Heat Tolerance and Flowering-heat-delay Sensitivity in Relation to Cell Membrane Thermostability in Chrysanthemum

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ABSTRACT. Flowering of many chrysanthemum [Dendranthema ×grandiflora (Ramat.) Kitam.] cultivars is reduced or delayed under high temperatures. Identification and rapid selection of heat-tolerant and flowering-heat-delay-insensitive chrysanthemum genotypes for commercial production is desirable. An electrolyte leakage technique was used to measure cell membrane thermostability of chrysanthemum cultivars. The relationship between the relative injury (RI) value occurring in leaf tissue discs and the treatment temperature was sigmoidal. The RI values at the approximate midpoint of the sigmoid response curve occurred at 47 to 53 °C for summer- and fall-flowering cultivars and at 45 to 46 °C treatments for winter- and spring-flowering cultivars. Regressing the delay in days to flowering for the cultivars grown at day/night temperature of 30/25 °C compared with those grown at 20/15 °C versus their associated RI values at 50 °C treatment showed a linear relationship. Reduced RI was more apparent in the heat-tolerant ‘Kaa Luoh-Lii’ than the heat-intolerant ‘Repulse’ after 30/25 °C treatment for 24 to 27 days. When 30/25 and 20/15 °C treatments were compared, the former did not alter leaf malondialdehyde (MDA) content in ‘Kaa Luoh-Lii’ but increased MDA content in ‘Repulse’.

For year-round production, growers in the subtropical areas conventionally divide chrysanthemum cultivars or lines into two groups: heat-tolerant (flowering during the natural summer and fall seasons) and heat-intolerant (flowering during the natural winter and spring seasons). In chrysanthemum, delayed anthesis is induced by temperatures of 26 to 32 °C (Karlsson et al., 1989; Whealy et al., 1987). Breeding heat-tolerant or heat-delay-insensitive genotypes is vital because the summer temperature is high whether in greenhouses or in the field. Shibata and Kawata (1987) measured differences in the degree of heat delay among Japanese chrysanthemum cultivars by growing the plants to flower in the summer; and they found that some genotypes may carry heat tolerance genes. Heat-delay-insensitive chrysanthemum genotypes exist, but screening for the desired seedlings by greenhouse or field evaluation techniques is slow (Anderson and Ascher, 2001; De Jong, 1989).

Cell membrane thermostability (CMT), measured as electrolyte leakage from leaf discs over a range of temperatures, is a sensitive and rapid method to evaluate heat tolerance in plants (Sullivan, 1972; Wu and Wallner, 1983). Several studies have shown the effectiveness of CMT testing in detecting genetic variability for heat tolerance among several crops (Chen et al., 1982; Lester, 1985; Martineau et al., 1979; Saadalla et al., 1990; Sullivan and Ross, 1979; Yeh and Hsu, 2004). Our previous report showed that, for summer- and fall-flowering chrysanthemum cultivars, those having a low relative injury value coincide with the greater CMT and shorter heat-induced delay to flowering in field conditions (Yeh and Lin, 2003). Little information on CMT in winter- and spring-flowering chrysanthemums is available at present.

Karlsson et al. (1989) reported that optimum temperatures in the growing periods from 1) start of short days to visible bud; 2) visible bud to disbud; 3) disbud to flower color; or 4) flower color to harvest were 21.3, 20.3, 23.1, and 19.1 °C, respectively, for chrysanthemums. However, they did not determine the difference in temperature effects on developmental phases in chrysanthemum cultivars with different heat tolerance or flowering-heat-delay sensitivity. Heat acclimation is required for detecting differences in heat tolerance among the lines or cultivars (Senthil-Kumar et al., 2003, 2007). Malondialdehyde (MDA) is a product of peroxidation of unsaturated fatty acids in phospholipids and is responsible for cell membrane damage (Halliwell and Gutteridge, 1989). It is hypothesized that heat-tolerant chrysanthemum cultivars would exhibit a greater adaptation to high growing temperatures and, hence, would have lower relative injury (RI) as well as MDA.

The objectives of this study were to 1) determine CMT of cultivars that flower in hot or cool seasons; 2) determine the effects of high temperature on the floral developmental phase in cultivars with different heat tolerance; and 3) compare RI and MDA of heat-tolerant versus heat-intolerant cultivars after heat treatment.

Materials and Methods

STOCK PLANTS. On 2 Aug. 2003, rooted tip cuttings of 10 commercial chrysanthemum cultivars were planted in 16-cm diameter plastic pots each containing sphagnum peat (Fafard No. 1; Conrad Fafard, Agawam, MA), perlite, and vermiculite mixed in equal volumes. To encourage later growth, shoot tips

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were pinched off 1 week after planting, leaving six leaves on each shoot. The plants were kept in a greenhouse at a mean daily temperature of 27 °C under 11.5- to 12-h natural day-length. Vegetative growth was maintained by a night break from 2200 to 0200 hr using incandescent lamps giving 2.2 μmol·m⁻²·s⁻¹ photosynthetic active radiation \([PAR (400 to 700 nm)]\) at plant level. The plants were fertilized weekly with water-soluble 20:0N–4.4P–24.9K (Scotts, Marysville, OH) at 300 mg·L⁻¹ nitrogen.

**Leaf cell membrane thermostability measurement for different cultivars.** On 24 Oct. 2003, fully expanded leaves (leaves three to six from apex) were harvested from stock plants of six summer- and fall-flowering cultivars (Fig. 1) and four winter- and spring-flowering cultivars (Fig. 2). These leaves were evaluated for CMT following procedures described by Yeh and Lin (2003). CMT was measured at 25, 30, 35, 40, 45, 50, 55, 60, 65, and 70 °C for 30 min. Each sample for assay consisted of five 6-mm diameter leaf discs (1.5 ± 0.3 mg dry weight) cut from a group of five leaves with a cork borer. Before assay, the leaf discs were rinsed thoroughly with three rapid changes of distilled water. Leaf discs were then placed in 25·150-mm test tubes containing 1 mL distilled water to prevent secondary water stress. Three tubes per treatment were placed in a heated, circulating water bath for 30 min at each treatment temperature. Distilled water (16 mL) was added to each tube after elevated temperature exposure. Samples were then placed at 10 ± 2 °C for 24 h before solution conductivity was measured with a conductivity meter (model SC-170; Suntex Instruments, Taipei, Taiwan). The tubes were capped with foil, autoclaved (121 °C, 1.2 kg·cm⁻²) for 15 min, cooled to 25 °C, and incubated for an additional 24 h before final conductivity measurements were taken. The degree of RI induced by the temperature treatment was calculated as follows: \[ RI(\%) = \left[ 1 - \frac{T_1}{C_1} \right] \times \left[ 1 - \frac{T_2}{C_2} \right] \times 100, \] where \(T\) and \(C\) refer to conductivity values for treatment and control (25 °C) vials, respectively, and subscripts 1 and 2 refer to initial and final conductivity readings, respectively. The relationship between RI value and water bath temperature treatments was determined with regression analysis using Sigma Plot (version 8.0; SPSS Inc., Chicago, IL). The inflection point, which is the midpoint of the sigmoid response curve, was calculated from the regression equation for each cultivar.

**Effect of temperature under 12- to 13.5-h daylength on floral development.** As a result of limited space, only five chrysanthemum cultivars (Table 1) were used. Rooted cuttings were obtained from the stock plants and planted in 16-cm diameter plastic pots. Medium and fertilizer application were the same as described previously. Plants with 18 to 20 fully expanded leaves were transferred on 8 Mar. 2004 to greenhouses under natural 12- to 13.5-h daylength during the experiment. The day/night temperatures were 20/15 and 30/25 °C for two treatments as measured at the top of plant canopy and recorded every 30 min by using a copper/constantan thermocouple (type T; Ming-Guan, Instruments, Chang-Hua, Taiwan) attached to a data logger (DL2e; Delta-T, Cambridge, UK). The time elapsed from the beginning of temperature treatment to visible bud [VB (2-mm diameter terminal bud)], show color (first appearance of color on flower bud), and anthesis were recorded at 3-d intervals for 120 d.

The experiment was arranged within temperature treatments in a completely randomized design with six replicate plants in each treatment and the data tested by analysis of variance. Means were separated by \(t\) test at \(P \leq 0.05\). The degree of heat delay was calculated following the method of Shibata and Kawata (1987) as the difference between days to flowering at 20/15 and at 30/25 °C. Regression analysis was used to determine the relationship
between the cultivars’ RI values and their associated degree of heat delay.

**Effect of Temperature Under 11- to 12-h Daylength on Floral Development, Leaf Relative Injury, and Malondialdehyde.** Rooted cuttings of ‘Kaa Luoh-Lii’ and ‘Repulse’ were planted in 11-cm diameter plastic pots on 15 Sept. 2007. After pinching, three axillary shoots were allowed to grow on each plant. Plants were initially kept in a greenhouse at a mean daily temperature of 27 °C under 11- to 12-h natural daylength. Vegetative growth was maintained by a night break from 2100 to 0300 hr using incandescent lamps giving 2.2 μmol·m⁻²·s⁻¹ PAR (400 to 700 nm) at plant level. Plants with 12 to 15 fully expanded leaves were transferred on 4 Oct. 2007 to greenhouses at 20/15 or 30/25 °C under natural 11- to 12-h daylength. Both greenhouses had an average noon photosynthetic photon flux of 700 μmol·m⁻²·s⁻¹ during the experiment. The experiment was arranged within temperature treatments in a completely randomized design with 30 replicate plants in each treatment. Leaf number below the terminal inflorescence and time required to complete VB, show color, and anthesis in each cultivar were determined. The lengths of time to reach each developmental phase for each cultivar were compared between two temperature treatments by t test.

From Day 0 to 27 after the beginning of temperature treatments, a random sample of 20 fully expanded leaves from six plants per treatment were taken at 3-d intervals for measurements of CMT and MDA. CMT was determined by measuring electrolyte leakages of leaf discs after water bathing at 25 and 50 °C for 30 min and RI values were calculated as described previously. MDA was measured by using the method of Heath and Packer (1968) with some modification. Samples of 0.1 g leaf tissue were homogenized with a mortar. A 1-mL aliquot of enzyme solution was added to a tube containing 4 mL of 20% (v/v) trichloroacetic acid and 0.5% (v/v) thiobarbituric acid. The leaf tissue was added to the tube and the mixture was heated in a water bath at 95 °C for 30 min and then quickly cooled in an ice bath followed by centrifugation at 10,000 g, for 10 min. The absorbance of the supernatant at 532 nm was determined with a spectrophotometer (model U-2800A; Hitachi, Tokyo, Japan) and the nonspecific absorbance at 600 nm was also determined and subtracted. The MDA content was calculated by the extinction coefficient of 155 m⁻¹ cm⁻¹ (Heath and Packer, 1968). RI and MDA values were presented as means ± SE.

**Results**

**Leaf Cell Membrane Thermostability of Different Cultivars.** The relationship between the RI value of leaf tissue discs and the treatment temperature was sigmoidal for all six summer- and fall-flowering chrysanthemum cultivars (Fig. 1). The RI values near the midpoint of the sigmoid response curve occurred at 47 to 53 °C water bath temperature for summer- and fall-flowering cultivars. A single temperature treatment at 50 °C for 30 min resulted in 72% and 69% RI in ‘Rong-Hung’ and ‘Huang Ching-Chin’, but only 23% and 9% RI in ‘Remix’ and ‘Hisao Hung-Niang’, respectively.

In winter- and spring-flowering cultivars, the relationship between the RI values of leaf tissue discs and treatment temperatures was also sigmoidal (Fig. 2). However, the RI of...
this group of cultivars peaked at the 50 °C treatment. The RI values near the midpoint of the sigmoid response curve occurred at 45 to 46 °C water bath temperature. These results suggest that differences in heat tolerance of leaf tissues exist between chrysanthemum cultivars and the differences are detectable by measuring CMT.

Effect of temperature under 12- to 13.5-h daylength on floral development. Plants of ‘Hisao Hung-Niang’ flowered at the same time regardless of two different growing temperatures (Table 1). Plants of cultivars Remix, Hung-Feng, and Huang Ching-Chin took longer to anthesis at 30/25 than at 20/15 °C. The flowering-heat-delay was not the result of longer time required to attain visible bud stage, but attributable to slower bud development at 30/25 °C. ‘Repulse’ failed to show reach visible bud stage in 120 d at 30/25 °C. ‘Huang Ching-Chin’, which had a relatively low temperature to attain the midpoint of the sigmoid response curve (Fig. 1), and a long delay to flowering under high temperatures (Table 1), looks more like a winter/spring cultivar than a summer cultivar.

Relationship between leaf cell membrane thermostability and heat delay. Heat delay-sensitive cultivars had higher RI values at 50 °C water bath (Fig. 1) and a greater degree of heat delay at 30/25 °C (Table 1). After a point for nonflowering in ‘Repulse’ at 30/25 °C (Table 1) was omitted, regressing the delay in days to flowering for planting at 30/25 °C versus their associated RI values at 50 °C water bath treatment in the cultivars showed a linear relationship (Fig. 3).

Effect of temperature under 11- to 12-h daylength on floral development, leaf relative injury, and malondialdehyde. Temperature treatments did not alter leaf number below the inflorescence, days to show color, and anthesis in ‘Kaa Luoh-Lii’ (Table 2). In contrast, plants of ‘Repulse’ had more leaves below the inflorescence and took longer to reach VB, show color, and anthesis at 30/25 than at 20/15 °C.

During the first 6 d after the start of 20/15 versus 30/25 °C temperature treatments, RI increased for both cultivars (Fig. 4A–B). From Day 6 to 27, the response of leaf RI to growing temperature differed between cultivars. Leaf RI of ‘Kaa Luoh-Lii’ declined from 90% to 70% at 20/15 °C and from 80% to 40% at 30/25 °C. RI of ‘Repulse’ ranged between 85% and 90% at 20/15 °C but decreased from 90% to 70% at 30/25 °C. Reduced RI was more apparent in ‘Kaa Luoh-Lii’ than ‘Repulse’ after 30/25 °C treatment for =24 to 27 d.

Leaf MDA content declined during the first 6 d after the start of temperature treatments for both cultivars (Fig. 4C–D). Leaf MDA content of ‘Kaa Luoh-Lii’ increased thereafter and ranged between 50 and 60 nmol·g⁻¹ fresh weight in both temperature treatments. Leaf MDA of ‘Repulse’ also decreased in the first 6 d; and MDA at 30/25 °C was consistently higher than that at 20/15 °C only after 12 d (Fig. 4D).

Discussion

The electrolyte leakage test is one of the most convenient methods of screening crops for heat tolerance (Martineau et al., 1979; Sullivan, 1972). The present study with chrysanthemum showed electrolyte leakage from leaf discs to have a sigmoidal response to increasing temperature (Figs. 1 and 2). Similar response curves have been reported for a number of agronomic crops (Chen et al., 1982; Ismail and Hall, 1999), fruit crops (Ingram and Buchanan, 1984), vegetable crops (Inaba and Crandall, 1988; Lester, 1985), and ornamentals (Yeh and Hsu, 2004; Yeh and Lin, 2003).

It would be tedious to derive injury response curves such as shown in Figures 1 and 2 for a lot of individual plants. From a practical perspective, it is easier to set up a single temperature bath for electrolyte test than to set up many different temperatures. The 50 °C water bath treatment followed by RI assessment resulted in the best differentiation between heat tolerance for summer cultivars (Fig. 1) but resulted in plateaued RI values for winter cultivars (Fig. 2). The present results agree with Yeh and Lin (2003) who showed that a single temperature treatment at 50 °C generated the greatest sensitivity in detecting genotypic differences in heat tolerance. CMT technique may be useful for growers to evaluate the heat tolerance of new cultivars or lines before scheduling commercial production and marketing. The present study and our previous results (Yeh and Lin, 2003) indicated that cultivars with leaf RI greater than 70% after a 50 °C water bath treatment for 30 min are not suitable for hot summer season production as a result of excessive flowering heat delay.

A positive linear relationship was found between leaf RI value and the degree of heat-induced flowering delay in chrysanthemums (Fig. 3). Thermotolerance being correlated...
between the young vegetative and flowering stages has been reported among spring wheat (*Triticum aestivum* L.) cultivars (Fokar et al., 1998), winter wheat genotypes (Saadalla et al., 1990), and cowpea (*Vigna unguiculata* (L.) Walp.) (Ismail and Hall, 1999). Measurement of leaf CMT is simple, quick, and less expensive than a whole-plant screening (Wu and Wallner, 1983). Therefore, breeders can use the CMT technique to screen large populations of plants for their heat tolerance at their early stage of development.

The cultivar Hisao Hung-Niang appeared to be heat-delay-insensitive (Table 1), indica ting the potential use for the introduction of its heat tolerance genes to new cultivars (Shibata and Kawata, 1987). Delayed anthesis occurred with the cultivars Remix, Hung-Feng, and Huang Ching-Chin at 30/25 °C (Table 1). Delayed anthesis of chrysanthemums with temperatures of 26 to 32 °C have been reported previously (Cockshull, 1979; Karlsson et al., 1989; Whealy et al., 1987). High temperatures that result in heat delay vary across ornamental species and/or cultivars. For example, *Schlumbergera truncata* (Haw.) Moran flower initiation was inhibited when average daily temperature reached <25 °C (Erwin et al., 1990). Increasing temperature from 20 to 30 °C increased days to flowering of 12 *Viola x Wittrockiana* Gams. cultivars (Warner and Erwin, 2006). Delayed anthesis in the cultivars Remix, Hung-Feng, and Huang Ching-Chin was attributed to delayed flower development after visible bud phase rather than to delayed flower initiation (Table 1). A similar heat delay of flower development has been reported for other chrysanthemum cultivars (Karlsson et al., 1989; Whealy et al., 1987).

Regardless of temperature treatments, plants of ‘Kaa Luoh-Lii’ had similar leaf number below the terminal inflorescence and days to anthesis (Table 2). In contrast, plants of ‘Repulse’ had more leaves below the inflorescence and took longer to anthesis at 30/25 °C than at 20/15 °C. These are consistent with previous results that cultivars with a lower leaf RI at the 50 °C treatment had no or shorter heat-induced delay to flowering (Figs. 1–3). Plants of ‘Repulse’ at 20/15 °C flowered earlier under 11.5 to 12 h than 12- to 13.5-h daylength (Tables 1 and 2), indicating a quantitative short-day plant for flowering. In the 30/25 °C treatment, ‘Repulse’ did not initiate flower buds under 12- to 13.5-h daylength after 120 d, but the plants flowered under 11.5- to 12-h daylength after 70 d (Tables 1 and 2). Cathey (1957) noted that high temperature has a great influence on critical daylength on flowering in this group of cultivars.

RI increased and MDA decreased during the first 6 d of temperature treatments for ‘Kaa Luoh-Lii’ and ‘Repulse’ cultivars (Fig. 4), suggesting the plants adapted to the new environmental changes. From Day 6 to 27, the response of leaf RI to growing temperature differed between cultivars. Differential heat acclimation was reported for kentucky bluegrass (*Poa pratensis* L.) (Liu et al., 2008), ‘Barlexas’ tall fescue (*Festuca arundinacea* Schreb.), and ‘Accent’ perennial ryegrass (*Lolium perenne* L.) under heat stress (Xu et al., 2006). High temperature at 30/25 °C for 27 d resulted in RI close to the value at Day 0 and did not alter MDA content in the heat-tolerant ‘Kaa Luoh-Lii’ (Figs. 4A and 4C), possibly as a result of adjustment of membrane structure and/or function under heat conditions (Xu et al., 2006). For the heat-intolerant ‘Repulse’, the 30/25 °C treatment for 27 d reduced leaf RI, but the reduced value was still higher than that at Day 0 (Fig. 4B). Leaf MDA content of ‘Repulse’ was higher at 30/25 than at 20/15 °C (Fig. 4D), indicating a higher membrane lipid peroxidation under heat (Halliwell and Gutteridge, 1989). MDA increased after the peak time of RI (Fig. 4), suggesting that MDA is a result of temperature-induced membrane damage and not the cause. Differences in MDA recovery occurred as a result of differences in temperature and cultivar. MDA recovery was faster at high temperatures because enzymatic-based membrane repair mechanisms would work faster. The heat-tolerant ‘Kaa Luoh-Lii’ might have better repair capabilities and thus recovered its MDA faster at
30/25 °C. This may partially explain why this cultivar grows and flowers better than heat-intolerant cultivars at higher temperatures. MDA acts as a biomarker because 1) as damaged membranes are repaired, MDA increases; 2) the more damage was done in heat-sensitive cultivars, the higher the MDA levels; and 3) heat-sensitive cultivars do not completely recover in the heat and, therefore, need continuous damage repair and show continuous MDA increase.

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