



## Galactinol synthase transcriptional profile in two genotypes of *Coffea canephora* with contrasting tolerance to drought

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### Abstract

Increased synthesis of galactinol and raffinose family oligosaccharides (RFOs) has been reported in vegetative tissues in response to a range of abiotic stresses. In this work, we evaluated the transcriptional profile of a *Coffea canephora* galactinol synthase gene (*CcGolS1*) in two clones that differed in tolerance to water deficit in order to assess the contribution of this gene to drought tolerance. The expression of *CcGolS1* in leaves was differentially regulated by water deficit, depending on the intensity of stress and the genotype. In clone 109A (drought-susceptible), the abundance of *CcGolS1* transcripts decreased upon exposure to drought, reaching minimum values during recovery from severe water deficit and stress. In contrast, *CcGolS1* gene expression in clone 14 (drought-tolerant) was stimulated by water deficit. Changes in galactinol and RFO content did not correlate with variation in the steady-state transcript level. However, the magnitude of increase in RFO accumulation was higher in the tolerant cultivar, mainly under severe water deficit. The finding that the drought-tolerant coffee clone showed enhanced accumulation of *CcGolS1* transcripts and RFOs under water deficit suggests the possibility of using this gene to improve drought tolerance in this important crop.

**Keywords:** coffee, drought stress, raffinose family oligosaccharides (RFOs).

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### Introduction

In Brazil, the cultivation of Conilon and Robusta coffee (*Coffea canephora* Pierre ex A. Froehner) has expanded to areas where water availability is the main limiting factor for production (DaMatta *et al.*, 2003). In contrast to Arabica coffee (*Coffea arabica*), *C. canephora* exhibits a broad range of drought tolerance. Among the populations representing the known *C. canephora* distribution and genetic groups, Guinean genotypes are considered to be the most tolerant to drought, and genotypes from the Congolese SG1 subgroup are more tolerant than those from SG2 (Montagnon and Leroy, 1993). Several clones of *C. canephora* 'Kouillou' (known in Brazil as 'Conilon') tolerant to drought have been identified in the germoplasm col-

lection of the Institute for Research and Rural Assistance of Espírito Santo State (INCAPER), Brazil (Ferrão *et al.*, 2000a). Some of these accessions have been well characterized with regard to the physiological and molecular mechanisms involved in their tolerance to water deficit (DaMatta *et al.*, 2003; Pinheiro *et al.*, 2005; Praxedes *et al.*, 2006; Marraccini *et al.*, 2012; Vieira *et al.*, 2013).

Drought stress triggers a series of plant responses involving transcriptional cascades and interactions among gene products that cause an important shift in the entire plant physiology, growth and development (Shinozaki and Yamaguchi-Shinozaki, 2007). One of the many mechanisms used to counteract the deleterious effects of drought in plants is the accumulation of compatible solutes such as amino acids, amines and several soluble sugars, *e.g.*, the raffinose family oligosaccharides (RFOs) (Molinari *et al.*, 2004; Alcázar *et al.*, 2010; Egert *et al.*, 2013). RFOs are extensively distributed in higher plants (Castillo *et al.*, 1990) and have important functions in carbon storage, photo-

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synthate translocation and seed physiology (Ayre *et al.*, 2003). RFOs may act as compatible solutes in abiotic stress protection (Haritatos *et al.*, 1996), and also appear to have an important role as antioxidants against damage induced by reactive oxygen species (ROS) under stress conditions (Nishizawa *et al.*, 2008; ElSayed *et al.*, 2014).

RFOs are synthesized from sucrose by the addition of galactose moieties donated by galactinol (O- $\alpha$ -D-galactopyranosyl-(1  $\rightarrow$  1)-L-myo-inositol). Galactinol synthase (GolS, EC 2.4.1.123) belongs to a family of glycosyltransferases involved in the first step of RFO biosynthesis; GolS catalyzes the transfer of UDP-D-galactose to myo-inositol (Schneider and Keller, 2009) and is considered the main regulator of this biosynthetic pathway (Peterbauer and Richter, 2001). Other oligosaccharides of this pathway (stachyose, verbascose and ajugose) are sequentially formed by the action of specific galactosyl transferases using galactinol as the galactosyl donor.

The expression of enzymes related to the biosynthesis of galactinol and RFOs and their intracellular accumulation in plant cells are closely associated with the responses to environmental stress (Taji *et al.*, 2002; Panikulangara *et al.*, 2004). As the key enzyme of a primary metabolite, GolS is not only closely related to carbohydrate metabolism, but also plays an important role in stress tolerance. Several studies have shown that the expression of *GolS* genes is consistent with a role in the response to diverse abiotic stresses. Ten *AtGolS* genes were identified in the *Arabidopsis* genome (Nishizawa *et al.*, 2008), three of which (*AtGolS1*, *AtGolS2* and *AtGolS3*) were up-regulated by abiotic stress (Taji *et al.*, 2002). Nishizawa *et al.* (2006) observed that transcription of the *AtGolS1* and *AtGolS2* genes in *A. thaliana* was induced by a combination of high light and heat stress or treatment with hydrogen peroxide. Transgenic tobacco plants overexpressing *Cucumis sativus* *CsGolS1* showed an increased accumulation of galactinol that led to increased tolerance to biotic stress, drought and high salinity (Kim *et al.*, 2008). *BhGolS1* transcripts of the resurrection plant *Boea hygrometrica* are induced by abscisic acid (ABA) and the overexpression of this gene in tobacco plants increased their tolerance to dehydration (Wang *et al.*, 2009). In *Brassica napus*, the accumulation of *BnGolS1* mRNA in developing seeds was associated with the acquisition of tolerance to desiccation and coincided with the formation of raffinose and stachyose (Li *et al.*, 2011).

We recently reported the differential regulation of three GolS isoforms in *C. arabica* (*CaGolS1*, *CaGolS2*, *CaGolS3*) under water deficit, high salt and heat shock conditions that enhanced raffinose and stachyose formation during these stresses (dos Santos *et al.*, 2011). Among these genes, *CaGolS1* was expressed in plants under normal growth conditions and was also the most stress-responsive GolS isoform when the plants were subjected to water deficit.

A limitation in experiments that use single genotypes, without comparing differences in transcription levels between drought-tolerant and drought-susceptible genotypes, is the impossibility to specify which differentially expressed genes are actually responsible for enhancing drought tolerance. As variability in drought tolerance is found in different genotypes of *C. canephora* (DaMatta *et al.*, 2003; Marraccini *et al.*, 2012), the study of the differential expression of *GolS* genes and their products in sensitive vs. tolerant clones may provide further insight into the role of GolS and RFOs in the tolerance of this important perennial tree crop to drought stress.

In this report, we describe a comparative gene expression analysis of a *C. canephora* isoform (*CcGolS1*) most similar to *C. arabica GolS1*, in two genotypes of *C. canephora* that differ in water deficit tolerance (clone 14: drought-tolerant – D<sup>T</sup> and clone 109A: drought-susceptible – D<sup>S</sup>). In addition, we examined the changes in the RFO content of plants under different conditions of water availability (from fully irrigated to a severe water deficit).

## Materials and Methods

### *In silico* analysis

Searches for *GolS* sequences in the HarvEST:*Coffea* v.0.16 platform (<http://harvest.ucr.edu/>) yielded only one assembled contig with full-length cDNA (Unigene 2798). This sequence was compared with those in the Brazilian Coffee Genome Project Consortium (Mondego *et al.*, 2011) and the NCBI database using BlastX (<http://www.ncbi.nlm.nih.gov/BLAST>) (Altschul *et al.*, 1997). The reference accession for *CcGolS1* in NCBI is GT646983.1 and Contig 7664 in the Brazilian Coffee Genome Project Consortium. The open reading frame (ORF) of the unigene was predicted using the ORF Finder program (NCBI). Multiple sequence alignments were done using CLC Main Workbench v.5.0 based on the deduced amino acid sequence of coffee *CaGolS1* and *CcGolS1*. A phylogenetic tree was constructed using the neighbor-joining method (Saitou and Nei, 1987) with Poisson-corrected distances and pairwise deletion in MEGA6 software (Tamura *et al.*, 2011). The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) were shown next to the branches (Felsenstein, 1985). The phylogenetic tree was drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the tree. The analysis involved 30 amino acid sequences from different organisms with 275 positions in the final dataset. The 3D structure of *CcGolS1* was generated using the I-TASSER server (Roy *et al.*, 2010).

### Plant material and experimental treatments

The drought stress experiment was done at the Agronomic Institute of Paraná (IAPAR – latitude 23°18' S, lon-

gitude 51°09' O, average altitude 585 m; Londrina, Paraná State, Brazil). Two *C. canephora* clones, D<sup>T</sup> (clone 14) and D<sup>S</sup> (clone 109A), with contrasting responses to water deficit stress (DaMatta *et al.*, 2003; Pinheiro *et al.*, 2004; Marraccini *et al.*, 2012; Vieira *et al.*, 2013) were obtained from the Espírito Santo State Institute of Research, Technical Assistance and Rural Extension (Incaper, Brazil) and grown under greenhouse conditions. The experiments were done twice with four biological replicates per experiment. Biological replicates are defined as pools of leaves at the same developmental stage.

### Water deficit stress treatment

The basic procedures for the drought stress treatments followed those of dos Santos *et al.* (2011). Ten 18-month-old plants from each *C. canephora* clone (D<sup>T</sup> and D<sup>S</sup>) were cultivated in pots placed in similar positions in relation to the incidence of solar radiation. The leaf water potential was monitored using thermocouple psychrometer chambers (model C-30, Wescor, Inc.) assembled with a datalogger (model CR-7, Campbell Scientific, Inc.). On each day of measurement, a sample ~2 cm<sup>2</sup> was collected between 9:30 and 10:00 a.m. from a fully mature leaf with no signs of injury or mineral deficiency and placed in the psychrometers. In all cases, the leaves were obtained from the central portion of plagiotropic shoots located in the middle of the plant. The stress conditions were defined based on the leaf water potential: irrigated ( $\pm$  -1.35 MPa), moderate stress ( $\pm$  -2.35 MPa), severe stress ( $\pm$  -4.3 MPa) and recovery (rehydrated plants 72 h after reaching the water potential established as severe stress).

### RNA isolation, cDNA synthesis and semi-quantitative RT-PCR analysis

Total RNA from leaves of the *C. canephora* clones D<sup>T</sup> and D<sup>S</sup> was isolated using the same procedures as dos Santos *et al.* (2011) and treated with DNase. The RNA concentration was determined using a NanoDrop<sup>®</sup> ND-100 spectrophotometer (Waltham, MA, USA). Complementary DNA (cDNA) was synthesized using SuperScript III reverse transcriptase (Invitrogen<sup>®</sup>), according to the manufacturer's instructions, in a final volume of 20  $\mu$ L containing 5  $\mu$ g of total RNA. Semi-quantitative RT-PCR analysis of *CcGolS1* was based on Maluf *et al.* (2009). Amplification was done using the following temperature profile: 2min initial denaturation at 94 °C followed by 30 cycles of 94 °C for 1 min, 60 °C for 1 min and 72 °C for 1 min, with a final extension of 3 min at 72 °C; after amplification, the samples were held at 4 °C. The primers were designed with Primer Express (version 3.0), based on parameters established by the software, to obtain amplicons of 100 bp with a T<sub>m</sub> of 60  $\pm$  1 °C. Primer sequences are 5'-CACAGGGTTGCATTGTTTCGT-3' (forward) and 5'-CGGAGCTTGAATAATTGATGAC-3' (reverse). The amplicons were separated on 2% agarose gels, stained

with ethidium bromide and photographed. The captured images were processed for densitometric analysis using ImageJ 1.43 U software, essentially as described by Freschi *et al.* (2009). The transcript level of *CcGolS1* in response to water deficit treatments was then normalized to the corresponding expression of EF1 $\alpha$  or *GAPDH*, as recommended by Goulão *et al.* (2012) and Carvalho *et al.* (2013). Semi-quantitative RT-PCR was repeated at least three times for each sample.

### HPLC analysis

Coffee leaves were lyophilized and submitted to an extraction process for obtaining low molecular weight oligosaccharides as described by Albini *et al.* (1994). The extracts were analyzed by high performance liquid chromatography (HPLC) using a Shimadzu system (Japan) equipped with a CBM-10A interface module, CTO-10A column oven, LC-10AD pump and RID-10A refractive index detector in conjunction with a Supelcogel Ca column (30 cm x 7.8 mm; Supelco, USA) and Supelcogel Ca pre-column (5 cm x 4.6 mm). The column was eluted with water at a flow rate of 0.5 mL/min and operating temperature of 80 °C. Calibration curves prepared with galactinol, raffinose and stachyose were used to quantify carbohydrates in the samples.

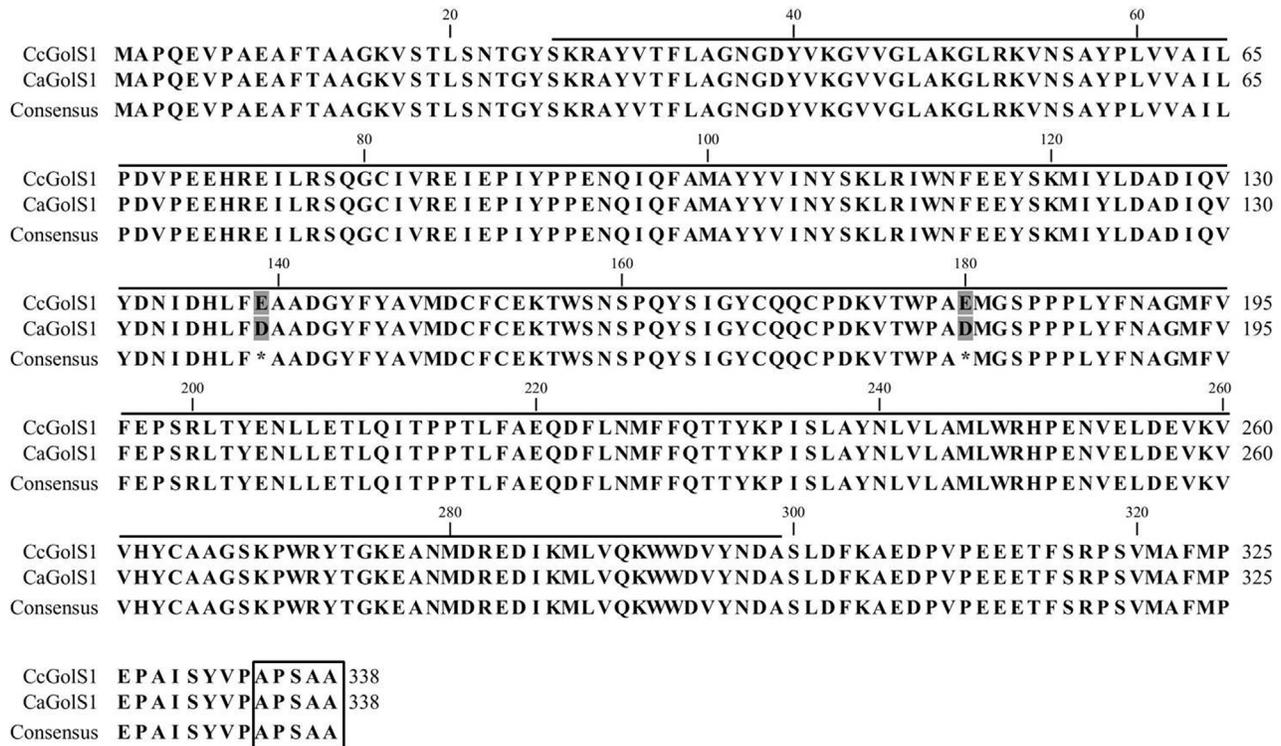
### Statistical analysis

Numerical results were expressed as the mean  $\pm$  SEM and statistical analyses were done using one-way ANOVA followed by Tukey's multiple comparisons test, with  $p < 0.05$  indicating significance. All data analyses were done using Sisvar software (Ferreira, 2011).

## Results

### *CcGolS1* annotation

*CcGolS1* encoded a 338 amino acid polypeptide with a predicted molecular mass of 38.43 kDa and an isoelectric point (pI) of 4.93. *CaGolS1* of *C. arabica* also encoded a 338 amino acid protein with a predicted molecular mass of 38.54 kDa and pI of 4.93 (dos Santos *et al.*, 2011) (Figure 1). The amino acid sequence of *CcGolS1* was nearly identical to that of *CaGolS1* and differed from the latter by only two amino acids at positions 139 and 180; the predicted *CcGolS1* protein sequence also contained the C-terminal hydrophobic pentapeptide APSAA, a common feature of galactinol synthases (Figure 1). The amino acid sequences of *CcGolS1* and other galactinol synthases were aligned and a phylogenetic tree was constructed to analyze the relationship among these enzymes. As shown in Figure 2, *CcGolS1* was very closely related to *CaGolS1* and there was a distinct separation between monocot and dicot sequences that probably reflected the absence of the ancestor sequence lost during evolution, as proposed by Sengupta *et al.* (2012).



**Figure 1** - Alignment of galactinol synthase amino acid sequences from *Coffea canephora* (CcGolS1 – Contig 7664) and *Coffea arabica* (ADM92588.1 – CaGolS1). The alignment was done using CLC Main Workbench v.5.0 software, with ClustalW default parameters. Asterisks indicate differences in amino acid residues (shaded in grey) at positions 139 and 180. The conserved glycosyltransferase domain is indicated by a black line above the amino acids (reviewed by Zhou *et al.*, 2012) and the C-terminal hydrophobic pentapeptide APSAA is boxed.

The CcGolS1 amino acid sequence was used to generate a 3D structure with the I-TASSER server (Roy *et al.*, 2010) (Figure S1). In agreement with the results of Sengupta *et al.* (2012), the best structural template for CcGolS1 was rabbit glycogenin glycosyltransferase - chain A (PDB ID 1ll0B), one of the two resolved crystal structures of the GT8 family. In our case, the percentage sequence identity of the template with the query sequence in the threading aligned region was 0.26, whereas the percentage sequence identity between the whole template chains and the query sequence was 0.23. The coverage of threading alignment was 0.76 and the normalized Z-score of the threading alignment was 1.92 for 1ll0B.

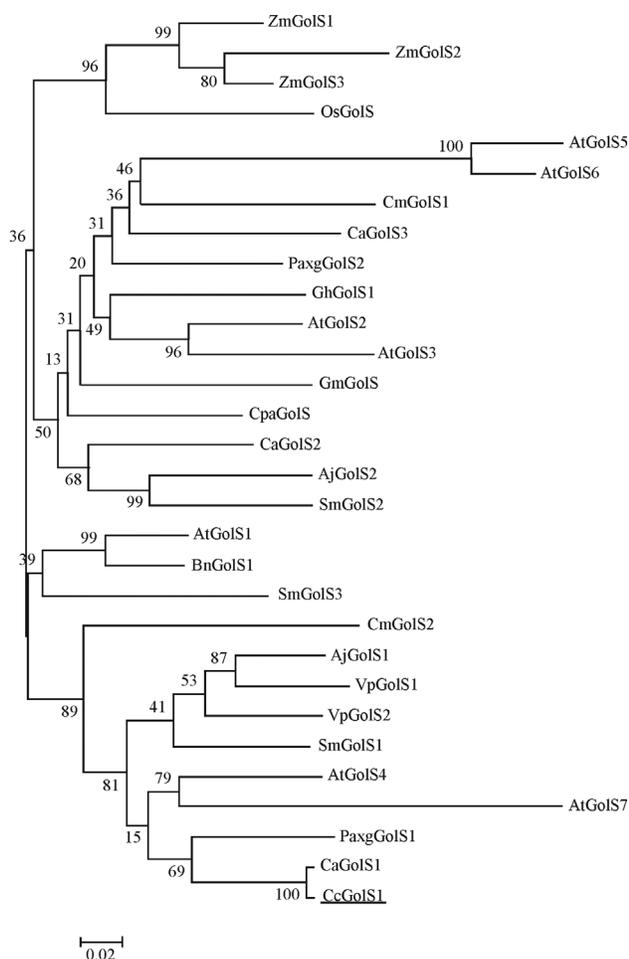
### Comparison of *CcGolS1* expression between genotypes

To examine the transcriptional profile of *CcGolS1* in *C. canephora* leaves, we performed semi-quantitative RT-PCR analyses using total RNA from the two clones under moderate and severe drought stress conditions. We also analyzed photosynthetic parameters from the two genotypes. There were differences in the photosynthetic rates of clones D<sup>T</sup> and D<sup>S</sup> after the fourth day of water deprivation, with the D<sup>S</sup> photosynthetic rates being lower from the 6<sup>th</sup> to the 10<sup>th</sup> day, after which both clones again showed the same levels of activity (Figure S2).

The transcript levels of *CcGolS1* in the D<sup>S</sup> and tolerant D<sup>T</sup> genotypes in non-stressed (irrigated) conditions were similar (Figure 3A,B) and reflected the constitutive expression of this gene under normal water supply. During water deficit (moderate and severe stress), *CcGolS1* expression was enhanced in the D<sup>T</sup> clone, in accordance with the stress level, while in D<sup>S</sup> plants the expression levels of this gene decreased under drought conditions. *CcGolS1* expression in the recovery phase (72 h after rehydration) was similar in both clones and was comparable to the irrigated plants (control) before water withdrawal (Figure 3A,B).

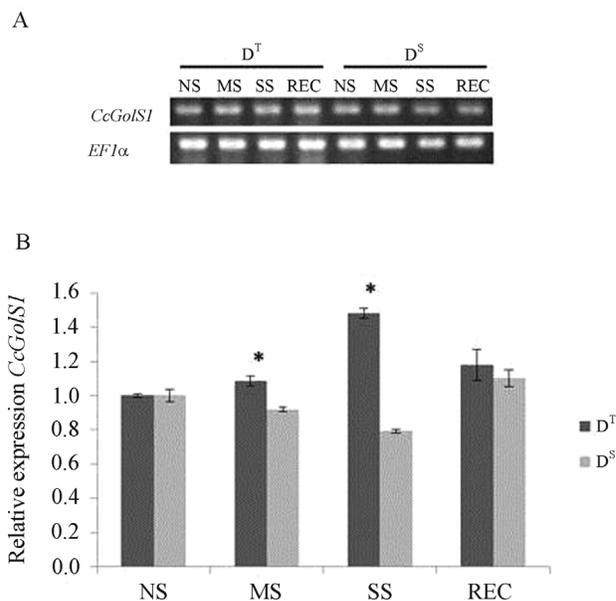
### Quantification of RFOs

In well-watered conditions, the leaves of D<sup>T</sup> plants had a lower galactinol content compared to D<sup>S</sup> plants (Figure 4A); the latter plants had a ~2.5-fold higher galactinol level than D<sup>T</sup> plants under normal irrigated conditions. With water deficit stress, the leaves of both clones showed a considerable decline in galactinol content, with no detectable difference between the genotypes under severe drought stress. After 72 h of recovery there was a significant increase in galactinol levels in both clones compared to the corresponding severe stress (SS) treatment and this increase was significantly greater for D<sup>S</sup> leaves (about 1.18 mg/g DW) compared to D<sup>T</sup> leaves (Figure 4A).



**Figure 2** - Unrooted phylogram of selected GolS genes. Sequence alignments were done using ClustalW and the tree was constructed with the neighbor-joining method using the program MEGA6. The numbers on the tree branches indicate bootstrap results from neighbour-joining analyses of the branches. The sequences were obtained through NCBI: *Ajuga reptans* (CAB51533.1 – AjGolS1; CAB51534.1 – AjGolS2), *Arabidopsis thaliana* (NP\_182240.1 – AtGolS1; NP\_176053.1 – AtGolS2; NP\_172406.1 – AtGolS3; NP\_176250.1 – AtGolS4; NP\_197768.1 – AtGolS5; NP\_567741.2 – AtGolS6; NP\_176248.1 – AtGolS7), *Brassica napus* (ACJ15472.1 – BnGolS1), *Capsicum annuum* (ABQ44212.1 – CpaGolS), *Coffea arabica* (ADM92588.1 – CaGolS1; ADM92590.1 – CaGolS2; ADM92589.1 – CaGolS3), *Coffea canephora* (CcGolS1 – Contig 7664; Mondego *et al.*, 2011), *Cucumis melo* (AAL78687.1 – CmGolS1; AAL78686.1 – CmGolS2), *Glycine max* (AAM96867.1 – GmGolS), *Gossypium hirsutum* (AFG26331.1 – GhGolS1), *Oriza sativa* (Os.2677.1S1\_at – OsGolS), *Populus alba x Populus grandidentata* (AEN74905.1 – PaxgGolS1; AEN74906.1 – PaxGolS2), *Salvia miltiorrhiza* (ACT34765.1 – SmGolS1; AEQ54920.1 – SmGolS2; AEQ54921.1 – SmGolS3), *Verbascum phoeniceum* (ABQ12640.1 – VpGolS1; ABQ12641.1 – VpGolS2) and *Zea mays* (AAQ07248.1 – ZmGolS1; AAQ07249.1 – ZmGolS2; AAQ07250.1 – ZmGolS3).

As with galactinol, the content of raffinose in leaves of  $D^S$  plants was significantly greater than in  $D^T$  leaves under irrigated (non-stressed) conditions (Figure 4B). However, after drought stress, the leaves of plants  $D^T$  showed an increase in raffinose levels that was greater than in  $D^S$  plants. In severe stress, the raffinose content of  $D^T$  plants was almost two-fold greater than in  $D^S$  plants (1.8 mg/g



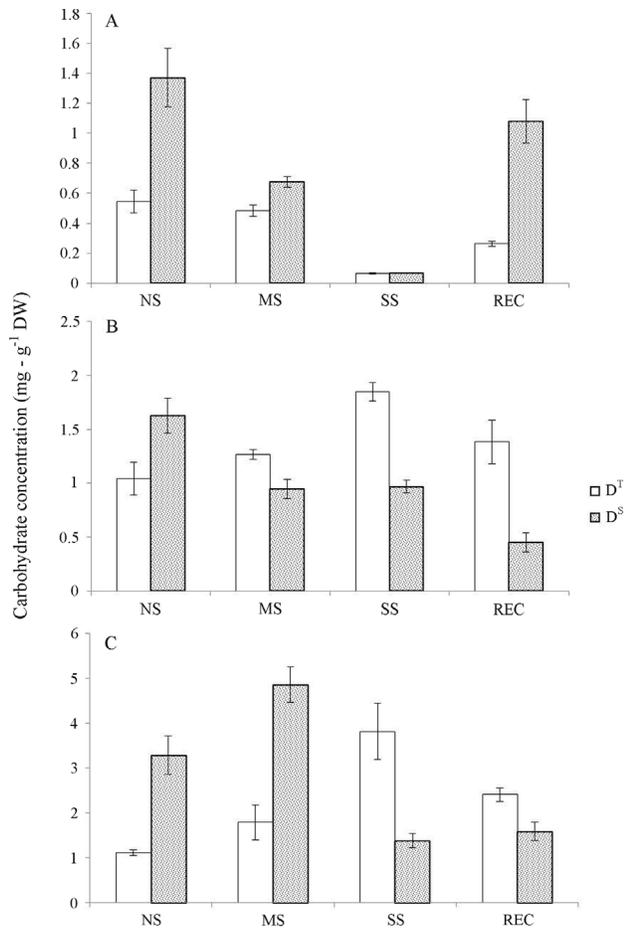
**Figure 3** - Transcriptional analysis of *CcGolS1*. (A) Semi-quantitative RT-PCR analysis using cDNA from *C. canephora* clones  $D^T$  (clone 14) and  $D^S$  (clone 109A). Water deficit treatments: non-stressed (NS;  $\pm$  -1.35 MPa), moderate stress (MS;  $\pm$  -2.35 MPa), severe stress (SS;  $\pm$  -4.3 MPa) and recovery (REC; 72 h following re-irrigation after severe stress); see Materials and Methods for details. (B) Densitometric analysis using ImageJ 1.43 U software (Freschi *et al.*, 2009). The *EF1α* gene was used as an internal control to normalize the expression level of the target gene (*CcGolS1*) among different treatments. The columns represent the mean  $\pm$  SEM ( $n = 3$ ). Asterisks indicate significant difference between genotypes ( $p < 0.05$ , ANOVA followed by Tukey test).

DW vs. 0.9 mg/g DW). After 72 h of re-irrigation, the raffinose content in the leaves of both clones declined, with the decrease in  $D^S$  plants being much greater than in  $D^T$  plants (mean REC levels of 1.5 and 0.5 mg/g DW for  $D^S$  and  $D^T$ , respectively) (Figure 4B).

The pattern of changes in stachyose content in both clones in the different conditions was generally similar to that of raffinose, with the noticeable exception of the moderate water deficit condition in which the leaves of clone  $D^S$  contained much more of this sugar (~2.5-fold more) than  $D^T$  leaves (Figure 4C).

## Discussion

*GolS* orthologs and paralogs have been studied in several plant species, *e.g.*, *Ajuga reptans* (Sprenger and Keller, 2000), *A. thaliana* (Taji *et al.*, 2002), *C. arabica* (dos Santos *et al.*, 2011), *Gossypium hirsutum* (Zhou *et al.*, 2012), *Populus trichocarpa* (Zhou *et al.*, 2014) and *Xerophyta viscosa* (Peters *et al.*, 2007), and in most cases are associated with multiple developmental and environmental responses. Alignment and phylogenetic analyses showed that the deduced *CcGolS1* protein sequence was highly similar (99%) to that of *CaGolS1* from *C. arabica* (Figures 1, 2). This high similarity reflects the fact that *C. arabica* is the result of a natural cross between the diploid



**Figure 4** - Galactinol (A), raffinose (B) and stachyose (C) concentrations in *C. canephora* D<sup>T</sup> and D<sup>S</sup> leaves, as determined by HPLC. The water stress conditions were: non-stressed (NS), moderate stress (MS), severe stress (SS) and recovery (REC), as defined in the Figure 3 legend. The columns represent the mean ± SEM (n = 3).

species *C. eugenoides* and *C. canephora* (Vidal *et al.*, 2010).

*CcGolS1* is a member of the GT8 glycosyltransferase family that catalyzes the first committed step in the biosynthetic pathway of RFOs. GolS represents a relatively small class of the eukaryotic glycosyltransferase family (GTs; EC 2.4.x.y.), specifically GT8. GT8 is a large enzyme family involved in the biosynthesis of a pool of diverse sugar conjugates with important roles in structure, storage, energy production and signaling. Despite the presence of diverse catalytic activities in the glycosyltransferases (Coutinho *et al.*, 2003), the existence of six monophyletic plant GT8 clades assembled into two widely divergent groups with a remote evolutionary relationship has been suggested (Yin *et al.*, 2010, 2011). Galactinol synthase (GolS) represents a monospecific clade within the GT8, with some common structural elements, *e.g.*, all of them use L-myo-inositol as the acceptor (Sengupta *et al.*, 2012). One common feature of almost all GolS is the C-terminal hydrophobic pentapeptide APSAA and a con-

served serine that serves as a site for phosphorylation (Sengupta *et al.*, 2012; Wang *et al.*, 2012). The 3D model of CaGolS1 showed the same structure as ZmGolS3 reported by Sengupta *et al.* (2012) (Figure S1).

Semi-quantitative RT-PCR analysis of *CcGolS1* transcripts using total RNA isolated from leaves of *C. canephora* D<sup>T</sup> and D<sup>S</sup> clones under water shortage and after recovery from stress suggested a possible involvement of this enzyme in the differential response of these genotypes to drought stress (Figure 3A,B). The increased transcriptional activity of *CcGolS1* under moderate and (particularly) severe stress was greater in D<sup>T</sup> leaves compared to D<sup>S</sup> leaves. Vieira *et al.* (2013) have also observed different regulatory responses to drought stress in D<sup>T</sup> plants.

In a study of three *GolS* genes from *C. arabica*, dos Santos *et al.* (2011) showed that *CaGolS1* was the only isoform that was highly up-regulated in all of the water deficit periods and also after rehydration, whereas *CaGolS2* and *CaGolS3* showed significant expression only under severe water deficit. As shown here, the transcriptional activity of *CcGolS1* was stimulated only in D<sup>T</sup> stressed plants, whereas the expression of this gene was markedly reduced in leaves of water-stressed D<sup>S</sup> plants (Figure 3A,B). The transcriptional modulation of *GolS* or its isoforms has been often reported as part of plant abiotic stress responses. Among the *GolS* genes studied in *Arabidopsis*, *AtGolS1* and *AtGolS2* were reported to be induced by drought and high salt, respectively, whereas *AtGolS3* was responsive to low temperatures (Taji *et al.*, 2002). In maize, *ZmGolS2* is induced primarily by dehydration, whereas *ZmGolS3* is up-regulated during heat stress (Zhao *et al.*, 2003, 2004). Wang *et al.* (2012) showed that of three *Salvia miltiorrhiza GolS* genes (*SmGolS1*, *SmGolS2* and *SmGolS3*), *SmGolS2* was the only isoform that responded predominantly to different stress treatments.

Several clones of *C. canephora* have been reported to be tolerant to drought. Ferrão *et al.* (2000a,b) found that D<sup>T</sup> and D<sup>S</sup> clones produced a good crop when grown under adequate irrigation, whereas with limited soil water, survival, productivity and maintenance of the tissue water status (DaMatta *et al.*, 2003) were impaired more in D<sup>S</sup> than in D<sup>T</sup>. However, little is known about the mechanism of drought tolerance in coffee at the biochemical level (DaMatta *et al.*, 2002a,b, 2003). Recently, Lima (Lima RB, 2013, PhD thesis, Universidade Federal do Paraná, Curitiba, PR, Brazil) studied the changes in the cell wall components of leaves from tolerant (D<sup>T</sup>) and susceptible (D<sup>S</sup>) genotypes under drought, heat and salt stress and found that D<sup>T</sup> plants showed stiffening of the cell wall in response to drought stress, while D<sup>S</sup> plants responded by loosening the cell wall; this characteristic could play an important role in the response to drought stress in D<sup>T</sup> plants.

Divergent results were observed between the level of *CcGolS1* transcripts and galactinol content in the leaves of non-stressed (irrigated) plants. Despite the similar trans-

criptional levels of *CcGolS1* in both clones grown with a normal water supply, the clone D<sup>S</sup> contained significantly greater amounts of galactinol in its leaves. The low concentration of galactinol during severe stress, combined with increased transcription of *CcGolS1*, indicated the need for increased production of high molecular mass RFOs to act against possible cell damage caused by stress, as described by dos Santos *et al.* (2011). McCaskill and Turgeon (2007) observed that inhibition of RFOs by RNAi silencing of the genes *VpGAS1* and *VpGAS2* in *Verbascum phoeniceum* negatively affected phloem transport and indicated that galactinol was important in the downstream metabolic pathway. In the latter case, galactinol was probably preferred as a substrate for the biosynthesis of oligosaccharide RFOs in D<sup>T</sup> and D<sup>S</sup> leaves under water deficit.

The accumulation of RFOs is often associated with stressful environmental conditions (Peterbauer and Richter, 2001; Peters *et al.*, 2007). Besides being part of a carbon storage mechanism, the accumulation of RFOs is involved in stress tolerance defense mechanisms in which these compounds act as osmoprotectors, antioxidants and signaling molecules in biotic and abiotic stress (ElSayed *et al.*, 2014). The higher levels of raffinose and stachyose detected during severe drought stress in clone D<sup>T</sup> may reflect a greater participation of this oligosaccharide in the response of *C. canephora* to extreme stress. The difference in the concentration of stachyose between clones D<sup>S</sup> and D<sup>T</sup> was quite pronounced in plants under stress (Figure 4C), which suggested that the greater increase in RFO content in clone D<sup>S</sup> possibly served as a defense against severe water deficit conditions. The lack of a strict correlation between RFO concentrations and *CcGolS1* transcript levels may reflect post-transcriptional and post-translational regulation, substrate availability and the involvement of different galactinol synthase isoforms in other biological processes, *e.g.*, phloem loading, carbohydrate transport, partitioning and storage that occur when plants are not subjected to drought stress.

In conclusion, we have shown that drought-tolerant and drought-susceptible *C. canephora* plants have different *GolS* transcriptional expression patterns and a differential accumulation of RFOs during water deficit. Given the complexity of the physiological processes involved in the mechanisms of drought tolerance, it is evident that various other physiological processes are required for survival and acclimation in adverse stress conditions. Nevertheless, the finding that a drought-tolerant coffee clone showed an accumulation of *CcGolS1* transcripts and greater RFO production during a water deficit provides further insight into the molecular mechanisms of drought tolerance in these plants. This finding also suggests the possibility of using this gene as part of a biotechnological strategy to improve drought tolerance in this important crop.

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## References

- Albini FM, Murelli C, Patriiti G, Rovati M, Zienna P and Finzi PV (1994) Low-molecular weight substances from the resurrection plant *Sporobolus stapfianus*. *Phytochemistry* 37:137-142.
- Alcázar R, Altabella T, Marco F, Bortolotti C, Reymond M, Koncz C and Tiburcio AF (2010) Polyamines: Molecules with regulatory functions in plant abiotic stress tolerance. *Planta* 231:1237-1249.
- Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W and Lipman DJ (1997) Gapped BLAST and PSI-BLAST: A new generation of protein database search programs. *Nucleic Acids Res* 25:3389-3402.
- Ayre BG, Keller F and Turgeon R (2003) Symplastic continuity between companion cells and the translocation stream: Long distance transport is controlled by retention and retrieval mechanisms in the phloem. *Plant Physiol* 131:1518-1528.
- Carvalho K, Besspalhok Filho JC, dos Santos TB, Souza SGH, Vieira LGE, Pereira LFP and Domingues DS (2013) Nitrogen starvation, salt and heat stress in coffee (*Coffea arabica* L.): Identification and validation of new genes for qPCR normalization. *Mol Biotechnol* 53:315-325.
- Castillo EM, De Lumen BO, Reyes PS and De Lumen HZ (1990) Raffinose synthase and galactinol synthase in developing seeds and leaves of legumes. *J Agric Food Chem* 38:351-355.
- Coutinho PM, Deleury E, Davies GJ and Henrissat B (2003) An evolving hierarchical family classification for glycosyltransferases. *J Mol Biol* 328:307-317.
- Cruz F, Kalaoun S, Nobile P, Colombo C, Almeida J, Barros LM, Romano E, Grossi-de-Sá MF, Vaslin M and Alves-Ferreira M (2009) Evaluation of coffee reference genes for relative expression studies by quantitative real-time RT-PCR. *Mol Breed* 23:607-616.
- DaMatta FM, Loos RA, Silva EA, Ducatti C and Loureiro ME (2002a) Effects of soil water deficit and nitrogen nutrition on water relations and photosynthesis of pot-grown *Coffea canephora* Pierre. *Trees* 16:555-558.
- DaMatta FM, Loos RA, Silva EA and Loureiro ME (2002b) Limitations to photosynthesis in *Coffea canephora* as a result of nitrogen and water availability. *J Plant Physiol* 159:975-981.
- DaMatta FM, Chaves ARM, Pinheiro HA, Ducatti C and Loureiro ME (2003) Drought tolerance of two field-grown clones of *Coffea canephora*. *Plant Sci* 164:111-117.
- dos Santos TB, Budzinski IG, Marur CJ, Petkowicz CL, Pereira LF and Vieira LG (2011) Expression of three *galactinol synthase* isoforms in *Coffea arabica* L. and accumulation of raffinose and stachyose in response to abiotic stresses. *Plant Physiol Biochem* 49:441-448.

- Egert A, Keller F and Peters S (2013) Abiotic stress-induced accumulation of raffinose in *Arabidopsis* leaves is mediated by a single raffinose synthase (RS5, At5g40390). *BMC Plant Biol* 13:e218.
- ElSayed AI, Rafudeen MS and Golladack D (2014) Physiological aspects of raffinose family oligosaccharides in plants: Protection against abiotic stress. *Plant Biol* 16:1-8.
- Felsenstein J (1985) Confidence limits on phylogenies: An approach using the bootstrap. *Evolution* 39:783-791.
- Ferrão RG, Fonseca AFA, Silveira JSM, Ferrão MAG and Bragança SM (2000a) EMCAPA 8141 - Robustão Capixaba, variedade clonal de café conilon tolerante à seca, desenvolvida para o estado do Espírito Santo. *Rev Ceres* 273:555-560.
- Ferrão RG, Fonseca AFAD and Ferrão MAG (2000b) Avaliação de clones elites de café conilon em condição de déficit hídrico no Estado do Espírito Santo. In: 1º Simpósio de Pesquisa dos Cafés do Brasil. Poços de Caldas, MG. Resumos Expandidos. Embrapa Café, Brasília, Brasília and Minasplan, Belo Horizonte, pp 402-404.
- Ferreira DF (2011) Sisvar: A computer statistical analysis system. *Ciênc Agrotecnol* 35:1039-1042.
- Freschi L, Nievola CC, Rodrigues MA, Domingues DS, Sluys MAV and Mercier H (2009) Thermoperiod affects the diurnal cycle of nitrate reductase expression and activity in pineapple plants by modulating the endogenous levels of cytokinins. *Physiol Plant* 137:201-212.
- Goulão LF, Fortunato AS and Ramalho JC (2012) Selection of Reference Genes for Normalizing Quantitative Real-Time PCR Gene Expression Data with Multiple Variables in *Coffea* spp. *Plant Mol Biol Rep* 30:741-759.
- Haritatos E, Keller F and Turgeon R (1996) Raffinose oligosaccharide concentrations measured in individual cell and tissue types in *Cucumis melo* L. leaves: Implications for phloem loading. *Planta* 198:614-622.
- Kim MS, Cho SM, Kang EY, Im YJ, Hwangbo H, Kim YC, Ryu CM, Yang KY, Chung GC and Cho BH (2008) Galactinol is a signaling component of the induced systemic resistance caused by *Pseudomonas chlororaphis* O6 root colonization. *Mol Plant Microbe Interact* 12:1643-53.
- Li X, Zhuo J, Jing Y, Liu X and Wang X (2011) Expression of a *galactinol synthase* gene is positively associated with desiccation tolerance of *Brassica napus* seeds during development. *J Plant Physiol* 168:1761-1770.
- Maluf MP, Silva CCD, Oliveira MDPAD, Tavares AG, Silvarolla MB and Guerreiro Filho O (2009) Altered expression of the caffeine synthase gene in a naturally caffeine-free mutant of *Coffea arabica*. *Genet Mol Biol* 32:802-810.
- Marraccini P, Vinecky F, Alves GSC, Ramos HJO, Elbelt S, Vieira NG, Carneiro FA, Sujii PS, Alekcevetch JC, Silva VA, *et al.* (2012) Differentially expressed genes and proteins upon drought stress in tolerant and sensitive genotypes of *Coffea canephora*. *J Exp Bot* 63:4191-4212.
- McCaskill A and Turgeon R (2007) Phloem loading in *Verbascum phoeniceum* L. depends on the synthesis of raffinose-family oligosaccharides. *Proc Natl Acad Sci USA* 104:19619-19624.
- Molinari HBC, Marur CJ, Besspalhok Filho JC, Kobayashi AK, Pileggi M, Leite Júnior RP, Pereira LFP and Vieira LGE (2004) Osmotic adjustment in transgenic citrus rootstock Carrizo citrange (*Citrus sinensis* Obs. x *Poncirus trifoliata* L. Raf.) overproducing proline. *Plant Sci* 167:1375-1381.
- Mondego JM, Vidal RO, Carazzolle MF, Tokuda EK, Parizzi LP, Costa GG, Pereira LF, Andrade AC, Colombo CA, Vieira LG, *et al.* (2011) An EST-based analysis identifies new genes and reveals distinctive gene expression features of *Coffea arabica* and *Coffea canephora*. *BMC Plant Biol* 11:e30.
- Montagnon C and Leroy T (1993) Réaction à la sécheresse de jeunes caféiers *Coffea canephora* de Côte-d'Ivoire appartenant à différents groupes génétiques. *Café Cacao Thé* 37:179-189.
- Nishizawa A, Yabuta Y, Yoshida E, Maruta T, Yoshimura K and Shigeoka S (2006) *Arabidopsis* heat shock transcription factor A2 as a key regulator in response to several types of environmental stress. *Plant J* 48:535-547.
- Nishizawa A, Yabuta Y and Shigeoka S (2008) Galactinol and raffinose constitute a novel function to protect plants from oxidative damage. *Plant Physiol* 147:1251-1263.
- Panikulangara TJ, Eggers-Schumacher G, Wunderlich M, Stransky H and Schöffl F (2004) Galactinol synthase 1: A novel heat shock factor target gene responsible for heat-induced synthesis of raffinose family oligosaccharides in *Arabidopsis*. *Plant Physiol* 136:3148-3158.
- Peterbauer T and Richter A (2001) Biochemistry and physiology of raffinose family oligosaccharides and galactosyl cyclitols in seeds. *Seed Sci Res* 11:185-197.
- Peters S, Mundree SG, Thomson JA, Farrant JM and Keller F (2007) Protection mechanisms in the resurrection plant *Xerophyta viscosa* (Baker): Both sucrose and raffinose family oligosaccharides (RFOs) accumulate in leaves in response to water deficit. *J Exp Bot* 58:1947-1956.
- Pinheiro HA, DaMatta FM, Chaves ARM, Fontes EPB and Loureiro ME (2004) Drought tolerance in relation to protection against oxidative stress in clones of *Coffea canephora* subjected to long-term drought. *Plant Sci* 167:1307-1314.
- Pinheiro HA, DaMatta FM, Chaves ARM, Loureiro ME and Ducatti C (2005) Drought tolerance is associated with rooting depth and stomatal control of water use in clones of *Coffea canephora*. *Ann Bot* 96:1001-108.
- Praxedes SC, DaMatta FM, Loureiro ME, Ferrão MAG and Cordeiro AT (2006) Effects of long-term soil drought on photosynthesis and carbohydrate metabolism in mature robusta coffee (*Coffea canephora* Pierre var. *kouillou*) leaves. *Environ Exp Bot* 56:263-273.
- Roy A, Kucukural A and Zhang Y (2010) I-TASSER: A unified platform for automated protein structure and function prediction. *Nat Protoc* 5:725-738.
- Saitou N and Nei M (1987) The neighbor-joining method: A new method for reconstructing phylogenetic trees. *Mol Biol Evol* 4:406-425.
- Schneider T and Keller F (2009) Raffinose in chloroplasts is synthesized in the cytosol and transported across the chloroplast envelope. *Plant Cell Physiol* 50:2174-2182.
- Sengupta S, Mukherjee S, Parween S and Majumder AL (2012) Galactinol synthase across evolutionary diverse taxa: Functional preference for higher plants? *FEBS Lett* 586:1488-1496.
- Shinozaki K and Yamaguchi-Shinozaki K (2007) Gene networks involved in drought stress response and tolerance. *J Exp Bot* 58:221-227.

- Sprenger N and Keller F (2000) Allocation of raffinose family oligosaccharides to transport and storage pools in *Ajuga reptans*: The roles of two distinct galactinol synthases. *Plant J* 21:249-258.
- Taji T, Ohsumi C, Iuchi S, Seki M, Kasuga M, Kobayashi M, Yamaguchi-Shinozaki K and Shinozaki K (2002) Important roles of drought- and cold-inducible genes for galactinol synthase in stress tolerance in *Arabidopsis thaliana*. *Plant J* 29:417-426.
- Tamura K, Peterson D, Peterson N, Stecher G, Nei M and Kumar S (2011) MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol Biol Evol* 28:2731-2739.
- Vidal RO, Mondego JMC, Pot D, Ambrósio AB, Andrade AC, Pereira LFP, Colombo CA, Vieira LGE, Carazzolle MF and Pereira GAG (2010). A high-throughput data mining of single nucleotide polymorphisms in *Coffea* species expressed sequence tags suggests differential homeologous gene expression in the allotetraploid *Coffea arabica*. *Plant Phys* 154:1053-1066.
- Vieira NG, Carneiro FA, Sujii PS, Alekcevetch JC, Freire LP, Vinecky F, Elbelt S, Silva VA, DaMatta FM, Ferrão MAG, et al. (2013) Different molecular mechanisms account for drought tolerance in *Coffea canephora* var. Conilon. *Trop Plant Biol* 6:181-190.
- Wang D, Yao W, Song Y, Liu W and Wang Z (2012) Molecular characterization and expression of three galactinol synthase genes that confer stress tolerance in *Salvia miltiorrhiza*. *J Plant Physiol* 169:1838-1848.
- Wang Z, Zhu Y, Wang L, Liu X, Liu Y, Phillips J and Deng X (2009) A WRKY transcription factor participates in dehydration tolerance in *Boea hygrometrica* by binding to the W-box elements of the galactinol synthase (*BhGolS1*) promoter. *Planta* 230:1155-1166.
- Yin Y, Chen H, Hahn MG, Mohnen D and Xu Y (2010) Evolution and function of the plant cell wall synthesis-related glycosyltransferase family 8. *Plant Physiol* 153:1729-1746.
- Yin Y, Mohnen D, Genelio-Albersheim I, Xu Y and Hahn MG (2011) Glycosyltransferases of the GT8 family. *Annu Plant Rev* 41:167-212.
- Zhao TY, Meeley RB and Downie B (2003) Aberrant processing of a maize galactinol synthase transcript is caused by heat stress. *Plant Sci* 165:245-56.
- Zhao TY, Thacker R, Corum III JW, Snyder JC, Meeley RB, Obendorf RL and Downie B (2004) Expression of the maize galactinol synthase gene family: (I) expression of two different genes during seed development and germination. *Physiol Plant* 121:634-646.
- Zhou J, Yang Y, Yu J, Wang L, Yu X, Ohtani M and Zhuge Q (2014) Responses of *Populus trichocarpa* galactinol synthase genes to abiotic stresses. *J Plant Res* 127:347-358.
- Zhou T, Zhang R and Guo S (2012) Molecular cloning and characterization of *GhGolS1*, a novel gene encoding galactinol synthase from cotton (*Gossypium hirsutum*). *Plant Mol Biol Rep* 30:699-709.

### Supplementary Material

The following online material is available for this article.

Figure S1 - Predicted 3D structure of *CcGolS1*.

Figure S2 - Photosynthetic rates of the two water-stressed *C. canephora* genotypes.

Figure S3 - Semi-quantitative RT-PCR analysis of *CcGolS1*.

This material is available as part of the online article from <http://www.scielo.br/gmb>.

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