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Wound-induced PAL activity is suppressed by heat-shock treatments that induce the synthesis of heat-shock proteins

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## Heat shock treatments delay the increase in wound-induced phenylalanine ammonia-lyase activity by altering its expression, not its induction in Romaine lettuce (*Lactuca sativa*) tissue

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Wounding lettuce (*Lactuca sativa* L., var. *Longifolia*) leaves induced an eight-fold increase in the activity of phenylalanine ammonia-lyase (PAL; EC 4.3.1.5), and the subsequent accumulation of phenolic compounds and tissue browning at 10°C. PAL is a key enzyme in the synthesis of phenolic compounds. A PAL cDNA was previously isolated by reverse-transcription PCR using total RNA from wounded lettuce leaves. RNA gel blots showed that maximum accumulation of both PAL mRNA and PAL enzyme activity occurred 24 h after wounding. A 2-min heat shock at 45°C administered within 5 min of wounding delayed the wound-

induced increase in PAL activity, but did not delay the increase in wound-induced PAL mRNA. Changes in the content of PAL protein were also followed by immunoblot using anti-PAL antibody raised against the bacterially expressed protein from the cDNA. Immunoblots showed that the level of PAL protein in wounded lettuce tissue was significantly reduced by the heat shock treatment. These data suggest that heat shock reduces the rise in wound-induced PAL enzyme activity by reducing the translation of wound-induced PAL mRNA, or by increasing the turnover of the induced PAL protein.

### Introduction

Physical wounding triggers several important changes in plant metabolism; for example, increased respiration, induced production of secondary metabolites, membrane disruption, increased production of ethylene, and activation of oxidative processes (Brecht 1995, Saltveit 1997). A pronounced change that occurs after wounding is the accumulation and oxidation of phenolic compounds that causes tissue browning (Ke and Saltveit 1989). Wounding induces an increase in the activity of phenylalanine ammonia-lyase (PAL; EC 4.3.1.5); a key enzyme in the synthesis of phenylpropanoid compounds (Hahlbrock and Scheel 1989). PAL activity is correlated with the accumulation of phenolic compounds that are oxidized by polyphenoloxidases (PPO) and peroxidases (POD) to dark-coloured pigments in wounded lettuce tissue (Ke and Saltveit 1988).

The physiology of minimally processed fruits and vegetables is similar to the physiology of wounded tissue since many forms of processing (e.g. abrading, cutting, shredding and slicing) are essentially types of wounding. Wound-induced changes can alter the appearance, organoleptic and nutritional quality of fruits and vegetables (Rolle and Chism 1987, King and Bolin 1989). In tissue in which the initial level of phenolic compounds is low (e.g. lettuce leaves), browning only occurs after wound-induced increases in phenolic metabolism and the accumulation of precursor compounds.

Treatments that interfere with the ability of wounding to increase PAL activity or the synthesis and accumulation of phenolic precursors could reduce browning (Hisaminato et al. 2001, Peiser et al. 1998). One such recently discovered

Abbreviations – CHX, cycloheximide; hsp, heat shock proteins; PAL, phenylalanine ammonia lyase; POD, peroxidase; PPO, polyphenyl oxidase

technique is the use of short, heat shock treatments to reduce PAL activity, the accumulation of phenolic compounds and tissue browning in wounded lettuce (Loaiza-Velarde et al. 1997, Loaiza-Velarde and Saltveit 2001, Saltveit 2001). Heat shocks also reduced PAL activity in transgenic tobacco (Moriwaki et al. 1999).

Exposure to temperatures above the normal growing temperature induces the synthesis and accumulation of a unique set of proteins called heat shock proteins (hsps). The heat shock response appears to be ubiquitous among all living organisms (Vierling 1991). The induction and synthesis of hsps alters the transcription and translation of other genes (Lindquist 1986, Vierling 1991). Exposure to high temperatures (e.g.  $45 \pm 5^\circ\text{C}$ ) for a short time (30–120 s) significantly reduces the wound-induced increase in PAL activity and subsequent tissue browning in lettuce leaves (Loaiza-Velarde et al. 1997, Loaiza-Velarde and Saltveit 2001). Heat shock treatments modified the synthesis of phenolic compounds and prevented tissue browning by reducing wound-induced increases in PAL activity. The presence of hsps had no effect on wound-induced PAL activity, but the heat-shock-induced synthesis of hsps (e.g. hsp 23) was correlated with the reduction of PAL activity (Kang and Saltveit 2003).

The objective of this research was to elucidate the mechanisms by which heat shock treatments block the rise in wound-induced PAL activity and subsequent tissue browning in wounded lettuce leaves. The possible effects of heat shock treatments on gene expression, protein synthesis and activity of PAL in wounded lettuce tissue are discussed.

## Materials and methods

### Plant materials

Heads of Romaine lettuce (*Lactuca sativa* L., var. Longifolia) were obtained from commercial sources, transported to the Mann Laboratory and held at  $0.5^\circ\text{C}$  until used. Complete leaves or 1-cm cross-sections of the trimmed mid-ribs were used in the experiments. Fully expanded, mature leaves were chosen; eliminating the damaged outer leaves and immature inner leaves. Pieces (approximately  $1\text{ cm} \times 2\text{ cm}$ ) were cut from the achlorophyllous mid-rib with a stainless steel razor blade and stored at  $10^\circ\text{C}$  for variable lengths of time before evaluating PAL activity, phenolic compounds, gene expression and protein accumulation.

### Phenolic compound determinations and colour evaluation

The concentration of phenolic compounds was measured as previously described (Ke and Saltveit 1988, Loaiza-Velarde et al. 1997). Briefly, 10 g of tissue was stored for varying lengths of time at  $10^\circ\text{C}$ , and then homogenized in 20 ml methanol (HPLC grade) with the Ultra-Turrax tissue homogenizer (Fisher Scientific, Sacramento, CA). The homogenate was filtered through four layers of cheesecloth and centrifuged at

$15\,000\text{ g}$  for 20 min. Absorbance of the supernatant was read at 320 nm (potential browning) and 437 nm (soluble *o*-quinones) using an UV-VIS spectrophotometer (UV-160 A; Shimadzu, Tokyo, Japan). The remnants of the extraction were placed in a Multiwell tissue culture plate (Falcon 3047; Becton Dickinson, Franklin Lakes, NJ) and colour evaluations were performed as described by Loaiza-Velarde and Saltveit (2001). The  $L^*$ ,  $a^*$  and  $b^*$  values were recorded using a colorimeter (CR-200; Minolta, Tokyo, Japan). In the Commission Internationale d'Eclairage standardize colour measurement, the  $L^*$   $a^*$   $b^*$  colour model defines  $L^*$  as the lightness of the colour, and  $a^*$  and  $b^*$  as the colour along a red/green and blue/yellow axis, respectively.

### Heat shock treatment application

Whole non-wounded leaves or 1-cm pieces of mid-rib tissue were immersed in  $45^\circ\text{C}$  water in a water bath (Fisher Scientific 9001) for up to 4 min. After the heat shock treatment, the leaves or tissue pieces were submerged in ice-cold water ( $0^\circ\text{C}$ ) for 10 s. The lettuce tissue was gently shaken (whole leaves) or spun in a salad spinner (tissue pieces) to remove excessive water from the surface. The samples were placed in plastic containers lined with moist paper towels to avoid dehydration, and stored in the dark at  $10^\circ\text{C}$ . The controls were whole leaves and tissue pieces treated in exactly the same way, but without the heat shock treatment.

### Protein extraction and quantification

Soluble proteins were extracted from lettuce mid-rib tissue as previously described by Ke and Saltveit (1986) with some modifications. Briefly, 4 g of tissue was homogenized with an Ultra-Turrax (high speed for 5 min) with 16 ml of 50 mM borate buffer (pH 8.5) containing 5 mM 2-mercaptoethanol and 0.4 g polyvinylpyrrolidone. The homogenate was filtrated through four layers of cheesecloth and centrifuge at  $20\,000\text{ g}$  at  $4^\circ\text{C}$  for 20 min. The supernatant was mixed 1:2 (v/v) with cold acetone ( $-20^\circ\text{C}$ ) and kept at that temperature for 1 h. The pellet was air dried and re-suspended in 50 mM PBS. The suspension was centrifuged in a bench top centrifuge at maximum speed for 5 min to remove the insoluble fraction. The protein content of the supernatant was quantified with the Bradford reagent (Bio-Rad, Hercules, CA) using bovine gamma globulin as a standard.

### PAL activity assay

Phenylalanine ammonia-lyase (PAL; EC 4.3.1.5) activity was measured in the protein extract as previously described by Ke and Saltveit (1986) with slight modification by Campos-Vargas et al. (2004).

## Protein electrophoresis and immunoblot

Protein separation was performed by SDS-PAGE in a 10% (w/v) acrylamide gels as described by Laemmli (1970). The total soluble proteins (10 µg) were loaded per well. After electrophoresis, the proteins were blotted, hybridized, washed and signal detected as described in Nonogaki et al. (2000). Briefly, the proteins were electroblotted on polyvinylidene difluoride (PVDF) membranes (Immobilon-P; Millipore, Billerica, MA) using a semi-dry blotter (Bio-Rad). The membranes were blocked for 1 h at 25°C or overnight at 5°C with 5% (w/v) skim milk in 50 mM phosphate buffer pH 7.2 containing 0.3 M sodium chloride and 0.5% (w/v) Tween 20 (PBST). The blots were incubated for 1 h with anti-PAL serum in a 1:1000 dilution at 25°C. The membranes were washed three times for 10 min each with PBST. A horseradish peroxidase-conjugated antirabbit goat IgG (whole molecule) (Sigma, St. Louis, MO) was used as a secondary antibody at 1:1000 dilution at 25°C. After washing, the signal was detected chemiluminescently by using Renaissance reagents (DuPont NEN, Boston, MA) on X-ray film (Fuji Super RX; Tokyo, Japan).

## Expression of the fusion protein

The expression and purification of the protein in *Escherichia coli* was performed following the procedure described by Nonogaki et al. (2000) with a small modification. Two primers were designed to the coding region of *LsPAL1*. The forward primer (5'-CGGAATTCATGGAGAACGGTAAT-3') included an *EcoRI* site, whereas the reverse primer (5'-CGTCTAGACTAACA-TATTGGAAG-3') incorporated an *XbaI* site. The PAL open reading frame was amplified by PCR and cloned into the *EcoRI* and *XbaI* sites in a maltose-binding protein (MBP) vector (pMALc) (New England Biolabs, Minneapolis, MN) and used for bacterial transformation. The transformed bacteria were incubated overnight at 37°C, and the synthesis of the fusion protein was induced by 2 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG) for 2 h. An aliquot of the overnight culture was used to inoculate an incubation broth for 4 h at 37°C. The cells were harvested by centrifugation and re-suspended in sonication buffer (Nonogaki et al. 2000). After freezing overnight, the cells were thawed and sonicated for 5–10 min to release a higher amount of soluble fusion protein. The soluble protein was purified as described in Nonogaki et al. (2000) and separated by electrophoresis in a 10% acrylamide gel. The bands were stained with Coomassie Brilliant Blue (Fisher) for approximately 1 h, and de-stained to visualize the major bands.

## Antibody preparation

The band of MBP-PAL fusion protein was excised from the gel, divided in small pieces as described by Nonogaki et al. (1995), extruded through a 25-gauge needle and

stored at 4°C until immunization of the rabbits. The protein preparation was injected subcutaneously into New Zealand rabbits at UC Davis Animal Resources Antibody Service. The first injection of the protein was performed with Freund's complete adjuvant and the subsequent injections were applied with the incomplete adjuvant. The rabbit received six immunizations with the antigen varying the concentration between 0.6 and 0.2 mg ml<sup>-1</sup>. The first five immunizations were performed with Factor Xa (New England Biolabs) cleaved fusion protein (1 µg Factor Xa per 50 µg fusion protein, at room temperature for 24 h). The last immunization was performed with fusion protein without protease treatment. The first five immunizations were injected at intervals of about 2 weeks. The last immunization was delayed until 4 weeks, and the exsanguination was performed by cardiac puncture 2 weeks later. The collected serum was aseptically filtered through 0.22 µm low protein retention membranes and stored at -20°C until used.

## RNA gel blots

Total RNA was extracted from achlorophyllous mid-rib lettuce tissue of mature leaves. A phenol extraction method was performed as described by Sambrook et al. (1989). The electrophoresis of total RNA was performed in 1.3% (w/v) agarose gels containing 7% (v/v) formaldehyde. The RNA was transferred overnight to Hybond N<sup>+</sup> membrane (Amersham Pharmacia, Piscataway, NJ) and UV cross-linked. The riboprobe was synthesized from 382 bp of the *LsPAL1* cDNA that was cloned in to pBSIKS vector (Stratagene, La Jolla, CA) using digoxigenin (DIG)-labelled NTP (Boehringer Mannheim, Indianapolis, IN) as described by Nonogaki et al. (2000). The membranes were prehybridized for 30 min in a buffer containing 5× standard saline citrate (SSC, 150 mM NaCl, 15 mM sodium citrate), 50% (v/v) formamide, 4% (w/v) blocking reagent (Boehringer Mannheim), 0.2% (w/v) SDS, 0.1% (w/v) *N*-lauroylsarcosine. The hybridization and washing of the membranes and the detection of the signal chemiluminescently was performed as described by Nonogaki et al. (2000). The quantification of the signal in RNA gel blots was performed by integrating densitometer scans (IS-1000 Digital Imaging System; Alpha Innotech Corporation, San Leandro, CA); normalizing the data with the integration of the ethidium bromide-stained ribosomal RNA of the same sample. The relative value was calculated for each signal using the signal from the non-wounded control as 100.

## Immunoprecipitation and Ouchterlony double diffusion assay

The immunoprecipitation experiments were performed using the enzyme solution prepared as described below. Fifty grams of 1-cm lettuce mid-rib tissue was stored for 24 h at 10°C. The tissue was ground and centrifuged at 20 000 g. The supernatant was mixed with two volumes

of cold acetone ( $-20^{\circ}\text{C}$ ) and incubated for 1 h to precipitate soluble proteins. The mixture was centrifuged for 20 min at 15000 *g* at  $4^{\circ}\text{C}$ . The pellet was air-dried and dissolved in 5 ml 50 mM PBS, pH 7.2. A 150- $\mu\text{l}$  aliquot of the dissolved pellet was mixed with 5  $\mu\text{l}$  of a serial dilution of anti-PAL serum. The serum cocktail (soluble proteins) was incubated for 24 h at  $4^{\circ}\text{C}$  (modified from Walter 1989). The mixture was centrifuged at maximum speed for 10 min in a bench-top centrifuge at  $4^{\circ}\text{C}$ . The supernatant (65  $\mu\text{l}$ ) was mixed with cold 50 mM borate buffer pH 8.5 to complete a volume of 1 ml. The resultant 1 ml solution was used to measure PAL activity. The activity was expressed as nmol of cinnamic acid per millilitre produced in 1 h.

### Identification of specific phenolic compounds by GC-MS

Two 2-g portions of lettuce mid rib were stored for 24 h at  $10^{\circ}\text{C}$  before being extracted as described for the PAL assay. The supernatant was mixed [1:2 (v/v)] with  $-20^{\circ}\text{C}$  acetone for 1 h, and the precipitated proteins were pelleted by centrifugation at 5000 *g* for 20 min at  $4^{\circ}\text{C}$ . The pellet was air-dried and dissolved in 1 ml 50 mM PBS buffer pH 7.2. A 100- $\mu\text{l}$  aliquot of the dissolved pellet was mixed with 10  $\mu\text{l}$  of pre-immunization or anti-PAL serum and was incubated for 1 h at  $30^{\circ}\text{C}$ , and overnight at  $4^{\circ}\text{C}$  (modified from Walter 1989). The mixture was centrifuged at maximum speed for 10 min in a bench-top centrifuge at  $4^{\circ}\text{C}$ , and 40  $\mu\text{l}$  of the supernatant was mixed with cold 50 mM borate buffer pH 8.5 to make 1 ml for the PAL assay. After 1 h, the assay was stopped by adding saturated NaCl solution to produce a final NaCl concentration of 1 *M*, and a drop of 1 *N* HCl to reduce the pH to about 2. The mixture was spun in a bench-top centrifuge at maximum speed, and the supernatant was mixed with 10  $\mu\text{l}$  of  $0.1\ \mu\text{g}\ \mu\text{l}^{-1}$  of syringaldehyde (Sigma, St Louis, MO). Two millilitres of chloroform was added to the mixture and shaken for 2 min. The aqueous phase was removed and the chloroform phase evaporated under a flow of nitrogen to dryness. The dried material was dissolved in 100  $\mu\text{l}$  of acetone.

A 1.8- $\mu\text{l}$  portion of this acetone solution was analysed by GC-MS as describe by Fritz and Moore (1987) with modifications. The phenolic compounds were separated on a HP-5MS capillary column (30 m  $\times$  0.25 mm  $\times$  0.25  $\mu\text{m}$ ) on a Hewlett Packard (Pleasanton, CA) 5890 gas chromatograph coupled to an HP 5973 mass spectrometer operating in electron impact mode. The initial column oven temperature was set to  $100^{\circ}\text{C}$ , with  $5^{\circ}\text{C}\ \text{min}^{-1}$  increment to get a final temperature of  $250^{\circ}\text{C}$  with 5 min hold at the maximum temperature. Peak areas were determined using HP CHEM STATION software adapted for mass analysis. Spectra were recorded at 70 eV with the source at  $200^{\circ}\text{C}$ . Authentic *trans*-cinnamic acid and syringaldehyde (Sigma) were used as standard and internal standard, respectively. Spectra of each were verified using the NIST spectral

library. The linearity of the area to mass relationship was confirmed with standards over a range from 0.001 to 1  $\mu\text{g}$ .

### Statistical design and analysis

Each experiment had at least three replicates of each treatment and all experiments were run at least twice with similar results. Measurements from all the replicates were combined and treatment effects subjected to an ANOVA, and 5% LSD values calculated when significant treatment differences were detected.

### Results

PAL activity increased 8.4-fold in wounded mid-rib pieces of Romaine lettuce held at  $10^{\circ}\text{C}$  for 24 h, while after 48 h, the concentration of phenolic compounds (i.e. absorbance of methanol extract at 320 nm) had increased 6.5-fold (Fig. 1) and the concentration of soluble *o*-quinones (absorbance 437 nm) had increased 4.3-fold (data not shown). These wound-induced increases in phenolic content occurred concomitantly with tissue browning (decreasing negative  $a^*$  values) (data not shown). A 1.5- to 4-min heat shock treatment at  $45^{\circ}\text{C}$  applied within 5 min of wounding reduced the wound-induced increase in PAL activity and the accumulation of phenolic compounds (Fig. 1) and soluble *o*-quinones (data not shown). In comparison with non-heat-shocked tissue, the browning potential and concentration of *o*-quinones in wounded lettuce was reduced by an average of 23 and 63%, respectively, by the heat shock treatments. The 1.5-min heat-shocked tissue showed a greater reduction in soluble *o*-quinones than in browning potential, while 3 and 4 min of heat shock treatment maximized the inhibition of browning. Excised tissue exposed to  $45^{\circ}\text{C}$  for

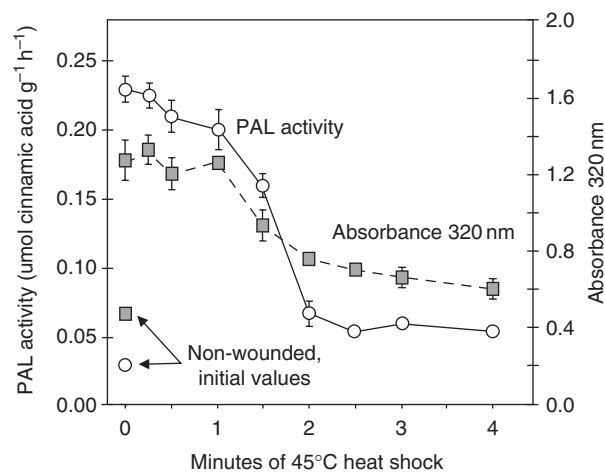


Fig. 1. Effect of heat shocks on PAL activity and phenolic content of wounded Romaine lettuce tissue. PAL activity was measured as the accumulation of cinnamic acid, whereas phenolic content was measured as the absorbance of a methanol extract at 320 nm. Vertical lines at means represent the standard error about that mean.

3–4 min were less crisp and more translucent than tissue exposed for shorter periods of times (data not shown). Because the tissue changes that occurred after the 3- and 4-min treatments were indicative of negative effect on tissue viability, the 3- and 4-min treatment were excluded, and the 2-min heat shock at 45°C was subsequently used as the standard heat shock treatment.

Wounding induced significant increases in PAL activity (Fig. 2A). After a 6-h lag, wound-induced PAL activity started to increase and reached a maximum at 24 h at 10°C, remained relatively constant for 12 h and then declined (Fig. 2A). The kinetics of induction was consistent with previous reports using Iceberg (crisp-head) lettuce (Ke and Saltveit 1989, Lopez-Galvez et al. 1996). PAL activity in non-wounded tissue remained consistently low throughout the 48 h of incubation. PAL activity in wounded lettuce leaves reached a

maximum at 24 h ( $0.27 \mu\text{mol g}^{-1} \text{h}^{-1}$ ) that was nine-fold higher than the average in non-wounded tissue ( $0.03 \mu\text{mol g}^{-1} \text{h}^{-1}$ ). The increase in PAL activity was delayed in wounded, heat-shocked lettuce, and the maximum PAL activity at 24 h was only 30% of that found in wounded tissue at the same time (Fig. 2A).

A wound-induced PAL (*LsPAL1*) was previously isolated from wounded lettuce leaves (Campos-Vargas et al. 2004). The riboprobe synthesized from the cDNA was used as a probe in a northern blot of total RNA extracted from wounded tissue (Fig. 2B). Transcript levels in wounded tissue increased 3.1- and 3.4-fold over non-wounded control after 12 and 24 h, respectively, before declining by 36 h. The maximum increase in transcript level at 24 h coincided with the maximum PAL activity (Fig. 2A). Interestingly, PAL activity was only 46% of the maximum at 12 h, whereas the level of PAL transcripts was 90% of the maximum. Between 24 and 36 h, PAL activity remained relatively constant (value at 36 h was 94% of maximum), while the level of PAL transcripts decreased 45%. By 48 h, PAL activity was still 67% of the maximum, while the level of PAL transcripts was 51% of the maximum. It appears that PAL activity lags transcript levels by about 12 h. Based on the accumulation of PAL mRNA and PAL activity in wounded tissues, we decided to use tissue incubated for 24 h to examine the effect of heat shock treatments on the accumulation of PAL mRNA in wounded tissue since the level of PAL transcripts was relatively constant 12 h before and PAL activity was relatively constant 12 h after this time.

The heat shock treatment had a significant effect on the accumulation of PAL mRNA in both non-wounded and wounded tissue (Fig. 3). Wounding increased transcript levels 3.4-fold over the non-wounded controls at 24 h (Fig. 3B). This increase in transcript level in wounded tissues is consistent with the nine-fold increase in PAL activity observed in the same tissues at 24 h (Fig. 2A). Densitometer scans showed that heat shock reduced transcripts by 37% in non-wounded tissue, and by 12% in wounded tissue. The minor reduction in the accumulation of PAL mRNA in wounded leaves caused by heat shock is in contrast to the large reduction in PAL activity in the same tissues (compare Figs 2A and 3A). This raises the question of how heat shock could significantly reduce the wound-induced increase in PAL activity without concomitantly reducing the increase in wound-induced PAL mRNA (Fig. 3B).

One possibility is that the reduction of PAL activity in heat-shocked tissue was due to changes in the level of PAL protein. To examine this possibility, we compared the contents of PAL protein in the wounded tissues before and after the heat shock treatment. For this purpose, we expressed *LsPAL1* protein in *E. coli* and raised the anti-PAL antibody using this protein as an antigen (see Material and Methods).

The specificity of the anti-PAL serum obtained was examined with immunoprecipitation experiments. The enzyme solution was prepared from lettuce tissue

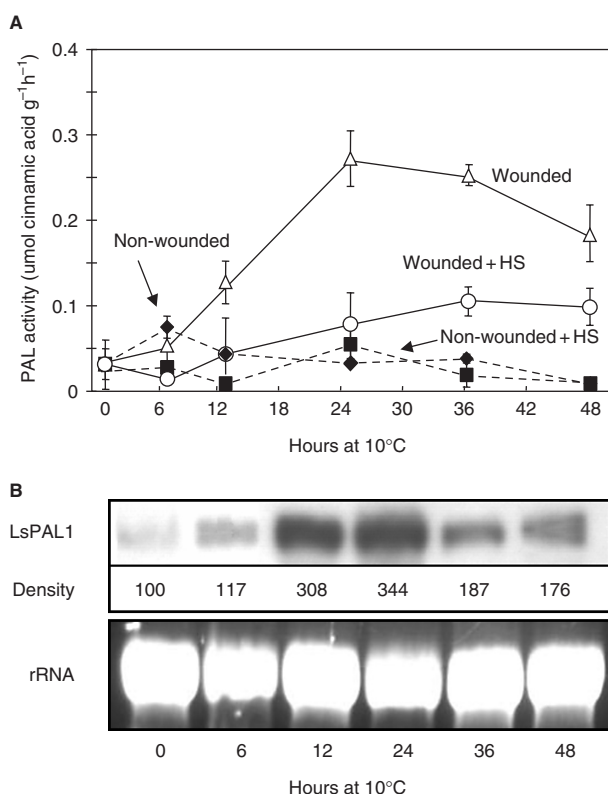


Fig. 2. Changes in PAL activity and *LsPAL1* gene expression in wounded and/or heat-shocked Romaine lettuce tissue. (A) Non-wounded tissue were intact leaves carefully excised from the head, while wounded tissue were 1 cm × 2 cm pieces excised from the mid-rib. Both were immersed in 20 or 45°C (HS) water for 2 min before being stored at 10°C for 48 h. PAL activity was assayed in extracts from tissue excised from the same region of the leaf. Vertical lines at means represent the standard error. (B) Northern blot of total RNA (4 μg) extracted from wounded tissue shown in panel A, and probed with *LsPAL1* riboprobe. An ethidium bromide staining ribosomal RNA is shown for comparison. Quantification of the signal in *LsPAL1* mRNA gel blots was normalized by the signals from ethidium bromide-stained ribosomal RNA. The relative value was calculated for each signal using the signal from the non-wounded control as 100.

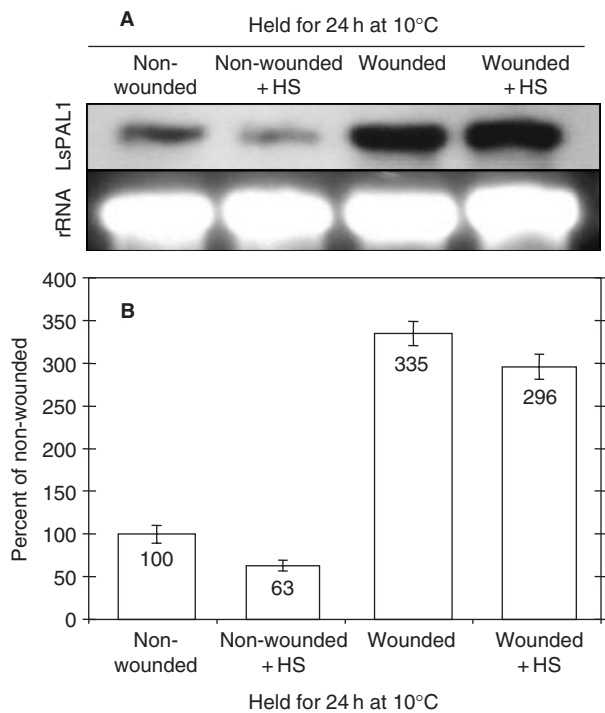


Fig. 3. RNA gel blot of total RNA (3  $\mu\text{g}$ ) from mid-rib segments of lettuce tissue stored at  $10^\circ\text{C}$  for 12 h (A), and the relative amount of hybridized signal in RNA gel blot analysed by densitometer scanning analysis (B). The treatments are non-wounded, non-wounded plus heat shock (HS), wounded, and wounded plus heat shock (HS). The blots were hybridized with *LsPAL1* riboprobe. An ethidium bromide staining ribosomal RNA is shown in panel A. The relative amount of the *LsPAL1* signal obtained by densitometer scanning analysis was normalized by the signals from ethidium bromide-stained ribosomal RNA. The relative value was calculated for each signal using the signal from the non-wounded control as 100.

wounded and stored at  $10^\circ\text{C}$  for 24 h. PAL activity in the enzyme solution was  $11.2 \text{ nmol ml}^{-1} \text{ h}^{-1}$  without the addition of serum, and was progressively reduced in the presence of increasing levels of antiserum; reaching zero activity with undiluted serum (Fig. 4).

The immunoprecipitation experiments were repeated, and the product of PAL activity, *trans*-cinnamic acid was quantified by GC-MS. This was done to confirm the substrate and product specificity of the reaction. Analogously to the spectrophotometric assay described above, re-dissolved acetone-precipitated proteins of wounded lettuce was incubated with pre-immunization and anti-PAL serum. The concentration of *trans*-cinnamic acid in wounded lettuce incubated with pre-immune serum was  $37 (\pm 1) \text{ nmol g}^{-1} \text{ h}^{-1}$  whereas it was  $8 (\pm 2) \text{ nmol g}^{-1} \text{ h}^{-1}$  when the anti-PAL serum was added. Incubation with anti-PAL serum reduced the production of *trans*-cinnamic acid by 77% compared with incubation with pre-immune serum. This indicated that PAL was recognized by the anti-PAL serum.

Immunoblots of total soluble protein extracted from wounded tissue were done using the anti-PAL antibody

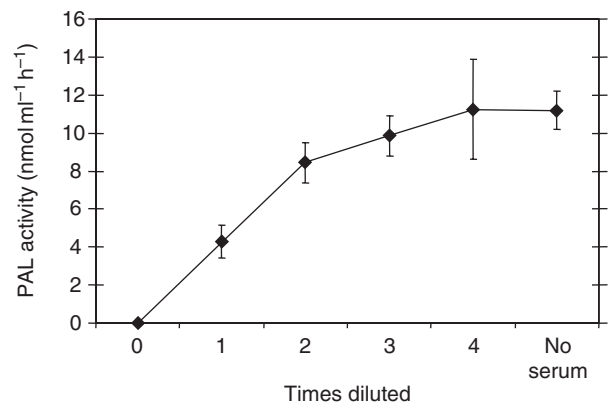


Fig. 4. Immunoprecipitation assay of PAL activity. (A) The extract from wounded lettuce tissues stored for 24 h at  $10^\circ\text{C}$  was concentrated by acetone precipitation and used as an enzyme solution for immunoprecipitation. The enzyme solution was incubated for 24 h at  $5^\circ\text{C}$  with serial dilution of anti PAL serum. After precipitating IgG by adding protein A and centrifugation, the PAL activity in the supernatant was measured as accumulation of cinnamic acid. Vertical lines at means represent the standard error. The control is the enzyme solution without the addition of serum.

(Fig. 5). The same time points were used for protein gel blot experiments as were used for the activity assay (Fig. 2). The immunoblot showed major bands with the molecular masses of about 80 kDa. This coincided with the predicted size of the PAL protein deduced from the *LsPAL1* cDNA sequence (approximately 77 kDa) (Campos-Vargas et al. 2004). In addition to the two major bands with the predicted molecular mass, we observed two minor cross-reactive bands (one with a larger and another with a smaller molecular mass compared to the major bands) (Fig. 5). These minor bands might be polypeptides of a PAL-related enzyme such as phenylalanine amino-transferase (Cunha 1988).

In the immunoprecipitation assay, PAL activity was completely precipitated to undetectable level by the anti-PAL antibody (Fig. 4). This suggests that the antibody cross-reacts with all the active PAL isoforms in the

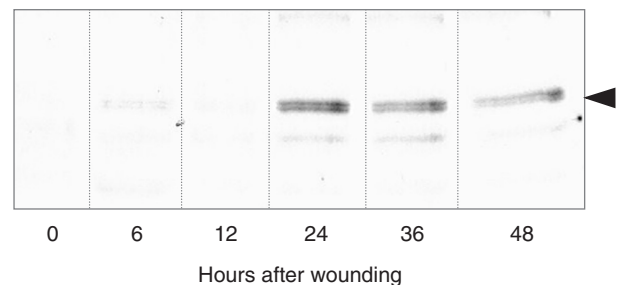


Fig. 5. Immunoblot of the protein extracts from wounded lettuce mid-rib stored at  $10^\circ\text{C}$  for 48 h. The proteins were extracted and concentrated by acetone precipitation. An equal amount of proteins (10  $\mu\text{g}$ ) were loaded per well. The blots were incubated with anti-PAL serum (see Materials and methods). The detection of signal was done using chemiluminescent substrates. The arrow to the right of the panel indicates the PAL polypeptide at a molecular mass of approximately 80 kDa.

extract. The two major polypeptides detected in the immunoblot probably represent most of the PAL activity in the crude extracts of wounded lettuce tissue. Changes in the intensity of each band were very similar and corresponded to the changes in PAL activity in lettuce tissue during incubation after wounding. These two immunoreactive bands were not evident during the first 12 h after wounding (Fig. 5), but were detectable 24 or 36 h after wounding when maximum PAL activity was observed (Fig. 2). The signal then declined 48 h after wounding (Fig. 5). Changes in the content of PAL polypeptides detected in the immunoblot assay were consistent with changes in PAL activity (Figs 2A and 5).

The effect of heat shock treatment on the contents of PAL protein was also analysed (Fig. 6). No signal was detected in immunoblots for non-wounded control tissue with or without heat shock. In contrast, wounding induced an eight-fold accumulation of PAL protein, while the accumulation was only 40% higher in wounded and heat-shocked tissue. The heat shock treatment of wounded tissue inhibited the wound-induced accumulation of PAL protein by 60%; to levels similar to the non-wounded controls. It appears that the heat shock treatment did not interfere with the wound-induced increase in PAL mRNA, but did prevent the synthesis and accumulation of wound-induced PAL proteins.

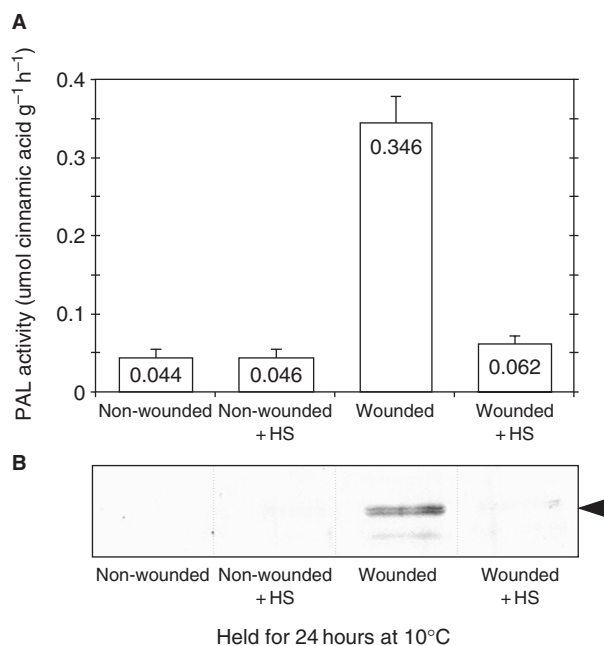


Fig. 6. PAL enzyme activity and PAL protein extracted from wounded and heat-shocked lettuce tissue. (A) Non-wounded tissue was intact leaves stored at 10°C for 48 h, wounded tissue was excised mid-rib tissue stored at 10°C for 48 h, both tissues were heat-shocked (HS) by immersion in 45°C water for 2 min (B) Immunoblot of the protein extracts from the tissues after 24 h at 10°C and probed with anti-PAL antibody. The arrow to the right of the panel indicates the PAL polypeptide at a molecular mass of approximately 80 kDa. Vertical line at top of each bar represents the standard error.

## Discussion

Heat shock responses are induced when tissues are exposed to temperatures 10–15°C above their normal growing temperature, or to an increase of 5°C when grown within a narrow range of temperatures (Lindquist 1986). Lettuce is a cool-season vegetable that grows best when the day/night temperature is 20/10°C (Rubatzky and Yamaguchi 1996). It follows that the 45°C heat shock treatment used in our experiments was sufficient to induce a heat shock response, and heat shock proteins have been detected in lettuce leaf tissue exposed to 45°C for 2 min (Kang and Saltveit 2003).

A few minutes exposure to 45°C modified the physiological response (e.g. phenylpropanoid metabolism) of lettuce tissue to wounding (Loaiza-Velarde et al. 1997, Loaiza-Velarde and Saltveit 2001, Kang and Saltveit 2003). Heat shock also modified changes in phenylpropanoid metabolism triggered by UV and fungal elicitors in parsley cell cultures (Walter 1989). Besides modifying stress-induced responses of secondary metabolism, heat shock treatments also induced the acquisition of tolerance to lethal high temperature, to chilling temperatures, and to high salt conditions (Lin et al. 1984, Lafuente et al. 1991, Saltveit 1991, 1997, 2001, Kuznetsov et al. 1993, Sabehat et al. 1998). It appears that exposure to one stress (e.g. heat shock) can increase the plant's tolerance to another stress; namely cross-tolerance. However, some cellular activities are not modified by exposure to prior stresses (Lancilotti et al. 1996).

There are a number of mechanisms by which heat shock could reduce wound-induced PAL activity. Heat shock could reduce accumulation of PAL mRNA (Belanger et al. 1986, Lancilotti et al. 1996), it could induce qualitative changes in ribosomes (i.e. phosphorylation of ribosome subunit proteins, Scharf and Nover 1982) or other organelles (i.e. endoplasmic reticulum, Belanger et al. 1986, Lancilotti et al. 1996), or it could cause the sequestering of mRNA in 'heat shock granules' (Nover et al. 1983, 1989). These modifications would reduce the capacity for protein synthesis or post-translational changes, or alter rates of protein turnover (Ferguson et al. 1994).

Heat shock induces degradation of mRNAs in yeast cells (Lindquist 1981) and the aleurone cells of cereal seeds (Belanger et al. 1986), but that was not the case with PAL mRNA in wounded lettuce leaves (Fig. 3A). The accumulation of PAL mRNA did not differ significantly between wounded heat-shocked and non-heat-shocked tissue (Fig. 3A), even though there were significant differences in PAL enzyme activity between tissue subjected to these two treatments (Fig. 2A). PAL activity is naturally low in the non-stressed control tissue used in our experiments, and only increased significantly after wounding. As the heat shock treatment was applied immediately after cutting when levels of PAL activity were low, the PAL synthesized in response to wounding was never exposed to temperatures above the incubation temperature of 10°C. The ability of heat shock



treatments to degrade mRNA (Lindquist 1981, Belanger et al. 1986) is therefore not relevant to this study of the effect of heat shock on the induction and accumulation of PAL mRNA that occurred hours after application of the heat shock treatment.

Difference in PAL activity induced by heat shock may have resulted from differences in the translation of wound-induced mRNA. Apuya and Zimmerman (1992) studied the effect of heat shock on developing carrots embryos. They found that the abundance of EF-1 $\alpha$  (translation elongation factor 1 $\alpha$ ) mRNA did not change when compared with non-heat-shocked plantlet and globular stages when gel blot analyses were done with total RNA. However, differences were apparent when polysomal RNA was examined. Heat shock appeared to affect the translation of mRNAs in treated cells. In wounded carrot root tissue, heat shock caused destabilization of secretory protein mRNA (Brodli and Ho 1992). The half-life of extensin mRNA was reduced from 4 to 0.5 h by the heat shock treatment, while a similar treatment reduced the stability of  $\alpha$ -amylase mRNA in barley aleurone layers (Lanciloti et al. 1996).

Heat shock treatments could induce mRNA modifications that would reduce translation. Storti et al. (1980) observed that the leaders of hsp mRNA are adenine-rich, thus making it easier for ribosomes to translate hsp mRNAs. Upon recovery from heat shock, this translation bias was lost and translationally sequestered normal mRNAs were reactivated. Incorporation of methionine into protein in corn seedlings was significantly reduced by heat shock treatments (Heikkila et al. 1984). Heat shock treatments that were effective in reducing PAL activity in wounded lettuce were also treatments that induced the synthesis of heat shock proteins (Kang and Saltveit 2003).

Alternatively, reduced accumulation of PAL protein in wounded, heat-shocked tissue could be explained by increased turnover of the PAL protein. Even if the capacity for translation of PAL mRNA was the same in wounded and wounded plus heat-shocked lettuce tissue, heat shock may have accelerated the turnover of PAL proteins so that heat-shocked tissues did not accumulate the protein and therefore had relatively low enzyme activity. Heat shock increases ubiquitin mRNA levels (Brodli and Ho 1992) and ubiquitin plays a major role in protein turnover (Callis and Vierstra 2000). However, PAL protein (Fig. 5) and PAL activity (Fig. 2) remained near their maximum values at 36 h, by which time the level of PAL mRNA (Fig. 2) had significantly declined.

Reduction in wound-induced PAL activity by heat shock treatments demonstrates the intricate changes and metabolic reorientation that occurs when an organism is exposed to sequential stresses. Hahlbrock et al. (1981) studied the effects of a fungal elicitor from *Phytophthora megasperma* var. *sojae* and white light on the enzymes of phenylpropanoid metabolism in parsley-cultured cells. They observed that the activ-

ities of PAL, acetyl-CoA carboxylase and chalcone synthase (CHS) in parsley cells exposed to both of the inducers (light and elicitors) simultaneously, were modified in a different fashion than when the inducers were assayed separately. Walter (1989) studied the modification in PAL and CHS in parsley cells by combinations of stresses including heat shock treatments, fungal elicitor (*Phytophthora megasperma* f. sp. *glycinea*) and UV light. He concluded that there might be a 'stress hierarchy', in which heat shock is the most important stress. Vayda and Schaeffer (1988) described a reduction in putative PAL protein synthesis when wounded potato tubers were exposed to hypoxia stress. Rumeau et al. (1990) reported the effect of wounding, pathogen and hypoxia on extensin and PAL gene in potato tubers. Hypoxia appears to be dominant over wounding since, wound-induced gene expression was reduced when both stresses were combined. Saltveit (1997) reviewed other examples of reduction of wound-induced changes by an additional stress, proposing a hierarchical response structure and suggesting the possibility of using heat shock to reduce wound-induced tissue browning. Saltveit (2000) speculated that the reduction in the synthesis of wound-induced proteins, and not the synthesis of heat-shock-like proteins was important in understanding the changes that occur in phenylpropanoid metabolism after heat shock treatments.

Heat shocks to Romaine lettuce leaves delayed and decreased the wound-induced increase in PAL activity. Romaine lettuce has a higher endogenous level of phenylpropanoid activity and phenolic content than iceberg lettuce (Tomás-Barberán et al. 1997). In iceberg lettuce, a similar heat shock treatment prevented any increase in wound-induced PAL activity (Loaiza-Velarde and Saltveit 2001). Heat shock-phenolics interaction seems to have a high degree of complexity. Loaiza-Velarde and Saltveit (2001) exposed iceberg lettuce tissue to combinations of heat shock and cyclohexamide (CHX). Heat shock or CHX treatments reduced wound-induced PAL activity and the development of tissue browning, but the heat shock treatment combined with CHX produced similar reductions in phenylpropanoid activity. These results suggested that both treatments may have similar modes of action, and that heat shock was triggering more than just the synthesis and accumulation of heat shock proteins.

The data presented in this paper supports the hypothesis that heat shock inhibits the increase in wound-induced PAL activity by inhibiting the accumulation of PAL proteins (and thereby increased enzyme activity) either by preventing the translation or accelerating the turnover of PAL proteins. It is clear that heat shock treatments cause profound and interconnected changes in the 'normal' physiology of plants in order to produce increased tolerance to other stress. There also appears to be a hierarchy in the tissue's response to stresses, such that the response to some stresses (e.g. heat shock) takes

precedence over the response to other stresses (e.g. wounding). Additional research is needed to differentiate among the ways by which heat shock alters the synthesis of various induced proteins.

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