PCR-Based Cloning of an α-Galactosidase Gene from Deacclimated Petunia (*Petunia* x *Hybrida*)

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Abstract

Previous studies of plant cold hardiness have focused mainly on the cold acclimation response, while studies on the deacclimation process have been limited. Alpha-galactosidase a key catabolic enzyme of the raffinose family oligosaccharides (RFO), cleaves the terminal-linked moiety from galactose-containing oligosaccharides. A cDNA clone petgal, was isolated from *Petunia* x *hybrida* 'Mitchell' leaf RNA by RT-PCR using degenerate oligosaccharide primers designed to amplify the mature α-galactosidase protein. The putative α-galactosidase cDNA sequence has high nucleotide sequence homology (>80%) to other known plant α-galactosidases. Southern blot analysis suggests that α-galactosidase represents a single gene family. Based on the amino acid alignment of the petunia α-galactosidase enzyme with other known α-galactosidases, it appears that the protein is conserved among species. Up-regulation of the α-galactosidase gene in response to deacclimation duration and temperature, suggests that warm temperature may regulate RFO catabolism by increasing the transcription of the α-galactosidase gene.

INTRODUCTION

Previous biochemical and physiological studies have demonstrated the accumulation of specific metabolites such as the raffinose family oligosaccharides (RFO) upon low temperature exposure (Bachmann et al., 1994). These studies of plant tolerance to low temperatures have focused primarily on the cold acclimation response, the process by which plants increase their tolerance to freezing in response to low non-freezing temperatures (Guy, 1990), while studies on the deacclimation process have been largely neglected.

Alpha-galactosidase (α-Gal), the key enzyme involved in the breakdown of RFOs, cleaves the terminal-linked moiety from galactose-containing oligosaccharides. Castonguay and Nadeau (1998) showed that α-Gal increased activity during spring dehardening at the time when RFO levels decreased rapidly.

α-Gal is widely distributed in microorganisms, humans and plants and cDNAs have been cloned from several sources including yeast (Liljestrom, 1985), human (Bishop et al., 1986), and tomato seed (Feurtado et al., 2001). This study reports on the cloning, sequence, and characterization of a cDNA clone encoding the petunia α-Gal to deduce its role in cold deacclimation in plants.

MATERIALS AND METHODS

Plant Materials and Treatments

Petunia x *hybrida* 'Mitchell' plants were grown from seed and maintained in a greenhouse at 25 °C with a 16/8 h photoperiod. Eight-week old plants were transferred to a growth chamber to induce cold acclimation by incubating at 15 °C for 7 d, 10 °C for 7 d, 5 °C for 7 d and subsequently at 3 °C for 3 d with a 12/12 h photoperiod under cool white fluorescent light at 60 µmole m^{-2} s^{-1}. Plants were deacclimated by incubating previously cold acclimated plants at 25 °C with a 16/8 h photoperiod for varying periods of time. In a separate experiment plants were deacclimated for 1 h at various temperatures

(25 °C to 40 °C). Five mm leaf discs samples were collected for electrolyte leakage tests to determine the approximate threshold temperature for heat-induced damage of petunia.

Isolation of Total RNA and Cloning of α-Galactosidase cDNA by RT-PCR

Total RNA was obtained from 8-week old deacclimated petunia plants using the TRIZOL reagent according to the manufacturers recommendations (Gibco, BRL, Rockville, MD). A putative α-Gal cDNA was isolated from petunia leaf total RNA using the Access RT-PCR System according to the manufacturers recommendations (Promega, Madison, WI). To amplify the α-Gal cDNA from petunia, reverse transcriptase polymerase chain reaction (RT-PCR) was performed using degenerate primers based on other plant α-Gal sequences to obtain the mature α-Gal protein from petunia. The primers used were as follows: aGal5' (5'ATGGGRT GGARYAGCTGGAAYCA 3') and aGal3' (5' CTDARWGGHCCDGCCCAWACCTC 3'). RT- PCR reactions were carried out in a volume of 50 μl containing (final concentration) 1X AMV T*fl* Reaction Buffer, 0.2mM dNTP mix, 5μM of each primer, 1mM $MgSO_4$, 0.1u/μl AMV RT, 0.1 u/μl T*fl* DNA polymerase, and 1μg tRNA sample. Cycling was performed in a Mastercycler (Eppendorf, Westbury, NY) under the following conditions: reverse transcription at 48 °C for 45 min, AMV RT inactivation and RNA/cDNA/primer denaturation at 94 °C for 2 min followed by 40 cycles of denaturation at 94 °C for 1 min, annealing at 65 °C for 1.5 min and extension at 72 °C for 2 min followed by a final extension at 72 °C for 7 min. The amplified product was purified and cloned into the pCR 2.1-TOPO expression vector, according to the manufacturers recommendations (Invitrogen, Carlsbad, CA). The putative clone designated petgal was sequenced using a Perkin Elmer 377 (ABI Prism, v.3.2) DNA sequencer.

Sequence Analyses and Amino Acid Alignment

Sequence analyses were performed using BLAST searches via the National Center for Biotechnology Information (NCBI) database (Altschul et al., 1997) and Vector NTI Suite software (Informax) for amino acid alignment.

DNA Extraction and Southern Blot Analysis

Genomic DNA was extracted using a hexadecyltrimethylammonium bromide (CTAB) method according to Murray and Thompson (1980). The petunia genomic DNA sample (10μg) was digested with *Xba* I, *EcoR* I and *Nco* I respectively. The DNA gel blot was performed according to Jones et al. (1995).

RNA Extraction and Northern Blot Analysis

Samples were collected from leaves of 8-week old acclimated and deacclimated plants at various temperatures and time points and from flowers, leaves, stems and roots of 8-week old plants. To check if α-Gal is developmentally regulated, samples were collected from the first eight true leaves. Leaves were sorted into four groups 1^{st}-2^{nd}, 3^{rd}-4^{th}, 5^{th}-6^{th} and 7^{th}-8^{th} according to their appearance order. Total RNA was extracted using the TRIZOL reagent according to the manufacturers recommendations (Gibco, BRL, Rockville, MD). RNA gel blots were performed according to Jones et al. (1995).

RESULTS AND DISCUSSION

Amplification and Sequencing of a cDNA Encoding Petunia α-Gal

The cDNA amplification was performed by the RT-PCR method. The cDNA encoding α-Gal was amplified as a single PCR product of the putative 855 bp fragment length .The product amplified by RT-PCR was cloned into the pCR 2.1 TOPO vector (designated petgal). The double stranded plasmid cDNA of the putative clone was sequenced.

Sequence Analysis and Comparison of Deduced Amino Acid Sequences

The nucleotide sequence of the respective cDNA clone (petgal) was sequenced and compared to known α-Gal sequences complied in the NCBI GenBank. The nucleotide sequence of the cloned petgal contains an ORF of 285 amino acids (Fig. 1a.). The calculated molar mass of the polypeptide is 31.3 KD with an isoelectric point of 4.86. The predicted protein has a compositional bias for Gly (10.88 %), Ser (9.47 %) and Asp (8.42 %). A comparison of the cDNA sequence to others in GenBank revealed striking homologies (between 42 % to 88 %) with human, microbial and plant α-Gal genes.

Based on the alignment of the petunia α-Gal with other known α-galactosidases, it appears that the protein is conserved among species (Fig. 1b.). Table 1. shows the nucleotide and amino acid identities between petunia α-Gal and those from other species.

Southern Blot Analysis

Southern hybridization using petgal as a probe suggests that the α-Gal represents a single gene in petunia. Only two fragments of the genomic DNA digested with the restriction enzymes were detectable (Fig. 2.). Single fragments were detectable in the *Xba*I and *Eco*RI lanes respectively but in the *Nco* I lane two fragments gave positive signals. These results suggest that the gene represented by the petgal clone sequence is a single copy gene with an intrinsic *Nco* I site. Similarly, only one gene is present for tomato seed α-Gal (Feurtado et al., 2001) while two different α-Gal genes are present in barley (Chrost and Krupinska, 2000). To our knowledge, the number of genes in other plant species has not been investigated, neither is the genomic sequence available for the plant enzyme.

Northern Blot Analyses of α-Gal

The cDNA clone described above was used to probe northern blots prepared with RNA from a range of tissues, i.e. flowers, leaves, stems, roots and leaves at four stages of development and from leaves of cold acclimated and deacclimated plants. Petunia α-Gal transcripts were detected at the same levels in flowers, leaves, stems and to a lesser degree in roots (Fig. 3.). This suggests that petgal is not tissue specific but rather is involved in non-specific degradation of raffinose. In tomato for example, EST clones were isolated from seed (Genbank accession number AF191823), ovary (Genbank accession number AI898528) and root (Genbank accession number BE451601). The presence of the enzyme in different plant tissues suggests that α-Gal is not only involved in raffinose degradation but plays a broader role in galactomannan remobilization during seed germination (Feurtado et al., 2001). In plants not synthesizing these oligo-saccharides, α-Gal seems to be involved in galactolipid metabolism of plastid membranes (Chrost and Krupinska, 2000).

Petunia plants grown for 4 weeks had eight or more leaves. There was a clear growth gradient, with younger leaves growing faster than older leaves. The activity of α-Gal showed no relationship with growth rate (data not shown) and neither did mRNA transcript accumulation (Fig 4.). Petgal is not developmentally regulated, as its pattern of mRNA transcripts is unchanged throughout plant development. Since α-Gal activity is fairly constant during various stages of development, the transcripts are used to replace enzyme turned over. Consistent with our findings is the observation that α-Gal activity in leaves remained constant at all stages of development (Thomas and Webb 1978).

Another interesting finding in this study is the rapid activation of petgal with the onset of deacclimation. As shown in Figure 5, α-Gal mRNA accumulated 1 h following deacclimation. α-Gal mRNA transcript levels remained relatively constant initially for 90 min but mRNA accumulation had decreased to control levels by 120 min.

Induction of α-Gal mRNA corresponding to petgal was detected in acclimated plants subjected to 25 °C and 30 °C (Fig. 6.). Transcript levels rapidly decreased when the temperature was increased from 35 °C to 40 °C, approximately the threshold temperature for heat-induced damage of petunia. This suggests transcript accumulation was inhibited due to heat injury as indicated by electrolyte leakage test (Fig. 7).

The data presented in this study are important for future research in devising experimental strategies to assess phenotypes of loss of function mutants and transgenic plants that over- and or under-express α-Gal.

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Tables

Table 1. Nucleotide and protein sequence homology between petunia α-galactosidase and those from six other plants.

Plants	Homology (% identities[1])	
	Nucleotide	Protein
Tomato	88	83
Coffee	85	83
Kidney bean	84	81
Soybean	83	80
Guar	85	78
Arabidopsis	81	76

[1] highest homologies selected

Figures

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  1 atgggnggaagaagcggaatcctttttggttgctatattgacgag
    M  G  G  R  S  G  I  L  F  G  C  Y  I  D  E
 46 aaaatgataagggaaacagctgatgcaatggtatacactgggctt
    K  M  I  R  E  T  A  D  A  M  V  Y  T  G  L
 91 tcttctcttggatacaaatacatcaatcttgatgactgttgggct
    S  S  L  G  Y  K  Y  I  N  L  D  D  C  W  A
136 gaactcaacagggactctcaggggaatatggttcctaaaggttca
    E  L  N  R  D  S  Q  G  N  M  V  P  K  G  S
181 actttccttctggaattaaagcactagcagattatgttcacaac
    T  F  P  S  G  I  K  A  L  A  D  Y  V  H  N
226 aaaggattgaacctcggaatttattctgatgctgggactcaaacg
    K  G  L  N  L  G  I  Y  S  D  A  G  T  Q  T
271 tgtagtaaagaaatgccaggttcattaggtcacgaagaacaagat
    C  S  K  E  M  P  G  S  L  G  H  E  E  Q  D
316 gcaaaaacttttgcttcctggggagttgattacttgaagtatgat
    A  K  T  F  A  S  W  G  V  D  Y  L  K  Y  D
361 aactgtaacaatgaaaatcgaagtccaagagaaaggtatcctaca
    N  C  N  N  E  N  R  S  P  R  E  R  Y  P  T
406 atgagcaaagctctacaaaactctggaagggctatattttattcc
    M  S  K  A  L  Q  N  S  G  R  A  I  F  Y  S
451 ctatgtgaatggggagatgatgatcctgccacttgggctttctct
    L  C  E  W  G  D  D  D  P  A  T  W  A  F  S
496 gttggaaatagttggagaactactggagatatttctgataactgg
    V  G  N  S  W  R  T  T  G  D  I  S  D  N  W
541 gacagtatgacatctcgggcggatcaaaatgataaatgggcatct
    D  S  M  T  S  R  A  D  Q  N  D  K  W  A  S
586 tatgctggtccaggaggctggaatgatccagacatgttagaagtt
    Y  A  G  P  G  G  W  N  D  P  D  M  L  E  V
631 ggaaatggaggaatgacaactgcagaatatcgttcacatttcagc
    G  N  G  G  M  T  T  A  E  Y  R  S  H  F  S
676 atatgggcattagcaaaagcgcctttaataattggttgtgatata
    I  W  A  L  A  K  A  P  L  I  I  G  C  D  I
721 cgatccatggacgaaactaccaaagaaatcctaagcaacaaaggg
    R  S  M  D  E  T  T  K  E  I  L  S  N  K  G
766 gtttttgcagttaaccaagataaacttggagttcaaggtaaaaaa
    V  F  A  V  N  Q  D  K  L  G  V  Q  G  K  K
811 gttaagagtgatagcggcttggaggtttgggccggaccactaagt 855
    V  K  S  D  S  G  L  E  V  W  A  G  P  L  S
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Fig. 1a. Nucleotide sequence and amino acid translation of the petunia α-galactosidase cDNA clone.

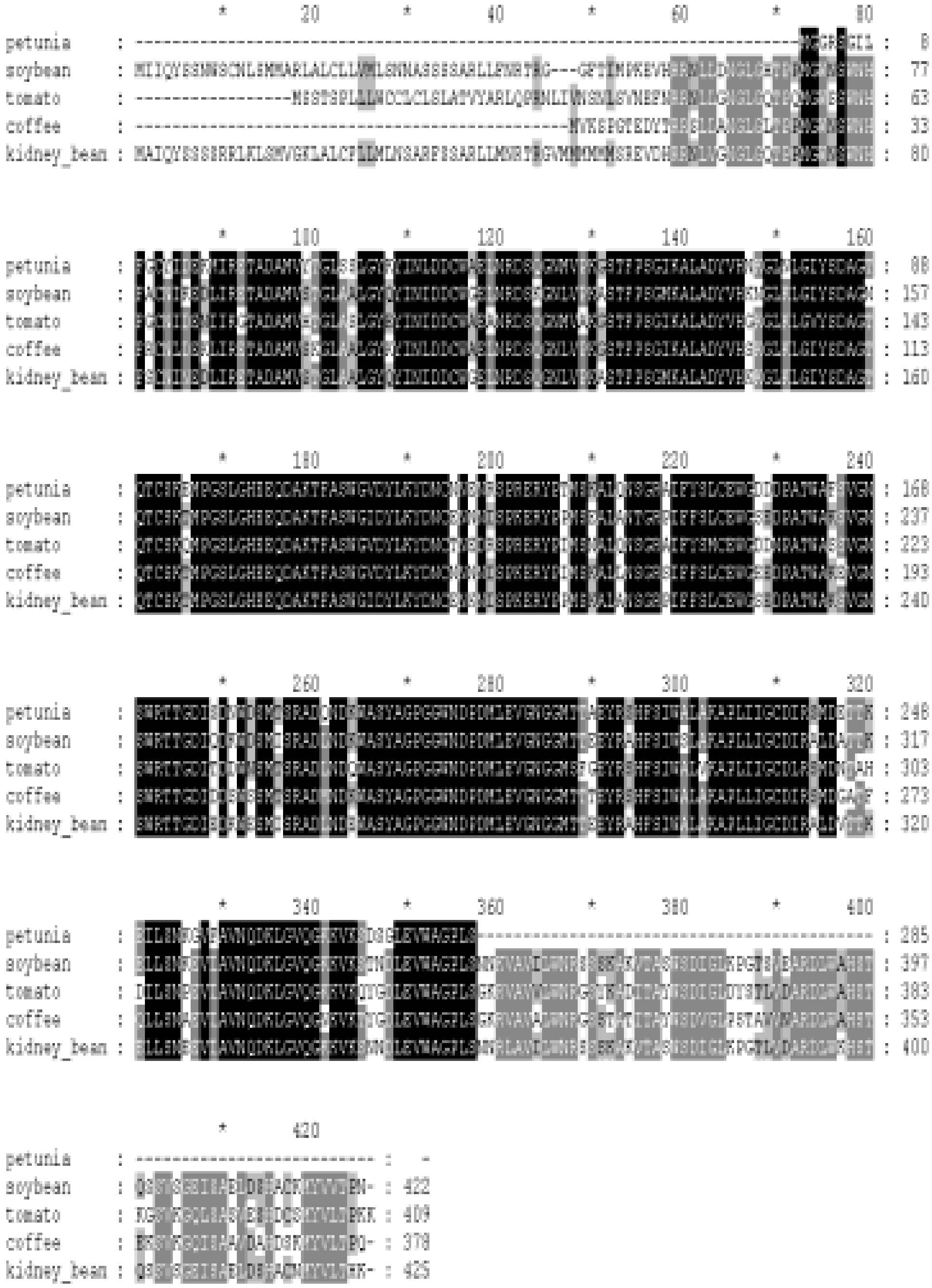

Fig. 1b. Amino acid sequence alignment of petunia (*Petunia* x *hybrida*) with soybean (*Glycine max*), tomato (*Lycopersicon esculentum*), coffee (*Coffee arabica*) and kidney bean (*Phaseolus vulgaris*). The alignment was performed using the Vector NTI Suite software (Informax). Identical amino acids are shaded in black and conserved substitutions are shaded in grey.

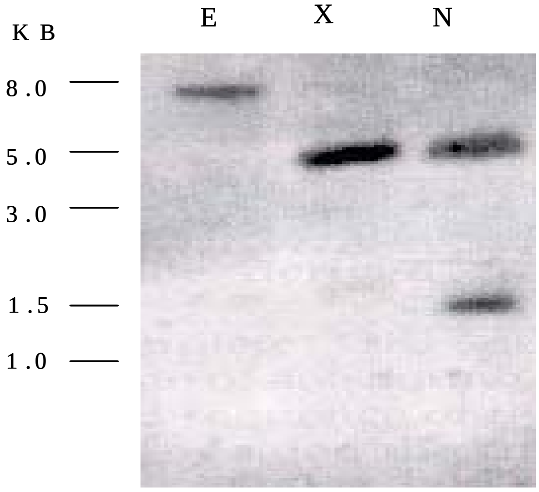

Fig. 2. Southern blot analysis of the petgal cDNA clone. Ten micrograms of total genomic DNA was digested with *EcoR* I (E), *Xba* I (X) or *Nco* I (N). The fragments were separated by electrophoresis in a 1 % agarose gel and transferred onto Nytran membrane which was hybridized with petgal as a probe. Molecular size standards in kilobases are shown to the left.

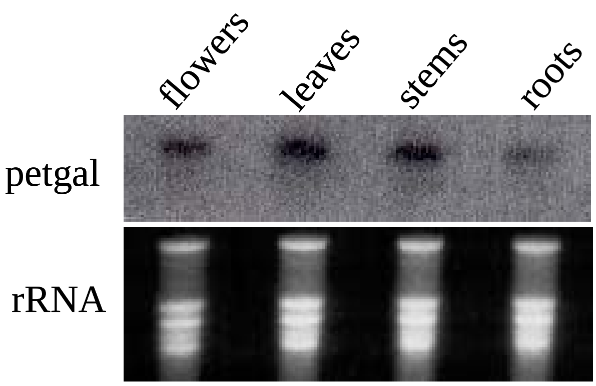

Fig. 3. Northern blot analysis of total RNA isolated from various tissues. Ten micrograms of total RNA was separated by electrophoresis through agarose and hybridized with α-^{32}P- labeled petgal. rRNA stained with ethidium bromide was used as a control for uniform loading of the gel.

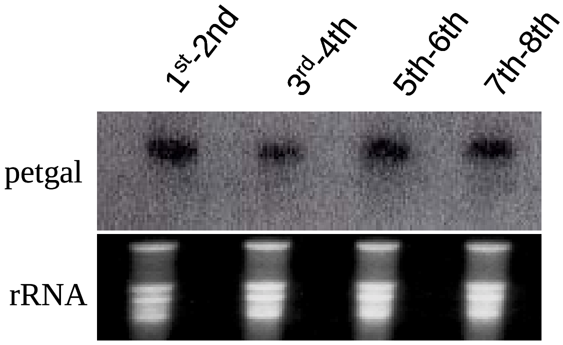

Fig. 4. Non-developmental regulation of petgal Northern blot analysis of the four leaf groups. Ten micrograms of total RNA was separated by electrophoresis through agarose and hybridized with α-^{32}P- labeled petgal. rRNA stained with ethidium bromide was used as a control for uniform loading of the gel.

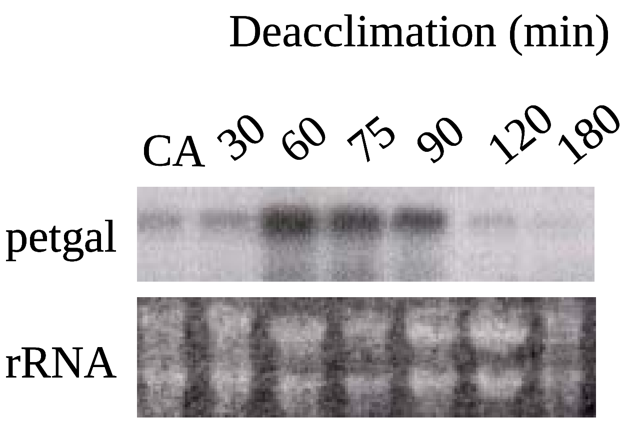

Fig. 5. Deacclimation-induced expression of petgal in petunia. Northern blot analysis of petunia plants deacclimated at 25 °C for indicated lengths of time. See M&M for cold acclimation (CA) regime. Each lane was loaded with 10 μg rRNA, separated by electrophoresis through agarose and hybridized with α-^{32}P-labeled petgal. rRNA stained with ethidium bromide was used as a control for uniform loading of the gel.

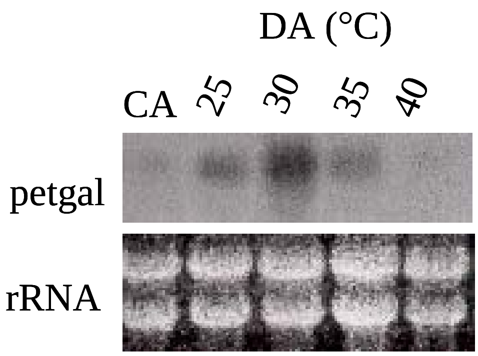

Fig. 6. Temperature dose response of petgal. Northern blot analysis of plants deacclimated (DA) for 1 h at the indicated temperatures. See M & M for cold acclimation (CA) regime. Each lane was loaded with 10 µg of rRNA, separated by electrophoresis through agarose and hybridized with α-^{32}P-labeled petgal. rRNA stained with ethidium bromide was used as a control for uniform loading of the gel.

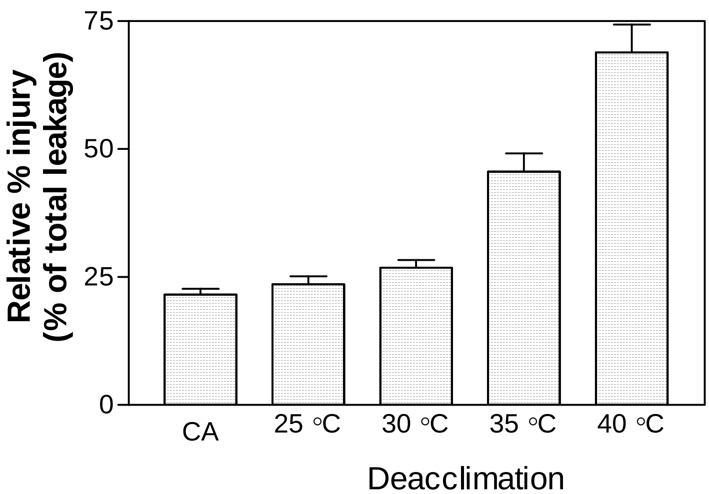

Fig. 7. Electrolyte leakage as a measure of heat stress on deacclimated petunia plants. Plants were deacclimated by incubating previously cold acclimated (CA) plants (see M&M for CA regime) for 1 h at the deacclimating temperatures indicated. Five mm leaf discs were sampled to determine electrolyte leakage. Relative % injury represents the mean leakage as a percentage of the mean total leakage from frozen-killed samples. Error bars are the standard deviation of 5 replicates.