Physiol Mol Biol Plants (February 2021) 27(2):203–212
https://doi.org/10.1007/s12298-021-00930-6

RESEARCH ARTICLE

The urea transporter DUR3 is differentially regulated by abiotic and biotic stresses in coffee plants

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Received: 25 March 2020 / Revised: 20 November 2020 / Accepted: 11 January 2021 / Published online: 4 February 2021

Abstract The high costs of N fertilizers in the coffee production emphasizes the need to optimize fertilization practices and improve nitrogen use efficiency. Urea is widespread in nature, characterizing itself as a significant source of nitrogen for the growth and development of several organisms. Thus, the characterization of genes involved in urea transport in coffee plants is an important research topic for the sustainable production of this valuable cash crop. In the current study, we evaluated the expression of the *DUR3* gene under abiotic and biotic stresses in coffee plants. Here, we show that the expression of a high-affinity urea transporter gene (*CaDUR3*) was up-regulated by N starvation in leaves and roots of two out of three *C. arabica* cultivars examined. Moreover, the *CaDUR3* gene was differentially expressed in coffee plants under different abiotic and biotic stresses. In plants of cv. IAPAR59, *CaDUR3* showed an increased expression in leaves after exposure to water deficit and heat stress, while it was downregulated in plants under salinity. Upon infection with *H. vastatrix* (coffee rust), the *CaDUR3* was markedly up-regulated at the beginning of the infection process in the disease susceptible Catuaí Vermelho 99 in comparison with the resistant cultivar. These results indicate that besides urea acquisition and N-remobilization, *CaDUR3* gene may be closely involved in the response to various stresses.

Keywords Coffea ssp · Nitrogen deficiency · Gene expression · Urea transporter

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Introduction

Coffee is one of the most valuable agricultural traded commodities worldwide and is grown mostly in developing countries (Tucker 2017). It is one of the most popular and frequently consumed beverage worldwide, being also an important product for trade in many non-tropical countries involved in the industrialization (Fridell 2014). The allotetraploid *Coffea arabica* L. (2n = 4x = 44) and the diploid *C. canephora* Pierre ex A. Froehner (2n = 2x = 22) are the most cultivated species in the world (International Coffee Organization, http://www.ico.org) among the 124 coffee species already described (Davis et al. 2011).

Coffee production depends on climate, soil quality and external inputs, such as agrochemicals and fertilizers. Nitrogen (N), one of the most important nutrients for crop growth and yield, are mainly available in many forms expensive commercial inorganic fertilizers (Bloom 2015). Urea [$CO(NH_2)_2$] has become the most commonly used N fertilizer in the world because of it's low production costs and high N content (http://faostat.fao.org). Urea is a small neutral molecule, which is widespread in nature, where it acts as a source of N for many organisms, including bacteria, fungi and plants (Wang et al. 2008). In coffee

production, urea is the major N form supplied for the full crop cycle.

Nitrate is the preferred inorganic N source for several plant species, though ammonium has been shown to be the preferential inorganic N source for N-starved coffee roots (dos Santos et al. 2017). Plants also possess a specific system for the direct uptake of urea from the environment (Fan et al. 2017). Once absorbed, urea is hydrolyzed to form ammonium, which in turn enters into the GS/GOGAT cycle (glutamine synthetase/glutamate synthase cycle) before being assimilated in amino acids (Tegeder and Masclaux-Daubresse 2018). Urea uptake in roots can be mediated by membrane intrinsic proteins such as NIP5;1 (Yang et al. 2015), but the most important high-affinity urea transporter in roots are DUR3 homologs.

DUR3 belongs to the sodium:solute symporter (SSS) family in plants (Kojima et al. 2007). Functional complementation of the yeast *dur3* mutant had been successfully used to identify and characterize the DUR3 orthologues and study the urea transport in plants (ElBerry et al. 1993; Zanin et al. 2014; Zhang et al. 2016). In *Arabidopsis thaliana* the *AtDUR3* gene is expressed in the roots under N deficiency, and its induction increases drastically when the roots come into contact with urea (Kojima et al. 2007). Studies in algae, rice and maize confirmed DUR3 as the major transporter involved in urea acquisition and its regulation by N supply in plants (Liu et al. 2015; Beier et al. 2019). Phylogenetic analysis by De Michele et al. (2012) demonstrated a reduction of genes encoding urea transporters throughout plant evolution, eventually down to a single copy in vascular plants, which is possibly a consequence of the preference for other nitrogen sources.

Despite the agronomic importance of urea fertilizer, the detailed molecular characterization of *DUR3* is restricted to model plants, such as *A. thaliana*, rice and maize (Kojima et al. 2007; Wang et al. 2013; Zanin et al. 2014). To the best of our knowledge, there is no study addressing urea transporters in woody species, such as in coffee, for example. Therefore, the objective of this study was to analyze transcriptional patterns of *CaDUR3* in *C. arabica* plants under nitrogen starvation, abiotic and biotic stresses. We compared the transcriptional levels of *CaDUR3* in three *C. arabica* cultivars under nitrogen starvation and investigated the expression patterns of the *CaDUR3* gene in coffee plants under abiotic (water deficit, heat and salinity) and biotic (coffee leaf rust) stresses.

Material and methods

In silico identification of the *CaDUR3* gene

We performed searches in the Brazilian Genome Project database (Vieira et al. 2006; Mondego et al. 2011) using the keyword urea transporter. A cDNA clone corresponding to a unique EST (expressed sequence tag/CA00-XX-IA2-016-H07-ECF) was identified and obtained from Cenargen/Embrapa (Brasília-DF). This sequence was compared by BLAST against Arabidopsis sequence databases (Altschul et al. 1997) and used as query sequence to find the complete *CaDUR3* sequence in the NCBI database (National Center for Biotechnology Information/ https://www.ncbi.nlm.nih.gov/genome/gdv/?org=coffea-arabica&group=lamiids). After confirming the identity of the *CaDUR3* gene, we used gene-specific primers (Fw *CaDUR3*—5′GGCCTCTTCTCCCACAATTT 3′ and Rev *CaDUR3* – 5′CCTCAA ACAGCTGCTTCCAT) for amplifying the probe for Southern blot and for transcriptional analyses.

Plant materials

We used the following *C. arabica* cultivars throughout this study, viz., cv. IAPAR59, cv. Catuaí Vermelho 99, cv. Mundo Novo, cv. Timor hybrid CIFC 832/2 (HT). We also used *C. canephora* var. Conilon (Cc) and *C. eugenioides* (Ce) in the Southern Blot analysis. The cultivars used are specified in each procedure. The plants were grown in a greenhouse and under field conditions at the experimental facility of the Agronomic Institute of Paraná (IAPAR—latitude 23°18′ S, longitude 51°09′ W, average altitude 585 m; Londrina, Paraná State, Brazil).

Southern blotting

The extraction of genomic DNA from leaves of *C. arabica* cv. Hybrid of Timor CIFC 832/2, *C. canephora*, *C. eugenioides* and *C. arabica* cv. IAPAR59 was performed using the protocol of Doyle and Doyle (1987). Aliquots (20 ug) of genomic DNA were digested overnight at 37 °C with the enzyme *Dra*I, fractionated on 0.7% agarose gel electrophoresis and transferred onto a positively charged Hybond N^+ membrane (Amersham Biosciences) according to the standard protocols. The probe was obtained by amplification of the clone CA00-XX-IA2-016-H07-EC.F (primers Fw *CaDUR3* 5′-GGCCTCTTCTCCCACAATTT-3′ and Rev *CaDUR3* 5′-CCTCAAACAGCTGCT TCCAT-3′) and random primed labeled with α-dCTP^{32} for 24 h at 37 °C. The image was captured using fluorescent image

analyzer FLA 3000—series (Fuji Photo Film CO, Lts. Tokyo, Japan).

Drought, saline and heat stresses

Detailed procedures of the stress experiments were described in previous studies from our group (dos Santos et al. 2011, 2015). For these experiments we used 20 months-old *C. arabica* cv IAPAR59 plants grown in 15 L pots equally filled with substrate (3 soil: 1 sand: 1 organic compound). Mature leaves in the drought stress experiment were collected according to the water potential readings: no stress (NS; ± −1.36 MPa), moderate stress (MS; ± −2.35 MPa) and severe stress (SS; ± −4.3 MPa).

In the saline stress experiment, the plants were irrigated on the first day of the treatment with 50 mM NaCl and with 100 mM NaCl on the second day to avoid osmotic shock. From the third day until the end of the experiment (25 days), the plants were irrigated daily with 150 mM NaCl. Mature leaves were sampled after 0, 6, 12 and 25 days after the beginning of the salt treatment. Both, the drought and saline stresses were performed in a greenhouse regulated to 25 ± 2 °C on natural day/night cycle.

Plants used for the heat stress experiment were cultivated in a growth chamber (12/12 h light/dark cycle, 25/23 °C day/night temperature, 45% humidity, and photosynthetic photon flux density of ~ 250 µmol $m^{-2}{\cdot}s^{-1}$, respectively. After initial acclimation (7 days), the temperature was elevated to 37 °C for five days with daily irrigation maintenance. The leaf samples were collected 3 and 5 days after the beginning of the stress. The three biological replicates were represented by pools of lateral roots and leaves at the same developmental stage. Subsequently, samples from all the treatments were immediately frozen in liquid nitrogen and stored at –80 °C until analysis.

N-starvation

The basic procedures of the N-starvation experiment using *C. arabica* cv. IAPAR59 plants were according to de Carvalho et al. (2013) and dos Santos et al. (2019). Initially, seeds were germinated in greenhouse using boxes containing autoclaved sand and were irrigated twice weekly with Clark nutritive solution (Clark et al. 1975). After 4 months, we selected uniform plants by size uniformity with 3 leaf pairs. Plants were then transferred to a nutritive solution system with constant aeration. In brief, plants were submitted to a 4-week period of acclimation. After this period, plants were transferred to a modified Clark solution without N. Lateral roots and leaf samples were harvested after 1 day and 10 days in the N-free solution. As experimental control for each time point, were used plants under normal N levels. Experiments were conducted twice with a minimum of three biological replicates per experiment. Biological replicates were represented by pools of lateral roots and leaves of at least nine plants at the same developmental stage. After harvesting, all samples were frozen immediately in liquid nitrogen and stored at –80 °C until further analysis.

Urea quantification in N-starved plants

For the urea determination, leaves and roots were macerated in liquid nitrogen, suspended in milliQ water autoclaved, homogenized and then centrifuged at 10.000 rpm at 4 °C for 10 min. The supernatant was filtered through a glass wool, transferred to a new tube and kept on ice until the time of analysis. The reaction mixture was composed of 750 µL phosphate buffer (KH_2PO_4) 0.1 M pH 7.5, 200 µL of plant extract and 50 µL of 50 mM urease solution. The reaction was incubated in a water bath at 37 °C for 40 min and quantified according to Weatherburn (1967) using urea as standard, and measuring absorbance at 625 nm.

Hemileia vastatrix infection

The experiment was conducted in a greenhouse at the Coffee Center "Alcides Carvalho", (Instituto Agronômico de Campinas—IAC, Campinas, Brazil) as described in Baba et al. (2020). In this experiment, six months-old plants with uniform leaf size and development were used. A suspension of *Hemileia vastatrix* race II uredospores (final concentration of 1 mg/mL) was inoculated on the abaxial surface of completely expanded leaves of *C. arabica* cv. IAPAR59 and cv. Catuaí Vermelho 99 which are resistant and susceptible to coffee rust, respectively. Leaves were also mock inoculated with water as controls. Subsequently, the plants were transferred to a humid chamber, covered by a plastic and maintained in the dark for 24 h, at 22 °C with high relative humidity. Samples were collected at 0, 12, 24 and 48 h post-inoculation (HPI), and each treatment was composed of three biological replicates represented by pools of leaves at the same developmental stage. All leaves collected were frozen immediately in liquid nitrogen and stored at –80 °C until further analysis.

RNA extraction and cDNA synthesis

Before initiating the N-starvation, abiotic an biotic stresses experiments, we sampled fully expanded leaves, flower buds, flowers, plagiotropic shoots, secondary roots, endosperm and pericarp from mature fruits of the *C. arabica* cv. IAPAR59 plants cultivated under field conditions in order

to have an overall assessment of the *CaDUR3* transcriptional profiles.

Total RNA extraction from all the samples was performed using the standard CTAB method using the same procedures of dos Santos et al. (2011). RNAs extracted were checked for integrity by agarose gel electrophoresis and then treated with DNase (RNase-free, Invitrogen) to remove genomic DNA contamination. The purity and concentration of the extracted RNAs were determined using a NanoDrop® ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). The cDNAs synthesis were carried out using a SuperScript III Reverse Transcriptase kit (Invitrogen, Carlsbad, CA, USA) in a final volume of 20 µL using 5 µg of total RNA, according to the manufacturer's instructions.

Expression analysis

CaDUR3-specific primers were designed using Primer Express v3.0.1. Specific primers (Tm = 60 °C) were designed from the EST (CA00-XX-IA2-016-H07-ECF) to ensure the amplification of a single ~ 85 bp PCR product (Fw *CaDUR3*—5′GGCCTCTTCTCCCACAATTT 3′ and Rev *CaDUR3*—5′CCTCAAACAGCTGCTTCCAT 3′). The specificity of the primers was confirmed by PCR and by melting curves to verify the presence of nonspecific products. The mean amplification efficiency of the primer pair (98%) was assessed by the program LinRegPCR (Ruijter et al. 2009). RT-qPCR was analyzed in a 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The PCR mixture (10 µL total volume) included 1 µL cDNA, 5 µL SYBR Green Master Mix (Life Technologies, USA), 0.5 µL sequence-specific forward primer (10 µM), 0.5 µL reverse primer (10 µM) and 3 µL water. The RT-qPCR program conditions an initial step of 95 °C for 10 min, followed by 40 cycles of 95 °C for 35 s and 60 °C for 60 s. The relative expression levels of the *CaDUR3* gene were calculated based on $(1 + E)^{-\Delta\Delta ct}$ method (Livak and Schmittgen 2001) and normalized using the gene *CaEF1α*, as recommended in de Carvalho et al. (2013). All RT-qPCR experiments were performed in triplicate in three independent experiments.

Statistical analysis

The experimental design used in all experiments (abiotic stresses, *H. vastatrix* infection and N starvation) was the completely randomized design with three biological replications. Data from RT-qPCR and urea content were submitted to analysis of variance (ANOVA) to determine differences between treatments at $p < 0.05$. Means were separated according to Duncan's test ($p < 0.05$). The data were normalized using the Box-Cox power transformation (Box and Cox 1964). All statistical analyses were performed using the easyanova (Arnhold 2013) and MASS (Venables and Ripley 2002) packages of R statistical software (R Core Team, 2019).

Results

CaDUR3 identification

Using urea transporter as the keyword, the *in silico* survey within the Brazilian Coffee Genome Database (http://www.lge.ibi.unicamp.br/cafe/; Vieira et al. 2006) resulted in only one EST (CA00-XX-IA2-016-H07-EC.F). This sequence corresponds to the accession number GW440609 deposited in NCBI database. Primarily, the analysis of the corresponding cDNA clone by the BLAST tools (http://www.ncbi.nlm.nih.gov/blast) showed that it matches the sequence of the *DUR3* gene of the sodium:solute symporter (SSS) family (Kojima et al. 2007). Using this sequence as query, we identified the complete sequence of the *DUR3* gene in the *C. arabica* genome deposited in the NCBI database (LOC113707287—NCBI Accession number), which is located on chromosome 8 and codes for a mature protein of 678 amino acids (XP_027085342). By comparing this sequence with the known sequences of *A. thaliana* genome (TAIR: https://www.arabidopsis.org), we verified that the *CaDUR3* presented high similarity (84%) with AT5G45380.1 (*AtDUR3*; Kojima et al. 2007).

The cDNA probe detected a single band on Southern blots of genomic DNA of *C. eugenioides* digested and two strong hybridization signals in *C. canephora*, *C. arabica* cv. IAPAR59 and in the Hybrid Timor accession with the restriction enzyme *Dra*I (Fig. 1). *C. arabica* is derived from an allopolyploidization (C^aE^a genome) event between the parental diploids *C. canephora* (C genome) and *C. eugenioides* (E genome) (Lashermes et al. 1999, 2000), therefore the extra band observed in the *C. arabica*, *C. canephora* and in the interspecific hybrid is due to differences in the enzyme restriction profiles of the genomic DNA between the two parental species. Indeed, the analysis of the *DUR3* gene sequences of *C. arabica* and *C. canephora* on the available databases showed a single internal restriction site for the enzyme *Dra*I, which resulted in the expected Southern Blot profiles. Therefore, according to the restriction pattern observed, the *DUR3* is likely present as a single copy gene within in all species examined here.

CaDUR3 expression in *C. arabica* organs

Initially, we verified the transcript levels of the *CaDUR3* gene in seven organs of *C. arabica* plants (cv. IAPAR59)

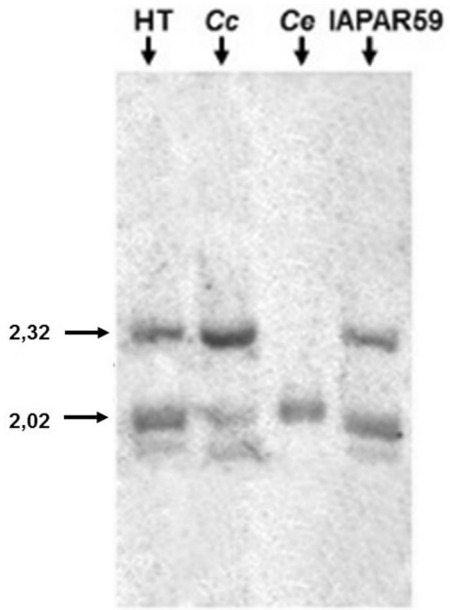

Fig. 1 Southern Blot analysis. The genomic DNA of *C. arabica* cv. Timor hybrid CIFC 832/2 (HT), *C. canephora* (Cc), *C. eugenioides* (Ce) and *C. arabica* cv. IAPAR59 were digested with the restriction enzyme *Dra*I. The probe used was the PCR product obtained from the amplification of the cDNA clone CA00-XX-IA2-016-H07-EC.F that corresponds to a partial sequence of the *CaDUR3* gene

cultivated under field conditions. The presence of *CaDUR3* transcripts was observed in all tissues, with particularly high-level expression in roots (Fig. 2).

Transcription patterns of *CaDUR3* under N-starvation

In order to quantify the variation in transcript abundance of *CaDUR3*, we performed RT-qPCR analysis on plants of three *C. arabica* cultivars subjected to N-starvation for 1 and 10 days (Fig. 3). There was a considerable variation in the expression of *CaDUR3* in leaves of the three cultivars (IAPAR59, Mundo Novo and Catuaí Vermelho 99). Nonetheless, our results showed that *CaDUR3* transcript levels increased after long-term N-starvation (10 days) when compared to the N-sufficient condition (day 0) in all cultivars, with the levels of *CaDUR3* expression being highest in leaves of the cv. IAPAR59 (approximately eight times more that the calibrator) (Fig. 3). We also showed that *CaDUR3* transcripts substantially increased in roots of this cultivar after long exposure to N-starvation (Fig. 4).

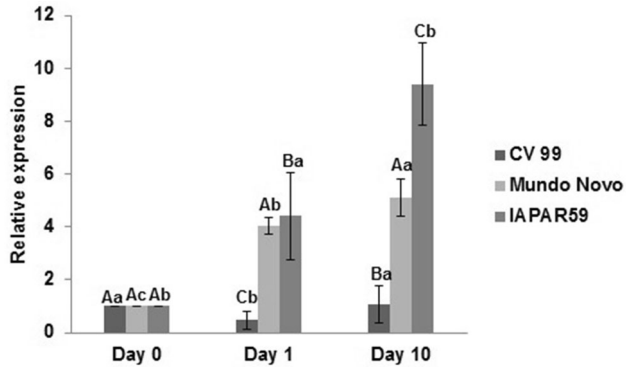

Fig. 3 Relative expression of *CaDUR3* gene in coffee leaves of plants under nitrogen starvation of the *C. arabica* cv. IAPAR59, *C. arabica* cv. Mundo Novo and *C. arabica* cv. Catuaí Vermelho 99. Transcript abundances were normalized against the expression of the *CaEF1α* gene and treatment day 0 was used as calibrator samples (relative gene expression = 1). Values are presented as mean ± standard deviation (n = 3). Averages followed by the same capital letter, compare differences between the *C. arabica* cultivars at the same day of N starvation treatment, did not differ significantly by the Duncan test ($p < 0.05$). Averages followed by the same lowercase letter, compare differences between different days of N starvation treatment within each cultivars, did not differ significantly by the Duncan test ($p < 0.05$)

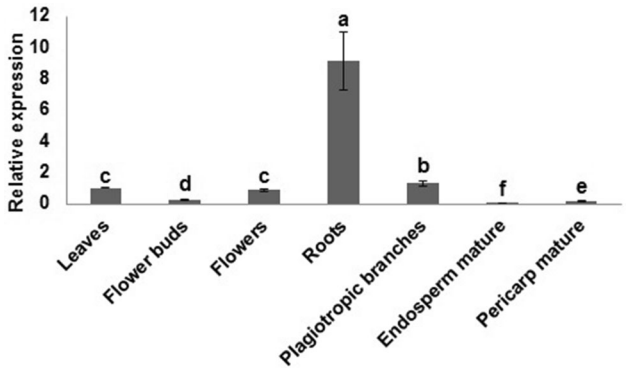

Fig. 2 Relative expression of *CaDUR3* in different tissues of the *C. arabica* cv. IAPAR59. The internal calibrator used in this experiment is it's leaf. Transcript abundances were normalized using the expression of the *CaEF1α*. Values are presented as mean ± standard deviation (n = 3)

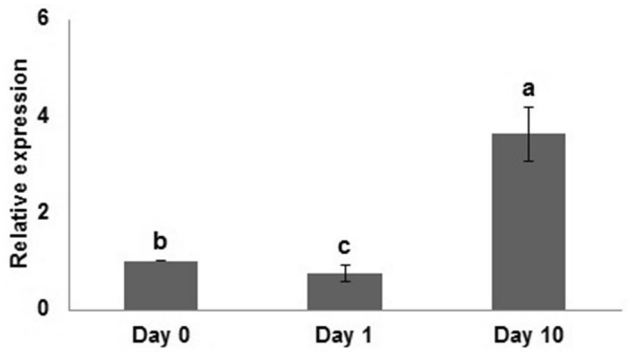

Fig. 4 Relative expression of *CaDUR3* gene in coffee roots of the *C. arabica* cv. IAPAR59 under nitrogen starvation. Transcript abundances were normalized using the expression of the *CaEF1α* gene and day 0 as calibrator sample (relative gene expression = 1). Values are presented as mean ± standard deviation (n = 3)

Urea content in leaves and roots of *C. arabica*

Regarding the leaf and root urea content under N-starvation conditions, we observed that the highest concentration was in leaf tissues of the *C. arabica* cv. IAPAR59 (Fig. 5). The N contents in roots were very low in all treatments. The concentrations were practically the same from the beginning to the end of the experiment.

Transcription patterns of *CaDUR3* under abiotic stresses

Abiotic stresses are among the main factors affecting food production worldwide and may affect nitrogen metabolism. In this sense, we sought to investigate the transcriptional patterns of the *CaDUR3* gene in leaves of *C. arabica* cv. IAPAR59 plants in response to salt stress, high temperature and drought (Fig. 6). We detected 2- and threefold increase in transcript levels in plants under moderate and severe water deficits treatments compared with those under normal irrigation conditions, respectively (Fig. 6a). In heat-stressed plants, the expression of the *CaDUR3* gene was upregulated when compared to the control treatment (day 0—no stress) (Fig. 6b). There were significant differences in the transcript levels, mainly at day 5 of heating at 37 °C. Under conditions of salt stress, the transcriptional profile of the *CaDUR3* gene after the different periods under salinity condition (150 mM NaCl solution) was differentially modulated. The results showed in Fig. 6c, demonstrated that in the first two sampling periods (6 and 12 days) there was a drastic reduction in the number of transcripts (downregulation). However, the expression level of *CaDUR3* increased moderately in leaf tissues in 25 days after NaCl treatment (Fig. 6c).

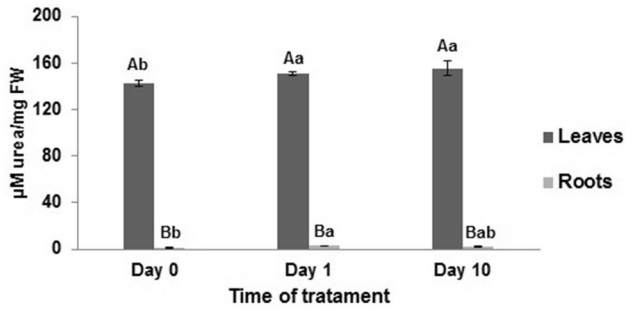

Fig. 5 Urea content in leaves and roots of *C. arabica* cv. IAPAR59 plants grown under N starvation. Values are presented as mean ± standard error (n = 3). Averages followed by the same capital letter, compare differences between leaves and roots at the same day of N starvation treatment, did not differ significantly by the Duncan test ($p < 0.05$). Averages followed by the same lowercase letter, compare differences between the tissues (leaves and roots) at different days of N starvation treatment, did not differ significantly by the Duncan test ($p < 0.05$)

Transcription patterns of *CaDUR3* after *H. vastatrix* infection

The infection of coffee leaves with race II of *H. vastatrix* modulated differential *CaDUR3* gene expression patterns depending on the cultivar tested (Fig. 7). In a rust-resistant cultivar (IAPAR59), a gradual decline of transcript abundance was observed until 48 h post-inoculation. On the contrary, the susceptible cultivar (Catuaí Vermelho 99) showed a peak of *CaDUR3* mRNA abundance in their leaves at the first sampling time (12 h post-inoculation), with a considerable decline in the next two sampling periods (24 and 48 h post-inoculation) (Fig. 7).

Discussion

Urea is mainly used as a source of N fertilizer in agricultural crops with high N demand (Karamos et al. 2014), such as in coffee. In plants, the first evidence of active transport of urea *in planta* was presented by Liu et al. (2003a). *DUR3* belongs to a family of transporters for several solutes found in eukaryotes and is presented as a single copy in the plant systems evaluated so far (De Michele et al. 2012). The *C. arabica* is an amphidiploid (C^aE^a genome) species originated from the natural hybridization (estimated 553,000 to 665,000 years ago) (Yu et al. 2011; Scalabrin et al. 2020; Bawin et al. 2020) between two diploid ancestral species (*C. canephora*—C genome and *C. eugenioides*—E genome), and in its genome there is the contribution of two sub-genomes (Lashermes et al. 1999, 2000; Vidal et al. 2010). As illustrated by the Southern blot, the results in the *Coffea* species tested (Fig. 1) are concordant with data in other plant genomes, such as in *Arabidopsis* (Liu et al. 2003a), rice (*OsDUR3*) and maize (*ZmDUR3*) (Wang et al. 2012; Zanin et al. 2014).

We were able to detect the presence a low level of *CaDUR3* transcripts in most analyzed tissues of the *C. arabica* cv. IAPAR59, indicating that the urea transporter is differentially expressed in different tissues of this species under normal field growth conditions (Fig. 2). However, the highest level of mRNA expression was observed in the roots, similarly to previous studies in other species (Zanin et al. 2014). The higher expression of this gene in root tissues most probably reflects its involvement in the mechanisms of N acquisition by the roots.

Although the roots absorb the N available in the soil solution (Witte 2011), some studies suggest that urea can be translocated from the roots to other parts of plants (Gerendás et al. 1998; Mérigout et al. 2008). In this study, changes in the transcript level of *CaDUR3* in leaves of three *C. arabica* cultivars under nitrogen starvation were

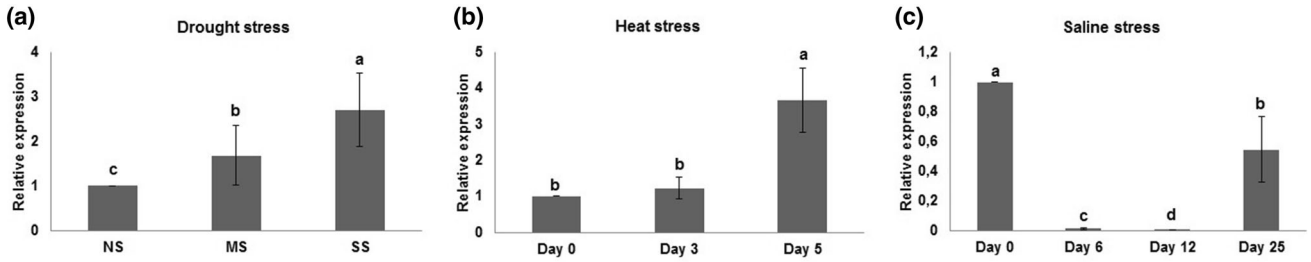

Fig. 6 Relative expression of *CaDUR3* in leaves of *C. arabica* cv. IAPAR59 under abiotic stresses. **a** Drought experiment: no stress (NS; ±-1.36 MPa), moderate stress (MS; ±-2.35 MPa), severe stress (SS; ±-4.3 MPa); **b** Heat experiment: no stress (Day 0), 3 and 5 days exposed to 37 °C; **c** Saline experiment: no stress (Day 0), 6, 12 and 25 days after onset of the saline stress (150 mM NaCl). Transcript abundances were normalized against the expression of the *CaEF1α* gene, and the treatments NS (drought stress) and day 0 (heat and saline stress) were used as controls

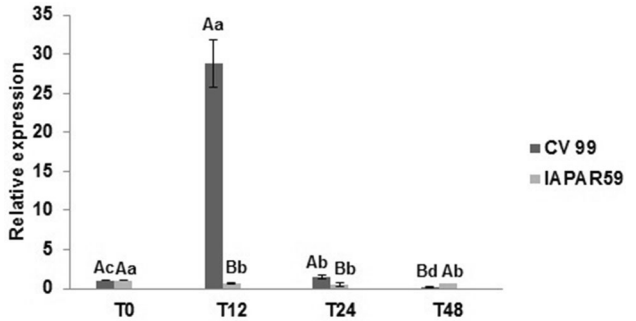

Fig. 7 Relative expression of *CaDUR3* gene in coffee leaves infected with *Hemileia vastatrix* race II after 0, 12, 24 and 48 hours post-inoculation in *C. arabica* cv. Catuaí Vermelho 99 (susceptible) and *C. arabica* cv. IAPAR59 (resistant) genotypes. Transcript abundances were normalized against the expression of the *CaEF1α* gene and T0 was used as calibrator samples (relative gene expression = 1). Values are presented as mean ± standard deviation (n = 3). Averages followed by the same capital letter, compare differences between the *C. arabica* cultivars at the same hours post-inoculation, did not differ significantly by the Duncan test ($p < 0.05$). Averages followed by the same lowercase letter, compare differences between hours post-inoculation within each cultivars, did not differ significantly by the Duncan test ($p < 0.05$)

detected (Fig. 3). We suggesting that this transporter has a great potential to be considered in breeding for optimization of urea use between different genotypes and foliar fertilization. Conceivably, genotypes maintaining higher *CaDUR3* gene expression may have better performance under low N availability conditions.

By observing the expression data in different coffee tissues, particularly in leaves (Fig. 2 and 3), we can reinforce the role of the *CaDUR3* gene in the process of remobilization of N in coffee tissues under stress conditions. As this element is easily redistributed in plants via phloem in the form of amino acids when the N supply is not sufficient, the N of the old leaves is remobilized to other leaves and tissues (Matiz et al. 2019). Indeed, other studies have reported that DUR3 triggered a mechanism of endogenous urea remobilization during leaf senescence and germination of seeds (Bohner et al. 2015; Liu et al. 2015). Additionally, Beier et al. (2019) suggested in their study that DUR3 participates in the N redistribution from leaves to panicles in rice.

Our study revealed a differentiated relationship of *CaDUR3* gene expression with urea content present in leaves of cv. IAPAR59 (Figs. 3, 5). Possibly, the urea was rapidly assimilated and therefore there was no difference expressive in content. Additionally, we can suggest that the coffee tree also showed a "facilitated diffusion" in the acquisition of urea under stress conditions. Our hypothesis is that the *CaDUR3* in coffee plants may be the limiting transporter for absorption of urea through the roots to the leaves. It is important to emphasize that the limiting factors for use efficiency of urea may be associated with its translocation and assimilation capacities. In the plant cell, the assimilation of urea is possibly slower than for ammonium or nitrate, as it depends on the action of the enzyme urease (Wang et al. 2008). Moreover, when perturbation occurs in N metabolism, urea levels may deliberately increase and be translocated to other tissues (Masclaux-Daubresse et al. 2010).

The expression of the *CaDUR3* gene increased almost fourfold in roots of cv. IAPAR59 after a prolonged N-deficiency period (Fig. 4), showing that *DUR3* is not only up-regulated by urea, but also by extended periods of nutrient depletion.

Drought, salinity and extreme temperatures are among the main environmental restrictions for coffee crop cultivation. Under such conditions, changes occur in the plant's ability to absorb, transport and assimilate the ions needed for its growth, including nitrogen.

Here, we were able to show that the *CaDUR3* is differentially modulated in coffee plants under the drought, heat and saline stresses (Fig. 3a–c). It is well known that abiotic stresses lead to major changes in nitrogen metabolism and are accompanied by significant changes in the expression of various genes involved in the plant's ability to absorb, transport and assimilate this nutrient. Previous

research showed that tonoplast intrinsic proteins (TIPs), and aquaporins that facilitate the movement of low-molecular weight molecules such as urea, boron and ammonia, among others (Chaumont and Tyerman 2017), play a role in the response to abiotic stresses.

Few studies to date have attempted to evaluate genes related to mineral nutrient uptake and defense response to pathogens, especially in perennial crops. However, it is known that nutritional imbalances associated with N can influence negatively to increase of disease in plants (Roca et al. 2018; Thalineau et al. 2018). Also, studies have demonstrated that the source of N can also influence the severity of a disease (Pageau et al. 2006; Gupta et al. 2013). Here, we showed that the expression of *CaDUR3* was higher in *H. vastratix* infected leaves of the susceptible *C. arabica* cultivar (CV 99) when compared with the tolerant genotype (IAPAR59) in almost all sampling points during infection (Fig. 7). This may indicate that the higher level of expression of *CaDUR3* gene may reflect the impaired physiological and metabolic status of the susceptible genotype during infection (Baba et al. 2020).

Overall, in this study we demonstrated the multiplicity of factors that modify the mRNA abundance of the *CaDUR3* gene. Although DUR3 play a fundamental role in the high affinity urea acquisition by the roots, the *CaDUR3* was shown to be transcriptionally present in other vegetative and reproductive tissues of *C. arabica*. Here, we also showed that this gene was upregulated by exposure of *C. arabica* plants to different abiotic stresses and depending on the genotype resistance, to *H. vastratix* infection. From an agricultural point of view, this gene may be considered a strong candidate for coffee breeding programs aiming at the efficient use of nitrogen with the objective of increasing the quality and production of coffee fruits.

Conclusions

To our knowledge, this is the first study on the transcriptional changes of the *DUR3* gene in *C. arabica* plants. Our expressional analysis of *CaDUR3* has provided new understanding for the molecular functions of this gene in *C. arabica* plants, especially under biotic and abiotic stresses. Considering the importance of urea as a source of nitrogen in coffee production, and that its absorption may be dependent on the combined performance of high transport (DUR3) and low affinity systems, further analysis using heterologous gene expression would be essential for determining the contribution of each transporter system in plants subject to abiotic and biotic stresses under field conditions.

Acknowledgments This research was supported by of the Brazilian Coffee Research Consortium and National Council of Technological and Scientific Development (CNPq). Special acknowledge to Dr. Anderson Rotter Meda for participating in the beginning of these studies.

Compliance with ethical standards

Conflict of interest The author declare that they have no conflict of interest.

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