Molecular and biochemical mechanisms associated with dormancy and drought tolerance in the desert legume *Retama raetam*

Lilach Pnueli[1], Elza Hallak-Herr[2], Mira Rozenberg[1], Mira Cohen[2], Pierre Goloubinoff[2], Aaron Kaplan[2] and Ron Mittler[3,*]

[1]*Department of Biology, Technion – Israel Institute of Technology, Technion City, Haifa 32000, Israel,*

[2]*Department of Plant Sciences and The Minerva Arid Ecosystem Research Center, The Hebrew University of Jerusalem, Jerusalem 91904, Israel, and*

[3]*Department of Botany, Plant Sciences Institute, Iowa State University, Room 353 Bessey Hall, Ames IA 50011 USA*

Summary

Dormancy is an important developmental program allowing plants to withstand extended periods of extreme environmental conditions, such as low temperature or drought. Seed dormancy, bud dormancy and desiccation tolerance have been extensively studied, but little is known about the mechanisms involved in the dormancy of drought-tolerant plants, key to the survival of many plant species in arid and semi-arid environments. Subtractive PCR cloning of cDNAs from *Retama raetam*, a C_3 drought-tolerant legume, revealed that dormancy in this plant is accompanied by the accumulation of transcripts encoding a pathogenesis-related, PR-10-like protein; a low temperature-inducible dehydrin; and a WRKY transcription factor. In contrast, non-dormant plants subjected to stress conditions contained transcripts encoding a cytosolic small heat-shock protein, HSP18; an ethylene-response transcriptional co-activator; and an early light-inducible protein. Physiological and biochemical analysis of Rubisco activity and protein in dormant and non-dormant tissues suggested a novel post-translational mechanism of regulation that may be controlled by the redox status of cells. Ultrastructural analysis of dormant plants revealed that air spaces of photosynthetic tissues contained an extracellular matrix that may function to prevent water loss. The cytosol of dormant cells appeared to be in a glassy state, limiting metabolic activity. A combination of biochemical, molecular and structural mechanisms, in association with metabolic suppression, may be key to the extreme drought tolerance *of R. raetam* and its acclimation to the desert ecosystem. These may enable plants to withstand long periods of drought, as well as rapidly to exit dormancy upon rainfall.

Keywords: desert, dormancy, drought, environmental stress, heat shock, post-translational regulation, *Retama raetam*, Rubisco, transcription factor.

Introduction

Dormancy in plants can be defined as a process by which physiological activities become capable of ceasing entirely, in a reversible manner. The plant is thus less dependent on the environment, and consequently becomes tolerant to adverse physical conditions (Koller, 1969). Different types of dormancy in plants have been characterized. They all involve the suppression of metabolic activities such as photosynthesis, transcription, translation, protein turnover and even respiration.

However, dormancy in seeds and buds is associated with the formation of specialized organs, tissues and structures, whereas dormancy in resurrection plants and some drought-tolerant plants is primarily associated with biochemical and molecular changes that accompany water loss, rather than structural changes (Hoekstra *et al.*, 2001; Raven *et al.*, 1992). The loss of water from dormant plant tissue appears to be the key to its resistance to environmental conditions. In the absence of water, many reactions

Table 1. cDNAs isolated by PCR subtractive cloning from dormant and non-dormant *Retama raetam* stems subjected to harsh environmental conditions

Clone homology	Number of repeats	Accession number
Dormancy-specific clones		
PR-10-like	12	AF439272
Dehydrin	3	AY039800
NADH-ubiquinone oxidoreductase	1	AF439273
WRKY-like transcription factor	1	AF439274
Unknown	1	AF439275
Non-dormant/stress-specific clones		
sHSP (cytosolic 18 kDa)	8	AF439277
Ethylene-transcriptional co-activator	4	AF439278
Early light-induced protein	2	AF439279
Lipid transfer protein	1	AF439280
Ribosomal S15 protein	1	AF439281
Chl *a*/*b*-binding protein	1	AF439282
PsbW	1	AF439283
PsaK	1	AF439284

Sampling of plant tissues from naturally grown plants and PCR subtractive cloning is described in Experimental procedures.

that might have had the potential to damage cells under adverse physical conditions, such as reactions involving reactive oxygen intermediates, are suppressed.

During moderate dehydration in which the bulk of cytoplasmic water is removed, cells accumulate a range of compatible solutes to replace water and stabilize enzymes, membranes and protein complexes. These include proline, glutamate, glycine-betaine, mannitol, sorbitol, fructans, polyols, trehalose, sucrose and oligo-saccharides. However, during severe dehydration, at a water content of <0.3 g H_2O g^{-1} dry weight, only sugars appear to be capable of replacing the dissipating water (Bohnert and Sheveleva, 1998; Hoekstra *et al.*, 2001). A number of different proteins that accumulate during water loss were also implicated in stabilizing and protecting cellular structures during dehydration. These include some members of the late embryogenesis-abundant (LEA) supergene family (Ingram and Bartels, 1996), and small heat-shock proteins (sHSP; Wehmeyer and Vierling, 2000). Some of these proteins contain a high number of polar residues, suggesting that they may coat cellular macro-molecules with a cohesive water layer. It is thought that sHSPs directly associate with membranes and membrane-bound multiprotein complexes, and protect them from unfolding and aggregation (Hoekstra *et al.*, 2001; Torok *et al.*, 2001). Small heat-shock proteins can also bind denatured proteins in a folding-competent state, for sub-sequent refolding by other molecular chaperones (Ehrnsperger *et al.*, 1997). Proteins such as sHSP and dehydrins (members of the LEAs) may also protect cells

during the course of dehydration, and during recovery when plants undergo a process of rehydration.

Retama raetam is a stem-assimilating, evergreen, C_3, desert legume, common to arid ecosystems around the Mediterranean basin. It uses an acclimation strategy of partial plant dormancy to resist long periods of water deficit (<30 mm rain per year; Merquiol *et al.*, 2002). Dormancy in *R. raetam* is characterized by a decrease in overall metabolism; inhibition of photosynthesis; and the apparent disappearance of many cellular proteins. Transcripts encoding these proteins remain at a high level within cells, and respiration is not suppressed. Upon rainfall the plant recovers, rapidly accumulates the 'miss-ing' proteins, and exits dormancy (Mittler *et al.*, 2001). The relative water content of dormant tissues (about 35%), as well as the maintenance of respiration during dormancy, suggest that *R. raetam* is not subjected to severe dehy-dration during dormancy, and may therefore be classified as a drought-tolerant, not a desiccation-tolerant or resur-rection plant.

Because dormancy appears to be essential for the acclimation of plants to a variety of harsh environments around the world (Koller, 1969; Raven *et al.*, 1992), we used a combination of molecular, biochemical and structural methods to further characterize the state of dormancy in drought-tolerant *R. raetam* plants growing naturally within their arid dune ecosystem.

Results

Subtractive cloning of dormancy- and stress-associated cDNAs

To survive their harsh environment, *R. raetam* plants use a combination of different avoidance and resistance strat-egies (Merquiol *et al.*, 2002; Mittler *et al.*, 2001; Streb *et al.*, 1997). Using a subtractive PCR hybridization protocol, we isolated two classes of cDNAs from *R. raetam*: (i) cDNAs induced at midday during summer when the photosyn-thetic activity is suppressed to ≈15–20% of maximal daily activity and ROI-detoxifying enzymes are induced (non-dormant/stressed); and (ii) cDNAs induced when water availability is very low and plants enter a state of dormancy (dormant).

We obtained 300 clones from each of the different subtraction assays, and placed them on nylon filters in an array configuration. Duplicate filters were then hybridized with radiolabeled non-dormant/stressed or dormant cDNAs, and clones that were highly expressed in dormant or stressed samples were sequenced. We identified and sequenced ≈25 differentially expressed clones from each of the different subtraction assays. As shown in Table 1, the genes that were specifically expressed in dormant *R. raetam* tissue were very different from those expressed
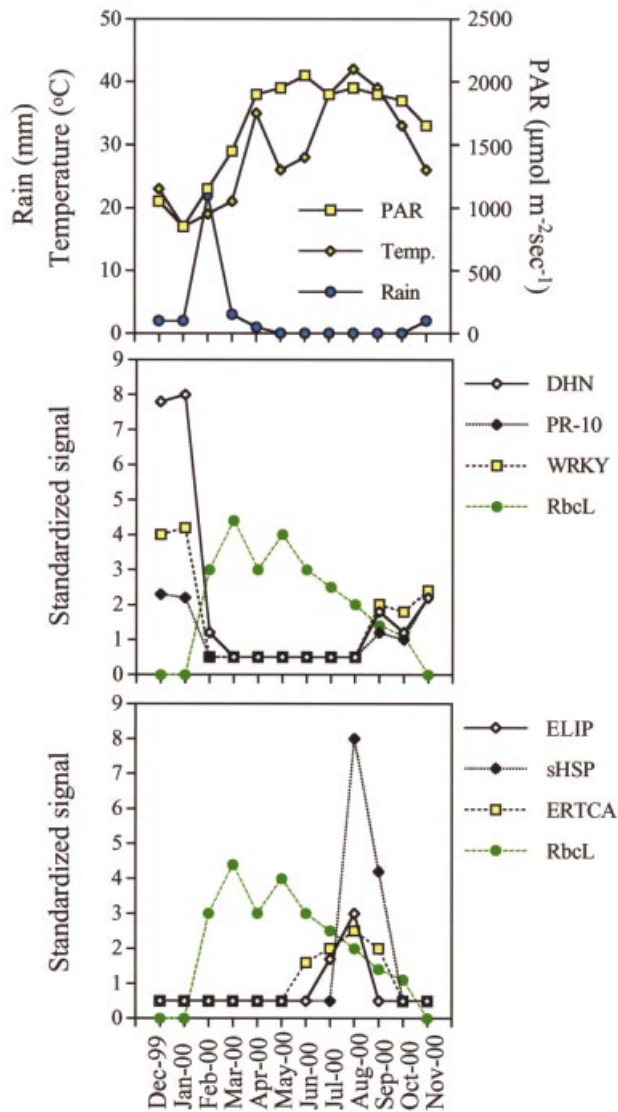
Figure 1. Seasonal changes in the expression pattern of cloned *Retama raetam* cDNAs in naturally grown plants.
Top: seasonal changes in environmental conditions during the year.
Middle: expression pattern of dormancy-associated transcripts during the year.
Bottom: expression pattern of non-dormant/stress-associated transcripts during the year.
The level of RbcL protein detected in protein extracts, as described by Mittler *et al.* (2001), is shown in the middle and bottom panels. The results shown were pooled from two independent plants sampled on a monthly base at midday (Merquiol *et al.*, 2002). Similar results were obtained with additional plants; however, these entered and exited dormancy at different dates, a result of the phenotypic plasticity of plants in the field (Merquiol *et al.*, 2002). Methods of tissue sampling, environmental data recording, protein and RNA analysis, and normalization of signals (to 18S rRNA or total protein) are described in Experimental procedures.

in non-dormant/stressed tissue. The transcript that we found to be most abundant in dormant tissue, using the PCR subtractive protocol, was a transcript encoding a PR-

10 homolog. We also cloned a WRKY transcription factor with a high degree of homology to the parsley transcription factor WRKY2. In parsley, this WRKY protein regulates the expression of PR-10 by directly interacting with a W-box located at the promoter of PR-10 (Eulgem *et al.*, 1999). We also cloned a dehydrin that we previously reported to be induced during dormancy in *R. raetam* (Merquiol *et al.*, 2002), and an unknown protein with a high degree of homology to an unknown *Arabidopsis* gene (AC007508.2). Although it was suggested that sHSPs are involved in protecting dormant plant tissues (Hoekstra *et al.*, 2001), we did not identify an sHSP in our screen of dormant *R. raetam* tissue. Transcripts encoding a cytosolic sHSP were, however, abundant in non-dormant/stressed *R. raetam* tissue. This finding was in agreement with our previous analysis of seasonal changes in gene expression in naturally growing *R. raetam* plants, in which we used an *Arabidopsis* cytosolic sHSP as a probe on RNA blots and did not detect sHSP transcripts in dormant tissue (Merquiol *et al.*, 2002). In addition to transcripts encoding cytosolic sHSP, transcripts encoding a homolog of ethylene-response transcriptional co-activator (ERTCA; Zegzouti *et al.*, 1999) and an early light-inducible protein (ELIP, a protein induced in plants in response to high light stress; Adamska *et al.*, 1993) were expressed in non-dormant/stressed *R. raetam* tissue (Table 1).

The WRKY and ERTCA cDNAs induced during dormancy and environmental stress in *R. raetam* had a high degree of homology to other members of their corresponding families, induced in response to pathogen attack or ethylene, suggesting that these transcription factors regulate diverse processes in plants and may be involved in different developmental programs such as dormancy, as well as in stress tolerance.

Seasonal changes in the expression pattern of R. raetam *cDNAs*

We tested the expression of some of the cDNAs shown in Table 1 in plants that grow naturally within their arid dune ecosystem. This analysis was performed on samples pooled from two independent plants once a month at midday between December 1999 and November 2000. The expression level of the different cDNAs was correlated with: the level of Rubisco large-subunit protein (RbcL) detected in protein extracts by protein blots, as a measure of plant dormancy (using an extraction buffer with a reducing agent as described below; Mittler *et al.*, 2001); the different growth conditions – temperature and photosynthetic active radiation (PAR) – at the research site during sampling; and monthly precipitation. Samples obtained in the different months used for this analysis adequately represent the overall conditions at the research site during the year (Merquiol *et al.*, 2002). As shown in Figure 1,
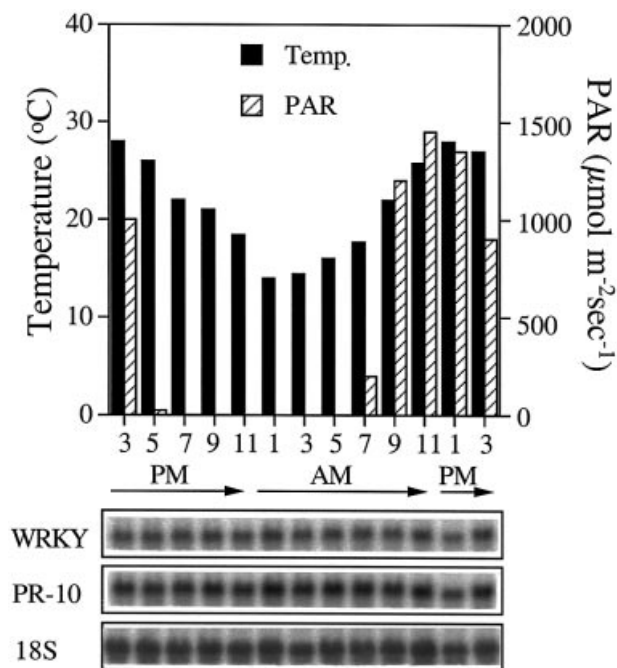
Figure 2. Diurnal change in the expression pattern of WRKY and PR-10 in dormant plants.
Top: environmental conditions recorded at times of sampling.
Bottom: RNA blots showing the expression pattern of WRKY and PR-10 in tissue samples obtained at the different times.
This analysis was performed on different plants during November 2001 with similar results. Methods of tissue sampling, environmental data recording and RNA analysis are described in Experimental procedures. A probe for 18S rRNA was used to ensure equal loading of RNA.

Figure 3. Diurnal change in the expression pattern of ERTCA and sHSP in non-dormant plants.
Top: environmental conditions recorded at the times of sampling.
Bottom: RNA blots showing the expression pattern of ERTCA and sHSP in tissue samples obtained at the different times.
This analysis was performed on different plants during July 2001 with similar results. Methods of tissue sampling, environmental data recording and RNA analysis are described in Experimental procedures. A probe for 18S rRNA was used to ensure equal loading of RNA.

transcripts encoding dehydrin, PR-10 and WRKY were elevated in plants between December 1999 and January 2000, and between September 2000 and November 2000, when the RbcL protein level was low. The level of these transcripts declined as plants exited dormancy following the rainy season in February, and increased late in the growing season when plants re-entered dormancy, after about 7 months without precipitation (Figure 1). In contrast, expression levels of ELIP, sHSP and ERTCA were induced between July 2000 and September 2000 when growth conditions at the sites were harsh and water availability was low. Expression of ELIP, sHSP and ERTCA declined when plants started to re-enter dormancy between September 2000 and November 2000 (Figure 1).

Diurnal changes in the expression pattern of *R. raetam* cDNAs

We tested diurnal changes in the expression pattern of some of the clones shown in Table 1, and compared them to the environmental parameters recorded during sampling. As shown in Figure 2, the expression of WRKY and PR-10 in samples obtained from dormant plants in
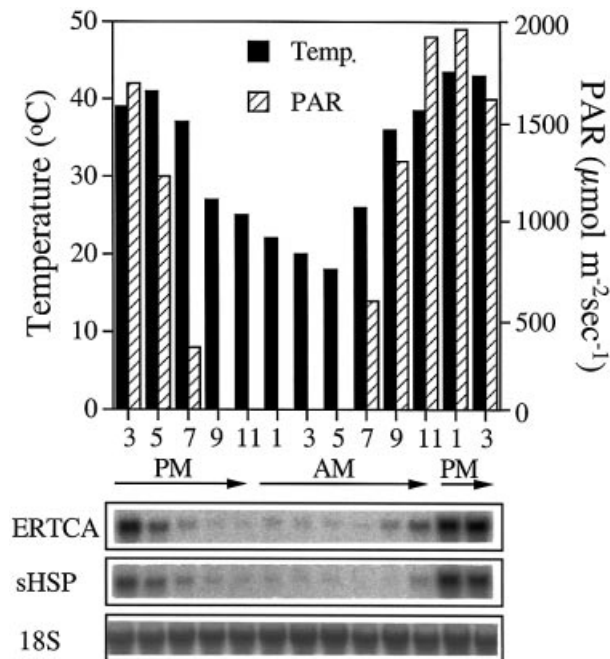
November 2001 did not change in response to the different growth conditions. In contrast, as shown in Figure 3, the expression of ERTCA and sHSP in samples obtained from the same plants in July 2001 (non-dormant) had a very distinct diurnal cycle. Thus ERTCA and sHSP were induced at midday when the conditions were harsh, and declined late in the afternoon and at night when the conditions were mild. WRKY and PR-10 were not expressed in July 2001, and sHSP and ERTCA were not expressed in November 2001 (not shown). The dormancy level in the samples obtained in July and November 2001 was comparable to the level of dormancy shown in Figure 1 for July and November 2000 (not shown).

Induction of cloned *R. raetam* cDNAs in laboratory-grown plants in response to heat shock and cold stress

A strong correlation between gene expression during drought and cold stress has previously been observed in plants (Seki *et al.*, 2001; Shinozaki and Yamaguchi-Shinozaki, 1996). We therefore tested the effect of cold stress on dormancy-associated transcripts in *R. raetam*. Cold treatment (4°C, 48 h) of *R. raetam* seedlings in the laboratory revealed that, although WRKY and dehydrin
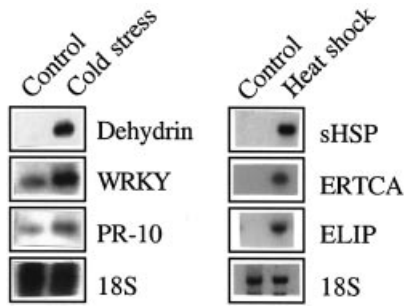
Figure 4. Induction of dormancy- and stress-associated transcripts during cold stress and heat shock in *Retama raetam* seedlings.
Fifty-day-old *R. raetam* seedlings grown under controlled conditions were subjected to cold stress (4°C, 48 h), or heat shock (44°C, 5.5 h) and assayed by RNA blots for the expression of the different *R. raetam* cDNAs. A probe for 18S rRNA was used to ensure equal loading of RNA. Growth conditions, stress treatments and RNA analysis are described in Experimental procedures.

Figure 5. Seasonal changes in the steady-state level of RbcL and APX protein and RNA in naturally growing plants.
The expression of APX and RbcL transcripts, determined by RNA blots, was compared to the level of APX and RbcL protein, detected in protein extracts as described by Mittler *et al*. (2001), during the year. This analysis was performed on plants growing naturally as shown in Figure 1. Methods of tissue sampling, and protein and RNA analysis are described in Experimental procedures.

transcripts were strongly elevated during cold stress, PR-10 transcripts were not similarly elevated (Figure 4, left panel). Cold stress did not result in the induction of sHSP or ERTCA, but caused an induction in the expression of ELIP (not shown). To confirm that the induction observed in dehydrin upon cold stress (Figure 4, left panel) corresponded to the dehydrin clone isolated from dormant plants (Table 1), we performed RT–PCR assays using primers specific to this clone. These studies confirmed that the dehydrin induced during cold stress in *R. raetam* seedlings was also induced during dormancy in mature plants grown in the field (not shown). Heat shock (45°C, 5.5 h) applied to *R. raetam* seedlings grown under controlled laboratory conditions resulted in the induction of sHSP, ERTCA and ELIP (Figure 4, right panel), but did not result in the induction of WRKY, PR-10 or dehydrin (not shown). It is, however, uncertain how the data obtained in the laboratory using *R. raetam* seedlings should be interpreted for mature plants growing in the field.

Discrepancy between RNA and protein levels in R. raetam *during dormancy*

To compare the expression patterns of the different cDNAs shown in Table 1 with those of known housekeeping and defense genes, we extended our seasonal analysis of gene expression shown in Figure 1 to include RbcL and APX. As shown in Figure 5, transcripts encoding RbcL and APX were present in dormant and non-dormant tissues. In contrast, the level of RbcL and APX protein in protein extracts obtained from dormant tissue with a denaturing buffer (see below) was very low. This finding extended our previous comparison of gene expression between dormant and non-dormant tissues (Mittler *et al*., 2001), and
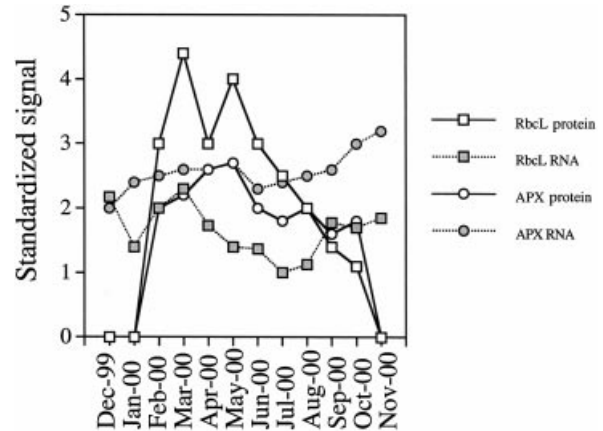
prompted us to examine the state of RbcL and APX protein in protein extracts obtained from dormant tissues.

Biochemical and physiological analysis of Rubisco in protein extracts obtained from dormant and non-dormant R. raetam *tissue*

In agreement with the very low level of RbcL protein found in dormant tissues, measurements of net CO_2 exchange performed under light or dark conditions, in field-grown dormant and non-dormant *R. raetam* plants during daytime, revealed that photosynthetic activity is almost completely suppressed in dormant tissue (Figure 6a, left; Mittler *et al*., 2001). However, when protein extracts were prepared from the same dormant tissues as were used for measuring net CO_2 exchange in the field (Figure 6a, left), for measuring *in vitro* Rubisco activity, it was found that dormant *R. raetam* plants contained active Rubisco protein (Figure 6a, right). Immunoblot analysis revealed that native protein extracts prepared for the determination of Rubisco activity from dormant and non-dormant plants contained RbcL protein (Figure 6b, right), whereas protein extracts prepared in a denaturing buffer from dormant tissues did not (Figure 6b, left). Protein extracts prepared in a denaturing buffer from non-dormant tissues contained RbcL (Figure 6b, left).

Figure 7 shows the different protein profiles obtained when total protein extracts from dormant and non-dormant *R. raetam* tissues were prepared with a native or a denaturing buffer and subjected to protein-blot analysis (left panel), or to SDS–PAGE and silver staining (right panel). In contrast to non-dormant tissue, the protein
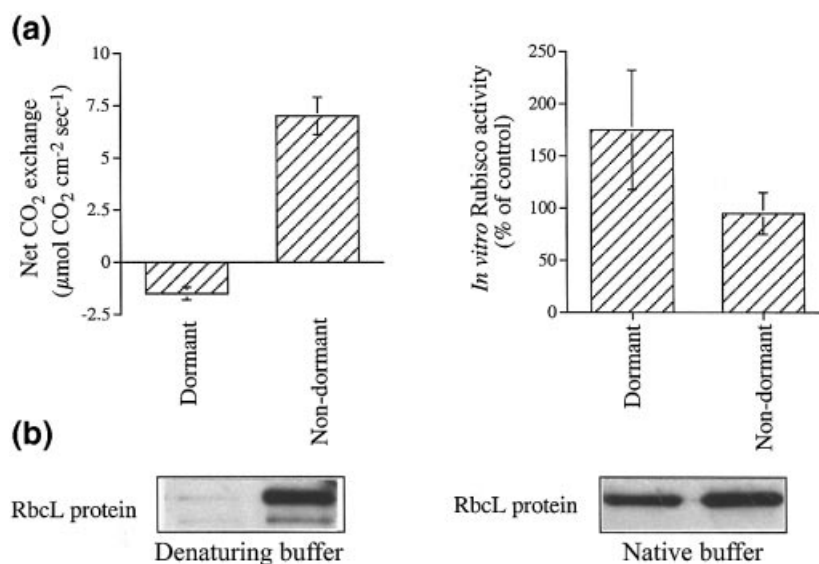
Figure 6. Analysis of Rubisco activity and protein in dormant and non-dormant tissues.
(a) Measurements of net CO_2 exchange in stems of dormant and non-dormant plants in the field (left), and measurements of *in vitro* Rubisco activity in protein extracts obtained from the same samples (right). The data shown are the average and standard deviation of 10 different measurements.
(b) Gel-blot analysis of RbcL protein in extracts obtained from field-grown plants [as in (a)] with a denaturing buffer (left) or a native buffer used for the determination of Rubisco activity (right). Protein isolation and measurements of Rubisco activity and net CO_2 exchange were performed as described in Experimental procedures.

profiles obtained from dormant tissue differed quite dramatically when extracted with the two different buffers (Figure 7, right panel). Proteins such as RbcL, APX and tubulin, which could not be detected by Western blots in protein extracts obtained from dormant tissues with a denaturing buffer, were found in protein extracts obtained from the same tissues with a native buffer (Figure 7, left panel). In contrast to these, at least SOD (cytosolic CuZnSOD) could be detected in protein extracts obtained with a denaturing buffer from dormant tissues, whereas chloroplastic HSP70 and sHSP (22 kDa) could not be detected in dormant tissues regardless of the type of extraction used. Membrane-associated proteins such as B6F and D1 could not be detected in native extracts because they precipitated with the membrane fraction during centrifugation.

Removing each of the different components of the denaturing buffer, and testing for the extractability of RbcL and APX from dormant tissue by protein blots, revealed that the presence of the reducing agent in the denaturing buffer (2-mercaptoethanol) was the cause of this phenomenon (not shown; see Figure 8). During protein extraction the tissue was ground to a fine powder in liquid nitrogen and dissolved in the extraction buffer (native or denaturing). Following centrifugation, the supernatant was loaded on SDS–PAGE gels and processed for immunoblot analysis or protein staining. We therefore examined whether RbcL and APX, two soluble proteins, were found in the centrifugation pellet following extraction

of proteins with a buffer containing a reducing agent from dormant tissue. As shown in Figure 8, the extraction of RbcL and APX from dormant tissue with a buffer containing a reducing agent was very minimal (left lane in RbcL and APX panels). When the reducing agent was removed from the buffer, RbcL and APX were extracted (middle lane in RbcL and APX panels). Re-extracting proteins with a buffer without a reducing agent from the centrifugation pellet obtained following extraction with a buffer with a reducing agent, revealed that at least some of the proteins that were not extracted by the buffer with the reducing agent were found in pellets (right lane in RbcL and APX panels). A similar re-extraction procedure performed on pellets obtained following isolation of protein extracts with a buffer without a reducing agent did not result in detection of RbcL and APX by Western blotting (not shown). This phenomenon was found to occur with different extraction buffers such as sodium phosphate (50 mM) or Tris (100 mM) both at pH 7; in the presence or absence of SDS (1–2%) or glycerol (10%); and with different reducing agents such as 2-mercaptoethanol (20–200 mM) and DTT (5–100 mM), but not glutathione (5–10 mM; not shown).

Ultrastractural analysis of dormant R. raetam tissue

A combination of molecular, biochemical and anatomical adaptations may be required for *R. raetam* acclimation to the desert ecosystem. Many anatomical adaptations of *R.*
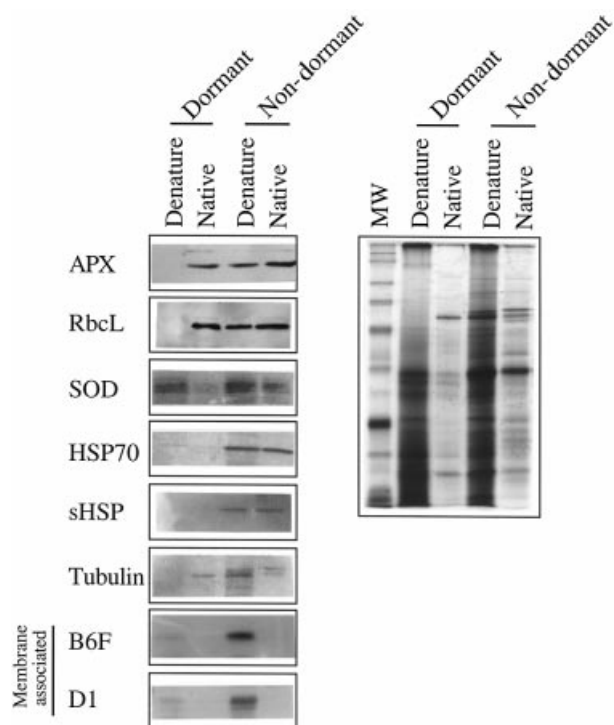
Figure 7. Differential extraction of proteins from dormant *Retama raetam* tissue in native or denaturing buffer.
Proteins were extracted with a native or a denaturing buffer from dormant and non-dormant tissues and subjected to protein-blot analysis (left) or stained with silver (right). Protein extraction and blot analysis were performed as described in Experimental procedures. Size of molecular weight markers (MW) from top to bottom (right) in kDa is: 143, 88, 64, 50, 36, 22, 16, 6.

Figure 8. Detection of RbcL and APX in pellets obtained following the extraction of proteins from dormant tissue with a denaturing buffer.
Protein-blot analysis showing the detection of RbcL (left) or APX (right) in protein extracts obtained with a reducing agent (left lane); in the same buffer but without a reducing agent (middle lane); and in pellets obtained following extraction of proteins with a reducing agent (right lane). Extraction buffers tested included sodium phosphate (50 mM) or Tris–HCl (100 mM), both at pH 7, with or without SDS (1–2%) or glycerol (10%), and with or without the reducing agents 2-mercaptoethanol (20–200 mM) and DTT (5–100 mM), with similar results.

raetam have been described previously (Fahn and Cutler, 1992). However, the finding that dormant *R. raetam* tissues may have very different biochemical features compared to non-dormant tissue (Figures 6–8) may indicate that, at the cellular level, dormant tissue is very different from non-dormant tissue. We previously used transmission electron microscopy (TEM) to study dormant *R. raetam* tissue (Mittler *et al.*, 2001). However, the use of TEM requires plant tissues to be fixed in an aqueous solution that may alter the features of the dehydrated dormant tissue. To avoid this complication, we used a freeze-fracture technique coupled with scanning electron microscopy (SEM). Using SEM, we found that cells of dormant stems have a very dense cytoplasm, indicative of low water content. They were smaller than non-dormant cells, did not contain vacuoles, and were very resistant to freeze-fracturing (compare Figure 9a, left and right panels). Indeed, the relative water content of dormant tissue was about 35%, compared to 80% of non-dormant tissue (Mittler *et al.*, 2001). The cytoplasm of dormant cells may therefore be in a state that may resemble the glassy state found in seeds
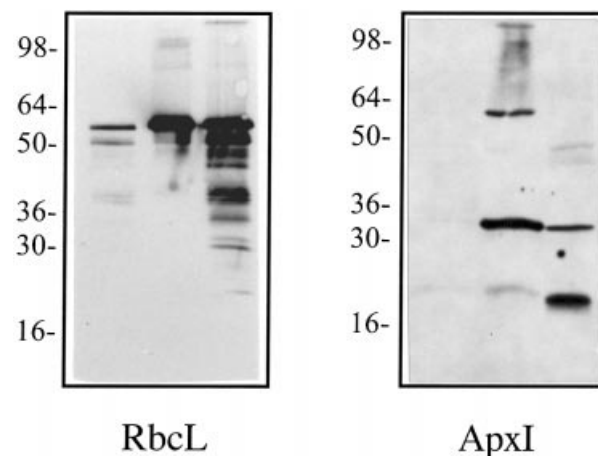
and some desiccation-tolerant plants (Buitink *et al.*, 2000; Sun, 2000).

We found that dormancy in *R. raetam* is accompanied by the accumulation of an intercellular substance at the inner-stem air spaces (Figure 9a, right panel). The substance, appearing as a polymer in SEM images (fiber diameter of 0.12–0.15 nm) was absent from air spaces of non-dormant tissue (Figure 9a, left). It was not soluble in aqueous solution, and it stained with periodic acid–Schiff (PAS), suggesting that it contained carbohydrates (not shown). As shown in Figure 9(b), the inner-stem air spaces of *R. raetam* are connected with the atmosphere via stomata (s) and contain photosynthetic mesophyll cells (m) similar to leaf tissue. Because mature *R. raetam* plants do not have leaves, this tissue represents the principle photosynthetic tissue of the plant. To prevent water loss, stomata found in specialized cavities within stems (Figure 9b; Fahn and Cutler, 1992) are mostly kept closed during periods of drought. However, dormant stems of *R. raetam* may have an added layer of protection against water loss. While the stomata are kept closed or partially closed during dormancy, the matrix within the air spaces (Figure 9a, right) may lower the diffusion rate of air and create a microenvironment that preserves humidity. Negative staining with trypan blue (not shown), the diameter and appearance of fibers, and the strong correlation between dormancy and fiber accumulation suggest that these fibers do not belong to a parasitic fungus.
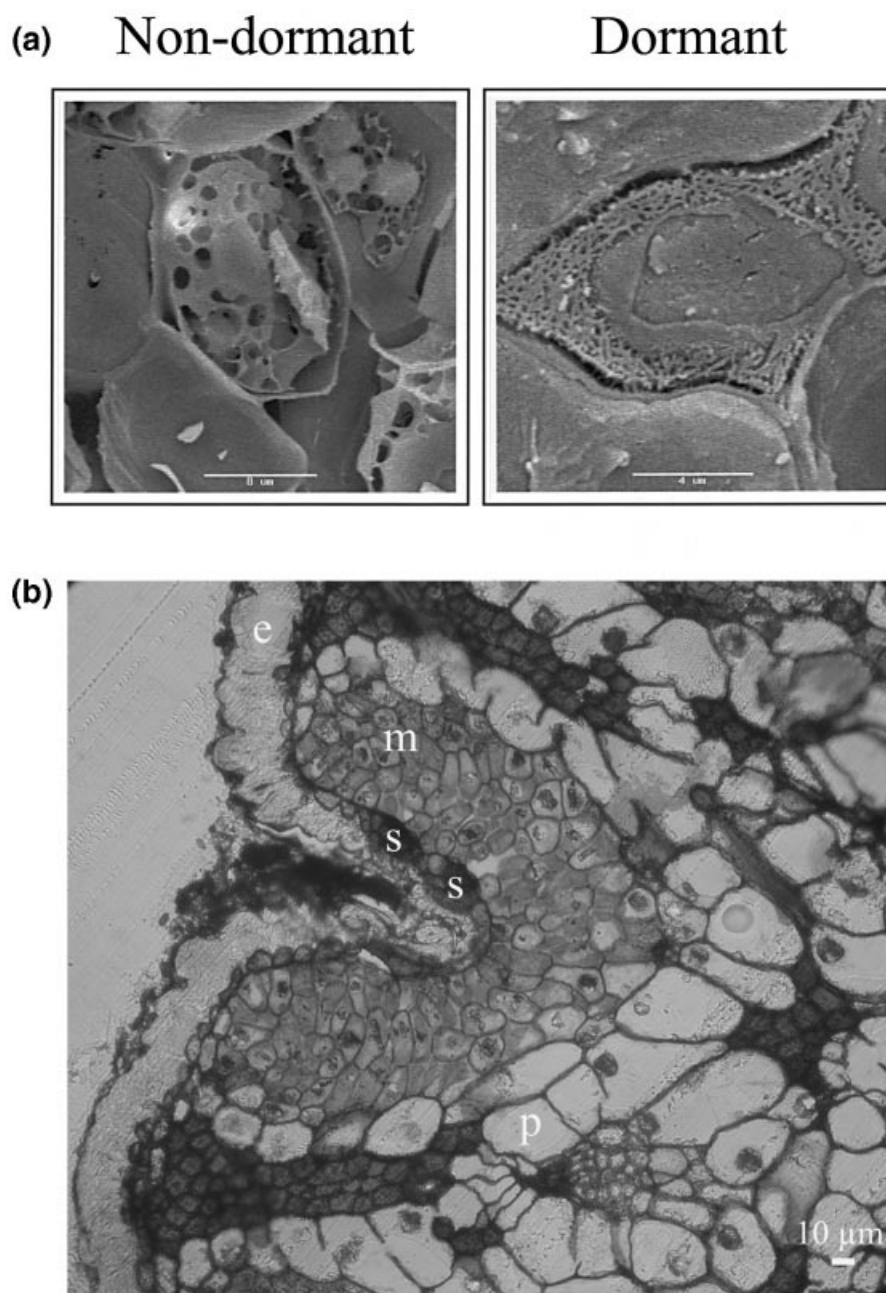
Figure 9. Structural analysis of dormant and non-dormant *Retama raetam* stems.
(a) SEM images showing cells within the inner-stem air spaces of non-dormant (left) and dormant (right) stems of *R. raetam*. The cytoplasm of cells from dormant tissue is shown to be condensed; air spaces are filled with a substance that may prevent water loss during dormancy.
(B) Part of a cross-section through a non-dormant *R. raetam* stem showing mesophyll cells (m) with air spaces, stomata (s), parenchyma cells (p), epidermis (e), and a cavity in which the stomata are located. Methods of tissue sampling and structural analysis with SEM (a) or light microscopy (b) are described in Experimental procedures.

Discussion

A combination of molecular, biochemical and structural adaptations may be critical for the acclimation of *R. raetam* to its arid desert environment. We cloned and characterized four different transcripts that accumulate in *R. raetam*

during dormancy. The most abundant transcript found in this tissue by PCR subtractive cloning was a transcript encoding a protein with a high degree of homology to the pathogenesis-related protein PR-10. In other plants, PR-10 is induced by drought (Dubos and Plomion, 2001); salt stress (Moons *et al.*, 1997); pathogen attack (McGee *et al.*,

2001); and the plant hormones abscisic acid and methyl jasmonate (Moons *et al.*, 1997; Wang *et al.*, 1999). At least in roots of white lupin, PR-10 was shown to have ribonucleolytic activity, and has been suggested to act as an RNase (Bantignies *et al.*, 2000). However, it is not known whether this is the only cellular function of PR-10, and examples of proteins with multiple cellular functions do exist. The PR-3 protein, which is induced in response to pathogen attack and functions as a chitinase, is also induced during cold stress and contains domains that function as an antifreeze protein (Pihakaski-Maunsbach *et al.*, 2001). In addition, osmotin, another PR protein that is induced during pathogen attack, is induced during abiotic stress and may play a dual defense role (Zhu *et al.*, 1995). It is possible that PR-10 may exhibit other cellular functions, in addition to its suggested ribonucleolytic function, that may be similar to dehydrin or sHSPs. This possibility gains support from the high number of polar residues per total number of side chains found in PR-10 (about 40%), compared to dehydrin (about 50%). Therefore PR-10 may function as an LEA-like protein to protect other proteins and cellular structures during dormancy, due to its high number of polar residues. The cloning of a group I WRKY transcription factor with a high degree of homology to a parsley WRKY, which binds to the promoter of PR-10 and activates its expression in parsley (Eulgem *et al.*, 2000), provided additional support for the induction of PR-10 during dormancy. Although the possibility cannot be completely discounted that PR-10 is induced in *R. raetam* in the field in response to a pathogen attack, it seems unlikely because PR-10 and WRKY were induced in different plants, located at different areas of the ecosystem and sampled at different times.

WRKY belongs to a family of transcription factors unique to plants, and may be involved in the response of plants to pathogen attack, as well as in the regulation of different physiological programs (Eulgem *et al.*, 2000; Yu *et al.*, 2001). The cloning of a WRKY transcription factor that is expressed during dormancy and cold stress provides additional evidence for the importance of this group of transcription factors in plants. The cloning of a homolog of the ethylene-response transcriptional co-activator protein from stressed non-dormant *R. raetam* tissue, its induction during heat shock, and its diurnal pattern of expression may suggest that this transcriptional co-activator is also involved in the regulation of gene expression in plants in response to environmental stress. Expression of the *R. raetam* WRKY or ERTCA in related legumes such as soybeans may result in the enhancement of drought, heat or cold tolerance, similar to the effect that expressing the *Arabidopsis* transcription factor DREB1A had on stress tolerance in transgenic plants (Kasuga *et al.*, 1999).

Previous analysis of drought and desiccation tolerance in plants suggests a role for sHSPs in defending plant tissues during desiccation. However, we were unable to detect sHSPs in dormant *R. raetam* plants (Figure 7; Merquiol *et al.*, 2002; Mittler *et al.*, 2001). It is possible that, due to the relatively low water content of dormant *R. raetam* tissues, the catalytic function of sHSPs is not needed and the protective/stabilizing function of sHSPs is replaced by dehydrins and compatible solutes. At least in this context, dormancy in *R. raetam* may be different from other studied examples of dormancy in plants that may extensively rely on sHSP for tissue protection (Hoekstra *et al.*, 2001). Alternatively, a novel class of sHSPs with a low degree of homology to known sHSPs may function to protect *R. raetam* tissues during dormancy.

Biochemical analysis of protein extracts obtained from dormant and non-dormant *R. raetam* tissues revealed that the presence of a reducing agent in the extraction buffer had a dramatic effect on the state of proteins extracted from dormant tissues. Under reducing conditions, some of the proteins previously described as 'missing' (Mittler *et al.*, 2001) were apparently present as a high molecular-weight complex that precipitated during extraction and did not resolve on protein gels. Extraction of proteins under non-reducing conditions released the proteins from this state. This finding may explain the rapid appearance of some of the 'missing' proteins following rehydration of dormant tissues (Mittler *et al.*, 2001). It is possible that reducing equivalents found in *R. raetam* during dormancy maintain proteins in a protected, multimeric form until rehydration. Rehydration dilutes these reducing equivalents and releases proteins into their soluble, active form. The presence of a reducing agent(s) in dormant tissue may be necessary to protect this tissue from oxidation that will result in cellular damage (Roberts, 1969). A number of studies link the prevention of oxidation processes and plant dormancy, supporting this possibility (Hendricks and Taylorson, 1975; Stacy *et al.*, 1999). We are in the process of analyzing dormant *R. raetam* tissue in an attempt to identify possible cellular compounds or proteins that may be involved in this phenomenon. Our new findings with dormant *R. raetam* tissue suggest that at least part of the regulation of gene expression in this plant during dormancy is not at the post-transcriptional level, as previously suggested (Mittler *et al.*, 2001), but rather at the post-translational level. For Rubisco this may be apparent from comparing the *in vivo* and *in vitro* analysis of its activity and protein (Figures 5–8). Thus, although Rubisco protein is found in dormant tissues in an active form (apparent from protein blots and *in vitro* assays of Rubisco activity in protein extracts obtained from dormant tissue in the absence of a reducing agent), it is kept inactive (apparent from *in vivo* measurements of net CO_2 exchange in the dark and in the light in dormant and non-dormant plants, and from protein blots performed with protein extracts prepared with a reducing agent). Rehydration of dormant

tissue may release Rubisco from its non-active/non-sol-uble form without a significant change in protein levels (Figure 6; Mittler *et al.*, 2001). This mode of regulation may not require *de novo* synthesis of new Rubisco protein, and may enable *R. raetam* to respond very rapidly to changes in growth conditions. Further molecular and biochemical studies are required to elucidate the mechanism(s) underlying this post-translational mode of regulation.

Ultrastructural analysis of dormant tissues using SEM revealed two interesting features. In general, cells of dormant tissue were very hard and could not be easily fractioned for SEM. When fractioned, they revealed a very dense cytoplasm that almost completely lacks vacuolar structures. Dormant seeds were shown to be in a state referred to as glassy, in which the cytoplasm is extremely dense and completely inhibits all metabolic activity due to lack of water (Hoekstra *et al.*, 2001). It is possible that dormant *R. raetam* tissues exist in a similar state, but – based on the relative water content of dormant *R. raetam* tissue and its maintenance of respiration during dormancy – it should not reach the level of desiccation found in seeds. Based on the model developed for seed dormancy (Leopold *et al.*, 1994), it is possible that reduced water content and condensation of the cytoplasm will lower metabolic activity, thus contributing to the stress tolerance of dormant *R. raetam* tissue. An unexpected result of our SEM analysis was the detection of a substance that filled the air spaces of dormant tissue (Figure 9a). The function of this substance is not known. Because its appearance correlated with plant dormancy and a decrease in relative water content, it is possible that it serves to trap humidity within the air spaces, a feature that will provide *R. raetam* with an advantage in the struggle to survive the harsh desert environment. A combination of molecular, biochemical and structural mechanisms is therefore required to induce dormancy and drought tolerance in *R. raetam*, and enables it to acclimate to the desert ecosystem.

Experimental procedures

Plant materials and sampling

All field experiments were performed with mature *Retama raetam* plants that grow naturally within two research sites of the Minerva Arid Ecosystem Research Center (Berkowicz *et al.*, 1995; http://aerc.es.huji.ac.il/). Environmental parameters were collected, stored and analyzed as described by Berkowicz *et al.* (1995). For cold stress and heat shock, *R. raetam* seedlings were planted in soil and grown for 50 days at 25°C under constant light (75 μmol photons m^{-2} sec^{-1}). Seedlings were then shifted to 4°C for 48 h (cold stress), or to 45°C for 5.5 h (heat shock), under the same light intensity. Control seeds were kept at 25°C. For biochemical and molecular analysis, stems were collected as previously described (Mittler *et al.*, 2001) and immediately frozen in liquid nitrogen. Photosynthetic activity of stems was measured in the field with a Licor LI-6400 apparatus using the following

measuring cell (6 cm^2) parameters: 26°C, 1500 μmol photons m^{-2} sec^{-1} or dark, and an air flow of 300 μl sec^{-1}. Relative water content (RWC) of stems was determined as described by Mittler and Zilinskas (1994). Dormancy of plants was defined by a combination of three different parameters: RWC < 40%; negative overall net CO_2 exchange rate in the light; and the failure to detect RbcL or RbcS protein by protein-gel blots in protein extracts obtained with a buffer that contained a reducing agent.

Subtraction PCR cloning

A cDNA subtraction library was prepared using Clontech PCR-select cDNA subtraction kit (K1804-1). Poly(A) RNA was isolated as described by Mittler *et al.* (1998). The tester RNA was poly(A) RNA prepared from dormant stems, and the driver RNA was poly(A) RNA from non-dormant stems sampled at the same time of day, as previously described (Mittler *et al.*, 2001). The library PCR fragments were cloned into pGem-T Easy vectors (Promega, Madison, WI, USA). Filter arrays were prepared from the library clones at the Hadassah Medical School DNA Facility of the Hebrew University. Duplicate filters were hybridized with radio-labeled cDNAs prepared from tester or driver poly(A) RNA (3 μg) using oligo-dT (Promega) and Superscript reverse transcriptase (Gibco BRL, Carlsbad, CA, USA), as suggested by the manufacturer. Hybridization conditions were as follows: 60°C, 5 × SSC, 5 × Denhart, 0.5% SDS, 100 μg ml^{-1} salmon sperm DNA, overnight. Washing conditions were as follows: 60°C, 2 × SSC, 0.1% SDS, for 20 min followed by 0.2 × SSC, 0.1% SDS, 60°C for 20 min. Following washes, the filters were exposed to X-ray films. Clones corresponding to RNA from dormant plants and not to that from non-dormant plants were chosen for further analysis, and sequenced at the Iowa State University Sequencing and Synthesis Facility. A similar design was used to clone non-dormant stress-specific cDNAs using tester RNA from non-dormant stressed plants, and driver RNA from non-dormant non-stressed plants sampled at the same time of day, as described by Merquiol *et al.* (2002); Mittler *et al.* (2001). Environmental conditions at the time of sampling for subtractive cloning are shown in Figure 1, with dormant tissues sampled in January 2000 and non-dormant tissues in August 2000.

Analysis of gene expression

For the analysis of gene expression, plant tissue frozen in liquid nitrogen was ground to a fine powder with a mortar and pestle, and protein and RNA were isolated and analyzed by RNA and protein-gel blots as described by Mittler and Zilinskas (1994); Mittler *et al.* (2001). RNA and protein gels were loaded based on an equal amount of protein or RNA (Mittler and Zilinskas, 1994). RNA gel blots were hybridized with radiolabeled cDNA probes, as described previously (Mittler *et al.*, 2001), and quantified by exposing blots to a phosphoimager (Fuji BAS1000, Kanagawa, Japan). In all cases the gene-specific signal was standardized to the level of ribosomal 18S RNA, detected as described previously (Merquiol *et al.*, 2002; Mittler and Zilinskas, 1994). Protein-specific signals were normalized to the amount of total protein loaded on gels (Merquiol *et al.*, 2002). Rubisco activity was assayed as described by Marcus and Gurevitz (2000) with the following modifications. The extraction buffer used was 100 mM sodium phosphate pH 7, 10 mM ascorbic acid, 5 mM glutathione, 2 mM PMSF, 1 mM EDTA. The reaction buffer used was 50 mM Hepes pH 8, 20 mM MgCl$_2$, 10 mM NaHCO$_3$ + NaHC^{14}O$_3$ pH 8, 1 mM DTT, 0.5 mM Rubp. Rubisco activity of *R. raetam* plants naturally

growing at the research sites was compared to the Rubisco activity of plants grown at the Hebrew University under non-stressful conditions (control, 100% activity).

Protein isolation under native or denaturing conditions

Protein extracts were prepared by grinding frozen plant tissue to a fine powder and dissolving the powder in the extraction buffer. Extraction buffers tested included sodium phosphate (50 mM) or Tris–HCl (100 mM), both at pH 7, with or without SDS (1–2%) or glycerol (10%), and with or without the following reducing agents: 2-mercaptoethanol (20–200 mM), DTT (5–100 mM), or reduced glutathione (5–10 mM). The extracts were then centrifuged for 10 min at 12 000 g and the supernatant subjected to SDS–PAGE for staining, or to protein gel blots as described previously (Mittler *et al.*, 2001).

Light and scanning electron microscopy

Cross-sections, ≈1 mm width, obtained from dormant and non-dormant stems of *R. raetam* plants were fixed for 12 h in 2.5% glutaraldehyde, 0.1 M sodium phosphate buffer pH 7.0, washed in 0.1 M sodium phosphate buffer, and post-fixed for 2 h with 1% osmium tetroxide, 0.1 M sodium phosphate buffer pH 7.0. Samples were then washed, dehydrated in a graded ethanol series, and embedded in epon resin. Thick sections (2 μm) were stained with 0.5% tuloidine blue (in the presence of 0.5% borax), 0.5% trypan blue, or PAS, as described below, and examined by light microscopy as described previously (Mittler *et al.*, 1995). For PAS staining, sections were placed on slides and incubated for 2 h at 55°C. Sections were then incubated with 0.5% periodic acid for 20 min, extensively washed with water, and incubated with Schiff reagent at 37°C for 20 min. Sections were then washed with 0.5% sodium metabisulfite (3 × 2 min), washed with water, covered with glycerol, and observed and photographed as described by Mittler *et al.* (1995). For SEM analysis, plant tissue was flash-frozen in the field in liquid nitrogen, fractured with an Oxford CT1500 crio-SEM, coated with gold, and viewed in a Jeol 5400 SEM.

Acknowledgements

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