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Research Note

Gene expression changes in response to drought stress in *Ilex paraguariensis* leaves

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Abstract

Ilex paraguariensis St. Hil. is recognized as a shade-tolerant tree, cultivated in north-eastern Argentina, south-eastern Brazil, and Paraguay. Its leaves and young shoots are industrialised and; hence, being used to prepare a traditional tea-like infusion (mate-tea) with various medicinal benefits. Under the prevailing agro-climatic conditions in NE Argentina and with the occurrence of high temperatures and scarce rains during spring or summer, *I. paraguariensis* plants are subjected to water stress, reducing their yield. Differential Display was used to compare differences in gene expression between stressed and unstressed (control) potted plants. We found 52 differentially expressed transcripts (DETs), analysing banding patterns of Differential Display polyacrylamide gels, in which 38 DETs were induced, and 14 DETs were repressed in response to drought. Sequence comparisons revealed that 19% of the fragments showed no homologies, whereas 40.5 % of the total number of fragments had strong similarities with database entries of known functions. The other DETs belonged to unclassified proteins or genes with unknown functions. Interestingly, 12% of the DNA sequences were related to signal transduction, and 2% of the sequences were identified as transcription factors. Approximately, 12% of the annotated sequences were involved in stress tolerance and metal homeostasis, while 7% of the DETs were associated with photosynthesis. Other groups, representing 12%, included those involved in secondary metabolism, lignin biosynthesis, cell walls and RNA stabilization/chaperone activity. When integrated with physiological data, our results suggest that the transcriptional activity of some of the studied genes could be related to the degree of drought tolerance/susceptibility of *I. paraguariensis*.

Keywords: Abiotic stress; gene expression; mRNA Differential Display; water deficit; yerba mate.
Abbreviations:ABA_abscisic acid; DD_mRNA Differential Display; DEGs_differentially expressed genes; DETs_differentially expressed transcripts; DW_dry weight; EDTA_ethylenediaminetetraacetic acid; FW_fresh weight; GSPs_gene-specific primers; LEA proteins_late embryogenesis abundant proteins; PPFD_photosynthetic photon flux density; RQ_relative quantitation; RWC_relative water content; SDH_succinate dehydrogenase; SW_satured weight; TDF_transcript derived fragment; Ψ_{soil}_soil water potential; Ψ_{leaf}_leaf water potential.

Introduction

Ilex paraguariensis St. Hil. (yerba mate) is a tree species native to South America. The leaves and young shoots of this species are manufactured to prepare a unique tea-like beverage, which is prized for its distinctive taste and stimulating properties and for the social practices related to its consumption. *I. paraguariensis* is used both as a source of caffeine, in parallel with tea and coffee, and as a therapeutic agent with proven pharmacological properties, including anti-inflammatory, anti-mutagenic, and lipid-lowering activities (Bracesco et al., 2011; Lima et al., 2014).

During the annual growing cycle, commercial crops are subject to periods of water deficit and high temperatures that negatively affect yields. Dry periods occur primarily during late spring and summer, which coincides with the period of greatest sprouting, when 60 to 80% of the total leaf area index is produced. Because drought tolerance is the result of interactions between genotypes and the environment, the characterization of tolerant cultivars is especially important for successfully transferring desirable traits into more sensitive varieties. In a previous study, we thoroughly characterized the metabolic and physiological response to drought and recovery after re-watering of the cultivar SI-49 (Acevedo et al., 2013). Among the many strategies utilized by plants to cope with drought, stomatal closure and osmotic adjustment have been observed in the leaves of this cultivar; consequently, leaf abscission is significantly reduced, which promotes rapid growth recovery when rainfall resumes. Given that, the harvestable products of yerba mate are the leaves. This trait confers a selective advantage compared with the dramatic leaf abscission that is characteristic of most commercial cultivars under stress. The stress response and adaptations of plants that are exposed to drought conditions form a complex network involving a cascade of genes (Rowley and Mockler, 2011). Thus, identifying the genes involved in the drought response process is of prime importance. Cellular sensors are initially sense and respond to

drought induced signalling, which in turn, triggers changes in gene expression in order to synthetize additional signal molecules such as abscisic acid (Xiong, 2009). Further signalling cascades are then initiated to signal new gene expression patterns that are thought to play a role in cellular adaptations to water-deficit stress (Bray, 2002; Shinozaki et al., 2003). There are two classes of proteins synthetized as a result of cellular sensing of drought stress. The first class is the regulatory proteins, including kinases, which are components of signalling transduction and amplification pathways (such as abscisic acid, ABA), and the transcription factors that activate genes encoding protective proteins. The second class of proteins (the functional proteins), includes proteins that have protective with chaperoning roles, such as late embryogenesis abundant (LEA) proteins, proteases, and proteins regulating water channels for turgor pressure (Rowley and Mockler, 2011).

Previously, we demonstrated that drought affects the expression of *IpSDH1*, which encodes the flavoprotein subunit of succinate: ubiquinone oxidoreductase (succinate dehydrogenase, SDH, EC 1.3.5.1) in the leaf. We provided evidence that *IpSDH1* expression is up-regulated by drought and ABA presence in the leaves of a drought-tolerant *I. paraguariensis* genotype. This up-regulation is correlated with a significant increase in SDH activity in the absence of mitochondrial damage (Acevedo et al., 2013). Recently, Debat et al. (2014), using NGS and homology sequence searches, have generated a draft transcriptome from an uncharacterized pool of *I. paraguariensis* leaves.

The goal of this work is to identify differentially expressed genes in *I. paraguariensis* leaves induced by drought as a strategy to investigate the molecular mechanisms underlying drought acclimation in the tolerant cultivar SI-49. We used mRNA Differential Display (DD) technique (Liang and Pardee, 1992) to compare the expression profile of stressed and unstressed plants and to isolate the transcript-derived fragments (TDFs) from differentially expressed genes. The obtained results were validated by real-time PCR. These data could be useful for improving the breeding efficiency of new drought tolerant cultivars.

Results

Detection of differentially expressed transcripts using mRNA Differential Display

During the 35-day experimentation period, the leaf water potential (Ψ_{leaf}) decreased from -1.73±0.03 MPa under well-watered conditions to -2.25±0.03, -2.78±0.02, and -2.85±0.05 MPa in response to a mild, moderate, and severe stress, respectively. As a consequence, relative water content (RWC) dropped by 78.1±3.1, 65.6±3.2, and 56.9±6.5 %, respectively. After recovering, both parameters were similar to those under the well-hydrated condition. The experiment demonstrated the ability of yerba mate (*Ilex paraguariensis*) to revive its normal functions, as measured by Ψ_{leaf} and RWC characteristics, in spite of suffering severe drought stress. Under severe stress, RWC was only 57.6 % of its fully hydrated value.

The mRNA Differential Display (DD) showed that a number of transcripts were up- and down-regulated in response to drought and during recovery (Tables 1, 2). For DD assay, we utilised a total of 200 different combinations of primer pairs, resulting from the mix of a decamer primer with an anchored primer. Of these, 142 PCR reactions amplified successfully. The analysis of the polyacrylamide gels indicated 52 differentially expressed transcripts (DETs) ranging in size from 179 to 1,172 bp; 38 DETs were induced (Table 1), whereas 14 DETs were repressed in response to drought compared to the well-watered conditions (Table 2). Additionally, Rapid Amplification of cDNA Ends (RACE) was used to amplify both the 5' and 3' ends of cDNA sequences shorter than 350 bp to improve the identification and annotation of differentially expressed genes.

Sequence comparison with data available from the National Centre for Biotechnology Information (NCBI) GenBank revealed that 19% of the fragments had no homologies, whereas 40.5% of the total number of fragments had strong similarities with database entries of known functions (proteins or characterized genes). Other sequences matched unclassified proteins or genes with unknown functions. Sequence analysis of selected clones and their putative gene products, including similarity and expected values, are listed in Table 1 and Table 2.

Functional categories of Ilex paraguariensis transcripts modulated by drought

The identified genes were assigned to specific functional categories based on a careful analysis of the scientific literature. Figure 1 displays the proportion of DETs assigned to each functional category. Interestingly, 12% of the DNA sequences were associated with signal transduction, and 2 % of the sequences were identified as transcription factors. Approximately, 12% of the annotated sequences were involved in stress tolerance and metal homeostasis, while 7% of DETs were associated with photosynthesis. Other groups of DETs, representing 12% of the total, included those involved in secondary metabolism, lignin biosynthesis, cell walls, RNA stabilization and RNA chaperone activity.

Validation of expression patterns using quantitative Real-Time PCR

Six transcripts with different expression patterns were chosen for further analysis of expression levels. The expression profiles of leaves from stressed and re-watered plants were compared with the equivalent sample from unstressed plants (Fig. 2). An arbitrary expression value of 1 was assigned to the well-watered treatment. For representing the overexpressed genes, the metallothionein type 2 (clone 10a), the GLK1-like transcription activator (clone E3), and an uncharacterized protein (clone B1) were chosen. Among them, the expression of clone 10a increased up to 4-fold in response to moderate stress. The transcription of clone E3 increased progressively until to reach a peak of 7-times under severe stress, while the transcription of B1 clone is mainly induced under moderate stress. After re-watering, all of them reduced their transcription rate to the fully hydrated stage. On the other hand, as representative of the repressed genes, the photosystem I P700 apoprotein A1 (clone B9), the enzyme isopiperitenol/carveol dehydrogenase (clone C2) and the protein phosphatase 2C 57 (clone B5) were selected. In this context, we detected that the expression of B5, C2 and B9 clones diminished under mild, moderate and severe stress, respectively. After 48 h from re-watering, the transcription level of B5 is recovered. Statistical analysis confirmed that there were significant differences in the relative levels of expression of the chosen DETs and validated the DD experiments.

Discussion

Previous studies (Acevedo et al., 2013) have indicated that the physiological response of cultivar SI-49 to an imposed

Table 1. Genes induced in *Ilex paraguariensis* leaves in response to moderate, mild and severe drought stress.

	Seq N°	Size (bp)	Accession N°	Gene annotation	NCBI BLAST Hit	e-value	Homology
Mild stress	10a	525	JX271039	Metallothionein type 2-like	ABD97258.1	4e-19	85%
	F11	1,375	KT428840	Chorismate mutase, chloroplastic-like	XP_007011720.1	1e-84	68%
	F13	1,420	KT428841	Stellacyanin-like	XP_010323477.1	6e-42	61%
	E3	1,449	KT428870	Transcription activator GLK1-like	XP_002275230.1	3e-138	63%
	G3	521	KT428842	Uncharacterized protein	CDP04700.1	2e-25	60%
	G5	326	KT428843	Uncharacterized protein	No similarity	—	—
	G10	1,733	KT428844	Uncharacterized protein At2g24330-like	XP_010647006.1	3e-165	72%
	G11	799	KT428845	Uncharacterized protein At5g01610-like	XP_009335705.1	4e-62	71%
	G14	1,171	KT428846	Uncharacterized protein At1g01500-like	XP_011650732.1	6e-153	70%
	G1	339	KT428847	EST from drought stressed leaves	No similarity	—	—
	G2	1,081	KT428848	EST from drought stressed leaves	No similarity	—	—
	G4	549	KT428849	EST from drought stressed leaves	No similarity	—	—
Moderate stress	D14	1,166	KT428850	Protein TIME FOR COFFEE-like	XP_008351235.1	1e-78	59%
	B4, P1	741	KT428851	glycine-rich cell wall structural protein-like	XM_002528173.1	2e-45	40%
	B17	422	KT428852	Ubiquitin-conjugating enzyme E2 35-like	XM_011008219.1	4e-14	72%
	F10	812	KT428853	Expansin-related protein 2 precursor	XP_002309385.1	3e-39	57%
	P7	1,263	KT428854	Protein of unknown function (DUF3353)	XP_002278026.1	5e-140	75%
	B16	630	KT428855	Uncharacterized protein	XP_009349676.1	2e-32	68%
	E12	1,190	KT428856	Uncharacterized protein	XP_007222563.1	1e-143	68%
	B1	1,286	KC505154	Uncharacterized protein	XP_009758931.1	6e-98	56%
	C4, C6	553	KT428857	Uncharacterized protein	XP_010694368.1	6e-09	92%
	B8	555	KT428858	Uncharacterized protein	CDP14956.1	2e-24	38%
	G15	1,183	KT428859	Uncharacterized protein	CDP02918.1	4e-35	35%
	G19	516	KT428860	Uncharacterized protein	CDO99352.1	2e-35	56%
	G20	582	KT428861	Uncharacterized protein	No similarity	—	—
	G21	779	KT428862	Uncharacterized protein At1g08160-like	XP_010644674.1	6e-91	66%
	B15	539	KT428863	EST from drought stressed leaves	No similarity	—	—
	C8	753	KT428864	EST from drought stressed leaves	No similarity	—	—
	F14	499	KT428865	EST from drought stressed leaves	No similarity	—	—
	F5	383	KT428866	EST from drought stressed leaves	No similarity	—	—
Severe stress	F2	1,854	KT428867	Heat shock 70 kDa protein 8	XP_011090717.1	0.0	83%
	B10	1,415	KT428868	Basic 7S globulin	XP_002272235.1	0.0	82%
	B12	993	KT428869	Replication protein A 32 kDa subunit B-like	XP_002268721.2	2e-152	76%
	G22	807	KT428871	Uncharacterized protein At4g22758-like	XP_011090002.1	2e-85	69%
	G18	314	KT428872	Uncharacterized protein	No similarity	—	—
	B14	554	KT428873	EST from drought stressed leaves	No similarity	—	—
	G8	625	KT428874	EST from drought stressed leaves	No similarity	—	—

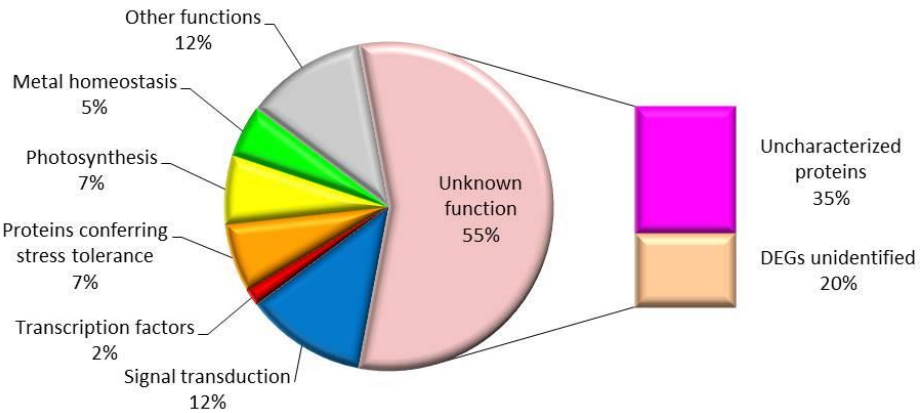

Fig 1. Functional categories of *Ilex paraguariensis* transcripts modulated by drought stress.

Table 2. Genes repressed in *Ilex paraguariensis* leaves in response to water stress.

Seq N°	Size (bp)	Accesion N°	Gene annotation	NCBI BLAST Hit	e-value	Homology
C2	995	KT441125	(-)-isopiperitenol/(-)-carveol dehydrogenase, mitochondrial-like	XP_009369359.1	3e-122	73%
F6	1,885	KT441126	protein WALLS ARE THIN 1-like	XP_011074627.1	0.0	85%
F12	570	KT441127	chromatin modification-related protein MEAF6-like	XP_011073527.1	8e-56	74%
D10	1,424	KT441128	Zinc/iron transporter-like	XP_009775150.1	1e-138	70%
D20	1,436	KT441129	E3 ubiquitin-protein ligase RING1-like	XP_010088395.1	1e-105	60%
F16	3,885	KT441130	Probable ubiquitin conjugation factor E4	XP_009589734.1	0.0	86%
F4	795	KT441131	Probable calcium-binding protein CML29	XP_008233918.1	4e-66	69%
E1	2,502	KT441132	Threonyl-tRNA synthetase-like	XP_002265302.1	0.0	84%
B5	1,329	KC491213	Protein phosphatase 2C 57-like	XP_011084521.1	0.0	74%
B9	1,275	KT441133	Photosystem I P700 chlorophyll a apoprotein A1	ADD30820.1	0.0	99%
F9	2,033	KT441134	ribulose bisphosphate carboxylase/oxygenase activase, chloroplastic	XP_009343792.1	0.0	80%
F15	2,152	KT441136	UPF0481 protein At3g47200-like	XP_011069627.1	3e-111	45%
G12	1,046	KT441137	Uncharacterized protein At2g34460-like	XP_009596108.1	2e-159	78%
G23	999	KT441138	Uncharacterized protein At4g14100-like	XP_012439068.1	2e-94	77%
F3	233	KT441139	EST from drought stressed leaves	No similarity	—	—
B16	630	KT428855	Uncharacterized protein	XP_009349676.1	2e-32	68%
E12	1,190	KT428856	Uncharacterized protein	XP_007222563.1	1e-143	68%
B1	1,286	KC505154	Uncharacterized protein	XP_009758931.1	6e-98	56%
C4, C6	553	KT428857	Uncharacterized protein	XP_010694368.1	6e-09	92%
B8	555	KT428858	Uncharacterized protein	CDP14956.1	2e-24	38%
G15	1,183	KT428859	Uncharacterized protein	CDP02918.1	4e-35	35%
G19	516	KT428860	Uncharacterized protein	CDO99352.1	2e-35	56%
G20	582	KT428861	Uncharacterized protein	No similarity	—	—
G21	779	KT428862	Uncharacterized protein At1g08160-like	XP_010644674.1	6e-91	66%
B15	539	KT428863	EST from drought stressed leaves	No similarity	—	—
C8	753	KT428864	EST from drought stressed leaves	No similarity	—	—
F14	499	KT428865	EST from drought stressed leaves	No similarity	—	—
F5	383	KT428866	EST from drought stressed leaves	No similarity	—	—

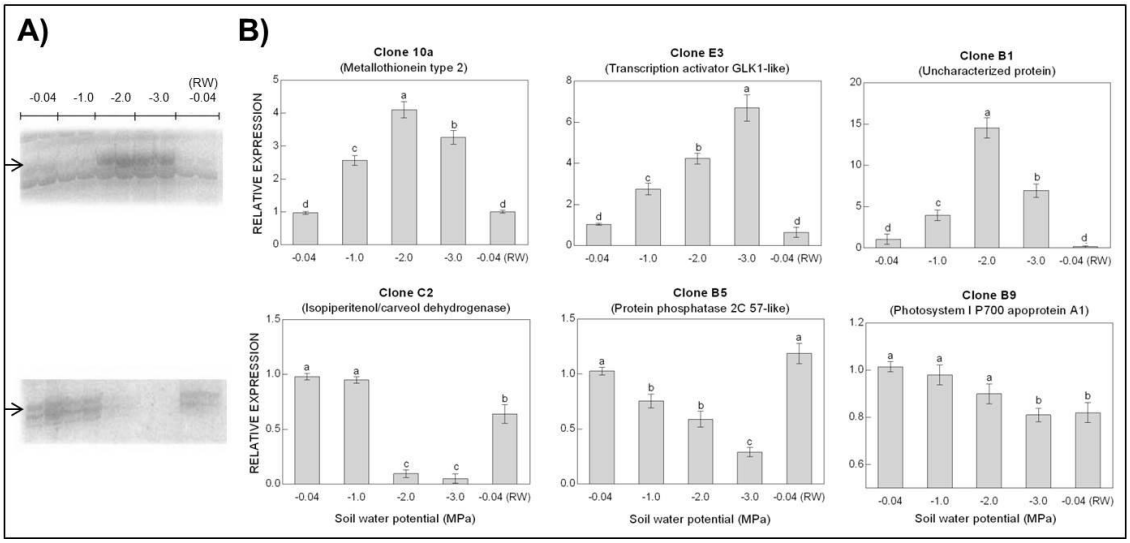

Fig 2. Transcript levels in leaves of *Ilex paraguariensis* cv SI-49 up- and down-regulated (top and bottom, respectively) by drought stress. **A)** Differential Display polyacrylamide gel patron. **B)** Quantitative Real-Time PCR to validate Differential Display expression profiles. *I. paraguariensis* β-tubulin was amplified as a reference gene to normalize expression. Bars represent the SEM calculated in triplicate on three plants. Different letters indicate significantly different ($P \leq 0.05$) values.

Table 3. Primers used for gene expression analysis by qRT-PCR.

Clone	Homologous protein	Primer sequence (5´- 3´)	Length bp	Tm(°C)	Amplicon
B1	Uncharacterized protein	F: ACGAGGGTAAGATTGGTGATTAAG R: AAACCCACCACCTTCCATTCT	24 21	61.2 60.6	150 pb
B5	Protein phosphatase 2C 57-like	F: AAGAAGAATGAGATGCTGGAGAAG R: CACAAGCCATCAGATGCTAATAAC	24 24	61.2 61.2	167 pb
B9	Photosystem I P700 chlorophyll a apoprotein A1	F: CACCATTTAGCGGGGCTACT R: GTGAAAAACGGGGTTGCTCC	20 20	61.5 61.8	200 pb
C2	Isopiperitenol / carveol dehydrogenase-like	F: AGGAGCAAACATGCGGACCGATTACA R: ACAACACAGCATCTGCGACGTGTCTG	26 26	66.2 67.7	245 pb
E3	Transcription activator GLK1-like	F: GGGTCATCCATCTGTCGACC R: GAAGGGTCTGATGGTGGTGG	20 20	64.5 64.5	147pb
10a	Metallothionein type2-like	F: CTGTGGAGGAAACTGTGGCT R: GGTGGTGCTCTCAGAGTAGC	20 20	62.4 64.5	100 pb
Reference gene	β-tubulin	F: GCAGTTTACGGCCATGTTCA R: TCCTCATCTGCAGTGGCATC	20 20	60.4 62.4	89 pb

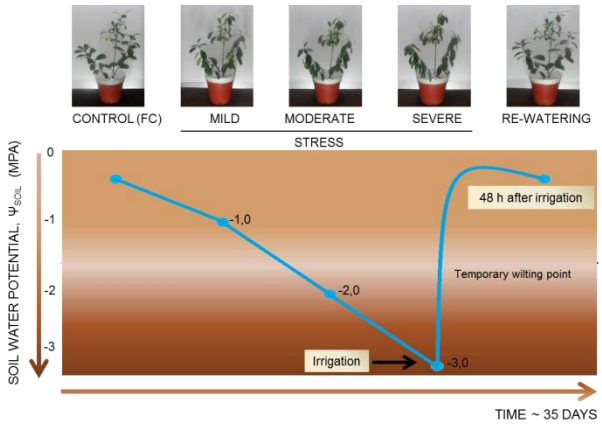

Fig 3. Drought imposition assay. Well-hydrated plants were deprived of irrigation until the soil water potential (Ψ_{soil}) reached -3 MPa. Subsequently, the plants were irrigated to overcome a severe drought stress. Ψ_{soil} versus time is outlined throughout the assay. The blue symbol indicates the time of sample collection. FC: field conditions.

water deficit might be related to the tendency of this species to minimize water losses through stomatal closure in the early stages of dehydration to avoid tissue desiccation. Because the leaf water potential diminished due to an increase in water restriction, leaf tissues reacted by making osmotic adjustments to sustain tissue metabolic activity, which enabled the recovery of photosynthesis upon re-watering. This experiment showed that yerba mate was able to recover, in terms of photosynthesis and water status, in spite of suffering severe drought stress.

Here, we characterized a number of genes that were up- and down-regulated in response to drought and recovery by using mRNA Differential Display. The expression levels of a transcription factor (transcription activator GLK-1-like, *GenBank: KT428870*) and a plethora of genes related to amino acid metabolism (chorismate mutase, *GenBank: KT428840*), antioxidant defence (metallothionein type 2-like, *GenBank: KX271039*), cell wall properties (stellacyanin, *GenBank: KT428841*), and other genes encoding uncharacterized proteins were up-regulated at an early stage of dehydration.

The Golden 2-like (GLK) transcription factors are functionally conserved within the plant kingdom and are required for chloroplast development (Fitter et al., 2002). These proteins include GLK-1- and GLK-2-like transcription factors, which encode the components involved in chlorophyll biosynthesis comprising the subunits of Mg-chelatase (Waters et al., 2009; Barajas-López et al., 2013). Both *GLK* genes respond to plastid retrograde signals and are sensitive to feedback signalling from the chloroplast (Woodson and Chory, 2012). The expression of GLK-1 in *I. paraguariensis* stressed leaves was induced 3- to 7-fold, depending on the severity of dehydration (Table 1). Savitch et al. (2005) have reported that cold stress and cold acclimation of *Brassica napus* plants is associated with a moderate accumulation of mRNA for *GLK-1* and *GLK-2*. Similarly, the amino acid composition is modified by environmental conditions and characterized by the elevated

accumulation of specific amino acids involved in plant stress tolerance (Planchet and Limami, 2015). In response to environmental constraints, amino acid metabolism plays an important regulatory role by potentially adjusting and signalling molecules and precursors for energy-associated metabolites, as well as numerous secondary metabolites that have several functions in plant growth and adaptive responses to stress. The enzyme chorismate mutase (CM) catalyzes conversion of chorismate to prephenate (Tzin and Galili, 2010). Chorismate is the final product of the shikimate pathway and is the initiator metabolite for the synthesis of aromatic amino acids and secondary metabolites derived from these amino acids, including auxins, salicylates, antimicrobial alkaloids, flavonoids, and polyphenolic compounds produced in response to environmental signals as part of a general defence response (Less et al., 2010). The overexpression of genes encoding aromatic amino acids may be related to the protective role of flavonoids during photosynthetic acclimation under moderate water stress (Watkinson et al., 2003). Our results also showed an increased expression of the gene encoding metallothionein type 2 protein (MT) in response to stress severity. In fact, the transcript levels in leaves, analysed by Real-Time PCR, revealed a ~2.5-fold increase during acclimation. A 4-fold increase was observed when plant was subjected to mild stress conditions. The transcript levels decreased (8-15%) under severe dehydration. MTs are a cysteine-rich protein family with low molecular weight (5-10 kDa) that are widely distributed in nature. The MTs are found in prokaryotes, yeast, protozoans, invertebrates, vertebrates and plants. The main features of these proteins come from their abundant thiol (-SH) groups, which enable them not only to join both physiological (Zn, Cu, Se) and xenobiotic (Cd, Hg, Ag, As) heavy metals but also to scavenge reactive oxygen species. Some genes encoding MTs are induced by Cu, Zn, Cd, Ag and Hg. The resulting proteins are able to bind these metals efficiently. Thus, MTs appear to participate in metal homeostasis, mechanisms leading to tolerance to high concentrations of metals, and antioxidant defence mechanisms (Cobbett and Goldsbrough, 2002; Guo et al., 2008; Usha et al., 2009). Furthermore, Mir et al. (2004) have demonstrated that metallothionein type 2-like is actively transcribed in *Quercus suber* cork cells in response to oxidative stress, both as a free radical scavenger, via its sulfhydryl groups and as a copper chelator. Zinc is considered an essential microelement in plant development because it acts as a cofactor for many enzymes and may be necessary for the activation of certain transcription factors by binding to zinc finger domains. When zinc enters a cell, it can be captured by MTs and transported to another part of the cell, where it is transferred to another protein or organelle. Thus, MT is a key component of the signalling system (Capdevila et al., 2012). Stellacyanins are Cu(II) glycoproteins that are members of the phytocyanin subclass of the cupredoxins. Type 1 copper proteins (cupredoxins) possess a mononuclear type 1 copper site, with the Cu transitioning between the oxidized Cu(II) form and the reduced Cu(I) form. Therefore, they are a key class of electron-transfer proteins found in both prokaryotes and eukaryotes (Nersissian and Shipp, 2002; Koch et al., 2005; Choi and Davidson, 2011). Stellacyanin has been known to function as an electron-transfer protein, which accepts electrons from the cytochrome b_6-f complex and donates them to photosystem I during photosynthesis (Müller et al., 1992). Although, stellacyanin genes are up-regulated by wounding and pathogens, expression is induced by ABA, drought, and salt stress in pepper leaves (Kong et al., 2002). Thus, the expression of stellacyanin transcripts in the leaves of yerba mate may correlate with increased ABA content during the early stages of drought acclimation (Acevedo et al., 2013).

When the soil water potential (Ψ_{soil}) reached -2 MPa (moderate stress) and the leaf RWC dropped to approximately 65%, a number of genes related to the circadian clock and metabolic homeostasis (*GenBank: KT428850*), including glycine-rich cell wall structural proteins (*GenBank: KT428851*), expansin (*GenBank: KT428853*), and ubiquitination-related genes (*GenBank: KT428852*), were up-regulated.

The circadian clock is a time-keeping mechanism used to coordinate the physiology of an organism to its surrounding environment (Sanchez et al., 2011). The circadian clock contributes to plant homeostasis by both ensuring a proper energy balance and priming responses to metabolic stress. Several reports have demonstrated signalling intercommunication between the circadian clock and stress responses (Legnaioli et al., 2009; Castells et al., 2010; Lai et al., 2012). Additionally, Sanchez-Villarreal et al. (2013) have used transcriptomic and metabolomic comparative analyses to determine that *TIME FOR COFFEE* (*TIC*), a circadian regulator involved in clock resetting at dawn, constitutes a central element in plant homeostasis that affects diverse developmental and metabolic pathways, such as starch and carbon metabolism, ABA-related responses, oxidative stress tolerance, and amino acid pools. The authors suggest that *TIC* acts as the central hub connecting the circadian clock, stress signalling, and metabolic homeostasis.

The plant glycine-rich proteins superfamily (GRP) is characterized by the presence of a glycine rich domain arranged in semi-repetitive glycine-rich motifs (Gly)n-X. The diverse but highly specific expression pattern of GRP genes, when considered together with the distinct sub-cellular localization of some GRP groups, clearly indicates that these proteins are implicated in several independent physiological processes, including plant defence against biotic and abiotic stress (Bocca et al., 2005). Several GRPs are modulated by ABA and osmotic stress (Mangeon et al. 2010). Furthermore, the RNA-binding activity of some GRPs (e.g. RB-GRP) has been biochemically demonstrated, and it is thought that these GRPs may be involved in RNA stabilization, processing and transport. In the recent years, the functions of *RB-GRPs* have been analysed in various plants, and expression of these genes is regulated by different environmental stresses (Zhang et al., 2014).

Expansins are a family of cell wall proteins proposed to play a key role in the regulation of tissue elongation and cell wall differentiation (Cosgrove, 2000). There is increasing evidence demonstrating that expansins are involved in the responsive mechanisms of plant species to water stress (Dai et al., 2012; Zhao et al., 2012; Barcia et al., 2014). Recently, Zhou et al. (2015) have suggested that water stress inhibits leaf growth; however, changes in expansin activity and cell wall susceptibility to expansin during times of water deficit could increase the growth rate after rehydration. Our results are consistent with this finding (Table 1), and they confirmed that moderate water restriction up-regulated the transcript F10 (an ortholog of *Populus trichocarpa GenBank: XP_002309385.1*) encoding an expansin-related protein 2 precursor.

Ubiquitination is a post-translational modification, whereby ubiquitin is attached to a substrate protein. Ubiquitination is known to regulate important functions in a wide variety of plant growth and developmental processes, including photomorphogenesis, vascular differentiation, flower

development, and biotic and abiotic stress responses (Zhang et al., 2015). Ubiquitination is carried out in three main steps, activation, conjugation, and ligation, which are performed by ubiquitin-activating enzymes (E1), ubiquitin-conjugating enzymes (E2), and ubiquitin ligases (E3), respectively. E3 ligases play important roles in governing the ubiquitin signalling pathway by transferring ubiquitin from E2 conjugation to specific protein substrates (Zhiguo et al., 2015). We observed that the expression of the transcript B17 (an orthologue of *Populus euphratica*, *GenBank: XM_011008219.1*, Table 1), which encodes the E2 enzyme, was induced in leaves of drought treated plants.

When water restrictions became more severe (at Ψ_{soil}= -3 MPa) and leaf RWC dropped by 56.9±6.5%, some genes encoding heat shock protein 70 kDa (*GenBank: KT428867*), Basic 7S globulin (*GenBank: KT428868*) and replication protein A 32 kDa subunit B (*GenBank: KT428869*) were up-regulated to reduce cell damage. The heat shock protein 70 (Hsp70) family is one of the five major families of Hsps/chaperones that is highly conserved. Hsp70 has essential functions in preventing aggregation and in assisting in refolding of non-native proteins under both normal and stress conditions. Thus, Hsp70 has a crucial role in protecting plants against stress by restoring the functional conformation of altered proteins and; therefore, of cellular homeostasis. This protein family is also involved in protein import and translocation processes and in facilitating the proteolytic degradation of unstable proteins by enabling the targeting of proteins by lysosomes or proteasomes (Saibil, 2008). A minimum of 18 genes in the *Arabidopsis* genome have been identified as encoding members of the Hsp70 kDa family. Some members are expressed in response to environmental stress conditions such as heat, cold and drought, as well as to chemical and other stresses. The overexpression of Hsp70 genes positively correlates with the acquisition of thermotolerance and results in enhanced tolerance to salt, water and high-temperature stress in plants (Wang et al., 2004; Sruthy et al., 2015). In addition to general chaperone functions, some members of the Hsp70 family have been implicated in the modulation of key enzymes in signal transduction cascades, such as protein kinases and protein phosphatases. Therefore, the Hsp70 chaperones might play a broader role by participating in modulation of the expression of many downstream genes in signal transduction pathways (Nollen and Morimoto, 2002). Hu et al. (2010) have suggested that HSP70 may play a crucial role in ABA-induced antioxidant defence of maize to the combination of drought and heat. Grigorova et al. (2011) have observed an increase in Hsp70 in wheat plants under drought or heat stress. This increase is a significantly greater when plants simultaneously experience both stresses, a situation that normally occurs in nature. Similarly, the mRNA level of the Basic 7S globulin (Bg7S), a cell wall protein primarily located in the middle lamella of soybean seeds, increases in response to heat stress (Fujiwara et al., 2014).

Replication protein A (RPA) is essential for DNA replication, repair, and recombination (Sakaguchi et al., 2009). In higher plants, three different types of replication proteins (types A, B and C), each displaying different functions in DNA metabolism, have been identified (Ishibashi et al., 2006; Sakaguchi et al., 2009). In addition, Xia et al. (2006) have reported that ROR1/RPA2A, a 31-kDA protein similar to RPA2, plays important roles in maintaining epigenetic gene silencing and regulating meristem development in *Arabidopsis*.

Among the DETs repressed by drought in *I. paraguariensis*, the expression of genes associated with photosynthesis (*GenBank: KT441133, KT441134, KC491213*), secondary metabolism (*GenBank: KT441125*), auxin transport (*GenBank: KT441126*), DNA damage repair (*GenBank: KT441127*), tRNA protection (*GenBank: KT441132*), protein stability (*GenBank: KT441129, KT441130*), and metal homeostasis (*GenBank: KT441128, KT441131*) were down regulated.

Osmotic stresses, including drought, substantially damage the photosynthetic machinery of plants (Gururani et al., 2015). Photosystem II (PSII) is one of the most susceptible components (Nath et al., 2013). Changes in the expression of genes related to photosystems and CO_2 assimilation were observed in the leaves of stressed plants. The mRNA level of protein phosphatase 2C 57-like (PPH1) declined significantly at an early stage of dehydration but increased to control levels after re-watering (Fig. 2). PPH1 is a chloroplast protein that is primarily associated with the stroma lamellae of thylakoid membranes. Using a reversible phosphorylation of key proteins in the photosynthetic membrane, plants can quickly adapt to changes in the intensity and the spectral quality of light conditions; a process called state transition (Shapiguzov et al., 2010). When tissue dehydration became more severe (Ψ_{soil}= -3 MPa), the transcription of B9 sequences encoding Photosystem I P700 apoprotein A1 significantly decreased. Part of the long-term response to excitation imbalances between photosystems involves a re-adjustment of photosystem stoichiometry to keep the net energy fixation as high as possible (Steiner et al., 2009). In conjunction with the decrease of stomatal conductance, the expression of the F9 sequence encoding ribulose 1,5-bisphosphate carboxylase/oxygenase activase decreased in response to moderate and severe stress. In higher plants, the active state of Rubisco is strictly dependent on Rubisco activase (RCA). RCA is a chaperone-like protein of the AAA+ family, which utilizes ATP hydrolysis to mechanically modify the conformation of Rubisco and facilitate dissociation of tight-binding inhibitors of the active site (Chao et al., 2014). Most species studied contain two isoforms of RCA, α- (46-48 kDa) and β-isoform (41-43 kDa), from a single alternatively spliced pre-mRNA that is transcribed from a single RCA gene (Bayramov and Guliyev, 2014). In *I. paraguariensis*, the mature mRNA of the long isoform lacks an intron that encodes a stop codon; thus, enabling the extension of the C-terminus by 37 amino acid residues more than the small isoform. Control of RCA expression is complicated under unstressed conditions and is regulated at various levels in different species. In *Pinus halepensis* needles, the RCA protein quantity is increased by drought (Pelloux et al., 2001). In contrast, in mature leaves of *Brachypodium distachyon*, proteins and transcript levels of both isoforms decrease in response to water deficits (Bayramov and Guliyev, 2014). However, mRNA levels of the RCA gene do not change in response to drought or re-watering in *Poa pratensis* (Xu et al., 2013).

Materials and Methods

Plant material and drought assay

Plants from *Ilex paraguariensis* cv. SI-49 were obtained from rooted stem cuttings using the protocol developed by Tarragó et al. (2005). Plants were grown in 4 L pots filled with lateritic soil under greenhouse conditions for twelve months. Before the experiment, all plants were pruned to obtain a similar leaf area index and transferred to a chamber with controlled environmental conditions (27±1ºC day, and 22±2ºC night, with a 14 h photoperiod under an irradiance of

420 µmol m^{-2} s^{-1} photosynthetic photon flux density from high pressure mercury lamps, 50-55 % relative humidity). 12-month-old plants were watered to the drip point (Ψ_{soil}= ~ -0.04 MPa) and subsequently subjected to a continuous soil drying episode by withholding water from pots until the Ψ_{soil} at pre-dawn reached either -1, -2 or -3 MPa (~ 35 days) considered mild, moderate, and severe stress, respectively. Pots were covered with aluminium foil to prevent evaporation. Well-watered (control at field capacity) and re-watered (48 h after reach Ψ_{soil}= -3 MPa) treatments were also included (Fig. 3).

Soil water potential was measured using a psychrometer (dew point microvoltimeter, model HR-33T, Wescor Inc., USA) with a PST-55 thermocouple according to the manufacturer´s instructions. To determine the leaf water content in relation to the imposed stresses, Ψ_{leaf} was determined *in situ* using an L-51 thermocouple. At each Ψ_{soil}, six fully expanded leaves were harvested per plant to give three leaves for RWC measurements ($RWC\ \% = [FW - DW]/[SW - DW]\ x100$; where FW, DW and SW, are fresh weight, dry weight and satured weight respectively) and three leaves for molecular analyses. For the molecular analyses, leaves were harvested during the daytime, immediately frozen in liquid nitrogen, and stored at -80 °C prior to RNA extraction. Samples were collected from three biological replicates for each Ψ_{soil} condition.

RNA isolation and synthesis of first-strand cDNAs

Total RNA was extracted from leaves using the SV Total RNA Isolation System (Promega, Madison, WI, USA) according to the manufacturer's protocol, which includes treatment with RNase-free DNase to eliminate contaminating genomic DNA. Each RNA sample was quantified by spectrophotometric methods. The 260/280 ratio of all RNA samples was approximately 2. The integrity of the RNA samples was determined by visualizing bands via agarose gel electrophoresis with ethidium bromide staining. For each sample, first-strand cDNA was synthesized from 1 µg of total RNA by SuperScript II Reverse Transcriptase (Invitrogen™, Waltham, MA, USA). One of four anchored oligo(dT) primers (DDT1: 5´-T_{12}VA-3´, DDT2: 5´-T_{12}VC-3´, DDT3: 5´-T_{12}VG-3´or DDT4: 5´-T_{12}VT-3´; where V represent A, C, or G) was used for the synthesis when cDNA was intended for analysis by mRNA Differential Display assays, and an oligo(dT_{18}) primer was used when cDNA was intended for Real-Time PCR.

mRNA Differential Display assay

mRNA Differential Display experiments were performed following the original protocol (Liang and Pardee, 1992) with minor modifications (Pessino et al., 2001; Akpinar et al., 2015). To create the pairs of primers needed for PCR, one arbitrary decanucleotide primer, obtained from the British Columbia University RAPD Primer Synthesis Project (set3, decamers 201 to 250) was used, in combination with the same anchored oligo(dT) primer used in cDNA synthesis (DDT1, DDT2, DDT3, or DDT4). Duplicate PCRs were prepared in final volumes of 25 µl containing 1X GoTaq® Reaction Buffer (Promega). The final concentrations of the buffer components after this addition were 1.5 mM $MgCl_2$, 200 µM dNTPs, 0.6 µM arbitrary decanucleotide primer, 4 µM anchored oligo(dT) primer, 2.5 U of GoTaq® DNA Polymerase (Promega, Madison WI, USA) and 2.5 µl of the reverse transcription reaction (previously diluted 1/20). Negative controls were performed using total RNA (non-reverse-transcribed) to verify the absence of genomic DNA in the RNA preparations. The thermocycler programme consisted of an initial step of 3 min at 94 °C, followed by 40 cycles of 20 s at 94 °C, 20 s at 38 °C and 30 s at 72 °C, with a final step of 5 min at 72 °C. The samples were mixed with denaturing loading buffer, treated for 3 min at 95 °C and separated on 6% polyacrylamide gels. The amplification products were silver-stained. Bands of interest (up- or down-regulated by drought) were excised, eluted in a buffer of 0.5 M ammonium acetate and 1 mM ethylenediaminetetraacetic acid at pH 8, precipitated in ethanol, and re-amplified using the same PCR conditions described above.

Cloning, sequencing, rapid amplification of 5´ and 3´ cDNA ends of TDFs and homology analysis

Each transcript derived fragment (TDF) with differential expression was cloned into the pGEM®-T Easy Vector System (Promega). Sequencing of the inserts was performed by Macrogen Inc. (Seoul, Korea). Full-length 5′ and 3′ ends of TDFs were obtained with the GeneRacer™ Kit (Invitrogen™) according to the manufacturer's protocol. Sequences of gene-specific primers (GSP) for each TDF were designed using Primer3Plus (http://www.primer3plus.com). The cDNA synthesized with the oligo(dT_{18}) was utilized as a template for the PCR reactions. To amplify the 5′ or 3′ end of the transcript of interest, GSP, in conjunction with the primer provided by the kit, were used in the PCR reactions. The enzyme mixture Platinum® Taq DNA Polymerase High Fidelity (Invitrogen™) was used in the PCR reactions. The amplified DNA fragments were cloned into the pGEM®-T Easy Vector System (Promega), and sequencing of the inserts was performed by Macrogen Inc. (Seoul, Korea).
To annotate DETs, homologies were searched using the BLAST tool of the NCBI (http://www.ncbi.nlm.nih.gov).

Real-Time PCR analysis

Quantitative Real-Time PCR analysis was carried out to validate the cDNA expression profiles. Gene-specific primers (Table 3) were designed on the basis of the DETs sequences using the primer design software Primer3Plus (http://www.primer3plus.com) and were subsequently used for PCR amplifications. Total RNA, extracted as described above, was used for first strand cDNA synthesis. Real-Time PCRs were performed on the cDNA templates using specific primers and SYBR Green PCR Master Mix (Applied Biosystems, California, USA), following the manufacturer's instructions. All reactions were prepared in triplicate and performed twice. The thermal cycle conditions included an initial denaturing step at 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min using the 7500 Real-Time PCR System (Applied Biosystems). The *I. paraguariensis* β-tubulin gene was used to estimate up- or down-regulation of the target gene for all Real-Time PCR analyses. The results were processed with the 7500 Software v2.0.1 (Applied Biosystems) using the comparative method CT (ΔΔCT) for calculating relative quantitation (RQ) of gene expression. Outputs were the means ± SE of the three biological replicates. Data were compared by one-way analysis of variance (ANOVA).

Conclusion

In response to applied water deficit stress, we identified 52 differentially expressed transcripts. This differential expression analysis provided the distribution of functional

categories and homologous genes. The availability of these sequences provides an opportunity to clone and characterize the corresponding full-length genes and associated promoter regions. This work will contribute to understanding of the relationship between gene expression and the molecular adjustments that *I. paraguariensis* makes in response to drought.

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