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Effect of heat shock on the chilling sensitivity of trichomes and petioles of African violet (*Saintpaulia ionantha*)

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Chilling at 6°C caused an immediate cessation of protoplasmic streaming in trichomes from African violets (*Saintpaulia ionantha*), and a slower aggregation of chloroplasts in the cells. Streaming slowly recovered upon warming to 20°C, reaching fairly stable rates after 4, 15, 25 and 35 min for tissue chilled for 2 min and for 2, 14 and 24 h, respectively. The rate of ion leakage from excised petioles into an isotonic 0.2 M mannitol solution increased after 12 h of chilling and reached a maximum after 3 days of chilling. A heat shock at 45°C for 6 min reduced chilling-induced rates of ion leakage from excised 1-cm petiole segments by over 50%, namely to levels near that from non-chilled control tissue. Heat-shock treatments themselves had no effect on the rate of ion leakage from non-chilled petiole segments. Protoplasmic streaming was stopped by 1 min of heat shock at 45°C, but slowly recovered to normal levels after about 30 min Chloroplasts aggregation was prevented by a 1 or 2 min 45°C heat-shock treatment administered 1.5 h before chilling, but heat-shock treatments up to 6 min only slightly delayed the reduction in protoplasmic streaming caused by chilling. *Tradescantia virginiana* did not exhibit symptoms associated with chilling injury in sensitive species (i.e. cessation of protoplasmic streaming in stamen hairs and increased ion leakage from leaf tissue).

Introduction

Chilling injury is a physiological disorder that affects plants indigenous to the tropics and subtropics when they are exposed to non-freezing temperatures below 10–12°C. (Lyons 1973, Saltveit and Morris 1990, Saltveit 2000). Economically important chilling-sensitive plants include avocado, banana, beans, corn, cucumber, rice, squash, sugarcane, sweet potato and tomato. Most ornamental plants indigenous to the tropics, such as African violets (*Saintpaulia ionantha*), are sensitive to chilling temperatures. Symptoms of chilling injury include the cessation of protoplasmic streaming, diminished seedling vigour, reduced plant growth, increased cellular leakage, impaired photosynthesis, excessive water loss, increased susceptibility to certain diseases, and enhanced senescence. For example, chilling reduced the rate of protoplasmic streaming in trichomes of *Tradescantia albiflora* in a manner consistent with that observed in chilling-sensitive plants such as

tomato (*Lycopersicon esculentum*) and watermelon (*Citrullus vulgaris*) (Patterson and Graham 1977).

The difference between a chilling and non-chilling temperature can be very narrow. For example, the subsequent elongation of 10-mm-long cucumber radicles is inhibited by a 2-day exposure to 7.5°C but not by exposure to 10°C (Rab and Saltveit 1996). The difference between an exposure that produces observable damage and one that does not depends on the tissue, its past exposure to stresses, the symptom being observed, and the duration of exposure. The response of sensitive tissue to chilling can be immediate or protracted. Days of exposure to chilling temperatures are usually necessary to induce increased rates of ion leakage, while the rate of protoplasmic streaming ceases almost immediately as the temperature falls below the threshold chilling temperature (Saltveit 2000).

Several methods have been developed to decrease chilling sensitivity and/or increase chilling resistance (Lyons and Breidenbach 1979, Saltveit 2000). These methods

Abbreviations – hsps, heat-shock proteins.

include conditioning at temperature near the chilling temperature, intermittent warming, storage in atmospheres low in oxygen and/or high in carbon dioxide, and exposure to abiotic shocks (e.g. heat and chemical shocks). Exposure to a 45°C heat shock for a few minutes reduced the subsequent chilling-induced inhibition of radicle elongation in a number of sensitive species (Mangrich and Saltveit 2000a, b). The physiological basis for this effect is unknown, although the synthesis of protective heat-shock proteins (hsps) has been correlated with the appearance and disappearance of heat-shock-induced chilling tolerance (Lafuente et al. 1991, Lurie and Klein 1991, Saltveit 1991, Collins et al. 1995, Lurie 1998).

Protoplasmic streaming appears to be much more sensitive than other measured aspects of chilling injury. Such a rapid response suggests that changes involved with the loss of protoplasmic streaming may be closer to the transduction of a chilling temperature into a physiological response than is the subsequent increased rate of ion leakage. Results reported in this paper characterize the chilling sensitivity of *S. ionantha*, and *T. virginiana*, and show that *T. virginiana* does not exhibit symptoms associated with chilling-sensitive species. The effect of heat shock and chilling on the rate of ion leakage, protoplasmic streaming and aggregation of chloroplasts is reported for *S. ionantha*.

Materials and methods

Plant tissue and microscopic observations

Young, expanding leaves of *S. ionantha* (about 5 mm long) were excised from the plant and cross-sections of the petiole, approximately 0.2 mm thick, were made in a drop of solution with a scalpel under a 6× dissecting microscope. The solution contained 1 mM KCl, 1 mM MgCl$_2$, 0.1 mM CaCl$_2$, and 5 mM HEPES at pH 7.0. Petroleum jelly was melted and drawn into a glass transfer pipette. An open ring about 1 cm in diameter was drawn in the centre of a 25 × 75 mm glass microscope slide by touching the tip of the pipette on the glass and quickly moving it in a circular motion. A drop of solution was placed within the circle and a petiole cross-section transferred to the slide. A cover slip was placed over the circle and pushed down to flatten the petiole section and to make a uniform seal with the circle of petroleum jelly. Care was exercised to make sure the ring remained open. Preliminary experiments showed that closure of the ring was followed within a day by an abnormal appearance of the trichomes, possibly caused by anaeriobiosis. When kept in a humid environment, petiole tissue on slides prepared with an open circle retained their characteristic colour and microscopic cellular structure for over 3 days at 20°C or 7 days at 6°C. Stamen hairs were excised from young buds of *T. virginiana* and prepared as described above.

Microscopic observations were carried out on a Reichert Zetopan microscope using Nomarski differential interference contrast optics. A 40× dry objective (NA = 0.65)

was the principal lens use for the observations. Images were acquired on photographic film (Kodak T-Max; ASA 400; Eastman Kodak Co., Rochester, NY, USA) with an Olympus automatic camera (PM-10AD)(Olympus Corp. of America, New York, NY, USA) which was regulated by the Olympus camera control unit (PM-CBAD).

The distance that a cellular organelle travelled in a given time was measured with a calibrated ocular micrometer, and the distance and time were used to calculate the rate of streaming. Chloroplast aggregation was subjectively assessed from observations and photomicrographs of tissue after various durations of chilling. The extent of aggregation before chilling was assigned a value of 0%, whereas the maximum extent of aggregation after chilling was assigned a value of 100%. Intermediate values were approximated using sample photomicrographs as references.

Petiole segments (1-cm long) were excised from fully expanded leaves with a scalpel, washed in three changes of distilled water and blotted dry. Twelve segments were randomly assigned to each 15 × 60-mm-diameter plastic Petri dish. Each dish contained a 5.5-cm circle of filter paper moistened with 1-ml of water.

Heat-shock treatments

Slides were immersed in a water bath heated to 45°C for 0–6 min The slides were held in a horizontal orientation at all times. The heat-shock temperature did not liquefy the petroleum jelly or alter the orientation of material on the slide. Petri dishes containing petiole segments were floated on the 45°C water for 0–20 min After the heat shock, slides or Petri dishes were placed on wet paper towels at room temperate for 1.5 h before continuing the treatments.

Chilling treatments

Petri dishes and slides were placed in 4-l translucent plastic boxes lined with wet paper towels. The boxes were held in a walk-in cooler maintained at 6°C for up to 6 days. Slides were placed on wire test-tube racks in the cooler for short-term (up to 60 min) chilling experiments.

Measurement of ion leakage

Petri dishes containing the 1-cm *S. ionantha* petiole segments, or 1-cm *T. virginiana* leaf segments were removed from the chilling temperature after the designated time and held at room temperature (approximately 20°C) for 2 h. Control segments were also held at a non-chilling temperature of 15°C for up to 6 days. After chilling or holding, three segments were placed in a 50-ml conical, plastic centrifuge tube containing 20 ml of 0.2 M mannitol. The tubes were gently shaken at 120 cycles min^{-1} between measurements. Conductivity of the solution was measured after 30, 90 and 150 min The tubes were capped and frozen. After warming to room temperature with shaking, the total conductivity was measured. The

increase in conductivity was linear ($R^2 > 0.96$) from 30 to 150 min The percentage of total conductivity was calculated by subtracting the 30 min value from the 150 min value and dividing by the total conductivity and the hours between the two measurements.

Statistical analysis

All experiments were repeated at least twice with similar results, and each experiment had at least three replicates. Where appropriate, data were subjected to an analysis of variance and means and standard deviations were calculated.

Results

Effect of chilling on ion leakage

Chilling injury is commonly quantified by measuring the rate of ion leakage from tissue immersed in an isotonic solution (Saltveit and Morris 1990, Saltveit 2000, 2002). A significant increase in the rate of ion leakage from excised 1-cm segments of *S. ionantha* petioles into a 0.2-*M* isotonic mannitol solution was not observed until after 12 h of chilling at 6°C (Fig. 1). The rate of leakage continued to increase with increasing duration of chilling, reaching a plateau at 3 days. Control tissue held at a non-chilling temperature of 15°C exhibited an increase in ion leakage after 3 days that became significant by 6 days. The increase in ion leakage at 6 days coincided with tissue discoloration and water logging (i.e. translucent areas), which may have indicated the onset of senescence or disease, although no growth of micro-organisms (i.e. fungal hypha or bacterial colonies) was observed.

It appears that *S. ionantha* petiole segments are more chilling sensitive than *Cucumis sativus*, *Zea maize* and *Oryza sativa* seedling radicles, or *Lycopersicon esculentum*

fruit pericarp discs in which significant increases in ion leakage did not appear until after 3 days at 2.5°C (Mangrich and Saltveit 2000a), 5 days at 2.5°C, 1.5 days at 5°C (Kang and Saltveit 2002), and 4 days at 2.5°C (Saltveit 2002), respectively.

In contrast with the pronounced effect of chilling on petioles of *S. ionantha*, chilling did not increase the rate of ion leakage from *T. virginiana* leaves. In fact, chilling caused a significant decrease in the rate of ion leakage from excised 1-cm leaf segments from 1.1 ± 0.4 to 0.3 ± 0.052 to $0.04 \pm 0.017\%$ of total conductivity per hour as the leaf segments were chilled for 0, 2 and 7 days at 6°C, respectively. Chilling-induced ion leakage is not an all-or-none response, and increased exposure and/or reduced temperatures may induce the chilling symptom of increased ion leakage. However, most extensively studied chilling-sensitive plants (e.g. avocado, cucumber, maize, pepper, rice, soybean, squash, tomato, etc.) exhibit increased ion leakage when chilled for less than 7 days (Saltveit and Morris 1990).

Effect of chilling on protoplasmic streaming

Chilling caused two gross morphological changes in petiole trichomes of *S. ionantha*; it stopped protoplasmic streaming and it caused the chloroplasts to aggregate. Before chilling, the chloroplasts, which were clearly evident in the cytoplasm of individual trichome cells, were randomly distributed within the cell (Fig. 2). As the duration of chilling at 6°C increased, cellular organelles (e.g. chloroplasts), which were initially dispersed throughout the protoplasm (0% aggregation), began to aggregate into clumps (Fig. 3). After 60 min of chilling there was slight aggregation of the chloroplasts (i.e. approximately 20%). Aggregation continued until most chloroplasts were clustered together after 3 h (100% aggregation).
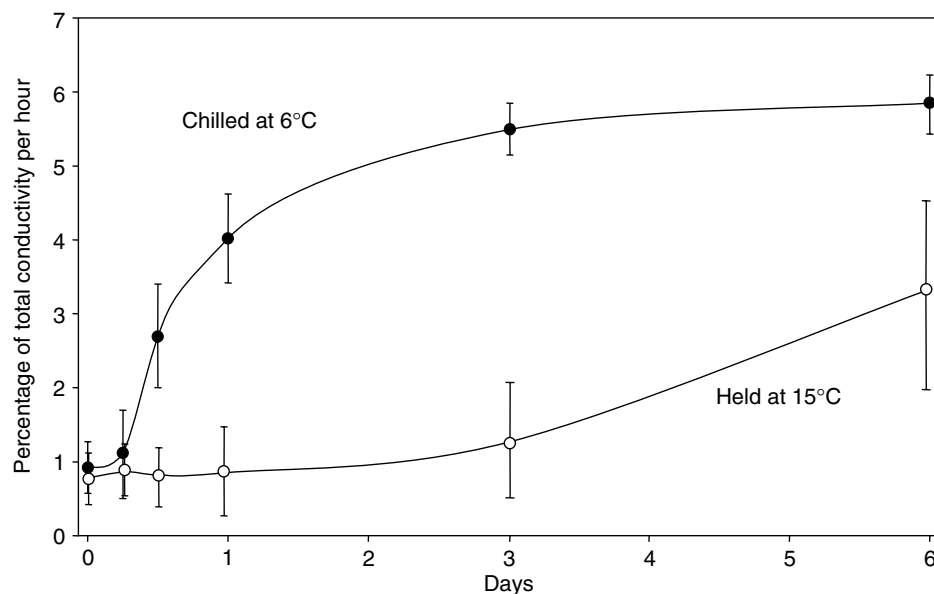

Fig. 1. Effect of chilling on the rate of ion leakage from 1-cm segments of *Saintpaulia ionantha* petioles. The vertical lines at each data point represent the standard deviation about that mean.
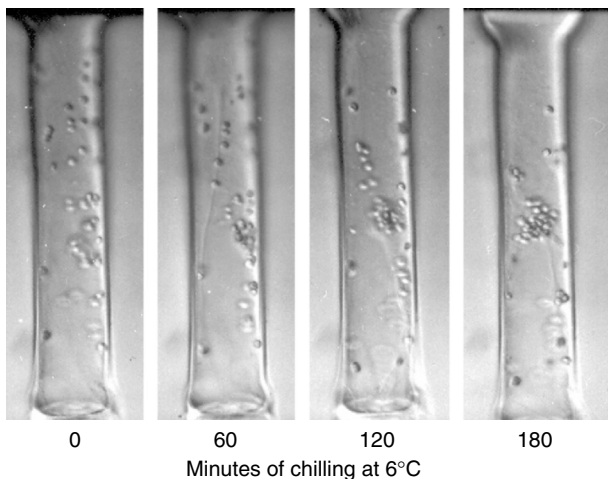
Fig. 2. Photomicrographs of petiole trichomes of *Saintpaulia ionantha* chilled at 6°C for 0, 60, 120, and 180 min. The sequential aggregation of chloroplasts is shown.

In contrast to the protracted clustering of the chloroplasts (Fig. 3), protoplasmic streaming was rapidly lost from chilled trichomes (Fig. 4). As the temperature dropped from 20 to 6°C, the rate of protoplasmic streaming gradually declined in stamen hairs of *T. virginiana* to around 40% of the initial rate, while it abruptly dropped from $4.2\,\mu m\,s^{-1}$ to zero within 30 s in trichomes of *S. ionantha* (Fig. 4). The rate of protoplasmic streaming increased to initial rates when the temperature was increased to 20°C within 4 min in trichomes of *S. ionantha*, whereas it took 8 min in stamen hairs of *T. virginiana* (data not shown).

Recovery of streaming took more time the longer the tissue was chilled (Fig. 5). After 2 min, and 2, 14 and 24 h

of chilling at 6°C, recovery took 4, 15, 25 and 35 min to reach fairly stable rates of 4.3 ± 0.5, 4.5 ± 0.4, 3.8 ± 0.4 and $3.2\pm0.5\,\mu m\,s^{-1}$, respectively. Rates of streaming continued to slowly increase, reaching 4.5 ± 0.4, 5.2 ± 0.6, 4.6 ± 0.7 and $4.2\pm0.7\,\mu m\,s^{-1}$ after 180 min for tissue chilled for 2 min, and 2, 14 and 24 h, respectively.

The rate of protoplasmic streaming in *T. virginiana* stamen hair cells declined with temperature (Fig. 4), but streaming was still vigorous, although slower than controls, after 7 days at 6°C. This observation contrasts with the precipitous decline reported by Patterson and Graham (1977) for *T. albiflora*. Our data on streaming and ion leakage clearly show that *T. virginiana* does not exhibit the symptoms (i.e. cessation of protoplasmic streaming in stamen hairs and increased ion leakage from leaf tissue) that are characteristic of chilling-sensitive species.

Effect of heat shock and chilling on ion leakage

A heat shock at 45°C for up to 20 min had no significant effect on the rate of ion leakage from non-chilled *S. ionantha* petiole segments held at room temperature for 24 or 48 h (Fig. 6). In contrast, it had a significant effect on tissue chilled at 6°C. Chilling for 24 and 48 h caused a 2.6- and 4.3-fold increase, respectively, in the rate of ion leakage. In tissue chilled for 48 h, the chilling-induced increase was significantly reduced by 34% from 4.3 to 3.2% total conductivity per hour and by about 50% to 2.1% total conductivity per hour by 2 and 4 min of heat shock, respectively. The optimum heat-shock treatment appeared to be around 10–12 min, at which time the rate of leakage from chilled tissue remained relatively constant, but slightly higher than that from non-chilled tissue. Heat-shock treatments that lasted longer than 12 min caused a slight, but non-significant
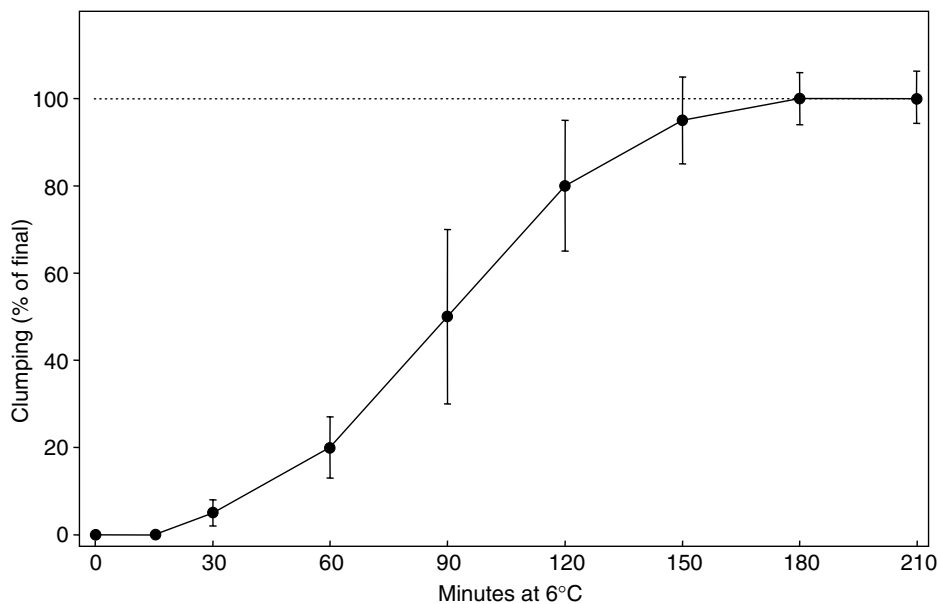

Fig. 3. Effect of chilling on the aggregation of chloroplasts in petiole trichomes of *Saintpaulia ionantha*. A subjective evaluation was made from observations and photomicrographs. The vertical lines at each data point represent the standard deviation about that mean.
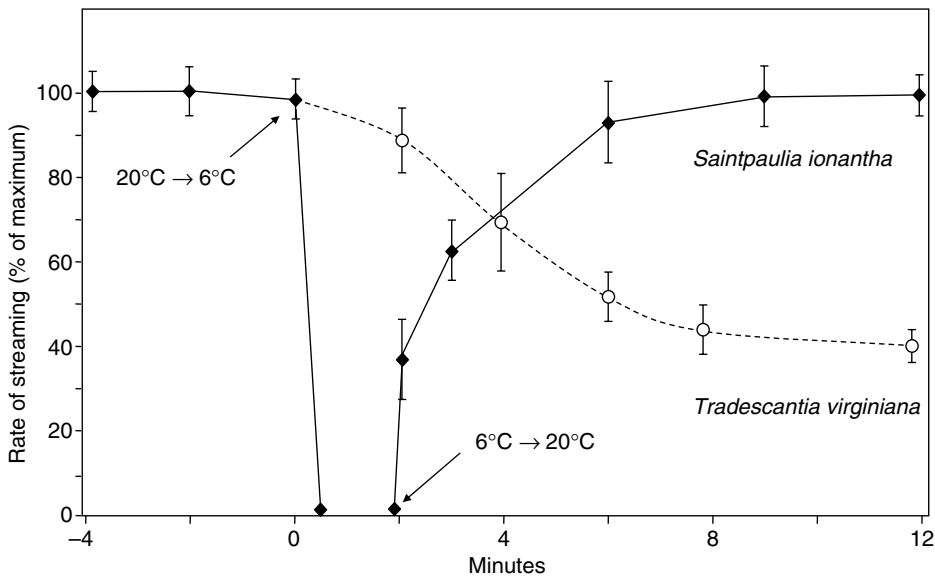
Fig. 4. Effect of changing the temperature from 20 to 6°C and from 6 to 20°C on the rated of protoplasmic streaming in stamen hairs of *Tradescantia virginiana* and petiole trichomes of *Saintpaulia ionantha*. Rates of streaming were converted to percentage of maximum so data from *Tradescantia virginiana* and *Saintpaulia ionantha* could be compared. The vertical lines at each data point represent the standard deviation about that mean.

increase from chilled tissue. A heat shock of 6 min at 45°C was selected for subsequent experiments.

Effect of heat shock and chilling on protoplasmic streaming

Chloroplasts did not aggregate in heat-shocked and chilled *S. ionantha* trichomes. A heat shock at 45°C for 1 min immediately stopped protoplasmic streaming. Protoplasmic strands were still evident in the trichome cells, but all directed movement was absent. Chilling at 6°C for up to 48 h failed to cause the aggregation of chloroplasts in *S. ionantha* trichomes that had been heat shocked for 1–6 min and held at approximately 20°C for 1.5 h before being chilled.

Recovery of protoplasmic streaming from a 6-min heat shock at 45°C took about 10 min in stamen hairs of *T. virginiana,* whereas it did not start until after 60 min in petiole trichomes of *S. ionantha* and took about 3 h to reach initial rates of streaming (Fig. 7). However, the chilling-induced loss of protoplasmic streaming (Fig. 8) was slower, and its recovery from chilling (Fig. 9) was faster following heat shock in trichomes of *S. ionantha*, than in untreated controls. Nevertheless, the differences were not as great as they were for reductions in ion leakage caused by similar heat-shock treatments (i.e. a 50% reduction). Shorter (2 and 4 min) and longer (8 and 10 min) exposures to 45°C did not significantly alter the inhibition or recovery of protoplasmic streaming in petiole trichomes of *S. ionantha* chilled at 6°C (data not shown).
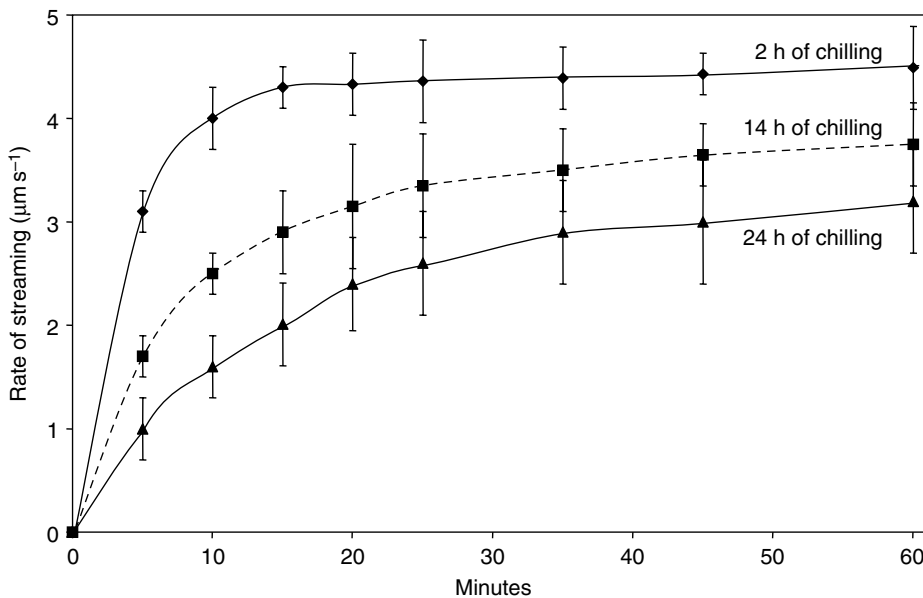

Fig. 5. Recovery of protoplasmic streaming in petiole trichomes of *Saintpaulia ionantha* at 20°C after chilling at 6°C for 2, 14 and 24 h. The vertical lines at each data point represent the standard deviation about that mean.
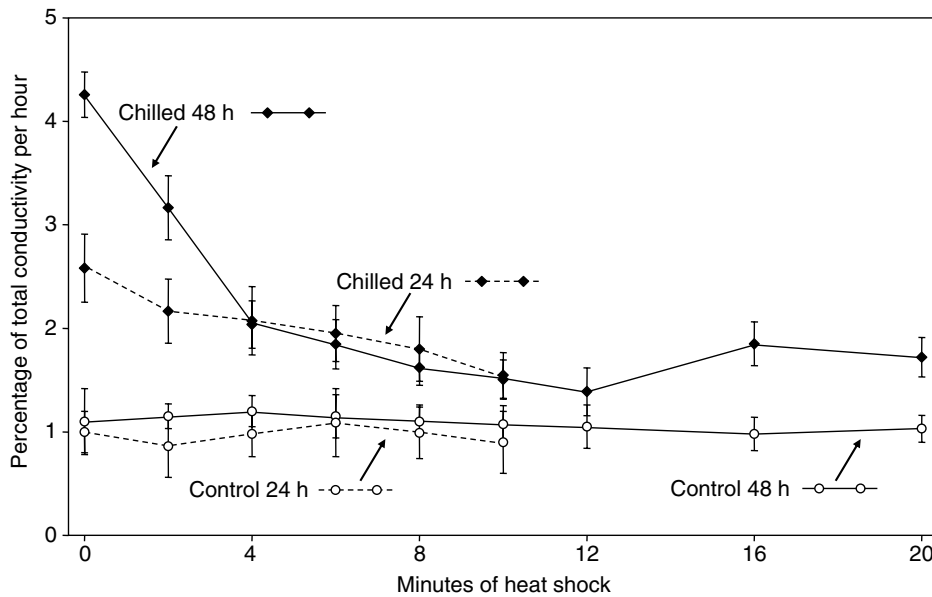
Fig. 6. Effect of heat shock at 45°C for 0–20 min on the subsequent rate of ion leakage from 1-cm segments of *Saintpaulia ionantha* petioles held at 20°C or chilled at 6°C for 24 or 48 h. The vertical lines at each data point represent the standard deviation about that mean.

Discussion

A 6-min heat shock at 45°C significantly reduced ($> 50\%$) the rates of ion leakage from *S. ionantha* petiole segments that were subsequently chilled at 6°C for 2 days. However, this same heat-shock treatment was far less effective at mitigating the inhibition of protoplasmic streaming in petiole trichomes of *S. ionantha* caused by chilling. Chilling-induced changes in membrane permeability (i.e. increased ion leakage) are thought to be caused by membrane phase transitions or by oxidative damage associated with metabolic dysfunctions caused by chilling. It is also widely recognized that chilling induces abrupt increases in the intracellular concentration of calcium ions (Ca^{2+}) (Knight et al. 1991, Knight

and Knight 2000). Indeed, the Ca^{2+} elevations may derive directly from the chilling-induced increase in membrane permeability, which given the much higher concentration of Ca^{2+} outside the cell than inside, will allow its rapid influx into the cytosol. Elevated cytosolic levels of Ca^{2+} are a well-known inhibitor of protoplasmic streaming. The ion may exert a direct effect on actin microfilaments and cause their fragmentation (Kohno and Shimmen 1988), or, as seems more likely, it may inhibit the actin-associated motor protein, myosin (Shimmen et al. 2000, Yokota 2000).

The chilling/calcium connection may not be the whole story since low temperatures also affect cytoplasmic pH, and acidification from seven to six blocks streaming
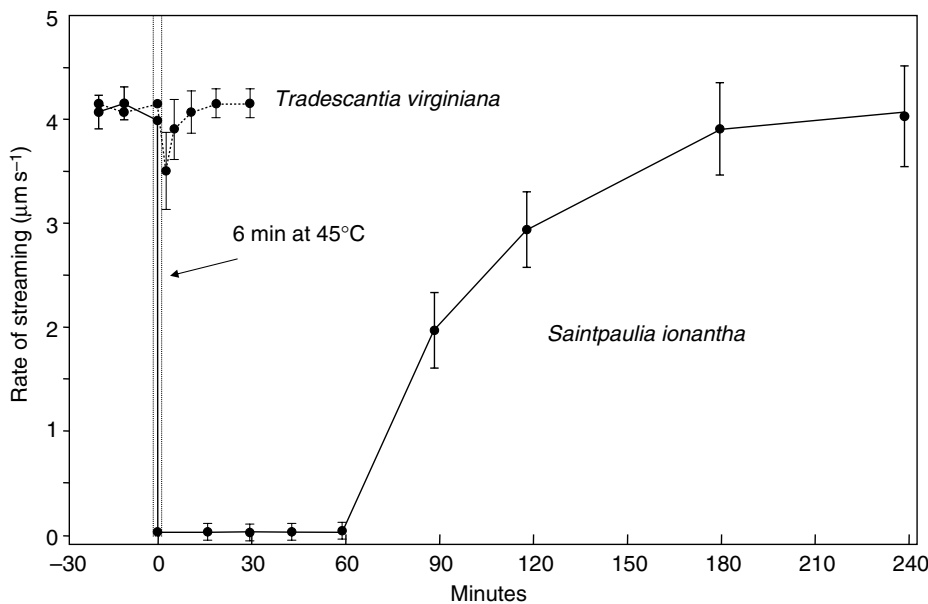

Fig. 7. Effect of heat shock at 45°C for 1 min on the subsequent rate of protoplasmic streaming in stamen hairs of *Tradescantia virginiana* and petiole trichomes of *Saintpaulia ionantha*. Tissue was heat shocked at 45°C for 1 min and then held at 20°C. The vertical lines at each data point represent the standard deviation about that mean.
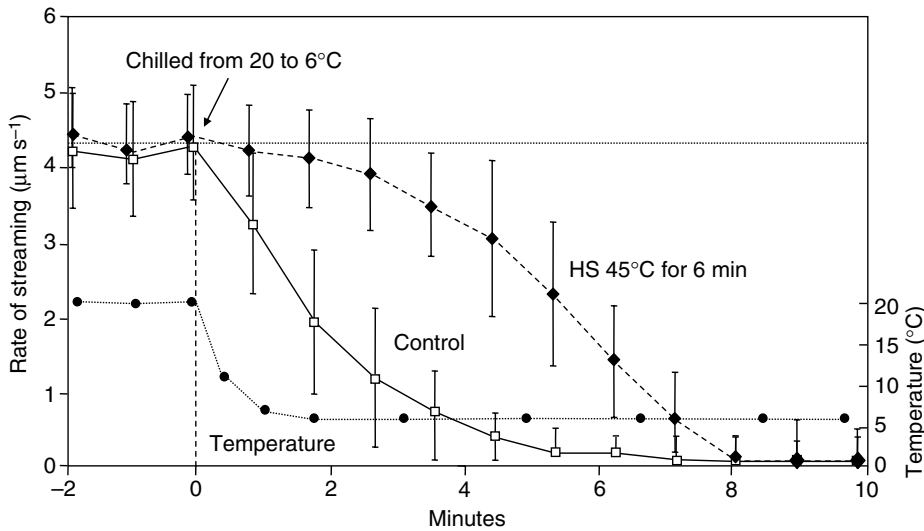
Fig. 8. Effect of heat shock and chilling on the subsequent rate of protoplasmic streaming in petiole trichomes of *Saintpaulia ionantha*. Petiole trichomes were heat shocked at 45°C for 0 (control) or 1 min and then chilled at 6°C. The vertical lines at each data point represent the standard deviation about that mean.

(Shimmen and Yokota 1994). Finally, it should be noted that, independently of its effect on acto-myosin, cold temperatures biophysically weaken hydrophobic interactions between tubulin dimers, and could contribute to microtubule depolymerization and associated changes in physiological processes (Inoue 1963).

Chilling causes an increase in intracellular calcium in both chilling-sensitive and chilling-tolerant plants. For example, chilling produced a transient rise in cytosolic calcium in chilling-tolerant *T. virginiana* stamen hair cells, which was associated with a loss of protoplasmic streaming (Hepler and Wayne 1985). A rise in cytosolic calcium and a reduction in protoplasmic streaming also occur in heat-shocked tissue. We found that chilling-tolerant tissue (e.g. *T. virginiana* stamen hair cells) was less affected and recovered more quickly than

chilling-sensitive tissue (petiole trichomes of *S. ionantha*) to both chilling (Fig. 4) and heat shock (Fig. 7).

The difference between our observation that *T. virginiana* is chilling tolerant, and that of Patterson and Graham (1977) that *T. albiflora* is chilling sensitive probably results from species differences. In their paper, Patterson and Graham (1977) also showed that two species of *Lysopersicon* (*L. esculentum* and *L. hirsutum*) exhibited different levels of chilling sensitivity, and that within *L. hirsutum*, chilling sensitivity was greater in plants collected from lower elevations (e.g. 400 m) with warmer environments than from plants collected from higher elevations (e.g. 3100 m) with colder environments.

A number of abiotic stresses (e.g. chilling, cold shock, heat shock, oxidative and touch) produce a transient rise in cytoplasmic calcium (Minorsky 1989, Knight et al.
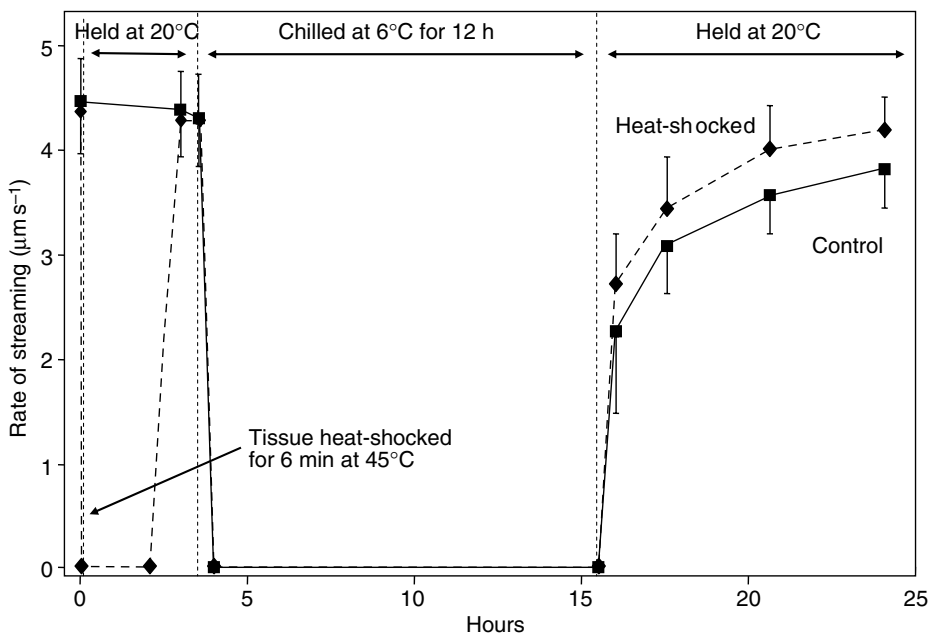

Fig. 9. Effect of heat shock at 45°C for 1 min on the subsequent rate of protoplasmic streaming in petiole trichomes of *Saintpaulia ionantha*. Petiole trichomes were heat shocked at 45°C for 0 (control) or 1 min and then held at 20°C for 4 h. They were chilled at 6°C for 12 h and then held at 20°C for an additional 18 h. The vertical lines at each data point represent the standard deviation about that mean.

1991, Braam 1992, Price et al. 1994). This rise in cytosolic calcium is thought to cause the cessation of protoplasmic streaming (Hepler and Wayne 1985). Different abiotic stresses appear to influence different pools of intracellular calcium. Price et al. (1994) found that inhibitors had differential effects on the increase in cytoplasmic calcium caused by cold shock, touching or oxidative stress in tobacco seedlings suggesting that these three stresses mobilized different pools of intra-cellular calcium.

In *S. ionantha*, chilling induced an immediate cessation of protoplasmic streaming in trichomes (Fig. 4), a slow aggregation of chloroplasts (Figs 2 and 3), and a later increase in ion leakage from petiole segments (Fig. 1). A 6-min heat shock at 45°C decreased chilling-induced ion leakage (Fig. 6) by about 50% and prevented chloroplasts aggregation, but had a minimal effect on the cessation (Fig. 8) or recovery (Fig. 9) of protoplasmic streaming. If rates of streaming can be used as a natural indicator of intracellular Ca^{2+} levels (Hepler and Callaham 1987, Shimmen and Yokota 1994), then it appears that the heat-shock treatment had minimal effect on the ability of chilling temperatures to cause an increase in cytoplasmic calcium, while significantly altering the physiological response to chilling that manifests itself as increased ion leakage. However, this conclusion needs to be tested directly using intracellular Ca^{2+} indicators in cells subjected to a heat shock/chilling regime.

Heat shock (and other abiotic shocks such as exposure to cold, ethanol, arsenite and hypo- or hyper-tonic solutions for a few minutes) induces cells to synthesize and accumulate a unique set of proteins (Vierling 1991). Proteins induced by heat shocks (i.e. hsps) are the most extensively studied group of these stress-induced proteins. The presence of hsps in previously heat shocked cells appears to confer resistance to subsequent thermal stresses, and some other abiotic stresses by inhibiting protein denaturing following exposure to extreme temperatures. The protective effects of the induced hsps may not extend to protecting those physiological pathways that control cytosolic calcium concentrations during periods of stress.

If chilling acts through an increase in cytosolic free calcium, as proposed by Minorsky (1989), then heat shock apparently has a very limited effect on the chilling-induced increase in cytosolic calcium since a heat shock treatment that substantially reduced chilling-induced ion leakage had a minimal effect on altering chilling-induced cessation of protoplasmic streaming. Heat shock appears to affect the intervening steps between the initial perception of chilling temperatures (e.g. alteration of membranes or ion channels that allow an increase in cytosolic calcium) and the development of observable physiological responses (e.g. increased ion leakage or reduced radicle elongation). Supporting this contention is the observation that heat-shock treatments are almost as effective in reducing the effects of chilling when administered after chilling as when applied before chilling (Saltveit 2001).

Chilling tolerance (e.g. reduced chilling-induced ion leakage, and other symptoms such as reduced radicle growth and fruit ripening) induced by heat shock may arise through a different set of physiological pathways than those controlling cytosolic calcium concentrations. Part of the ability of tissue to tolerate chilling (and possibly other abiotic stresses such as heat shock) could reside in the tissues' ability to quickly reverse the rapid rise in cytosolic calcium that appears to be ubiquitous among the tissues' initial response to these stresses.

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