *Cc10_g04710*, *Cc07_g06220 and Cc08_g07780* were induced by all stresses with the highest expression values for heat, drought and cold, respectively. The highest expression values of this subgroup were in the genes *Cc06_g12520* and *Cc10_g10960*. The *Cc06_g12520* gene was highly induced by the drought condition, but also presented induction for cold. This gene showed the highest expression values of this subgroup. For the *Cc10_g10960* gene, heat was the main influencing stress, but also presented some induction for cold (Figure 14).

Subgroup III

Of the 12 genes of this subgroup, it was possible to analyze the expression of five genes. The genes *Cc02_g24810*, *Cc04_g02760* and *Cc08_g09520* were induced by all stresses being more induced by photo-oxidative, heat and drought stresses, respectively. The gene *Cc02_g03430* showed the highest expression values of this subgroup and was highly induced by the drought condition. The gene *Cc02_03420* was highlighted for the drought condition (Figure 15).

Subgroup IV

Of the eight genes of this subgroup, it was possible to analyze the expression of four genes. The genes *Cc10_g14160*, *Cc06_g00780, Cc02_g05970* and *Cc10_g14150* were induced by all stresses being more induced by heat, exogenous application of ABA, drought and heat stresses, respectively. Of the four *DREB* subgroups, this was the one with the lowest expression values (Figure 16).

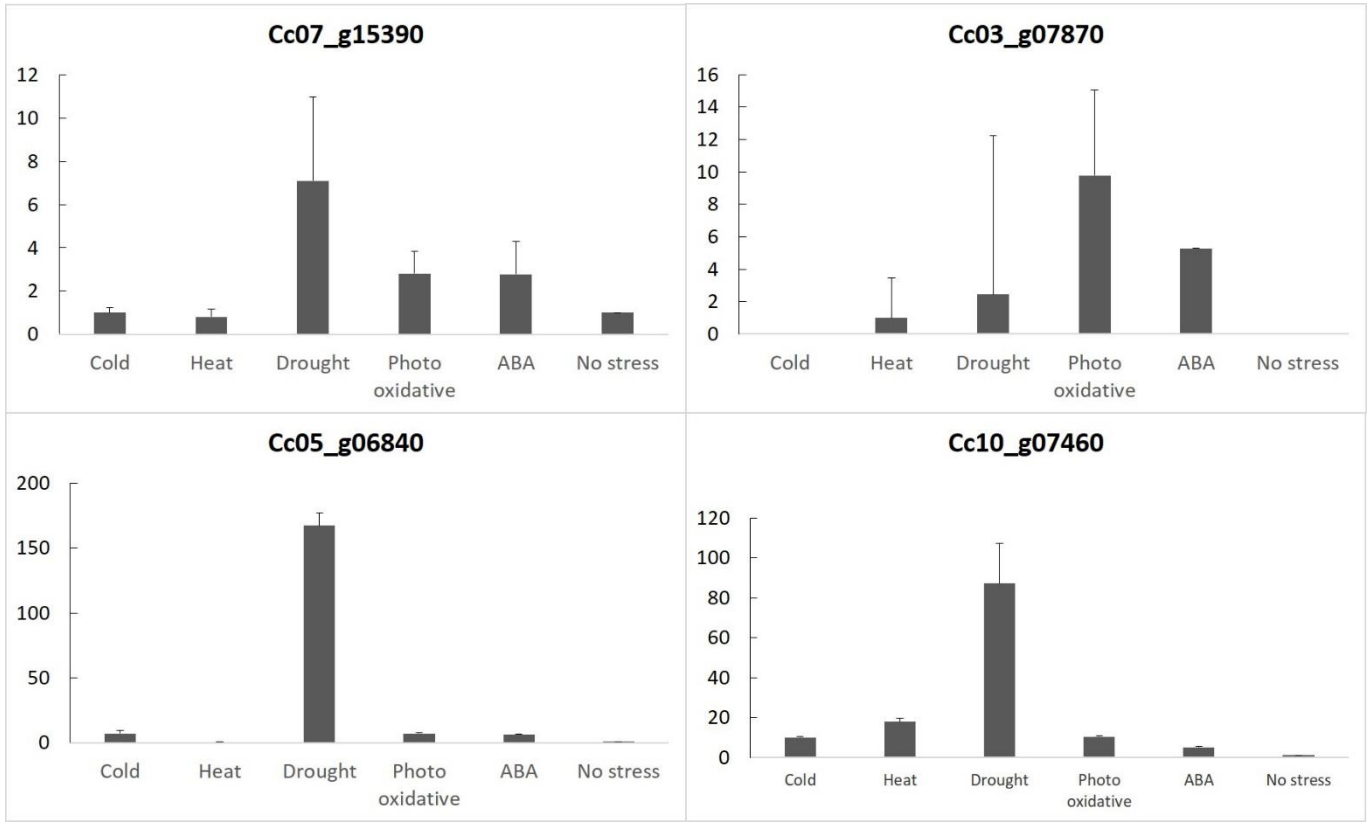

Figure 13 - Relative expression values of *DREB* subgroup I genes up-regulated in leaves of *C. arabica* by RT-qPCR. Relative quantification of each transcript in each stress was normalized against *GAPDH*. The condition with the lowest expression (No stress = Control) was used to calibrate the relative value between the stress. *Bars* represent the standard deviation values.

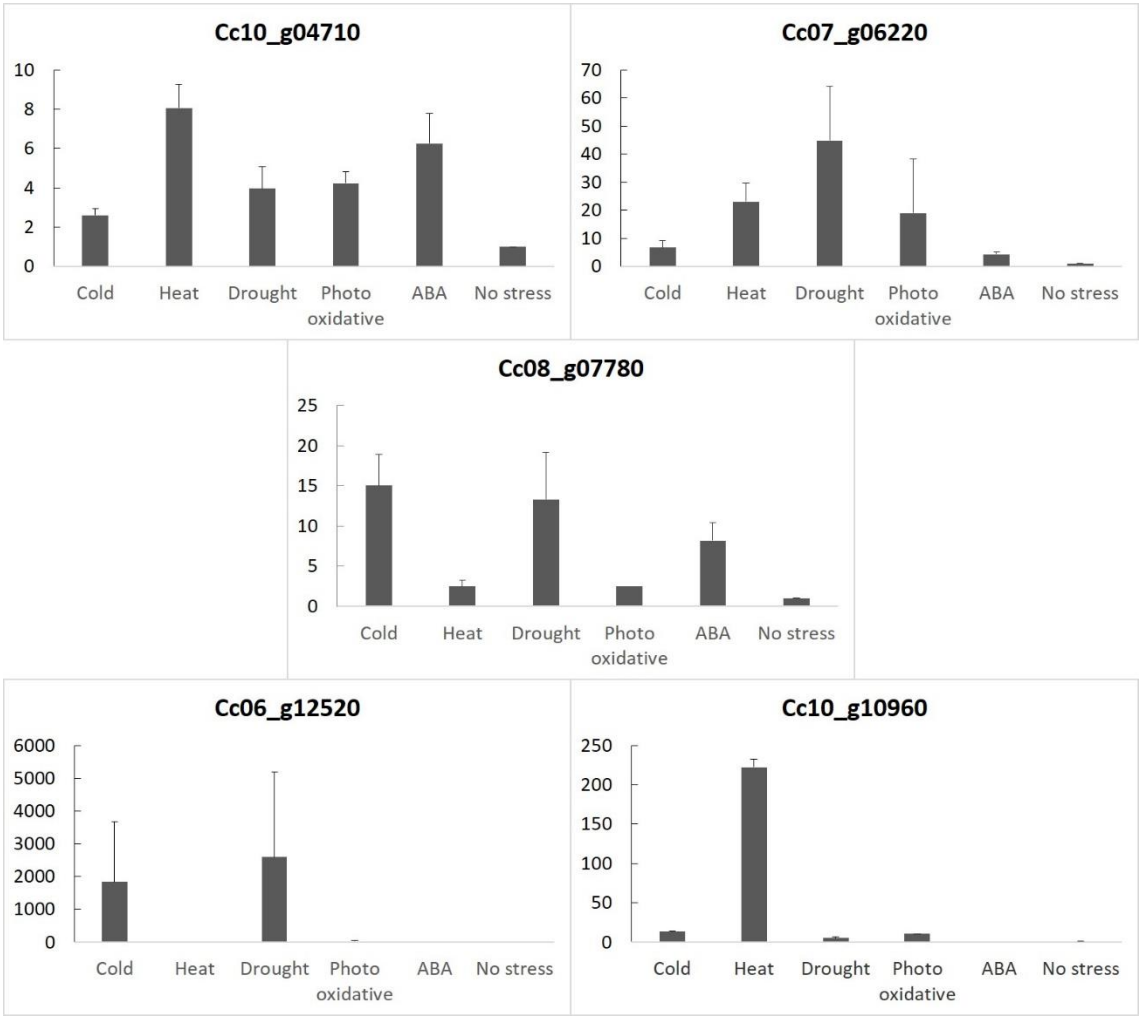

Figure 14 - Relative expression values of *DREB* subgroup II genes up-regulated in leaves of *C. arabica* by RT-qPCR. Relative quantification of each transcript in each stress was normalized against *GAPDH*. The condition with the lowest expression (No stress = Control) was used to calibrate the relative value between the stress. Bars represent the standard deviation values.

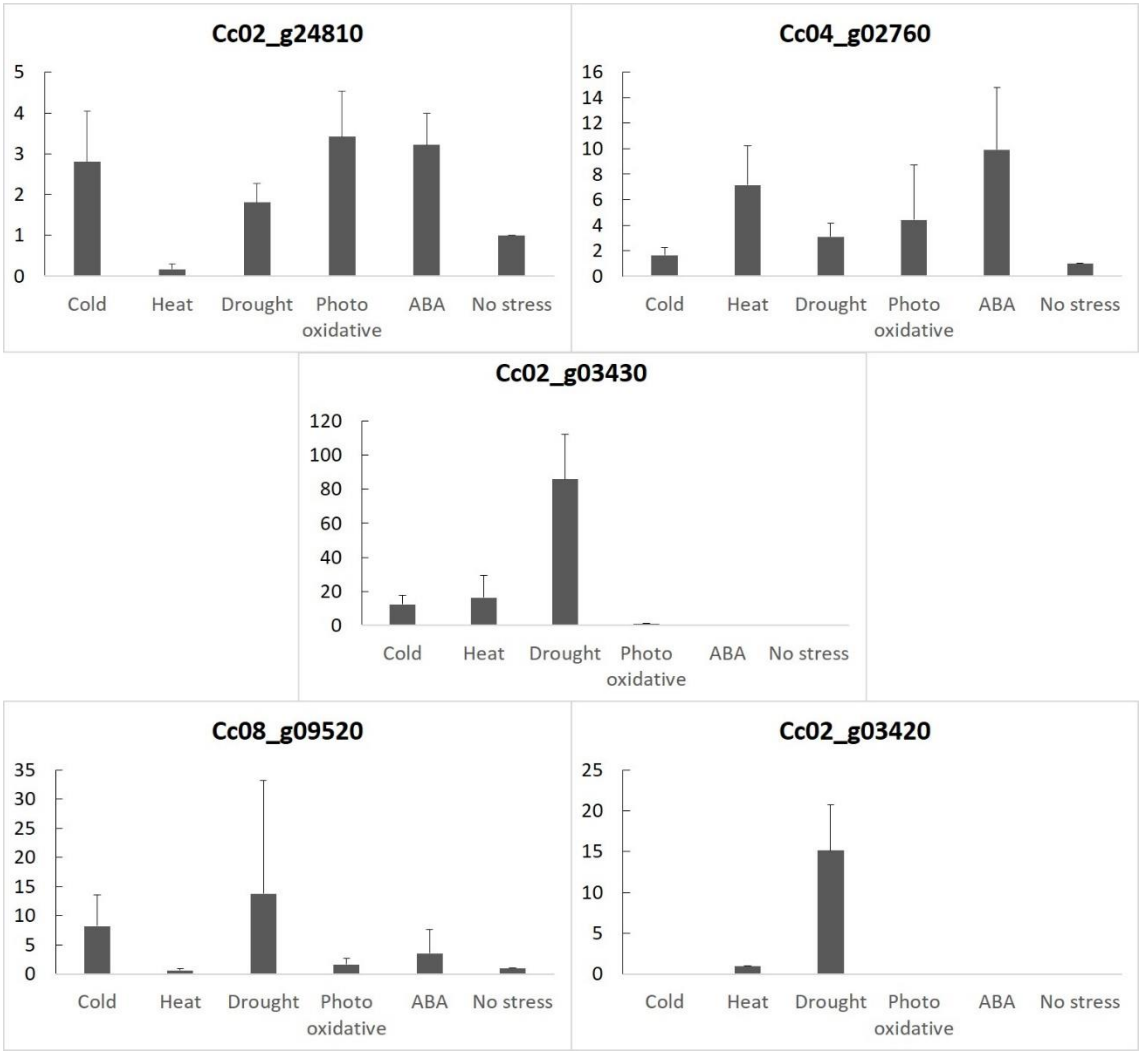

Figure 15 - Relative expression values of *DREB* subgroup III genes up-regulated in leaves of *C. arabica* by RT-qPCR. Relative quantification of each transcript in each stress was normalized against *GAPDH*. The condition with the lowest expression (No stress = Control) was used to calibrate the relative value between the stress. Bars represent the standard deviation values.

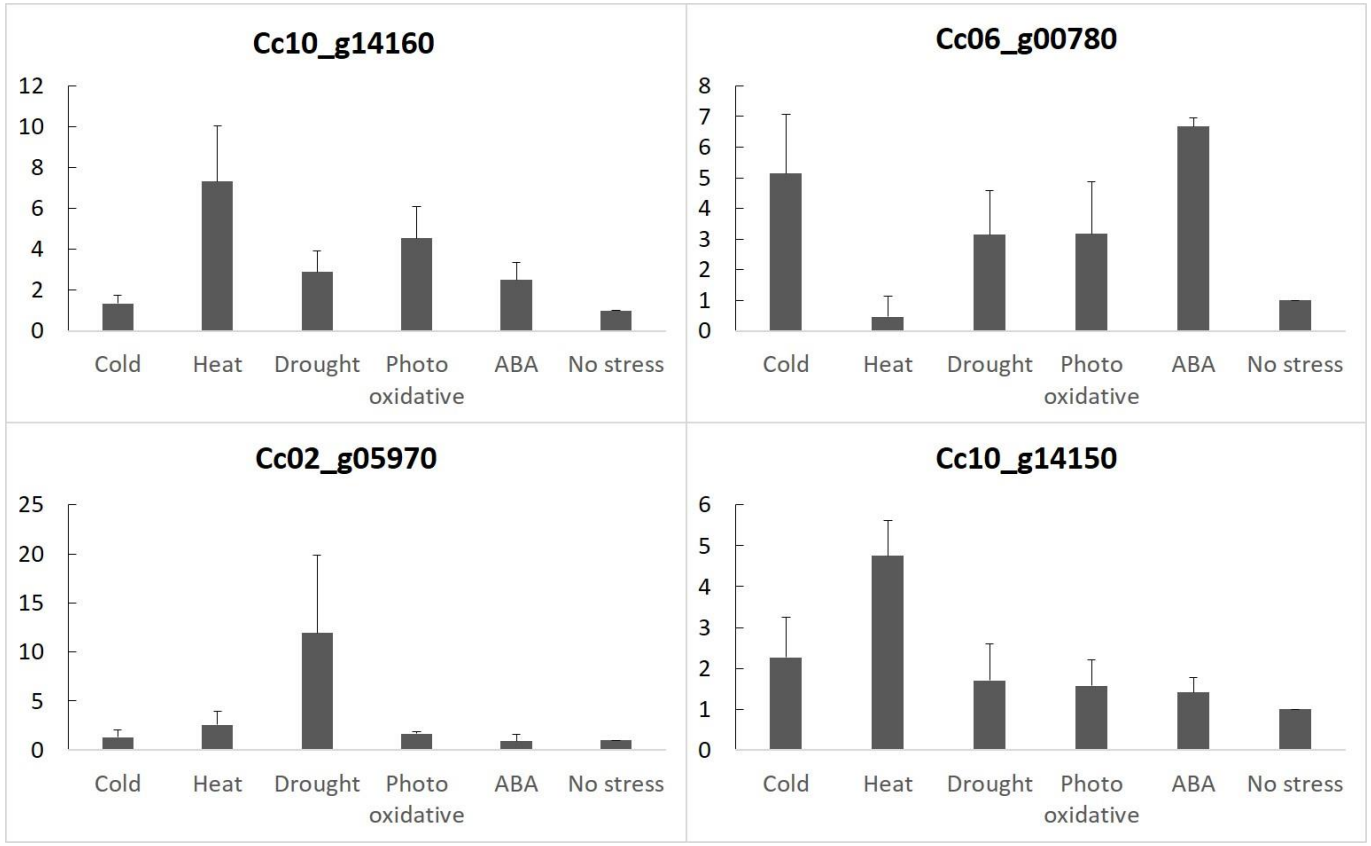

Figure 16 - Relative expression values of *DREB* subgroup IV genes up-regulated in leaves of *C. arabica* by RT-qPCR. Relative quantification of each transcript in each stress was normalized against *GAPDH*. The condition with the lowest expression (No stress = Control) was used to calibrate the relative value between the stress. Bars represent the standard deviation values.

DISCUSSION

Characterization of differentially expressed genes

GO annotations in the *C. arabica* transcriptome found genes associated with metabolic process and cellular process in the biological process category, in commum for all stresses. A previous study in *C. arabica, C. canephora and C. eugenioides* reported similar categories (Mondego et al. 2011). In *C. eugenioides*, GO annotations found genes associated with macromolecule metabolic process and primary metabolic process in the biological process category (Yuyama et al. 2015).

These genes could reflect expression associated with basal processes, thereby reinforcing information also observed in *C. arabica* (Vidal et al. 2010) which identified the contributions of subgenome CaCe in *C. arabica* proteins associated with the citric acid cycle, pentose-phosphate shunt and photosynthesis.

Because the ultimate goal of this study was to identify potential candidates for abiotic stress responses in coffee leaves, some genes were characterized from the comparisons with databases and selected for validation using qPCR. In this study, *Cc00_g07510* presented high expression in leaves and was annotated as a dehydrin (DH1a). Dehydrins (DHNs) play a fundamental role in plant response and adaptation to abiotic stresses and have already been described to be induced by drought stress in coffee (Marraccini et al. 2012). They accumulate typically in maturing seeds or are induced in vegetative tissues following salinity, dehydration, cold and freezing stress. Possible functions for selected plant dehydrins in numerous transgenic studies revealed a positive effect of dehydrin gene expression on plant stress tolerance, mainly drought (and variations) and cold (Hanin et al 2011).

Other gene with a high expression in leaves was *Cc02_g32610,* and annotated as Galactinol synthase. Plant cells use myo-inositol to synthesize a variety of low molecular weight compounds and sugar alcohols such as the galactinol, a key element in the formation of raffinose family oligosaccharides. Nishizawa et al. (2008) found that plants with high galactinol and raffinose contents were less susceptible to oxidative stress. In *C. arabica*, up-regulation of *CaGolS* genes involved in galactinol biosynthesis was reported in leaves of plants subjected to severe drought (dos Santos et al. 2011; Marraccini et al. 2012).

Many of the drought-induced stress genes identified in this work for *C. arabica* were also identified in *C. canephora* in previous studies (Vinecky 2012; Marraccini et al. 2012; Vieira et al. 2013; Moffato et al. 2016).

In that case, the expression of genes encoding glycine-rich proteins, heat shock proteins, dehydrins, ascorbate peroxidase, as well as trans-acting factors (such as *DREB1D*), for example, increased under drought conditions (Mofatto et al. 2016).

The results of the RT-qPCR analysis of the induced stress genes corroborated with data previously obtained with the RNA-seq experiments, in which differential expression was observed for each of the genes tested. These same genes have been reported in the literature with the same function that was identified in this work.

RT-qPCR from the pre-selected *DREB* genes as candidate genes responsive to different stresses, in RNA-Seq experiments

From the analyses of RNA-seq data, a total of 31 genes belonging to the *DREB* gene family were selected for primer-pairs design aiming to perform the RT-qPCR experiments. The results of the RT-qPCR analysis of these genes, responsive to certain abiotic stresses, largely corroborated data previously obtained with the RNA-seq experiments, where for most of the genes tested, differential expression was observed, mainly between the stresses of drought, low and high temperatures.

Genes that did not obtain similar results in the two analyses (*in silico* and *in vivo*) were not totally validated. The genes with very discrepant results have not been validated under any conditions, and are as follows: *Cc06_g12520, Cc07_g06220, cC02_g03420, C02_g05970* and *Cc07_g15390*. The fully validated genes were as follows: Subgroup I - *Cc10_g07460*, Subgroup II - *Cc10_g04710, Cc08_g07780*, Subgroup III - *Cc02_g24810*; Subgroup IV - *Cc10_g14150, Cc10_g14160*.

The genes validated in part, that is, those with similar results for the two analyzes in particular stress, were the following: Subgroup I - *Cc03_g07870* (heat, drought, photo oxidative and ABA), *Cc05_g06840* (drought, photo oxidative, ABA and cold); Subgroup II - *Cc10_g10960* (drought and photo oxidative); Subgroup III - *Cc08_g09520* (cold and drought), *Cc04_g02760* (drought, photo oxidative and ABA), *Cc02_g03430* (cold, heat and drought); Subgroup IV - *Cc06_g00780* (cold, drought, photo oxidative and ABA).

For genes that were not fully validated, we can explain this difference in expression because the gene sequences of the *DREB* subgroups are very similar, so that during the amplification the genes are ringed in very regions similar to those which should have been originally ringed, causing this difference in expression.

DREB genes act under abiotic stress conditions and can be regulated by specific sequences to improve plant fitness under a variety of conditions (Mizoi et al. 2012). This

property of transcription factors, such as *DREB*, makes them an attractive category of genes for the manipulation of abiotic stress tolerance (Akhtar et al. 2012). Although *DREB1/CBF* and *DREB2* are well characterized as the main positive regulators of responses to abiotic stresses, other *DREBs* have been reported to be responsive and to confer tolerance to these stresses (Mizoi et al. 2012).

In vivo and *in silico* expressions of *DREB* subgroup genes

In silico data from leaves of drought-tolerant clones of *C. canephora* submitted to drought conditions showed expression for the gene *Cc03_g07870*, *Cc05_g06840* and *Cc10_g07460* (Reichel 2016), the same results found for arabica, which allows us to state these genes as likely candidates for drought tolerance in the two coffee genotypes.

For arabica, the gene *Cc07_g15390* did not present expression in the condition of water deficit, being the greatest expressions for conditions of low and high temperatures. Similarly for *C. canephora*, this gene was more expressed in the irrigated condition, that is, when it was not in water deficit (Reichel 2016). It is assumed the participation of this gene when in conditions of low and high temperatures.

The *Cc05_g06840* gene presented the highest expression value of this subgroup in both *in silico* and *in vivo* analyzes, which makes this gene a strong candidate for dry tolerance of subgroup I.

In the subgroup II, the *Cc10_g04710* gene was coincident in the two analyzes with the highest expression value in the heat condition, which leads us to consider this gene as a probable candidate for response to high temperatures. For the *Cc08_g07780* gene, higher expression value was found in the low temperature condition, for both analyzes, considering this gene to play a role in such condition.

The gene with the highest *in vivo* expression values of subgroup III was the *Cc02_g03430* gene for the drought condition, and with lower levels of expression, upon cold and heat. This expression in the *in silico* results coincides only in the cold condition. These divergent results were also present for *C. canephora* (Reichel 2016). This gene is also named *DREB1D/CBF4*, and was observed to be highly expressed in *C. canephora* conilon when subjected to drought conditions, being considered as a candidate gene in such condition (Marraccini et al. 2012).

The gene *Cc02_g24810* showed coincident expressions of higher value for the high luminosity condition and exogenous application of ABA for both analyzes, being, therefore, a

probable candidate gene to tolerate these adverse conditions. Another likely candidate gene found was *Cc04_g02760*, for the ABA condition.

Although *DREB1/CBF4* genes are generally induced by the low temperature, there are divergences in relation to these characteristics (Zhou et al. 2010), as it was concluded in this work. Other studies have also reported expression in response to drought, such as Marraccini et al. (2012), where the water deficit increased the expression of *CcDREB1D* in the leaves of Conilon. Futhermore, Alves et al. (2017), investigated the responses of different haplotypes of the *CcDREB1D/*CBF4 promoters of *C. canephora* to PEG and low RH treatments by analyzing their ability to regulate the expression of the *uidA* reporter gene in stably transformed *C. arabica* plants.

In others species, the overexpression of *AtDREB1B* in tomato may increase tolerance to water deficit (Hsieh et al. 2002) and overexpression of rice *OsDREB1F* increases drought tolerance in *Arabidopsis* and rice (Wang et al. 2008).

In the subgroup IV, the *Cc10_g14160* gene was shown to be a probable candidate for tolerance to high temperature and luminosity conditions, as it obtained the highest expression values in both analyzes. In *C. canephora*, this gene presented higher levels of expression in response to drought, when subjected to ceased irrigation conditions (Reichel 2016), being totally different its expression in arabica, in which this gene presented very low expression. Very discrepant differences lead us to assume that the transcriptional activity of this gene under drought conditions is genotype-specific.

The gene with the highest *in vivo* expression value of this subgroup was *Cc02_g05970*, and the most expressed condition was for drought, cold and heat. All conditions allowed expression of the gene, and the results of the two analyzes are not coincident, as was observed in *C. canephora* (Reichel 2016). This reinforces what has already been said about the gene sequences of the *DREB* are very similar, so that during the amplification the genes are ringed in very regions similar to those which should have been originally ringed, causing this difference in expression.

The *DREB* genes belong to a family involved in responses to various abiotic stresses beyond of the drought, such as high salinity and low temperature, for example. Thus, members of different subgroups of *DREB* may play multiple roles in plants, depending on the genotype and conditions tested (Zhou et al. 2012).

In general, the functional differences observed among members of subgroups I to IV may be due to the various target genes that are present in the genome, their distinct

transcriptomic responses and the ability of the transcription factor to activate or repress each target gene (Hussain et al. 2011).

CONCLUSIONS AND PERSPECTIVES

Through the *in silico* and *in vivo* analyzes presented in this work it was possible to select several genes, which are candidates to respond to different stresses in coffee. In addition to the induced stress genes, it was possible to analyze the behavior of the *DREB* genes in their respective subgroups, according to the applied stress.

The identification and characterization of these genes make it possible to carry out further studies, such as analyzing the differential expression between plants grown in the field and in the greenhouse, or prospecting the natural variability using different genetic materials, which enable identification and validation of polymorphisms and specific alleles for the development of molecular markers associated with tolerance to various abiotic stresses.

Meanwhile, further studies on transcriptomic data are recommended to be performed, such as RT-qPCR validation of all induced stress genes and bioinformatics analysis to confirm the existence of crosstalks and allele-specificity expression in Arabica plants submitted to different stresses conditions.

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GENERAL CONCLUSIONS AND PERSPECTIVES

In the last 20 years, considerable amount on basic research of environmental coffee physiology focusing in water relations and drought tolerance mechanisms has been undertaken, but much less research efforts has been devoted to identify responses and tolerance mechanisms to unfavorable temperatures.

Through the *in silico* and *in vivo* analyzes presented in this work it was possible to identify and select several genes, which are putative candidates to respond to low or high temperatures, drought, photo-oxidative and abscisic acid supply stresses in *Coffea arabica*. In addition to the induced stress genes, it was possible to analyze the *DREB* genes behavior in their respective subgroups, according to the applied stress in the coffee plants.

The set of results presented in this thesis provided important insights towards the characterization of the coffee homolog gene *DREB1D* and to induced stress gene in *C. arabica*. It is important to emphasize that our work is the first one realized in coffee to perform a transcriptome analysis of a complete gene family under different stresses, besides in induced stress gene.

To our knowledge, the functional characterization of *CcDREB1D* promoters, performed by stable genetic transformation of *C. arabica*, is the first work to analyze simultaneously homologous *DREB* promoters in a range of stresses and highlight the expression of a *DREB* gene in guard cells and plant tissues. The results presented here clearly detected GUS activity in stomatal guard cells of *CcDREB1D*pro:GUS constructs, mainly for the HP16L haplotype suggesting the existence of an direct pathway to activate *DREB1D* gene specifically in this guard cells and plant tissues. Such results contribute to diversify the range of functional activity reported to *DREB* genes.

For the first time in the literature the identification of candidate genes in *C. arabica* under different conditions was made. The identification and characterization of these genes make it possible to carry out further studies, such the analysis of the differential expression between plants grown in the field and in the greenhouse, or prospecting the natural variability using different genetic materials, which allow identification and validation of polymorphisms and specific alleles for the development of molecular markers associated with tolerance to different abiotic stresses.

Meanwhile, further studies on transcriptomic data are recommended to be performed, such as RT-qPCR validation of all induced stress genes and bioinformatics analysis to confirm

the existence of crosstalks and allele-specificity expression in Arabica plants submitted to different stresses conditions.