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ORIGINAL PAPER

Expression of *hsp70*, *hsp100* and *ubiquitin* in *Aloe barbadensis* Miller under direct heat stress and under temperature acclimation conditions

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Abstract
Key message **The study determined the tolerance of Aloe vera to high temperature, focusing on the expression of *hsp70*, *hsp100* and *ubiquitin* genes. These were highly expressed in plants acclimated at 35 °C prior to a heat shock of 45 °C.**
Abstract *Aloe barbadensis* Miller (Aloe vera), a CAM plant, was introduced into Chile in the semiarid IV and III Regions, which has summer diurnal temperature fluctuations of 25 to 40 °C and annual precipitation of 40 mm (dry years) to 170 mm (rainy years). The aim of this study was to investigate how Aloe vera responds to water and heat stress, focusing on the expression of heat shock genes (*hsp70, hsp100*) and *ubiquitin*, which not studied before in Aloe vera. The LT_{50} of Aloe vera was determined as 53.2 °C. To study gene expression by semi-quantitative RT-PCR, primers were designed against conserved regions of these genes. Sequencing the cDNA fragments for *hsp70* and *ubiquitin* showed a high identity, over 95 %, with the genes from cereals. The protein sequence of *hsp70* deduced from the sequence of the cDNA encloses partial domains for binding ATP and the substrate. The protein sequence of ubiquitin deduced from the cDNA encloses a domain for interaction with the enzymes E2, UCH and CUE. The expression increased with temperature and water deficit. *Hsp70* expression at 40–45 °C increased 50 % over the controls, while the expression increased by 150 % over the controls under a water deficit of 50 % FC. The expression of all three genes was also studied under 2 h of acclimation at 35 or 40 °C prior to a heat shock at 45 °C. Under these conditions, the plants showed greater expression of all genes than when they were subjected to direct heat stress.

Keywords Thermotolerance · CAM plants · Lethal temperature · Water deficit · Heat shock · Acclimation

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Introduction

There are plants naturally adapted to grow in deserts where water is scarce or almost inexistent. Many of these plants are called "succulent" plants due to their capacity to store water in their leaves and stems. In addition, they have Crassulacean Acid Metabolism (CAM plants, Borland and Taybi 2004; Ceusters et al. 2009; Ceusters and Borland 2011). The plant opens the stomata for CO_2 uptake at night when there is less evaporative demand. The enzyme which catalyzes the first reaction of photosynthesis is not RUBISCO as in most plants, but rather phosphoenol pyruvic carboxylase (PEP-carboxylase), which has an affinity for HCO_3^- an order of magnitude greater than RUBISCO for CO_2 ($Km_{[HCO_3^-]}$ 0.5 μM in PEP-carboxylase *vs.* Km_{CO_2} 12 μM in RUBISCO, Vaasen et al. 2006; Cardemil 2008). As in C4 plants, CO_2 assimilation is very efficient; all the CO_2 that enters the leaf is converted into sugars. Unlike C3 plants, there is little photorespiration in CAM plants (Borland and Taybi 2004; Cardemil 2008; Herrera 2009). Indeed, we have shown that Aloe vera under water deficit conditions increases the amount of total and soluble sugars as well the amount of proline and fructans (oligo- and polyfructans), all molecules considered to be osmolytes, to achieve the osmotic adjustment of the plant (Delatorre-Herrera et al. 2010). Due to this increase in

osmolyte synthesis, the water use efficiency of the plant increases with increasing water deficit (Silva et al. 2010; Delatorre-Herrera et al. 2010).

Since sugar synthesis is efficient in these plants, sugars can be used to perform osmotic adjustment (Pierce et al. 2002; Van den Ende et al. 2004; Delatorre-Herrera et al. 2010). In CAM plants, much of the sucrose, which is the final product of the Calvin cycle, is used for the synthesis of complex polysaccharides. This is the case of fructans (oligo and polyfructans) which increase under water restriction (Delatorre-Herrera et al. 2010), helping the plant to perform osmotic adjustment and to protect the cell membranes (Van den Ende et al. 2004; Valluru and Van den Ende 2008). Other polysaccharides such as acetylated glucomannan (acemannan) are synthesized to accumulate water in the leaves and stems, giving the plant its succulent phenotype (Mohnen 2008; Moreira and Filho 2008; Hisano et al. 2008). The efficient osmotic adjustment of Aloe vera under water restriction explains the high water use efficiency (WUE) of this species when water is restricted (Silva et al. 2010; Delatorre-Herrera et al. 2010).

To achieve efficient synthesis of carbohydrates and high WUE, Aloe vera must protect the enzymes involved in sugar and polysaccharide synthesis. There are two main stress conditions present in the arid zones, water deficit and large fluctuations in temperature between day and night with high temperatures at midday. Parts of the enzyme protection mechanisms are given by heat shock proteins (HSP, Richter et al. 2010) and by selective protein degradation via ubiquitin (Callis et al. 1995; Sivamani and Qu 2006).

The heat shock proteins (also called molecular chaperones) are the quality control system of the proteome, because they remodel the regulatory and signaling proteins of the cell. The HSP constitute a family of proteins, which are named according to their molecular weight (HSP70, HSP40, HSP100). They participate in non-covalent folding and/or unfolding, playing a fundamental role in the assembly and disassembly of in vivo macromolecular structures. Chaperone HSP70 participates in the correction of protein structure of unfolded proteins during stress conditions and during protein synthesis and translocation to cellular compartments (Melnikov and Rotanova 2010; Richter et al. 2010); prevention of the aggregation of incorrectly folded or unfolded proteins is accomplished mainly by HSP100 (Melnikov and Rotanova 2010; Richter et al. 2010; Luján et al. 2009). When there is a stress condition such as an increase in temperature, proteins lose their native conformation, becoming dysfunctional. Unfolded proteins may have two fates; if the HSPs recover the structure of the damaged proteins they again become functional in the cell, while if the proteins are not recovered by the chaperones they need to be degraded via ubiquitin, since it is better for the cell not to have the damaged protein than rather to have a dysfunctional protein that can cause cell death. Ubiquitin is a small protein encoded by a polygene with tandem repeats. In *Arabidopsis thaliana,* the *ubiquitin* genes belong to a gene family of 14 members, 5 of which are *polyubiquitin* genes (Callis et al. 1995). In rice, there are six tandem repeat *ubiquitin* genes (Wang et al. 2000; Sivamani and Qu 2006), while in yeast one of four *ubiquitin* genes has been characterized as a *polyubiquitin* type (Ozkaynak et al. 1987). When the enzyme structure is irreversibly lost, ubiquitin binds to the protein as a cellular tag to be transported to the proteasome for final degradation (Biedermann and Hellmann 2011). Both the HSP and ubiquitin protection mechanisms are very conserved in all living organisms. Therefore, in Aloe vera, these two molecular protections probably help the plant to survive under the stressing conditions of arid and semiarid zones.

The purpose of this study was to evaluate the expression of the *hsp* (*hsp70* and *hsp100*) and *ubiquitin* genes under temperatures of 35 °C and above 35 °C by semi-quantitative RT-PCR. The expressions of these genes were also evaluated under induced thermotolerance, in which the plant is acclimated to a sublethal temperature before being exposed to a higher heat shock of 45 °C. The amplified fragments of cDNA were sequenced to corroborate that they correspond to putative *hsp70* and *ubiquitin* genes from Aloe vera.

Materials and methods

Plant material

Three-year-old plants of Aloe vera were grown in Las Cardas Experimental Station, located in the community of Coquimbo, IV Region of Chile, at about 31° south latitude. The plants were grown in rows of 50 plants each with a programmed irrigation every 15 days of 1.6 L/h for 1 h per plant, which corresponded to 100 % field capacity (FC). For temperature experiments, plants were grown in pots in a greenhouse in the Facultad de Ciencias, Universidad de Chile in Santiago with controlled environmental conditions; the average temperature was 25 °C and the average relative humidity was 50 %. The soil of the plants grown in pots was 1/3 organic soil and 2/3 sand, a proportion that simulates the natural soil of Las Cardas Experimental Station.

Physiological experiments

Plants of Aloe vera were subjected to water or to thermal stress separately. For water stress, plants in Las Cardas

were watered with different irrigation treatments using previous information on the atmospheric evaporative demand and the water characteristics of the soil (Silva et al. 2010). Four irrigation treatments were designed, with 20, 15, 10, and 5 % of the reference evapotranspiration (ET0) of the experimental site.

These four treatments were named T1, T2, T3, and T4, respectively. The rows of control plants (T1) were watered every 15 days, with a flow rate of 1.6 L/h for 1 h per plant (100 % field capacity, FC). Treatments T2, T3, and T4 were separate rows of 50 plants watered with the same flow rate as the control plants but with decreasing time periods. T2 corresponded to 75 % FC (45 min irrigation), T3 to 50 % FC (30 min irrigation) and T4 to 25 % FC (15 min irrigation).

For thermal experiments, 18-month-old plants of Aloe vera grown in pots in a greenhouse at 25 °C with photoperiod of 16 h light were transferred to growth chambers and incubated at 25 °C (control plants). For thermal stress, three different plants were subjected to 35, 40, and 45 °C for 2 h. In the case of acclimation experiments, groups of three plants were treated with sublethal temperatures to acclimate them to a more severe temperature stress. For this, plants grown at 25 °C were subjected for 2 h at 35 or 40 °C prior to an exposure of 3 h at 45 °C.

In the water and temperature stress experiments, samples were collected from the tips of leaves 70 cm long and 13 cm wide. The tip was chosen because it is the photosynthetic part of the leaf containing greater numbers of chloroplasts per cell than the leaf base, which is the growing region of the leaf where water and nutrients are stored. Samples of leaves were taken by sectioning 10 cm from the leaf tip. Samples were frozen in liquid N_2 and kept at −80 °C until they were processed.

LT_{50} determinations

LT_{50} is defined as the temperature at which there is 50 % membrane damage, determined by the conductivity of the water surrounding the tissue treated at different temperatures. The LT_{50} was determined by electrolyte leakage, measuring the electric conductivity of the solution when leaves of Aloe vera were incubated at different temperatures (Ortiz and Cardemil 2001). The percentage of membrane damage was calculated according to the following equation:

$$\%\ \text{membrane damage} = \frac{C_x - C_c}{C_m - C_c} \times 100$$

where C_x is the conductivity of the water in which the samples are incubated at different temperatures for 2 h, C_c is the conductivity of the water of the sample at 25 °C and C_m is the maximum conductivity of the water when the samples were treated at 80 °C overnight to kill the leaf tissue (Ortiz and Cardemil 2001).

Leaf temperature determinations

The leaf temperature was determined using a copper constantan thermocouple. The thermocouple was inserted in the central section of each leaf.

Primer selection

Primers were designed from known conserved DNA sequences of other related monocot species, since Aloe vera is a monocotyledonous plant. For hsp70, we also considered three sequences from *Arabidopsis thaliana*, since in this species the hsp70 family has 12 members (Sung et al. 2001), in order to have a wider spectrum of *hsp70* gene sequences. For the *hsp100* gene, a sequence of *A. thaliana* was chosen (Sharp and Li 1987). For the *ubiquitin* gene, the sequences of three dicotyledonous plants were considered because the gene has high nucleotide conservation, which was corroborated by the analyses performed. The regions with a higher level of nucleotide similitude were determined for primer construction by performing a multiple alignment using ClustalW (Labarga et al. 2007). The primers used for the *18S* rRNA housekeeping gene were from carrot (*Daucus carota*, access number GQ380561). Since the primer selections were designed using conserved regions of the genes, the amplicons obtained were of different sizes. Besides, the genes are also of different sizes; *ubiquitin* is a small gene of 227 bp, encoding a protein of 8.5 kD. Table 1 shows the plant species and the respective accession numbers from which the primer sequences were selected for these three genes.

mRNA analyses

RNA extraction and DNAse treatment

RNA extraction was performed using the RNA extraction kit Invisorb Spin Plant RNA Mini Kit (Invitek, Berlin, Germany), according to the manufacturer's recommendations, using 100 mg of frozen leaf powder.

The amount of RNA obtained was quantified by spectrometry (ND-1000 V3.3, NanoDrop Technologies, Inc., Wilmington, DE, USA) with 2 % error. The integrity of the RNA was verified by gel electrophoresis using 1 % agarose gels. For DNAse treatment of RNA, 90 ng of RNA were treated with 1U DNAse (Fermentas, Glen Burnie, MD, USA) in the corresponding buffer containing 10 U RNAse inhibitor Ribolock™ (Fermentas). The volume was adjusted with nanopure water with 1 % DEPC. The

Table 1 Plant species from which *hsp70*, *hsp100* and *ubiquitin* gene sequences were chosen to design the primers

hsp70		*hsp100*		*Ubiquitin*	
AF005993.1	*Triticum aestivum*[a]	AF174433.1	*Triticum aestivum*[a]	AY862401.1	*Triticum aestivum*[a]
X67711.2	*Oryza sativa*[a]	AJ970536.2	*Triticum turgidum subsp. Durum*[a]	AY072820.1	*Oryza sativa*[a]
AY914601.1	*Zea mays*[a]	AK105433.1	*Oryza sativa*[a]	M73787.1	*Zea mays*[a]
AY059885.1	*Arabidopsis thaliana*	AF332981.1	*Oryza sativa*[a]	DQ793134.1	*Arabidopsis thaliana*
AY054183.1	*Arabidopsis thaliana*	AF133840.1	*Zea mays*[a]	D16248.1	*Glycine max*
AY054190.1	*Arabidopsis thaliana*	ATU13949.1	*Arabidopsis thaliana*	DQ445914.1	*Gladiolus grandiflorus*

The GenBank access numbers of the gene sequences are also shown. For the *18S* rRNA gene the primers used were from carrot (*Daucus carota),* access number GQ380561

[a] Monocot

reaction mix was incubated at 37 °C for 15 min, adding EDTA to a final concentration of 2.5 mM.

RT-PCR analyses

The analyses were performed using a MyCycler™ (BIO-RAD, Berkeley, CA, USA) thermocycler. The reaction mix contained 45 ng of RNA treated with DNAse and 0.5 mM of the primer oligo-dT. This mix was incubated at 70 °C for 5 min and placed on ice for another 5 min. After this reverse transcriptase buffer (1×) was added, which contained $MgCl_2$ 6.5 mM, dNTPs 0.5 mM, 20 U of the RNAse inhibitor Ribolock™ and 1 U of the reverse transcriptase RT-Improm II (Promega, Madison, WI, USA). The negative control was the same reaction mix without enzyme, which was replaced by DEPC-treated water. The temperature program was incubation at 25 °C for 5 min, at 42 °C for 60 min, 70 °C for 15 min and final incubation at 4 °C.

To evaluate the primer efficiency in cDNA synthesis, a reverse transcription was performed using oligo-AP primers (the oligo-AP is a consense sequence for the AP-1 gene which encodes a transcription factor. The oligo-AP primer sequence was: 5′-TTT TTT TTT TTT TTT TTT TTA GCT TCG-3′). However, for the semi-quantitative expression analyses the primers used for *hsp70*, *ubiquitin*, *hsp100*, and *18S* were the reverse primers (*hsp70*R, *ubiquitin*R, *hsp100*R, and *18S*R). Unlike the oligo-AP primers, the reverse primers gave better qualitative, quantitative and more repeatable results in the PCR analyses. The reverse primer sequences are shown in Table 2.

In these analyses, the expression of *hsp* and *ubiquitin* genes was compared with the expression of *18S* rRNA, which is a constitutive gene. In the analysis, therefore, the *18S* expression does not change under stress conditions and was used as an internal control (Goidin et al. 2001; Stürzenbaum and Kille 2001). The reverse and forward primers for the *18S* rRNA gene were designed from the carrot gene (*Daucus carota*). To make the analysis quantitative, the optimum number of cycles for each pair of primers was determined using enough cycles to observe a discrete cDNA fragment band in the gel but not enough to give the intensity of the saturated phase of amplification. In other words, the number of cycles was chosen to give band intensities in the exponential phase of amplification, and standardized with respect to the exponential phase of *18S* rRNA.

PCR amplification

For the PCR reactions, the conditions were optimized according to the characteristics of the pair of primers selected and the size of the fragment to be amplified. For the *hsp* and *ubiquitin* genes the following PCR program was run: the incubation step at 94 °C for 5 min, 36 cycles with temperature intervals of 95 °C for 50 s, 50.5 °C for 50 s and 72 °C for 40 s, with a final elongation at 72 °C for 10 min and a final incubation at 4 °C. The reaction mix included 2 μL cDNA, 2.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.2 mM of each primer, 1 U of Taq polymerase (Favorgen, Ping-Tung, Taiwan) and its respective 1× buffer. To amplify the internal standard, the 18S gene, only 0.5 μL of cDNA and 24 amplification cycles were used.

Quantification of gene expression

To quantify gene expression, the agarose gels containing the PCR products resolved by electrophoresis and were photographed with the *GeneSnap* (Syngene, Cambridge, UK). To estimate the amount of cDNA, the images were analyzed with the Software ImageJ (Abràmoff et al. 2004) program, which allows comparing the band intensities with respect to the intensity of the *18S* rRNA standard.

In the case of *ubiquitin*, the *ubiquitin*F and *ubiquitin*R primers amplified multiple bands, since *ubiquitin* is a polygene. For the *ubiquitin* expression analyses, only the three smallest bands (monomer, dimer, and trimer) were considered, because these were more intense and better resolved.

Table 2 Primer sequences for synthesis of cDNA from total mRNA

Primer	Sequence	Amplified fragment	Annealing temperature (°C)
oligoAP	5′-TTT TTT TTT TTT TTT TTT TTA GCT TCG-3′	–	42
*hsp70*F	5′-GGT TTG AGG AGC TCA ACA TGG A-3′	609	50.5
*hsp70*R	5′-GTG ATG GTG ATC TTG TTC TTC TG-3′		
*hsp100*F	5′-CTC AAG GAG AAG TAC GAG GG-3′	560	52.5
*hsp100*R	5′-CCA RCG GCT CAC CAC CTC-3′ (R = A/G)		
*ubiq*F	5′-GGC AAG ACC ATC ACC CTG GA-3′	165	55.5
*ubiq*R	5′-ACT CCT TCT GGA TGT TGT AGT C-3′		
*18S*F	5′-TTG ATT ACG TCC CTG CCC TTT-3	197	50.5
*18S*R	5′-ACA ATC ATC CTT CCG CAG GT-3		

Primers were used for the semi-quantitative RT-PCR analysis of gene expression of *hsp70*, *hsp100* and *ubiquitin* and for sequencing the amplified cDNA fragments of *hsp70* and *ubiquitin*. The table also shows the primers for *18S* RNA amplification, used as internal control

All data were expressed relative to the load control *18S* rRNA and normalized with respect to the control experimental condition (100 % FC and 25 °C).

DNA extraction

DNA extraction was performed to amplify the sequences enclosed by the selected primers. The objective of this was to evaluate if the genome amplicons were identical to those of the cDNA amplicons. The extraction was performed by the CTAB-buffer methodology (Cetiltrimethyl Ammonium Bromide) according to Murray and Thompson (1980). The buffer contained 2 % CTAB, 1.4 mM NaCl, 20 mM EDTA and 100 mM Tris. 100 mg of frozen powder was homogenized in 3 µL of β-mercaptoethanol, then 600 µL of CTAB buffer was added to the homogenized tissue and incubated at 70 °C for 30 min. After incubation, another 400 µL of CTAB was added and incubated at 70 °C for 15 min. Next, 500 µL chloroform: isoamyl alcohol 24:1 was mixed with the homogenate to separate the proteins and polysaccharides from the DNA. The mix was centrifuged for 10 min at 15,000×*g* and the supernatant containing the DNA was saved. The DNA was precipitated with 750 µL of isopropanol and incubated at −20 °C for 30 min. The mix was again centrifuged for 10 min at 15,000×*g* and the precipitate was washed with 700 µL of cold 70 % ethanol, vortexing vigorously. This mixture was centrifuged for 5 min at 15,000×*g*, leaving the precipitate at room temperature. The DNA precipitate was resuspended in 50 µL of nanopure water. 1 µL of this DNA extract was used for all the electrophoretic analyses.

Agarose gel electrophoresis of the PCR products

The electrophoresis was performed in 1 % agarose gels in TAE buffer (40 mM Tris, 19 mM glacial acetic acid and 1 mM EDTA, pH 8), with 0.5 µg/mL ethidium bromide. The samples were loaded on the gel with the buffer containing bromophenol and xylencyanol (Sambrook et al. 1989). The visualization of the bands was performed in a Syngene transilluminator, MultiGenius model. To estimate the sizes of the bands a 100 bp to 1 Kb molecular size standard was used (Ladder Gene Ruler™ from Fermentas). The electrophoresis was run at 70 V for RNA analysis and at 100 V for DNA analysis. The content of the RNA or DNA in the bands was quantified with the program GeneSnap (Syngene). For this, the gels were photographed and the amount of DNA was estimated from the images by the intensity of the band compared to the standard. The analyses were performed with the GeneTools program (Syngene).

Sequencing the amplified cDNA fragments

To confirm that the amplified cDNA fragments corresponded to bona fide *hsp70* and *ubiquitin* sequences the two fragments were purified, cloned in the pGEM®-T vector and sequenced. The cDNA fragment was inserted in the pGEM®-T Easy Vector, cloned in competent *Escherichia coli* (DH5α strain) as described by Sambrook et al. (1989), and sequenced by the Centro de Biotecnología de la Universidad de Chile, from an aliquot containing 500 ng of plasmid DNA. The sequencing was performed in an ABI PRISM 3100-Avant Genetic Analyzer (Applied Biosystems, Carlsburg, CA, USA) using the sequencing DYEnamic™ ET terminator kit (Amersham, Little Chalfont, UK), and the direct and reverse universal primers pUC/M13F and pUC/M13R, respectively.

Sequence analyses

The nucleotide sequences were translated to protein sequences with the Expasy program (Gasteiger et al. 2003).

The nucleotide and protein sequences were compared with the database information of the National Center for Biotechnology Information (NCBI) using BLAST (Altschul et al. 1990, 1997) to find the genetic similarity with genes and proteins of other species. The amino acid sequences were also analyzed to find the corresponding functional protein regions, using the ClustalW program (Labarga et al. 2007) and searching the conserved motifs with the InterProScan (Quevillon et al. 2005).

Statistical analysis

Analyses were carried out using one-way analysis of variance (ANOVA) in GraphPad Prism 5 to test for differences among leaf temperature treatments and between the water treatments. Significant differences among treatments indicated by the ANOVA were further tested with Tukey's multiple range tests; differences were considered significant at $P < 0.05$.

Results

Lethal temperature and leaf temperature of Aloe vera

Lethal temperature of Aloe vera was determined as described in "Materials and methods". Figure 1 shows the LT_{50} for the plants determined by the membrane damage in leaves of plants subjected to different temperatures. The LT_{50} found was 53.2 °C, indicating that Aloe vera is a plant ranked among the most tolerant plants to heat shock (Ortiz and Cardemil 2001; Luján et al. 2009).

In the experiments, the temperature of leaves was determined and it was always the greenhouse temperature, around 25 °C. When plants were subjected to temperature stress, the leaves of the plants reached the temperature of the incubation chamber after 1 h of exposure to that temperature. Therefore, for temperature experiments the time of 2 or 3 h of temperature exposure was considered after the leaves of the plants reached the chamber temperature.

cDNA bands amplified by the primers

The primers for *hsp70*, *hsp100* and *ubiquitin* amplified bands of the expected molecular sizes of the genes in Aloe vera. Figure 2 shows the expected amplified cDNA fragments and the genomic DNA fragments for *hsp70*, *hsp100* and *ubiquitin*. In the case of *ubiquitin*, two or more bands were amplified frequently because *ubiquitin* is a polygene transcribed with several tandem RNA copies. In this analysis, two bands were amplified; the band of greater molecular size, 495 bp, probably corresponds to an amplified dimer.

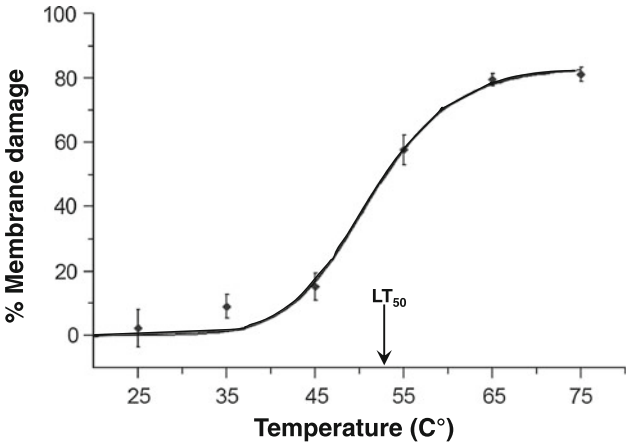

Fig. 1 Determination of the lethal temperature of Aloe vera. The LT_{50} was determined through the electrolyte leakage from the leaves of plants subjected to different temperatures. The arrow indicates the LT_{50}. Each point corresponds to the average of three determinations ± SD

cDNA fragment sequences of *hsp70* and *ubiquitin*

To confirm that the amplified sequences corresponded to fragments of the *hsp70* and *ubiquitin* genes, these were cloned using the vector pGEM®-T Easy (Promega). The sequences of these cDNA fragments and their respective dendrograms are shown for *hsp70* (Fig. 3a) and for *ubiquitin* (Fig. 3b). The *hsp70* and *ubiquitin* sequences have 98–99 % identity with monocots and 82–87 % identity with dicots.

The principal domains of the HSP70 and ubiquitin proteins encoded by the *hsp70* and *ubiquitin* genes are shown in Fig. 4. The figure also shows the protein fragments encoded by the amplified *hsp70* and *ubiquitin* cDNA fragments. The protein fragment encoded by the amplified cDNA of *hsp70* contains partial sequences of the ATP and substrate domains. The cDNA fragment of ubiquitin encodes protein sequences for interaction with the E2 and ubiquitin carboxy-terminal hydrolase (UCH) enzymes, and for coupling ubiquitin conjugation to endoplasmic reticulum degradation (CUE) (Hicke et al. 2005).

We did not sequence the amplified cDNA fragment from *hsp100*. However, the amplified fragment was of the expected size of 560 bp and its expression increased with temperature. The fragment was treated with restriction enzymes to determine if there were conserved restriction sites in this sequence. When the fragment was restricted with *Xho*I, a band of 341 bp was observed that is present in the *hsp100* sequence from corn and wheat, while *Pst*I gave a band of 311 bp present in wheat and rice.

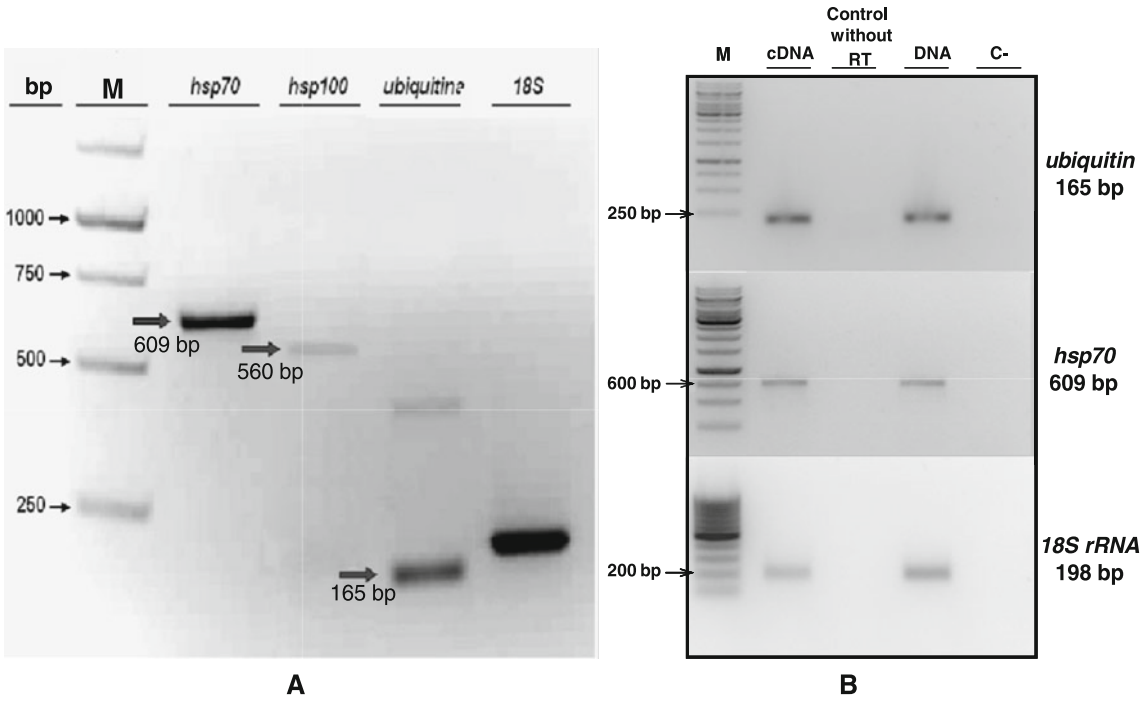

Fig. 2 cDNA (**a**) and genomic DNA (**b**) amplified bands using the designed primers. C- in **b** is a control without RNA. *Black arrows* show bands of 609 bp for the *hsp70*, of 560 for *hsp100* and of 165 for *ubiquitin*. The*18S* gene of 198 bp was run as an internal standard

Expression of *hsp70*, *hsp100* and *ubiquitin* in plants subjected to temperature stress

The expression of the *hsp70* gene in leaves of Aloe vera plants subjected to direct temperature stress is shown in Fig. 5a. The electrophoretic analysis gave a single amplified band of 609 bp. The relative expression of *hsp70* (Fig. 5e) at temperatures of 40 and 45 °C was 50 % greater than in the control plants, with no significant difference between these two groups of plants.

A single band of 560 bp was detected by agarose gel electrophoresis for *hsp100* (Fig. 5b). In the case of *ubiquitin* multiple bands of mRNA appeared; monomers, dimers, trimers, etc. (Fig. 5c). As in the case of *hsp70*, the expression of *hsp100* and *ubiquitin* increased with increasing temperature. However, the expression of *hsp100* increased significantly (70 %) only at 45 °C, while the expression of *ubiquitin* increased by 75 % at 35 °C, by 85 % at 40 °C and by 115 % at 45 °C (Fig. 5e).

Expression of *hsp70* and *ubiquitin* in plants under water deficit

Plants of Aloe vera were subjected to water stress of 75 % (T2), 50 % (T3) and 25 % (T4) FC; the control plants were irrigated with 100 % (T1) FC. The results of this experiment are shown in Fig. 6. Under water deficit one single band of *hsp70* cDNA fragment of 609 bp (Fig. 6a) and two bands of *ubiquitin* cDNA fragments of 165 and 495 bp were detected (Fig. 6c).

Hsp70 and *ubiquitin* expression increased with increasing water deficit (Fig. 6b, d). For *hsp70*, the expression was 150 and 130 % greater than in the control when plants were irrigated with 50 % FC (T3) and 25 % FC (T4), respectively. Under T3 water irrigation the relative expression of *hsp70* was three times greater than the expression of the gene in plants subjected to normal irrigation (100 % FC).

For *ubiquitin*, the expression increased 50 and 65 % when plants were treated with 75 % (T2) and 50 % (T3) of FC (Fig. 7d). In T4, the expression increased by 25 %, with no significant difference compared to control plants. Comparing the expression of the ubiquitin gene under water and temperature stress conditions, at 45 °C the gene was expressed 50 % more than in control plants (25 °C), while the expression was only 75 % of the control plant expression (100 % FC) under a water restriction of 50 % FC.

Expression of *hsp* and *ubiquitin* genes in acclimated plants

Plants of Aloe vera were acclimated for 2 h to a moderate temperature stress of 35 or 40 °C before being subjected to a higher heat stress of 45 °C. The expression of *hsp70*, *hsp100*, and *ubiquitin* under these conditions is shown in Fig. 7. In the case of *hsp70* and *hsp100* a single band was

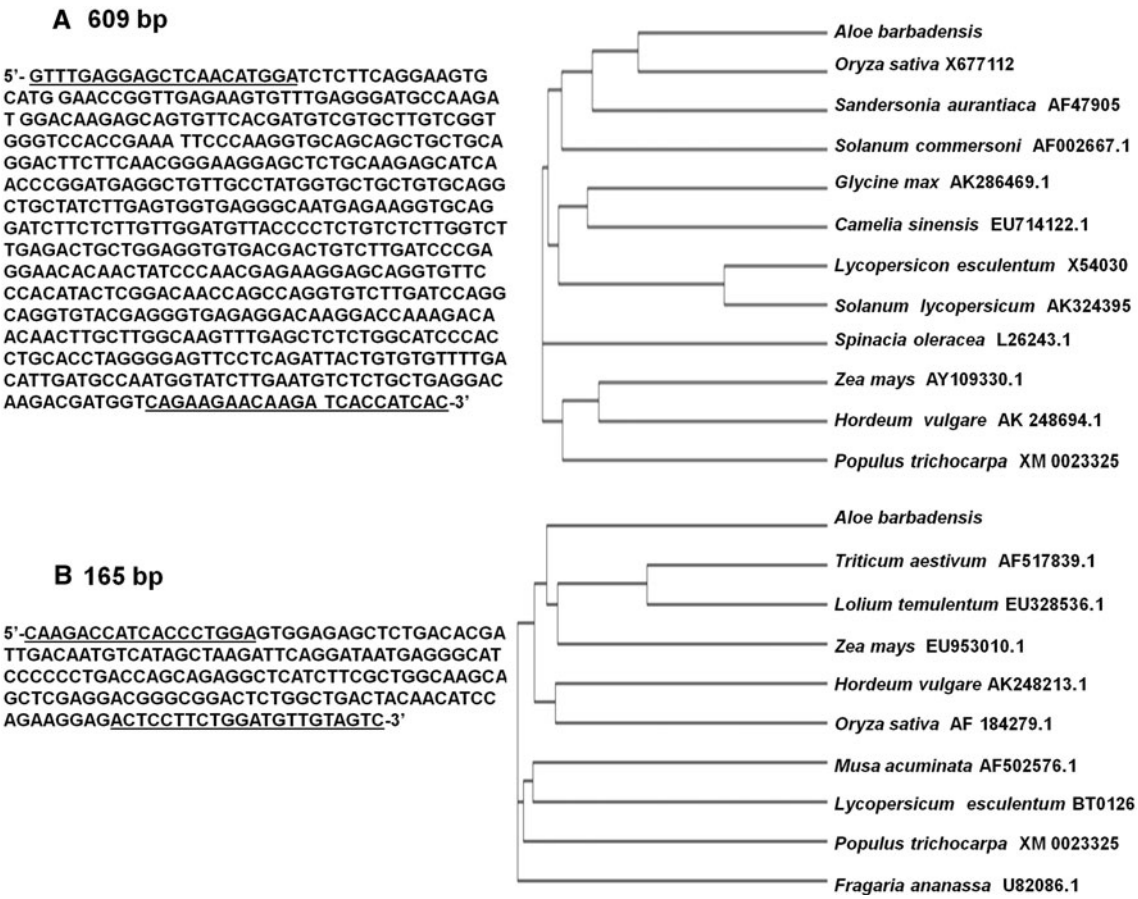

Fig. 3 Sequences of the amplified cDNA fragments of *Aloe barbadensis* Miller corresponding to the genes *hsp70* (a) and *ubiquitin* (b) and their respective dendrograms. The amplified fragments correspond to the most conserved regions of these genes found in monocotyledonous plants. Primers selected are shown as *underlined sequences*. The alignment to construct the dendrograms was performed with the ClustalW program (http://www.ebi.ac.uk/clustalw/)

detected (Fig. 7a, c), while with *ubiquitin* several bands were amplified (Fig. 7b), as in the direct heat shock experiments.

The results of the acquired thermotolerance experiments indicated that the expression of all three genes was greater under an acclimation condition of 2 h at 35 °C than under acclimation at 40 °C and greater than under direct heat shock conditions. In the case of *hsp70*, the expression increased by 140 % over the control in plants subjected to 35 °C prior to being treated at 45 °C, and 55 % over those plants acclimated to 40 °C prior to a treatment of 45 °C. In the case of *hsp100*, the expression was 120 % greater than the controls in plants subjected to an acclimation of 35 °C compared to an increase of 60 % over the control group when the acclimation was at 40 °C. In the case of *ubiquitin* the expression at 45 °C was 160 % greater than the control if the plants were previously acclimated to 35 °C, while the increase of expression was 140 % greater than the control if the acclimation was at 40 °C (Fig. 7d).

Discussion

Tolerance to abiotic stress is a new challenge in plant breeding research, due to global climate change which has endangered many animal and plant species. High temperature and drought are abiotic factors involved in the global changes. If the temperature continues to increase with loss of water availability, many species will not be able to tolerate these extreme conditions (Hall 2001). Heat stress by itself can have an impact on plant metabolism, reproduction, development, and growth, all of which affect agricultural yield. To counteract these conditions, plants express genes with a protective role against temperature and water stress. Some of these genes encode the chaperones, whose role is to recover protein structure when this is lost or partially lost during the stress.

The expression of heat shock genes under different environmental stress conditions is a conserved response from prokaryotes to eukaryotes, and therefore is probably a very ancient survival mechanism acquired during the

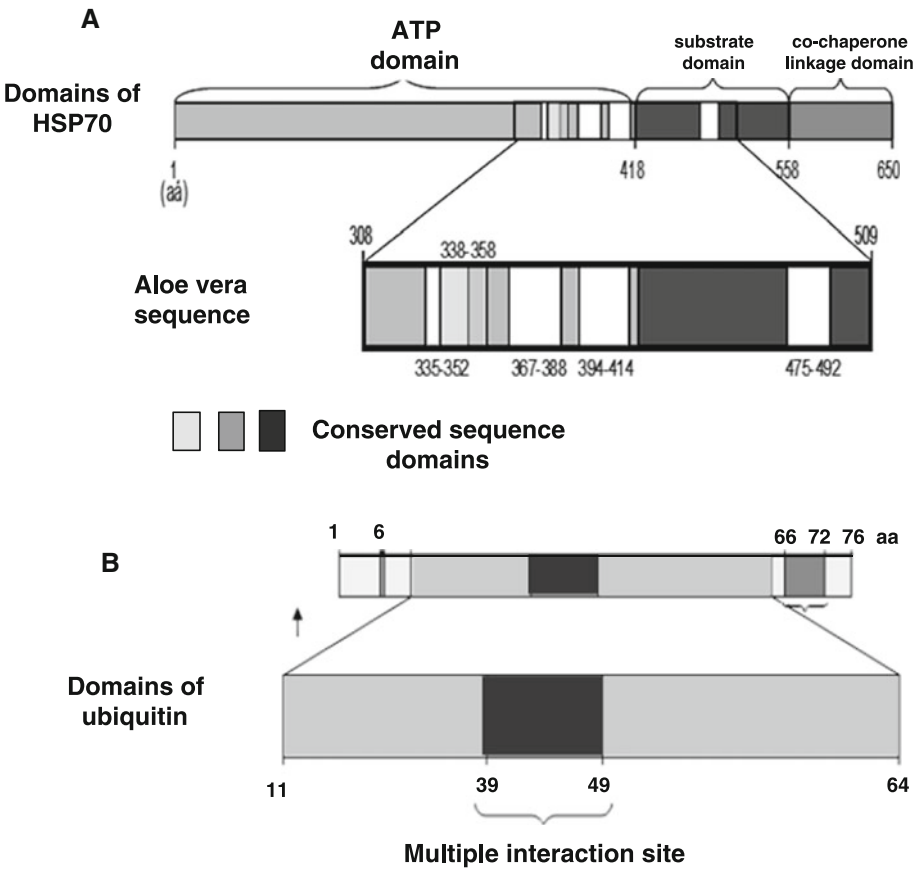

Fig. 4 Principal domains of the HSP70 and ubiquitin proteins encoded by the amplified cDNA fragments. **a** The HSP70 proteins encoded by *hsp70* genes have a binding site for ATP, the substrate and the co-chaperones. The corresponding region encoded by cDNA fragment cloned from Aloe vera are also shown containing portion of ATP and substrate binding domains. **b** The ubiquitin protein encoded by the *ubiquitin* gen contains interaction sites to bind the target proteins, E2 enzyme, the ubiquitin carboxy-terminal hydrolase (UCH) and for coupling ubiquitin conjugation to endoplasmic reticulum degradation (CUE). The corresponding region encoding by the cDNA fragment cloned from Aloe vera contains, between amino acids 39 and 49, a multiple interaction site that binds E2, UCH and CUE. The corresponding regions were obtained from the translation of the cDNA fragment sequences shown in Fig. 3 using the ClustalW and InterProScan programs

evolution of living organisms (Melnikov and Rotanova 2010; Richter et al. 2010). However, not all organisms display this protective mechanism with the same magnitude and efficiency, which creates the difference between a tolerant species and one susceptible to abiotic stress. This is especially true in plants, since they are immobile organisms and cannot escape from heat or lack of water. Plants will survive these stresses if they have a better expression of *hsp* genes (Mittler et al. 2012). The development of cultivars tolerant to heat stress will be of crucial importance if the temperature continues to increase in the planet in the near future.

CAM plants are adapted to semiarid and arid regions of the planet, and therefore are naturally tolerant to water deficit and high temperatures. This is the case of Aloe vera, which has an LT_{50} of 53.2 °C, similar to other plants adapted to arid and semi-arid regions, such *Prosopis chilensis* which has a LT_{50} of 53.3 °C (Ortiz and Cardemil 2001) and *Agave tequilana* whose LT_{50} is 55 °C (Luján et al. 2009). The LT_{50} of these species implies that the foliar tissue is more tolerant to heat stress than the foliar tissue of soybean, whose LT_{50} is 47.2 °C (Ortiz and Cardemil 2001) and many other plants (Martineau et al. 1979; Schaff et al. 1987).

The results of leaf temperature measurements appear to indicate that Aloe vera does not open its stomata during the day, since the temperature of the leaf was always the chamber temperature after 1 h of exposure to 35, 40 or 45 °C. At night, Aloe vera opens the stomata, as shown by the sap flow rate that increases considerably at night (Delatorre-Herrera et al. 2010).

Aloe vera was introduced into Chile in the year 1990 to reinforce the agriculture of arid and semi-arid regions of Chile. Although the plant is adapted to aridity, it was

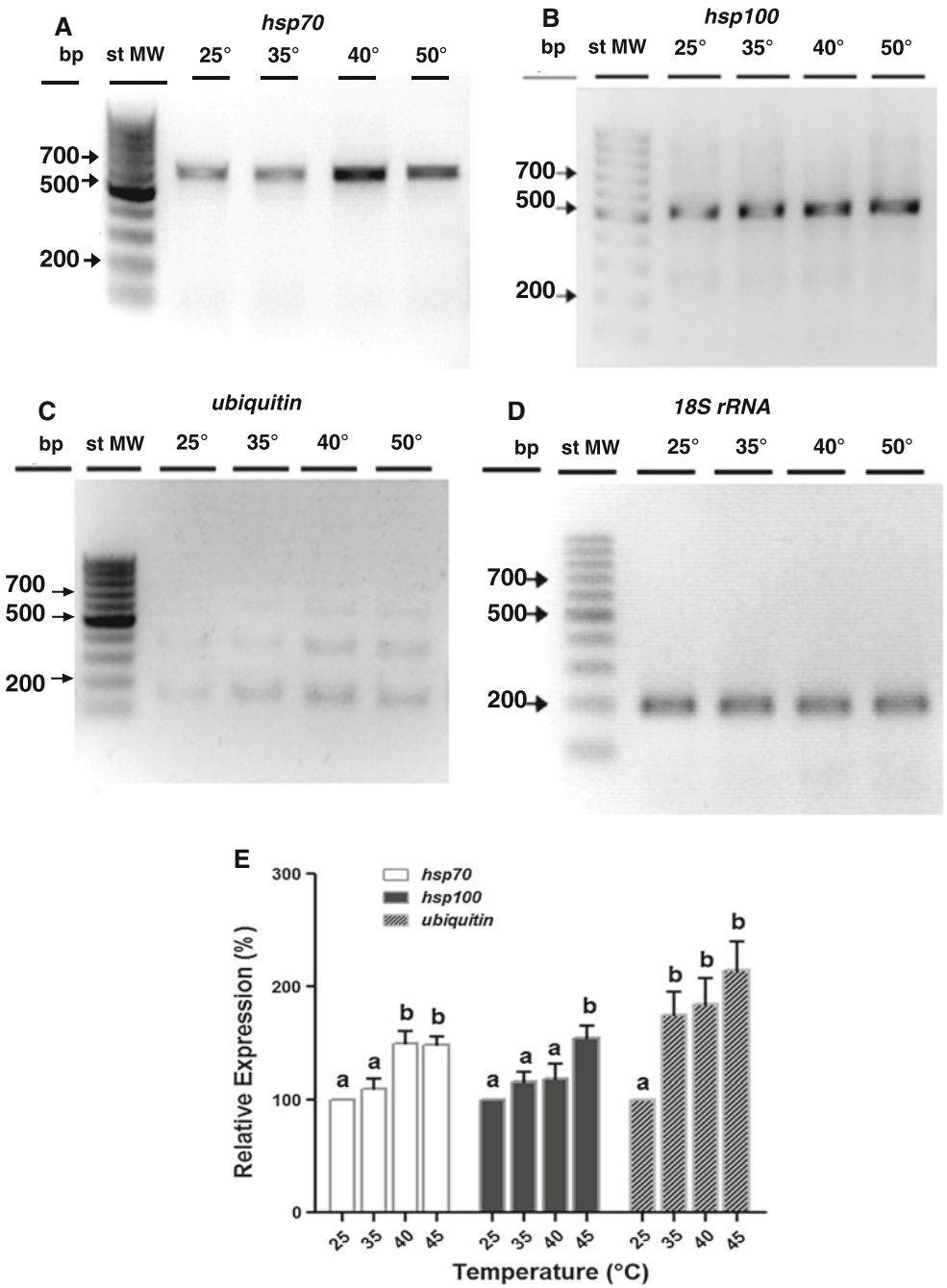

Fig. 5 Relative expression of *hsp70, hsp100* and *ubiquitin* in leaves of Aloe vera plants subjected to different temperature treatments. Three-year-old individuals were subjected to temperatures of 35, 40 and 45 °C for 2 h; control plants were maintained at 25 °C. Total RNA was extracted and quantified from the leaves by semi-quantitative RT-PCR analysis using the *18S* ribosomal gene as an internal control. **a** Agarose gel electrophoresis of the amplified cDNAs of *hsp70*, **b** agarose gel electrophoresis of the amplified cDNAs of *hsp100*, **c** agarose gel electrophoresis of the amplified cDNAs of *ubiquitin*, **d** the expression of *18S* RNA was run as internal control, **e** the relative expression of *hsp70*, *hsp100* and *ubiquitin* genes under different temperature treatments. The levels of expression were determined by the intensity of the cDNA bands considering the expression of the leaves of control plants as 100 %. The visualization of the bands and their intensity determinations were made using the GeneTools software (Syngene) using the Software ImageJ program to compare the band intensities respect to the intensity of the *18S* rRNA standard. Each bar of the graphs represents the average of three independent determinations with its standard deviation. *Different letters* denote significant differences between treatments (Tukey's test, $P \leq 0.05$)

necessary to evaluate the tolerance of this species to water deficit and to high temperature in the IV Region of Chile. The tolerance to water deficit was reported in previous papers of our group (Silva et al. 2010; Delatorre-Herrera et al. 2010). However, in these reports, we did not evaluate the expression of heat shock genes and *ubiquitin* under

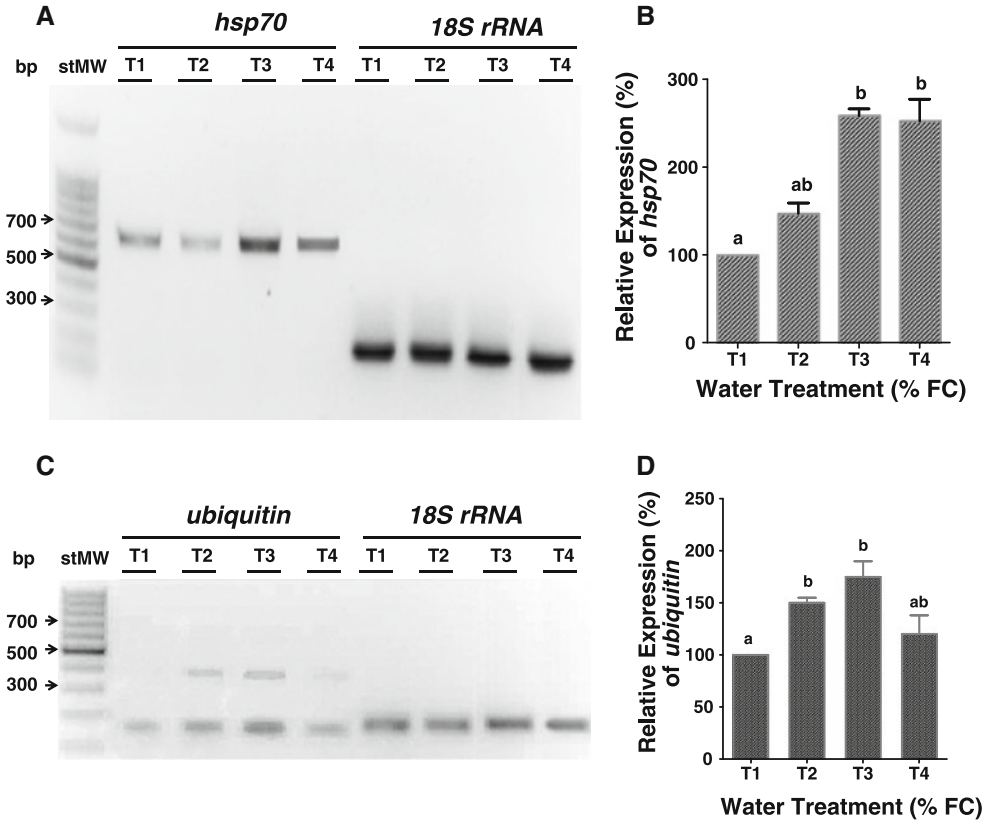

Fig. 6 Relative expression of *hsp70* and *ubiquitin* in leaves of Aloe vera plants subjected to different water treatments. Three-year-old individuals were subjected to water treatments of 100 % FC (*T1* control plants), 75 % FC (*T2*), 50 % FC (*T3*) and 25 % FC (*T4*), for 3 months. Total RNA was extracted from the leaves for semi-quantitative RT-PCR analysis using the *18S* ribosomal gene as an internal control. **a** Agarose gels electrophoresis of the amplified cDNA for *hsp70*. **b** Relative expression of *hsp70* as determined by the intensity of the cDNA bands. **c** Agarose gels electrophoresis of the amplified cDNA for *ubiquitin*, **d** Relative expression of *ubiquitin* as determined by the intensity of the cDNA bands. The relative expression was determined considering as 100 % the gene expression of the leaves of control plants watered with 100 % FC. The visualization of the bands and their intensity determinations were made using the GeneTools software (Syngene) using the Software ImageJ program to compare the band intensities respect to the intensity of the *18S* rRNA standard. Each *bar* of the graph represents the average of three independent determinations with its standard deviation. *Different letters* denote significant differences between treatments (Tukey's test, $P \leq 0.05$)

water and heat stress conditions. Our results indicate that Aloe vera is well adapted to heat shock and water stress with a good expression of *hsp70*, *hsp100* and *ubiquitin* when plants are subjected to direct heat stress of 35, 40 or 45 °C or direct water deficit of 75, 50, or 25 % FC. The expression of these genes probably contributes to the elevated LT_{50} of the leaves.

The expression of *hsp100* increased only at 45 °C, probably because aggregations of proteins are formed at this temperature. The *hsp100* genes include two families of genes encoding proteins which use ATP to promote changes in protein folding and assembly. Each of these families has eight subfamilies (Schirmer et al. 1996; Vierling 1997). The genes probably are expressed under different stress conditions. However, all are strongly expressed under heat shock and seem to be crucial in thermotolerance acquisition (Gulli et al. 2007). In *Agave tequilana,* a CAM plant related to Aloe vera, the expression of two genes of the *hsp100* family was studied (Nieto-Sotelo et al. 1999, 2002; Luján et al. 2009), *hsp99* and *hsp101*. An increase in expression of these two genes was found at 44 °C in the leaves and in the spike of this plant, with almost no expression of the *hsp101* at temperatures lower than 30 °C. This is similar to the *hsp100* expression of Aloe vera that was observed at 45 °C; however, this gene is also expressed at temperatures below 30 °C. We do not know if the *hsp100* cDNA fragment is a protein domain present in all HSP100; we did not sequence the cDNA amplified fragment of Aloe vera. However, the fragment obtained was of the expected 560 bp size and the expression increased with temperature. Restriction of this fragment with the enzyme *Xho*I gave a 341 bp fragment which is present in corn and wheat genes. Restriction with *Pst*I produced a 311 bp fragment which is present in rice and wheat. These results appear to indicate that the amplified fragment is part of a putative *hsp100* of Aloe vera.

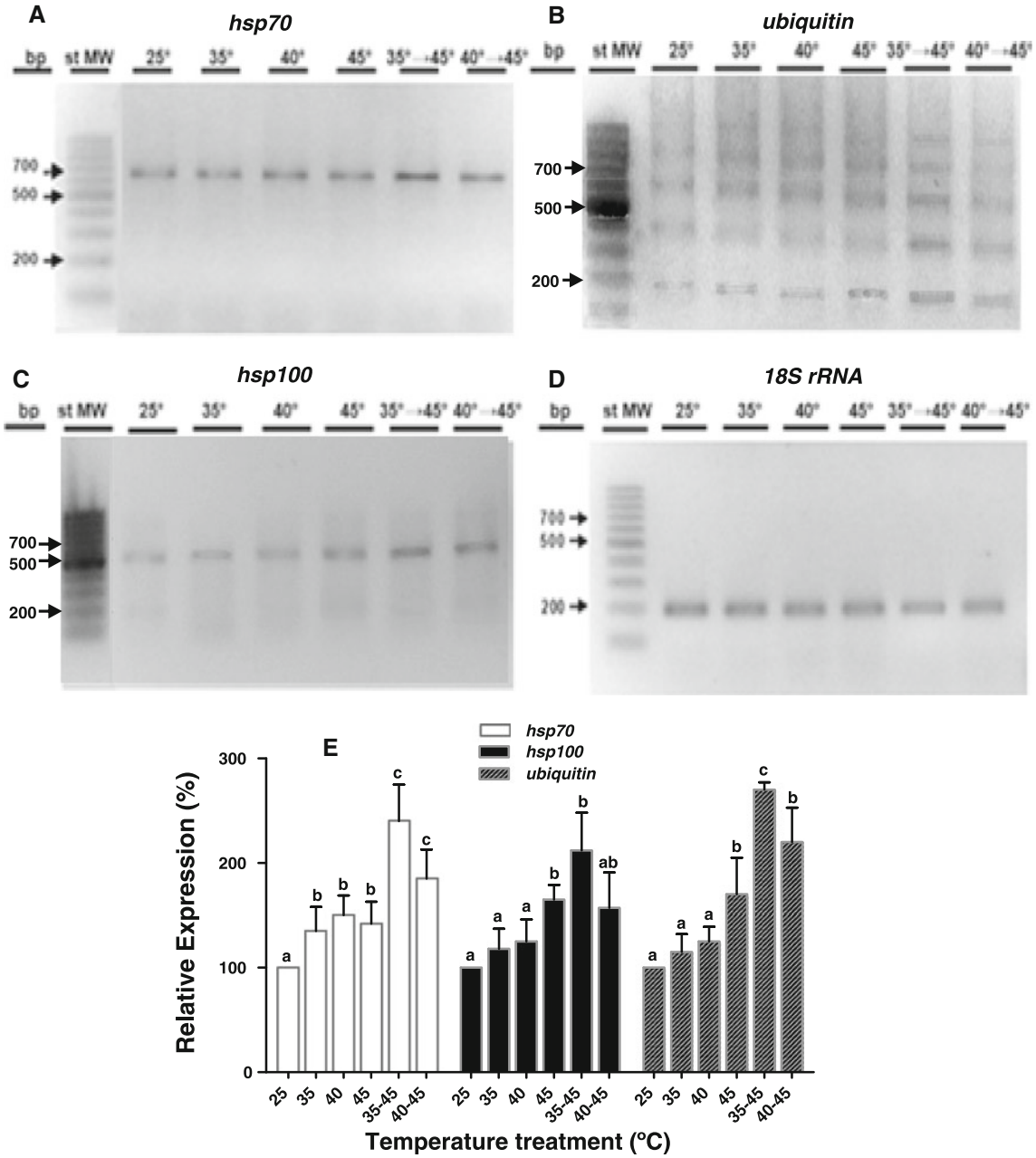

Fig. 7 Relative expression of *hsp70*, *hsp100* and *ubiquitin* genes in leaves of Aloe vera plants subjected to an acclimation temperature of 35 °C prior to a temperature of 40° (35 °C → 40 °C) or 45 °C (35° → 45 °C). Three-year-old individuals were subjected to acclimation conditions for 2 h at 35 °C prior to exposure to temperatures of 40° or 45 °C. The expression was compared with plants subjected to a direct heat stress of 35°, 40° and 45 °C. Plants at 25 °C were the control plants. Total RNA was extracted from the leaves for semi-quantitative RT-PCR analysis using the *18S* ribosomal gene as an internal control. **a** expression of *hsp70*, **b** expression of *ubiquitin*, **c** expression of *hsp100*, **d** expression of *18S* rRNA. To estimate the sizes of the bands a molecular size standard (MW) was used. The relative expression (*E*) was determined considering the gene expression of leaves from plants grown at 25 °C as 100 %. The visualizations of the bands and their intensity determinations were made using the GeneTools software (Syngene) using the Software ImageJ program to compare the band intensities respect to the intensity of the *18S* rRNA standard. Each bar of the graphs represents the average of three independent determinations with its standard deviation (Tukey's test, $P \leq 0.05$)

The expression of *ubiquitin* was greater than the expression of *hsp70* and *hsp100* under all the temperatures of the direct heat stress experiment (ANOVA, $P < 0.005$). The expression of ubiquitin begins to be significantly greater in plants treated at 35 °C compared to control plants; which appears to indicate that selective protein degradation takes place in Aloe vera at a medium temperature of 35 °C. The maximum expression of *ubiquitin*

was found at 45 °C, although there were no significant differences with the expressions at 35 and 40 °C. Therefore, it appears that in Aloe vera selective protein degradation begins at a lower temperature than protein aggregation.

The *hsp70* and *ubiquitin* genes increased their expression in plants subjected to increased water deficit. In both cases, the amplified cDNA fragments were of identical size and sequence. Interestingly, the expression of *hsp70* was 150 and 130 % greater than the expression of control plants under a water deficit of 50 or 25 % FC, respectively, while under an increase in temperature from 25 to 40 °C the magnitude of the expression was only 50 % greater than the expression of controls at 25 °C. We do not know, however, if the same *hsp70* gene is expressed under heat shock and under water deficit. The amplified cDNA fragment is the same, and the sequences present in the expressed genes are the same. However, the amplified cDNA fragment of the *hsp70* gene is from a conserved region present in all *hsp70*. The sequence of the fragment obtained had 98–99 % identity with *hsp70* from other monocots and over 82 % identity with dicots. The sequence itself, when translated in silico, corresponds to a partial ATP binding domain including part of the substrate-binding domain adjacent to it; therefore, this sequence probably is present in all *hsp70* genes.

The expression of the *ubiquitin* gene was only 50 and 75 % greater than the control in plants under water treatments T2 and T3, respectively, while under temperature stress the expression of these genes was 75–115 % more than in control plants when the temperature increased from 35 to 45 °C. In plants subjected to water restriction the magnitude of *ubiquitin* expression was less than the magnitude of expression under temperature stress. At a water restriction of 50 % FC, the expression was 65 % of the expression of control plants, while under temperature stress of 45 °C the expression was 130 % of control plants (25 °C). Temperature stress probably demands more protein degradation than water stress in Aloe vera plants.

As in the case of *hsp70*, *ubiquitin* is a much-conserved gene in all organisms, encoding a small protein of 8.5 kDa composed of 76 amino acids. In plants, the *ubiquitin* genes are a gene family, many of which are polyubiquitin genes with tandem repeats (Callis et al. 1995; Wang et al. 2000; Sivamani and Qu 2006; Ozkaynak et al. 1987; Groettrup et al. 2008). All *polyubiquitin* genes are transcribed as a poly-mRNA for ubiquitin, which is detected as multiple bands in the RT-PCR amplification analysis (Perales et al. 2008). The protein sequence deduced from the amplified cDNA fragment has a domain for ubiquitin interaction with the E2 enzyme, a ubiquitin-conjugating enzyme to carry the ubiquitin to the E3 enzyme that binds ubiquitin to the target proteins for its final degradation (Biedermann and Hellmann 2011). The domain also interacts with UCH, a ubiquitin carboxy-terminal hydrolase, to separate ubiquitin-protein conjugates in the ubiquitin–proteasome system (Wang et al. 2008), and with CUE for coupling ubiquitin conjugation to endoplasmic reticulum degradation (Hicke et al. 2005). This important domain is conserved in all ubiquitin genes. In other plants *ubiquitin* expression began to increase at 28 °C, declining at temperatures greater than 44 °C (Luján et al. 2009). It has been proposed that the *ubiquitin* proteasome pathway should be more diverse in plants than in other organisms, since plants have to be adapted more efficiently to changes in the environment than mobile organisms. Perhaps being sessile, plants need to be more versatile at the molecular, biochemical and physiological levels (Gagne et al. 2002; Biedermann and Hellmann 2011).

There has not been much research on heat shock responses in CAM plants. The Luján group found that *Agave tequilana* is exceptional for its high tolerance to heat stress; it tolerates temperatures higher than 48 °C. However, the high tolerance of *A. tequilana* might be due not only to *hsp* gene expression but also to the fact that the plant opens its stomata during the day when the temperature is extremely high (Luján et al. 2009), allowing the plant to refresh the leaves. This might happen in leaves of Aloe vera under some conditions. However, the temperature of the leaves during heat shock experiments remained at the chamber temperature after they reached that temperature, indicating that evapotranspiration was not refreshing the leaf. Therefore, the stomata of the leaves appear to have remained closed.

The expression of these genes was also studied in Aloe vera plants under acclimation conditions, when plants were subjected to an intermediate heat shock at 35 or 40 °C prior to an exposure to 45 °C. Under these conditions, the expression of all three genes increased compared to the direct heat shock stress experiment. The expression was greater when the acclimation occurred at 35 °C than when it took place at 40 °C, indicating that 35 °C is a sublethal temperature better for induction of acquired thermotolerance. The induction of acquired thermotolerance has been reported for other species of the arid regions of Chile (Ortiz and Cardemil 2001). This acquired thermotolerance of Aloe vera probably allows the plant to survive temperatures above 40 °C in the arid and semiarid regions. In The III and IV Regions of Chile where Aloe vera is cultivated, the temperature starts decreasing in the evening, and reaches temperatures of 6 °C at 4–5 a.m. The temperature increases gradually to reach 35–40 °C at midday in summer days. The acclimation condition of 35 °C may induce in Aloe vera an increase in the transcription levels of *hsp* genes and *ubiquitin*, or an increase in the accumulation of transcripts with higher levels of heat shock and ubiquitin

protein synthesis. These will contribute to a more efficient repair of the protein damage in acclimated plants.

In summary, this study leads to the conclusion that Aloe vera is a plant with a good response to temperature stress, as it has been reported to be a plant also tolerant to water deficit (Silva et al. 2010; Delatorre-Herrera et al. 2010). Under both stresses, there is an increase in expression of *hsp70*, with a greater expression of *hsp70* under water deficit than under a temperature of 40–45 °C. The sequence of the amplified fragment of this gene codes for a conserved region of the protein containing part of an ATP binding domain adjacent to the substrate-binding domain. *Ubiquitin* and *hsp100* also increased expression at temperatures over 35 °C. As in the case of *hsp70*, the amplified sequence of *ubiquitin* encodes a portion of the protein containing a domain for interactions with E2 and UCH and for coupling ubiquitin conjugation to endoplasmic reticulum degradation. Aloe vera acquires thermotolerance by increasing the expression of these three genes under an acclimation condition of 35 °C, prior to a higher heat shock.

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