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Expression of *DREB*-Like Genes in *Coffea canephora* and *C. arabica* Subjected to Various Types of Abiotic Stress

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Abstract

The aim of this work was to study the regulation of coffee *DREB*-like genes in leaves of *C. arabica* subjected to cold, heat, low relative humidity, exogenous abscisic acid and high light stress, as well as in leaves and roots of drought-tolerant and drought-susceptible clones of *Coffea canephora* subjected to water limitation. In *C. arabica*, *CaERF017* was the most expressed gene under low temperatures and relative humidity, while low humidity and high temperatures up-regulated the expression of *CaERF053* and *CaERF014*, respectively. Under water limitation, *CcDREB1B*, *CcRAP2.4*, *CcERF027*, *CcDREB1D* and *CcTINY* were the most expressed genes mainly in leaves of drought-tolerant *C. canephora*. On the other hand, expression of the *CcERF016*, *CcRAP2.4* and *CcDREB2F* genes was highly up-regulated under water limitation in the roots of drought-susceptible *C. canephora* clone 22. We previously reported fine-tuned regulation of *CcDREB1D* promoter haplotypes (HP15, HP16 and HP17) in transgenic *C. arabica* subjected to low humidity. Here, we investigated the regulation of these haplotypes under high light, cold, heat, and abscisic acid (ABA) stress. In apical buds and leaf guard cells, GUS-stained percentages were higher in pHP16L-transformed plants subjected to low humidity, high light and ABA stress than in pHP17L- and pHP15L-transformed plants. We also reported up-regulated expression of the endogenous *CaDREB1D* gene for both the cold and low humidity in leaves of pHP16L-transformed *C. arabica* suggesting a key role of this gene in controlling the responses of coffee plants to abiotic stress probably through an ABA-dependent pathway.

Keywords Abiotic stress · Abscisic acid - coffee - *DREB* - gene expression - promoter

Key message: *DREB*-like genes are differentially expressed in drought-tolerant and susceptible clones of *C. canephora* subjected to different abiotic stress. In *C. arabica*, both cold, low humidity and ABA up-regulate *CaDREB1D* gene expression.

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Introduction

As for many other plant commodities, climate change is likely to affect coffee production in the coming decades by increasing abiotic stress periods (drought, high temperatures), pests and diseases (Jaramillo et al. 2011), reducing flowering (Imbach et al. 2017), altering plant development (DaMatta and Ramalho 2006), as well as fruit ripening and quality (Silva et al. 2005; Vinecky et al. 2017), thereby making it increasingly difficult to maintain coffee in many traditional coffee regions (Davis et al. 2012; Bunn et al. 2015; Van der Vossen et al. 2015). For these reasons, research on coffee adaptation to abiotic stress is an urgent priority, as is the need for alternative and faster breeding methods (Andrade 2018).

Plants react to biotic and abiotic stress by producing hormones such as abscisic acid (ABA), salicylic acid and jasmonic acid. These hormones promote the expression of transcription factors (TFs), which themselves activate the expression of many genes involved in cell protection, detoxification and repair, among other things (Atkinson and Urwin 2012). Of these TFs, DREB (Dehydration Responsive Element Binding) proteins have been extensively studied because they respond to a variety of stresses (Shinozaki and Yamaguchi-Shinozaki 2007; Khan 2011). Their corresponding genes belong to the AP2/ERF (APETALA 2/ethylene-responsive factor) family characterized by the presence of conserved protein domains used for their classification in higher plants (Sakuma et al. 2002; Nakano et al. 2006; Canella et al. 2010). In terms of their regulation, *DREB* genes are commonly divided into two homologous gene families, *DREB1* and *DREB2*, the first being induced mostly by cold and the second by dehydration, high salinity and heat stress, for example (Shinozaki and Yamaguchi-Shinozaki 2007; Lata and Prasad 2011). Based on the completion of the *C. canephora* genome (Denoeud et al. 2014), and as previously observed for *AtDREB* genes in *Arabidopsis* (Nakano et al. 2006), Alves (2015) recently identified thirty-one coffee *DREB*-like genes phylogenetically divided into four subgroups (I, II, III and IV) containing five, six, twelve and eight genes, respectively (Supplementary Fig. S1).

In order to study the genetic determinism of drought tolerance in coffee, we previously reported the identification of more than eighty candidate genes in different tissues (leaves, roots, plagiotropic buds) of drought-tolerant (D^T) and drought-susceptible (D^S) plants of *C. canephora* (Vinecky et al. 2012; Marraccini et al. 2012; Vieira et al. 2013) and *C. arabica* (Freire et al. 2013; Mofatto et al. 2016) displaying differential expression profiles under drought stress. Among them, *CcDREB1D* (also known as C-repeat-binding factor 4 - CBF4) attracted particular attention because it was highly up-regulated under drought stress in the leaves of D^T clones (mainly in clone 14 and to a lesser extent in clones 73 and 120) of *C. canephora* Conilon, while its expression was barely increased under water limitation in the leaves of D^S clone 22 (Marraccini et al. 2012; Vieira et al. 2013). In higher plants, *DREB1D* gene expression has been reported to be up-regulated by development and several stress conditions, such as drought in *Arabidopsis* (Haake et al. 2002), as well as by low temperatures, drought, and salinity in *Vitis* (Xiao et al. 2008; Zandkarimi et al. 2015) and in *Medicago truncatula* (Li et al. 2011). In several genetically engineered plants, overexpression of the *DREB1D*/*CBF4* gene has been shown to increase tolerance of drought, cold and/or salt (Haake et al. 2002; Li et al. 2011; Guttikonda et al. 2014), highlighting its key role in plant responses to abiotic stress. In coffee, recent studies reported up-regulated expression of the *DREB1D* gene in *C. canephora* leaves subjected to a sharp drop in relative humidity (RH) (Thioune et al. 2017), as well as in *C. arabica* leaves subjected to low RH, and to high and low temperatures (Alves et al. 2018). An in-depth study of the *CcDREB1D* promoters in D^T clone 14 and D^S clone 22 revealed the existence of three haplotypes (HP15, HP16 and HP16) diverging from each other in several single nucleotide polymorphisms (SNPs) and insertions/deletions (INDELs), with HP15 being common to the two clones, while HP16 and HP17 were specific to clones 14 and 22, respectively (Alves et al. 2017). The genetic diversity of *DREB1D* promoters demonstrated that HP15 and HP16 haplotypes clustered in *C. canephora* genetic sub-group 1 (SG1) of the Congolese group, considered highly tolerant to drought, while HP17 was closer to genotypes of Congolese sub-group 2 (SG2) considered as susceptible (Alves et al. 2018). A functional analysis of these haplotype transgenic plants of *C. arabica* var. Caturra subjected to water limitation (mimicked by low RH and polyethylene glycol [PEG] osmotic treatments) also showed that HP16 was able to drive expression of the *uidA* reporter gene in leaf mesophyll and guard cells more strongly and earlier than the HP15 and HP17 haplotypes (Alves et al. 2017). Using the same constructions in transgenic tobacco, up-regulated expression of the *uidA* reporter gene under the control of the HP15 and HP16 haplotypes was observed under cold stress, but not under dehydration and heat shock (de Aquino et al. 2018). The nucleic polymorphism detected between these promoter haplotypes might explain differences in the fine-tuning of their regulation under water limitation in both *C. arabica* and *N. tabacum* transgenic plants.

Based on this information, the main objectives of this work were (i) to study the regulation of previously identified coffee *DREB*-like genes in leaves and roots of D^T and D^S clones of *C. canephora* subjected to water limitation, and in leaves of *C. arabica* subjected to cold, heat, low RH, exogenous ABA and high light stress (mimicked by high irradiance) treatments and (ii) to gain a clearer understanding of the tissue location, regulation and activity of *CcDREB1D* promoter haplotypes by analysing their ability to regulate the expression of the *uidA* reporter gene in *C. arabica* transgenic plants subjected to the similar abiotic stress.

Materials and Methods

DREB-Like Gene Nomenclature

The thirty-one *DREB*-like orthologous genes were previously identified in the *C. canephora* genome and manually edited in the Coffee Genome Hub (http://coffee-genome.org/) (Alves 2015). These genes were divided into four subgroups (SG) named I, II, III and IV (Supplementary Fig. S1 and Table 1): 5 in SG-I (*Cc03_g07870* [*ERF062*], *Cc05_g06840* [*ERF053*], *Cc07_g15390* [*ERF060*], *Cc08_g15980* [*ERF061*] and *Cc10_g07460* [*RAP2.4*]), 6 in SG-II (*Cc06_g12520* [*ERF017*], *Cc07_g06220* [*RAP2.10*], *Cc08_g07780* [*ERF016*], *Cc10_g04710* [*RAP2.1*], *Cc10_g09120* [*ERF013*] and *Cc10_g10960* [*ERF014*]), 12 in SG-III (*Cc01_g09680* [*DREB1C*], *Cc02_g03420* [*ERF027*], *Cc02_g03430* [*DREB1D*], *Cc02_g24810* [*ERF034*], *Cc02_g39490* [*ERF023*], *Cc04_g02760* [*ERF043*], *Cc06_g05340* [*ERF024*], *Cc06_g10260* [*DREB3*], *Cc08_g09520* [*TINY*], *Cc08_g12160* [*ERF039*], *Cc08_g13960* [*DREB1B*] and *Cc08_g13970* [*ERF025*]) and 8 in SG-IV (*Cc02_g05970* [*DREB2A.1*], *Cc02_g06230* [*ABI4*], *Cc06_g00780* [*DREB2C*], *Cc06_g16660* [*DREB2F*], *Cc09_g03140* [*DREB2G*], *Cc10_g02270* [*DREB2D*], *Cc10_g14150* [*DREB2A.2*] and *Cc_10g14160* [*DREB2A.3*]). The nomenclature for *DREB*-like genes uses the *Ca* and *Cc* prefixes for *C. arabica* and *C. canephora*, respectively.

Plant Materials

The drought-tolerant (D^T: 14, 73 and 120) and drought-susceptible (D^S: 22) clones of *C. canephora* Conilon were selected at the Institute for Research and Rural Assistance (Incaper, Vitoria, Espirito Santo, Brazil) (Ferrão et al. 2000), propagated by cuttings and grown (in 2009) in greenhouse (Federal University of Viçosa-UFV, Minas Gerais, Brazil) conditions [25 °C, 70% relative, average mid-day photosynthetic photon flux (PPF) of 900 μmol m^{-2} s^{-1} / sodium lamps] individually in pots of 12 l of a mixture of soil, sand, and manure (3:1:1, *v*/v/v). After 6 months, plants were subjected to water limitation (WL) by water withdrawal (NI) until reaching a Ψ_{pd} (predawn leaf water potential) of −3.0 MPa (Marraccini et al. 2011, 2012; Vieira et al. 2013). For *C. arabica* var. Caturra, young plants (around 4 cm in height and with 4–5 leaf pairs) transformed by the pHP15L, pHP16L, pHP17L, pBI121 (CaMV35S::*uidA*, positive control) and pBI101 (*uidA*-promoterless, negative control) constructions were previously described by Alves et al. (2017) and cultivated in growth chambers (in 2014) at IRD (Institut de recherche pour le développement, Montpellier, France). For each construction and transgenic line, only plants harbouring a single insertion were conserved for further bioassays. Prior to stress, *C. arabica* plants were cultivated in Gerber flasks on MS (Murashige and Skoog 1962) medium in a growth chamber under a 12-h-light/12-h-dark (light from 08:00 am to 08:00 pm) photoperiod (70 μmol m^{-2} s^{-1} photon flux density / LED lamps) at 26 °C and 80% relative humidity (RH), as described by Etienne (2005).

Abiotic Stress Experiments on *C. arabica*

For the low relative humidity (LH), cold (LT, low temperature), heat (HT, high temperature), high light stress (HL) and abscisic acid (ABA) experiments, four plants (one plant per Gerber flask forming an independent replicate) derived from independent transformation events were studied. To minimize possible effects of the circadian clock, all these experiments began at around 10:00 am (after a 2 h light period) and were applied for 12 h, except for the ABA treatment, which was applied for periods of 24 and 48 h. The stress conditions were as follows:

- Low relative humidity assay (LH): the a low (9%) humidity was created using 500 ml of potassium hydroxide (KOH) supersaturate solution poured into the lower compartment of a temporary immersion bioreactor (Matis®, CID Plastiques, France) (Supplementary Fig. S2). Transformed coffee plants were placed in the upper compartment over 55 mm Petri dishes with their upper part exposed to the outside environment and their radicles immersed in MS medium with activated charcoal (1 g l^{-1}) through a small hand-made hole in the Petri dish cover (one plant per Petri dish). To avoid any exchange of water vapour between the MS medium and the atmosphere outside the bioreactor, the hand-made hole was closed with high-vacuum silicone grease (Dow Corning®, Sigma) and the Petri dishes were sealed with plastic film. Batches of 10 plants were incubated in the bioreactor at 9% RH and 26 °C. Leaf samples were harvested at 10:00 pm (after 2 h of dark).
- Cold stress (LT: low temperature): plants were transferred from the growth chamber (26 °C) into a cold chamber at 5 °C without lighting. Leaf samples were harvested after being 12 h in dark.
- Heat stress (HT: high temperature): plants were transferred from the growth chamber (26 °C) to an oven heated to 40 °C without lighting. Leaf samples were harvested after being 12 h in dark.
- High light stress (HL): the light intensity of the growth chamber was suddenly increased from 70 to 200 μM m^{-2} s^{-1} (without a spectrum change). Leaf samples were harvested at 10:00 pm (after 2 h of dark).
- Exogenous abscisic acid (ABA): plants were transferred from the 'M' maturation medium (Etienne 2005) into Gerber flasks containing the same medium supplemented with 10 μM ABA (Sigma Aldrich, St Louis, USA).

Table 1 *DREB*-like gene information and their corresponding primers used for RT-qPCR experiments

N°	*DREB*-SG	Locus ID	Protein	Gene name	GB	Primer names	*DREB* primer sequences	Cc leaf	Cc root	Ca leaf
1	I	*Cc03_g07870*	GSCOCP00033164001	*ERF062*	nd	Cc03g07870-F1	5′ TGCTCAAGGGCTATGATGCA 3′	+	+	-(1)
						Cc03g07870-R1	5′ TCAACGGAATGTGGCTTCCT 3′			
						Cc03g07870-F2	5′ TTCCGGACCTGAAACACCAA 3′	-(1)	-(1)	+
						Cc03g07870-R2	5′ GCAGGCAGGCCTTGTGATAT 3′			
2	I	*Cc05_g06840*	GSCOCP00042199001	*ERF053*	GW488015(3)	Cc05g06840-F1	5′ GGCGAGAATGCTAGGCTCAA 3′	+	+	-(1)
						Cc05g06840-R1	5′ AGGGTCCGAGGCTGATGAAT 3′			
						Cc05g06840-F2	5′ ACCCTCCAACTCCCCATGAC 3′	-(1)	-(1)	+
						Cc05g06840-R2	5′ TGGCAGCTCTGGGATGTACA 3′			
3	I	*Cc07_g15390*	GSCOCP00036481001	*ERF060*	GT005857(3)	Cc07g15390-F1	5′ TCCAAACCTTCGGCATCAAT 3′	+	+	-(1)
					KF743541(4)	Cc07g15390-R1	5′ GAATCGGCCAAGCTTTGACA 3′			
						Cc07g15390-F2	5′ GTTCCAACCACAGCCCACAT 3′	-(1)	-(1)	+
						Cc07g15390-R2	5′ TTGAACCACCACCTGATTGCT 3′			
4	I	*Cc08_g15980*	GSCOCP00035545001	*ERF061*	GT689001(3)	Cc08g15980-F	5′ CTGAAGCAGCAGCGTATGCA 3′	+	+	-(1)
						Cc08g15980-R	5′ TCGCGAAGATTGGGAAAATT 3′			
5	I	*Cc10_g07460*	GSCOCP00034518001	*RAP2.4*	DV696821(3)	Cc10g07460-F	5′ AGGGCTCACTTGGGCTCAAT 3′	+	+	+
					KF743540(4)	Cc10g07460-R	5′ TCGTTCTGGAGGCTGAATCG 3′			
6	II	*Cc06_g12520*	GSCOCP00041527001	*ERF017*	CF589189(3)	Cc06g12520-F	5′ GGTGGTCCAGTTGGAGAGTGA 3′	+	+	+
						Cc06g12520-R	5′ TGGGTGGCACAAAAAAATCA 3′			
7	II	*Cc07_g06220*	GSCOCP00039919001	*RAP2.10*	GW436790(3)	Cc07g06220-F1	5′ TGATGCCGTTCAAAGTTCCA 3′	+	+	-(1)
					KF743542(4)	Cc07g06220-R1	5′ CTGAGTCGGTGTTGCATGGT 3′			
						Cc07g06220-F2	5′ GAGGGAGGTGGAGGAATTGG 3′	-(1)	-(1)	+
						Cc07g06220-R2	5′ TCTAATCTCCGCCACCCATT 3′			
8	II	*Cc08_g07780*	GSCOCP00011870001	*ERF016*	nd	Cc08g07780-F	5′ TGTCTTCGTGGCAAGAATGC 3′	+	+	+
						Cc08g07780-R	5′ GGTTGGCGTATTCAGATGCA 3′			
9	II	*Cc10_g04710*	GSCOCP00034903001	*RAP2.1*	GW446706(3)	Cc10g04710-F1	5′ TTCAACAAACTGCGGTGCAT 3′	+	+	-(1)
						Cc10g04710-R1	5′ CCAGGCGAGGAGGAGTAGAA 3′			
						Cc10g04710-F2	5′ GCCCTTCTGCGAGGCTTAAC 3′	-(1)	-(1)	+
						Cc10g04710-R2	5′ TAGCAGCCGCAGACAAGTCA 3′			
10	II	*Cc10_g09120*	GSCOCP00034294001	*ERF013*	nd	nd	nd	-(2)	-(2)	-(2)
11	II	*Cc10_g10960*	GSCOCP00016651001	*ERF014*	GW430236(3)	Cc10g10960-F	5′ TCACCGGACTCACCATCTCA 3′	+	+	+
						Cc10g10960-R	5′ GGAGGATGATCAGGTGGTGAA 3′			
12	III	*Cc01_g09680*	GSCOCP00024032001	*DREB1C*	nd	nd	nd	-(2)	-(2)	-(2)
13	III	*Cc02_g03420*	GSCOCP00020226001	*ERF02*	DV712429(3)	Cc02g03420-F	5′ ATCACGACGGCATCCTCATT 3′	+	+	+
						Cc02g03420-R	5′ CCAGCCATATGCGCGTAGTT 3′			
14	III	*Cc02_g03430*	GSCOCP00020227001	*DREB1D*	DV712804(3)	Cc02g03430-F	5′ ATGGATGAGGAGGCGGTTTT 3′	+*	+**	+**
					KM281283(4)	Cc02g03430-R	5′ TCATCCCCAACTGCACATTG 3′			
15	III	*Cc02_g24810*	GSCOCP00028837001	*ERF034*	GT001600(3)	Cc02g24810-F1	5′ GCGAAGATCAATGGCAATGA 3′	+	+	-(1)
					KF743543(4)	Cc02g24810-R1	5′ CCCCTGTAGGTCGGATGCTT 3′			
						Cc02g24810-F2	5′ GCCCGATCTTTCCTTTGATG 3′	-(1)	-(1)	+
						Cc02g24810-R2	5′ GATGATGAACCGTGGAAGCA 3′			
16	III	*Cc02_g39490*	GSCOCP00027708001	*ERF023*	nd	nd	nd	-(2)	-(2)	-(2)
17	III	*Cc04_g02760*	GSCOCP00021957001	*ERF043*	GW429993(3)	Cc04g02760-F	5′ CGGCAACAAGCATCCAGTTT 3′	-(1)	-(1)	+
						Cc04g02760-R	5′ GAGCCATATGCGCGATTTTT 3′			
18	III	*Cc06_g05340*	GSCOCP00043080001	*ERF024*	nd	nd	nd	-(2)	-(2)	-(2)
19	III	*Cc06_g10260*	GSCOCP00041808001	*DREB3*	GW442525(3)	nd	nd	-(2)	-(2)	-(2)

Table 1 (continued)

N°	*DREB*-SG	Locus ID	Protein	Gene name	GB	Primer names	*DREB* primer sequences	Cc leaf	Cc root	Ca leaf
20	III	*Cc08_g09520*	GSCOCP00026893001	*TINY*	DV690143(3)	Cc08g09520-F	5′ TCCCCATTATCGCCAAACAC 3′	+	+	+
						Cc08g09520-R	5′ TTGGACTCTGCATCCTGATTGT 3′			
21	III	*Cc08_g12160*	GSCOCP00030518001	*ERF039*	GW468381(3)	nd	nd	-(2)	-(2)	-(2)
22	III	*Cc08_g13960*	GSCOCP00030281001	*DREB1B*	GW469717(3)	Cc08g13960-F	5′ GCCCAAAGAGCCATCAATTC 3′	+	+	-(1)
						Cc08g13960-R	5′ CTTCCTCCCAGCTCGCTTCT 3′			
23	III	*Cc08_g13970*	GSCOCP00030280001	*ERF025*	GT678195(3)	Cc08g13970-F	5′ GGCTTGGCTGGAGAAGATGA 3′	+	+	-(1)
						Cc08g13970-R	5′ GGCCATGTCCACAAGCAAAT 3′			
24	IV	*Cc02_g05970*	GSCOCP00039118001	*DREB2A.1*	GT722424(3)	Cc02g05970-F	5′ GGGCAAATGGGTAGCTGAAA 3′	+	-(1)	+
						Cc02g05970-R	5′ CCTCAACTGCAGTCCCAAATG 3′			
25	IV	*Cc02_g06230*	GSCOCP00039149001	*ABI4*	nd	nd	nd	-(2)	-(2)	-(2)
26	IV	*Cc06_g00780*	GSCOCP00023278001	*DREB2C*	GW446113(3)	Cc06g00780-F	5′ AGCAGCCACCAGAGAACCAA 3′	-(1)	-(1)	+
						Cc06g00780-R	5′ GCACCAACAGGATCGTCGAT 3′			
27	IV	*Cc06_g16660*	GSCOCP00030654001	*DREB2F*	nd	Cc06g16660-F	5′ GCAATCCCAAGAGCGACGTA 3′	+	+	-(1)
						Cc06g16660-R	5′ TGCTCCGCTTCGTCATTATTT 3′			
28	IV	*Cc09_g03140*	GSCOCP00017585001	*DREB2G*	GR995250(3) JQ687375(4)	nd	nd	-(2)	-(2)	-(2)
29	IV	*Cc10_g02270*	GSCOCP00024726001	*DREB2D*	DV684300(3)	nd	nd	-(2)	-(2)	-(2)
30	IV	*Cc10_g14150*	GSCOCP00012002001	*DREB2A.2*	GW441162(3)	Cc10g14150-F	5′ GCACAGCTCAACCTCCCAAA 3′	+	+	+
						Cc10g14150-R	5′ GCAAACTCTGCATGGGAAGTG 3′			
31	IV	*Cc10_g14160*	GSCOCG00012004001	*DREB2A.3*	GT001480(3)	Cc10g14160-F	5′ TGGCACTGCAAGGTCGATAA 3′	+	+	+
						Cc10g14160-R	5′ GCCAAGACAGTGGGAACCAA 3′			

DREB-like genes found in *C. canephora* genome are listed according to their organisation in sub-groups (SG) (Alves, 2015). Locus identity (ID) and protein numbers corresponded to those found in the Coffee Genome Hub (http://coffee-genome.org/). *DREB*-like names were given according to orthologous genes of *Arabidopsis thaliana*, *Solanum lycopersicum* and *Solanum tuberosum*. For expression studies in *C. canephora* (Cc: roots and leaves) and *C. arabica* (Ca: leaves), RT-qPCR experiments (-) were not performed for the following reasons: (1) primer pair with low efficiency and/or specificity in this tissue, (2) none of the primer pairs designed and tested in these tissues/species permitted transcript detection. (+): gene expression successfully determined by RT-qPCR experiments. GB: GenBank accession numbers of homologous ESTs (3) or cDNA sequences (4). *DREB1D* gene expression profiles already reported by (*) Marraccini et al. (2012) and Vieira et al. (2013), and (**) Alves et al. (2018)

β-Glucuronidase (GUS) Staining

Following stress, transgenic coffee plants (four biological replications for each construction) were tested by GUS staining. Leaves, apical buds and roots were immersed in staining solution (100 mM sodium phosphate buffer, pH 7.2, 10 mM sodium ethylenediaminetetraacetic acid, 0.1% Triton X-100, 1 mg ml^{-1} 5-bromo-4-chloro-3-indolyl-D-glucuronic acid [Sigma] and 2.5 mM potassium ferricyanidine), followed by vacuum infiltration for 10 min, incubation at 37 °C for 24 h, then rinsing with 70% ethanol. The GUS-stained samples were fixed in fixative (50% methanol and 10% acetic acid) at 4 °C for 24 h, rinsed with water, 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 sectioning 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 kept 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, the tissues were rinsed with 70% ethanol and assembled on microscope slides. Whole leaves were examined using a DM600 Leica microscope. 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 × 36 mm areas distributed in six pre-delimited leaf zones. For each pHP construction, four leaves from plants of four independent transformation events were sampled for each type of abiotic stress.

RNA Extraction and Real-Time Quantitative PCR Assays

For expression analyses in *C. canephora*, roots and fully expanded leaves (8–15 cm long) from the third or fourth pair from the apex of plagiotropic branches localised in the third upper part of the plant canopy, were collected at daytime (between 10:00 am and noon). Expression analyses in *C. arabica* var. Caturra were performed in pHP16L-transformed plants using well-developed orthotropic leaves (1–2 cm long). All these samples were taken in triplicate, immediately frozen in liquid nitrogen and stored at −80 °C for RNA extraction. Total RNA was extracted from tissues ground in liquid nitrogen and treated as described by Marraccini et al. (2011) for *C. canephora* (root and leaf) and Breitler et al. (2016) for *C. arabica* (leaf). RNA was quantified using a NanoDrop™ 1000 Spectrophotometer (Waltham, MA, USA). For real-time quantitative PCR, the first-strand cDNA was synthesized using 1 µg of total RNA, the ImProm-II Reverse Transcription System and oligo(dT15) according to the manufacturer's recommendations (Promega, Madison, WI, USA). Real-time quantitative PCR (RT-qPCR) experiments were carried out using the protocol recommended for the use of a 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) as previously described by Marraccini et al. (2012). *DREB* primers (Table 1) were designed using Primer Express software (Applied Biosystems) and were preliminarily tested for their specificity and efficiency against a cDNA mixture from roots and leaves. Data were analysed using 7500 Fast Software v2.0.6 (Applied Biosystems) to determine cycle threshold (Ct) values. Gene expression levels were normalized to the expression level of the *CaGAPDH* (de Carvalho et al. 2013) and *CcUBQ10* (Barsalobres-Cavallari et al. 2009) reference genes (Table 2), in *C. arabica* and *C. canephora*, respectively.

Statistics

The significance of expression level differences was evaluated using the pairwise Wilcoxon rank test (nonparametric test).

Table 2 List of primers used in this study. Primer pairs used in RT-qPCR experiments to analyse the expression of the *uidA* (GUS-F/R) and *CaDREB1D* (DREBA09-F/R, Alves et al. 2017) genes in pHP16L-transformed plants of *C. arabica* var. Caturra (see Fig. 8). The primer pair GAPDH-F/R and BUBI-F/R were used to amplify the transcripts of the *CaGAPDH* and *CcUBQ10* used as reference genes in *C. arabica* and *C. canephora*, respectively

Primers	Sequences
GUS-F	5′ GCACTAGCGGGACTTTGCAA 3′
GUS-R	5′ CGCGAAGCGGGTAGATATCA 3′
DREBA09-F	5′ CAATGCCTGCAAAGCCAATTA 3′
DREBA09-R	5′ TTTTCCTGCCTGCACGTTTC 3′
GAPDH-F	5′ TTGAAGGGCGGTGCAAA 3′
GAPDH-R	5′ AACATGGGTGCATCCTTGCT 3′
BUBI-F	5′-AAGACAGCTTCAACAGAGTACAGCAT-3′
BUBI-R	5′-GGCAGGACCTTGGCTGACTATA-3′

Results

Expression of *DREB*-Like Genes in *C. arabica* and *C. canephora* Subjected to Different Types of Abiotic Stress

The expression profiles of *DREB*-like genes were analysed (1) in leaves of *C. arabica* subjected to cold (LT: low temperature), heat (HT: high temperature), low humidity (LH), ABA and high light (HL) treatments and (2) in leaves (L) and roots (R) of drought-tolerant (D^T) and drought-susceptible (D^S) clones of *C. canephora* subjected (NI) or not (I: control) to water limitation (WL).

Whatever the primer pairs tested, we were unable to detect expression of *ERF013*, *DREB1C*, *ERF023*, *ERF024*, *DREB3*, *ERF039*, *ABI4*, *DREB2G* and *DREB2D* genes in either *C. arabica* or *C. canephora* (Table 1). While expressed in *C. canephora*, expression was not detected for *CaERF061*, *CaDREB1B*, *CaERF025* and *CaDREB2F* genes in *C. arabica*. On the contrary, expression of *ERF043* and *DREB2C* genes was observed in *C. arabica* but not in *C. canephora*. For the remaining *DREB*-like genes, the gene expression profiles obtained in *C. arabica* (L) and/or *C. canephora* (L and R) are described below.

- ***Expression of DREB-like genes in C. arabica leaves subjected to cold, heat, low humidity, ABA and high light treatments***

The expression of *DREB*-like genes in leaves of *C. arabica* var. Caturra transformed by pHP16L and subjected to different abiotic stress was analysed by RT-qPCR (Fig. 1). Under stress-free conditions (NS), the expression of all *DREB*-like genes was barely detected. For some

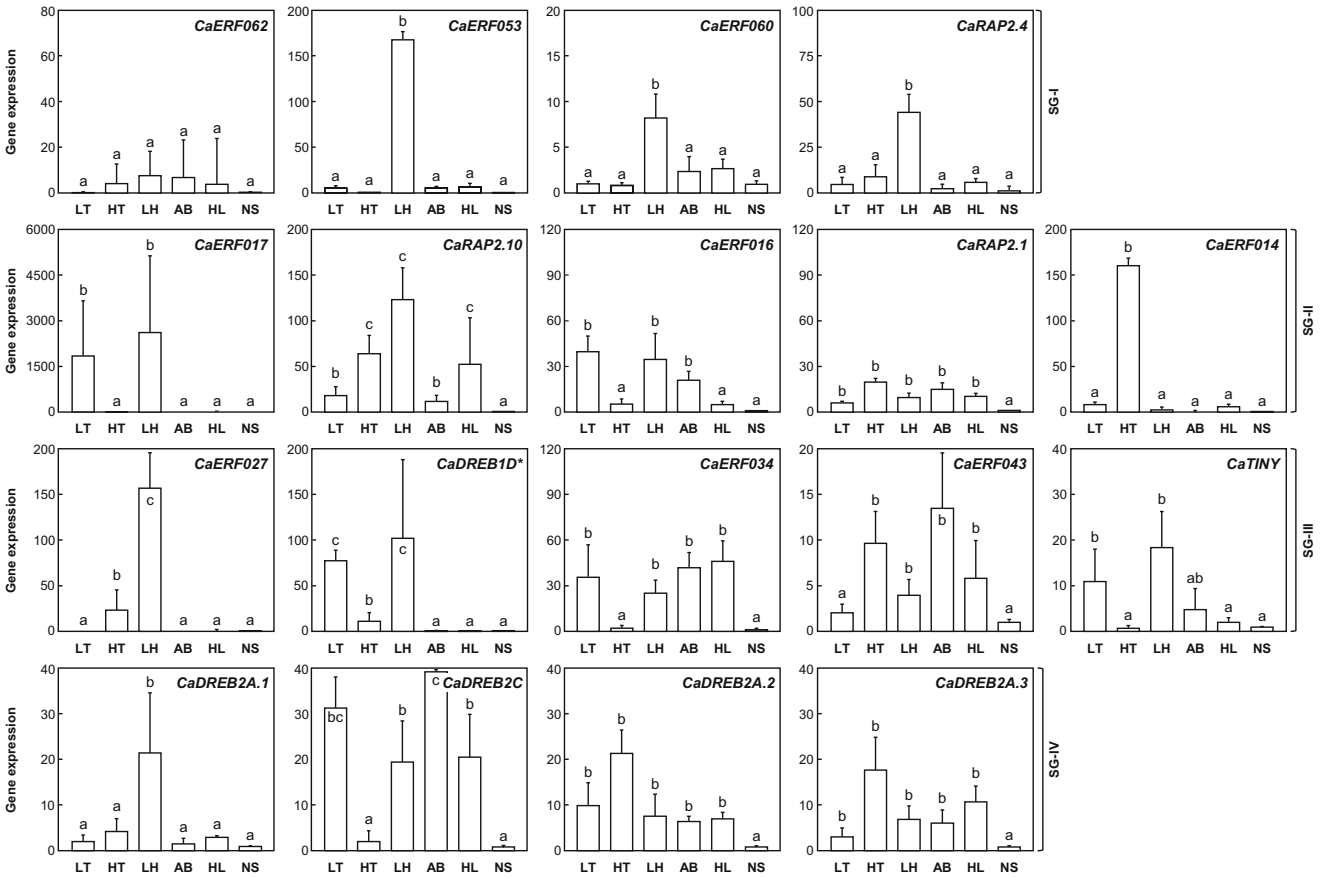

Fig. 1 Expression of *DREB*-like genes in leaves of *C. arabica* var. Caturra transformed by pHP16L and subjected to low (LT) and high (HT) temperatures, low humidity (LH), ABA treatment (AB) and high light stress (HL). Gene names are indicated in the histograms. Expression values corresponding to the mean of three biological and technical replications (± SD) are expressed in fold change relative to the expression level of NS (no stress) sample used as the reference sample (relative expression = 1). *DREB* sub-groups (SG) are indicated. Transcript abundances were normalized using the expression of the *CaGAPDH* gene as the endogenous control. (*): the *CaDREB1D* expression profile, already published by Alves et al. (2018), is shown in order to be compared with expression profiles of other *DREB*-like genes. Treatments sharing the same letter are not significantly different

genes, expression was up-regulated by a unique stress. This was the case of SG-I genes *CaERF053,CaERF060*, *CaRAP2.4* and SG-III gene *CaDREB2A.1* which were up-regulated specifically by LH treatment and of *CaERF014* gene specifically up-regulated by HT. *CaERF027* was the unique gene up-regulated by both LH (highly) and HT (moderately). LT and LH conditions highly up-regulated *CaERF017* that presented the highest levels of gene expression. In addition to LT and LH treatments, *CaDREB1D* and *CaTINY* genes were also weakly up-regulated by HT and ABA, respectively. Regarding the genes moderately induced by stress, *CaRAP2.10* was up-regulated by HT, LH and HL treatments, *CaERF016* by LT, LH and ABA, and *CaERF034* by LT, LH, ABA and HL. The expression profiles of remaining genes were considered as weakly up-regulated by the different abiotic treatments.

- ***Expression of DREB-like genes in C. canephora subjected to water limitation***

Expression of *DREB*-like genes was analysed independently in leaves and roots of drought-tolerant (D^T) clones 14, 73 and 120, and drought- susceptible (D^S) clone of *C. canephora* subjected (NI) or not (I) to water limitation (WL).

In the leaves of *C. canephora* clones, the genes most expressed were *CcDREB1B* and *CcTINY*, which were up-regulated under WL conditions in both D^T and D^S clones (Fig. 2). However, WL up-regulated the expression of *CcERF053*, *CcRAP2.4*, *CcERF017*, *CcERF014*, *CcERF027* and *CcDREB1D* specifically in leaves of all D^T clones but not in those of D^S clone 22. An inverse situation was observed for *CcERF062* for which its expression was higher in clone 22 than in the D^T clones under WL. Water limitation also up-regulated the expression of *CcRAP2.10* and *CcERF014* genes in leaves of D^T clone 14 but not in those of D^T clones 73 and 120. For the remaining genes, expression profiles did not undergo significant variations by WL.

In roots, *CcRAP2.4*, *CcERF016* and *CcDREB2F* were the most overexpressed *DREB*-like genes under WL (Fig. 3). Under this condition, it is worth noting that the expression of these genes was weak (or even undetectable) in all the D^T clones, but greatly and specifically induced in D^S clone 22. Water limitation also up-regulated the expression of *CcERF061* in both D^T and D^S clones, *CcTINY* in D^T clone 14, and *CcDREB2A.2* in D^T clone 120. On the other hand, and whatever the clones, gene expression was considered as not significantly altered by WL for all remaining genes.

GUS Enzymatic Activity Regulated by the *DREB1D* Promoter Haplotypes under Different Types of Abiotic Stress

Regulation of the HP15, HP16 and HP17 promoter haplotypes of the *CcDREB1D* gene was studied by analysing GUS enzyme activity in leaves, apical buds and roots of *C. arabica* transgenic plants subjected to different types of abiotic stress (Fig. 4). Whatever the stress applied, strong GUS staining was observed in the leaves and apical buds of the pBI121-transformed coffee plants used as a positive control (Supplementary Fig. S3). In contrast, and whatever the abiotic stress conditions applied, GUS activity was not detected in pBI101-transformed coffee plants (negative control) as well as in roots of the three *CcDREB1D* promoter haplotypes (Supplementary Table S1).

- ***Low relative humidity***

Faint GUS staining was observed around the secondary veins of leaves of pHP15L- (Fig. 4 A1) and pHP16L-transformed coffee plants (Fig. 4 A3) subjected to LH conditions. Under higher magnification, several GUS-stained guard cells were also seen in the leaves of these two transgenic lines (Fig. 4 A7 and A9). However, GUS activities were not detected in the leaves of pHP17L-transformed coffee plants (Figs. 4 A5 and A11). In apical buds, GUS staining was stronger in pHP16L-transformed plants (Fig. 4 A4 and A10) than in those of plants transformed by pHP15L (Fig. 4 A2 and A8). However, no GUS staining was observed in apical buds of pHP17L-transformed plants (Fig. 4 A6 and A12).

- ***Cold stress***

No GUS staining was observed in the leaves of plants transformed by pHP15L (Fig. 4 B1 and B7) and pHP16L (Fig. 4 B3 and B9) subjected to a low temperature. Conversely, weak GUS activity was observed in the leaves of pHP17L-transformed coffee plants (Fig. 4 B5 and B11). Faint GUS staining was observed in the apical buds of plants transformed by pHP15L (Fig. 4 B2 and B8) and pHP17L (Fig. 4 B6 and B12), while moderate GUS staining was noticed in pHP16L-transformed plants (Fig. 4 B4 and B10).

- ***Heat stress***

Whatever the *CcDREB1D* promoter haplotype, no GUS expression was seen in either the leaves (Fig. 4 C1, C3, C5, C7, C9 and C11) or the roots of transformed plants subjected to heat stress (Supplementary Table 1). Moderate GUS staining was observed in the apical buds of plants transformed by pHP15L (Fig. 4 C2 and C8) and pHP16L (Fig. 4 C4 and C10).

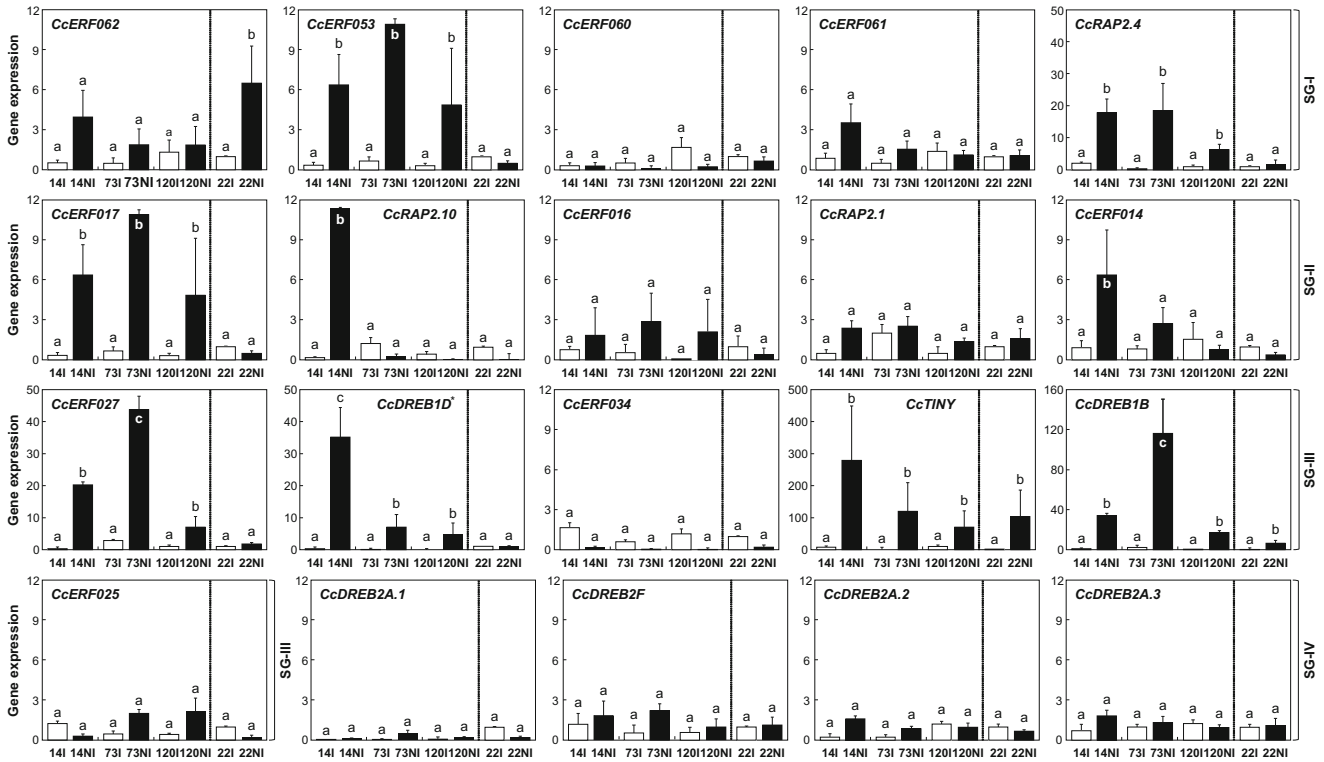

Fig. 2 Expression profiles of *DREB*-like genes in leaves of D^T (14, 73 and 120) and D^S (22) clones of *C. canephora* Conilon subjected to water limitation. I: irrigation (white isobars). NI (not irrigated): water limitation (black isobars). The D^T and D^S clones are separated by a vertical dotted line. Gene names are indicated in the histograms. Expression values corresponding to the mean of three biological and technical replications (± SD) are expressed in fold change relative to the expression level of the sample 22I (D^S irrigated) as the reference sample (relative expression = 1). *DREB* sub-groups (SG) are indicated. Transcript abundances were normalized using the expression of the *CcUBQ10* gene as the endogenous control. (*): the *CcDREB1D* expression profile, already published by Marraccini et al. (2012) and Vieira et al. (2013), is shown in order to be compared with expression profiles of other genes. Treatments sharing the same letter are not significantly different

Compared to these haplotype responses, higher GUS staining was detected in the apical buds of pHP17L-transformed plants (Fig. 4 C6 and C12).

- ***ABA assay***

After 24 h of ABA treatment, faint GUS staining was systematically observed in the leaves of coffee plants transformed by pHP15L (Fig. 4 D1 and D7), pHP16L (Fig. 4 D3 and D9) and pHP17L (Fig. 4 D5 and D11). GUS staining was considered as weak, medium and strong in the apical buds of plants transformed by pHP15L (Fig. 4 D2 and D8), pHP16L (Fig. 4 D4 and D10) and pHP17L (Fig. 4 D6 and D12), respectively. Strong GUS staining was also observed in the roots of pHP15L-transformed plants (Fig. 5a and b), whereas no GUS activity was detected in the roots of plants transformed by the pHP16L and pHP17L constructions (Supplementary Fig. S3 and Supplementary Table 1). After 48 h of ABA treatment, GUS staining was considered as weak in the leaves of pHP16L-transformed plants (Fig. 4 E3 and E9) and as moderate in the leaves of pHP15L-transformed plants (Fig. 4 E1 and E7). In the apical buds, moderate and strong GUS activity was observed in plants transformed by pHP15L (Fig. 4 E2 and E8) and pHP16L (Fig. 4 E4 and E10), respectively. However, no GUS staining was detected in the pHP17L-transformed plants (Fig. 4 E5, E6, E11 and E12).

- ***High light stress***

Under high light (HL) conditions, intense GUS staining was observed in apical buds, particularly in plants transformed by pHP16L (Fig. 4 F4 and F10) and pHP17L (Fig. 4 F6 and F12) and to a lesser extent, in those transformed by pHP15L (Fig. 4 F2 and F8). Weak GUS staining was also observed in leaves of plants transformed by pHP15L (Fig. 4 F1 and F7) and pHP17L (Fig. 4 F5 and F11), whereas no GUS activity was observed in the leaves of pHP16L-transformed plants (Fig. 4 F3 and F9).

Proportion of GUS-Stained Guard Cells in Leaves of *C. arabica* Subjected to Different Types of Abiotic Stress

In order to assess how *CcDREB1D* promoter haplotypes were regulated, the activity of these sequences was evaluated by

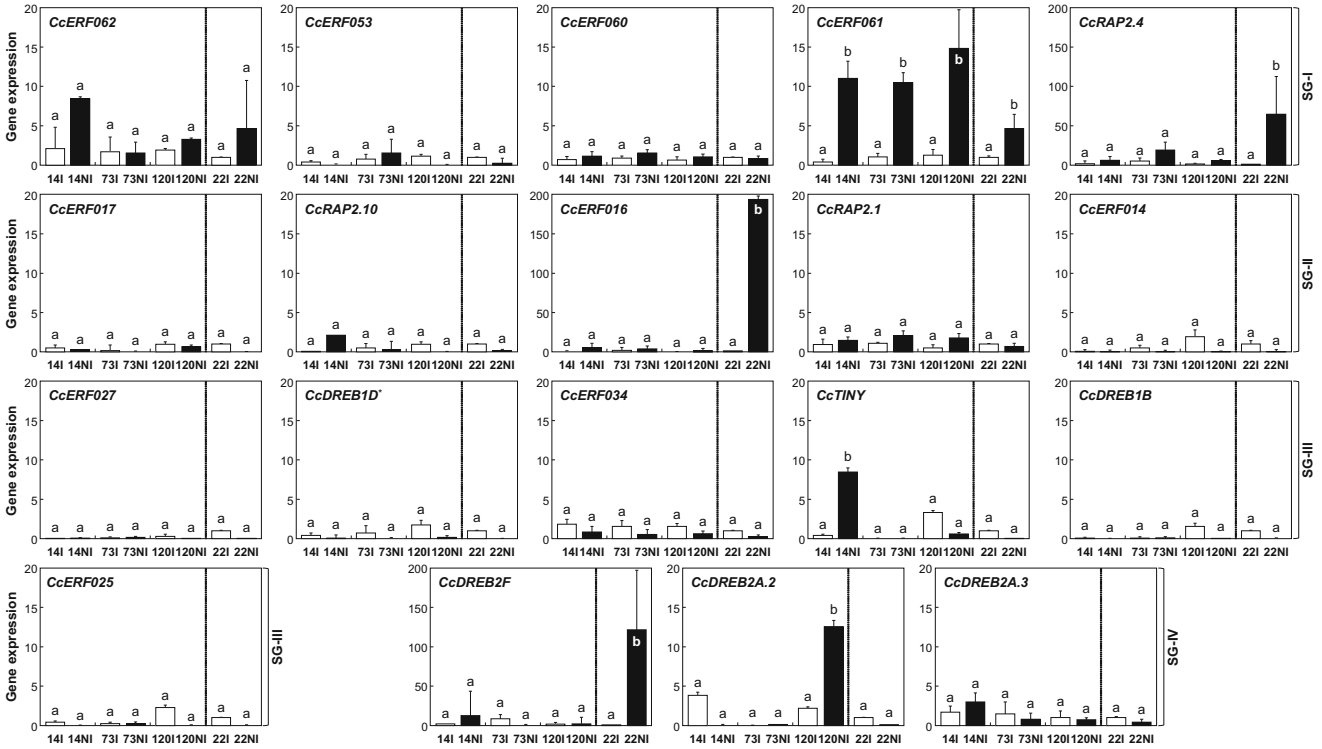

Fig. 3 Expression profiles of *DREB*-like genes in roots of D^T (14, 73 and 120) and D^S (22) clones of *C. canephora* Conilon subjected to water limitation. I: irrigation (white isobars). NI (not irrigated): water limitation (black isobars). The legend corresponds to that described in Fig. 2. (*): the *CcDREB1D* expression profile, already published by Alves et al. (2018), is shown in order to be compared with expression profiles of other genes. Treatments sharing the same letter are not significantly different

analysing the proportion of GUS-stained guard cells in the leaf abaxial regions of pHP-transformed coffee plants subjected to different types of abiotic stress. As expected, a large proportion of GUS-stained guard cells was observed in the leaves of pBI121-transformed coffee plants (positive control), for example under LH condition (Fig. 6a) but also under all other abiotic stress tested and stress-free conditions (Supplementary Fig. S3). On the other hand, no GUS-stained guard cells were observed in pBI101-transformed plants, in untransformed (WT) coffee plants subjected to LH conditions (negative controls, Fig. 6b and c), as well as in pHP-transformed plants under stress-free conditions (Supplementary Table S1).

However, GUS-stained guard cells were observed in the leaves of coffee plants transformed by pHP15L (Fig. 6d) and pHP16L (Fig. 6e) subjected to LH as well as in those of pHP16L-transformed plants subjected to 48-h ABA (Fig. 6f) treatments, for example. In pHP16L-transformed plants, the proportion of GUS-stained guard cells was higher under LH than under ABA treatments. In addition, the proportion of GUS-stained guard cells under LH was also higher in pHP16L- than in pHP15L-transformed plants. Photographs of blue-stained guard cells observed for the cold (Fig. 6g) and high light (Fig. 6h) treatments in pHP17L-transformed plants are also provided as examples of observed GUS staining.

The proportions of GUS-stained guard cells were then measured for each construction and stress condition (Fig. 7). The highest proportion of GUS-stained guard cells was observed in pHP16L-transformed plants, particularly for the low humidity (± 47%), and 24 and 48 h ABA treatments (± 20%), but also to a lesser extent for the high light and cold treatments (< 7%). In pHP17L-transformed plants, the largest proportions of GUS-stained guard cells were observed for the cold (13%) and high light (8%) treatments. On the other hand, and whatever the stress conditions, the proportion of GUS-stained cells was always relatively low (< 6%) in pHP15L-transformed plants. As a positive control, the proportion of GUS-stained guard cells was always large (> 80%) in pBI121-transformed plants, whereas no GUS-stained guard cells were detected in pBI101-transformed plants.

Fig. 4 Histochemical location of GUS activity in transgenic plants of *C. arabica* var. Caturra transformed independently by *CcDREB1D* promoter haplotype constructions, called pHP15L, pHP16L and pHP17L, and subjected to low humidity (A), cold (B), heat (C), ABA (D, 24 h; E, 48 h), and high light (E) stress treatments. For each construction and stress condition, GUS staining of leaves (even numbers) and apical buds (odd numbers) was analysed by a 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 longitudinal- or cross-sections of the organs (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


pHP15L
pHP16L
pHP17L
Low humidity (A)
Cold (B)
Heat (C)
ABA 24h (D)
ABA 48h (E)
High light (F)
1
2
3
4
5
6
7
8
9
10
11
12

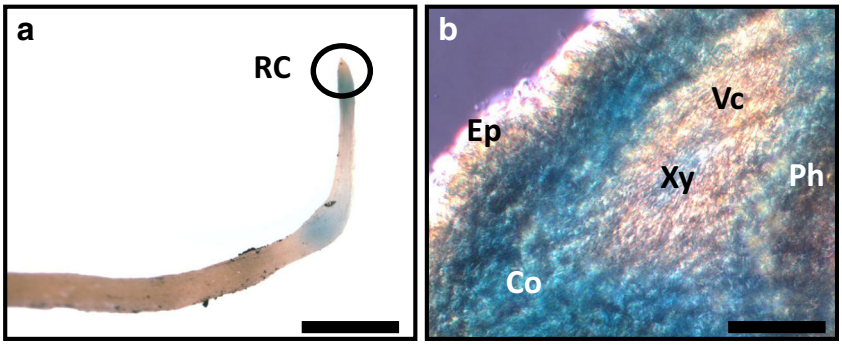

Fig. 5 Histochemical location of GUS activity in roots of *C. arabica* plants transformed by the *CcDREB1D* promoter haplotype pHP15L and subjected to the ABA for a period of 24 h. **a** Binocular loupe image (bar = 1.5 mm). **b** Bright field microscopy of root tissues in cross-section (bar = 40 μm). Tissue abbreviations: Rc: root cap; Ep: epidermis; Co: cortex; Xy: xylem; Ph: phloem; Vc: vascular cylinder

Expression of *uidA* and Endogenous *CaDREB1D* Genes

Since the largest proportions of GUS-stained guard cells were observed in pHP16L-transformed plants, these plants were used to check expression in the leaves of the *uidA* reporter gene and *CaDREB1D* endogenous gene for the cold (LT), heat (HT), low humidity (LH), high light (HL) and 24-h ABA treatments by RT-qPCR experiments (Fig. 8). Under stress-free (NS) conditions, *uidA* and *CaDREB1D* transcripts were

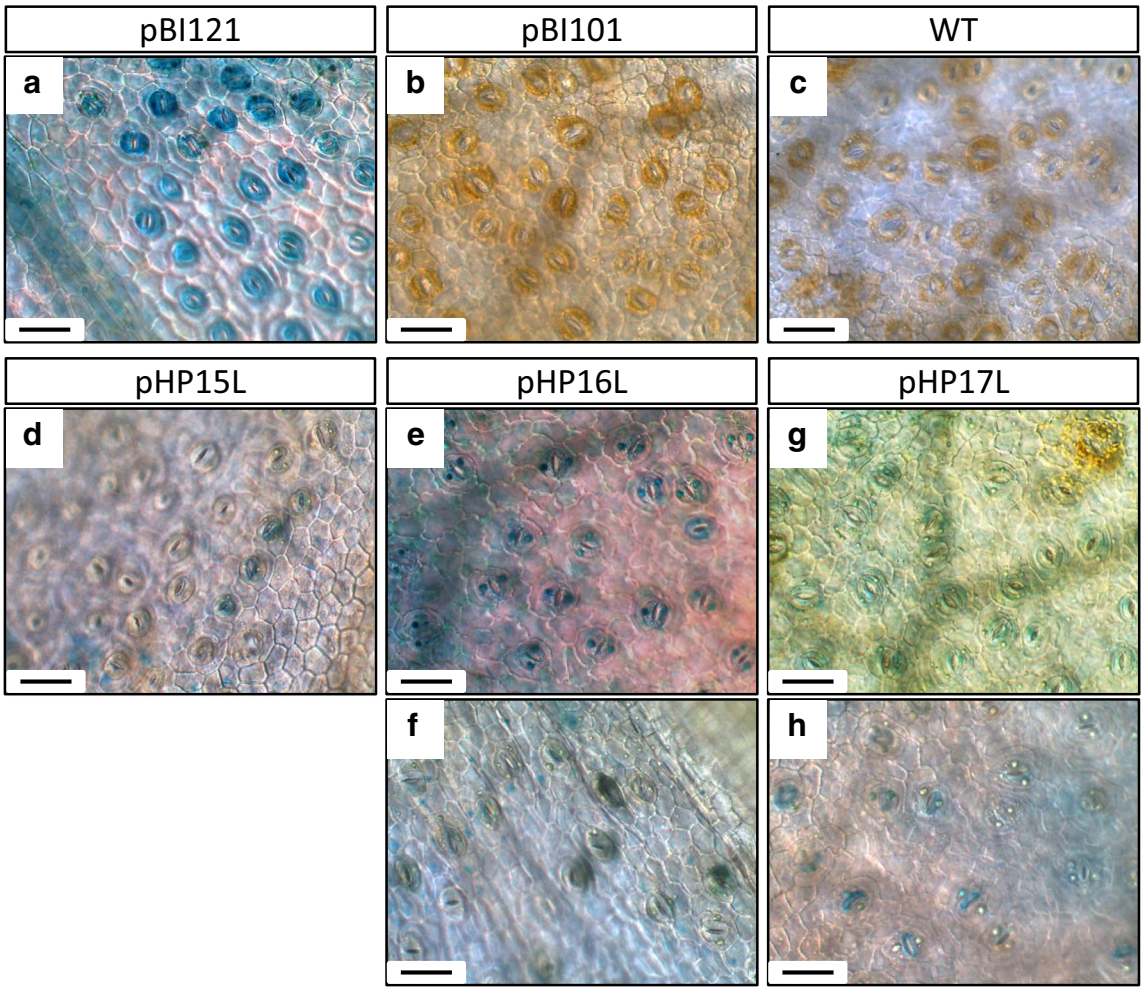

Fig. 6 Examples of GUS staining observed in guard cells of *C. arabica* plants transformed by the *CcDREB1D* promoter haplotype (pHP) and subjected to abiotic stresses. Stomata were visualized by bright field microscopy in the abaxial region of leaves of untransformed (WT = wild type) coffee plants and in those of plants transformed by pBI101 (negative control), pBI121 (positive control), pHP15L and pHP16L, pHP17L subjected to low humidity (**a**, **b**, **c**, **d** and **e**), 48 h ABA (**f**), cold (**g**) and high light (**h**) treatments. Bars represent 80 μm

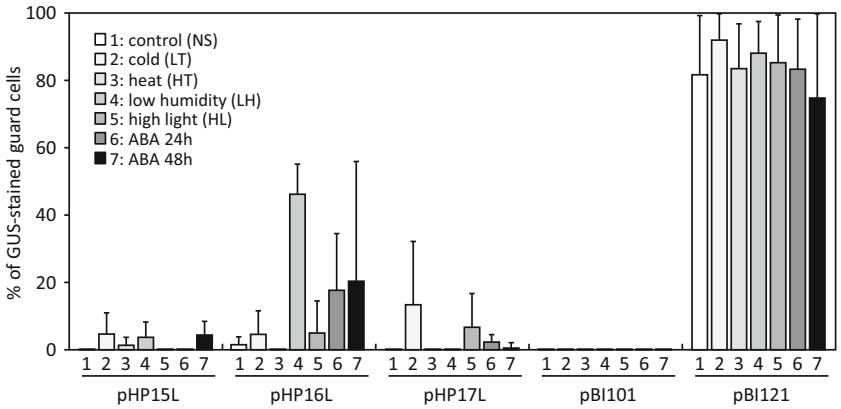

Fig. 7 Proportion of GUS-stained guard cells in leaves of *C. arabica* plants transformed by the *CcDREB1D* promoter haplotype (pHP) subjected to different types of abiotic stress. The colours used for each type of abiotic stress (cold, heat, low humidity, high light and ABA 24 h/48 h) are defined in the fig. NS corresponds to the control (no stress). The proportions of GUS-stained guard cells in pBI121- and pBI101-transformed coffee plants were used as positive and negative controls, respectively

barely detected. Conversely, the transcripts of these two genes were highly accumulated with the LT and LH treatments, and to a lesser extent under heat stress (HT). Compared to these conditions, a low but significant increase in transcript levels was also detected for *uidA* and *CaDREB1D* under high light (HL) and for *uidA* under ABA treatment.

Discussion

Expression of *DREB*-Like Genes in *C. arabica* and *C. canephora*

Whatever the primer pairs, or the tissues and conditions tested during this study, the results presented here did not enable us to detect expression of the *ERF013*, *DREB1C*, *ERF023*, *ERF024*, *DREB3*, *ERF039*, *ABI4*, *DREB2G* and *DREB2D* genes in the *C. arabica* and *C. canephora* species by RT-qPCR. The fact that coffee ESTs with similarity to *ERF013* and *ERF024* were not present in the public nucleic databases suggests that these genes correspond to pseudogenes (never expressed) or to genes expressed in very specific conditions or tissues, not studied in previous coffee cDNA library sequencing projects (Lashermes et al. 2008; Vieira et al. 2006; Mondego et al. 2011). Dussert et al. (2018) recently reported *CaABI4* gene expression in the embryos during the latest stages of coffee bean development therefore indicating that this gene is functional. This also seems to be the case for the *ERF016* and *ERF062* genes which were weakly up-regulated by abiotic stress in the leaves of *C. arabica* and by drought in the leaves of *C. canephora*.

A different situation was observed for the *ERF023*, *DREB3*, *ERF039*, *DREB2G* and *DREB2D* genes, for which

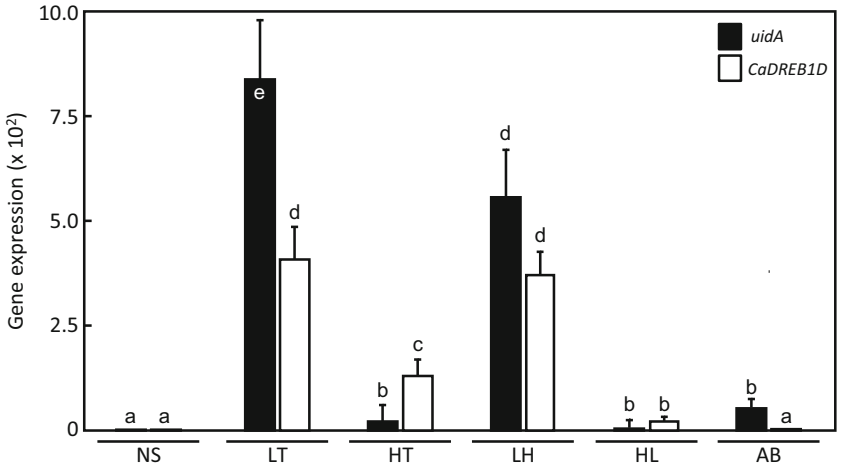

Fig. 8 Expression profiles of the *uidA* and *CaDREB1D* genes in leaves of *C. arabica* var. Caturra plants transformed by pHP16L subjected to low (LT) and high (HT) temperatures, low humidity (LH), ABA (AB) and high light (HL) conditions. Expression was analysed by RT-qPCR using the GUS-F/R and DREBA09-F/R primer pairs (Table 2) for the *uidA* (black isobars) and *CaDREB1D* (white isobars) genes, respectively. Expression values corresponding to the mean of three biological and technical replications (±SD) are expressed in fold change relative to the expression level of NS (control, no stress) sample used as the reference sample (relative expression = 1). Transcript abundances were normalized using the expression of the *CcUBQ10* gene as the endogenous control. Treatments sharing the same letter are not significantly different

ESTs displaying high sequence similarity were found in different coffee cDNA libraries (e.g. from fruits and leaves). In a recent study, the expression of *DREB2G* gene was reported to be highly up-regulated in the endosperm during the DT (desiccation tolerance) phase occurring at the end of *C. arabica* bean development (Dussert et al. 2018). If we consider that all the primer pairs tested for these genes were appropriate (in terms of specificity and efficiency), we concluded that these five genes were not expressed under the different conditions tested in our study.

For the remaining twenty-two genes, sixteen were expressed in both coffee species, four (*ERF061*, *DREB1B*, *ERF025* and *DREB2F*) were expressed in *C. canephora* but not in *C. arabica* and two (*ERF043* and *DREB2C*) were expressed in *C. arabica* but not in *C. canephora*. The limited number of corresponding ESTs to these genes found in the cDNA libraries of *C. arabica* (Vieira et al. 2006; Mondego et al. 2011) and *C. canephora* (Lin et al. 2005; Poncet et al. 2006) demonstrates their low expression and probably explains the difficulty in detecting their transcripts.

The fact that some genes were expressed in one species but not in the other, could also reflect the occurrence of different regulations of their homeologs (Vidal et al. 2010; Marraccini et al. 2011). Indeed, all primers used in this study were deduced from the *C. canephora* genome sequence. However, *C. arabica* is an amphidiploid species (originating from a cross between *C. canephora* and *C. eugenioides*) and its transcriptome is a mixture of genes expressed from both *C. eugenioides* (*CaCe*) and *C. canephora* (*CaCc*) sub-genomes (Vidal et al. 2010). In this context, it is possible that the primers used in this study recognized only the *DREB*-like genes of *CaCc* sub-genome and not those of *CaCe*. The access to the *C. arabica* genome sequence (Mueller et al. 2015) should solve this point through the design of primers able to analyze the expression of each homeologs.

The absence of gene expression in one species, but not the other, might also be explained by the fact that plant growth and drought stress conditions were not similar between *C. arabica* and *C. canephora*. Indeed, the *C. arabica* plants were cultivated in vitro and subjected for 12 h to low RH (Alves et al. 2017), while *C. canephora* clones were grown under greenhouse conditions with water withdrawal for several days before being stressed (Marraccini et al. 2011, 2012). Under these conditions, the expression profiles of *DREB*-like genes presented here for *C. arabica* and *C. canephora* could be considered as rapid and late responses, respectively. Several authors have already reported rapid expression (within a few hours) of *DREB*-like genes under drought (Sun et al. 2008; Wang et al. 2008; Li et al. 2011). More recently, Thioune et al. (2017) reported a peak of *CcDREB1D* transcripts after only 45 min, followed by a decrease, in *C. canephora* leaves subjected simultaneously to a 30% RH and heat shock (35 °C), characterizing a fast and transient expression of this gene. On the other hand, the expression of *DREB* genes over longer periods (from 24 h up to several days) of drought was also reported in maize (Liu et al. 2013), *Pisum sativum* (Jovanović et al. 2013), *Brassica oleracea* (Li et al. 2017) and grape (Zandkarimi et al. 2015), for example.

DREB-Like Genes Are Differentially Expressed in *C. arabica* under Abiotic Stress

Differential expression of *DREB* genes under various types of abiotic stress has already been reported in many plants (Lata and Prasad 2011). In *Arabidopsis*, expression of *DREB1A/CBF3*, *DREB1B/CBF1* and *DREB1C/CBF2* was up-regulated by cold (Gilmour et al. 1998), while *DREB2A* responded to drought, salt and ABA (Liu et al. 1998), *DREB2C* responded to salt, mannitol and cold (Lee et al. 2010) and *DREB1D/CBF4* responded to drought and ABA (Haake et al. 2002). The gene expression profiles presented here in leaves of *C. arabica* showed that the *CaERF017*, *CaDREB1D* and *CaTINY* genes were highly up-regulated by both cold and low humidity. The expression of *CaERF053*, *CaRAP2.4*, *CaERF027* and *CaDREB2A.1* was also up-regulated specifically by low humidity. Of all the genes tested, *CaERF014* was the unique gene up-regulated only by high temperature.

DREB-Like Genes Are Differentially Expressed in Leaves and Roots of D^T and D^S *C. canephora* Clones Subjected to Water Limitation

In *C. canephora*, it is worth noting that gene expression levels in leaves under WL conditions were always higher in D^T clones than in D^S clone 22. This was particularly the case of *CcERF053*, *CcRAP2.4*, *CcERF017*, *CcERF027* and *CcDREB1D* which were over-expressed under WL in D^T but not in the D^S clones. Some differences in expression levels were also observed between D^T clones. For example, leaf expression of *CcERF027* and *CcDREB1B* under WL was greater in D^T clone 73 than in D^T clones 14 and 120. On the other hand, expression of *CcRAP2.10* and *CcDREB1D* was significantly greater in D^T clone 14 than in D^T clones 73 and 120.

In roots, WL up-regulated the expression of *CcERF061* (mainly in D^T clones), *CcTINY* (in D^T clone 14) and *CcDREB2A.2* (in D^T clone 120). More interestingly, expression of the *CcRAP2.4*, *CcERF016* and *CcDREB2F* genes was highly induced under WL conditions, specifically in roots of D^S clone 22. In these cases, gene expression levels were among the highest measured in roots and opposite to those observed in leaves, where they were very low. These results back the idea that roots are key organs in the adaptation of coffee plants to water deficit (Pinheiro et al. 2005). These results confirmed those previously reported in *C. canephora* showing the existence of different mechanisms among the D^T clones of

C. canephora as regards water deficits (Marraccini et al. 2012; Vieira et al. 2013). They also highlighted the differential expression of *DREB*-like genes occurring in leaves and roots of D^T and D^S coffee clones. Similar results were already described when comparing leaf and root expression profiles in D^T and D^S varieties of *Vitis vinifera* (Zandkarimi et al. 2015) and lines of *Zea mays* L. (Zhang et al. 2017), for DREB/CBF and ERF encoding genes, respectively. Cohen et al. (2010) also reported high up-regulation of several orthologues of dehydration-responsive-element binding protein such as *DREB2A*, *DREB1A* and *DREB1D* in roots of *Populus deltoides* subjected to drought. Altogether, these results clearly suggest that the specific up-regulation of these *DREB* genes in response to WL, particularly in the roots of these plants, might participate in enhancing their response and/or adaptation to limited soil water content (Joshi et al. 2016).

CcDREB1 Promoter Haplotypes Are Differentially Regulated in Leaves of *C. arabica* Subjected to Different Types of Abiotic Stress

Among the genes involved in plant responses to stress, the expression of many of them was shown to be under the control of DREB transcription factors (Lata and Prasad 2011). Despite the importance of these genes, a limited number of *DREB* promoters have been analysed using transgenic approaches. We recently reported the differential expression of three *CcDREB1D* promoter haplotypes under water deficit in transgenic coffee plants (Alves et al. 2017). these promoter haplotypes through their capacity to control the expression of the *uidA* reporter gene in transgenic plants of *C. arabica* subjected to other abiotic stress.

The GUS staining results presented here showed that the HP15, HP16 and HP17 haplotypes of the *CcDREB1D* promoter did not function in the leaves, apical buds and roots of unstressed coffee plants. However, blue-stained tissues were detected in transgenic coffee plants transformed by all haplotypes when those plants were subjected to low humidity, cold, heat, high light and ABA treatments. For example, strong GUS staining was observed in the roots of pHP15L-transformed coffee plants under 24 h of ABA treatment, but also in the apical buds of pHP16L-transformed plants subjected to 48 h ABA and low humidity, as well as in those of pHP17L-transformed plants subjected to 24 h ABA, heat and high light 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) harboured all the *cis*-regulatory elements (CREs) required to correctly regulate the expression of the *uidA* reporter gene in transgenic plants. Moreover, the fact that high accumulation of the *uidA* transcripts in pHP16L-transformed plants subjected to cold and low humidity overlapped the increase of *CaDREB1D* endogenous transcripts also indicated that the transcriptional machinery of allotetraploid *C. arabica* correctly regulated the HP16 haplotype of *CcDREB1D* promoter from the diploid *C. canephora*.

The GUS staining results presented here also emphasized the fact that the HP15, HP16 and HP17 haplotypes responded in different ways to the same abiotic treatments. For example, the proportion of GUS-stained guard cells was larger in pHP16L-transformed plants subjected to LH and exogenous ABA (after both 24 h and 48 h of treatment), than the proportion measured in plants transformed by pHP15L and pHP17L and subjected to the same stress. These results are similar to those previously reported in the same plants by Alves et al. (2017) showing a larger proportion of GUS-stained guard cells in pHP16L-transformed plants than in coffee transformed by pHP15L and pHP17L subjected to low humidity mimicked by PEG treatment.

The increased proportion of GUS-stained guard cells observed in pHP16L-transformed coffee plants subjected to abiotic stress was confirmed by analysing expression of the *uidA* reporter gene. In that case, the amount of *uidA* transcripts increased under cold and low humidity, as well as (but to a lesser extent) under 24 h of ABA. Our results also showed that the *uidA* transcripts were not detected after 3 h of heat and high light treatment in pHP16L-transformed plants, thereby confirming the small proportion (and even the absence) of GUS-stained guard cells for these treatments. However, the low *uidA* gene expression observed after 3 h of ABA treatment did not match 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* occurring after 3 h of ABA treatment could explain this situation. In that sense, it is worth noting that *Arabidopsis* genes containing the DREB1A/CBF3 motif in their promoters were mainly upregulated after 6 h of ABA treatment (Huang et al. 2007).

Several guard cell-specific genes have already been reported (Wang et al. 2011; Virlouvet and Fromm 2015). Interestingly, the guard cell transcriptome is particularly rich in transcription factor-encoding genes including *DREB*, *WRKY*, *MYB* and *MYC* (Hachez et al. 2011; Baldoni et al. 2015). In the great majority, functional analyses of their promoters have shown that expression was not restricted to guard cells, but was in fact guard cell-preferred, with expression often 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 in *Arabidopsis* promoters of *CYP86A2* and *MYB60* genes (Galbiati et al. 2008; Yang et al. 2008; Cominelli et al. 2011). In addition to its guard cell specificity, the activity of the *AtMYB60* promoter, but also of the *VvMYB60* promoter from *Vitis vinifera*, was also shown to be rapidly down-regulated by ABA (Galbiati et al. 2011;

Rusconi et al. 2013). Computational analyses have also shown that the G-box containing the classical ABA-regulated elements (ABRE) was overrepresented in guard cell-specific promoters that were ABA-upregulated (Leonhardt et al. 2004; Wang et al. 2011). Interestingly, in their long (L) versions, the HP15, HP16 and HP17 *CcDREB1D* promoter haplotypes all contained several ABRE-like motifs (Alves et al. 2017).

To our knowledge, the results presented here are the first demonstrating guard cell up-regulated expression of a *DREB1D* promoter in response mainly to ABA and low humidity in coffee plants. However, cold, heat shock and high light treatments were less efficient in up-regulating the expression of this haplotype. The fact that DREB genes responded in different ways to abiotic stress was already reported in the literature. For example, the expression of *AtDREB1D* (also known as *AtCBF4*) was up-regulated by drought but not by cold in *Arabidopsis* (Haake et al. 2002). However, cold and drought treatments up-regulated the expression of *DREB1D* in *Vitis* sp. (Xiao et al. 2008; Zandkarimi et al. 2015) and *Medicago truncatula* (Li et al. 2011).

Studies demonstrating differential expression of haplotypes (or alleles) of the same promoter are also very limited in plants (de 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, clearly demonstrated that the three *CcDREB1D* promoter haplotypes responded differentially to abiotic stress. Since the HP16 haplotype was isolated from *C. canephora* D^T clone 14, this might explain why *CcDREB1D* gene expression was highly up-regulated by WL in the leaves of this clone, but not in those of D^S clone 22 harbouring the HP15 and HP17 haplotypes (Marraccini et al. 2012).

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 a higher root-to-shoot ratio was observed in transgenic plants overexpressing *DREB* genes also demonstrates the key role of these genes in root system development (Janiak et al. 2016). Interestingly, Pinheiro et al. (2005) showed that *C. canephora* D^T clone 14 had deeper roots than D^S clones, suggesting that root architecture contributes to drought tolerance in coffee. Even though expression of *CcDREB1D* was barely detected in the roots of *C. canephora* D^S and D^T clones subjected to WL, the results presented here clearly showed ABA-induced GUS staining in the roots of pH15L-transformed plants. In addition to the peak of *CcDREB1D* transcripts observed in leaves *C. canephora* subjected to a humidity shock treatment, Thioune et al. (2017) also reported concomitant expression of *CcNCED3* (encoding a protein involved in ABA synthesis) and *CcATAF1* (encoding a putative protein ortholog of AtATAF1 from Arabidopsis known to regulate *AtNCED3* promoter activity). The expression of the first gene was already reported to be induced by drought in leaves of *C. arabica* (Simkin et al. 2008) but also in roots of *C. canephora* (Costa 2014). Altogether, the results presented here highlight the capacity of *CcDREB1D* promoter haplotypes in regulating (in different ways) the expression of the *uidA* reporter gene in the guard cells of transgenic coffee plants, and suggest a key role of ABA in controlling *CcDREB1D* gene expression in response to WL in coffee. They also showed that *DREB*-like genes reported here to be differentially expressed between clones and/or under different abiotic stress could be used as (positive and/or negative) markers in future breeding programmes aiming to generate new coffee cultivars better adapted to climate change (Bertrand et al. 2016).

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Author's Contribution LFT, TR, SOA, KED, TSC, JCB, MGC and TSC extracted RNA samples, performed qPCR experiments and analysed the results. LFT, ED, HE and GSCA carried out genetic transformation, applied abiotic stress on *C. arabica* and performed GUS staining and microscopy analyses with the help of MC. HE, ACA and PM designed the study, drew up the experimental design and implemented it. LFT, ATS, LECD, LVP, ACA, HE and PM wrote the manuscript. All authors read and approved the final manuscript.

Compliance with Ethical Standards

Conflict of Interest All authors declare that they have no conflict of interest.

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