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Heat-induced protection against death of suspension-cultured apple fruit cells exposed to low temperature

C. Y. WANG,* J. H. BOWEN, I. E. WEIR, A. C. ALLAN & I. B. FERGUSON

The Horticulture and Food Research Institute of New Zealand, Private Bag 92169, Auckland, New Zealand

ABSTRACT

Death of suspension-cultured apple fruit cells (*Malus domestica* Borkh. cv. Braeburn), resulting from exposure to low temperature (1 °C), was reduced by a prior 1 h/38 °C treatment. Pre-heated chilled cells produced less ethylene, indicating lower chilling injury. Heat treatment resulted in increased transcripts for a low molecular weight (hsp17) and a 70 kDa heat shock protein (hsp), but expression weakened within 24 h following the transfer of cells to 25 °C. When cells were instead transferred to 1 °C after heat, elevated hsp gene expression persisted for up to 3 d. Flow cytometric analysis of apple cell protoplasts showed that the 1 °C treatment resulted in a subpopulation of protoplasts undergoing cell death, not observed with the 38 °C pre-treatment. Chromatin condensation did not occur with the heat treatments. These results suggest that heat-induced reduction of cell death at low temperatures may involve persistence of hsps, and low-temperature susceptibility is less likely to be associated with programmed cell death. All cells do not respond in the same way, their tolerance being associated with different states of metabolism or of the cell cycle.

Key-words: Apoptosis; cold stress; flow cytometry; heat shock proteins; programmed cell death.

INTRODUCTION

Cell death from low temperature has usually been associated with membrane dysfunction, enzyme inactivation, and disruption of a range of other physiological and biochemical processes, although no complete, definitive description of the death mechanisms has been provided. Recent interest in the process of programmed cell death (PCD) is leading to closer examination of the mechanisms of death in a range of circumstances. Cell death in mammalian cells can occur either through necrosis, a traumatic physical process not under molecular control, which includes collapse of membrane function and cell metabolism, cell swelling, loss of compartmentation, loss of selective permeability of the plasma membrane, and no obvious early changes in DNA (Dive *et al*. 1992), or by a genetically programmed process, which includes apoptosis. This latter process comprises chromatin condensation, cell shrinkage, nucleosomal DNA fragmentation with disintegration of the nucleus, dissipation of cell contents into discrete apoptotic bodies, and ultimately death. Various parts of this process have been demonstrated in plant cells (Pennell & Lamb 1997), and the temporal progress of the nuclear and signalling changes has been charted in our recent flow cytometric studies (O'Brien, Reutlingsperger & Holdaway 1997; O'Brien *et al*. 1998a,b).

The importance of a description of cell death in response to stresses such as extreme temperatures, lies in our need to understand tolerance mechanisms. We have recently suggested that observed reversibility of early stages of PCD may be associated with stress tolerance (O'Brien *et al*. 1998b). The ability of cells to recover from early increases in chromatin condensation (and associated loss in transcriptional capacity) may be related to the ability to recover from permissive stress levels. We can hypothesize that there is an association among induction of specific protective proteins such as heat shock proteins (hsps) or protein products of cold-resistant genes, recovery from immediate changes in general protein breakdown and synthesis (e.g. Ferguson, Lurie & Bowen 1994), and recovery from changes in nuclear DNA status.

Low-temperature tolerance can be induced in plants in a number of ways, including acclimation or preconditioning (mostly demonstrated in relation to freezing tolerance; Hughes & Dunn 1996; Thomashow 1998, 1999), and treatment with regulators such as abscisic acid and methyl jasmonate (Wang & Buta 1994). Although induction of tolerance is considered to involve specific proteins (Hughes & Dunn 1996; Thomashow 1998), or metabolites such as polyamines (Kramer & Wang 1989) and antioxidants (Van Breusegem *et al*. 1999), specific mechanisms of protection remain largely speculative.

Chilling injury in fruits such as tomatoes (Saltveit 1991; Lurie & Sabehat 1997), avocados (Woolf *et al*. 1995) and persimmons (Woolf *et al*. 1997), and in other plant tissues (e.g. mung bean hypocotyls; Collins, Nie & Saltveit 1995), can be reduced by prior treatment with high temperatures. As expected, preconditioning heat treatments of fruits results in induction or up-regulation of hsp gene expression and accumulation of the gene products (Lurie 1998). Although such proteins are known to provide protection, often acting as chaperones, or as in the case of ubiquitin,

Correspondence: Dr Ian Ferguson. Fax: + 64 9815 4202; e-mail: iferguson@hortresearch.co.nz

*Permanent address: Beltsville Agricultural Research Center, ARS, USDA, Bldg 002, 10300 Baltimore Ave, Beltsville, MD 20705-2350, USA.

facilitating removal of heat-damaged proteins, there have been suggestions that they also provide protection against low-temperature damage. The up-regulation of hsp transcripts after heat treatment of both tomatoes and avocados is sustained during subsequent low-temperature storage (Lurie *et al*. 1993; Woolf *et al*. 1995; Sabehat, Weiss & Lurie 1996). A similar stimulation and maintenance has been found in apple fruit in the field experiencing high afternoon and subsequent cooler night temperatures (Ferguson *et al*. 1998). Heat shock proteins themselves have also been shown to persist during low-temperature storage of tomatoes after heat treatment (Sabehat *et al*. 1996). Aside from the effect of heat, RNA levels of members of the hsp70 family in tomato and spinach leaves can be enhanced within 24–48 h by low temperature (5 °C) alone (Anderson *et al*. 1994; Li, Haskell & Guy 1999), and there are other examples of cold-induction of hsp genes (Thomashow 1999).

Low-temperature tolerance therefore is likely to be the result of a number of interlinked responses, including specific proteins, metabolites and physical membrane changes. In studying further the interaction between high temperature and tolerance to low temperatures, our aims were twofold. The first was to see whether high temperature reduces low-temperature-induced cell death in cultured apple fruit cells, thus providing a model system for what has been described in whole fruit. The second was to use our current knowledge of PCD and of temperature-tolerance mechanisms to see whether a cytological approach to low-temperature response could provide new information on mechanisms of tolerance and cell death. For the latter work, we have used shorter-term experiments with apple cell protoplasts and flow cytometry, which allows for single cell analysis of intracellular events, and identification of subpopulations of cells responding in different ways.

MATERIALS AND METHODS

Plant cells and protoplasts

Apple fruit cells (*Malus domestica* Borkh. cv. Braeburn) were maintained in suspension culture in the same medium and under the same conditions as for pear cells (Ferguson *et al*. 1994). Protoplasts were isolated and washed according to O'Brien & Lindsay (1993) except that cellulase (Worthington Biochemical Corp, Lakewood, NJ, USA) was used instead of Cellulysin (Calbiochem, La Jolla, CA, USA) due to possible DNAse activity in the latter. Protoplasts were held in the wash solution (O'Brien & Lindsay 1993) for all experiments and for labelling with fluorescent markers. Early log phase cells were used for all experiments.

Temperature treatments

Cells (20 mL) from the suspension cultures were placed in 50 mL Erlenmeyer flasks, in triplicate. When protoplasts were used, 5 mL of protoplasts were held in 25 mL Erlenmeyer flasks. The following four temperature treatments were used: 25, 1, 38 °C for 1 h then transfer to 25 °C, and 38 °C for 1 h and then transfer to 1 °C. The 25 °C temperatures were provided by the normal culture conditions. For the heat treatment, the flasks were held in a heated shaking water bath (38 °C ± 0·5 °C), and the low-temperature treatment provided by shaking the flasks on a platform shaker in a cold room (1 °C ± 0·5 °C). Temperatures were monitored using thermistor probes and a data-logger (Squirrel CM-UU-V5–1; Model 1206; Grant Inc. Cambridge, UK). The 38 °C/1 h treatment was chosen from preliminary work as a permissive heat treatment which these apple cells survive, yet which induces thermotolerance allowing the cells to survive higher temperatures such as 46 °C (Bowen 2000). It is also a permissive heat shock temperature for similarly cultured pear fruit cells (Ferguson *et al*. 1994).

Cell and protoplast viability was measured by placing 100 μL of cells on a microscope slide together with 5 μL of Evan's blue dye (0·5% in 4% v/v glycerol). The percentage of live cells (those remaining unstained) was determined from a minimum of five replicate microscope fields at each sampling time. We have found that viability measured with Evan's blue was similar to that recorded by growing cells after stress treatment and measuring growth (Bowen 2000). Further viability data were provided by flow cytometric measurements using fluorescein diacetate (FDA) and propidium iodide (PI) (see below).

To measure ethylene production in cells and protoplasts, the flasks were sealed with rubber septa, and after times of up to 1 h, headspace gas samples were taken by syringe. Ethylene concentrations were determined by gas chromatography with a flame ionization detector and alumina column (Philips PU4500; Unicam, Cambridge, UK).

Flow cytometry

Protoplasts were analysed using a Coulter EPICS Elite ESP flow cytometer (Beckman Coulter, Hialeah, FL, USA) fitted with a 140 μm quartz flow cell tip to enable analysis of large cells. Throughout this study, the air-cooled 15 mW 488 nm argon laser was used. All protoplasts were labelled in protoplast wash solution (O'Brien & Lindsay 1993). Fluorescent probes were purchased from Molecular Probes Inc (Eugene, OR, USA) and prepared according to the manufacturer's specifications. Loading into cells was confirmed by fluorescent microscopy using a Leitz Fluovert FS microscope equipped with a high-pressure 50 W mercury arc illumination source (Leica Microsystems AG, Wetzler, Germany).

For all protoplast measurements, unstained protoplasts were used as autofluorescence controls in order to set baseline fluorescence. Forward scatter (FS) is a measure of the amount of laser light which passes through the cell at the point of interrogation, and is proportional to cell size; the greater the FS, the larger the cell. Side scatter (SS) indicates the granularity of the cellular contents, the greater the SS, the more dense or granular the cell.

Chromatin condensation and DNA breakdown were measured using the intercalatory DNA probe propidium iodide (PI), with fluorescence measured using a 610 bp fil-

ter. Nuclei were isolated by cell lysis using Triton X-100 (O'Brien *et al*. 1998a), prior to labelling with 50 μg mL^{-1} of PI. The histograms depict fluorescence peaks for G_0/G_1, with the channel numbers referring to quantitative measures of PI fluorescence intensity.

Protoplast viability was measured by dual staining with FDA (1 μg mL^{-1}) and PI (5 μg mL^{-1}). FDA is taken up by cells and cleaved by intracellular esterases, with resulting fluorescence and cleavage products retained in viable cells. PI is only taken up into cells when membranes become dysfunctional. Therefore these probes provide information on the integrity of the plasma membrane, and thus cell viability. The protoplasts were labelled for 10 min, with fluorescence measured using a 525 bp filter (FDA) and a 610bp filter (PI). Fluorescence compensation was used to subtract 10% of PMT-4 fluorescence (610 bp) from PMT-2 (525 bp).

To detect annexin V binding to phosphatidylserine, protoplasts were incubated for 5 min with fluorescein-labelled annexin V protein (APOPTEST™-FITC, NeXins Research BV, Hoeven, The Netherlands), in modified protoplast wash containing 3 mol m^{-3} $Ca(NO_3)_2$ (O'Brien *et al*. 1997), and PI (5 μg mL^{-1}). The protoplasts were analysed by flow cytometry with FITC fluorescence measured using a 525 bp filter and PI using a 610 bp filter. Fluorescence compensation was used to subtract 10% of PMT-4 fluorescence (610 bp) from PMT-2 (525 bp).

All flow cytometric measurements were carried out on duplicate samples, and experiments were repeated at least twice.

Northern analysis

Cells were harvested by filtration, frozen in liquid nitrogen, and stored at –80 °C. Total RNA was extracted (Lopez-Gomez & Gomez-Lin 1992), denatured, fractionated and transferred to a Hybond®-N + membrane (Veierskov, Ferguson & Lay-Yee 1992). The following cDNA clones, all from apple fruit, were used for gene expression analysis: Mdhsp1 (Genbank accession number AF161179) with homology to Class 1 low molecular weight hsps; Mdhsp2 (AF161180) with homology to cytosolic hsp70; pBG1, encoding ubiquitin (U74358). The cDNA inserts were labelled with ^{32}P using a random primers DNA labelling system. Hybridization was carried out according to the Amersham Hybond®-N + protocol. After hybridization, the membrane was washed twice for 10 min in 2 × sodium chloride/sodium citrate (SSC) and 0·1% sodium dodecyl sulphate (SDS) at room temperature, then twice in 1 × SSC and 0·1% SDS at 65C for 15 min. The membrane was exposed to Kodak XAR film with intensifying screens at –80 °C. RNA loadings for the membranes were determined by hybridization with an 18S ribosomal probe (Simon & Weeden 1992). Autoradiographs were processed using densitometry software to quantify the observed signal. Signal intensities were adjusted for differences in RNA loadings, and the normalized values are presented as bar graphs.

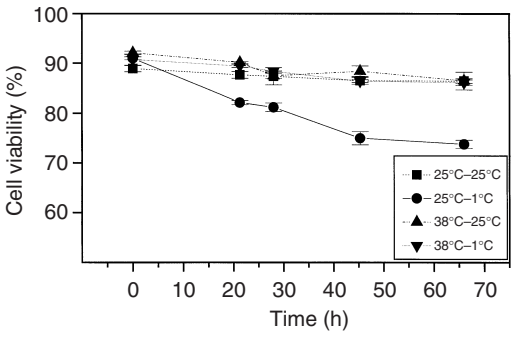

Figure 1. Viability of apple fruit cells under different temperature regimes. Viability was assessed by staining with Evan's blue. Error bars denote ±SE.

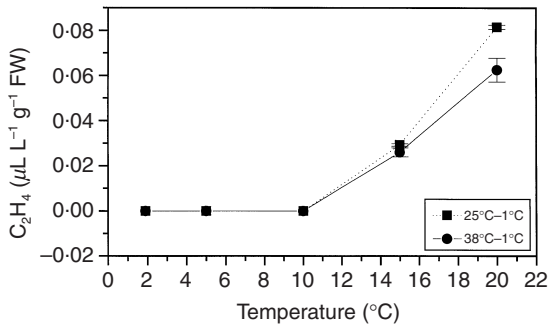

Figure 2. Ethylene production by apple fruit cells measured after removal from being held at 1 °C for 3 d. Temperatures along the *x*-axis are those measured as the cells warmed up. Error bars denote ±SE.

RESULTS

Cell survival and low-temperature response

Cell viability, as measured by staining with Evan's blue, declined from 90 to 73% over 65 h at 1 °C (Fig. 1). There was little change in viability over this time in cells held at 25 °C. A 1 h 38 °C heat treatment prior to transfer to 1 °C protected cells from dying at low temperature, viability (86% at 65 h) being no different from that of cells at 25 °C, or transferred to 25 °C after the 38 °C (Fig. 1). This latter treatment showed that there was no specific effect of the heat treatment on viability at normal growth temperatures

A characteristic chilling response in fruit tissues is stimulation of ethylene production upon warming, probably an indication of low-temperature injury to cells (Wang & Adams 1982). After being held at 1 °C for 3 d, apple cells were removed and allowed to warm up. Ethylene production by the cells at temperatures below 10 °C was not detected (Fig. 2). As the cells warmed up, ethylene accumulated to detectable concentrations by the time 15 °C was reached (30 min), and continued to increase as the cells warmed to room temperature (60 min). A 1 h 38 °C treatment prior to chilling resulted in slightly lower levels of ethylene production during this warm-up period.

Table 1. Viability of apple cell protoplasts after high- and low-temperature treatments. Viability was measured with Evan's blue staining. Viability of the control (25 °C) protoplasts was unchanged over the 24 h period

Treatment	Viability (%)
25 °C	92[a]
After 1 h at 38 °C	89[a]
25 °C	81[b]
After 24 h at 1 °C	64[c]
After 1 h at 38 °C prior to 24 h at 1 °C	79[b]

Data are from different experiments, and for either experiment, different letters denote significant differences at the 5% level.

Protoplasts

The suspension-cultured apple cells cannot be used directly for flow cytometry since they clump and will not pass through the flow cytometer as single cells. Protoplasts, however, can be used with considerable facility, since not only are they individual, but are more easily loaded with fluorescent markers. Nuclei can also be prepared from protoplasts in a few minutes; to prepare them from whole cells, protoplasts have to be made first, precluding short time intervals from treatment to analysis. Apple cell protoplasts treated with the same temperature regimes as described above for the cells, produced the same heat/low-temperature effects, although the time scale was reduced to 24 h. Protoplast data from four different experiments showed that at 1 °C, viability decreased over 24 h from 81 to 64%, whereas a 1 h treatment at 38 °C protected protoplasts from death (Table 1). There was no decrease in viability over the first 4 h at low temperature (data not shown), or immediately after the heat treatment (Table 1). Experiments were not carried on further than 24 h because of possible loss of viability in control protoplasts.

Flow cytometric analysis of viability

Integrity and survival of cells and protoplasts can be assessed with the use of fluorescent markers. FDA is taken up by all protoplasts in a non-polar esterified form, and is then de-esterified by non-specific esterases in the cytosol. The resulting polar fluorescein will be retained longer in cells with intact functional membranes, but quickly leaks out of dead or dying cells. PI is not taken up by cells or protoplasts which have fully functional and structurally intact plasma membranes, but is taken up when there is a loss of selective permeability.

Control protoplasts at 25 °C displayed a single, normally distributed population stained with both FDA and PI [Fig. 3 (A2,A4)]. The plot of SS against FDA and PI shows a population centred with relatively low SS properties [Fig. 3 (A1,A3)]. After 24 h at 1 °C, a large subpopulation of cells showed up as a second peak with greater fluorescence [Fig. 3 (B2,B4)], and low SS properties, suggesting less dense protoplasts [Fig. 3 (B1,B3)]. A heat treatment of 38 °C for 1 h prior to 24 h at 1 °C prevented this subpopulation from forming [Fig. 3 (C1–C4)]. The subpopulation appearing with the low temperature treatment constituted about 50% of the total protoplast population. These protoplasts were also larger since they had relatively high FS properties [Fig. 3 (D)]; they can be identified by back-gating from a forward scatter/side scatter (FS/SS) plot. Back-gating the subpopulation designated as 'o' [Fig. 3 (D)] showed that the new subpopulation [Fig. 3 (B)] comprised these larger cells.

When the protoplasts were warmed up to 25 °C for 4 h after the 24 h period at 1 °C, the subpopulation that showed greater fluorescence with FDA in the no-heat treatment (Fig. 4a) was largely lost (Fig. 4b). FDA staining of the control and pre-heated protoplasts was unchanged over this period (data not shown).

Chromatin condensation

PI staining of isolated nuclei provides a measure of the degree of chromatin condensation or coiling of DNA on the histone backbone. There was no change in intensity of the G_0/G_1 PI fluorescence peak in response to low temperature (data not shown). This would be indicated by a change in peak position along the *x*-axis (reduction in fluorescence to the left). However, treatment for 1 h at 38 °C resulted in an increase in fluorescence, signified by a shift in the G_0/G_1 peak to the right (compare Fig. 5a,b). This shift suggests a relaxing of DNA coiling due to the heat treatment, which is maintained over the subsequent low-temperature period. The shifts can also be expressed in terms of channel numbers along the *x*-axis (the total being 1024). Directly after the 1 h treatments, the G_0/G_1 peak was 263 for the 25 °C treatment (Fig. 5a) and 279 for the heat treatment (Fig. 5b). Similar differences were found in repeat experiments.

Annexin V binding

Binding of annexin V to phosphatidylserine exposed on the outer surface of the plasma membrane is an early indicator of apoptosis in both animal and plant cells (O'Brien *et al.* 1997). We could not find any positive binding of annexin V in protoplasts under the different low-temperature treatments (data not shown). Protoplasts treated with hydrogen peroxide were used as a positive control (O'Brien *et al.* 1997).

Gene expression

The 38 °C heat treatment resulted in elevated levels of transcripts of both the low molecular weight (LMW) and hsp70 RNAs (Fig. 6).These elevated levels were found directly after the heat treatment (Fig. 6 Cont) and declined after 24 h in cells held at 25 °C. By contrast, holding the cells at 1 °C after the heat treatment resulted in maintenance of the elevated hsp RNA levels over the 3 d of the experiment. There was some variability in expression of the hsp RNAs at 25 °C over the 3 d of the experiment. When cells held at 1 °C for

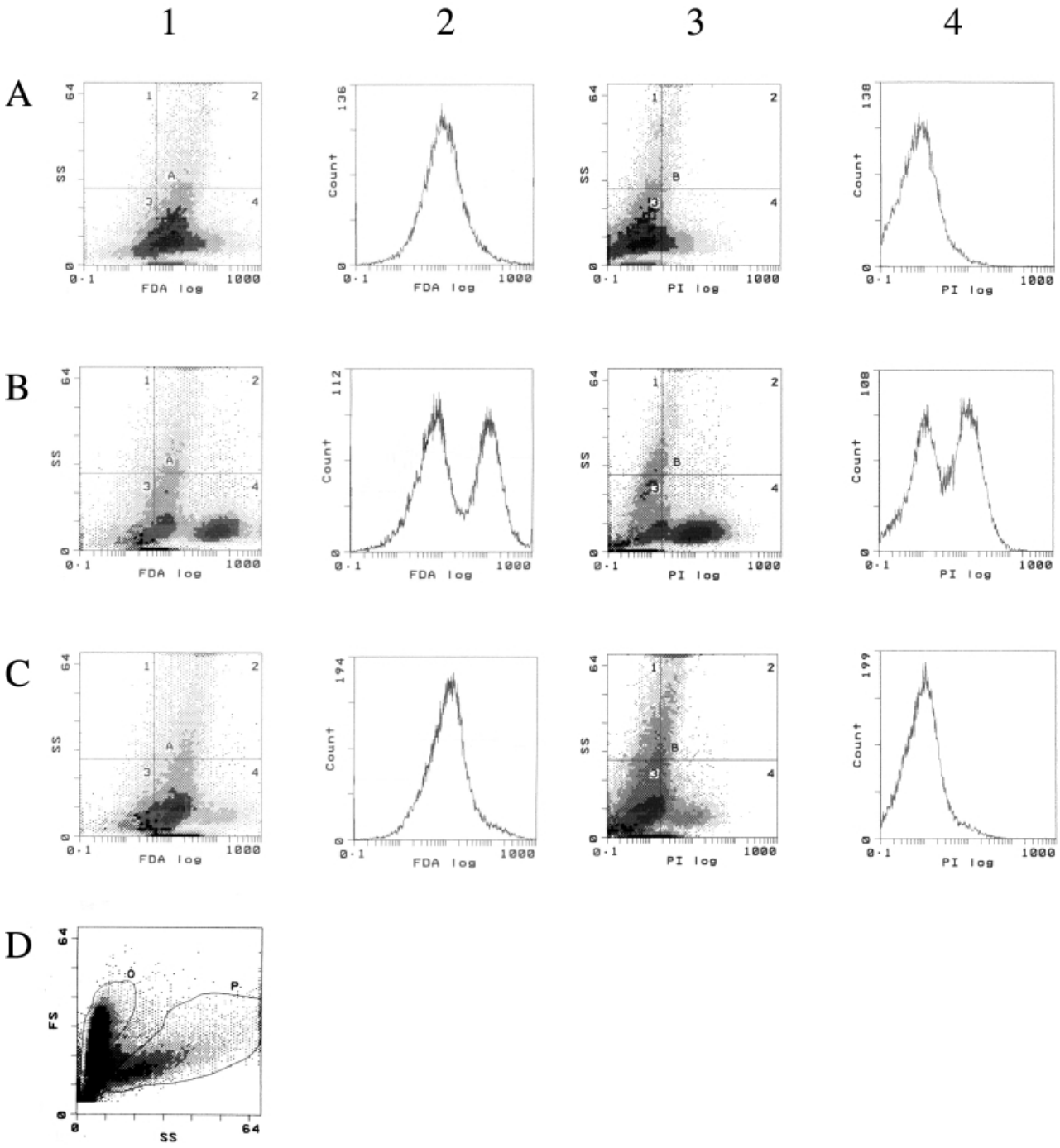

Figure 3. Fluorescence in protoplasts stained with FDA (1,2) and PI (3,4) after 24 h at 25 °C (A), 1 °C (B), and 1 °C with a prior treatment of 1 h at 38 °C (C). The 2-D plots (1,3) show side scatter (SS) properties against FDA and PI, and the histograms (2,4) show equivalent fluorescence distribution and intensities. Backgating the populations at 1 °C (D) showed that the protoplasts with high forward scatter (FS), gated as 'o', were related to the new necrotic population in quadrant 4 (B1,3).

3 d were moved to 25 °C, the elevated hsp transcripts were also maintained. Ubiquitin was strongly expressed constitutively (Fig. 6), and transcripts were not markedly higher with the heat treatment. Transcript levels declined with age of the cells.

DISCUSSION

Heat and cell survival

A heat treatment of 1 h at 38 °C provided protection from cell death at 1 °C. This effect confirms at a cellular level,

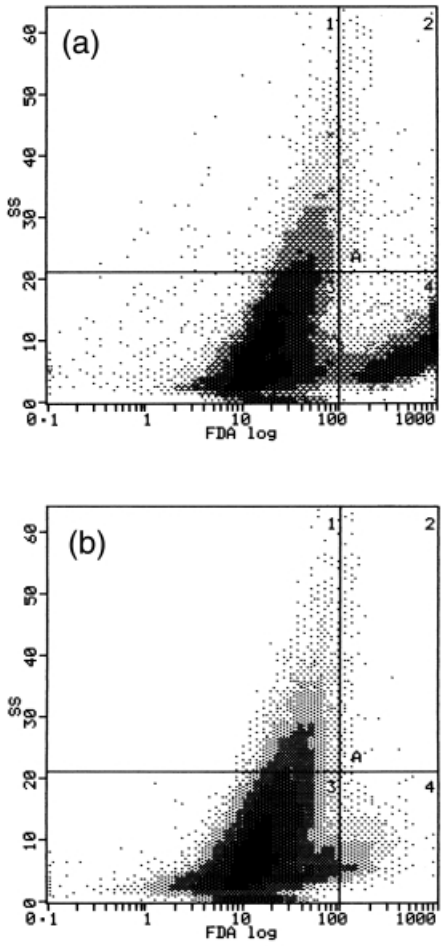

Figure 4. Fluorescence in protoplasts stained with FDA after 24 h at 1 °C (a) and after a further 4 h at 25 °C (b). The 2-D plots show side scatter properties against FDA.

what has generally been found for whole fruit – that a high temperature pre-treatment can protect against chilling injury. Low-temperature injury symptoms in whole fruit which can be reduced by heat treatments are various, including a failure to ripen (tomatoes, Lurie & Klein 1991), external skin browning (avocados, Woolf *et al*. 1995), flesh gelling (persimmons, Woolf *et al*. 1997), and surface pitting (zucchini, Wang & Qi 1997). This range of symptoms suggests either an equivalent range of protective physiological and biochemical effects from high temperature, or protection acquired at a basic cellular level (e.g. membranes or proteins), common to many tissues and manifest in different responses. Heat at 38 °C has been shown to be a permissive temperature (for up to 2 h) both for these apple cells (Bowen 2000) and suspension-cultured pear fruit cells (Ferguson *et al*. 1994). At 42 °C, cells begin to die, and temperatures above 44 °C are generally lethal.

As distinct from these effects at the whole tissue level, our results from analysis of cell or protoplast populations raises the question of individual cell response. The results suggest that low-temperature injury is occurring unequally in the cell population. Given that the culture is not synchronous and will have cells of differing ages, this may be expected (although it is not always appreciated). The effect of high temperature may be considered in two ways. It may be on the whole population, being protective where cells are predisposed to chilling injury. Alternatively, high temperature has a specific protective effect only on those cells identified as the subpopulation with high FDA signals, which have a particular sensitivity to low temperature. One interesting possibility is that this population of cells is at a different stage of the cell cycle.

The defining properties of this subpopulation are unknown, although physically sorting these cells in the future will help to identify specific features of them. We have observed in the past (unpublished results) that over the short time span of the assay, an increase in FDA fluorescence can be observed before there is a decay in signal with subsequent outward leakage. McAinsh *et al*. (1996) have also shown that at relatively high concentrations of externally applied H_2O_2 (> 10^{-5} M), membrane damage occurs in guard cells accompanied by diffuse fluorescence from FDA. It is also possible that there is increased non-specific FDA-cleaving esterase activity in susceptible cells

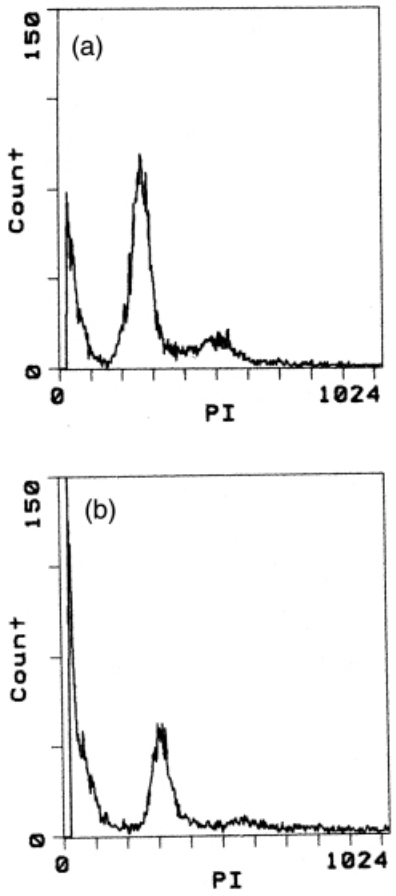

Figure 5. PI fluorescence of nuclei from apple cells extracted and stained after 1 h at 25 °C (a) or 38 °C (b). The peak in (a) has shifted to the right (more fluorescence), denoted by an increase in channel number (279 versus 263 for the 25 °C treatment).

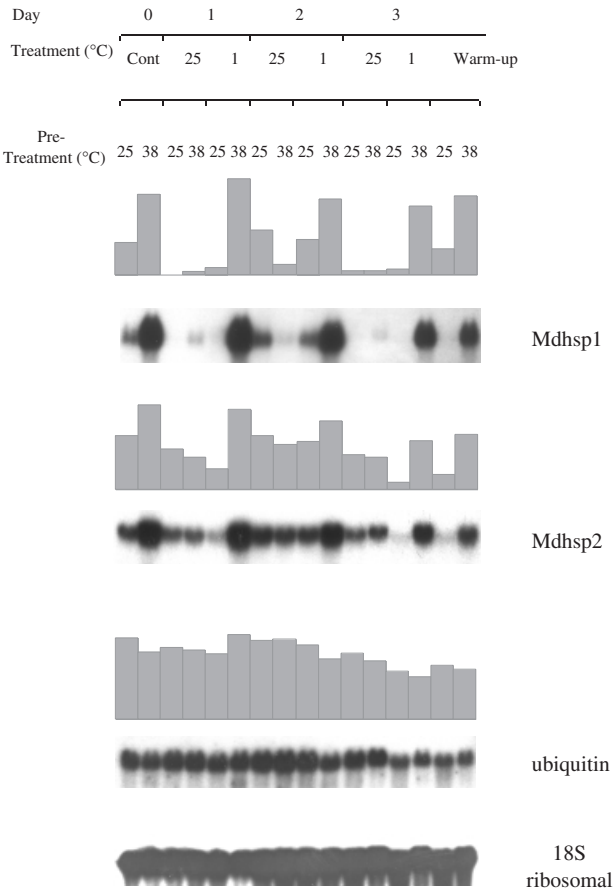

Figure 6. Northern analysis of apple low molecular weight hsps (Mdhsp1) and hsp 70 (Mdhsp2) transcripts and ubiquitin. Gene expression was followed over 3 d. Over this time, cells were treated at either 25 °C or 1 °C, and then warmed up to 25 °C after day 3. Prior to these treatments cells were given 1 h pre-treatments at either 25 or 38 °C. Thus the histograms and Northern blots refer to cells at either 25 or 1 °C which had previously been treated at 25 or 38 °C. Histograms show normalized signal intensities adjusted for differences in RNA loadings determined from ribosomal RNA levels.

which are becoming damaged. FDA is also pH sensitive, and changes in pH of the cytosol may influence fluorescence levels. However, we might expect some acidification with low temperature; FDA has been used to measure a reduction in intracellular pH with low temperature in cultured mung bean cells (Yoshida 1994). An increase in vacuolar pH has also been found with low temperature in cultured mung bean cells (Yoshida 1995), due to inhibition of the vacuolar H^+-ATPase. The data of Yoshida (1994) suggest that it is unlikely that there is any migration of fluorescent marker into the vacuole in the short time of the labelling and assay. We did not see, under the microscope, any label in the vacuole.

The loss of identity of the subpopulation after the 4 h warming to room temperature suggests that either these protoplasts collapsed and were unable to be labelled, or that the changes they underwent were reversible, and they recovered during this time. We have no evidence for reversibility, and such a process would mean that changes in membrane permeability would also have to be considered as reversible. It is more likely that when we observe the sensitive subpopulation, it is heading down a necrotic pathway. This is supported by the positive PI staining, and the fact that these cells have also become swollen, a characteristic feature of necrosis as compared to apoptosis.

In summary, the sensitive subpopulation of cells able to be protected from damage by heat, are larger, react to FDA more strongly, and are becoming necrotic. Whatever the reason, the marked effect of the heat treatment on this subpopulation provides a focus for understanding the beneficial effects of high temperature.

DNA changes

The mammalian paradigm for apoptosis includes chromatin condensation, visualized in flow cytometry by reduced fluorescence of extracted nuclei stained with PI. This fluorescence shift has been observed in plant cells responding to a number of cytotoxic reagents (O'Brien *et al*. 1998a,b). Such a change in itself is not enough to suggest the operation of the apoptotic process, but if observed, serves as an apoptotic indicator, and would suggest that it be worthwhile to further investigate other aspects such as DNA fragmentation.

The results from PI staining of nucleic DNA in response to low temperature and the protective heat treatment do not show an apoptotic-like increase in condensation. The only direct effect from the heat was a slight increase in fluorescence, suggesting some relaxation of DNA coiling. It is possible that with longer times, more breakdown or condensation would be evident. We could not identify any nuclear change associated with the protective effect of the heat treatment, other than the slight DNA relaxation. This may represent an increase in translational capacity. Certainly, the status of the DNA would be crucial to the efficiency of transcription factor binding, as for instance with heat-shock factors.

In cultured carrot cells, a heat-shock treatment of 55 °C for 10 min resulted in DNA fragmentation (assayed by *in situ* TUNEL) and cell death after cell shrinkage (McCabe *et al*. 1997). However, this treatment appears to have been a lethal treatment, as shown by the eventual 90% death rate, rather than permissive. DNA fragmentation was also found in tobacco protoplasts after 2 h at 44 °C and 24 h at 25 °C (Chen, Zhou & Dai 2000). Cell death rates were not given in this latter work, and it is difficult to determine whether the cells surviving the treatment were undergoing PCD if still alive after 24 h. In our earlier work (O'Brien *et al*. 1998b), we have suggested that stress tolerance may be manifest in the ability of a cell to recover from early stages of PCD, particularly early stages of chromatin condensation. However, there is nothing in the present results which suggest that heat-induced low-temperature tolerance is related to this. Given that there is other evidence for some PCD response to both high and low tempera-

tures in plant cells (McCabe & Leaver 2000), and the possibility of an interaction between hsp induction and inhibition of apoptosis in both animal and plant cells (Weir, personal communication; Charette *et al*. 2000), there is a need to look further at temperature/time combinations in different cell types to clarify the possible involvement of PCD. Few experiments have looked at this in a systematic way.

Hsp gene expression

The maintenance of elevated levels of hsp transcripts during low-temperature storage of fruit after heat treatments is a notable feature of experiments with tomatoes (Sabehat *et al*. 1996) and avocados (Woolf *et al*. 1995; Woolf, Bowen & Ferguson 1999). This has also been shown to occur in apple fruit on the tree where elevated transcript levels were maintained during lower night temperatures (Ferguson *et al*. 1998). In apple cells, low temperature after heat either maintained mRNA synthesis at the stimulated levels, or reduced mRNA breakdown; the levels of LMWhsp and hsp70 transcripts stimulated by 38 °C for 1 h, returned to control levels within 24 h at 25 °C (Fig. 6). Given the general acceptance that hsp mRNA and the proteins have a limited half-life after initial stimulation, both with return to normal and at sustained high temperatures, the most likely reason for the low-temperature effect is a reduction in hsp mRNA decay. There are recent reports that low temperatures themselves are implicated in enhanced hsps transcript levels (Anderson *et al*. 1994; Li *et al*. 1999). Although this raises the possibility of the low-temperature stimulation of hsps, there is no evidence under the sampling regime (24 h) in our experiments for a specific effect of low temperature (Fig. 6).

Heat-induced low-temperature tolerance

A complete explanation for the protective effect of heat treatments on chilling injury in plant tissues is still elusive. Hsps are clearly induced with effective heat treatments, and certainly hsp transcripts are maintained at elevated levels during subsequent low-temperature conditions in a range of tissues (Lurie 1998). Although at least one hsp itself has been observed at relatively high levels at low temperature (Sabehat *et al*. 1996), and recent evidence suggests that some hsps can be induced by chilling (Li *et al*. 1999), an exact role is still speculative. We might suggest that the protective, chaperone-like properties of hsp are of benefit at low temperatures where protein damage can also occur, but there is, as yet, no direct evidence for this.

Our data do suggest that perhaps more physical affects of low temperature on cell membrane structure and function may be retarded by heat, and this would fit with effective heat treatments on avocado fruit, where skin browning at low temperature (presumably due to phenol oxidation resulting from membrane damage and loss of compartmentation) is inhibited by prior heat treatment (Woolf *et al*. 1995).

We have no evidence that the features of PCD which we have investigated in model tobacco cells under cytotoxic stress (O'Brien *et al*. 1998a,b) play a role in heat-induced tolerance. An attractive theory arising from our earlier work is that stress tolerance is related to the reversibility of early stages of PCD (chromatin condensation, DNA breakdown). However, these processes are not prominent in low-temperature responses.

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