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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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Wounding lettuce leaves induces the de novo synthesis of phenylalanine ammonia-lyase (PAL, EC 4.3.1.5), the accumulation of phenolic compounds, and subsequent tissue browning. A brief heat-shock at 45°C reduces the rise in wound-induced PAL, the accumulation of phenolic compounds, and tissue browning. The activity of PAL measured 24 h after wounding and the content of phenolic compounds (absorbance of methanol extract at 320 nm) measured 48 h after wounding was highly correlated ($R^2 > 0.90$) in tissue developing the normal wound response and in tissue subjected to 0–180 s of heat-shock after wounding. The synthesis of a unique set of proteins called heat-shock proteins (hsps) is induced by these heat-shock treatments. Western-blot analyses of proteins isolated from wounded and heat-shocked Iceberg and Romaine lettuce mid-rib leaf tissue was done using antibodies against hsp 23. Only those heat-shock treatments that were effective at inducing the synthesis of hsp 23 were effective in reducing the activity of PAL induced by wounding and the subsequent accumulation of phenolic compounds. Hsps induced in non-wounded, whole leaves by exposure to 45°C for 150 s did not significantly interact with PAL previously synthesized in non-heat-shocked wounded leaves to limit its activity. The preferential synthesis of hsps over that of wound-induced PAL, rather than the presence of hsps, may be responsible for the ability of a heat-shock treatment to reduce the wound-induced increase in PAL activity. Our results support this novel concept, and the possibility that heat-shock treatments can have significant physiological effects on the response of the tissue to other stresses, not because of the specific genes they induce or repress, or the products they cause to be synthesized, but by their secondary action of influencing the synthesis of other proteins (e.g. PAL) by the suppression of non-hsps protein synthesis.

Introduction

Wounding lettuce leaves induces the synthesis of specific enzymes and the accumulation of specific phenolic compounds associated with tissue browning (Ke and Saltveit 1989, Brecht 1995, Tomás-Barberán and Espin 2001). Non-stressed Iceberg and Romaine lettuce leaves contain low levels of phenolic compounds (Tomás-Barberán et al. 1997). When wounded, phenylalanine ammonia-lyase (PAL, EC 4.3.1.5), the first committed step in the synthesis of phenylpropanoid compounds (Hahlbrock and Scheel 1989) is synthesized de novo, phenolic compounds (e.g. chlorogenic and caffeoltartartic acids) are synthesized and accumulate, and tissue browning occurs (Tomás-Barberán et al. 1997). A brief heat-shock (90 s at 45°C) disrupts the wound-induced increase in PAL activity, and delays and diminishes the accumulation of phenolic compounds and tissue browning (Loaiza-Velarde et al. 1997). The heat-shock treatment is most effective if administered immediately before or after wounding, and the effect diminishes as the time of application after wounding increases (Loaiza-Velarde and Saltveit 2001).

Heat-shocks induce the synthesis of a unique set of proteins called heat-shock proteins (hsps) (Lindquist 1986, Vierling 1991). The presence of hsps in induced cells is thought to make them more resistant to the deleterious effects of subsequent stresses. The accumulation and persistence of hsps has been correlated with increased chilling tolerance in chilling sensitive tissue (Lafuente et al. 1991, Whitaker 1994, Collins et al. 1995, Sabehat et al. 1996). The induced synthesis of hsps is usually accompanied by a reduction or cessation in the synthesis of other proteins (Apuya and Zimmerman 1992, Brostrom and Brostrom 1998). Therefore, the ability of heat-shock treatments to reduce wound-induced browning may not be the result of

the accumulated hsps, but the result of the decreased synthesis of wound-induced PAL brought on by the preferential synthesis of hsps over that of wound-induced proteins (Saltveit 1997, 2000). This possibility is supported by the report that heat-shock treatments were equally effective in suppressing the development of chilling injury symptoms when administered either before or after chilling (Saltveit 2001).

Research reported in this paper examines the relationship between heat-shock-induced production of hsps and the repressed activity of PAL in wounded lettuce leaves. We show that those heat-shock treatments that are effective in inducing the synthesis of hsps are effective in reducing the increase in wound-induced PAL activity and tissue browning.

Materials and methods

Plant material

Heads of Iceberg and Romaine lettuce (*Lactuca sativa* L.) were obtained from commercial sources, transported to the laboratory and held at 0.5°C until used within a few days. Fully expanded leaves were chosen from the middle of the head; eliminating the damaged outer leaves and immature inner leaves. Pieces (approximately 2×2 cm) were cut from the achlorophyllous mid-rib with a stainless steel razor blade and stored at 10°C for 24 h before analysis of PAL activity and hsp synthesis, and for 48 h before analysis of phenolic content.

Heat shock (HS) treatments

Pieces of mid-rib tissue were immersed in a water bath set at 45°C (Fisher Scientific 9001, Sacramento, CA, USA) for 0 (control), 30, 60, 90, 120, 150, and 180 s. Tissue pieces were then submerged in water (20°C) for 1 min and subsequently blotted with paper towels to remove excessive surface water. Around 4 g of tissue were then immediately placed in 15×100 mm diameter plastic Petri dishes, which were placed in plastic containers lined with moist paper towels to avoid tissue dehydration, and stored in the dark at 10°C for 24 or 48 h.

Assay for PAL activity

Phenylalanine ammonia-lyase activity was measured as previously described by Ke and Saltveit (1989).

Assay for phenolic compounds

The concentration of phenolic compounds was measured as described by Ke and Saltveit (1989) and modified by Campos-Vargas and Saltveit (2002). Briefly, the absorbance of a clarified aliquot of a methanol extract (2 ml methanol per g FW) was read at 320 nm (Loaiza-Velarde et al. 1997).

Protein extraction

Soluble proteins were extracted from lettuce mid-rib tissue that had been held for 24 h at 10°C after treatment (e.g. control, heat shock, wounding, or heat shock and wounding). Samples were homogenized in sample buffer (100 m*M* KCl, 5 m*M* KH_2PO_4, 2 m*M* EDTA, 1 m*M* $MgCl_2$, 40 m*M* HEPES at pH 6.8). The supernatant was mixed 1:2 (v/v) with cold acetone (−20°C), kept at that temperature for 1 h, and then centrifuged at 10 000 *g* for 5 min at 4°C. The pellet was air dried and re-suspended in sample buffer. The suspension was centrifuged at 10 000 *g* for 5 min at 4°C to pellet insoluble material. Protein content was determined using BSA as a standard, according to the method of Bradford (1976).

Immunoblot analyses

Proteins were concentrated using acetone at −20°C. The concentrated proteins were solubilized by the addition of an equal volume of 2× Laemmli buffer (9% (w/w) SDS, 6% (v/v) s-mercaptoethanol, 10% (v/v) glycerol, and a trace amount of Bromophenol Blue dye in 0.196 *M* Tris/HCl at pH 6.7). Samples containing 10 µg protein were electrophoresed through 10% SDS-PAGE and then transferred to Immobilon-P membranes (Millipore Corp., Bedford, MA, USA) using a wet transfer system (Bio-Rad, Richmond, CA, USA). Prestained SDS-page protein standards (5 µg; Bio-Rad, Hercules, CA, USA) were run in each gel. Following blocking in 5% skim milk dissolved in 20 m*M* Tris HCl, pH 7.6, 137 m*M* NaCl and 0.1% Tween-20 for 2 h, the blots were incubated with primary polyclonal antibodies raised against 23 kDa heat shock protein at 1:10 000 dilution for 1 h. The small heat shock protein (hsp 23) antibody was a gift from Dr Choo Bong Hong at the Institute for Molecular Biology and Genetics, Seoul National University.

Immunodetection was performed using horseradish peroxidase-conjugated antirabbit IgG (Sigma, St Louis, MO, USA) as secondary antibodies for 30 min. All incubations were done at room temperature (approximately 22°C) unless otherwise mentioned. After 3 washes of 5 min each, blots were treated with Enhanced Chemi-Luminescence reagents (NEN life science products, Inc. Boston, MA, USA) and the heat shock protein were detected by autoradiography for 5 min with Kodak X-Omat film (Eastman Kodak, Rochester, NY, USA). Quantification of the Western blot was done by measuring the spot integrated density value of each band, corrected for film background, using a IS-1000 digital imaging system (Alpha Innotech Corporation, San Leandro, CA, USA). The results are shown as means ± SE of four different blots per group.

Statistics

Each experiment was repeated at least twice with similar results. All treatments were replicated at least six times within each experiment. Means and standard errors were calculated from pooled data. When presented in the figure, the vertical line associated with each point or atop each bar represents the standard error.

Results

The wound that resulted from excision of mid-rib leaf tissue induced a 5.6- and a 11.7-fold increase in PAL

activity after 24 h at 10°C in Iceberg and Romaine lettuce, respectively (Fig. 1A,B). After 48 h, the level of extractable phenolic compounds (absorbance at 320 nm g^{-1}) had increased 2.5- and 3.0-fold for Iceberg and Romaine lettuce, respectively. These levels of induction are similar to those previously reported for these two types of lettuce (Loaiza-Velarde et al. 1997, Tomás-Barberán et al. 1997, Campos-Vargas and Saltveit 2002).

Exposing wounded lettuce mid-rib tissue to a 45°C heat-shock caused a progressive decline in wound-induced PAL activity and extractable phenolic compounds for both types of lettuce as the duration of treatment increased from 0 to 180 s (Fig. 1A,B). For this range of treatment times, PAL activity is given for Iceberg lettuce by the equation 0.298 – (s of heat-shock × 0.00143), with an R^2 of 0.96, while for Romaine lettuce it is given by the equation 0.342 – (s of heat-shock × 0.00189), with an R^2 of 0.98.

There was a high degree of correlation between the level of PAL activity measured 24 h after wounding and the level of extractable phenolic compounds (absorbance at 320 nm g^{-1}) measured 48 h after wounding for excised tissues receiving the heat-shock treatments (Fig. 2A,B). For Iceberg lettuce, the level of wound-induced PAL activity is given by the equation (absorbance at $320g^{-1}$ × 0.294) – 0.073; with an R^2 of 0.93, while for Romaine lettuce it is given by the equation (absorbance at 320 g^{-1} × 0.337) – 0.149; with an R^2 of 0.98. A similar correlation was found between PAL activity in wounded Romaine mid-rib leaf tissue and the level of phenolic compounds (absorbance at 320 nm g^{-1}) extracted 12 h later (Campos-Vargas and Saltveit 2002). The level of PAL activity, whether altered because of natural wound-induced synthesis or because of heat-shock suppression of wound-induced synthesis, is highly correlated with the subsequent accumulation of phenolic compounds.

Heat-shocking excised mid-rib tissue from both types of lettuce at 45°C induced a large increase in the synthesis of hsp 23 measured 24 h after treatment (Fig. 3A,B). Low molecular weight hsps (i.e. hsp 23, hsp 25) have been associated with the protective effects of similar heat-shock treatments (Lafuente et al. 1991, Sabehat et al. 1996). The density of the Western blots for hsp 23 reached a maximum at 150 s of treatment and then declined as the duration of the treatment increased. The only difference between the induction of hsp 23 in Iceberg and Romaine lettuce was the significant increase in Iceberg from the 15 s treatment that was lacking in Romaine lettuce.

There was a high degree of correlation between the density of the Western blots for heat-shock-induced hsp 23 and the level of wound-induced PAL activity for the 0–180 s treatments (Fig. 4A,B). In both types of lettuce the level of wound-induced PAL activity was depressed by the same treatment that increased the synthesis of heat-shock-induced hsp 23. For Iceberg lettuce, the level of wound-induced PAL activity is given by the equation 0.324 – (0.0292 × density); with an R^2 of 0.87, while for Romaine lettuce it is given by the equation 0.350 – (0.0296 × density); with an R^2 of 0.91. It is apparent that as the synthesis of hsp 23 increased due

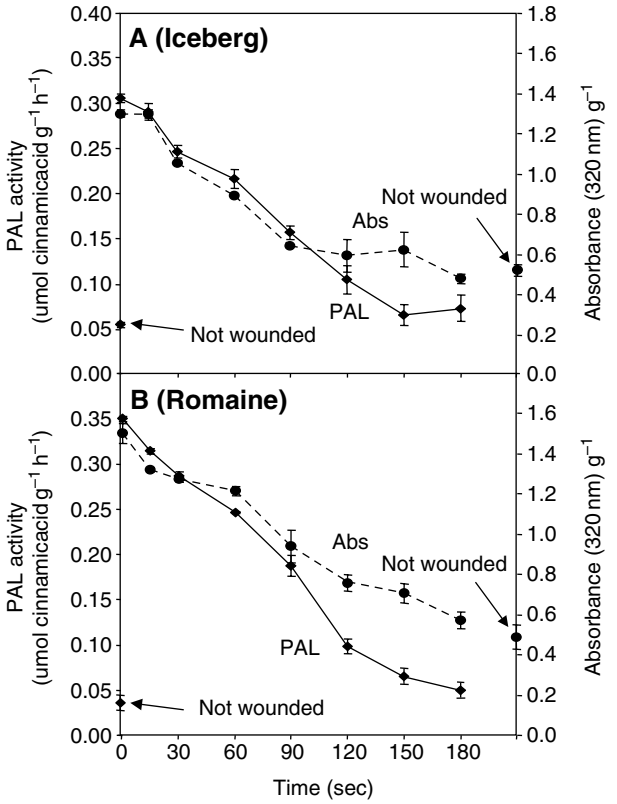

Fig. 1. Effect of heat-shock treatments on the activity of phenylalanine ammonia-lyase (PAL) and the concentration of phenolic compounds (abs; absorbance at 320 nm) in (A) Iceberg and (B) Romaine lettuce. Mid-rib tissue was excised and subjected to a 45°C heat shock for 0–180 s. PAL and absorbance were measured after holding at 10°C for 24 and 48 h, respectively. Non-wounded tissue was assayed immediately after excision. Vertical bars represent the standard error about that mean (n = 4).

to greater induction by lengthening the duration of the 45°C heat-shock treatment, there was a concomitant decrease in the subsequent synthesis of wound-induced PAL.

Reduced PAL activity brought on by the heat-shock treatment could have resulted from decreased synthesis or inactivation of wound-induced PAL, or from a direct effect of the newly synthesized hsps on the activity of wound-induced PAL. To test the last hypothesis, PAL was assayed in Iceberg and Romaine mid-rib tissue 24 h after wounding, after heat-shock, after wounding and heat-shock, and in a mixture of equal parts heat-shocked and wounded tissue (Fig. 5). PAL activity increased 6.0- and 9.7-fold as the result of wounding in Iceberg and Romaine mid-rib tissue, respectively. A 150-s 45°C heat-shock treatment reduced the increase in wound-induced PAL activity by 75% for Iceberg and 85% for Romaine; to levels close to those of the non-wounded tissue. Heat-shocking non-wounded whole leaves of Iceberg and Romaine increased PAL activity by 33% and 50%, respectively. This was a significant, but relatively small increase, compared to that induced by wounding. The activity of PAL in an assay using equal

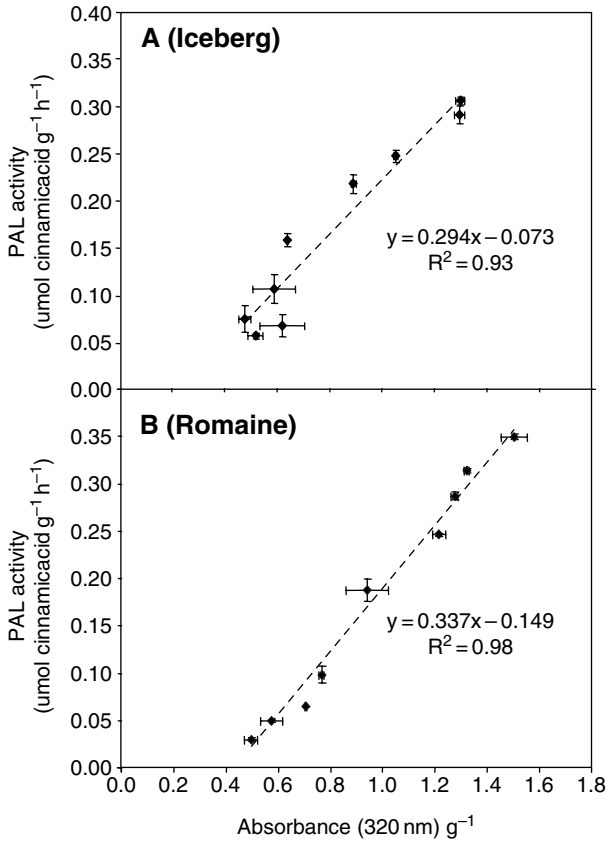

Fig. 2. Relationship between the activity of phenylalanine ammonia-lyase (PAL) measured 24 h after excision and the concentration of phenolic compounds (abs; absorbance at 320 nm) measured 48 h after excision in (A) Iceberg and (B) Romaine lettuce mid-rib tissue. The tissue was held at 10°C after being subjected to the various heat-shock treatment indicated in Fig. 1. Horizontal and vertical bars represent the standard error about that mean (n = 4).

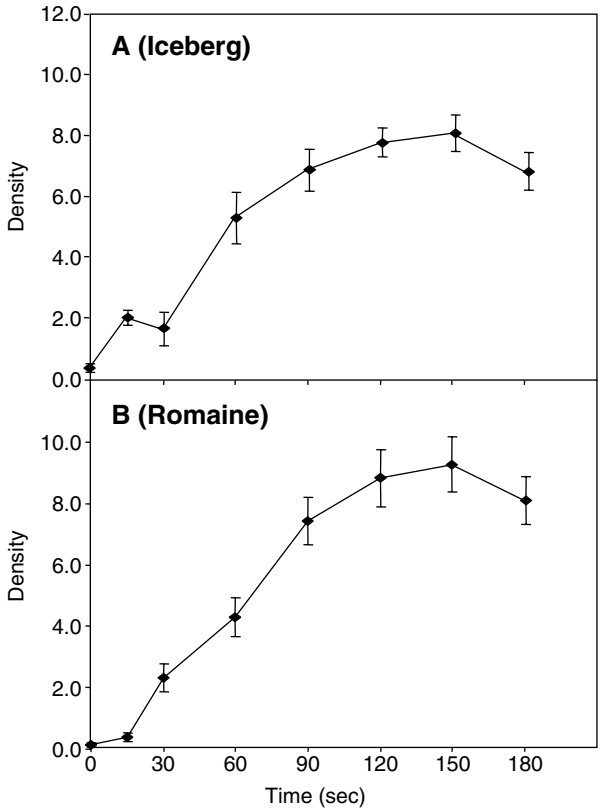

Fig. 3. Western-blot analyses of soluble proteins isolated from wounded and heat-shocked Iceberg and Romaine lettuce mid-rib leaf tissue with antibodies against hsp 23. Proteins were extracted from tissue held for 24 h at 10°C after treatment. Density of the blots for protein isolated from excised (A) Iceberg and (B) Romaine lettuce tissue that was heat shocked at 45°C for 0–180 s. Vertical bars represent the standard error about that mean (n = 4).

weights of non-heat-shocked wounded tissue and non-wounded heat-shocked tissue was the arithmetic average of its activity in the two tissues; $(0.11 + 0.49)/2 = 0.30$ μmol cinnamic acid g^{-1} h^{-1} compared with 0.30 ± 0.01 μmol acid g^{-1} h^{-1} for Iceberg lettuce, and $(0.08 + 0.50)/2 = 0.29$ μmol acid g^{-1} h^{-1} compared with 0.29 ± 0.01 μmol acid g^{-1} h^{-1} for Romaine lettuce.

Discussion

Wounding induces the de novo synthesis of PAL that is the first enzyme in phenylpropanoid metabolism (Hahlbrock and Sheel 1989). In wounded lettuce, or lettuce exposed to hormonal levels of ethylene (e.g. 1 μl l^{-1}; Abeles et al. 1992), this induced pathway produces phenolic compounds that accumulate and cause tissue browning (Tomás-Barberán et al. 1997). The concentration of phenolic compounds is initially low in non-stressed lettuce tissue. The activity of PAL, synthesized in response to wounding, results in the accumulation of phenolic compounds that contribute to subsequent tissue browning. Heat-shock treatments that reduce tissue browning by reducing the synthesis of wound-induced PAL, cannot act by heat inactivating PAL, since the heat treatments are applied hours before there is an increase in the synthesis and activity of wound-induced PAL (Loaiza-Velarde and Saltveit 2001).

The synthesis and accumulation of hsps appear necessary for the effect they have on protecting induced cells from subsequent heat stress (Lindquist 1986, Vierling 1991). Induced cells preferentially synthesize hsps, with a general inhibition of the synthesis of other proteins (Zhang et al. 1984, Ougham and Stoddart 1986, Somers et al. 1989, Ferguson et al. 1994, Brostrom and Brostrom 1998). There may be systems in which the preferential synthesis of hsps over that of other proteins is the basis of the protective effect, rather than accumulation of the synthesized hsps themselves. Two such systems may be heat-shock induced chilling tolerance (Lafuente et al. 1991), and heat-shock induced reductions in wound-induced browning in lettuce described in this paper.

The preferential synthesis of hsps, not their accumulation and presence, may contribute to the ability of heat-shock treatments to increase the chilling tolerance of sensitive tissue. Initial evidence seems to show a

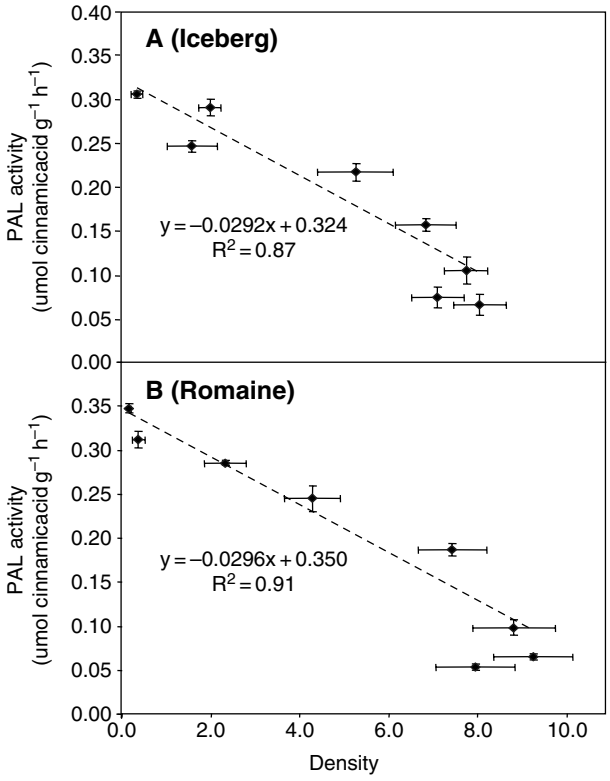

Fig. 4. Relationship between the activity of phenylalanine ammonia-lyase (PAL) measured 24 h after excision and the density of Western-blot analyses of proteins isolated from wounded and heat-shocked (A) Iceberg and (B) Romaine lettuce mid-rib tissue. The tissue was held at 10°C after being subjected to the various heat-shock treatments indicated in Fig. 1. Horizontal and vertical bars represent the standard error about that mean (n = 4).

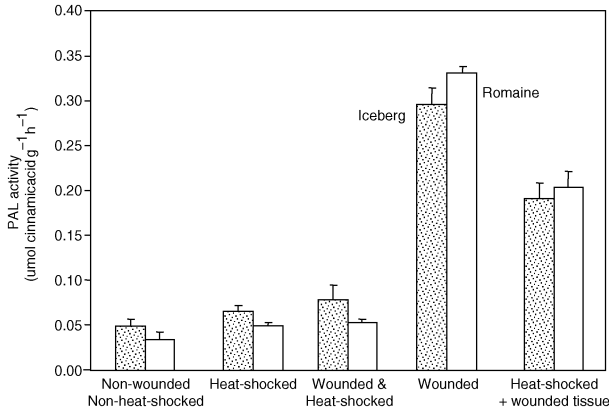

Fig. 5. Activity of phenylalanine ammonia-lyase (PAL) in mid-rid tissue of Iceberg and Romaine lettuce. Samples were analysed from non-wounded, non-heat-shocked tissue (control), heat-shocked tissue, wounded and heat-shocked tissue, wounded tissue, and equal parts of tissue that were heat-shocked or wounded. Excision of the mid-rib tissue from Iceberg and Romaine lettuce was the wound treatment, and the heat-shock treatment was exposure to 45°C for 150 s. The vertical line atop each bar represents the standard error about that mean (n = 4).

relationship between accumulation and protection. Lafuente et al. (1991) were the first to show that the induction (6 h at 37 or 42°C) of hsps with apparent molecular mass of 25 and 70 were correlated with increased chilling tolerance in cucumber (*Cucumis sativus*) cotyledons. Later, Collins et al. (1995) showed that the level of heat-shock induced (3 h at 40°C) hsp 70 fell to control levels at the same time as mung bean (*Vigna radiata*) hypocotyl tissue lost chilling tolerance induced by the heat-shock treatment. The appearance and persistence of a 23-kDa protein (48 h at 38°C) was also shown to increase the chilling tolerance of whole tomato (*Lycopersicon esculentum*) fruit (Sabehat et al. 1996). However, Saltveit (2001) recently questioned whether hsps present during chilling were necessary to protect tissue from developing chilling injury symptoms after chilling. He showed that a heat-shock treatment reduced chilling injury in cucumber (3 min 45°C) and rice (12 min 45°C) seedling radicles and in tomato (16 min 45°C) pericarp discs when applied either before or after chilling. While the induction and persistence of hsps has been correlated with heat-shock induced chilling tolerance, the effectiveness of heat-shock treatments applied after chilling suggests that the hsps need not be present during chilling, and may be acting through either the preferential synthesis of hsps or the presence of hsps during the development of chilling injury symptoms. In either case, the synthesis or presence of induced hsps would be interfering with the development of chilling injury symptoms rather than interfering with the primary transduction of chilling into a physiological injury.

The synthesis of, rather than the presence of hsps, appears to be important in the ability of heat-shocks to reduce wound-induced increases in PAL activity. Clearly, the hsps in the heat-shocked, non-wounded tissue had no significant effect on the activity of PAL from wound-induced, non-heat-shocked tissue. If the hsps were the cause of the reduced PAL activity in wounded and heat-shocked tissue, then an extract produced from equal weights of heat-shocked tissue (in which there was no wound-induced PAL) with wounded lettuce (in which there were no induced hsps) should have significantly lower PAL activity below their simple arithmetic average. If the presence of hsp 23 interferes with PAL activity, then the hsp 23 in the heat-shocked, non-wounded tissue should have reduced the activity of PAL in the wounded, non-heat-shocked tissue. Yet PAL activity in the mixture was simply the arithmetic average of PAL from the two tissues.

The effectiveness of heat-shock treatments that reduced wound-induced increases in PAL activity was highly correlated with the induced synthesis of hsp 23. Since hsp 23 by itself did not interfere with the activity of wound-induced PAL (Fig. 5), the preferential synthesis of hsp 23 over that of wound-induced PAL may account for the effectiveness of the heat-shock treatments. Characterization of two wound-induced PAL genes and the induced synthesis of wound-induced PAL protein will be reported in a subsequent paper.

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