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Programmed cell death induced by heat shock in mung bean seedlings

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ABSTRACT. The effect of heat shock (HS) on death, weight and membrane leakage of mung bean seedlings as well as on early biochemical markers of PCD (cytochrome c release from mitochondria and internucleosomal DNA fragmentation) was studied. It was found that heat shock ranging from 48-55°C stimulated the releasing of cytochrome c from mitochondria to cytosol and the nuclear DNA fragmentation occurred during the first six hours of heat treatment. Sublethal heat shock (48°C) gives rise to non-ladder DNA degradation. Lethal heat shock (50 and 55°C) generates two types, ladder and non-ladder, DNA degradation. Internucleosomal (ladder) DNA fragmentation and cytochrome c releasing suggest the programmed cell death occurs in mung bean seedling induced by heat shock.

Keywords: Cytochrome c; DNA fragmentation; Heat shock; Programmed cell death.

INTRODUCTION

Programmed cell death (PCD) occurred during plant developmental processes such as flower development, embryogenesis, seed germination, and vessel and trachea formation was reported before (Pennell and Lamb, 1997; Mittler, 1998; Egorova et al., 2010). In addition, PCD can be also triggered in plant cells infected by pathogens, wound by physical treatments or damaged by low dosage of toxic compounds (Buckner et al., 2000).

One of the earliest markers of animal PCD is the releasing of cytochrome c from mitochondria to cytosol (Reed, 1997; Reape and McCabe, 2010). By analogy with mammalian apoptosis, plant mitochondria have been suggested to play a pivotal role in the integration of environmental and developmental signals that trigger cell death (Lam et al., 2001). Few studies, however, have explored the possible involvement of cytochrome c and mitochondria in plant PCD (Balk et al., 1999; Balk and Leaver, 2001; Yu et al., 2002; Reape and McCabe, 2010). Nuclear DNA degradation, which occurs following the initial stage of chromatin condensation, is an early feature of both animal and plant PCD (Krishnamurthy et al., 2000). The orderly degradation of genome during PCD is contrast to random decay of DNA that follows other types of cell death such as injury-induced necrosis (Kerr et al., 1995). In plants, nucleosomal DNA degradation has been observed during certain development events (Young and Gallie, 2000; He and Kermode, 2003), after induction by different stresses (Koukalova et al., 1997), and during pathogen-induced death (Mittler and Lam, 1997). DNA is first cleaved into large fragments of about 300 and/or 50 kb (Oberhammer et al., 1993), and these are further digested between nucleosomes resulting in DNA fragments that are multimers of about 180 bp monomers (Lagarkova et al., 1995). It is currently believed that these changes in genomic DNA are incompatible with cell survival and mark the point of no return in the execution stage of the PCD pathway.

In this work, we studied the effect of heat shock (HS) on death, weight and membrane leakage of mung bean seedlings as well as on early biochemical markers of PCD (cytochrome c release in cytosol and DNA fragmentation). It was found that all temperatures applied for heat shock (48-55°C) increase the releasing of cytochrome c from mitochondria to cytosol and causes a concomitant nuclear DNA fragmentation during the first six hours of heat shock at 50°C and 55°C. The sublethal heat shock (48°C) gave rise to non-ladder DNA fragmentation and lethal heat shock (50°C and 55°C) exhibited both ladder and non-ladder DNA degradation.

MATERIALS AND METHODS

Plant material

Mung bean seeds (*Vigna radiata* L. (Wilczek) cv. Tainan) were grown as previously described (Dai et al., 1998). To induce HS, three-day-old seedlings were incubated for 12 h in a dark growth chamber (humidity 67%) at 48°C, 50°C, and 55°C (Altschuler and Mascarenhas, 1982). Con-

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trol seedlings were incubated at 28°C. Fifteen seedlings from the HS-induced and control treatments, respectively, were harvested at successive time points, frozen in liquid nitrogen, and stored at -80°C ready for further extraction and subfractionation in order to carry out DNA and immunoblotting analysis.

Viability assay

Seedling death was assessed by both morphological (wilting symptoms) and physiological criteria. Quantitative experiments were repeated three times and more then 500 seedlings were examined in each experiment. The percentage of dead seedlings was calculated from the total seedling scored after subtracting the basal amount of dead seedlings (which was assessed in the same manner prior to the beginning of each experiment). Only seedling populations with less then 5% initial death were chosen for assay (Elbaz et al., 2002).

Fresh weight determination

Fifty mung bean seedlings were collected and weighted in 0, 2, 6, and 12 h along heat shock at 48°C, 50°C, and 55°C (28°C as control). The average weight of 50 seedlings was determined and standard error (SE) was less than 8% among three repeats.

Membrane leakage

Measurement of ion leakage from leafs was performed essentially as described (Xu and Hanson, 2000) with some modification. For each measurement, eight leaves (two primary leaves from four independent seedlings) were floated in 15 ml of double distilled water for 4 h at room temperature. This solution was tested for the "sample conductivity". Then the leaves were frozen in 15 ml of double distilled water at -20°C overnight and this solution was use to obtain the "subtotal conductivity". Membrane leakage is represented by the "relative conductivity", which was calculated as sample conductivity divided by total conductivity (the last one is sum of sample conductivity and subtotal conductivity). Conductivity of the solutions was measured with conductivity meter (model 30, Denver Instrument, USA).

DNA extraction and degradation analysis

At successive time points during 12-h HS, three seedlings (approximately 700 mg) were ground to a fine powder in liquid nitrogen, and DNA was isolated according to a modified cetyltrimethylammonium bromide method (Fulton et al., 1995). DNA samples were digested with DNase-free RNase for 1 h at 37°C, and the DNA content was estimated. 15 µg DNA was analyzed by electrophoresis on a 2% (w/v) agarose gel in a standard TAE buffer. The DNA was transferred to a nylon membrane (Hybond-N, Amersham, Ireland) and hybridized with a radioactive probe prepared from mung bean total genomic DNA. The Southern blot analysis was the same as described previously (Dai et al., 1998).

Isolation of proteins

HS exposed and control seedlings were frozen in liquid nitrogen and ground to a fine powder. After adding of the extraction buffer: 220 mM mannitol, 68 mM sucrose, 50 mM PIPES-KOH, pH 7.4, 50 mM KCl, 5 mM EGTA, 2 mM $MgCl_2$, 1 mM dithiothreitol, and 1 tablet of EDTA-free protease inhibitor (Rocher) for each 50 ml of buffer, the samples were incubated for 30 min at 4°C (Bossy-Wetzel et al., 1998). Cell debris was pelleted by a quick centrifugation step during which the rotor was stopped as soon as it reached 6000 × *g*. The supernatant was recentrifuged at 14000 × *g* for 15 min to remove mitochondria, and the remaining cytosolic supernatant was stored at -80°C for further analysis. A modified Lowry method was used for protein quantitation in the cytosolic fractions (Larson et al., 1986). Equal amounts of total protein were used in all the experiments.

Western blot analysis

30 µg of total cytosolic protein was separated on 12.5% SDS-PAGE gel. The protein was transferred to a PVDF membrane (Millipore, Bredford, MA) and immuno-blotting analysis against cytochrome c antibody (BD Biosciences) was the same as previously described (Dai et al., 1998).

RESULTS AND DISCUSSION

It was known from previous report that plants could activate either HS responses (Altschuler and Mascarenhas, 1982) or PCD (McCabe et al., 1997; Vacca et al., 2006) depending on the severity of heat shock. Temperature known to induce the HS response (45°C) had no influence on plant survival (Lebel et al., 1993). However, plant cells subjected to 55°C for 10 min can activate a cell death program that results in the series distinctive morphological and biochemical changes (Balk et al., 1999).

The degree of HS effect on plants is generally exemplified by the changes in a set of physiological parameters, including weight and cellular membrane leakage (Mittler and Lam, 1995; Panavas et al., 1998) as well as by the percentage of plant death. The time dependences of these changes were examined in our work. Proper number of mung bean seedlings was pooled at each time point during each individual HS experiment, and at least three independent HS experiments were carried out for analyzing the value of each parameter.

Exposure of three-day-old mung bean seedlings to 12-h HS at 48°C did not affect viability of the seedlings throughout the experiment (Figure 1A, the percentage dead seedlings remains less then 5%), while the treatment of seedlings for 12 h at 50°C as well as for 6 and 12 h at 55°C induced death of almost all seedlings: the percentage of dead seedlings reached 92% at 50°C and 100% at 55°C (Figure 1A). The survived seedlings showed no visible wilting after 12-h HS at 48°C, 6-h HS at 50°C and 2-h HS at 55°C. Furthermore, they continued to grow normally

after being returned to 28°C .

As shown in Figure 1B, fresh weight of seedlings exposed to 12-h HS at 48°C only decreased slightly. On the other hand, a fast decline in fresh weight was observed along 50°C and at 55°C treatment (Figure 1B). This result is consistent with the traditional concept that the loss of water content in seedling tissues contributes to wilting symptoms and finally brings about the death of seedlings (Kimpei and Key, 1985).

Plant cell death is also associated with an increase in cellular membrane leakage (Mittler and Lam, 1995) which can be measured by ion leakage (Xu and Hansen, 2000). In seedlings exposed to 48°C for 12-h HS, membrane leakage remains constant as control through 12 h treatment period. Heat shock at 50°C on seedlings, the conductivity kept as low as control for first 4 h treatment, a quick increases was observed after 4 h heat incubation. When temperature elevates up to 55°C, the conductivity increase continuously up to 12 h heat treatment (Figure 1C). This data show a close correlation and a good agreement with death percentage (Figure 1A) as well as with decrease in weight of seedlings (Figure 1B) effected by HS on plant seedlings.

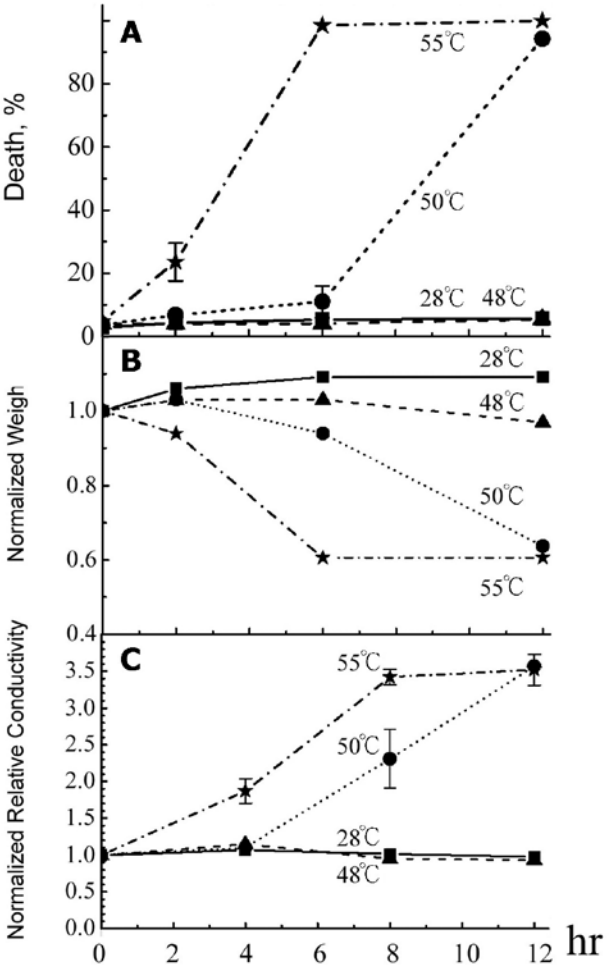

Figure 1. Influence of heat shock on three-day old mung bean seedlings. Temperature and duration used for heat shock treatment are shown in the figures. Vertical bars represent ±SE (n=3). Panel A. The average percentage of dead seedlings. For each treatment, 500 seedlings was examined for each time point. Three repeated experiments were carried out. Death of seedlings was assessed morphologically; Panel B. Effects of heat shock on seedling fresh weight. For each time point of every heat treatment, 50 seedlings were used; Panel C. Change in relative conductivity during heat shock caused by leakage of ions from leaf disks. Relative conductivity is the ratio of sample conductivity to total conductivity (see text).

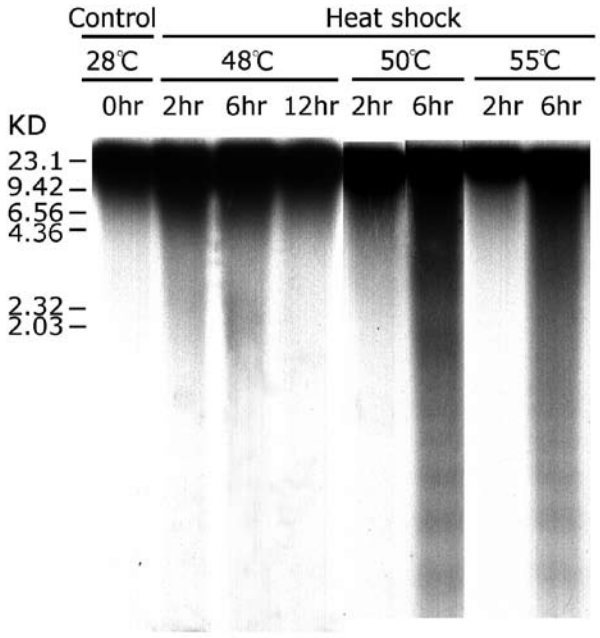

Figure 2. Southern blot analysis of genomic DNA from 3-day-old mung bean seedlings exposed to 2, 6 and/or 12 h-heat shock, respectively. Temperature and time periods of exposure to heat shock are indicated. Fifteen micrograms of DNA was fractionated on a 2% (w/v) agarose gel, transferred to a nylon membrane, and hybridized to radioactive probe prepared from mung bean total genomic DNA.

One of the hallmarks of plant PCD is the organized degradation of the genome DNA caused by internucleosomal fragmentation resulting in the appearance of ladder DNA fragments formed by 180-200 bp multimers resolved by agarose gel electrophoresis (Barry and Easman, 1992; Collins et al., 1992; He and Kermode, 2003). To examine whether similar PCD events may occur in heat shocked mung bean seedlings, DNA was purified gently from seedlings after the heat treatment of consecutive time points (Figure 2) and analyzed on agarose gel. In order to increase the sensitivity of detection, DNA was transferred to nylon membrane and Southern hybridization was carried by using purified mung bean nuclear DNA as a probe. The result in Figure 2 shows that the majority of DNA molecules from control seedlings as well as from heat shocked seedlings stay in the well exhibited its high molecular weight. Besides the main high molecular weight band, all temperatures of heat shock treatment induce DNA degradation and this degradation is most prominent

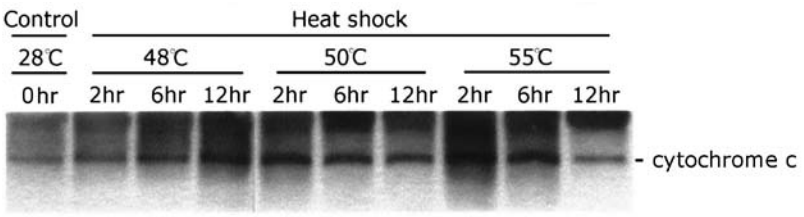

Figure 3. Immunodetection of cytochrome c in the cytosolic fraction of 3-day-old mung bean seedlings during heat shock. Each sample containing mitochondria-free cytosolic proteins was separated by SDS-PAGE followed by a Western blot analysis against anti-cytochrome c antibody.

at 6 h heat shock treatment. The degradation does not form a ladder DNA in the case of 48°C heat shock treatment. The reversal of DNA degradation was shown after further 6 h 48°C heat treatment. These data together with the data on seedling survival, seedling weight and leaves conductivity show in Figure 1 indicate that HS at 48ºC induces a weak reversible DNA degradation.

Both internucleosomal DNA fragmentation (ladder of nucleosomal multimers) as well degradation of non-nucleosomal fragmentation (non-ladder) are clearly observed at 6-h of heat treatment at 50°C and 55°C. This result suggests that heat shock for 6 h at 50°C and at 6 hr at 55°C trigger PCD on molecular level in some cells of seedlings or in some seedlings.

It has been demonstrated the translocation of cytochrome c from the mitochondria to the cytosol upon induction of PCD in plant (Yu et al., 2002; Reape et al., 2008; Reape and McCabe, 2010). To investigate cytochrome c relocation in cytosol during heat stress, control and HS exposed seedlings were fractionated to remove mitochondria from cytosol by centrifugation. The Western blot analysis on cytosol purified from different temperatures treated seedlings reveal that the cytochrome c is increased in cytosol for all heat shock treatments at different time intervals (compare lanes of heat shock to lane of 28°C control in Figure 3). Obviously, the cytochrome c increasing in cytosol of 48°C treated sample do not cause the DNA fragmentation. However, the increasing of cytochrom c was correlated with DNA fragmentation of 50°C/55°C, 6 h treated seedlings. The cytochome c was increasing with prolonged 48ºC heat treatment but neither DNA fragmentation was observed nor increasing of DNA degradation exhibited consecutively correlated to increasing amount of cytochrome c. The immunoblotting analysis was repeated 3 times and similar data were obtained. In order to obtain the accurate result, the amount of cytosol protein was measured three repeats and the average was taken in above experiment.

Taken together, our result presents that: 1. Heat shock at 48°C will not change seedling survival, leaf conductivity but some minor change is shown in seedling weight. Heat shock at 50ºC caused significant change of above three criteria after 6 h treatment time. The drastic change occurred right after heat treatment at 55°C. 2. For 48ºC treatment, no DNA fragmentation was found but smear DNA degradation appears during 2 and 6 h heat treatment. The reversal of DNA degradation was exhibited at 12 h of heat treatment. 3. The internucleosomal DNA fragmentation was occurred during heat shock at 50°C /55°C for 6 h. 4. Induction of cytochrome c was shown in all heat treating seedlings. For 48°C treatment, no DNA fragmentation was detected caused by increasing of cytosolic cytochrome c. There is also no correlation between amount of DNA degradation in smear form and amount of cytochrome c increasing. For 50°C and 55ºC heat shock treatments, the correlation between increasing of cytosolic cytochrome c and DNA fragmentation at 6 h heat shock time point was found. We conclude here that additional cytochrome c releasing from mitochondria to cytosol during heat shock in mung bean seedling may not be a sole factor essential for DNA fragmentation in PCD.

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熱處理誘發綠豆幼苗的細胞凋亡

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熱處理影響綠豆幼苗的死亡、重量及其膜滲漏。同時也可引起早期細胞凋亡的生化指標，包括細胞色素 C 在細胞質中的增加及核 DNA 之片段化。在 48℃至 55℃之熱處理下，皆有誘導綠豆幼苗細胞質中細胞色素 C 增加的現象。核 DNA 片段化則在 50℃及 55℃熱處理六小時的綠豆幼苗中發生。未達致死量之 48℃熱處理可造成 DNA 非片段化的降解。致死性之熱處理（50℃及 55℃處理六小時）可造成核 DNA 片段化降解及非片段化降解。本研究證實熱處理可誘發綠豆幼苗的細胞凋亡。

關鍵詞：細胞色素 C；DNA 片段化；熱處理；細胞凋亡。