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Research article

A succinate dehydrogenase flavoprotein subunit-like transcript is upregulated in *Ilex paraguariensis* leaves in response to water deficit and abscisic acid

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ABSTRACT

Ilex paraguariensis plants were subjected to progressive soil water deficit, and differential display (DD) was used to analyse gene expression in leaves to characterise physiological responses to mild and severe water deficits. A cDNA fragment showing strong homology with the flavoprotein subunit (*SDH1*) of succinate:ubiquinone oxidoreductase (succinate dehydrogenase, SDH, EC 1.3.5.1) was upregulated in plants exposed to drought. Quantitative real-time PCR revealed that the *SDH1*-like transcript level began to increase when the leaf relative water content (RWC) decreased to 78% and peaked when the RWC dropped to 57%. A correlation between abscisic acid (ABA) concentration and variations in transcript levels was assessed by GC-SIM. After rehydration, *SDH1* mRNA and ABA returned to their initial levels. In stressed leaves sprayed with ABA *SDH1* mRNA accumulated in greater levels compared to stressed leaves that did not receive ABA. Moreover, the enzymatic activity of succinate dehydrogenase increased 1.5-fold in the mature leaves of ABA-treated plants. This physiological response may be related to the tendency of this species to minimise water losses through stomatal closure in the early stages of dehydration to avoid tissue desiccation. As the leaf water potential diminished due to an increase in water restriction, *I. paraguariensis* leaf tissues reacted by making osmotic adjustments to sustain tissue metabolic activity, which enables the recovery of photosynthesis upon re-watering. These results provide new insights concerning the linkage between plant respiration and photosynthetic metabolism that could be potentially further used in breeding programs aiming water tolerant genotypes.

1. Introduction

Ilex paraguariensis St. Hil. (yerba mate) is a shade-tolerant tree that is cultivated either via agroforestry systems or monoculture in northeastern Argentina, southeastern Brazil, and Paraguay [1,2]. Its leaves and young shoots are used to prepare a traditional tea-like infusion (mate-tea) that is greatly appreciated for its flavour, stimulant properties, and various medicinal benefits [3]. *I. paraguariensis* is typically found in forests under the shadow of *Araucaria angustifolia*, where conditions of low photosynthetic photon flux density (PPFD) and high humidity predominate. Few physiological studies of this species' ability to adapt to new environments such as fields have been performed, particularly regarding the increase in sunlight irradiance and the resulting increase in water demand due to evapotranspiration [4]. However, proper characterisation of the drought stress response and identification of drought-tolerant genotypes is imperative for yerba mate cultivators. Most plants that are grown in field conditions experience water stress during part of their annual growing cycle, especially at mid-day and early afternoon in the summer, when drought negatively affects growth and yield in conjunction with high temperatures and radiation. During the growing season (spring to autumn), yerba mate plants undergo

Abbreviations: ABA, abscisic acid; DD, Differential display; Ψ_{leaf}, leaf water potential; $\Delta\Psi_{\pi}^{100}$, osmotic adjustment; Ψ_{π}^{100}, osmotic potential; PPFD, photosynthetic flux density; RWC, relative water content; Ψ_{soil}, soil water potential; SDH, succinate dehydrogenase.

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three periods of rapid shoot elongation that alternate with intervals of little or no growth [5]. This phenomenon, known as "periodic" or "flushing" growth [6], is characterised by a perfectly coordinated growth process that begins with a bud burst stage, continues with an internode elongation stage and ends with a leaf expansion phase. In this context, the generation of drought-tolerant yerba mate varieties is now one of the priorities of local breeding programs [7,8]. Considering that drought tolerance is the result of interactions between genotype and environment, the characterisation of tolerant cultivars is especially important for successfully transferring desirable traits into more sensitive varieties.

The *I. paraguariensis* cultivar San Isidro 49 was identified during a drought period and is more tolerant to water deficit than other commercial cultivars from Argentina. Among the many strategies utilised by plants to cope with drought, stomatal closure and osmotic adjustment have been observed in the leaves of this genotype; consequently, leaf abscission was reduced, which favours rapid growth recovery when rainfall resumes. Given that the harvestable product of yerba mate is the leaves, this trait confers a selective advantage compared with the dramatic leaf abscission that is characteristic of most commercial cultivars under stress.

Water deficit, defined as an imbalance between soil water availability and evaporative demand, decreases carbon accumulation and tissue expansion and reduces cell number. The plant's response to this stress involves a large number of genes, enzymes, hormones, and metabolites [9]. The products of drought stress-inducible genes can be classified into two groups [10,11]; the first group includes functional proteins whereas the second group comprises regulatory proteins involved in signal transduction and gene expression (e.g., transcription factors, protein kinases and protein phosphatases) as well as diverse effectors involved in signalling (e.g., calmodulin-binding protein) [12]. Hundreds of genes that are induced under drought conditions have been identified, and a range of functional tools are being used to study their specific role in plant acclimation and adaptation to water deficit [13].

Many of these stress-inducible genes are activated by abscisic acid (ABA), and their products may improve tolerance to water stress [14]. Some of the genes that are upregulated by drought/ABA encode enzymes that function in the biosynthesis of compatible solutes that could lower leaf water potential (Ψ_{leaf}) and facilitate water uptake and retention. Others encode enzymes that can directly detoxify reactive oxygen species (ROS) [15]. Certain stress-responsive genes encode polypeptides that may help to restore the native structure of abnormally folded proteins. The list of genes that are upregulated by ABA also includes various components of the proteolysis pathway, which may promote the degradation of unfolded proteins that cannot be repaired, thus avoiding the negative effects of their accumulation on cellular activities [14]. Although drought alone can activate these stress-responsive genes, ABA can synergistically enhance their expression, enhancing the ability of the plants to respond to stress [16]. Marracini and co-workers [17] examined the molecular mechanisms underlying drought acclimation in coffee plants by identifying candidate genes and concluded that the response involved a complex network of responses involving ABA and nitric oxide as the proposed major molecular determinants, which might explain the increased efficiency in controlling stomata closure and transpiration display by drought-tolerant clones of *Coffea canephora*.

While conducting differential display experiments aimed at identifying *I. paraguariensis* genes regulated in leaves during water deficit periods, our group isolated a candidate that was homologous to the flavoprotein subunit of mitochondrial complex II: the *sdh*1 transcript. The objective of the present work was to (1) characterise the expression of this particular transcript in the leaves of water-stressed plants, (2) to determine the influence of ABA on its mRNA level and (3) to determine the enzymatic activity of SDH.

2. Results

2.1. Physiological characterisation of stressed and non-stressed plants

I. paraguariensis St. Hil. plants of the drought tolerant cv. *San Isidro 49* were subjected to a 35-day treatment in which drought was applied gradually by restricting evaporation. This approach helped to circumvent artificial changes in gene expression and physiological responses that may be induced by a shock treatment. The Ψ_{leaf} declined in response to the drought treatment (Table 1) compared to plants grown at Ψ_{soil} −0.04 MPa (field capacity) due to the development of an osmotic constraint in the stressed leaves. Ψ_{leaf} and Ψ_{π}^{100} decreased from −1.73 and −1.03 to −2.85 and −1.6 MPa, respectively, when Ψ_{soil} decreased to −3 MPa. A strong reduction in the leaf RWC (from 99 ± 0.3 to 57 ± 6.5%) was correlated with a significant difference in stomatal conductance between non-stressed and stressed plants (0.96 ± 0.1 and 0.04 ± 0.01 mol H_2O m^{-2} s^{-1}, respectively).

Additionally, endogenous ABA levels were quantified in the mature leaves of control and stressed plants (see Materials and methods). When Ψ_{soil} decreased to −1 MPa, foliar ABA levels were more than three-fold higher compared to the control treatment (Table 1). As water stress continued to increase, the situation was magnified by a further decrease in ABA levels. Stressed plants subjected to Ψ_{soil} −1, −2 or −3 MPa exhibited osmotic adjustment during soil drying with $\Delta\Psi_{\pi}^{100}$ of 0.41, 0.56, and 0.57 MPa, respectively. Two days after re-watering, the stressed plants displayed similar leaf RWC (97 ± 0.4%) and Ψ_{leaf} with respect to the control plants. However, Ψ_{π}^{100} remained 0.2 MPa lower than the control, indicating that the solute concentration had changed

Table 1
Effect of drought and re-watering on relative water content, water potential, osmotic potential, osmotic adjustment, ABA content, and MDA level of leaves from plants growing under different soil water conditions.

	Soil water potential (MPa)				
	−0.04 (**FC**)	−1	−2	−3	−0.04 (**RW**)
Ψ_{leaf} (MPa)	−1.73 ± 0.03	−2.25 ± 0.03***	−2.78 ± 0.02***	−2.85 ± 0.05***	−1.81 ± 0.04
Ψ_{π}^{100} (MPa)	−1.03 ± 0.01	−1.44 ± 0.03**	−1.59 ± 0.04**	−1.60 ± 0.03**	−1.23 ± 0.03*
$\Delta\Psi_{\pi}^{100}$ (MPa)	–	−0.41 ± 0.02***	−0.56 ± 0.04***	−0.57 ± 0.07***	−0.20 ± 0.03
Leaf RWC	98.97 ± 0.3	78.10 ± 3.1*	65.63 ± 3.2***	56.97 ± 6.5***	97.17 ± 0.4
g ($molH_2O$ m^{-2} s^{-1})	0.96 ± 0.10	0.33 ± 0.02***	0.08 ± 0.01***	0.04 ± 0.01***	0.59 ± 0.15*
A ($molCO_2$ m^{-2} s^{-1})	9.85 ± 0.36	7.37 ± 0.20***	6.88 ± 0.22***	5.71 ± 0.17***	9.39 ± 0.34
Leaf ABA (ng gr^{-1} DW)	218	690	187	402	100
Oxidative lipid injury (nmol MDA gr^{-1} FW)	60.1 ± 2.6	59.9 ± 3.8	65.1 ± 4.0	64.3 ± 5.0	63.5 ± 2.8

Values = mean ± SEM; $n = 6$; simple, double and triple asterisks indicate significant differences respect to the control at $P < 0.05$, 0.01 and 0.001, respectively; Dunnett's multiple comparison test. **FC**, field capacity; **RW**, re-watering.

during the drought, while the leaf ABA content decreased more than six-fold with respect to the greatest value achieved when $\Psi_{soil} = -1$ MPa.

Finally, considering that membranes are subject to rapid damage when water stress intensifies due to an uncontrolled increase of reactive oxygen species that cause lipid peroxidation [18], we decided to measure the production of malondialdehyde (MDA), which is a decomposition product of the polyunsaturated fatty acids in biomembranes [19]. Our results revealed that the accumulation of MDA in the mature leaves of *I. paraguariensis* slightly increased (by 8%) after exposure to severe water deficit ($\Psi_{soil} = -2$ and -3 MPa) but did not show significant variation from the non-stressed plants (Table 1).

2.2. Differential expression of a SDH1-like gene under water deficit conditions

For the analysis of gene expression modulation, leaf samples were harvested from stressed plants at mid-day after Ψ_{soil} reached -1, -2 and -3 MPa and two days after a recovery period (re-watering). Control samples were obtained from non-stressed plants. Differential display screenings led to the identification of a band showing a polymorphic pattern associated with the drought treatment (Fig. 1). Amplification was detected in samples collected from plants corresponding to the -1, -2 and -3 MPa Ψ_{soil} stress levels, but not from those grown under humid field conditions. After re-watering, no amplification was observed. Although DD is not considered to be a quantitative technique, a gradual increase in the transcript from -1 MPa to -3 MPa was evident to the naked eye (Fig. 1).

Cloning and sequencing of the corresponding DNA fragment revealed that it had been produced via random decamer priming from both the 5′ and 3′ flanks, and it corresponded to a 391 bp segment.

BLAST searches in public databases using the 391 bp nucleotide sequence as a query revealed strong similarity to the succinate dehydrogenase flavoprotein subunit from various species. The candidate being investigated was therefore called *IpSDH1* (GenBank ID: JX261967). Characterisation of the deduced amino acid sequence for *Ip*SDH1 indicated the presence of a motif with similarities to the *fumarate reductase flavoprotein C-term* conserved domain (Succ DH flav C; Pfam ID: PF02910). This family contains fumarate reductases, succinate dehydrogenases and L-aspartate oxidases. Because the amino acid sequences for *SDH1* were not available at the level of genus, family, order or super-order from the taxonomic relations of *I. paraguariensis*, the sequences was compared with the subclass Magnoliidae. The relationships between the species are shown in a distance tree (Fig. 2). Unexpectedly, *IpSDH1* revealed higher amino acid identity with the monocotyledonous *Zea mays* (100% identity, GenBank ID: EU970939) and *Oryza sativa* (95% identity, GenBank ID: NP001058845) than dicotyledonous species. This is due to a high degree of sequence conservation of the flavoprotein subunit and the iron-sulphur subunit, in contrast with subunits 3 and 4, which show high variability across species [20]. In addition, *IpSDH1* has high similarity to *Litchi chinensis* (91% identity, GenBank ID: HQ667569), a woody plant species growing in the centre of origin of the genus *Ilex* [21]. Compared with *Arabidopsis thaliana* flavoprotein subunits, *IpSDH1* shows greater identity with isoform 1 (81% identity, GenBank ID: NM126074.2) than the isoform 2 (78% identity, GenBank ID: NM127401.2).

To validate the differential expression of the *SDH1* candidate, transcript accumulation was examined in the mature leaves of plants subjected to different soil water potentials (Ψ_{soil}) using quantitative RT-PCR. *IpSDH1* was significantly induced by dehydration (Fig. 1, bottom); it began to accumulate when leaf RWC reached 78% and peaked at 57%. In response to the rehydration treatment, *SDH1* mRNA decreased to pre-dehydration levels.

2.3. Differential expression of a SDH1-like gene under water deficit and ABA treatment

To analyse the plant response to ABA, on the first day after pruning (day 1), several plants were sprayed with distilled water

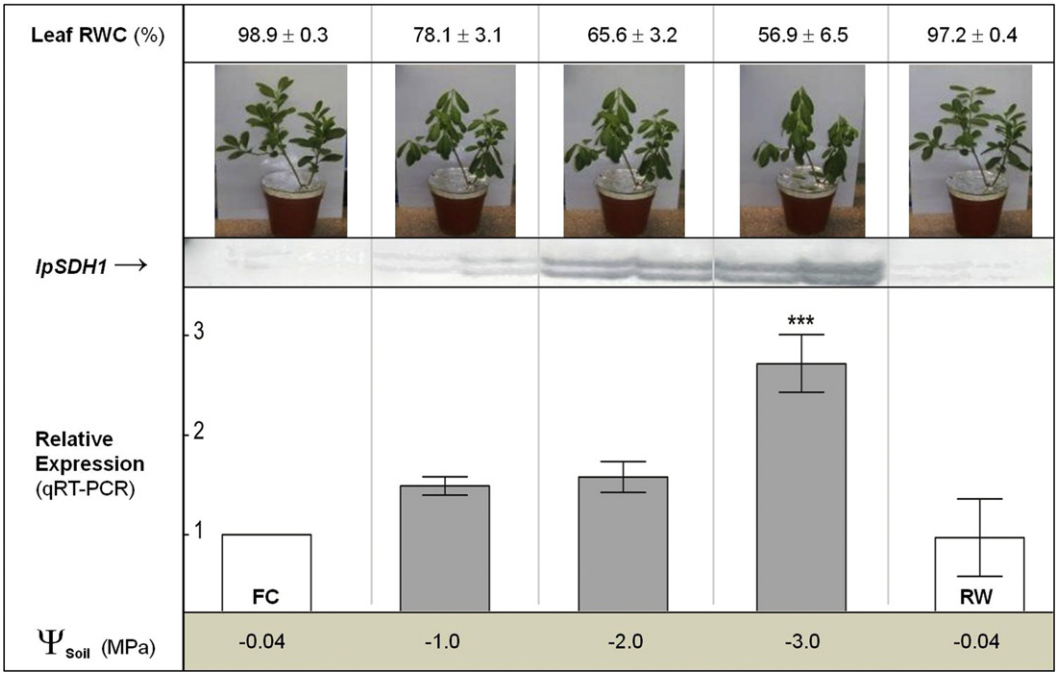

Fig. 1. Patterns of *IpSDH1* differential display amplification and transcript levels determined by qRT-PCR in the mature leaves of *I. paraguariensis* plants grown at various soil water potentials. DD assays were conducted in duplicate (technical replicates). An arrow indicates the polymorphic band corresponding to the *SDH1*-like gene fragment. **FC**, soil humidity under field conditions; **RW**, re-watering.

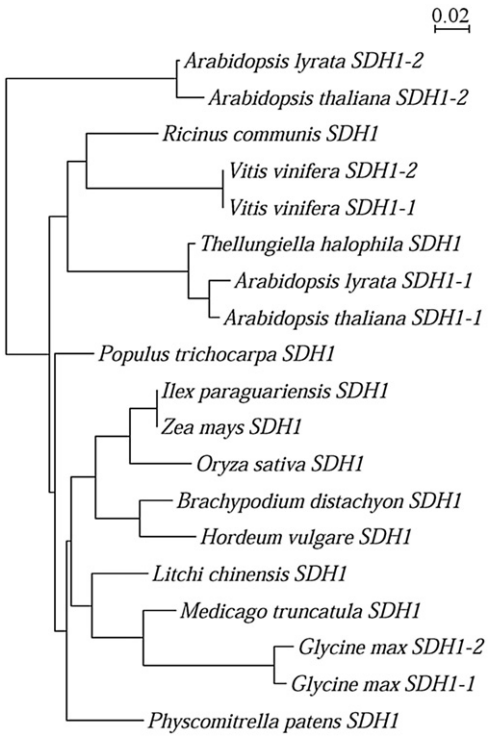

Fig. 2. Distance tree of amino acids sequences of flavoprotein subunit of SDH retrieved from GenBank through the BLASTx algorithm using the *IpSDH1* nucleotide sequence as query. SDH1 amino acids sequences were aligned using the software package ClustalX 2.1.

(control) or ± cis, trans-ABA (2 mM) until incipient runoff (see Materials and methods). Spraying was repeated once a week until Ψ_{soil} reached −2 MPa. Leaf samples were then collected to quantify the *IpSDH1* fragment by qRT-PCR analysis and the SDH-specific enzymatic activity by measuring the decrease in absorption at 600 nm due to the reduction of 2,6-dichlorophenolindophenol (DCPIP) (see Materials and methods). The results are presented in Fig. 3. The accumulation of *IpSDH1* mRNA in the ABA-treated plants

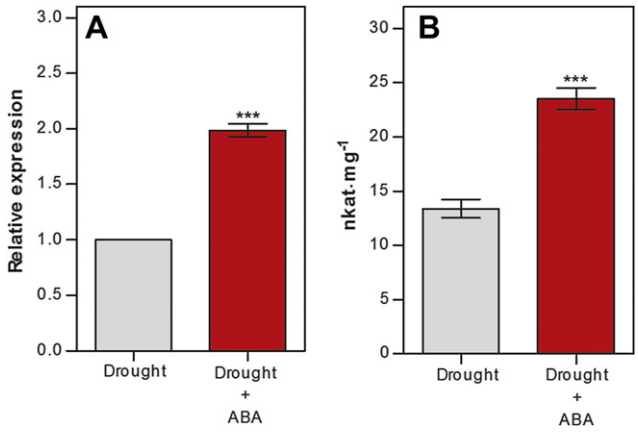

Fig. 3. Effect of the combination of drought and exogenous ABA on the expression of succinate dehydrogenase. A, transcript level of *SDH1* in the mature leaves of plants sprayed weekly with distilled water or ± cis, trans-ABA until the Ψ_{soil} reached −2 MPa. In both cases, the values were normalised using β-tubulin as a reference gene. B, specific succinate dehydrogenase activity in the mature leaves of plants subjected to $\Psi_{soil} = -2$ MPa. The results are shown as means ± SEM from three biological replicates. Asterisks indicate significant differences from the stressed non-ABA treatment ($p < 0.01$, Dunnett's multiple comparison test).

doubled compared to the non-ABA-treated plants (Fig. 3A). Concurrently, the specific activity of succinate dehydrogenase increased significantly (Fig. 3B) in the mature leaves of ABA-treated plants. Our results indicated that ABA induced an increase in both the mRNA level and the enzymatic activity of SDH in plants subjected to a severe water deficit ($\Psi_{soil} = -2$ MPa).

3. Discussion

In this work, we report the isolation of an *SDH1*-like partial cDNA clone from a drought-tolerant cultivar of *I. paraguariensis* that was upregulated following a water-stress treatment. The cDNA encodes the flavoprotein component of the succinate:ubiquinone oxidoreductase complex, which plays an unknown role in the abiotic stress response. Succinate dehydrogenase (SDH, EC 1.3.5.1), often referred to as complex II, has a dual function and plays a significant role in both the tricarboxylic acid cycle and the aerobic respiratory chain by catalysing the oxidation of succinate to fumarate and the reduction of ubiquinone to ubiquinol, respectively [22]. Multimeric SDH consists of the following four subunits encoded by the nuclear genome: a flavoprotein (*SDH1*) harbouring the FAD co-factor and the substrate-binding site, an iron-sulphur protein (SDH2) containing three Fe–S clusters, and two small integral membrane proteins (SDH3 and SDH4), which form the ubiquinone-binding site within the membrane [23]. In addition to these four classical subunits, *Arabidopsis* has four additional subunits that are presumably plant-specific; the presence of these subunits, termed SDH5, 6, 7, and 8, may indicate that this complex has a secondary or peripheral function. However, none of these proteins exhibit homology to proteins of known function in the sequence databases [24,25].

To date, only a few functional genetic analyses have been employed to evaluate the physiological role of complex II and its constituent subunits in plants. Araújo and co-workers [22] have recently found that antisense repression of the iron-sulphur subunit of SDH (SDH2) in tomato results in a combined increase in stomatal conductance, photosynthetic rate and growth. Fuentes et al. [26] analysed SDH-deficient *Arabidopsis* plants that exhibited compromised expression of the flavoprotein subunit of SDH (*SDH1*) and demonstrated that a mild reduction in mitochondrial SDH activity had a positive impact on the photosynthetic performance of plants subjected to controlled environmental conditions that preclude water stress.

Here, we detected an increase in *SDH1*-like transcript in a drought stress-tolerant *I. paraguariensis* genotype that was correlated with an increase in ABA concentration. Although drought was applied gradually, the erratic changes in ABA levels (decreasing at −2 and increased again at −3 MPa) may explain the possible increase in ABA-oxidase activity observed in stress situations [27]; this explanation is confirmed by the fact that ABA levels dropped well below the control levels after rehydration, which was also observed in grape plants grown *in vitro* and exposed to UV-B radiation after they were removed from stressful conditions [28]. The mRNA level of *IpSDH1* increased further after treatment with exogenous ABA, and this treatment also enhanced its specific enzymatic activity. Additionally, no structural damage at the level of cell membranes was detected despite severe drought conditions. This physiological response could be a strategy used by this genotype to avoid tissue desiccation by minimising water loss through stomatal closure in the early stages of dehydration. Subsequently, as leaf water potential diminished due to greater stress conditions, the plant reacted by making an osmotic adjustment to sustain tissue metabolic activity and enable the recovery of photosynthesis upon the cessation of drought conditions (Table 1). Many evergreen shrubs and trees in arid or semi-arid regions combine high solute

concentrations in living cells with low rates of photosynthesis and stomatal conductance [29]. A large osmotic adjustment must be made by *I. paraguariensis* plants growing in the dry environment of cultivated farmlands, considering that the natural habitat of this species is the subtropical forest under the canopy of *Araucaria angustifolia*, where conditions of low photosynthetic photon flux density and high humidity predominate [4].

Although it is well known that plant mitochondria are involved in metabolic processes implicated in cell adaptation to abiotic stresses, the molecular basis of this response remains poorly characterised [30,31]. In addition to the major complexes of the mitochondrial respiratory chain that are common to most eukaryotes, plants possess additional energy-dissipating components, including type II NAD(P)H dehydrogenases, the alternative oxidase, and uncoupling proteins [32–34]. Each of these components either circumvents or short-circuits proton translocation pathways in *A. thaliana* in response to abiotic stress [35]. The regulation of respiration occurs at the transcriptional and post-translational levels during normal development as well as during the stress response, and its regulation has specialised roles in leaves associated with photosynthetic efficiency and redox regulation [36]. Phytochrome A participates in the modulation of mitochondrial respiration through its effect on SDH expression. This activity is considered to be an important mechanism for the regulation of mitochondrial respiration under light conditions. Gleason and co-workers [37] recently provided genetic evidence that SDH participates in the localisation of mitochondrial ROS that regulate plant stress and defence responses, and they concluded that complex II is a source of mitochondrial H_2O_2 that contributes to plant defence against fungal and bacterial pathogens. The present study provides evidence that *IpSDH1* expression is upregulated by drought and ABA in the leaves of a drought-tolerant *I. paraguariensis* genotype, and this upregulation is correlated with a significant increase in succinate dehydrogenase activity in the absence of mitochondrial damage. Future functional analysis studies should be performed on gain-of-function transformants with enhanced SDH activity to determine whether the manipulation of SDH activity could lead to the development of drought-tolerant genotypes.

4. Materials and methods

4.1. Plant growth

Two-year-old plants of the drought-tolerant *I. paraguariensis* St. Hil. cv *San Isidro 49* (kindly provided by Establecimiento Las Marías S.A.C.I.F.A.) were grown in 4 L plastic pots filled with lateritic soil under controlled environmental conditions (27 ± 1/22 ± 2 °C day/night, 14 h photoperiod, 420 μmol m^{-2} s^{-1} PPFD from high pressure mercury lamps, 50–55% relative humidity). Plants were pruned at the beginning of the experiment to obtain foliage of a similar size.

4.2. Drought assay

Drought treatments were 35 days in duration. Plants were watered to the drip point ($\Psi_{soil} = -0.04$ 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. Pots were covered with aluminium foil to prevent evaporation. Well-watered (control) and re-watering (at $\Psi_{soil} = -3$ MPa) treatments were included. Ψ_{soil} was measured with a PST-55 thermocouple placed horizontally at the root zone 8 cm below the soil surface close to the centre of the pot. Ψ_{leaf} was performed *in situ* using an L-51 thermocouple psychrometer connected to an HR-33T dew point microvoltimeter (Wescor Inc., South Logan, Utah, USA). At each Ψ_{soil}, six mature leaves were harvested per plant to give three leaves for osmotic potential (Ψ_π^{100}) measurement and three leaves for RWC measurement. The mean value from the two sub samples per plant was used to characterize each plant. For Ψ_π^{100} measurement, the leaves were re-hydrated to constant fresh weight by placing it in a beaker of distilled water under controlled environmental conditions, then placed in a syringe, frozen in liquid N_2 and kept at −80 °C pending further analysis. Syringes were thawed until samples reached room temperature, and the Ψ_π^{100} of expressed sap was measured with a C-52 thermocouple. Leaf RWC was determined using the following formula: leaf RWC (%) = (FW-DW)/(SW-DW) × 100. Leaf osmotic adjustment ($\Delta\psi_\pi^{100}$) during the drying episode was computed as the difference between pre-drought and post-drought Ψ_π^{100}. Leaf gas exchange rates were measured in intact, fully expanded mature leaves with infrared gas analysers built into a leaf cuvette in an open-flow gas exchange system (Li-Cor LI-6400) with the following conditions: PPFD, 1500 μmol m^{-2} s^{-1}; airstreams, 350 μmol $CO_2 \cdot mol^{-1}$; leaf temperature, 27 °C; leaf-to-air vapour pressure deficit: 1.6 kPa. Mean stomatal conductance (g) was also measured with an LI-6400. Measurements were taken at mid-day using healthy, fully expanded leaves at the same developmental stage. Sample data were calculated from three biological replicates for each Ψ_{soil} condition.

4.3. Exogenous ABA treatments

To analyse the response to ABA, on the day after pruning (day 1), ten plants were sprayed with distilled water (control) or ± 2 mM cis, trans-ABA (99% purity, Sigma–Aldrich, St. Louis, MO, USA) until incipient runoff (approximately 10 mL of aqueous solution per plant). Both solutions included Triton® (0.1%) as surfactant, and a minimum amount of ethanol used to dissolve the ABA. Sprays were repeated once a week until the Ψ_{soil} reached −2 MPa.

4.4. ABA quantification

ABA was quantified in the mature leaves of control and stressed plants following the procedure of Volmaro et al. [38] with modifications. Plant material was collected at mid-day, frozen in liquid nitrogen and dehydrated. Leaf tissue (1 g DW) was extracted for 16 h at 4 °C with methanol:water:acetic acid (80:19:1, v/v) and 200 mg L^{-1} of BHT (Fluka) as antioxidant. Subsequently, 200 ng of [$^2H_6$] ABA (kindly provided by Dr. J. D. Cohen, University of Minnesota, USA) was added as internal standard. After 1 h (to allow isotope equilibration), the sample was filtered, and the methanol was evaporated under low pressure. The remaining aqueous fraction was filtered and centrifuged for 15 min at 8000 rpm. The supernatant was adjusted to pH 3.0 and partitioned four times with ethyl acetate saturated with aqueous acetic acid (1%). The pooled ethyl acetate fractions were then submitted to reverse phase HPLC (C_{18} μ-Bondapack, 300 × 3.9 mm, Waters Associates, Milford, Massachusetts, USA) and eluted with a methanol: water (1% acetic acid) gradient from 10 to 73% methanol at a flow rate of 2 mL min^{-1}. The fraction that co-eluted with authentic ABA was converted to its methyl derivative using fresh diazomethane and analysed by capillary gas chromatography-selected ion monitoring (GC-SIM) on a Hewlett–Packard A 5890 GC (HP-5 column, 25 m, 0.22 mm i.d., 0.25 μm film thickness) connected with a direct interface to an HP B 5970 MSD. The gas carrier was He at 1 mL min^{-1}. The quantity of ABA was assessed by comparing the areas of m/z 194/166 and 190/162 for deuterons and endogenous ABA, respectively. Measurements were performed using duplicates of two different extractions.

4.5. RNA extraction

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. The tissue was ground in liquid nitrogen to a fine powder using a mortar and pestle. 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 visualising bands via ethidium bromide staining after agarose gel electrophoresis. Leaves were collected at mid-day using healthy, fully expanded leaves at the same developmental stage.

4.6. Differential display analysis

Differential display experiments were conducted according to the general protocol reported by Liang and Pardee [39] with minor modifications [40]. First-strand cDNA was synthesised from 1 μg of total RNA from each sample by SuperScript® II Reverse Transcriptase (Invitrogen) according to the supplier's manual using an anchored oligo(dT) primer (DDT1) corresponding to the sequence 5′$T_{(12)}$(ACg)A-3′. A decamer (5′-GGGAAAGCAG-3′) obtained from the British Columbia University RAPD Primer Synthesis Project (set 3) was used in combination with DDT1 to create a primer pair. Duplicate PCRs were prepared in final volumes of 25 μl containing 1× Taq activity buffer (Promega), 1.5 mM $MgCl_2$, 50 μM dNTPs, 0.7 μM arbitrary primer, 2.5 μM DDT1, 2 U of Taq DNA polymerase (Promega) 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 chromosomal DNA in the RNA preparations. The thermocycler program 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. 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 following the DNA silver staining system procedure (Promega) and recorded in a silver sequence APC film (Promega). Differential fragments were excised, eluted in a buffer of 0.5 M ammonium acetate and 1 nM EDTA (pH 8), precipitated in ethanol, and re-amplified using the same PCR conditions described above. The fragments were cloned with the aid of the pGEM®-T Easy Vector System (Promega). Sequencing of the differential display cDNA clones was performed by Macrogen Inc. (Seoul, Korea). An analysis of DNA similarity was performed using the BLAST tools at NCBI (http://blast.ncbi.nlm.nih.gov) and the TAIR webpage (www.arabidopsis.org). Sequence alignment was performed with the Clustal Omega Multiple Sequence Alignment tool (http://www.ebi.ac.uk/Tools/msa/clustalo/). Phylogenetic analysis was performed with the ClustalW2 phylogeny analysis tool (http://www.ebi.ac.uk/Tools/phylogeny/clustalw2_phylogeny) using the neighbour-joining method.

4.7. Quantitative real-time PCR expression analysis

Real-time qRT-PCR reactions were prepared with SYBR Green PCR Master Mix (Applied Biosystems, California, USA) following the manufacturer's instructions. The specific *sdh1* primer sequences were 5′-CTGGGGTACTGGGAGAACGAGAA-3′ (forward) and R 5′-GCGCCTTTGGTGGGAATGA-3′ (reverse). *I. paraguariensis* β-tubulin was amplified as a reference gene to normalise expression. The β-tubulin primer sequences were 5′- CTGGAGAAGGGATGGATGAA-3′ (forward) and 5′-GGCATCCTGATATTGCTGGT -3′ (reverse). Two biological replicates were amplified in triplicate. The thermocycler program included the following steps: an initial denaturation (95°/10 min) followed by 40 cycles of 95 °C/15 s and 60 °C/1 min using the 7500 Real-Time PCR System (Applied Biosystems). The results were processed using 7500 Software v2.0.1 (Applied Biosystems) to determine the relative expression level and the significance of the measurements.

4.8. Analysis of homologous sequences

Protein sequences from subclass Magnoliidae, corresponding to the flavoprotein subunit of SDH were retrieved from GenBank through the BLASTx algorithm using the *IpSDH1* nucleotide sequence as query, and were aligned with the software package ClustalW2 [41] using default parameters. The accession numbers of the sequences used in the analysis are: *Arabidopsis lyrata SDH1-1*: XM002866695, and *SDH1-2*: XM002886125; *A. thaliana SDH1-1*: NM126074, and *SDH1-2*: NM127401; *Brachypodium distachyon SDH1*: XM003558525; *Glycine max SDH1-1*: XM003517186, and *SDH1-2*: XM003537535; *Hordeum vulgare SDH1*: AK376855; *I. paraguariensis SDH1*: JX261967; *L. chinensis SDH1*: HQ667569; *Medicago truncatula SDH1*: XM003611938; *Oryza sativa SDH1*: NP001058845; *Physcomitrella patens SDH1*: XM001780288; *Populus trichocarpa SDH1*: XM002310189; *Ricinus communis SDH1*: XM002530436; *Thellungiella halophila SDH1*: AK352814; *Vitis vinifera SDH1-1*: XM002269371, and *SDH1-2*: XM003631831; *Z. mays SDH1*: EU970939. A distance tree was constructed using Neighbour-Joining method and edited using SEAVIEW V4.4.0 software [42]. The distances between sequences were calculated using pairwise deletion and *observed correction* for multiple hits, and bootstrap values were obtained with 1000 pseudoreplicates.

4.9. Determination of SDH activity

One gram of frozen leaves was homogenised in cold extraction medium (pH 7.2) consisting of 100 mM potassium phosphate, 300 mM mannitol, 2.5 mM EDTA, 5 g of PVPP and 1 g of XAD-4. The extract was filtered and centrifuged at 1700×*g* for 5 min at 4 °C. The supernatant was further centrifuged at 12,000×*g* for 12 min at 4 °C. The pellet (containing mitochondria) was resuspended in cold incubation medium (pH 7.8) containing 20 mM potassium phosphate, 350 mM mannitol, 5 mM $MgCl_2$ and 10 mM sodium succinate. The specific activity of SDH was calculated by measuring the decrease in absorption at 600 nm due to the reduction of 2,6-dichlorophenolindophenol (DCPIP) caused by 1 mg of total protein of the mitochondrial resuspension at 30 °C. The reaction medium (pH 7.8) consisted of 50 mM potassium phosphate, 0.08 mM DCPIP, 1 mM phenazine methosulphate (PMS), 4 mM sodium azide and 100 mM sodium succinate. Blanks were performed by replacing the succinate with 100 mM sodium malonate. Total protein concentration was measured using the Quick Start™ Bradford Protein Assay (Bio-Rad) with bovine serum albumin as a standard. The experimental molar absorption coefficient obtained in the reaction medium was 20 L $mmol^{-1}$ cm^{-1}.

4.10. Malondialdehyde estimation

Malondialdehyde (MDA) content was estimated indirectly by the T-BARS method described by Hodges et al. [43]; however, minor modifications were made due to the interference of anthocyanins. Forty mg of fully expanded leaf tissue was homogenised in 1 mL of an ethanol:water (80:20 v/v) solution. The homogenate was centrifuged at 3000×*g* for 15 min. Subsequently, a 0.2 mL aliquot was heated to 95 °C for 25 min in a thermal bath with or without 0.2 mL of thiobarbituric acid (TBA) in trichloroacetic acid (TCA) (0.65% TBA w/v, TCA 20% w/v). Next, the volume was adjusted to 1 mL with distilled water, and the sample was centrifuged at 3000×*g* for 15 min. The absorbance of the

supernatant was determined at 440, 532, and 600 nm. Data were expressed as equivalents of malondialdehyde and were calculated as follows: A: (Abs532 + ATB − Abs600 + ATB) − (Abs532 − ATB − Abs600 − ATB); B: (Abs440 + ATB − Abs600 + ATB) 0.0571. MDA equivalents (nmol/mL) = (A − B/157000) 106.

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