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Reversible or Irreversible Change of Molecular Dynamics of Water in Pea Seedlings Exposed to Heat Stress

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Influences of heat stress on epicotyls of pea (*Pisum sativum* L.) plants were investigated by using non-destructive method. NMR spin-lattice relaxation time (T_1), indicating molecular dynamics of water, was determined in tall type, cv. Alaska and dwarf type, cv. Progress No. 9 seedlings grown under light condition. In no-elongation zone for both fresh cultivars a rapid decrease in T_1 occurred in the tissues exposed to 20–30 °C while rapid increase followed by gradual decrease occurred in T_1 for the tissues exposed to 20–40 °C during a heat-cool cycle. On the other hand, in heat-denatured dead tissues, T_1 linearly corresponded. Further, when pea plants were heated for 5 h at 20–30 °C or 20–40 °C thermal hysteresis changes in T_1 corresponding temperature in the subsequent heat-cool cycles was notably different. Epicotyls exposed to 30 °C treatments did not show temperature dependency while those of the tissues exposed to 40 °C indicated temperature dependency. After the subsequent heat-cool cycles, the former was alive, the latter indicated tissue necrosis. Therefore, the change in T_1 of epicotyls observed within 1 h was considered a fast adaptation or mortality in cells to heat stress, and a temperature dependency of T_1 in the subsequent heat-cool cycles clearly reflected tissue viability in pea epicotyls. In conclusion, T_1 on thermal response can be used as an indicator of reversible or irreversible injury in an intact plant.

INTRODUCTION

Heat stress induces various physiological changes in organelles of pea (*Pisum sativum* L.) plants (Galvis *et al.*, 2001; Salvucci *et al.*, 2001, references therein). A 3 h treatment at 40 °C of pea (var. Douce Provence) plants induces production and accumulation of a small heat-shock protein, HSP22, in the matrix compartment of mitochondria (Lenne and Douce, 1994). Furthermore, a partial HSP21 complex purified from heat-stressed pea leaves contained no proteins other than HSP21 (Suzuki *et al.*, 1998). On the contrary, treatment at 40 °C to pea plants led to a serious injury of the photosynthetic apparatus as seen in a sharp increase of the ground Chl fluorescence Fo and a decrease of the variable Chl fluorescence Fv and the ratios Fv/Fm while the changes in Chl fluorescence parameters at 35 °C were reversible (Georgieva and Lichtenthaler, 1999). Further, 38 °C treatment caused an immediate decline of net D1 synthesis in pea plants (Franco *et al.*, 1999). Therefore, temperatures around 40 °C seem to set the tem-

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perature limits for studies on the heat acclimation or heat injury mechanisms of pea plants.

Besides, temperature response in pea seedlings was determined by using a diagnostic tool for monitoring the primary response of cells (Kaku and Iwaya-Inoue, 1988). A thermal hysteresis for nuclear magnetic resonance (NMR) spin-lattice relaxation time (T_1) in pea seedlings following a slow cool-warm cycle, between 20 to 0 °C, indicated that pea plants were chilling resistant. Additionally, there was no phase change in T_1 of epicotyls for pea plants while a phase transition was observed in chilling sensitive plants such as *Vigna radiata* and *V. mungo* seedlings (Iwaya-Inoue *et al.*, 1989). NMR allows nondestructive determination of each unique type of an NMR-active atom present in a molecule, how many of those atoms are present in the molecule. And thus both T_1 and spin-spin relaxation time (T_2) were used as indicators of molecular dynamics of water in tissues (Ishida *et al.*, 2000; Iwaya-Inoue and Nonami, 2003a, references therein). By using the parameter, sensitivity to various stresses of each species appears to be related to the severity of conditions in its natural habitats; leaves of azalea species with higher sensitivity to an environmental stress also exhibit higher sensitivity to other stresses and vice versa (Kaku, 1993). We have reported that dwarf type of pea was more drought resistant compared to the tall type (Iwaya-Inoue *et al.*, 2003b).

Otherwise, when pea plants were heated for 10–60 min at 42 °C, a reversible shift of the prooxidant-antioxidant equilibrium towards stimulation of lipid peroxidation, observed in chloroplasts after around 15-min heat treatment, is considered a fast adaptive response of cells to hyperthermia (Kurganova *et al.*, 1999). In addition, pea (cv. Feltham First) exposed to a heat stress of 37 °C for 6 h accumulated 22 kDa- HSPs and the response to heat shock was rapid with protein expression detected within 45 min (Wood *et al.*, 1998). Although mechanism of thermo-tolerance in pea plants has been extensively studied, little is known about time dependent changes in intact pea plants. Therefore, it is important to study dynamic states of water which affect cellular metabolism and to address the possibility of the primary response in the epicotyl tissues subjected to heat stress with non-destructive method. This study has two objectives: first, to determine characteristics of T_1 in intact fresh tissues of pea exposed to heat stress; and, second, to determine characteristics of T_1 in relation to dwarfism during subsequent heat-cool thermal hysteresis.

MATERIALS AND METHODS

Plant materials

Tall type of pea (*Pisum sativum* L.) cv. Alaska obtaining from commercial sources was used. Furthermore, seeds of dwarf genotype, cv. Progress No. 9, were provided by Dr. M. Katsumi. Two cultivars of pea were cultivated in a growth cabinet (Koitotron HNB-10A) at about 22 °C with a photoperiod of 14 h light/10 h dark. Fluorescent lamps (FLR20S W/M 20W×4) were used as a luminous source. 10 to 12 day-old seedlings were used as materials. 100 μM GA_1 was sprayed on an apex of 5 day-old seedlings of cv. Progress No. 9 grown under light condition. Epicotyls of the second node from the apex part as elongation zone or basal part as no-elongation zone were cut from the intact pea seedlings and they were used following experiments.

Measurements of proton T_1 relaxation times

Epicotyl tissues (each about 20 mm in length) were packed into a 7.5 mm diameter NMR tube. 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 epicotyls were measured by inversion recovery method using a $180°-\tau-90°$ pulse sequence (Farrar and Becker, 1971). The probe temperature was controlled by a thermostat (Lauda Kryo-SK65) connected to the sample chamber of the spectrometer, and NMR tubes without caps to allow air ventilation were used. Thus both measurements of T_1 and temperature treatments were carried out under aerobic conditions.

Heating and cooling treatment

For the slow heat-cool cycle, the temperature of the epicotyls was elevated gradually from 20 to 40 °C and *vice versa*. Control experiment was carried out in the temperature range from 20 to 30 °C and *vice versa*. Temperature was increased from 20 to 40 °C or 20 to 30 °C in 30 min, subsequently samples were allowed to equilibrate for 60 min at 40 or 30 °C, respectively (Figs. 1 and 2). They were kept at these equilibrated temperatures for 30 min and then they were treated to the second and third subsequent heat-cool cycles, and the thermal hysteresis lasted about 5 h (Fig. 3). The probe temperature and heating-cooling 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. It was done four replications for each cultivar and Figures indicate typical profiles in individual experimental series.

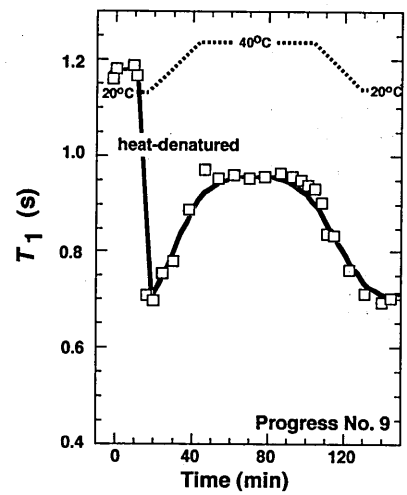

Fig. 1. Typical profile of thermal hysteresis of T_1 during a slow heat-cool cycle for pea (*Pisum sativum* L.) cv. Progress No. 9 epicotyls. Broken lines indicate temperature cycle for treatment as follows: before treatment (20 °C) followed by heat treatment (40 °C) and cool treatment (20 °C).

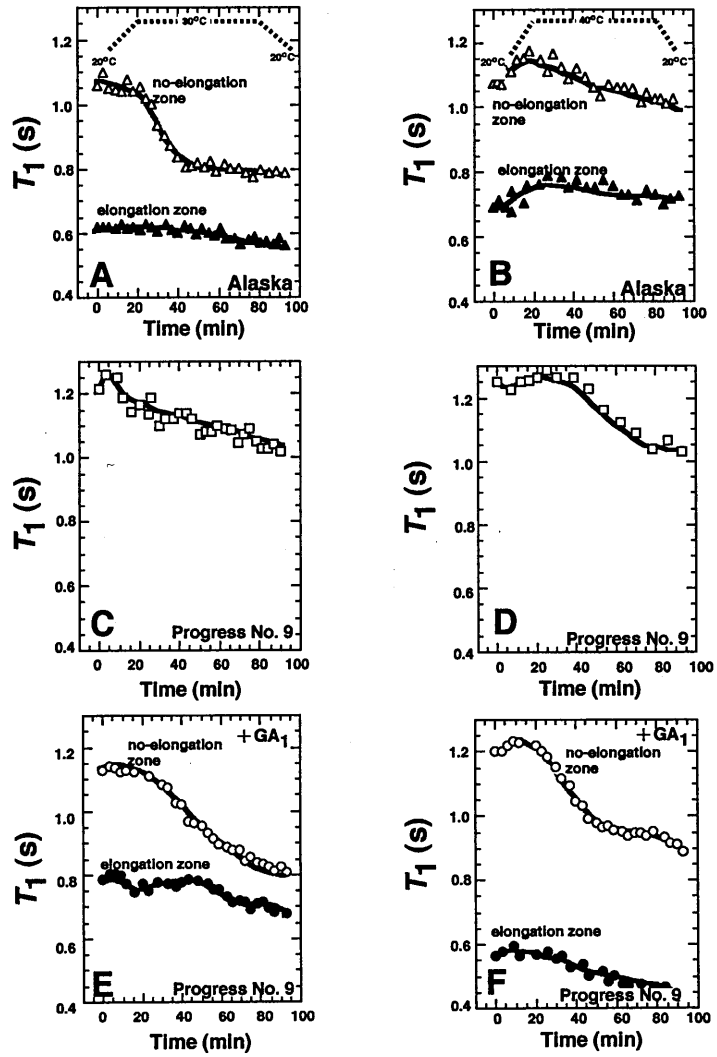

Fig. 2. Typical profiles of thermal hysteresis of T_1 during a slow heat–cool cycle for pea plant cultivars. (A) T_1 of cv. Alaska during 20–30 °C cycle. (B) T_1 of cv. Alaska during 20–40 °C cycle. (C) T_1 of cv. Progress No. 9 during 20–30 °C cycle. (D) T_1 of cv. Progress No. 9 during 20–40 °C cycle. (E) T_1 in GA_1–dosed cv. Progress No. 9 during 20–30 °C cycle. (E) T_1 in GA_1–dosed cv. Progress No. 9 during 20–40 °C cycle. Open symbols indicate no–elongation zone and closed symbols indicate elongation zone in each cultivar. Triangles indicate T_1s of Alaska, squires indicate those of Progress No. 9 and circles indicate those of GA_1 treated Progress No. 9. Broken lines in (A) indicate temperature cycle for treatment as follows: before treatment (20 °C) followed by heat treatment (30 °C) and cool treatment (20 °C), and the same treatment is not shown in both C and E. Broken lines in (B) indicate temperature cycle for treatment as follows: before treatment (20 °C) followed by heat treatment (40 °C) and cool treatment (20 °C), and the same treatment is not shown in both D and F.

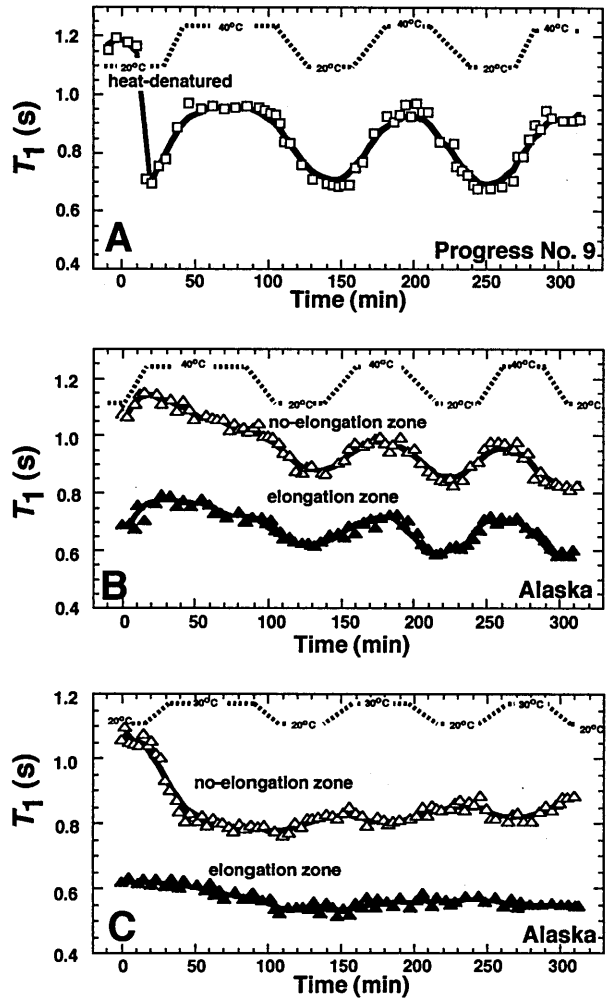

Fig. 3. Typical profiles of thermal hysteresis of T_1 during subsequent slow heat–cool cycles for pea epicotyls. (A) T_1 of cv. Progress No. 9 in 20–40 °C cycles. (B) T_1 of cv. Alaska in 20–40 °C cycles. (C) T_1 of cv. Alaska in 20–30 °C cycles. Broken lines in (A and B) indicate temperature cycles for treatment as follows: before treatment (20 °C) followed by heat treatment (40 °C) and cool treatment (20 °C). Broken lines in (C) indicate temperature cycles for treatment as follows: before treatment (20 °C) followed by heat treatment (30 °C) and cool treatment (20 °C).

RESULTS AND DISCUSSION

Thermal hysteresis of T_1 in heat-denatured pea seedlings

NMR spin-lattice relaxation time (T_1), indicating molecular dynamics of water, was determined in tall type, cv. Alaska and dwarf type, cv. Progress No. 9 in pea (*Pisum sativum* L.) epicotyls. The influence of the heat-denatured at 120 °C for 15 min on T_1 in intact epicotyl tissues of seedlings from cv. Progress No. 9 is indicated (Fig. 1). After the heat treatment, T_1 values in intact tissues markedly decreased from 1.2 s to 0.7 s. A similar tendency was observed in both elongation and no-elongation zones of epicotyls for pea cv. Alaska (data not shown), hypotoyls of mung bean (*Vigna ratidata* L.) (Iwaya-Inoue *et al.*, 2000) and frog lens tissues (Neville *et al.*, 1974). Change in T_1s as function of temperature in the heat-denatured tissues exposed to 20–40 °C thermal hysteresis is shown in Fig. 1. The T_1 values increased gradually with heating and decreased upon cooling. Therefore, a positive temperature dependency was clearly observed in T_1 for the heat-denatured dead tissues. In frozen-thawed sweet potato (*Ipomoea batatas* (L.) Lam.) tuber tissues, a positive temperature dependency of the Arrhenius plots was also clear in T_1 determined at temperatures ranging between 0 to 30 °C (Iwaya-Inoue *et al.*, 2004a). These results were supported by the fact that temperature dependency in T_1 was observed in solutions *in vitro* (Iwaya-Inoue *et al.*, 2004a). Therefore, the T_1 dependency in the dead plant tissues seems to solely reflect the mobility of water in the tissues.

Changes in T_1 of tall and dwarf types for pea seedlings exposed to 30 or 40 °C thermal hysteresis

Initial values of T_1 in no-elongation zone of epicotyls for tall type pea, cv. Alaska, were significantly higher than those of elongation zone, respectively (Fig. 2, A, B). In addition, the initial values in T_1 of epicotyls for dwarf type, cv. Progress No. 9 (Fig. 2, C, D)., were higher than those of elongation zone of cv. Alaska. It was indicated that the elevated T_1 value in dwarf type was not related to water content but to osmotic concentration (Iwaya-Inoue *et al.*, 2003b). Thermal response on T_1 in both tall and dwarf types of pea plants exposed to 20–30 °C heat-cool cycle is shown in Fig. 2 A and C, respectively. T_1 in no-elongation zone of epicotyls of cv. Alaska was initially 1.1s and it was almost constant or slightly decreased between 20 and 30 °C (Fig. 2A). When the tissues were kept at 30 °C T_1 markedly decreased to about 0.8s and indicated constant value while T_1s in elongation zone of cv. Alaska did not change during heat-cool cycle. In Progress No. 9, T_1 slightly increased and it gradually decreased during 20–30 °C thermal hysteresis (Fig. 2C).

On the other hand, T_1 in no-elongation zone of cv. Alaska epicotyls was initially 1.1s and it linearly increased between 20 and 40 °C, subsequently it gradually decreased at temperature equilibrium at 40 °C followed by temperature decrease (Fig. 2B). Although the initial value of T_1 in elongation zone of cv. Alaska was about 0.7s and it was markedly lower than that of no-elongation zone, a similar tendency on temperature change was observed when they were heated to 40 °C. Additionally, 40 °C-thermal response on T_1 in epicotyls of cv. Progress No. 9 is shown in Fig. 2D. T_1s of the dwarf cultivar kept the initial value and then gradually decreased during 20–40 °C thermal hysteresis.

In pea seedlings exposed to the heat stress, there was no clear relationship between T_1 values and water contents (data not shown). T_1 values of mung bean seedling exposed

to 40 °C for 4 h decreased by 10 to 15%, while water content decreased slightly (by less than 2%) after the stress (Iwaya-Inoue *et al.*, 1993). The decrease in T_1 was also observed in leaves of 6 wks-old perennial ryegrass exposed to 50 °C, however, water contents decreased (Iwaya-Inoue *et al.*, 2004b). These results suggested that relationship between T_1 and water content was affected by morphological characteristics and growth stage in the plant tissues.

GA_1-dose response on T_1s in dwarf type of pea seedlings exposed to heat stress

Epicotyls of cv. Progress No. 9 grown under light condition are extremely short compared to those of tall type, cv. Alaska, and exogenous GA_1 on dwarf pea seedlings induces shoot growth. Red light suppresses the growth of the shoots of pea seedlings via a phytochrome-mediated response (Sponcel, 1986). It has been stated that A-2s were responsible for red light-induced growth inhibition in cv. Progress No. 9 (Noguchi and Hashimoto, 1990). Further, it was indicated that red light inhibited the shoot elongation of cv. Progress No. 9 much more than that of cv. Alaska and the difference in GA 3β-hydroxylase between these cultivars was one replacement of alanine with threonine (Kato-Noguchi, 2002). Exogenous GA_1 on cv. Progress No. 9 seedlings caused marked changes in T_1 level; GA_1-dose induced decreased initial T_1 value in elongation zone while it increased T_1 in no-elongation zone (Iwaya-Inoue *et al.*, 2003b). GA_1-dose response on T_1 with temperature dependency is indicated by epicotyls of light-grown dwarf pea plants (Fig. 2 E, F). Changes of T_1 in epicotyls of GA_1-treated cv. Progress No. 9 indicated basically similar tendency observed in those in cv. Alaska during both 20-30 °C and 20-40 °C thermal hysteresis, respectively (Fig. 2 A, B). Kaku (1993) stated that leaves of azalea species with higher sensitivity to heat stress also exhibit higher sensitivity to drought stress, and *vice versa*. Although dwarf type indicated drought resistance (Iwaya-Inoue *et al.*, 2003b), there was no marked difference in T_1 changes and heat sensitivity among tall type, dwarf type and GA_1-dosed dwarf type.

Reversible or irreversible change in T_1s of pea seedlings exposed to subsequent heat-cool cycles

In the heat-denatured dead pea epicotyls T_1 was dependent on temperature during the subsequent heat-cool cycles for 5 h; it linearly decreased with decreasing temperature while it increased with increasing temperature (Fig. 3A). T_1s for water and sugar solutions clearly reflected thermal response (Iwaya-Inoue *et al.*, 2004a). Fig. 3B indicates T_1s in fresh epicotyls of both elongation and no-elongation zones of cv. Alaska exposed to 20-40 °C replicated thermal hysteresis during 5 h-treatment. T_1 linearly increased and it gradually decreased during 1 h while the subsequent thermal hysteresis, the second and the third exposure to the tissues, caused similar tendency observed in those of the heat-denatured dead tissues in the first heat-cool cyle (Fig. 1). On the contrary, T_1s in fresh epicotyls of pea exposed to 30 °C indicated noticeable difference in thermal response compared to those of 40 °C treatment; T_1s in fresh epicotyls of no-elongation zone of cv. Alaska kept marked shortening values at the subsequent thermal hysteresis during 5 h (Fig. 3C). In addition, there was no temperature dependency in elongation zone of cv. Alaska exposed to the same duration. Similar tendency was observed in both elongation and no-elongation zones of GA_1-dosed cv. Progress No. 9. From these results,

it was indicated that molecular dynamics of water in both heat-denatured and fresh tissues exposed to 40 °C exhibited temperature-dependent hysteresis while pea epicotyls exposed to 30 °C was temperature independent at subsequent heat exposure for 5 h-treatment.

It has been reported that treatment at 40 °C to pea plants led to a serious injury of the photosynthetic parameters (Georgieva and Lichtenthaler, 1999). In this study, epicotyls of both cvs. Alaska and Progress No. 9 after the subsequent thermal hysteresis of 20–40 °C cycles also indicated tissue necrosis. In contrast, the shortening of T_1 was observed at 40 °C in perennial ryegrass leaves and these tissues indicated no severe injury (Iwaya-Inoue *et al.*, 2004b). Additionally, a 3 h treatment at 40 °C of pea (var. Douce Provence) plants induced production and accumulation of HSP22 in the matrix compartment of mitochondria (Lenne and Douce, 1994). Further, heat shock treatment (40 °C for 4 h) caused decreasing T_1 values in hypocotyls of mung bean and it induced a 70 kDa protein and they were not severely injured (Iwaya-Inoue *et al.*, 1993). Members of heat shock protein, HSP70 family has been shown in the same species (Kawata and Yoshida, 1988). Further, pea (cv. Feltham First) exposed to 37 °C for 6 h accumulated 22 kDa-HSPs and the response to heat shock was rapid with protein expression detected within 45 min (Wood *et al.*, 1998). In yeast cells HSP70 family might stabilize denatured proteins and it might play a role as a surfactant in cells (Komatsu *et al.*, 1990). These results suggested that shortening of T_1 and no temperature dependency in intact pea epicotyls exposed to 30 °C-thermal hysteresis might reflect cell adaptation to heat stress.

Conclusion

When pea plants were heated for 5 h at 20–40 °C or 20–30 °C thermal hysteresis, a reversible or irreversible change in T_1 was observed. The change in T_1 of epicotyls within 1 h was considered a fast adaptation or mortality in cells to heat stress. The T_1 corresponding temperature in the subsequent heat-cool cycles was notably different; epicotyls exposed to 30 °C treatments, which were alive, did not show temperature dependency while those of the heat-denatured and fresh tissues exposed to 40 °C, which indicated tissue necrosis, were temperature dependency. Therefore, reversible changes in T_1 corresponding temperature reflected occurrence of tissue injury in pea epicotyls. From these results, T_1 on thermal response can be used as an indicator of tissue viability in an intact plant.

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