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An integrated analysis of mRNA and sRNA transcriptional profiles in *Coffea arabica* L. roots: insights on nitrogen starvation responses

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Abstract

Coffea arabica L. is an important agricultural commodity, accounting for 60% of traded coffee worldwide. Nitrogen (N) is a macronutrient that is usually limiting to plant yield; however, molecular mechanisms of plant acclimation to N limitation remain largely unknown in tropical woody crops. In this study, we investigated the transcriptome of coffee roots under N starvation, analyzing poly-A+ libraries and small RNAs. We also evaluated the concentration of selected amino acids and N-source preferences in roots. Ammonium was preferentially taken up over nitrate, and asparagine and glutamate were the most abundant amino acids observed in coffee roots. We obtained 34,654 assembled contigs by mRNA sequencing, and validated the transcriptional profile of 12 genes by RT-qPCR. Illumina small RNA sequencing yielded 8,524,332 non-redundant reads, resulting in the identification of 86 microRNA families targeting 253 genes. The transcriptional pattern of eight miRNA families was also validated. To our knowledge, this is the first catalog of differentially regulated amino acids, N sources, mRNAs, and sRNAs in Arabica coffee roots.

Keywords Coffee · Nitrogen transport · Differential gene expression · RNA-seq · microRNA

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Introduction

Coffee is one of the most important agricultural commodities in the world, representing a significant source of income for several tropical developing countries (van der Vossen et al. 2015). Two species are economically important: *Coffea arabica* L. (Arabica coffee) and *Coffea canephora* Pierre ex Froehner (Conilon or Robusta coffee). *C. arabica* represents nearly 60% of traded coffee worldwide (van der Vossen et al. 2015).

With the development of functional genomics, large-scale transcriptome analysis based on expressed sequence tag (EST) sequencing, microarray hybridization, and RT-qPCR analysis of key genes have been extensively used to investigate the molecular basis of several features in Arabica coffee during fruit development stages (Joët et al. 2009, 2014; Ivamoto et al. 2017a; Ivamoto et al. 2017b), responses to abiotic (Marraccini et al. 2012), and biotic (Cardoso et al. 2014) stresses. More recently, next-generation sequencing technologies for quantitative determination of RNA levels (RNA-seq) have been incorporated into studies on the coffee transcriptome. mRNA sequencing (RNA-seq) allowed significant progress to improve our knowledge of the transcriptional profile of coffee genes under biotic and environmental challenges (Fernandez et al. 2012; Bertrand et al. 2015; Mofatto et al. 2016). However, most of these studies have focused on leaf or fruit development, while the transcriptional profile of below ground organs remains poorly understood. For instance, less than 2% of all Arabica coffee publicly available ESTs have originated from root cDNA libraries (Vieira et al. 2006; Mondego et al. 2011). Thus, there is a need for large-scale studies that unravel the repertoire of transcriptionally active genes in *C. arabica* roots.

Nitrate and ammonium are the main sources of N for agricultural systems. Both substances can be absorbed by the roots through specific transporters for ammonium (AMT1 and AMT2 families), and nitrate (NRT1 and NRT2 families) coded by multigenic families (Rennenberg et al. 2010; Xu et al. 2012). Léran et al. (2014) reviewed and proposed a new nomenclature for nitrate transporters, where NRT1/PTR family were denominated as NPF members. On average, angiosperms have five members of each *AMT* family, four *NRT2*, and more than 50 *NRT1* genes (von Wittgenstein et al. 2014). Several studies have shown that these transporters are transcriptionally modulated by N availability (Scheible et al. 2004; Cai et al. 2012; Wei et al. 2013), and can also affect N net fluxes in roots (Sorgona et al. 2011; Luo et al. 2013). After uptake, nitrate is reduced to nitrite in the cytoplasm by nitrate reductase (NR) enzyme. Nitrite is then reduced to ammonium in the plastid by nitrite reductase (ammonium can enter directly at this step) for assimilation by glutamine synthetase (GS) enzyme (Coleman et al. 2012; Castro-Rodríguez et al. 2015). Additionally, N assimilation processes can also be transcriptionally regulated under N starvation (Ruffel et al. 2008; Krapp et al. 2011).

Gutiérrez et al. (2007) were the first to suggest that N metabolism could also be regulated by non-coding short RNAs, like microRNAs (miRNAs). For example, in *Arabidopsis thaliana*, miR156, miR169, and miR398 responded to N limitation (Pant et al. 2009; Zhao et al. 2011), whereas Liang et al. (2012) identified five miRNA families (miR160, miR780, miR826, miR842, and miR846) that are induced by N deficiency in *Arabidopsis* using small RNA deep sequencing analysis. Additionally, miR393 and one of its targets, the auxin receptor AFB3, act as molecular players mediating NO_3^- regulation of primary and lateral root growth (Vidal et al. 2010). Thus, it is now well accepted that miRNAs play important roles as regulators of nutrient metabolism in plant responses to low N stress (Kulcheski et al. 2015; Nath and Tuteja 2016). RNA-seq can also be used to analyze the small RNA (sRNA) component of the transcriptome when libraries are prepared from low-molecular weight RNA fractions. To date, large-scale analyses of *C. arabica* miRNAs have been based only on Sanger ESTs, and genome survey sequences (GSSs) (Akter et al. 2014; Devi et al. 2016), but with a clear bias imposed by the scarcity of available data from roots. Based on these informations, and knowing that integrated mRNA-seq and sRNA-seq analysis can make important contributions to understand root molecular responses to N starvation (Vidal et al. 2013), our study used high-throughput sequencing technologies to characterize a *C. arabica* root transcriptome in control conditions, with and without N supply. To support our analysis, we conducted experiments to address N uptake, assimilation, and amino acid profiles in coffee roots. This is the first study to simultaneously profile the mRNAs and miRNAs transcriptome in Arabica coffee and to perform a molecular characterization of Arabica coffee roots using bioinformatics and biochemical and molecular approaches.

Material and methods

Plant material and experimental conditions

Basic procedures for obtaining N-starved roots followed previous studies from our research group (de Carvalho et al. 2013; dos Santos et al. 2017). Arabica coffee seeds (*Coffea arabica* L. cv. IAPAR59) were germinated in greenhouse using boxes containing sterilized sand (Fig. S1). Seedlings were irrigated twice weekly with distilled water in the first 30 days, and with Clark nutritive solution (Clark et al. 1975) with pH adjusted to 5.5–6.0 for the remainder of the study. After 4 months, plants were selected by size uniformity (plants with three leaf pairs). Roots were washed thoroughly with distilled water and the plantlets were transferred to 4 L pots (three plants per pot) with nutrient solution, constant aeration, and 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/s). Plants were grown in nutrient solution containing 800 μM K_2SO_4, 250 μM, $MgSO_4$, 200 μM KH_2PO_4, 500 μM $CaCl_2$, 4000 μM NH_4NO_3, 100 μM NaFeEDTA, 5 μM H_3BO_3, 3 μM $MnSO_4$, 2.5 μM $ZnSO_4$, 0.1 μM $CuSO_4$, and 0.7 μM NaMoO with pH 5.8. The pH of the nutrient solution was adjusted to 5.5–6.0 every 2 days using sodium hydroxide or hydrochloric acid, and renewed weekly. After 4 weeks, lateral roots were harvested with a minimum of three biological replicates per sampling at 0, 1 day (24 h), and 10 days after transfer into an N-free nutrient solution. Sampling was always done between 9:30 and 10:30 h. Biological replicates were represented by pools of lateral roots of at least nine plants at the same developmental stage. After harvesting, all samples were immediately frozen in liquid nitrogen and stored at -80 °C until use. These samples were used for all analyses, except for the ^{15}N influx experiments.

Total N concentration

Total nitrogen content in roots was analyzed by spectrophotometry according to Miyazawa et al. (1999).

Glutamine synthetase activity

GS activity was measured using procedures similar to those used in previous studies of coffee plants (Pompelli et al. 2010). Plant material was homogenized in buffer containing 25 mM Tris-HCl (pH 7.6), 1 mM $MgCl$, 1 mM EDTA, 14 mM β-mercaptoethanol, and 1% (w/v) polyvinylpyrrolidone (PVP). The homogenate was centrifuged at 25,000 g for 30 min at 4 °C. Glutamine synthetase activity was determined using hydroxylamine as a substrate and the formation of γ-glutamyl hydroxamate (γ-GHM) was determined using acidified ferric chloride (Wallsgrove et al. 1979; Cruz et al. 2004). The GHM γ-glutamine was quantified as standard and the absorbance was measured at 540 nm. For each time point, GS activity was measured in three biological replicates.

Nitrate reductase activity

In vitro nitrate reductase (NR) activity was measured according to Rossa (1999) with modifications made by Dovis et al. (2014). Approximately 70 mg of tissue were ground in liquid N in a mortar, suspended in an extraction buffer (0.2 M sodium phosphate buffer, pH 8.0 [2.8% (w/v) and $NaH_2PO_4.H_2O$ and 97.2% (w/v) Na_2HPO_4. 7 H_2O]; 5 mM EDTA; 1 mM DTT; 0.3% (w/v) BSA), and centrifuged at 12,000 rpm and 4 °C for 15 min. The supernatant was collected and incubated in the reaction buffer (0.2 M sodium phosphate buffer, pH 8.0; 6 mM KNO_3, 0.5 mM $MgSO_4$) at 25 °C, and 0.04 mM NADH was then added to start enzymatic reaction. Controls without the addition of NADH were included for each treatment. The

reaction was stopped with 1.4 mM ZnSO and 43% (v/v) ethanol and the material centrifuged at 12,000 rpm and 4 °C for 10 min. The nitrite produced by NR activity was analyzed spectrophotometrically at 543 nm using a 1-cm polypropylene optical path cuvette after adding 9.6 mM sulfanilamide and 0.7 mM N-(1-naphthyl) ethylenediamine dihydrochloride (NED) to the extract. A 0.1 mM NaNO solution was used for the preparation of the standard curve. The in vitro activity of NR (NRA) was calculated according to the formula:

$$NRA = (EBt \times NO2^- \text{ produced})/(EBi \times t \times FW)$$

Where EBt = total volume of crude extract, EBi = volume of crude extract incubated, t = incubation time, and FW = fresh weight. The result is expressed as U g^{-1}, assuming that the activity of one NR unit (U) corresponds to 1 μmol nitrite produced per minute (Chapman and Harrison 1988) at a constant temperature of 25 °C. Three biological replicates were analyzed per time point.

Total protein content

To determine total protein concentration, frozen samples were homogenized in 2 ml of 50 mM potassium phosphate buffer (pH 7.3), 1% PVP (w/v), and 0.1 mM EDTA previously cooled to 4 °C. After centrifugation for 10 min at 4 °C and 7500 rpm, the supernatant was transferred to a different tube and kept on ice until the time of analysis. The extracts were quantified by the Bradford method (1976) using bovine serum albumin (BSA) as standard.

Total free amino acids content

The stored roots (-80 °C) were macerated in liquid nitrogen using a mortar and pestle and added 80% ethanol. Next, the mixture was homogenized, incubated at 80 °C for 20 min, and centrifuged at 10,000 rpm for 10 min. Total amino acid concentrations were determined based on the methodology described by Praxedes et al. (2006). The reaction mixture contained 50 μl of 1 kmol m^{-3} citrate-NaOH buffer (pH 5.2) with 0.2% (w/v) ascorbic acid, 100 ml of ethanolic extract, and 70 ml of 1% (v/v) ninhydrin in 70% (v/v) ethanol. The free amino acids concentration was estimated using a standard curve with an equimolecular mixture of glycine, glutamic acid, phenylalanine, and arginine in 70% (v/v) ethanol. The absorbance of the mixture was measured spectrophotometrically at 570 nm.

Individual amino acids analysis by UPLC-MS

For amino acids analysis, ground samples (100 mg) were extracted with 1 ml of methanol/water (50:50 v/v) solution for 30 min in ultrasonic bath. Next, the mixture was centrifuged at 7000 rpm for 10 min. The supernatant was collected into a vial

and directly analyzed by ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS). The samples were analyzed in an Acquity UPLC-MS system (Waters, Milford, MA USA) and data were acquired by injection of 7 µl of supernatant. Chromatographic separation was performed in a Waters Acquity C_{18} BEH analytical column (150 mm × 2.1 mm i.d., 1.7 µm) at 30 °C. Methanol (A) and 0.1% formic acid in water (B) were used as the mobile phase at a flow rate of 0.2 ml/min. The initial gradient condition was kept at 1% A and 99% B for 2.5 min, followed by a change to 50% A and 50% B for 2.5 min and back to the initial condition at 8 min. ESI(+)-MS and tandem ESI(+)-MS/MS were obtained under the following conditions: capillary, 3.5 KV; cone, 30 V; source and desolvation temperature, 150 and 300 °C, respectively. For ESI(+)MS/MS, the energy for the collision-induced dissociations (CID) was 15 eV. Data were acquired in the 50–300 m/z range. Ions were identified by the comparison of their m/z, retention time, and ESI(+)MS/MS dissociation patterns with pure standards.

^{15}N influx experiment

^{15}N uptake in coffee roots was measured using ^{15}N-labeled ammonium nitrate as N source with either nitrate (K^{15}NO) or ammonium (^{15}NH$_4$SO$_4$). To measure high and low-affinity N transport, ^{15}N-labeled ammonium nitrate was added to the N-free nutrient solution at two concentrations (0.2 mM and 2.0 mM) (Kojima et al. 2007; Camañes et al. 2009). During the acclimation period of the *C. arabica* L. cv. IAPAR59, seedlings were grown hydroponically under non-sterile conditions in a greenhouse with the following regime: 14/10 h light/dark and 28/18 °C temperature. Plants were grown in nutrient solution containing 1 mM KH$_2$PO$_4$, 1 mM MgSO$_4$, 250 µM K$_2$SO$_4$, 250 µM CaCl$_2$, 100 µM Na-Fe-EDTA, 50 µM KCl, 50 µM H$_3$BO$_3$, 5 µM MnSO$_4$, 1 µM ZnSO$_4$, 1 µM CuSO$_4$, and 1 µM NaMoO$_4$ (pH adjusted using 2 mM MES, pH 5.8; Sigma-Aldrich, St. Louis, MO, USA). The nutrient solution was replaced every 2 days. After a 1-week acclimation period, plants were supplied with N-sufficient (+ N, 2 mM NH$_4$NO$_3$) or N-starvation (− N, without N supply) nutrient solution for 3 days. Influx measurements of ^{15}N-labeled N in plant roots were conducted after rinsing the roots in 1 mM CaSO$_4$ solution for 1 min followed by incubation for 10 min in nutrient solution containing 0.2 mM of ^{15}N-labeled with the equimolar concentration of either K^{15}NO$_3$ (98.18 atom% ^{15}N) or ^{15}(NH$_4$)$_2$SO$_4$ (60 atom% ^{15}N) as the sole nitrogen source, and washing in 1 mM CaSO$_4$ solution. Roots were harvested and stored at − 70 °C until use. Each sample was ground in liquid N and dried at 55 °C for 5 days. ^{15}N and % N determination was performed by isotope ratio mass spectrometry (IRMS) (ANCA-SL, Sercon, Crewe, CHS, UK) with 5 mg of dried samples. The results are presented as the mean and standard error (SE) of three biological replicates ($n = 3$).

RNA extraction

Total RNA from *C. arabica* L. cv. IAPAR59 roots was extracted using an adapted CTAB method for plants (Chang et al. 1993; Tong et al. 2012). RNA integrity was examined by 1% agarose gel electrophoresis and RNA preparations were treated with DNase (RNase-free; Invitrogen, Carlsbad, CA, USA). Absence of DNA contamination was confirmed by PCR using *GAPDH* primers and approximately 100 ng of RNA (data not shown). All samples were purified using the PureLink™ Micro-to-Midi Total RNA Purification System (Invitrogen, Carlsbad, CA, USA) to have an A260/A280 > 2.0. RNA concentration and absorbance ratios were determined using a NanoDrop™ 1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

High-throughput sequencing: mRNA and sRNA libraries

mRNA was isolated from 20 µg of total RNA using DynaBeads mRNA purification kit (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. RNA was quantified using the Quant-iT RiboGreen RNA Reagent (Invitrogen) and assessed for integrity by electrophoresis using an Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA, USA).

Five hundred nanograms of mRNA were used for cDNA library construction and sequencing. First, the mRNA was fragmented using ZnCl$_2$ solution at 70 °C for 30 s. The first-strand cDNA was synthesized applying random primers according to the standard cDNA Synthesis System (Roche Diagnostics, Basel, Switzerland). The cDNA was then subjected to end-repair followed by adaptor ligation using the cDNA Rapid Library Prep kit (Roche Diagnostics). cDNA libraries were purified with Agencourt AMPure XP beads (Beckman Coulter Inc., Brea, CA, USA) and subjected to emulsion PCR amplification by applying two molecules of cDNA per bead (Roche Diagnostics) according to the manufacturer's instructions. Beads with clonally amplified cDNA library were selected and deposited in a picotiter plate for pyrosequencing using Titanium Sequencing Chemistry (Roche Diagnostics) with 200 flow cycles on a GS Junior 454 Sequencing System (Roche Diagnostics) following the manufacturer's protocols. In total, six libraries were constructed and sequenced, two for each time point (0, 1, and 10 days without N supply).

sRNA libraries were constructed using 1 µg of total RNA. The sRNAs were ligated with 3′ and 5′ adaptors, and RT-PCR was performed using the TruSeq Small RNA kit (Illumina, San Diego, CA, USA) according to the manufacturer's instructions. Library quality and quantity were tested using an Agilent 2100 Bioanalyzer (Agilent). Following purification, the cDNA from sRNA libraries was sequenced (50 bp [base

pairs], single-end) on an Illumina Genome Analyzer II at LC Sciences (Houston, USA). In total, nine sRNA libraries (three for each time point) were sequenced. Sequencing data containing data from mRNA-seq and sRNA-seq libraries have been deposited at the European Nucleotide Archive (ENA) (https://www.ebi.ac.uk/ena) under accession number ERP017352.

mRNA-seq: data processing, assembly, annotation, and differential expression analyses

Fastq-mcf version 1.04.662 (Andrews 2010) was used to process and trim reads from the 454 platform. We applied a quality filter to trim reads with mean Phred quality scores < 25, reads covered by more than 90% of homopolymers and low complexity sequences, reads with at least 5 Ns or 5 poly-A/Ts and adapters at the ends, and removing reads with minimum length of 40 after trimming.

De novo transcriptome assembly was initially performed using three assemblers: Newbler, CLC Genome Suite, and Trinity (data not shown). We opted to use Trinity assembly for further analyses because it yielded in the highest mean, median, and N50 contig length values and the lowest singleton number. Assembly with Trinity was performed using default parameters (Grabherr et al. 2011), except for min_contig_length = 200, and included the CuffFly parameter. We further checked the quality of our assembled transcripts by comparing their sequences to a core set of eukaryotic genes using BUSCO (Simão et al. 2015). The comparison revealed 71.1% of BUSCO genes, indicating that assembly quality was similar to that of high-quality plant transcriptome assemblies (Xu et al. 2015).

Differential expression analysis mostly followed the methods of Ranjan et al. (2014). We used RNA-seq by expectation maximization (RSEM), which allows for an assessment of transcript abundances based on the mapping of trimmed RNA-seq reads to the assembled transcriptome, for transcript abundance estimation of the de novo assembled transcripts (Li and Dewey 2011). Reads from individual libraries were mapped to final transcripts using default RSEM parameters using the script un_RSEM_align_n_estimate.pl, followed by joining RSEM-estimated abundance values for each sample using merge_R-SEM_frag_counts_single_table.pl. Lastly, differential expression analysis was performed using run_DE_analysis.pl as implemented in the Bioconductor package EdgeR in the R statistical environment (Robinson and Oshlack 2010; R Development Core Team 2011). Differentially expressed genes (available at https://doi.org/10.7910/DVN/OIN1UW) were selected using two criteria: fold change > 2 and < − 2 and p value < 0.05.

For annotation, Blast2GO (Conesa and Gotz 2008) was first used to obtain Gene Ontology (GO) attribution of the assembled transcripts. Next, the GO functional classifications of unigenes were performed with Web Gene Ontology Annotation Plot (WEGO) (Ye et al. 2006). Assembled contigs were also annotated using TRAPID (Van Bel et al. 2013) to obtain overall functional statistics, protein domains, closest species homologs, and comparative analyses.

Transposable elements identification

All assembled contigs were compared to the RepBase (Jurka et al. 2005) plant nucleotide database (version 21.08; http://www.girinst.org/repbase/) and to reference transposable elements (TEs) from the *C. canephora* genome (Denoeud et al. 2014; Guyot et al. 2014) using tBLASTx. Contigs were considered related to TEs when there was a minimum alignment of 100 bp, a score > 150, 75% of identity, and a 1e-05 e value in tBLASTx. Intersections between the two databases were highlighted using InteractiVenn (Heberle et al. 2015).

Identification of non-coding mRNA transcripts

The coding potential of assembled contigs was estimated using coding potential calculator (CPC) to discriminate coding and non-coding RNAs (Kong et al. 2007). Contigs were considered as non-coding sequences if they had a CPC score < 0 in both senses and returned "no hit" when submitted to tBLASTx analysis against Uniref90 protein database.

sRNA-seq: data analysis, miRNA prediction, and target prediction

Basic analyses were performed based on previous studies that aimed to identify microRNAs in plants (Guzman et al. 2012; Katiyar et al. 2015; da Silva et al. 2016). All procedures for miRNA prediction used the UEA sRNA workbench V3.2-Plant version (http://srna-tools.cmp.uea.ac.uk/) (Stocks et al. 2012). Low-quality reads (FASTq values < 13), low complexity sequences (sequences with < 3 distinct nucleotides), and 5′ and 3′ adapter sequences were all trimmed using the "sequence file pre-processing tool." Plant t/rRNAs from Rfam (excepted miRNAs), *Arabidopsis* tRNAs from "The Genomic tRNA Database," and plant t/rRNA sequences from "EMBL" release 95 were removed using the "filter pipeline" from UEA sRNA workbench. Sequences shorter than 18 nt and larger than 25 nt were also excluded from further analysis. For the identification of miRNAs, all unique reads obtained after filtering were submitted to the miRCat pipeline from UEA sRNA toolkit-Plant version, and default criteria were used to predict specific and conserved miRNAs. Reads were first mapped against the *C. canephora* genome (Denoeud et al. 2014). The remaining reads were re-mapped against the de novo assembled transcriptome. Potential precursors were

identified following the criteria for plant microRNAs annotation (Meyers et al. 2008) to recover the potential miRNA candidates. To identify conserved miRNAs, all the predicted miRNA sequences were mapped to known mature plant miRNAs deposited in miRBase v21 (Kozomara and Griffiths-Jones 2014) using an in-house Pearl script. A maximum of three mismatches were allowed during the alignment. Sequences with more than three mismatches or no homology to any previously known plant miRNAs were considered as novel miRNAs. We used the assembled root transcriptome as input reference for the identification of putative miRNAs targets in psRNATarget (http://plantgrn. noble.org/psRNATarget/) (Dai and Zhao 2011) with default settings: maximum expectation: 3; length for complementarity scoring: 20; maximum energy to unpair the target site (UPE): 25; flanking length around target site for target accessibility analysis: 17 bp; and range of central mismatch leading to translation inhibition: 9–11 nt.

Clean reads fully matching other RNAs, including repeat RNA, rRNA, snRNA, snoRNA, and tRNA, were excluded from the analysis. For newly discovered miRNAs, we included only those with their miRNA's co-existing in at least one library. To further validate the existence of miRNA precursors, we aligned miRNAs against the *C. canephora* genome (Denoeud et al. 2014) and *C. arabica* transcriptome datasets (Mondego et al. 2011).

RT-qPCR analysis

Total RNA extraction of root cDNA was performed as described in the "RNA isolation" section. Complementary DNA (cDNA) was synthesized using the SuperScript III Reverse Transcriptase kit (Invitrogen) following the manufacturer's instructions and 5 μg of total RNA for a final volume of 20 μl. For each 1 μg of total RNA extracted, 500 ng of oligo (dT) 12–18 and 1 mM dNTP mix were used. Removal of the original RNA was performed by incubating this reaction at 37 °C for 20 min with two units of RNase H (Invitrogen). The final cDNA products were diluted in 50 ng for use in RT-qPCR.

The transcriptional levels of six differentially expressed genes and six N transporters were evaluated by quantitative PCR (RT-qPCR) on a 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Primers specific for each gene were designed using Primer Express (Tables S1 and S2). Thermal conditions were 95 °C for 10 min, followed by 40 cycles at 95 °C for 30 s, and 60 °C for 60 s. All reactions were performed in triplicate for each of the three biological replicates, following the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines (Bustin et al. 2009). Dissociation curves were constructed to determine the presence of nonspecific products and primers were used only in the case of a single

peak, with amplicons checked in an agarose gel. The mean amplification efficiency of each primer pair was checked using the LinRegPCR program (Ramakers et al. 2003). Data were analyzed using the $(1 + E)^{-\Delta\Delta ct}$ method (Livak and Schmittgen 2001; de Carvalho et al. 2014), where E represents the efficiency, with day 0 used as calibrator. RT-qPCR reactions were normalized using the *CaEF1α* gene (de Carvalho et al. 2013).

cDNA synthesis for miRNAs

Basic procedures for cDNA synthesis followed those used in a recent transcriptional evaluation of miRNAs in coffee plants (Chaves et al. 2015). Stem-loop reverse transcription primers were designed according to Chen et al. (2005) and Varkonyi-Gasic et al. (2007). For cDNA synthesis reaction, we used 1 μg of total RNA, 1 μl of 10 mM dNTP, 2 μl of the specific primer for each miRNA, and 1 μl of oligo (dT) (500 ng/μl). The samples were incubated at 70 °C for 10 min for denaturation of the secondary structures and later incubated at 4 °C for 10 min. Next, we added 5 μl of 5X First-Strand Buffer (Invitrogen), 1.2 μl of 25 mM MgCl, 0.6 μl of RNase out (Invitrogen), and 1 μl of SuperScript III Reverse Transcriptase (Invitrogen). Reactions were run in a thermocycler under the following conditions: 16 °C for 30 min followed by 60 cycles at 30 °C for 30 s, 42 °C for 30 s and 50 °C for 1 s and 70 °C for 5 min. cDNA products were diluted in ~ 750 ng/μl for use in RT-qPCR analyses.

miRNAs transcriptional profile by RT-qPCR

We determined the transcriptional profile of eight miRNAs (miR159, miR166, miR167, miR169, miR171, miR393, miR858, and miRcar1; Table S3) in coffee roots by pulsed stem-loop RT-qPCR (Varkonyi-Gasic et al. 2007; Chaves et al. 2015). All reactions were run on a 7500 Fast Real-Time PCR System (Applied Biosystems). Each PCR reaction (10 μl) included 1 μl of cDNA, 5 μl of SYBR Green Master Mix (Life Technologies), 0.5 μl of sequence-specific forward primer (10 μM), 0.5 μl of universal reverse primer (10 μM), and 3 μl of water. The reaction conditions were 5 min at 94 °C followed by 15 s at 95 °C and 40 cycles of amplification at 60 °C for 10 s and 72 °C for 15 s. For all reactions, dissociation curve analysis was performed to determine the presence of nonspecific products. *CaEF1α* was used as the reference gene (de Carvalho et al. 2013). Dissociation curves were constructed for biological and technical triplicates. Amplification efficiency was evaluated using LinRegPCR (Ramakers et al. 2003). The relative expression levels of miRNAs were calculated according to the cycle threshold (Ct) method ($[1 + E]^{-\Delta\Delta ct}$) (Livak and Schmittgen 2001; de Carvalho et al. 2014) using day 0 as calibrator.

Statistical analysis

Statistical tests were performed using SISVAR software (Ferreira 2011) and means were compared by the Tukey's test at 5% significance. The Duncan, Dunnett's, and Student's t tests were used for amino acid analysis by UPLC-MS in SAS software (SAS Institute 2004). Data for differentially expressed gene candidates, genes regulated by N starvation, and miRNAs were analyzed by analysis of variance (ANOVA) and the means were compared by the Student's t test ($p < 0.05$) using ASSISTAT software (Silva and Azevedo 2013).

Results

Amino acid levels and N influx increase in coffee roots in response to N starvation

To characterize the root-specific response to N starvation, coffee plants grown in nutrient solution under a long-day cycle and high N supply were transferred to N-free nutrient solution for 10 days. After short-term (1 day) and long-term (10 days) N starvation, levels of N assimilation-related metabolites, total amino acids, total protein, and the activity of key enzymes involved in N metabolism, namely nitrate reductase (NR) and and glutamine synthetase (GS), were measured to characterize root responses to N starvation.

Total N content in coffee roots during N starvation showed a slight increase of ~8% after 1 day followed by a reduction of ~2% at 10 days after N restriction (Fig. S2a). Although NR and GS activity in roots increased slightly, no significant differences were detected after 1 and 10 days of N restriction, indicating that N assimilation in the roots of N-limited coffee plants was stable (Figs. S2b and S2c).

High-affinity transport systems (HATS) regulate N influx to the roots as an adaptive response to N starvation (Forde 2000; Loqué and von Wirén 2004; Glass et al. 2002). To measure the temporal regulation of HATS during N starvation, nitrate and ammonium influxes were measured at a concentration of 0.2 mM ^{15}N-labeled in plants grown in N-free nutrient solution for 1, 3, and 10 days. Short-term influx analysis after 1 and 3 days of N deprivation showed that ^{15}NO$_3$ uptake increased 1.8- and 2-fold in coffee roots, respectively, and was below half that of N-sufficient roots after prolonged N-deficiency (10 days) (Fig. 1). HATS activity for ammonium showed a transient, 5-fold ^{15}NH$_4$ influx increase in the roots after 1 and 3 days of N starvation. In contrast, extended N starvation reduced ^{15}NH$_4$ influx to roughly the levels of N-sufficient roots, with only a 1.4-fold increase in ^{15}NH$_4$ influx relative to the onset of N starvation (Fig. 1). These results indicate that for both inorganic N forms, HATS activity is
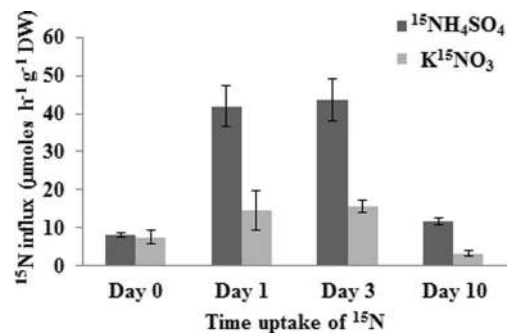

Fig. 1 Measurement of labeled nitrogen uptake in coffee roots along nitrogen starvation period. The coffee plants were pre-cultivated hydroponically in 2 mM NH$_4$NO$_3$ for 3 days before influx measurements of labeled ^{15}N. Values are means ± SE of three replicates

transiently induced under N deficiency, resulting in a temporal increase in N uptake capacity in coffee roots. In addition, at the beginning of N starvation, ^{15}NH$_4$ influx exceeded that of ^{15}NO$_3$ by 65%, and at 10 days of N deficiency, HATS activity for ammonium represented approximately 70% of the total ^{15}NO$_3$ uptake (Fig. 1), suggesting a relative preference of coffee roots for uptake of NH$_4^+$ over NO$_3$ when N availability is limited.

Total amino acid levels increased by ~5% after 1 day of starvation and remained constant after long-term starvation (Fig. 2b). The initial increase in total amino acids (TAA) concentration in roots may have been caused either by a remobilization of amino acids from the shoots to the roots or by the degradation of proteins in the roots. Indeed, total protein levels in roots increased by ~10% only 10 days after N deprivation (Fig. 2a), indicating that amino acids transported from the shoots may support the synthesis of essential proteins.

UPLC-MS analysis revealed that amino acid depletion was affected in response to N starvation (Fig. 2c). The most abundant amino acids in coffee roots were asparagine (Asn) and glutamate (Glu), whose concentrations increased by ~5 and ~10%, respectively, after 10 days of N starvation (Fig. 2c). Rapid and transient increases were observed for glutamine (Gln) and aspartate (Asp) levels after 1 day, but their concentration was reduced to ~60% of the initial levels after 10 days (Fig. 2c). Alanine (Ala), proline (Pro), and serine (Ser) showed lower absolute levels compared to other amino acids, but their concentration increased during short-term starvation (1 day) (Fig. 2c). Other minor amino acids are not shown. These changes in amino acid levels likely reflect the metabolic changes of coffee roots to adjust N supply in response to N limitation. The changes in TAA and protein levels described above indicate that N starvation in coffee roots leads to a temporal adaptation that relies on inducing HATS activity and amino acid transport to cope with N-limited conditions.
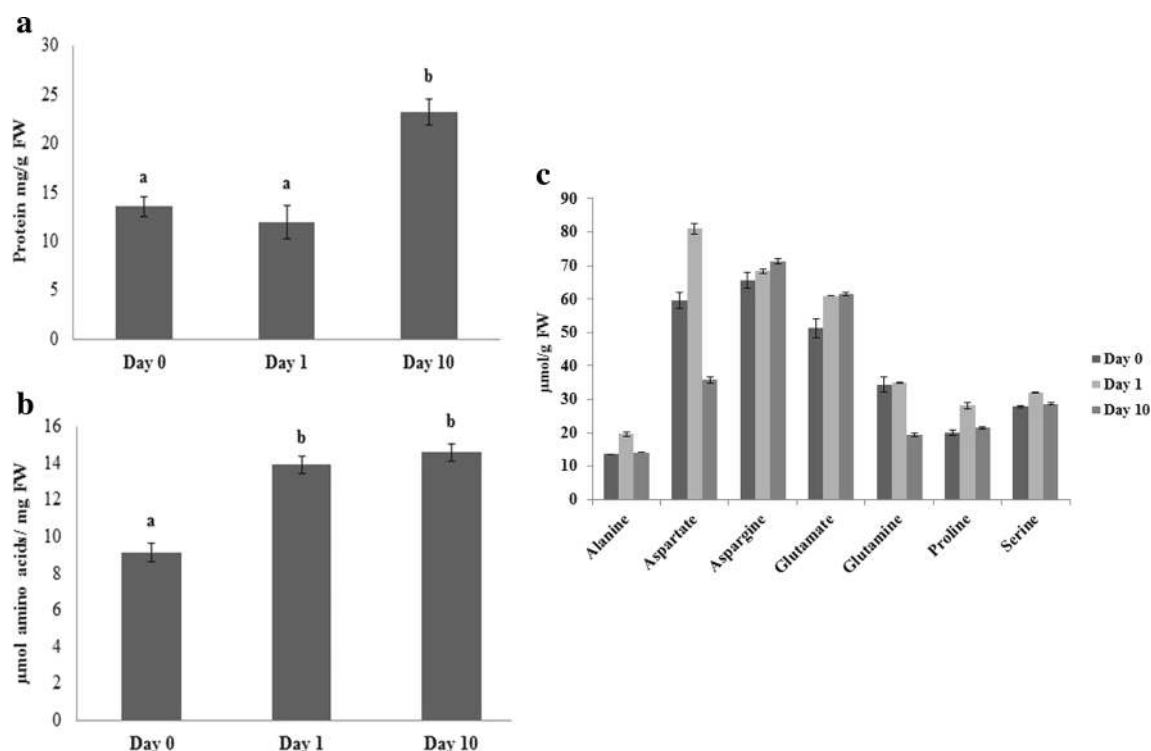
Fig. 2 Amino acid quantification results during nitrogen starvation experiment. **a** Total protein content and **b** total free amino acid concentration in coffee roots under nitrogen suppression. **c** Amino acid levels during nitrogen starvation. Values are means ± SE. Different letters above the bars indicate statistically significant differences ($p < 0.05$; t test). *FW* fresh weight

mRNA-seq of Arabica coffee roots under N starvation

In total, 809,517 raw reads were generated from roots of control plants and plants grown for 1 and 10 days without N supply (Table 1). To perform the de novo assembly, we used 774,529 high-quality reads, generating 34,654 contigs. Our dataset (34,654 contigs, Table 1) is comparable to most coffee transcriptomic resources available: previous studies reported 35,113 contigs for *C. arabica* (Mondego et al. 2011) and 36,935 contigs for *Coffea eugenioides* (Yuyama et al. 2016).

Contigs ranged from 200 to 8514 bp, with a mean contig length of 966 bp, an average open reading frame (ORF) length of 569 bp, a N50 of 1314 bp, and a contig GC content of 43.4% (Tables 1 and 2). Most contigs had BlastX hits in the nr database with an *e* value cut-off of 1 e^{-5} (Fig. 3a). Almost 85% of all assembled genes have one or two isoforms (Fig. 3b).

We used BlastX tool to compare, annotate, and study the conservation of our transcriptome contigs from coffee roots to other plant species genome sequences. A total of 25,026 contigs had correspondents in sequenced plant genomes. The top three species with first hits to Arabica coffee sequences were *Vitis vinifera* (23.1%, 5774 contigs), *Theobroma cacao* (16.3%, 4080 contigs), and *Manihot esculenta* (11.8%, 2947 contigs) (Fig. 3c). Only 1.1% of the total contigs had their first hit in *A. thaliana*.

We mapped contigs of *C. arabica* roots against Gene Ontology using BlastX analysis. We were able to classify 20,963 contigs in GO terms and 22,501 transcripts had a protein domain. A summary of the contigs annotated according to the GO terms classification is shown in Fig. 3d.

Table 1 Statistical summary of mRNA-seq sequencing for *Coffea arabica* roots

Statistics for assembly in *C. arabica* transcriptome	
Contig number	34.654
Mean contig lenght	966
Maximum contig lenght	8.514
Minimum contig lenght	379
GC% content	43

Table 2 Size distribution of assembled transcripts and BlastX hits

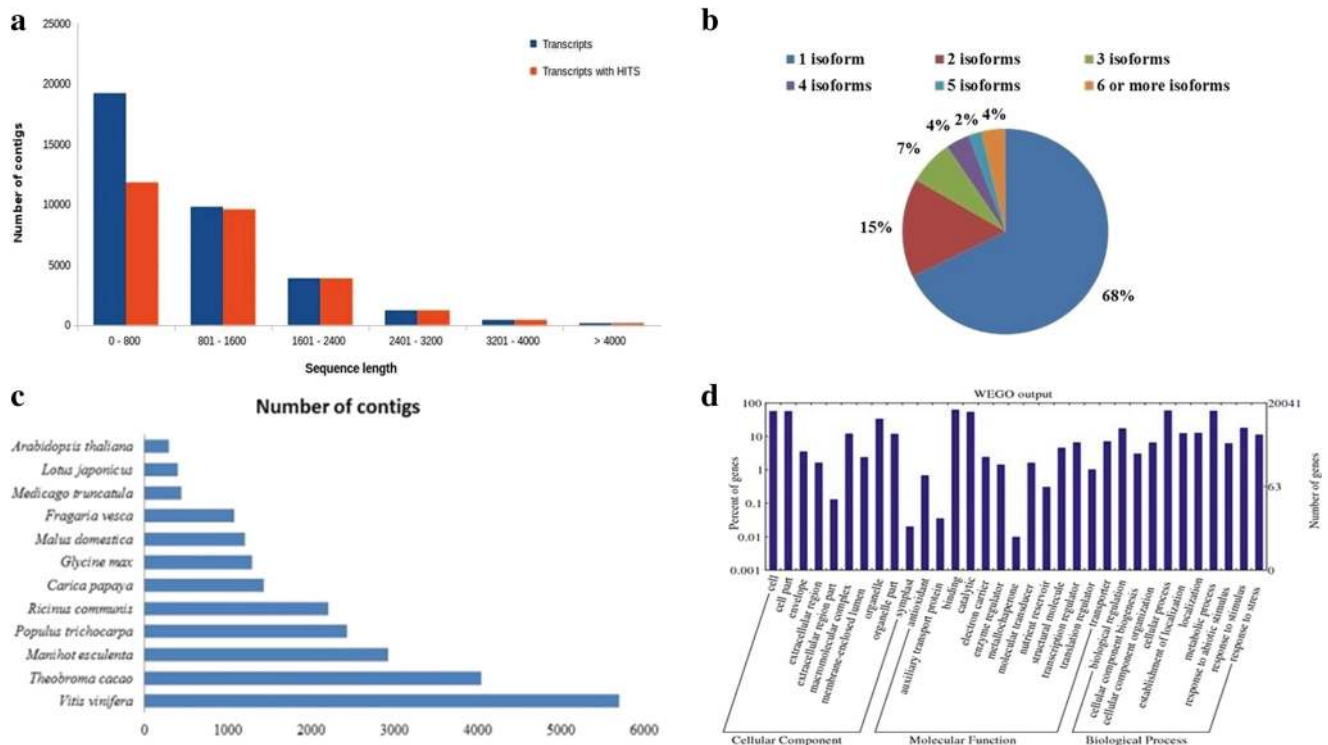|	Transcripts	Transcripts with BlastX Hits
0–800	19,229	10,662
801–1600	9783	9207
1601–2400	3874	3824
2401–3200	1202	1199
3201–4000	412	412
>4000 bp	154	154

Fig. 3 Coffee roots transcripts general information. **a** Length distribution of assembled contigs. Histogram of assembled transcripts and transcripts showing BlastX hits. **b** Isoform abundance in the assembled transcriptome. **c** The best 12 top-hit species distribution observed during *C. arabica* roots transcriptome annotation process. **d** Functional annotation of coffee root transcriptome based on WEGO classification. Classes were divided into three main categories: "cellular component," "molecular function," and "biological process." The x-axis indicates different categories described, the y-axis on the left indicates the percentages of genes involved, and the y-axis on the right indicates the number of genes

Validation of differentially expressed genes

To validate the differential expression of genes among libraries, we selected six differentially expressed genes (Table 3). Contigs comp10027_seq3 (TE-related transcript) and comp11089_seq8 (expansin) were selected in the 0×1 day combination; comp5715_seq2 (glycosyltransferase) and comp10285_seq3 (peroxidase) were differentially expressed at 0 and 10 days; and comp10246_seq1 (cytochrome P450) and comp8633_seq2 (plant kinase) were chosen based on their differential expression between days 1 and 10. The transcriptional validation of these genes using RT-qPCR revealed that this expression pattern is in agreement with the in silico expression analysis (Fig. 4).

Transcriptional analysis of N transporter genes

To investigate the transcriptional patterns of N transporter genes by RT-qPCR, we selected three ammonium transporters (AMTs) and three nitrate transporters (NRTs) based on a recent study by our research group

Table 3 Differentially expressed genes in mRNA-seq validated by RT-qPCR

| Gene identifier	InterPro domain	*Arabidopsis thaliana*	*Coffea canephora*	Up ▲ or downregulated ▼
comp10027_seq3	Not found (transposable element)	AT5G12010	Cc06_g11710	0×1 ▼ and 0×10 ▲
comp11089_seq8	Expansin	AT2G03090	Cc11_g15080	0×1 ▲
comp5715_seq2	Glycosyl transferase, family 3	AT1G70570	Cc04_g11160	0×1 ▼ and 0×10 ▲
comp10285_seq3	Plant peroxidase	AT5G05340	Cc01_g15110	0×1 ▲ and 0×10 ▲
comp10246_seq1	Cytochrome P450	AT4G31940	Cc10g_09170	0×1 ▲ and 0×10 ▼
comp8633_seq2	Protein kinase-like domain	AT2G40980	Cc06_g22710	0×1 ▼ and 0×10 ▲
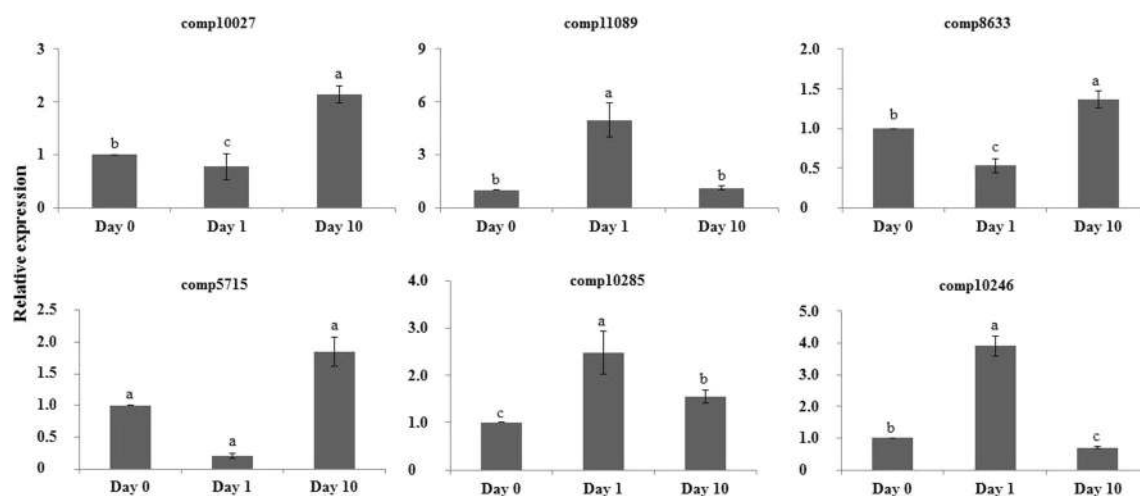
Fig. 4 Transcriptional analysis of differentially expressed genes in coffee roots under nitrogen starvation. Transcript levels were normalized using $CaEF1\alpha$ gene and day 0 as calibrator (relative gene expression = 1). Values are means ± standard errors (bars). Statistically significant differences are indicated by different letters (Student's t test; $p < 0.05$)

(Table 4; dos Santos et al. 2017). Most N transporters analyzed showed higher transcript abundance under long-term N starvation when compared to the N-sufficient condition (day 0) (Fig. 5). In particular, expression of *CaAMTa* was more than eight times higher in roots without N supply for 10 days than in control roots. We also observed similar upregulation for the *CaNRTc* gene (Fig. 5).

Transposable elements and non-coding transcripts

We identified 862 contigs in our analysis that contained TE fragments (Table S4). Most hits (806) were related to *C. canephora* transposable elements, whereas 35 contigs were related to TEs uniquely based on RepBase reference TEs. In addition, 21 contigs were identified by both approaches (Fig. S3). Most annotated contigs (86%, 744 contigs) were identified as belonging to class I elements (retrotransposons), which is I agreement with most analyses of transcriptionally active TEs in plants (Vicient 2010; Santos et al. 2015). In addition, 6192 transcripts had a CPC score < 0 and no hits in the Uniref database (Online Resource 1) and can be considered putative long ncRNAs.

Identification of miRNAs in N-deficient coffee roots by small RNA library sequencing

Nine small RNA libraries were prepared from roots maintained in N-sufficient and N-free nutrient solution. Small RNA libraries were sequenced using Illumina Hiseq2000, yielding a total of 86,826,830 redundant reads and 25,498,103 unique reads. After cleaning and trimming, 8,524,332 reads were retained for miRNA analysis. For redundant reads and unique reads, 21 nt was the most frequent length followed by 24 nt, similarly to what was reported for *Arabidopsis* roots under N starvation (Fig. 6; Vidal et al. 2013).

We identified 86 miRNA families when considering only sequences in the same library with miRNA and miRNA* strands and with precursors in the *C. canephora* genome or in our transcriptome. Among these families, 30 correspond to previously known miRNA families, whereas 29 families were found in the *C. canephora* genome (Table S5) and one family was found in the assembled contigs (Table S6). Fifty-six novel miRNA candidates were identified based on the presence of mature miRNA and miRNA* sequences in the same library using the miRCat pipeline; only one new family was

Table 4 N transporter genes analyzed by RT-qPCR

| Gene	InterPro domain	*Arabidopsis thaliana*	*Coffea canephora*
CaAMTa	Ammonium transporter	AT4G13510	Cc03_g06810
CaAMTb	Ammonium transporter	AT4G13510	Cc01_g14140
CaAMTc	Ammonium transporter	AT2G38290	Cc07_g19360
CaNRTa	Oligopeptide transporter	AT1G69850	Cc02_g36020
CaNRTb	Oligopeptide transporter	AT2G26690	Cc06_g08580
CaNRTc	Oligopeptide transporter	AT5G62680	Cc04_g15770
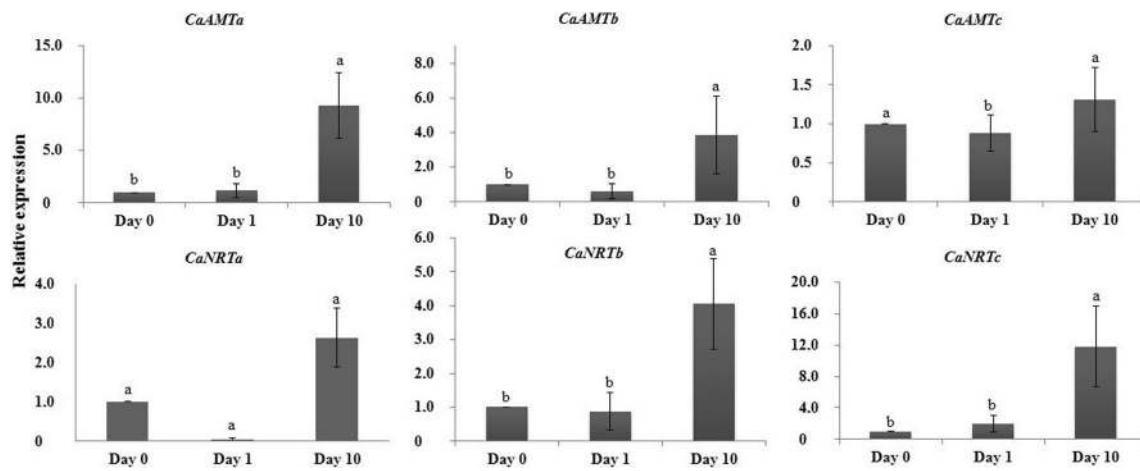
Fig. 5 Transcriptional analysis of N transporters. Transcript levels were normalized using *CaEF1α* gene and day 0 as calibrator (relative gene expression = 1). Values are means ± standard errors (bars). Statistically significant differences are indicated by different letters (Student's t test; $p < 0.05$)

identified in transcripts (Table S7) and 55 families were identified in the genome (Table S7). Putative hairpins are shown in Figs. S4, S5, S6, and S7.

Predicting miRNA targets

Subsequently, we identified targets for miRNAs using psRNATarget (Dai and Zhao 2011). For all miRNA families, we identified 253 target genes. These genes were functionally annotated using WEGO classification and according to their

respective cellular component, molecular function, and biological process results (Fig. S8).

Transcriptional profile of miRNAs in response to N starvation

We selected eight miRNA families for RT-qPCR transcriptional analyses (Table 5). Seven families were selected because they represent conserved miRNA families in plants with implications in N homeostasis (miR159,

Fig. 6 The total amount and reads length of small RNAs identified in our coffee root transcriptome analysis: **a** day 0, **b** day 1, and **c** day 10. Numbers in the *x*-axis indicate read size in nucleotides
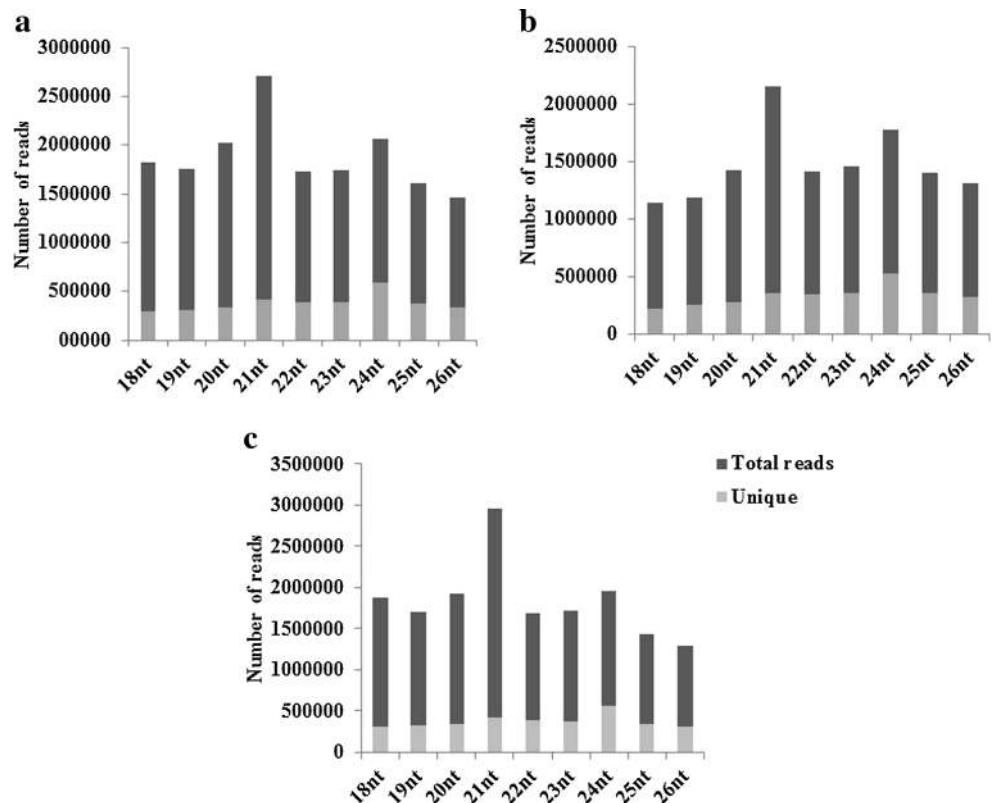
Table 5 microRNAs families and their possible targets in *Coffea arabica* roots identified by Illumina sequencing

MicroRNA family	Possible targets[a]—mRNAseq	Start-end position of target[a]	InterPro domain	First hit	
				Arabidopsis thaliana	*Coffea canephora*
miR159	comp6070_c0_seq2	539–548	Unavailable	AT5G24050	No hit found
	comp14548_c0_seq1	35–54	Peptidase C48, SUMO/Sentrin/Ubl1	AT5G60190	Cc01_g11690
	comp17045_c0_seq1	666–684	Unavailable	AT4G32190	No hit found
miR166	No hit found	–	–	–	–
miR167	comp16985_c0_seq1	23–42	Unavailable	AT4G30990	No hit found
	comp15125_c0_seq1	432–451	Cation/H+ exchanger	AT2G19600	Cc08_g10510
miR169	comp6797_c0_seq1	874–893	CCAAT-binding transcription factor, subunit B	AT1G72830	Cc08_g01380
	comp2134_c0_seq1	1433–1452	CCAAT-binding transcription factor, subunit B	AT1G72830	Cc04_g01480
	comp12135_c0_seq1	913–932	HAD-superfamily hydrolase, subfamily IIA, CECR5	AT3G45740	Cc01_g11880
	comp3298_c0_seq1	485–504	Protein of unknown function DUF620	AT3G19540	Cc02_g19530
miR171	comp9382_c0_seq1	1291–1310	GAGA binding-like	AT5G42520	Cc07_g10550
miR393	comp7250_c0_seq2	1736–1757	Cyclin-like F-box	AT3G26810	Cc07_g01170
	comp9341_c0_seq1	567–586	Uncharacterised protein family UPF0172	AT5G55940	Cc05_g15200
	comp10764_c0_seq2	1036–1055	Glycoside hydrolase, family 16	AT1G32170	Cc02_g26270
miR858	comp2343_c0_seq1	89–109	Myb transcription factor	AT2G47460	Cc01_g21250
miRcar1	comp8098_c0_seq1	852–872	Ethylene insensitive 3	AT1G73730	Cc01_g04200
	comp8659_c0_seq7	1217–1237	Unavailable	AT5G60360	Cc02_g25940
	comp7758_c0_seq2	1914–1933	Leucine-rich repeat	AT3G50690	Cc07_g09340
	comp12494_c0_seq1	159–178	RNA recognition motif, RNP-1	AT2G27790	Cc08_g15830
	comp14944_c0_seq1	1314–1334	Uncharacterised protein family UPF0120	AT3G55510	Cc02_g11370
	comp6487_c0_seq2	1196–1216	WD40 repeat 2	AT4G34280	Cc07_g06660
	comp3654_c0_seq1	372–391	Serine/threonine-protein kinase-like domain	AT5G61560	Cc02_g04620
	comp23850_c0_seq1	291–311	Unavailable	AT1G25320	no hit found
	comp17225_c0_seq1	25–45	Peptidyl-prolyl cis-trans isomerase, FKBP-type	AT1G07910	Cc05_g15330

[a] Start and end position of miRNA and target alignment

miR166, miR167, miR169, miR171, miR393, and miR858), and we validated the transcriptional profile of one new miRNA family (miRcar1) by stem-loop RT-qPCR (Table S8). The relative miRNAs expression profiles were transiently altered in coffee roots subjected to N deficiency (Fig. 7). Five miRNA families (miR169, miR171, miR167, miR393, and miR858) were upregulated after short-term N starvation (1 day). In contrast, miR159 was significantly overexpressed in roots after 10 days of N starvation, whereas miRcar1 was down-regulated after prolonged N restriction (Fig. 7). These results reveal distinct expression patterns of N-responsive miRNAs modulating the adaptive response to N fluctuations in coffee roots.

Discussion

Adaptive responses to N starvation in coffee roots

Nitrate and ammonium are taken up by roots and assimilated according to the plants demand for N. When N becomes limiting, the synthesis and degradation of individual amino acids are essential to provide N to sustain C metabolism to support plant growth and development (Stitt and Krapp 1999; Stitt et al. 2002). In the current study, TAA levels in coffee roots increased in the first day of starvation and remained high until 10 days of N restriction, and a similar response was observed for total protein levels in N-starved root at day 10 (Fig. 2a). The increase in total protein content likely reflects protein
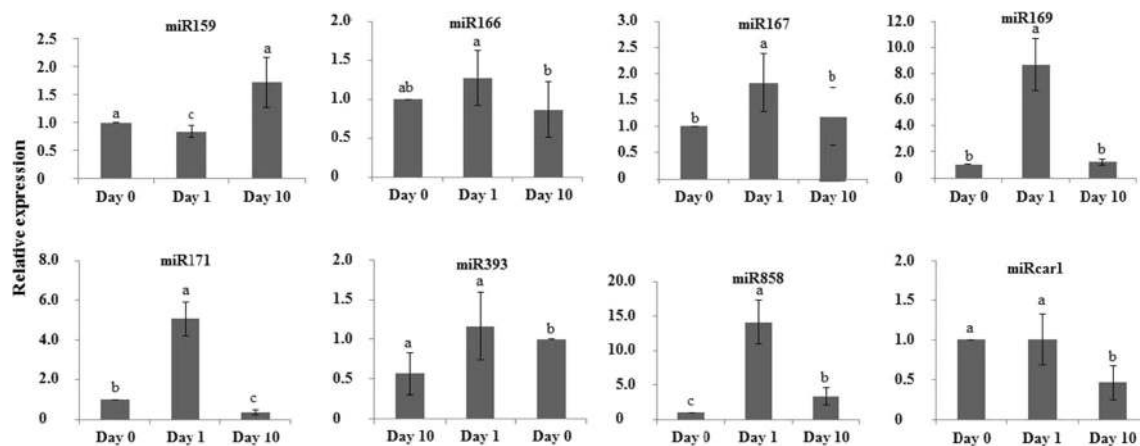
Fig. 7 Validation of the miRNAs transcriptional profile modulated by N starvation using RT-qPCR. miRNAs transcriptional levels were normalized with respect to the mean transcript level of the *CaEF1α* gene. Values are means ± standard errors (bars). Statistically significant differences are indicated by different letters (Student's *t* test; $p < 0.05$)

synthesis in roots as a result of the amino acids being exported from the shoots to the roots (Krapp 2011). Alternatively, the initial increase in TAA levels in coffee roots may be related to the induction of ammonium and nitrate HATS activity in N-starved roots after 1 day (Fig. 1). Induction of HATS activity for ammonium and nitrate production is a common response in plant roots to N limitation (Crawford and Glass 1998; Yuan et al. 2009); however, an intimate relationship between N uptake rates and N-assimilation was not observed in coffee roots, because NR and GS activity were not correlated with transient increases in N influxes (Fig. S2b and S2c). In addition, analysis of individual amino acids in coffee roots after 1 day of N starvation revealed that Gln remained at levels similar to those of N-sufficient plant roots, whereas Glu levels increased rapidly at 1 day of N restriction and remained high after 10 days (Fig. 2c), which supports the lack of GS activity at 1 day (Fig. S2c). Other nitrogen-containing metabolites such as Asp and Asn exhibited a different accumulation pattern in response to N starvation (Fig. 2c), confirming the alteration of the plants N status and suggesting a role for Asp, Asn, and Glu as N sources in the biosynthesis of other amino acids when N availability is limited in coffee roots (Forde and Lea 2007; Nunes-Nesi et al. 2010). Interestingly, the early accumulation of amino acids and N remobilization occurred before N was depleted in roots (Fig. 2b), clearly revealing a metabolic adaptation in this organ. A similar adaptive mechanism was previously shown to support root growth in *Arabidopsis* roots under long-term N deficiency (Krapp et al. 2011).

The lack of regulation of NR activity in coffee roots under N-limited conditions (Fig. S2b) indicates that nitrate assimilation occurs in the shoots (Carelli and Fahl 1991; Queiroz et al. 1991, 1993; Carelli et al. 2006). Moreover, accumulation of N metabolites such as Gln and Glu, as observed in N-starved coffee roots (Fig. 2c), has a negative feedback regulatory effect on NR activity in different plant species (Stitt et al. 2002;

Miller et al. 2007; Krapp et al. 2011). These results suggest that the nitrate taken up via HATS activity by coffee roots under N deficiency (Fig. 1) may be transported to the shoots to be assimilated or may act as signal to modulate N metabolism in the roots (Wang et al. 2000; Scheible et al. 2004; Krouk et al. 2010).

Altogether, these results indicate that N starvation induces a distinct temporal adaptation pattern in coffee roots through induction of nitrate and ammonium HATS activity and a rapid response for accumulation of amino acids via N remobilization before N is depleted in roots.

Differentially expressed genes

This is the first study to use mRNA-seq to understand the transcriptional patterns of genes under N starvation in coffee. We analyzed six differentially expressed genes that were modulated in Arabica coffee roots in response to N starvation (Table 3). The transcriptional pattern of comp10027, comp8633, and comp5715 revealed by RT-qPCR showed that the three contigs were upregulated in roots after 10 days of N deprivation (Fig. 4). Contig comp5715_seq2, an UDP-glycosyltransferase, has a homolog with higher expression in roots (At1g70570), whereas comp8633 is a zinc-finger kinase that is reported to have diverse functions in the regulation of specific genes (Noguero et al. 2013).

Expression of contig comp10246_seq, a P450 gene, was induced only in the first day of N deprivation. Cytochromes P450 is one of the largest gene super families of enzyme proteins and is found in the genome of all plants (Nelson 2011). P450 genes are often recruited in the biosynthesis of secondary metabolites and thus play important roles in several metabolic processes (Bak et al. 2012). Because At4g31940, the closest homolog of this gene in *Arabidopsis*, is preferentially expressed in roots (Ensembl Baseline Plant Expression Atlas, https://www.ebi.ac.uk/gxa/home; data not shown), we

can speculate that this P450 is possibly involved in the production of secondary metabolites during short-term N starvation. However, the transcriptional profile of comp11089 and comp10285 revealed that both contigs were specifically expressed during the first day of N starvation (1 day). Thus, we believe that Arabica coffee may also have different adaptations to short-term N stress.

AMTs and NRTs transcriptional patterns in coffee roots

We transcriptionally validated three AMT genes that were found in our mRNA-seq analysis of Arabica coffee roots under N starvation (Fig. 5). The AMT genes have been characterized in several plant species including, among others, Citrus spp. (Camañes et al. 2009), Solanum lycopersicum L. (Graff et al. 2011), Puccinellia tenuiflora (Bu et al. 2013), Medicago truncatula (Straub et al. 2014), Pyrus betulaefolia Bunge (Li et al. 2016), and Brassica alboglabra L. (Song et al. 2017).

The relative gene expression levels of all AMT and NRT family members analyzed in our study increased at 10 days, indicating that in short-term N deprivation, uptake is regulated at protein level, although both low- and high-affinity systems were induced by N deprivation. Interestingly, the only gene repressed at 1 day (Fig. 5) was the low-affinity transporter CaNRTa (dos Santos et al. 2017).

Even though this study was not aimed at assessing the role of ammonium and nitrate in root development, our results indicate that these ions transporters may play different roles in N assimilation (Fig. S2). Of the six genes that we transcriptionally analyzed (Fig. 5), AMT1 family members CaAMTa and CaAMTb were identified as candidate high-affinity NH_4^+ uptake transporters, whereas CaAMTc was classified as a low-affinity transporter based on dos Santos et al. (2017). According to Loqué et al. (2006) and Yuan et al. (2007), AMT1 family members account for approximately 70–80% of the high-affinity ammonium uptake capacity in roots. HATS activity is transiently induced under N deficiency (Fig. 1), resulting in a temporal increase in N uptake capacity in coffee roots, probably due to changes in the phosphorilation status of AMT transporters. Phosphorylated AMT transporters have restricted ammonium uptake capacity (Lanquar et al. 2009); in this sense, the dephosphorylation triggered by kinases is probably the first molecular transition in response to N deprivation. Nevertheless, a more precise characterization of the number and expression pattern of other AMT homologs in other C. arabica tissues is required.

We also analyzed the relative expression of three NRTs in coffee roots during N starvation. Based on the transcriptional pattern observed in our study, the CaNRTs examined are likely to be classified as members of the NRT1/PTR family in coffee (dos Santos et al. 2017). However, further research is needed

to determine the role of CaNRTs in coffee plants and measure the expression of this spatiotemporally regulated gene family. Hence, with all the presented results, we suggested a model for the ammonium and nitrate transporters in coffee roots under N starvation summarized in Fig. S9.

N starvation altered the expression pattern of miRNAs in coffee roots

The families of miRNA studied in our work, as already described in dicots and monocots plants, might play an important process role in response to the uptake and assimilation according to the availability of nitrogen in coffee roots. We observed an increase in the expression of miR159 after 10 days (Fig. 7), suggesting that this miRNA may play an important role in coffee roots under N starvation. miR159 has been shown to play an important role in regulating MYB and TCP transcription factors that are active in roots (Zhao et al. 2012; Wang et al. 2013). In potato (Solanum tuberosum), a reduction in miR159 expression resulted in increased expression of GAMyb-like genes possibly in response to drought stress (Yang et al. 2014).

In the analysis of novel and conserved miRNAs in C. arabica and C. canephora, Loss-Morais et al. (2014) identified eight miRNA sequences belonging to the miR166 family. This family of miRNAs is suggested to play vital roles in numerous biotic and abiotic stress responses. For example, miR166 was upregulated by cold treatment in S. lycopersicum (Valiollahi et al. 2014) and was involved in soybean defense against phytophthora root resistance (Wong et al. 2014). Here, we showed that miR166 is downregulated in coffee roots after long-term N starvation (10 days) (Fig. 7).

Similarly, Boualem et al. (2008) also reported that miR166 is significantly downregulated in M. truncatula roots grown under N stress. miR167 was significantly upregulated in coffee roots at the beginning (1 day) of the N starvation period (Fig. 7). In Arabidopsis and maize, this miRNA was reported to be responsive to N deficiency, indicating a conserved role for miR167 in regulating plant adaptation to N (Liang et al. 2012; Xu et al. 2011; Zhao et al. 2012). miR167 has two transcription factors regulated by auxin as targets (ARF6 and ARF8), which are involved in lateral root development (Gutierrez et al. 2009). Chaves et al. (2015) also identified and characterized miR167 in coffee plants and reported a high complementarity to the ARF8 sequence in Arabidopsis and to the putative ARF8 gene in coffee.

Several studies have shown that miR169 is downregulated by N deficiency, triggering the expression of its target genes NFYA2, NFYA3, NFYA5, and NFYA8 (Hsieh et al. 2009; Liang et al. 2012; Pant et al. 2009; Zhao et al. 2011; Sorin et al. 2014). In other plant species such as maize and soybean, miR169 has been implicated in the response to N deficiency (Trevisan et al. 2012; Xu et al. 2013; Zhao et al. 2013). In

coffee roots, this miRNA was upregulated after 1 day, returning to baseline expression levels after 10 days of N starvation (Fig. 7).

The role of miR858 in plants is still poorly understood. miR858 expression has been reported in Arabidopsis (Rajagopalan et al. 2006), apple (Xia et al. 2012), cotton (Guan et al. 2014), and, more recently, tomato (Jia et al. 2015). The current study is the first to provide information about the transcriptional changes of miR858 under N starvation (Fig. 7), and the first to present a transcriptome study of miRNAs in coffee roots. It has been shown that this microRNA participates in the regulation of MYB transcription factors involved in the biosynthesis of anthocyanins (Sharma et al. 2016), which can be affected by nitrogen supply (Soubeyrand et al. 2014). Thus, the modulation of miR858 observed in our study indicates that, in coffee plants under N starvation, the regulation of transcription factors in the MYB family may be dependent on miR858 regulation.

Conclusions

This study provides novel data on expressed sequences of coffee roots under N starvation, which may contribute to improve genome-wide research and analysis in this perennial species. In addition, we identified microRNAs and their putative targets, providing relevant information on gene transcriptional regulation of N metabolism in *C. arabica*. Finally, the information generated here provides a biotechnological basis for the characterization of genes and gene expression regulators that will guide future research aimed at developing coffee plants that are more adept at growing under suboptimal nutritional conditions.

Author contribution statement TBS, LGEV, and LFPP conceived and designed the research, performed transcriptional experiments, wrote and revised the final manuscript; ARM assisted in designing the experiment; JEL performed experiments using ^{15}N and wrote the manuscript; STI RT-qPCR statistical analysis, revised the final manuscript; JDMS and VYB RT-qPCR and statistical analyses; SGHS performed GS and NR analyses; JBM and PM performed the amino acid analysis; MYNJ, JCS, ORJ, APRL, and ARP bioinformatic analysis; ILMJA and UCO library construction and mRNAseq sequencing; RG and DSD transposable element analysis, coordinated the research, and revised the final manuscript. All authors read and approved the manuscript.

Compliance with ethical standards

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

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