PHYSIOLOGIA PLANTARUM 120: 556–562. 2004
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Effect of acetaldehyde, arsenite, ethanol, and heat shock on protein synthesis and chilling sensitivity of cucumber radicles

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Received 11 July 2003; revised 12 September 2003

Exposure to a chilling temperature of 2.5°C for 96 h inhibited the subsequent growth of cucumber seedling radicles at 25°C by 92%. Exposing seedling with 5 ± 1 mm long radicles to acetaldehyde vapour (275 $\mu l\,l^{-1}$) or to an aqueous ethanol solution (0.6 *M*) for 2 h, or to 45°C for 10 min before chilling, increased chilling tolerance so that the chilling treatment reduced growth by only 47, 39 or 36%, respectively. All of these effective treatments induced the synthesis of a number of proteins, and suppressed *de novo* protein synthesis (i.e. the incorporation of [^{35}S]-methionine) by about 70%. In contrast, treatment for 2 h with an aqueous arsenite solution (100 μM) had no effect on chilling sensitivity or the incorporation of [^{35}S]-methionine, yet it induced the synthesis of a complement of proteins that were similar to that induced by the effective heat-shock treatment. A unique protein or set of proteins may be responsible for heat-shock-induced chilling tolerance, but none was detected. The ability of various abiotic stresses to suppress protein synthesis may be more important in increasing tolerance to chilling injury than their ability to induce the synthesis of specific proteins.

Introduction

Chilling injury occurs in many plants of tropical and subtropical origin when exposed to non-freezing temperatures below approximately 10°C (Saltveit and Morris 1990). Symptoms of chilling injury include increased water loss, respiration, ethylene production, and disease susceptibility, abnormal ripening of fruit, and loss of seedling vigour. Symptoms are slow to develop during chilling, but develop rapidly after transfer to a non-chilling temperature.

Several techniques are used to reduce chilling injury symptoms in harvested fruits and vegetables (Saltveit and Morris 1990). The temperature of plant tissue immediately before chilling can significantly increase or decrease its chilling sensitivity (Saltveit 1991). The effects of chilling can also be modified by dips in calcium solutions before chilling, intermittent warming or reduced levels of oxygen and/or elevated levels of carbon dioxide during chilling, and storage in high relative humidity after chilling. Since a phase transition at the chilling temperature of some crucial membranes is thought to be involved in the development of chilling injury, attempts have been made to alter the fatty acid composition of membranes in chilling-sensitive plants. For example, tobacco plants that were engineered to have a higher proportion of *cis* unsaturated fatty acids in their chloroplast membrane (and therefore a lower phase transition temperature) exhibited greater chilling resistance than the non-transformed plants (Murata et al. 1992).

Chilling sensitivity can be modulated by prior temperature exposures. A brief thermal shock (e.g. 10 min at 45°C) conferred tolerance to an otherwise lethal high temperature stress (Lin et al. 1984), and a cold-shock-induced tolerance to subsequent chilling temperatures (Goto et al. 1984). Responses induced by exposure to temperature shocks are also cross protective (Saltveit 1991, Sabehat et al. 1998a). For example, a 6-h heat-shock treatment decreased the chilling sensitivity of cucumber cotyledons (Lafuente et al. 1991). Heat-shock-induced chilling tolerance is not unique to cucumber but is also reported for mung bean hypocotyls (Collins et al.

Abbreviations – hsps, heat shock protein; lmwp, low molecular weight proteins.

1993), seedling radicles of corn, cucumber, mung bean, and tomato (Rab and Saltveit 1996), and rice (Saltveit 2001), and harvested tomato fruit (Saltveit and Cabrera 1987, Lurie and Klein 1991, Sabehat et al. 1998a).

The presence of shock proteins induced by brief exposures to heat, cold and various chemicals are thought to confer tolerance to subsequent stress. For example, heat shocks induce the synthesis of a unique set of proteins called heat-shock proteins (hsps) (Linquist 1986, Vierling 1991, Waters et al. 1996). Accumulation of hsps appears to render induced cells and whole plants more resistant to subsequent thermal stresses. The accumulation and persistence of specific hsps has been correlated with increased chilling tolerance in chilling-sensitive cultured apple fruit cells (Wang et al. 2001), avocado fruit (Woolf et al. 1995), cucumber cotyledons (Lafuente et al. 1991), mung bean hypocotyls (Collins et al. 1995), and tomato fruit tissue (Whitaker 1994, Sabehat et al. 1996, 1998b, Kadyrzhanova et al. 1998, Ding et al. 2001).

If similar protective proteins are induced by different shock treatments, their identification could provide a powerful tool to engineer plants that would be more chilling tolerant. A problem with using induced proteins as a tool for genetic manipulation, however, is that many different proteins are induced and it is essential that a good correlation exist between the specific protein(s) induced and the induced level of chilling tolerance. In this study we used two-dimensional gels to characterize the induced proteins, and used other treatments that induce the synthesis of various levels of these proteins (i.e. acetaldehyde, arsenite, ethanol, and heat shock) to narrow the number of candidate induced proteins that are associated with increased chilling tolerance. We show that a reduction in total protein synthesis is more closely correlated with induced chilling tolerance than is the production of specific induced proteins.

Materials and methods

Plant material and growth conditions

Seeds of cucumber (*Cucumis sativus*, cv. Poinsett 76; Petoseed Co., Inc., Saticoy, CA, USA) were imbibed in distilled water with aeration for 16 h at 25°C and grown on sheets of water-saturated paper towels held vertically between two sheets of Plexiglas at 25°C as previously described (Rab and Saltveit 1996). Seven seedlings with radicals 5 ± 1 mm in length were used as units of replication in all experiments. Temperature and chemical treatments were selected that did not adversely affect the subsequent growth of the cucumber radicles (Jennings and Saltveit 1994, Rab and Saltveit 1996).

Heat-shock treatments

Cucumber seedlings were placed in the bottom of a 10 mm × 100 mm-diameter plastic Petri dish upon one layer of Whatman no. 1 filter paper (Fisher Scientific, Pittsburgh, PA) wetted with 1.5 ml of deionized water. The covered Petri dish was floated on water in a heated water bath. Cucumber seedlings were exposed for 0, 2.5, 5, 10 or 12.5 min at 45°C. Immediately following the heat-shock treatment approximately 4 ml of 5 m*M* MES/KOH buffer pH 6.7 was added to the Petri dish to maintain hydration of the seedlings while they were held at 25°C for 2 h.

Ethanol and arsenite treatments

Ethanol and arsenite treatments were given by placing cucumber seedlings in a 10 mm × 100 mm-diameter plastic Petri dish upon two layers of Whatman no. 1 filter paper wetted with 8 ml of 0.6 *M* ethanol in 5 m*M* MES/KOH buffer pH 6.7, or 50–500 μ*M* sodium arsenite in 5 m*M* MES/KOH buffer pH 6.7. Treatment duration was for 2 h at 25°C.

Acetaldehyde treatment

Seedlings were placed in 10 mm × 100 mm diameter plastic Petri dishes with one layer of Whatman no. 1 filter paper wetted with 3 ml of 5 m*M* MES pH 6.7. The uncovered dishes were placed in a 20-l jar through which air containing acetaldehyde (0, 140 ± 19, 275 ± 25, 420 ± 50 μl l^{-1}) flowed for 2 h at 20°C as previously described (Beaulieu and Saltveit 1997, Beaulieu et al. 1997).

Measurements of chilling injury

The chilling sensitivity of the treated and control cucumber seedlings was determined as previously described (Rab and Saltveit 1996). In summary, after the 2 h treatments, the seedlings were placed on moist paper towelling between vertically orientated 7 cm × 14 cm Plexiglas sheets and chilled at 2.5°C for 96 h. Subsequent radicle growth at 25°C was measured with a ruler to the nearest millimetre by inspection of individual seedling through the transparent Plexiglas after 72 h.

Protein labelling

Seedlings were labelled with [^{35}S]-methionine (Amersham; specific activity > 1000 Ci $mmol^{-1}$) (Amersham, Piscataway, NJ) during the final hour of the 2-h treatment period at 25°C. One hour following the heat-shock treatment, 10 cucumber seedlings were blotted dry and their radicles placed into 30 μl of 5 m*M* MES/KOH pH 6.7 containing 1.8×10^6 Bq [^{35}S]-methionine for 1 h; only the radicles were immersed. Likewise, 10 cucumber seedlings for the ethanol and arsenite treatments were blotted dry and placed into 30 μl 5 m*M* MES/KOH pH 6.7 plus 0.6 *M* ethanol or 100 μ*M* sodium arsenite, respectively, along with 1.8×10^6 Bq [^{35}S]-methionine.

Following the labelling period, 2 mm were cut from the distal end of the radicals, washed five times in 10 ml 5 m*M* MES/KOH pH 6.7, and homogenized in 100 μl extraction buffer [2% (w/v) SDS, 5% (v/v) β-mercaptoethanol, 62 m*M* Tris-HCl pH 6.8, 10% (v/v) glycerol].

Samples were boiled for 3 min and centrifuged to remove cellular debris. Aliquots of the soluble fraction were taken to ascertain uptake of the applied label by the tissue. Incorporation into protein was determined by the procedure of Mans and Novelli (1961).

Electrophoresis

Two-dimensional electrophoresis was conducted by following the method described by Bollag and Edelstein (1991). Samples for isoelectric focusing were precipitated by adding 9 volumes of ice-cold ethanol, holding at −20°C over night, and the supernatant was removed after centrifugation. The precipitate was re-suspended in loading buffer (9 *M* urea, 4% Nonidet-40, 4% β-mercaptoethanol, 0.8% pH 3–10 ampholytes, 1.6% pH 4–8 ampholytes, 1.6% pH 5–7 ampholytes). Samples containing equal amounts of [^{35}S]-methionine-incorporated protein were loaded on a 0.2-mm thick slab gel run at 6°C for 3500 V. The second dimension was run on a 12% (w/v) polyacrylamide gel using a discontinuous system (Laemmli 1970). Low molecular weight standards (Bio-Rad, Hercules, CA) were used to calibrate the gels. After electrophoresis the gels were stained with Coomassie Brilliant Blue, destained, dried and exposed to Fuji RX medical X-ray film (Fuji, Stamford, CT).

Statistical analysis

All experiments were repeated at least twice with similar results. Each of the three replicate per experiment contained seven seedlings with radicals 5 ± 1 mm in length for growth measurements, and 10 radicles for labelling and protein extraction. Data were subjected to an analysis of variance and means and standard deviations calculated.

Results

Effects of chilling on subsequent growth

The 5 ± 1 mm long non-chilled cucumber radicles grew to a length of 8.4 ± 0.6 cm in 72 h at 25°C. Chilling them at 2.5°C for 96 h before growth at 25°C reduced subsequent growth by 92% to 0.67 ± 0.33 cm (Fig. 1).

Treatment effects upon chilling tolerance

Arsenite

Radicle growth was unaffected by 2 h exposure to 50 or 100 μ*M* arsenite, whereas 250 or 500 μ*M* inhibited growth by about 50% (Fig. 2). However, none of these concentrations conferred any chilling tolerance to the seedlings (Figs 1 & 2). For example, the radicles of non-chilled seedlings treated with 100 μ*M* arsenite for 2 h grew to 8.17 ± 0.66 cm in length. In contrast, the radicles of chilled seedlings grew to a length of 0.71 ± 0.08 cm irrespective of the concentration (i.e. 0–500 μ*M*) of applied arsenite. This inhibition amounted to a 91% reduction in growth for the 100 μ*M* treatment.

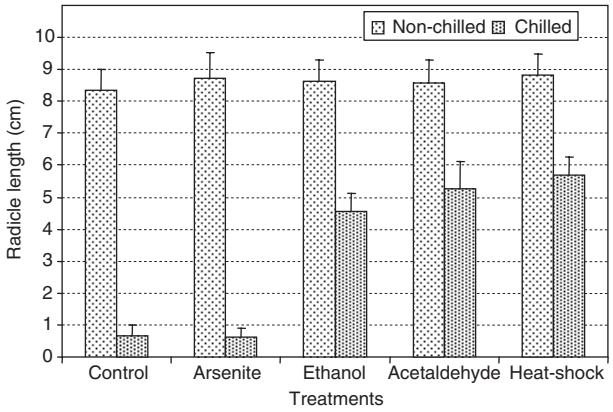

Fig. 1. Effect of exposing 5 mm long cucumber seedling radicles to arsenite (100 μ*M*), ethanol (0.6 *M*), or acetaldehyde (275 μl l^{-1}) for 2 h, or to a 45°C heat shock for 10 min on the subsequent growth of cucumber seedling radicle for 72 h at 25°C after holding at non-chilling (12.5°C) or chilling (2.5°C) temperatures for 96 h. The line at the top of each bar represents the standard deviation about that mean (n = 21).

Ethanol

Immersing seedling radicles in 0.6 *M* ethanol for 2 h did not reduce subsequent radicle growth, but it did significantly increase chilling tolerance (Fig. 1). This concentration and duration of treatment induced an optimal level of chilling tolerance (Jennings and Saltveit 1994). The chilling treatment reduced subsequent radicle growth by 47% from 8.64 ± 0.67 to 4.55 ± 0.58 cm. Since some of the physiological effects of ethanol may result from its conversion to acetaldehyde by alcohol dehydrogenase (Beaulieu et al. 1997), the ability of acetaldehyde to induce chilling tolerance was also tested.

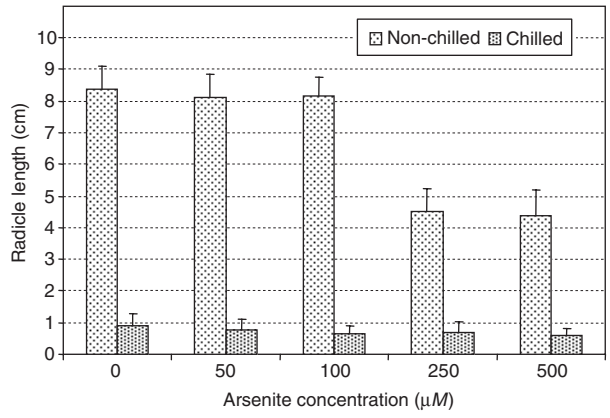

Fig. 2. Effect of exposing 5 mm long cucumber seedling radicles to aqueous arsenite solutions for 2 h on their subsequent growth at 25°C for 72 h after holding at non-chilling (12.5°C) or chilling (2.5°C) temperatures for 96 h. The line at the top of each bar represents the standard deviation about that mean (n = 21).

Acetaldehyde

Exposing seedlings to acetaldehyde vapours ($275 \pm 25\,\mu l\,l^{-1}$) for 2 h at 20°C did not significantly reduce the subsequent growth of their radicles, but it did significantly increase their chilling tolerance (Fig. 1). The chilling treatment reduced subsequent radicle growth by 39% from 8.59 ± 0.69 to 5.27 ± 0.85 cm. Higher concentrations and longer exposures inhibited the subsequent growth of non-chilled radicles (data not shown). The acetaldehyde vapour treatments were not continued because of the difficulty in consistently applying the vapour, and because the more easily applied ethanol treatment gave similar results and was shown to act through the endogenous production of acetaldehyde (Beaulieu et al. 1997).

Heat shock

Cucumber seedlings given a 5-min heat-shock treatment at 45°C exhibited increased tolerance to subsequent chilling as determined by radical growth (Fig. 1). Chilling reduced subsequent growth by 36% from 8.83 ± 0.67 to 5.69 ± 0.58 cm. The 5- and 10-min heat-shock treatments increased tolerance by about the same amount (data not shown).

Two-dimensional gels

The heat-shock treatment induced the de novo synthesis of proteins that were either not present, or present in much lower levels in control tissue (Fig. 3). Most of the newly synthesized proteins were of low molecular weight with masses around the 21 kDa standard. It appeared that proteins with similar characteristics were also synthesized in radicles treated with ethanol and arsenite. The 2-D pattern of labelled proteins on the gel from heat-shock and arsenite-treated radicles were surprisingly similar, whereas many of the proteins present in these two gels were absent from the gel of ethanol-treated and control radicles. A unique individual or set of hsps was not detected that was present in the heat-shocked and ethanol-treated tissue and was completely absent from the control or arsenite-treated tissue.

Equal amounts of label were applied to each of the four gels displayed in Fig. 3. Slight differences in the amount of label applied, exposure of the autoradiograph, development and printing, scanning the prints, and merging the images into the composite shown in Fig. 3 may account for the perceived differences in intensity among the gels. While it appears from a visual examination of the four gels that the heat-shock and arsenite treatments had greater levels of label incorporated into protein than the other two treatments, a measure of incorporation showed that the two treatments that were effective in promoting chilling tolerance (i.e. heat shock and ethanol) actually had significantly lower rates of incorporation than did the control or arsenite treatments which were very similar.

Incorporation of label

Treatments that conferred chilling tolerance reduced the incorporation of [^{35}S]-methionine into protein (Fig. 4). Cucumber radicles exposed to a heat shock of 45°C for 10 min, or to 0.6 *M* ethanol for 2 h incorporated only 33 ± 9.9 and $30 \pm 7.5\%$, respectively, of the control, whereas radicles exposed to 100 μM arsenite for 2 h incorporated $108 \pm 3.6\%$ of the control. Unlike the heat-shock and ethanol treatments, arsenite did not reduce the rate of incorporation of [^{35}S]-methionine into protein. Aliquots of the soluble fraction indicated that uptake of the applied label was slightly variable within and among the treatments (i.e. $\pm 6.5\%$), but not significantly different among the treatments.

The correlation between reduced incorporation of label and increased chilling tolerance was highly significant across all treatments (Fig. 5). The reduction in the incorporation of label could be predicted from the decrease in the inhibition of radicle growth as a percentage of non-chilled control (i.e. the increase in chilling tolerance). As chilling tolerance increased (i.e. the decline in the inhibition of radicle growth from 90 to 30% of the control), the incorporation of label declined from around 100 to 20% of the control. The relationship is given by the equation: Incorporation $= (1.23 \times$ inhibition of radicle growth$) - 17.65$ with an R^2 of 0.95 (Fig. 5).

Discussion

The chilling tolerance of cucumber seedling radicles was increased by a number of abiotic shocks. The 92% reduction in subsequent radicle growth caused by exposure to 96 h of chilling at 2.5°C was reduced by heat-shock, ethanol and acetaldehyde treatments to 36, 39 and 47%, respectively, of their non-chilled controls. Similar levels of chilling tolerance have been induced in this same cultivar of cucumber by a number of abiotic shocks including heat, cold, methanol, ethanol, salts and osmotic stress (Jennings and Saltveit 1994). They used longer durations of heat stress (e.g. 1 h at 40°C) and reported that chilling tolerance was increased in seedling radicles immersed in 0.4 *M* ethanol for 4 h. Our results confirm their observations and extend the treatments to shorter heat-shock and ethanol treatments, and treatments with acetaldehyde and arsenite.

Arsenite induces the synthesis of many of the same low molecular weight proteins induced by heat shock (Vierling 1991, Wollgiehn and Neumann 1999, Ding et al. 2001). The patterns of low molecular weight hsps induced by heat-shock and arsenite and analysed by 1- and 2-dimensional SDS-PAGE were similar to one another (Edelman et al. 1988). We showed that a 2-h treatment of cucumber seedling radicles with 100 μM arsenite did not inhibit radicle growth (Fig. 2), and did not induce a significant level of chilling tolerance (Figs 1 and 2) even though it did induce many of the same low molecular weight proteins induced by heat shock (Fig. 3).

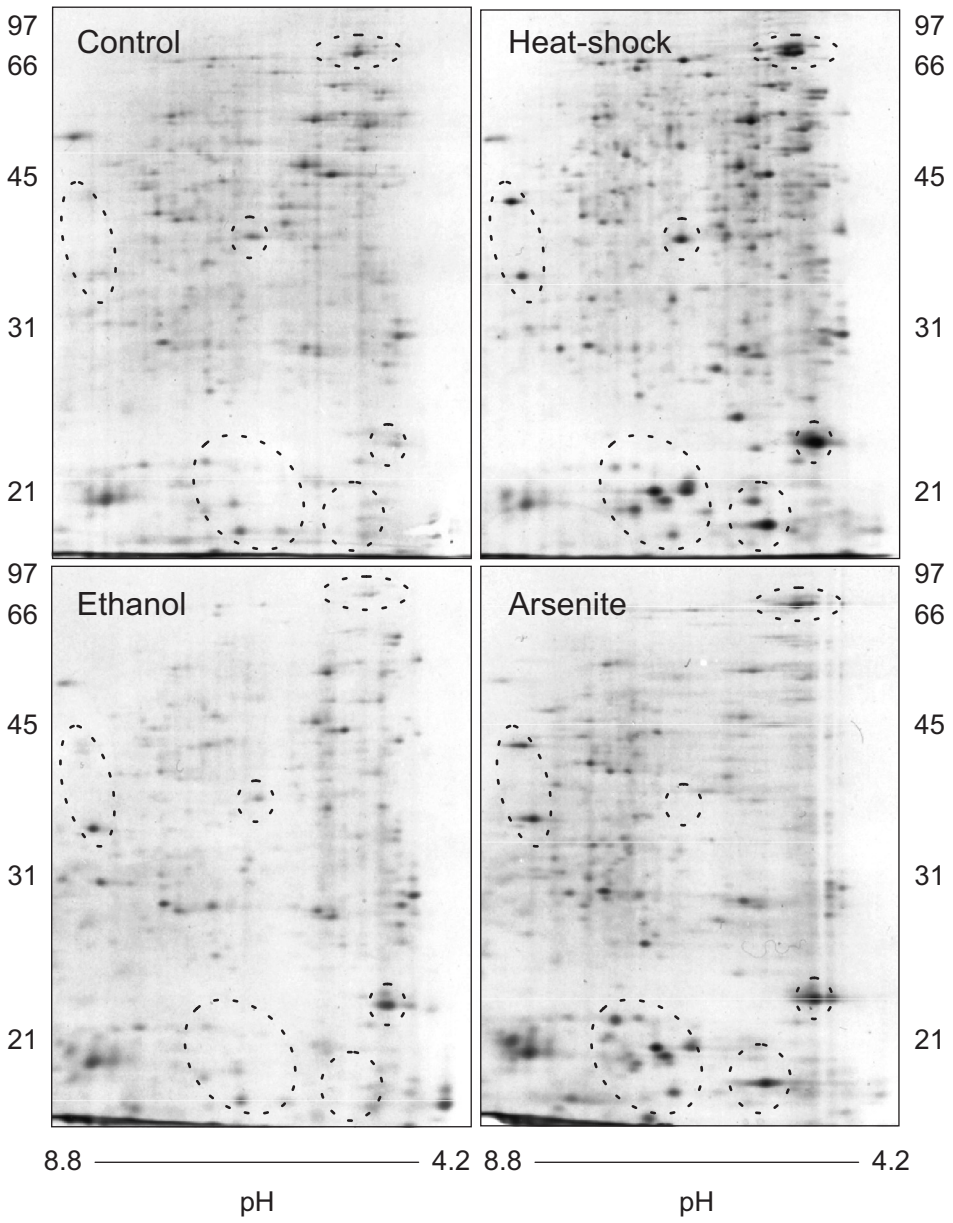

Fig. 3. Autoradiographs of two-dimensional gels of protein extracted from control, heat-shock, ethanol- and arsenite-treated cucumber radicles (see Materials and methods for details).

The ability of heat shock to induce chilling tolerance has been correlated with the appearance and persistence of heat-shock proteins (Lafuente et al. 1991, Collins et al. 1995, Sabehat et al. 1996; 1998c, Wang et al. 2001). However, the induced and synthesized hsps may not need to be present during chilling for these treatments to exert their protective effect since application of a protective heat-shock treatment after chilling was almost as effective as applying it before chilling (Saltveit 2001).

Three lines of evidence suggest that the presence of hsps may not be necessary for various abiotic stresses to induce increased chilling tolerance. First, similar proteins are induced by heat shock and arsenite (Fig. 3), yet only heat-shock-induced chilling tolerance (Fig. 2). Ethanol does not appear to induce the same type or level of proteins as heat shock (Fig. 3), yet both induce similar levels of chilling tolerance (Fig. 2). There may be a unique hsp that is induced by heat shock and ethanol, but not induced by arsenite, yet no such protein was identifiable in the 2-D gels. Second, in other studies, heat-shock treatments were almost as effective in protecting against chilling-induced reductions in radicle growth and increases in the rate of ion leakage if applied after chilling as they were if applied before chilling (Saltveit 2001) and third, there was a strong correlation ($R^2 = 0.95$) between the ability of any shock treatment to induce chilling tolerance and to reduce protein synthesis (i.e. incorporation of labelled methionine) (Fig. 5).

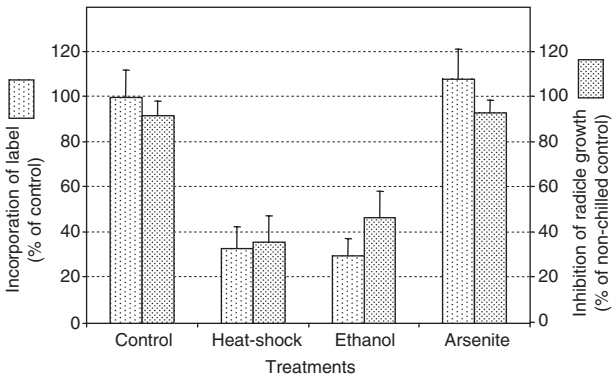

Fig. 4. Effect of exposing 5 mm long cucumber seedling radicles to a 45°C heat shock for 10 min, or to an aqueous solution of ethanol (0.6 *M*) or arsenite (100 μ*M*) for 2 h on incorporation of label and chilling inhibition of subsequent radicle growth for 72 h at 25°C after holding at non-chilling (12.5°C) or chilling (2.5°C) temperatures for 96 h (See Materials and methods for description of treatments). The vertical line at the top of the each bar represents the standard deviation about the means (for incorporation of label, n = 3, for radicle growth, n = 21).

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). The beneficial effects of the various abiotic shock treatments may not be the result of the accumulated induced proteins (e.g. hsps), but the result of the decreased synthesis of some proteins induced by the stress and responsible for the appearance of chilling injury symptoms (Saltveit 1997, 2000). The ability of various abiotic stresses to suppress protein synthesis may be more important in increasing tolerance to chilling injury than their ability to induce the synthesis and accumulation of specific proteins. Engineering plants to have elevated levels of specific hsps may not produce plants with elevated levels of tolerance to stresses such as chilling.

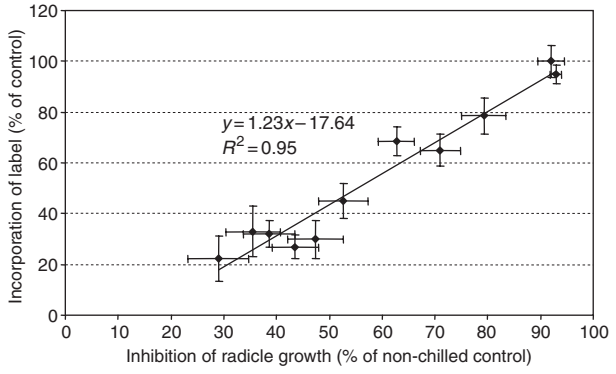

Fig. 5. Effect of various abiotic stresses on the relation between chilling inhibition of cucumber seedling radicle growth and incorporation of label in synthesized proteins (See Materials and methods for description of treatments). The vertical and horizontal lines represent the standard deviation about the means (for radicle growth, n = 21; for incorporation of label, n = 3).

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Edited by C. Guy