Effect of high temperature on protein expression in strawberry plants

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Abstract

Strawberry plants (*Fragaria* × *ananassa* Duch.) cvs. Nyoho and Toyonoka were exposed to temperatures of 20, 33, and 42 °C for 4 h, and protein patterns in leaves and flowers was analyzed by 2-dimensional polyacrylamide gel electrophoresis and immunoblotting. In leaves and flowers of both cultivars, the content of most proteins decreased, but a few new proteins appeared in response to heat stress. These heat shock proteins (Hsps) were detected in the range of 19 - 29 kDa in leaves, and 16 - 26 kDa in flowers. The intensity of a 43 kDa protein spot increased in response to heat stress in Nyoho flowers, but not in Toyonoka flowers. The peaHsp17.7 antibody recognized one band at approximately 26 kDa in leaves, and two bands at approximately 16 and 17 kDa in flowers of both cultivars. These results show that the effects of heat stress on Hsp synthesis in strawberry plants differ between plant organs and between cultivars.

Additional key words: *Fragaria* × *ananassa*, heat shock protein, immunoblotting.

Introduction

When plants are exposed to supraoptimal temperature, the synthesis of normal cellular proteins is decreased and the synthesis of heat shock proteins (Hsps) is induced (Vierling 1991, Parsell and Lindquist 1993). Hsps normally function as molecular chaperones by enhancing protein refolding or preventing thermal aggregation of denatured proteins (Hartl 1996, Boston *et al.* 1996). They are generally divided into the low-molecular mass (15 to 30 kDa) Hsps or small Hsps and the high molecular mass (60, 70, 90 and 110 kDa) Hsps (Lindquist 1986). Small Hsps are further classified by their location within cells, immunological cross-reactivity, and the homology of their amino acid sequences (Waters 1995). There are cytosolic small Hsps (Class I and II), endoplasmic reticulum and chloroplast small Hsps (Class III and IV), mitochondrial small Hsps (Class V), and Class VI Hsps which are proposed to be located in the endoplasmic reticulum (Lafayette *et al.* 1996).

The synthesis of Hsps under high temperature is linked to the acquisition of thermotolerance in some plants. Malik *et al.* (1999) showed that carrot plants over-expressing the *Hsp17.7* gene are more thermotolerant than the control lines and transgenic plants that did not express the *Hsp* gene. The same observation was reported with *Arabidopsis* transgenic lines that overexpress the *Hsp101* gene (Quietsch *et al.* 2000). On the other hand, the repression of Hsp synthesis results in the loss of thermotolerance in sorghum seedlings (Howarth and Skot 1994).

Strawberry plants are temperate crops with an optimum growth temperature between 10 and 26 °C. Although temperatures higher than 30 °C are known to reduce fruit size and fruit mass, there have been few studies of the effect of high temperature on protein expression, particularly Hsps synthesis. Civello *et al.* (1997) found that proteins whose molecular masses correspond to those of Hsps are synthesized in mature fruits heat-stressed at 42 and 48 °C. Medina-Escobar *et al.* (1998) reported, however, that mRNA corresponding to small Hsps is present in non-heat stressed fruits at various stages of development. As far as we know, however, the synthesis of Hsps in the leaves

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Abbreviations: BCIP - 5-bromo-4-chloro-3-indoylphosphate *p*-toluidine salt; 2-D PAGE - two-dimensional polyacrylamide gel electrophoresis; DTT - dithiothreitol; EDTA-Na_2 - ethylenediaminetetraacetic acid disodium salt; Hsp - heat shock protein; IEF - isoelectric focusing; NBT - *p*-nitroblue tetrazolium chloride; PMSF - phenylmethylsulfonyl fluoride; PVPP - polyvinylpolypyrrolidone; SDS - sodium dodecyl sulfate.

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and flowers of strawberry has not been reported.

The objectives of this study were *1*) to determine the effect of short periods of heat stress on protein expression in strawberry leaves and flowers, *2*) to determine the ability of strawberry plants to synthesize heat shock proteins, and *3*) to determine cultivar differences in the heat shock response.

Materials and methods

Heat shock treatment of strawberry leaves: Runners of strawberry (*Fragaria* × *ananassa* Duch.) cvs. Nyoho and Toyonoka were rooted in pots (7 cm in diameter) containing growth medium (*SoilMix*, *Sakata*, Yokohama, Japan) on July 2000. On September 2000, 5 plants from each cultivar were placed in different growth chambers, and the temperatures were raised at a rate of 4 °C h^{-1} from 20 °C to either 33 or 42 °C. The plants were kept at either 33 or 42 °C for 4 h, and then fully expanded leaves were collected and immediately frozen in liquid nitrogen (N_2).

Heat shock treatment on strawberry flowers: Runners of cvs. Nyoho and Toyonoka were rooted in the same medium on August 2000. Rooted plants were then exposed to a temperature of 10 °C under a 10-h photoperiod (irradiance of 80 μmol m^{-2} s^{-1}) for three weeks in order to induce flowering. At anthesis of the primary or secondary flowers, plants were placed in a growth chamber and subjected to high temperature treatment at either 33 or 42 °C for 4 h after increasing the temperature from 20 °C at a rate of 4°C h^{-1}. After the 4-h treatment, flowers were collected and immediately frozen in liquid N_2.

Protein extraction: About 2 g of leaves and flowers (stored at -80 °C) were ground to a fine powder in liquid N_2 with a prechilled mortar and pestle. Protein extraction buffer was added at a rate of 4 cm^3 of buffer to 1 g of tissue. The extraction buffer was composed of 60 mM Tris, 2 % sodium dodecyl sulfate (SDS), 2 % mercaptoethanol, 1 mM ethylene diamine tetraacetic acid disodium salt (EDTA-Na_2), 1 mM phenylmethylsulfonyl fluoride (PMSF), 5 % sucrose, 1.5 % polyvinyl-polypyrrolidone (PVPP), and the pH was adjusted to 8.0 with 1 M HCl. The suspension was centrifuged twice at 15 000 *g* for 20 min at 4 °C, and the supernatant was stored at -80°C until further analysis.

Electrophoresis: For 2-dimensional polyacrylamide gel electrophoresis (2-D PAGE), protein was precipitated by adding 100 % acetone containing 0.7 % 2-mercapto-ethanol to the supernatant at a 1:4 ratio. The mixture was kept at -18 °C for 2 h, and then centrifuged at 3 000 *g* for 15 min. The supernatant was discarded, and the precipitate was washed 3 times with 80 % acetone containing 0.7 % 2-mercaptoethanol and air-dried. The protein pellet was then dissolved in isoelectric focusing (IEF) sample buffer consisting of 9 M urea, 2 % Nonidet P-40 (NP-40), 100 mM dithiothreitol (DTT), 2 % *Pharmalyte 3-10* (*Amersham-Pharmacia*, Tokyo, Japan) and bromophenol blue.

The first dimension IEF was run using *Immobiline drystrips pH 3-10NL* on a *Multiphor II* unit (*Amersham-Pharmacia*) according to the manufacturer‘s instructions with some modifications. Voltage was changed according to the following program: 50 V for 1 h, 150 V for 2 h, 300 V for 2 h, 600 V for 2 h, 900 V for 2 h, 1 200 V for 2 h, and 3 000 V for 16 h. The second dimension (SDS-PAGE) was run on the vertical *Hoefer 660* (*Amersham-Pharmacia*) unit using 10 % acrylamide gels. Proteins were then visualized by silver staining.

Immunoblotting: Immunoblotting was performed using the *Amplified Alkaline Phosphatase Goat Anti-Rabbit Immun-Blot^R Assay Kit* (*BioRad*, Tokyo, Japan). Protein samples from all treatments were first separated on 12 % SDS polyacrylamide gels then electroblotted onto a nitro-cellulose membrane (45 μm). The membrane was blocked overnight in 5 % non-fat dry milk and then incubated overnight with either peaHsp17.7 or peaHsp18.1 antibodies (kindly provided by Dr. Vierling, University of Arizona) diluted 1:1000 in TTBS. The membrane was washed, incubated with the biotinylated goat anti-rabbit antibody, washed, then incubated with the streptavidin-biotinylated alkaline phosphatase complex. After thoroughly washing the membrane, the 5-bromo-4-chloro-3-indoylphosphate p-toluidine salt/p-nitroblue tetrazolium chloride (BCIP/NBT) colour development solution was used to visualize the reaction.

To ensure reproducibility of the results, the heat shock experiments on leaves and flowers were conducted twice, and the extracted proteins were analyzed three times by 2-D PAGE.

Results

Protein expression in leaves: In Nyoho leaves, many of the protein spots visible at 20 °C (control, Fig. 1*A*) either disappeared or decreased in intensity following heat shock treatment at 33 or 42 °C (Fig. 1*B*,*C*). In the 33 °C treated leaves, however, four proteins not visible at 20 °C could be detected (Fig. 1*B*, *black arrows*). These 4 heat-

shock induced proteins were still visible at slightly darker intensities in the 42 °C treated leaves, where three other proteins appeared (Fig. 1*C*, *white arrows*). The estimated molecular masses of these proteins ranged from 19 to 29 kDa, with pIs from 5.5 to 7.0. In the higher molecular mass range (30 - 94 kDa), heat shock treatment decreased the content of most proteins, and no heat shock-induced proteins were detected (data not shown).

In Toyonoka leaves, the content of many proteins decreased with increasing temperature, similarly, as was observed in Nyoho leaves (data not shown), but the expression of heat shock-induced proteins was different from that in Nyoho (Fig. 1*D,E*). In Nyoho leaves (Fig. 1*D*, *white arrow and circles*), 2 proteins with molecular masses of approximately 19.5 kDa and 4 others with molecular masses between 23 and 27 kDa were clearly visible at 42 °C. In Toyonoka leaves, these proteins were either faintly visible or absent altogether (Fig. 1*E*, *white arrows and black circles*). On the other hand, one protein of approximately 26.6 kDa was expressed more intensely in Toyonoka than in Nyoho (Fig. 1*E*, *black arrows*), and 3 other heat shock-induced proteins between 25 and 26.9 kDa were detected only in Toyonoka (Fig. 1*E*, *white circles*).

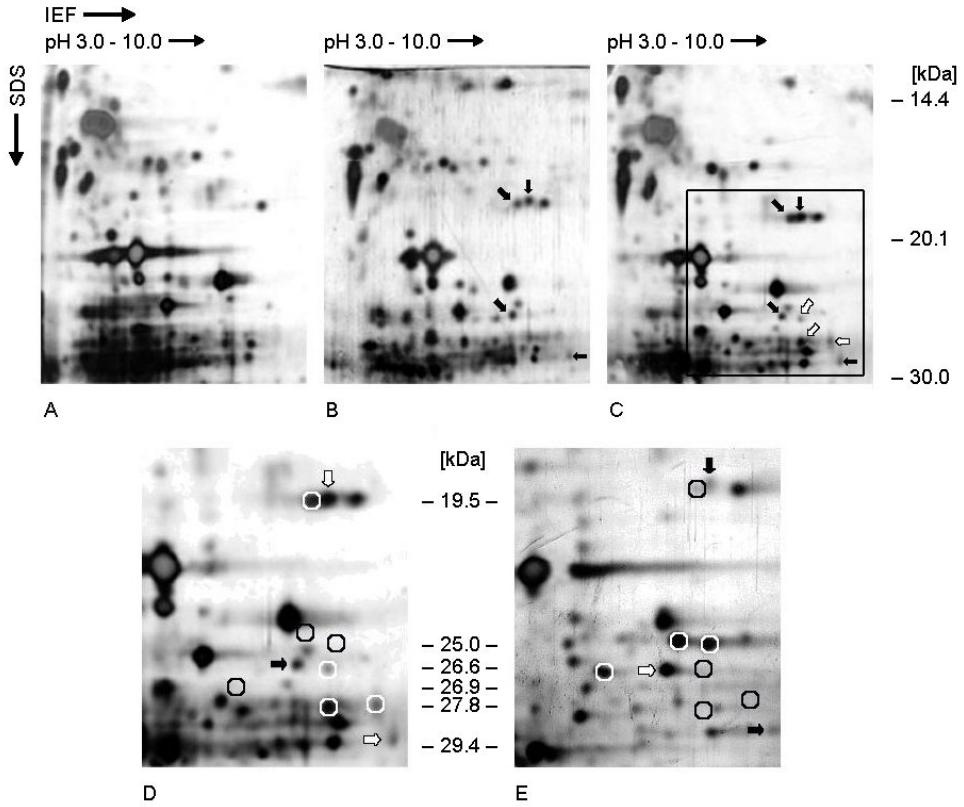

Fig. 1. Two-dimensional polyacrylamide gel electrophoresis (2-D PAGE) of low molecular mass (LMM) proteins from leaves. *A* - Nyoho at 20 °C (control); *B* - Nyoho at 33 °C; *C*, *D* - Nyoho at 42 °C; and *E* - Toyonoka at 42 °C. The rectangular area in *C* is enlarged in *D* and the corresponding area to *D* in Toyonoka is shown in *E*. *Arrows* in *B* and *C* indicate proteins that appeared only at the high temperature treatments. Molecular mass markers in kDa are shown to the right. *White arrows* in *D* and *E* indicate proteins that were clearly visible in heat-stressed Nyoho leaves but only faintly visible in Toyonoka, while *dark arrows* indicate the opposite. *White circles* indicate proteins that were specific to either cultivar, and *black circles* correspond to positions at which specific proteins in the other cultivar appeared. The molecular masses in kDa of some of these proteins are indicated.

Protein expression in flowers: Heat shock treatments also decreased the content of most proteins in Nyoho flowers, but the content of three proteins increased with increasing temperature (Fig. 2*A,B,C*, *white arrows*). Two proteins not visible at 20 °C appeared at 33 °C (Fig. 2*A,B*, *black arrows*). When the temperature was increased to 42 °C, these 2 protein spots increased in intensity and 3 more heat shock-induced proteins became visible (Fig. 2*C*, *black arrows*). The molecular masses of these heat shock-induced proteins were estimated at 16 to 26 kDa, with pIs from 5.5 to 7.0.

The response to heat shock treatment differed between Nyoho and Toyonoka flowers. In Nyoho, 7 heat shock-induced proteins with molecular masses of 16 to 26.8 kDa were detected at 42 °C (Fig. 2*D*, *black arrows*). The same proteins were only faintly visible in Toyonoka (Fig. 2*E*) and were not visible following the 33 °C treatment (data not shown). One protein of 19 kDa was visible

only in Nyoho (Fig. 2*D*,*E*, *black circle*).

In the range from 30 to 94 kDa, the content of two proteins of approximately 43 kDa increased in Nyoho flowers as the temperature increased (Fig. 3*A*-*C*, *black arrows*), but no heat shock-induced proteins were found in Toyonoka flowers in this size range (data not shown).

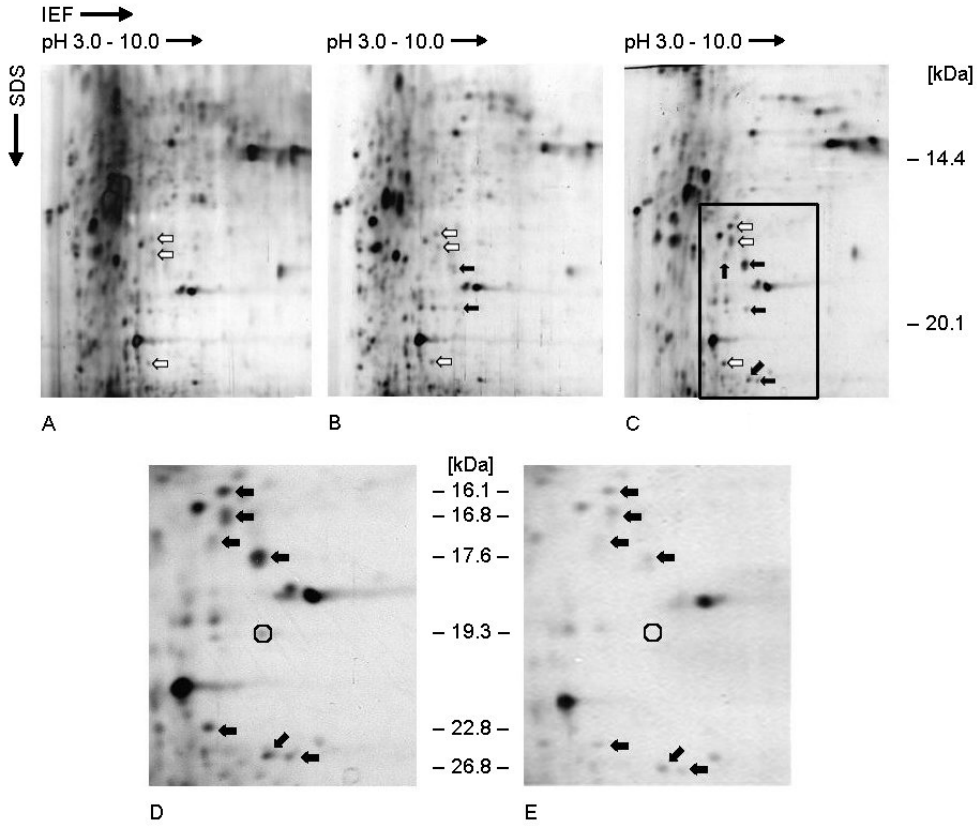

Fig. 2. Two-dimensional polyacrylamide gel electrophoresis (2-D PAGE) of low molecular mass (LMM) proteins from flowers. *A* - Nyoho at 20 °C (control); *B* - Nyoho at 33 °C; *C*,*D* - Nyoho at 42 °C; and *E* - Toyonoka at 42 °C. The rectangular area in *C* is enlarged in *D* and the corresponding area to *D* in Toyonoka is shown in *E*. *White arrows* in *A*, *B*, and *C* indicate proteins that increased in intensity with increasing temperature. *Black arrows* in *B* and *C* indicate proteins that appeared only at the high temperature treatments. Molecular mass markers in kDa are shown to the right. *Black arrows* in *D* and *E* indicate proteins clearly visible in heat-stressed Nyoho flowers but only faintly visible in Toyonoka. The *circle* in *D* and *E* indicate a protein that is specific to Nyoho and is not visible in Toyonoka. The molecular masses in kDa of some of these proteins are indicated.

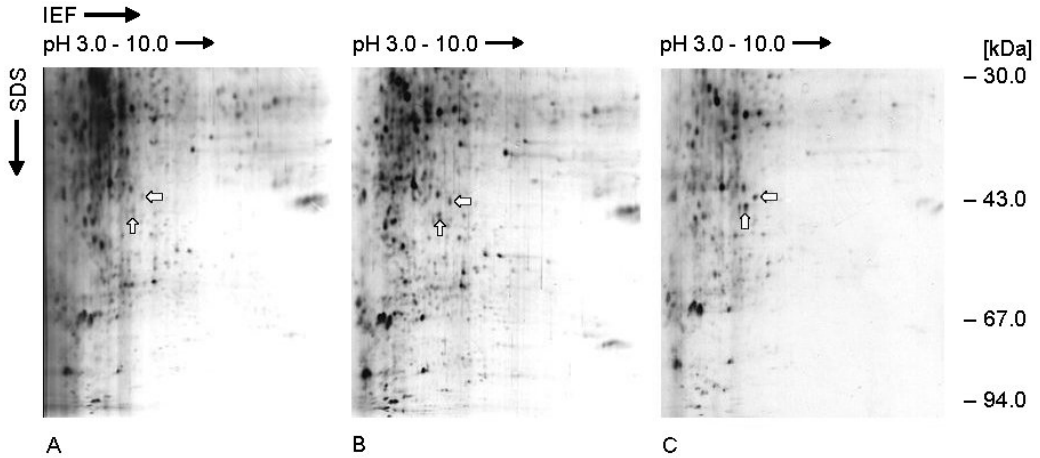

Fig. 3. Two-dimensional polyacrylamide gel electrophoresis (2-D PAGE) of high molecular mass (HMM) proteins from Nyoho flowers. *A* - 20 °C (control); *B* - 33 °C; and *C* - 42 °C. *White arrows* indicate proteins whose intensities increased as the treatment temperatures increased. Molecular mass markers in kDa are shown to the right.

Immunoblotting: The pea Hsp17.7 antibody recognized 3 distinct bands with approximate molecular masses of 16, 17, and 26 kDa (Fig. 4*A-D*). In Nyoho leaves (Fig. 4*A*), the 26 kDa band was clearly visible at 33 °C, but its intensity decreased at 42 °C. In Toyonoka leaves (Fig. 4*B*), the same band was clearly visible at 33 °C, and with greater intensity at 42 °C. In flowers of both cultivars, the 16 and 17 kDa Hsp bands were visible at 20, 33, and 42 °C (Fig. 4*C,D*). The intensities of these 2 bands increased as the heat shock temperatures were increased, and were greater in Nyoho than in Toyonoka at all temperatures. Pre-immune (PI) serum did not react with any of the leaf or flower proteins in either cultivar (Fig. 4*B,D*). We also did not detect any cross-reaction between the peaHsp18.1 antibody and proteins in flowers or leaves of either cultivar (data not shown).

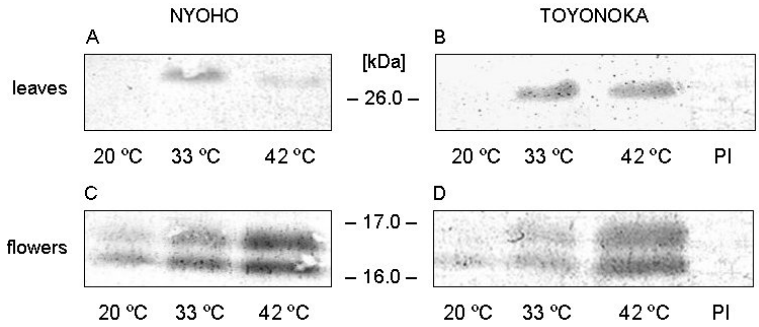

Fig. 4. Immunoblots of leaf (*A* and *B*) and flower (*C* and *D*) proteins of Nyoho (*A* and *C*) and Toyonoka (*B* and *D*) plants exposed to heat shock treatments of 20 °C (control), 33 °C and 42 °C. Strawberry proteins were reacted with the peaHSP17.7 antibody. PI is pre-immune serum from the same rabbit from which the pea HSP antibodies were obtained. Molecular mass markers in kDa are indicated.

Discussion

The appearance of Hsps at high temperatures is a typical response to heat shock. It has been reported that plants such as soybean, pea, maize, and wheat begin to synthesize small Hsps if they are exposed to temperatures above 32 - 33 °C (reviewed in Vierling 1991). The synthesis of small Hsps is maximum at temperatures that are just below lethal. In our study, mild heat shock treatment at 33 °C was enough to induce the expression of some proteins in strawberry leaves and flowers, although severe heat shock treatment at 42 °C intensified and/or induced the synthesis of a few more proteins. The estimated molecular masses of these proteins were in the range of 15 to 30 kDa, the same as those of small heat shock proteins.

Immunoblotting experiments revealed that the peaHsp18.1 antibody did not react with any of the Hsps in the leaves or flowers of Nyoho and Toyonoka. The peaHsp17.7 antibody, however, reacted with the bands of 3 small Hsps (Fig. 4). One of the 3 bands (26 kDa) was located in leaves, while the other 2 bands (16 and 17 kDa) were located in flowers. The 26 kDa Hsp in leaves was detected only after heat shock treatment (33 and 42 °C), but the 16 and 17 kDa Hsps in flowers were detected even at the normal temperature (20 °C), although their content increased with increasing temperature.

PeaHsp17.7 is a Class II cytosolic small HSP and peaHsp18.1 is a Class I small HSP. Lee *et al.* (1995) reported that Class I and Class II cytosolic small Hsps share amino acid similarity and identity, but they have different functions. DeRocher *et al.* (1991) also reported that Class I and Class II antibodies react only with small Hsps belonging to their specific class. It is likely, therefore, that the small Hsps detected by the peaHsp17.7 antibody in strawberry leaves and flowers are the Class II cytosolic small Hsps. Possibly, there are other small Hsps that belong to different classes in strawberry plants, and these Hsps were visible in the 2D PAGE gels (Figs. 1*D,E*, 2*D,E*).

The small Hsp with molecular mass of 26 kDa detected by the peaHsp17.7 antibody is probably a precursor form of an organelle-bound small Hsp. Organelle-localized proteins, including Hsps, are synthesized as precursor proteins in the cytoplasm before they are processed into their mature sizes during transport into a specific organelle, such as chloroplasts, ER or mitochondria (Vierling 1991). These precursor proteins are typically 5 to 7 kDa larger than their mature state (Lenne *et al.* 1995). Although the function of small Hsps in leaves is not completely understood, the production of a chloroplast Hsp24 is reported to be significantly related to photosynthetic thermotolerance in several genotypes of tomato (Preczewski *et al.* 2000). It has also been reported that chloroplast small Hsps protect the electron transport system in photosystem 2 during heat stress (Suzuki *et al.* 1998).

Immunoblotting experiments (Fig. 4*C,D*) showed that 2 small Hsps are present in the flowers of both strawberry cultivars even under non-heat stress conditions (20 °C). Small Hsps normally appear in response to heat stress in

vegetative organs, but can be detected under both heat and non-heat stress conditions in the reproductive organs of some crops (Vierling 1991). Therefore, it is hypothesized that small Hsps play a role in flower development (Tsukaya *et al.* 1993, Rigola *et al.* 1998), pollen development and germination (Atkinson *et al.* 1993, Žárský *et al.* 1995), somatic embryogenesis (Gyorgyey *et al.* 1991), and seed development (Coca *et al.* 1994, DeRocher and Vierling 1994).

There are conflicting reports about the synthesis of Hsps in response to heat stress in heat tolerant and sensitive cultivars. A thermotolerant line of wheat synthesizes an isoform of Hsp26 under heat stress, while a thermosensitive line does not (Joshi *et al.* 1997). The synthesis of a 45 kDa Hsp was found in a heat and drought tolerant maize phenotype, but not in the heat sensitive type (Ristic *et al.* 1997). In cotton plants, both heat sensitive and tolerant lines synthesize small Hsps under heat stress conditions, although several of these Hsps are specific to one line or the other (Fender and O'Connell 1989). The same results were also found in sorghum (Jorgensen *et al.* 1992). In creeping bentgrass, heat sensitive and tolerant lines synthesize similar small Hsps, but the heat tolerant line synthesizes 3 additional small Hsps (Park *et al.* 1996).

The present study shows that there are differences in the synthesis of Hsps between leaves and flowers, and among cultivars. In leaves, both cultivars synthesize unique Hsps, while in flowers only Nyoho synthesizes unique heat shock proteins (19.3 kDa and 43 kDa) in response to heat shock (Figs. 1*D*,*E*, 3). However, the immunoblotting experiments show that Hsps cross-reacting with the peaHsp17.7 antibody are present in flowers at 20 °C and that these Hsps are expressed more clearly in Nyoho than in Toyonoka as the temperature increases. Regarding Hsps that do not crossreact with the peaHsp17.7 antibody, their expression increases as the temperature increases, similar to Hsps that crossreact with the peaHsp17.7 antibody.

There are few reports concerning the differences in heat tolerance among Japanese strawberry cultivars. We found that fruit set was very low at day/night temperatures of 30/25 °C in Toyonoka, but no difference was found between 30/25 °C and 23/18 °C treatments in Nyoho (unpublished results). This suggests that Toyonoka is more sensitive to high temperature than Nyoho. Therefore, it is possible that the greater expression of small Hsps in response to moderate heat stress in Nyoho flowers is associated with a higher fruit set percentage at 30/25 °C compared with Toyonoka. The failure of fertilization at high temperatures has been explained by the fact that high temperature interferes with pollen function (reviewed in Mascarenhas and Crone 1996). It would be interesting to investigate whether the differences in fruit set percentage between Nyoho and Toyonoka is related to the synthesis of small Hsps in their pollen in response to high temperature stress.

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