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## Primary Response in Chilling Sensitive Crop Leaves Evaluated by Arrhenius Plots of $^1\text{H}$ -NMR Relaxation Times

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Temperature dependency of  $T_1$  of water protons in leaf tissues of chilling-sensitive crops was investigated by pulse nuclear magnetic resonance (NMR) spectroscopy. Arrhenius plots of the  $T_1$  of tomato and cucumber leaves indicated biphasic with temperature drop from 20 to 0°C. “Breaks” in Arrhenius plots of  $T_1$  of fresh leaves in tomato and cucumber occurred at about 12.5°C and 10°C, respectively, while those of chilling-insensitive clone of gloxinia indicated no break point during the same process. Furthermore, thermal hysteresis of  $T_1$  was observed during a slow cool-warm cycle in these crop leaves. The relation between the restoration process of  $T_1$  upon warming and the degree of chilling injury is summarized as follows: 1) a slight prolongation of  $T_1$  or no hysteresis was observed when chilling injury did not occur; 2) a marked prolongation of  $T_1$  was observed when injury occurred; 3) a marked shortening of  $T_1$  occurred from the earlier stage of the warming process when tissues were severely injured. In conclusion, Arrhenius plots of  $T_1$  in leaves provide a sensitive and non-invasive information for evaluating plant response to chilling stress in crops.

### INTRODUCTION

Many plants acclimate to low temperatures and tolerate cold stress (Thomashow, 1999 references therein). For chilling injury studies a variety of biological reaction rates have been plotted in Arrhenius plots, and non-linear temperature dependency or the presence of discontinuity has been seen as a diagnostic phase transition of membrane lipid and other parameters (Lyons, 1973; Martin, 1986; Queiroz *et al.*, 2000; Wan *et al.*, 2001). The physical state of water in plant cells is also a subject of interest relative to plant cold hardiness (Levitt, 1980). The degree of physiological activity in the tissue reflects the level of water binding.  $^1\text{H}$ -NMR provides a method of research water and water interactions in biological systems (Budinger and Lauterbur, 1984; Ishida *et al.* 2000). Previously, we resolved that the question determining phase transition point in NMR relaxation times of water proton of chilling sensitive seedlings for *Vigna radiata*, *V. mungo* and chilling insensitive *Pisum sativum* (Iwaya-Inoue *et al.*, 1989). And besides it was indicated that thermal hysteresis for  $T_1$  in seedlings of these three species following a slow cool-warm cycle was used as a diagnostic tool for monitoring the primary response of cell to chilling (Kaku and Iwaya-Inoue, 1988). Furthermore, the temperature dependence of  $T_1$  of water protons in azalea flower buds and thermal hysteresis were also observed for  $T_1$  following a slow freeze-thaw cycle (Kaku *et al.*, 1985). A similar thermal

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hysteresis in  $T_1$  and  $T_2$  relaxation times was observed in human breast cancer cells. Beall (1982) suggested that thermal hysteresis of NMR relaxation times may be useful in evaluating freezing damage to cells.  $T_1$  ratio (the ratio obtained from the difference between the original  $T_1$  value in an unfrozen sample and the final  $T_1$  after a freeze-thaw treatment, both at 20 °C, divided by the original  $T_1$ ) was closely correlated with the viability of azalea florets. Thus it was suggested that the  $T_1$  ratio may be useful in detecting the degree of chilling injury in plant tissues. However, there are a few information about NMR relaxation times of water protons in relation to low temperature stress in chilling-sensitive crops at growing stage. In this study the temperature dependency of  $T_1$  of water protons in gloxinia, tomato and cucumber leaves was determined in Arrhenius plots in cool-warm cycle and relationship between chilling sensitivity and  $T_1$  relaxation behavior will be briefly discussed.

## MATERIALS AND METHODS

### Plant materials

Tomato (*Lycopersicon esculentum* Mill. var. *cerasiforme* cv. minicarol), cucumber (*Cucumis sativus* L. cv. Ochiai) and gloxinia (*Sinningia speciosa* hybrids) obtaining from commercial sources were used. They were cultivated in growth cabinet at about 20 °C and the 7th leaves in individual clones from three plant species were used as materials. Gloxinia leaves were cut into 4 to 5 leaf sections (each about 25 mm in length  $\times$  4 mm in width) and bound by cotton treads. Intact whole leaves of tomato and cucumber were rolled and bound by cotton threads suitably for insertion into an NMR probe.

### Measurements of proton $T_1$ relaxation times

NMR measurements were made at 20 MHz on a Bruker Minispec PC20 pulsed NMR spectrometer as described previously (Iwaya-Inoue *et al.*, 1993). The  $T_1$  values of leaves were measured using a  $180^\circ\text{-}\tau\text{-}90^\circ$  pulse sequence (Farrar and Becker, 1971). Leaf tissues were packed into a 7.5 mm diameter NMR tube.  $T_1$  determination was done 10 to 15 times for each sample. The probe temperature was controlled by a thermostat (Lauda Kryo-SK65) connected to the sample chamber of the spectrometer.

### Cooling and warming treatment

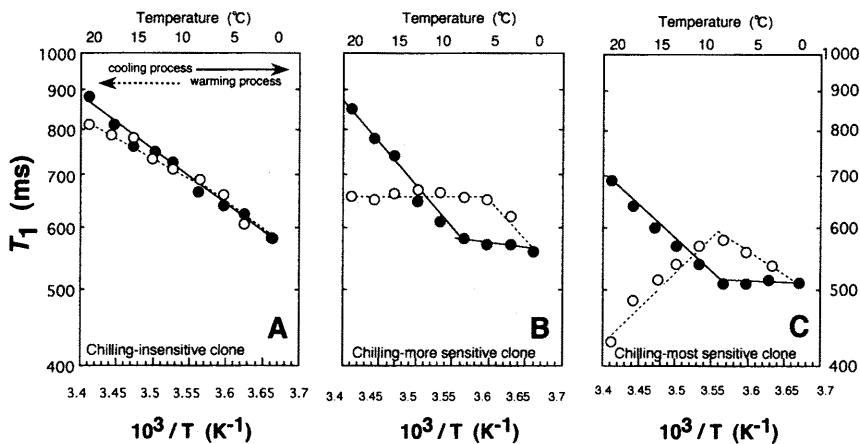
For the slow cool-warm cycle, the temperature of the leaves was lowered in 2.5 °C steps from 20 to 0 °C and *vice versa*. The cooling-warming rate was constant at 0.5 °C/min, and samples were allowed to equilibrate for 10 min at each predetermined temperature. The probe temperature and cooling-warming rate were obtained by a programmatic thermal regulator (Chino JP series) and thyristor regulator (Chino SF-V 22) attached to the refrigerated thermostat described above. The slow cool-warm cycle for the determination of thermal hysteresis lasted about 8 hours. Arrhenius plots of  $T_1$  were made by log  $T_1$  values versus reciprocal of absolute temperature. It was done five to ten individual measurements for each species and Figures indicate typical profiles in each experiment series. Additionally, tomato and cucumber leaves were exposed to 5 °C before cool-warm cycle (20 to 0 °C) for 1 day or 2 days. The injury of leaf pieces was determined by degree of changing in leaf color as described previously (Kaku and Iwaya-Inoue, 1987).

## RESULTS AND DISCUSSION

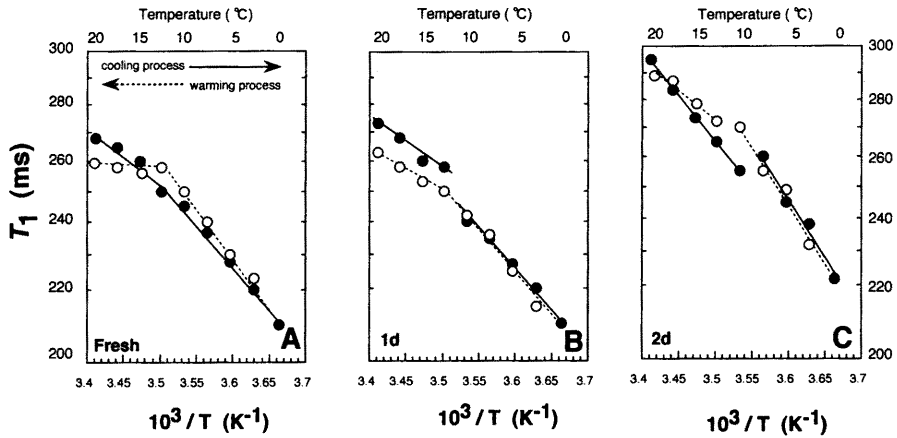
**Arrhenius plots of relaxation times in crop leaves**

The spin–lattice relaxation time ( $T_1$ ) and spin–spin relaxation time ( $T_2$ ) in intact plant tissues are valuable and non–invasive parameters for studying molecular dynamics of water (Ishida *et al.*, 2000, references therein). In chilling sensitive plant leaves Arrhenius plots of  $T_1$  in cooling process from 20 to 0 °C were shown as closed symbols (Figs. 1, 2 and 3). Arrhenius plots of  $T_1$  in cooling process indicated straight line in chilling–insensitive gloxinia leaves (Fig. 1A). On the other hand, “break” in  $T_1$  plots occurred at about 7.5 °C in both chilling–more sensitive and the most sensitive clones (Fig. 1B and C). Furthermore, an incline change in Arrhenius plots of  $T_1$  of fresh tomato leaves was observed in cooling process at about 12.5 °C (Fig. 2A). In contrast, abrupt biphasic with discontinuity occurred in tomato leaves exposed to 5 °C for 1 day and those for 2 days (Fig. 2B, C). Break points shifted to slightly lower temperature, between 12.5 and 10 °C in both treated leaf tissues. In cucumber leaves, distinct  $T_1$  changes in gradient after the “break” in fresh tissues occurred at about 10 °C in cooling process (Fig. 3A). On the contrary, the abrupt change in the incline disappeared in the leaves exposed to 5 °C for one day (Fig. 3B). Although  $T_1$  of pure water decreases linearly with temperature drop from 20 to 0 °C (data not shown), the temperature dependency of  $T_1$  disappeared in the cucumber leaves stored at 5 °C for 2 days (Fig. 3C).

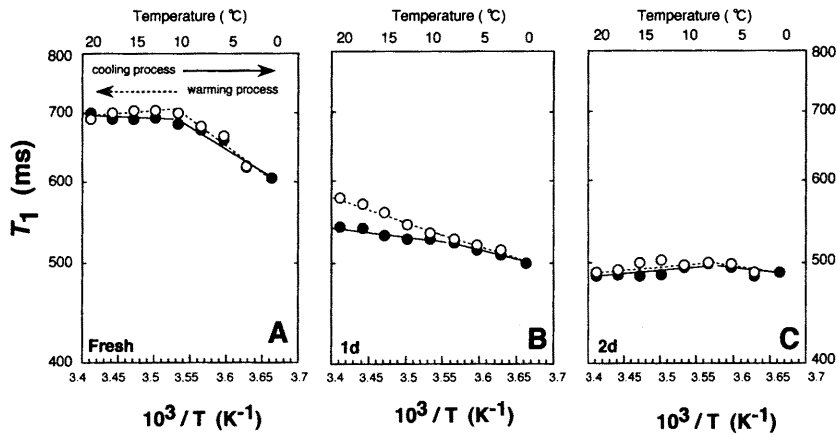
In previous report,  $T_1$  values of hypocotyl of *Vigna radiata* generally depended on temperature from 20 to 0 °C (Kaku and Iwaya–Inoue, 1988). Furthermore, break points occurred at 7.5 °C for *Vigna radiata* seedlings and at about 12.5 °C for *V. mungo*, respectively, and break point was not observed in *Pisum* seedlings (Iwaya–Inoue *et al.*, 1989).



**Fig. 1.** Typical profiles of thermal hysteresis of  $T_1$  relaxation times during slow cool–warm cycle (20 to 0 °C) for fresh gloxinia leaves. A profile for chilling insensitive clone (A), more sensitive clone (B) and the most sensitive clone (C). ●, cooling process; ○, warming process.



**Fig. 2.** Typical profiles of thermal hysteresis of  $T_1$  relaxation times during slow cool-warm cycle (20 to 0°C) in tomato leaves. A profile for fresh leaves (A), 1d-prechilled leaves (B) and 2d-prechilled leaves (C). Days (1d and 2d) indicated are the duration of prechilling at 5°C before cool-warm cycle. ●, cooling process; ○, warming process.



**Fig. 3.** Typical profiles of thermal hysteresis of  $T_1$  relaxation times during slow cool-warm cycle (20 to 0°C) in cucumber leaves. A profile for fresh leaves (A), 1d-prechilled leaves (B) and 2d-prechilled leaves (C). Days (1d and 2d) indicated are the duration of prechilling at 5°C before cool-warm cycle. ●, cooling process; ○, warming process.

In this study, an inflection was also determined on the Arrhenius plots with respect to the temperature dependency of the  $T_1$  in tomato and cucumber fresh leaves (Figs. 2A, 3A). On the other hand, break point for gloxinia leaves of chilling-insensitive clone was absent in cooling process (Fig. 1A).

An Arrhenius plots of  $T_1$  revealed a break in the line at specific temperature in chilling

sensitive plants such as tomato and cucumber, suggesting the occurrence of a metabolic transition at this temperature. Previous studies showed that the “break” in the Arrhenius plots of membrane thermal behaviors was considered to be due to a phase transition of membrane lipids, and it occurred in chilling-sensitive plants and was absent in chilling-resistant plants (Lyons, 1973; Levitt, 1980). This transition in the structure of phase state of the lipids in bilayers of cell membrane results in contraction of the membrane layer, and membrane-bound protein molecules are probably compressed and suffer a conformational change. In this study, the results suggested that “break” points of Arrhenius plots of water mobility in cooling process from 20 to 0 °C corresponded the chilling sensitivity in chilling sensitive and insensitive crops.

### **Thermal hysteresis of $T_1$ in crop leaves**

Typical profiles of  $T_1$  hysteresis in gloxinia, tomato and cucumber leaves during a cool-warm cycle (20 to 0 °C) are shown in Figs. 1, 2 and 3, respectively. Gloxinia leaves showing no thermal hysteresis indicated no injury after a cool-warm cycle (Fig. 1A).  $T_1$  values indicated shortening followed by marked prolongation in a warming process (Fig. 1B). The clone leaves showed about 50% of chilling injury after a cool-warm cycle. When a gloxinia clone showed marked shortening of  $T_1$ , about 80% of the leaf areas were killed after the same cool-warm cycles (Fig. 1C). These results indicate that the existence of differences in the thermal hysteresis of  $T_1$  in chilling sensitivity in gloxinia clones.

When thermal hysteresis in  $T_1$  was not clearly observed in fresh gloxinia, tomato and cucumber leaves (Figs. 1A, 2A, 3A), direct symptoms of chilling injury were not observed in these tissues after a slow cool-warm cycle. On the contrary, some tomato leaves, exposed to 5 °C for 1 and 2 days before such cool-warm treatment, showed black spotting (Fig. 2B, C). Manifested thermal hysteresis did not occur in these leaves when they were compared to that of the most sensitive gloxinia clone (Fig. 1C). Typical profiles of  $T_1$  hysteresis during slow cool-warm treatment in cucumber leaves are shown in Fig. 3.  $T_1$  prolongation in the warming process occurred in all experimental plots in fresh leaves (Fig. 3A). When cucumber leaves exposed to 5 °C for 1 and 2 days, respectively, visible injury symptoms were not observed. These leaf tissues after a slow cool-warm cycle indicated  $T_1$  prolongation in the warming process (Fig. 3B, C).

Chilling sensitivity of cucumber and tomato leaves differed from that of gloxinia and the  $T_1$  hysteresis is specific in individual plants. However, the basic relationship between the extent of  $T_1$  hysteresis and the degree of chilling injury was consistently observed in these leaves. Especially the restoration trends of  $T_1$  values in the warming process correlated with the degree of chilling damage or lesion in gloxinia leaf tissues (Kaku and Iwaya-Inoue, 1987). The relation between the restoration process of  $T_1$  upon warming and the degree of chilling injury is summarized as follows: 1) a slight prolongation of  $T_1$  or no hysteresis was observed when chilling injury did not occur; 2) a marked prolongation of  $T_1$  was observed when injury occurred; 3) a marked shortening of  $T_1$  occurred from the earlier stage of the warming process when tissues were severely injured.

### **Evaluation of $T_1$ hysteresis as related to chilling sensitivity in crop leaves**

It is assumed that the relaxation time of tissue water in biological systems is influenced by abnormal states in cells and tissues because of the water status in tissues,

and the structure of water will be altered by such physiological disturbance (Budinger and Lauterbur, 1984; Ishida *et al.*, 2000). More precisely, it was shown that the  $T_1$  of water protons in biological systems can be affected by a variety of changes in conformational state of macromolecules, water-membrane and water-protein interactions (Chang *et al.*, 1981; Mathur-De Vre, 1984). Distinct  $T_1$  changes in the gradient after the "break" in *Vigna* two species occurred might suggest the beginning of the  $T_1$  prolongation, assuming that the break does not occur and a linear drop of  $T_1$  continues over the entire temperature range, and if the  $T_1$  values between the actual data and the predicted value derived from the regression line for the first straight line segment (Iwaya-Inoue *et al.*, 1989). Additionally,  $T_1$  values of *Vigna radiata* seedlings gradually increased after the tissues were exposed to 0°C for 1h, and decrease in pH caused  $T_1$  prolongation *in vitro* (Iwaya-Inoue *et al.*, 1993). In the same species, it was shown that low temperature-induced acidosis of the cytoplasm at the initial stage of chilling stress was caused by a conformational change in H<sup>+</sup>-ATPase (Yoshida *et al.*, 1989). Thus it seems likely that  $T_1$  prolongation depends upon changes in cytoplasmic pH changes. Ling (1984) suggested that  $T_1$  of the water proton should reflect the multilayer state of normal cell water and it would be expected to increase with cell death and deterioration.

On the other hand, final post-thaw  $T_1$  value at 20°C in the thermal hysteresis following a freeze-thaw cycle of azalea flower buds was always lower than the original  $T_1$  value of the non-frozen buds, and we assumed that this may be due to the freezing in flower buds during membrane injury and loss of cell compartmentation would occur (Kaku *et al.*, 1985). Therefore, if severe chilling injury occurs in plant leaves (Fig. 1C), a marked shortening of  $T_1$  upon warming could happen just at the case of freeze-thawed samples in chilling-resistant plants. Thus there might be a similarity in relaxation behaviors of water protons between tissues severely damaged by low temperature of chilling-sensitive plants, and freeze-thawed tissues of chilling-resistant plants. In addition,  $T_1$  values determined at the initial temperature of the prechilled cucumber leaf tissues were around 500 ms (Fig. 3B, C), while  $T_1$  value of the fresh tissues was about 700 ms (Fig. 3A). Otherwise, cucumber leaves exposed to 3°C for 1d and warmed for another 1d at 28/22°C (Day/Night) indicated chilling injury in relation to involvement of oxygen radical generation (Shen *et al.*, 1999). Although fresh cucumber leaves after a cool-warm cycle and leaves exposed to 5°C for 1 or 2 days were not conspicuously injured, for the samples chilled over 4 days necrotic area in leaf tissues became visible following 2 days at 25°C (data not shown). From these results, Arrhenius plots of  $T_1$  during cool-warm cycle in leaves provides a sensitive and non-invasive information for evaluating plant response to chilling stress.

In conclusion, it appeared that measurement of thermal hysteresis of  $T_1$  following a cool-warm cycle provides a potential practical application as chilling temperature is a major limiting factor of geographical locations suitable for growing crops.

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