Cold- or Heat-Tolerance of Leaves and Roots in Perennial Ryegrass Determined by $^1$H-NMR

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Abstract: Dynamic states of water in the leaves and roots of perennial ryegrass (Lolium perenne L.) exposed to cold and heat stresses were studied by using $^1$H-NMR. NMR spin-lattice relaxation times ($T_1$) of leaves increased as temperature decreased from 20 to –20°C. However, spin-spin relaxation times ($T_2$) of the leaves decreased as temperature decreased below 0°C. The $T_2$ value of the long fraction (associated with vacuole) in leaves decreased to about 600µs at –25°C, but that of the short fraction was about 10µs, and the relative value of signal intensity of the long fraction decreased to about 0.2 at –25°C. The $T_2$ values of the two fractions in roots decreased to about 1ms at –10°C. Judging from $T_2$ and electrolyte leakage, both vacuolar and cytoplasmic compartments of leaves and roots froze at these temperatures. $T_1$ of the leaves decreased slightly as temperature increased from 20 to 40°C but greatly decreased as temperature increased further. The levels of electrolyte leakage from leaves exposed to the above heat stress was low suggesting that membrane was not severely injured. In conclusion, analysis of Arrhenius plots of $T_1$ and $T_2$ is a sensitive and non-invasive method to evaluate primary responses of perennial ryegrass organs to the temperature stresses.

Key words: Arrhenius plots, Cold stress, Dynamic states of water, Heat stress, NMR relaxation times ($T_1$, $T_2$), Organ, Perennial ryegrass, Water compartment.

Perennial ryegrass (Lolium perenne L.) has been widely cultivated as pasture plants in cool temperate region and it is important to select cold-tolerant perennial ryegrass genotypes for adaptation to northern climates (Ebdon et al., 2002). Many investigators assessed low-temperature tolerance using whole-plant survival and electrolyte leakage, and frost hardiness of stems of English ryegrass varieties has been studied by electrical impedance spectroscopy (Repo and Pulli, 1996). The over-wintering ability was mainly determined by the concentration of nonstructural carbohydrates in the roots rather than in the tops in four temperate perennial grasses including perennial ryegrass (Tamura and Moriyama, 2001). On the contrary, heat stress is also a major factor limiting the growth of the grasses during summer. Decline in photosynthetic rate, photochemical efficiency (Fv/Fm), and relative water content and the increase in electrolyte leakage occur in perennial ryegrass leaves exposed to heat stress (Jiang and Huang, 2001). To resolve these problems and maintain high productivity of perennial ryegrass through the year, it is important to elucidate the physiological response to temperature stresses of perennial ryegrass organs such as roots and leaves.

Although the mechanism of thermo-tolerance in plants has been extensively studied, little is known about dynamic states of water which affect cellular metabolism. Water is the major constituent of tissues in living cells. Since many metabolic processes such as enzymatic reactions, transportation and accumulation of materials occur in the cytosol, water in living tissues is considered to play an important role in their physiological condition. Therefore, the physical states of water reflect cellular activity. Nuclear magnetic resonance (NMR) allows nondestructive determination of changes in the state of water, and spin-lattice ($T_1$) and spin-spin ($T_2$) relaxation times are used as indicators of dynamic states of water in biological tissues since they reflect the motion of water molecules (Farrar and Becker, 1971). Since the mobility and characteristic of cell-associated water is closely related to the condition of the cells, NMR images represent physiological maps of the tissues (Kano et al., 1997; Ishida et al., 1997; 2000). In cases of disordered tissues in animals, relaxation times are greatly enhanced compared to those in the corresponding healthy ones (Damadian, 1971; Williams et al., 1980). Furthermore,
$T_1$ and $T_2$ of water protons have been applied to the studies of higher plant tissues exposed to thermal stresses (Kaku et al., 1985; Abass and Rajashekar, 1991; Iwaya-Inoue et al., 1993; Yoshida et al., 1997; Maheswari et al., 1999).

In chilling-injury studies Arrhenius plots of a variety of biological reaction rates have been examined, and non-linear temperature dependency or the presence of discontinuity has been seen as a diagnostic phase transition of membrane lipids and other parameters (Iwaya-Inoue et al., 1989; Caldwell, 1993; Schreiber, 1991; Iwaya-Inoue et al., 1993; Yoshida et al., 1997; Maheswari et al., 1999). In this study, we have characterized motional modes of tissue water in perennial ryegrass to determine cold and heat stress sensitivities of organs such as leaves and roots by using $^1$H-NMR spectroscopy.

**Materials and Methods**

1. **Plant material**

Perennial ryegrass (Lolium perenne L. cv. Friend) is a cold-tolerant pasture plant widely cultivated in north Japan (Yukijirushi Shubyo Co. Ltd.). Seeds were sown in September in 2001 and February in 2002 in pots (11cm in diameter×10cm height) filled with a mixture of (1 : 1 : 1, v/v/v) vermiculite/kokuryuhabido (meadow chernozen soil)/"Akadama" soil (reddish brown silty soil) and grown in a greenhouse at a rate of 0.5g L$^{-1}$ at sowing, and the plants were kept well-watered. A compound fertilizer was N-P-K : 16-16-16. After 3 weeks these pots were moved to a growth chamber (CR-41LC, Hitachi Ltd., Tokyo, Japan) and grown under a light of 25µmol m$^{-2}$ s$^{-1}$, with a 12h/12h day/night cycle at 15°C for 3 to 4 weeks before experiments.

2. **$^1$H-NMR analysis**

A $^1$H-NMR spectrometer with a magnet operating at 25MHz for $^1$H (Mu25A, JEOL Ltd., Tokyo, Japan) was used for the measurements of $^1$H-NMR spin-lattice relaxation time ($T_1$) and spin-spin relaxation time ($T_2$). Ten to twelve leaf pieces and 0.10 to 0.15g roots were used for the measurements of NMR relaxation times. Sample was put into an NMR tube (10mm in diameter) which was then set in the NMR spectrometer. For chilling treatment, the temperature of the samples was lowered in steps of 5°C from 20 to −15°C (or −25°C). The probe temperature was controlled with a thermostat connected to the sample chamber of the spectrometer using liquid nitrogen. For heat treatment, the temperature was raised in steps of 5°C from 20 to 50°C. The measurements were carried out after the NMR tubes were held at each temperature for 5 minutes.

For $T_1$ measurements, the saturation recovery method (90°-$\tau$-90° pulse sequence) was used (Figs. 1, 3, 5). In this method, $T_1$ is determined from $M_i = M_e[1 - \exp(-\tau/T_1)]$, where $M_i$ is the magnetization amplitude of proton at interval time $\tau$, and $M_e$ is the magnetization amplitude of proton in the equilibrium state. In this experiment, a free induction decay (FID) signal at every interval time $\tau$, was obtained by the accumulation of 4 scans. For measurement of $T_2$, the repetition time of the sequence was always kept more than five times of $T_2$. $T_2$ was measured by the Carr-Purcell-Meiboom-Gill (CPMG) method (Figs. 2, 6, 7 Phases 1, 2, Fig.4). $T_2$ is determined from $M_{m}=M_0\exp(-2\pi T_2\tau)$, where $M_0$ in the magnetization amplitude of the proton signal occurring at time 2$\tau$ after the initial 90° pulse in CPMG (90°-$\tau$-180°-$\tau$-180°-$\tau$...) pulse sequence. The $T_2$s were calculated based on 500 echo signals acquired by accumulation of 16 scans. The solid-echo (90°-$\tau$-90°) method was also applied for measurement of $T_2$ below 1ms (Figs. 2, 6, 7 Phase3). For this $T_2$ measurement, repetition time of the pulse sequence was also kept more than five times of $T_2$.

The solid echo signal was obtained by accumulation of 128 scans. $M(t) = \Sigma a_i \times \exp[-(t/T_i)^\alpha]$ where $a_i$ is the Weibull coefficient, and $\alpha$ is the signal intensity in each fraction. The relative value of fraction ratio, ($f_i$) is calculated by $f_i=a_i/\Sigma a_i$ (Sato, 1994).

A decay curve of echo signal was analyzed by using a non-linear least-square method on semi-log plots of signal intensity (Braga et al., 1997; Kumamoto et al., 1999). For detailed analysis, two component analysis was carried out. Changes in Arrhenius plots in organs exposed to cold stress were expressed by the $T_1$ value and relative value of signal intensity in two components (Fig. 5), by $T_2$ for organs (Fig. 6) and by $T_2$ for individual samples (Fig. 7).

3. **Measurement of water content**

Fresh and dry weights of leaves and roots were measured after drying in an oven at 80°C for 24h. The samples exposed to cold or heat-stress were also measured. Relative water content was expressed as the ratio of the amount of water to dry matter (g g$^{-1}$ dry matter).

4. **Leakage of electrolytes**

After the NMR measurement the leaves cut into pieces (5 × 5mm) and roots (about 0.05 g) were used for measurement of electrolyte leakage. Fresh samples were used as controls. The pieces were immersed in 20mL of distilled water and shaken at 120 reciprocates min$^{-1}$ for 5h, and leakage of electrolytes was measured with an electrolyte conductivity meter (Toa conductivity meter, Model CM-20E, Toa Electronics Ltd., Tokyo, Japan) and the extent of leakage of electrolytes was expressed as the percentage of the total electrolytes leaked from each sample, to those from the samples killed by a cycle of freezing and thawing.
Results

1. Changes in NMR relaxation times ($T_1, T_2$) in leaves and roots exposed to cold stress

Mean value of $T_1$ in leaves and roots was about 497 ms and 732 ms, respectively, at 20°C (Table 1). $T_1$ was markedly shorter in leaves than in roots. Fig. 1 shows Arrhenius plots of $T_1$ in the leaves and roots of perennial ryegrass exposed to cold stress. In the temperature ranging from 20 to 0°C (Phase 1), $T_1$ in leaves and roots linearly decreased with the drop in temperature. $T_1$s in the leaves gradually increased when the temperature was further lowered from 0 to –20°C and could not be determined at –25°C (Fig. 1A Phase 2). On the other hand, $T_1$ in roots could not be determined even at –5°C.

$T_2$ values in roots were also longer than those in leaves like $T_1$. $T_2$ values were almost constant or slightly increased with the decrease in temperature from 20 to 0°C in both leaf and root tissues (Fig. 2 Phase 1). $T_2$ in leaves gradually increased when the temperature decreased from 0 to –20°C (Fig. 2A Phase 2), but that in roots gradually increased from 20 to –5°C and decreased when the temperature decreased from –5 to –10°C (Fig. 2B Phase 2).

Mean values of $T_2$ determined at 20°C in leaves and roots were 181 ms and 279 ms, respectively (Table 1). $T_2$ values in roots were also longer than those in leaves like $T_1$. $T_2$ values were almost constant or slightly increased with the decrease in temperature from 20 to 0°C in both leaf and root tissues (Fig. 2 Phase 1).

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$T_2$s in the leaves linearly decreased with the drop in temperature. $T_2$s in the leaves and roots linearly increased when the temperature was further lowered from 0 to –20°C and could not be determined at –25°C (Fig. 1A Phase 2). On the other hand, $T_2$ in roots could not be determined even at –5°C.

Mean values of $T_2$s determined at 20°C in leaves and roots were 181 ms and 279 ms, respectively (Table 1). $T_2$s values in roots were also longer than those in leaves like $T_1$. $T_2$s values were almost constant or slightly increased with the decrease in temperature from 20 to 0°C in both leaf and root tissues (Fig. 2 Phase 1).

The extent of injury is closely related with the increase of electrolyte conductivity in temperature-stressed perennial ryegrass tissues (Jiang and Huang, 2001; Ebdon et al., 2002). Leakage of electrolytes from the ryegrass leaf and root tissues after the abrupt decrease of $T_2$ which occurred at –25 and –15°C, respectively, was about 100% (Table 2). Judging from the ion leakage, these organs were severely damaged after the exposure to these temperatures. From these results, the drastic decrease in $T_2$ reflected the freezing of tissue water in leaves between –20 and –25°C, but in roots at –10°C in the perennial ryegrass cultivar ‘Friend’.

Table 1. $T_1$ and $T_2$ shown as one component model and two component model in leaves and roots of perennial ryegrass cv. ‘Friend’ exposed to 20°C.

| Component | One component model | Two component model |
|-----------|---------------------|---------------------|
|           | Value, ms           | Value, ms          | Ratio |
| Leaves    | $T_1$ 497.3 ± 53.6 | 750.1 ± 75.8        | 0.57 ± 0.11 |
|           | $T_2$ 181.0 ± 8.7   | 246.8 ± 11.4        | 0.58 ± 0.00 |
| Roots     | $T_1$ 732.2 ± 50.3  | 1172.5 ± 129.2      | 0.49 ± 0.04 |
|           | $T_2$ 279.0 ± 17.1  | 408.3 ± 9.8         | 0.49 ± 0.03 |

$a$ Individual data indicate mean and S. D. of four replications.

Table 2. Electrolyte leakage and water content from leaves and roots in perennial ryegrass cv. ‘Friend’ exposed to cold or heat stress.

| Water content (g H2O g\textsuperscript{-1} dry wt.) | fresh\textsuperscript{a} | cold stress\textsuperscript{b} | heat stress\textsuperscript{c} |
|--------------------------------------------------|---------------------------|-------------------------------|-------------------------------|
| leaves                                           | 8.3 ± 2.0                 | 4.5 ± 0.5                     | 5.8 ± 0.5                     |
| roots                                            | 8.3 ± 0.3                 | 4.9 ± 0.5                     | 5.1 ± 1.5                     |

| Electrolyte leakage (%)                         | fresh | cold stress | heat stress |
|------------------------------------------------|-------|-------------|-------------|
| leaves                                         | 12.5 ± 2.3 | 99.6 ± 1.6 | 27.2 ± 4.8 |
| roots                                          | 30.5 ± 12.1 | 93.0 ± 1.0 | 56.3 ± 13.9 |

\textsuperscript{a}Exposed to 20°C (control)

\textsuperscript{b}After exposure to –15 or –25°C

\textsuperscript{c}After exposure to 50°C
2. Changes in NMR relaxation times ($T_1, T_2$) in leaves and roots exposed to heat stress

Fig. 3 shows Arrhenius plots of $T_1$ in leaves and roots exposed to the rise in temperature from 20 to 50°C. $T_1$ in leaves was almost constant or slightly decreased from 20 to 35°C and clearly from 35 to 40°C (Fig. 3A), but that in roots decreased in linearly 20 to 50°C (Fig. 3B). On the other hand, $T_2$ values in both leaves and roots of perennial ryegrass decreased linearly with an increase in temperature (Fig. 4). Ion leakage in the leaves and roots after exposure to the heat stress was 27 and 56 %, respectively (Table 2). These results
suggested that leaves were more tolerant to heat than roots.

3. Changes in water compartments in ryegrass leaves and roots exposed to cold stress

$T_1$ and $T_2$ values analyzed for the tissue water as one component (Figs. 1,2,3,4) were each divided into two fractions (short and long) for the two-compartment analysis (Figs. 5,6). $T_1$ values of the two fractions in leaves were constant or linearly decreased as the temperature was lowered from 20 to 0°C (Fig. 5A Phase1) but $T_1$ of the long fraction gradually increased.

![Fig. 3](image1.png) Arrhenius plots of $T_1$ of leaves (A) and roots (B) of perennial ryegrass cv. Friend exposed to an increase in temperature from 20 to 50°C. Arrows indicate heating process.

![Fig. 4](image2.png) Arrhenius plots of $T_2$ of leaves (A) and roots (B) of perennial ryegrass cv. Friend exposed to an increase in temperature from 20 to 50°C. Arrows indicate heating process.
as the temperature decreased from 0 to −10°C (Phase 2). $T_1$ could not be determined at −20°C. The relative value of signal intensity in all leaves tested was almost constant at the temperature between 20 and −15°C (Fig. 5C). On the other hand, $T_1$ values of the two fractions in roots linearly decreased as temperature decreased from 20 to 0°C (Fig. 5B), and the relative value of signal intensity did not change in this temperature range (Fig. 5D). $T_1$ in roots could not be determined below −5°C. These results indicated that changes in $T_1$s of leaves and roots shown by one component analysis (Fig. 1) did not depend on the change in signal intensity but on the change in $T_1$ values (Fig. 5).

$T_2$ values of the two fractions in the leaves were constant at temperatures between 20 and 0°C (Fig. 6A Phase 1). In the temperature range from 0 to −20°C (Phase 2), gradual change of the $T_2$ value of the long fraction increased slightly, but that of the short fraction decreased linearly. $T_2$ values of the long fractions and those of the short fractions were less than 1ms and 30µs, respectively at −25°C (Fig. 6A Phase 3). Additionally, the relative value of signal intensity in the long fraction gradually increased as the temperature decreased from 0 to −20°C (Fig. 6C Phase 2), but it markedly decreased at −25°C (Phase 3). In all roots tested, $T_2$ values of both long and short fractions were constant at temperatures between 20 and 0°C (Fig. 6B Phase 1). In Phase 2...
temperatures ranging from 0 to –10°C, the $T_2$ value of both fractions decreased slightly. The relative value of signal intensity in the long $T_2$ fraction increased at –5°C but decreased at –10°C (Fig. 6D Phase 2). During the observation of an NMR signal the determination could not be carried out by the CPMG method. Therefore, $T_2$ was determined by the solid echo method at –10 and –15°C. In Phase 3, $T_2$ values of long fractions and those of short fractions were less than 5ms and 1ms, respectively (Fig. 6B Phase 3).

Discussion

1. Influences of cold stress on $T_1$

Water in plant tissues is often characterized by different proton relaxation times (Ishida et al., 1987; Isobe et al., 1999; Iwaya-Inoue and Nonami, 2003). $T_1$ and $T_2$ have been used to detect the water components in parenchyma tissue of apple (Snaar and Van As, 1992). In morning glory (Pharbitis nil Chois.) seeds, three components of water fractions characterized by different $T_1$s and chemical shifts were observed (Isobe et al., 1999). $T_1$ of the two components in ryegrass leaves and roots linearly decreased as the temperature decreased from 20 to 0°C (Fig. 5 Phase 1). $T_1$ values of seedlings in Pisum sativum linearly decreased as the temperature decreased from 20 to 0°C (Iwaya-Inoue et al., 1989). On the contrary, $T_1$ values of the two components in ryegrass leaves gradually increased as the temperature decreased from 0 to –10°C (Fig. 5A Phase 2). Seasonal changes in $T_1$ were closely correlated with water content and supercooling ability of azalea florets (Kaku et al., 1984). The water content of ryegrass fresh leaves was 8.3 g H$_2$O g$^{-1}$ dry wt, and it decreased to 4.5 g H$_2$O g$^{-1}$ after the cold stress (Table...
Therefore, changes in $T_1$ of leaves exposed to cold stress could not be solely ascribed to the change in water content.

$T_1$ values of chilling-sensitive *Vigna radiata* hypocotyls have been reported to show a reversible gradual increase after the tissues were exposed to 0°C for 1h (Iwaya-Inoue et al., 1993). It is known that proteins are reversibly denatured at low temperatures as a result of weakening of hydrophobic bonds. A reversible change in conformation of H'-ATPase in the tonoplast of hypocotyls of *Vigna radiata* at 0°C, may cause cytoplasmic acidification (Yoshida et al., 1989). There was a close correlation between pH and $T_1$ in vitro; when the pH of a solution of paramagnetic ions and protein, in particular, Mn$^{2+}$ and bovine serum albumin (BSA), was lowered from 7.5 to 6.0, the $T_1$ value increased (Iwaya-Inoue et al., 1993). The range of pH values that affected $T_1$ significantly corresponded to the pH of the cytoplasm in *Vigna* cells in the culture exposed to 0°C (Yoshida et al., 1989). Therefore, it seems likely that the gradual prolongation of $T_1$ in plant tissues exposed to chilling stress depends partly on changes in cytoplasmic pH.

2. **Influences of cold stress on $T_2$ in organs**

The difference in $T_2$ values of biological tissues can also be interpreted in terms of the differences of the ratio of "free water" to "bound water" (Walter et al., 1989). Typical $T_2$ change in two water components in an individual measurement is shown in Fig. 7. The temperature dependency of the leaf tissues was not clear in $T_2$ values of both long and short fractions between 20 and 0°C (Fig. 7A Phase 1). $T_2$ changes in relation to chilling temperature have been discussed in animal cells; when chicken eggs were stored between 5 and 8°C for over 2 weeks, $T_2$ increased in comparison to unchilled eggs (Schwagele et al., 2001). $T_2$ of sugar solution depends less on temperature but $T_1$ linearly decreased as the temperature decreased from 30 to 0°C (Iwaya-Inoue et al., 2004). Thus, it was assumed
that the suppression of water mobility determined by $T_2$ was more intensified at higher temperatures.

In the leaf tissues, $T_2$ values in the long fraction increased but those in the short fraction linearly decreased as temperature decreased from 0 to $–20^\circ$C (Fig. 7A Phase2). $T_2$ in the long fraction indicating over 200 ms is thought to be mainly associated with vacuole and the fraction with the short $T_2$ less than 60ms is thought to be associated with the cytosol and apoplastic region in the leaves (Chen and Gusta, 1978). The prolongation of $T_2$ in the long fraction was accompanied by a gradual increase in signal intensity in the temperature range from 0 to $–20^\circ$C. In roots, $T_2$ values of the two components did not change when the temperature decreased from 20 to 0$^\circ$C but they decreased at $–10^\circ$C (Fig. 7B Phase2). The relative value of signal intensity of the long $T_2$ fraction increased at $–5^\circ$C but it decreased at $–10^\circ$C.

Abrupt decrease in $T_2$ value of the two components in both leaves and roots was accompanied with the decrease in signal intensity of the long fraction (Fig. 7 Phase3). The $T_2$ value of the long fraction in leaf tissues decreased to about 600$\mu$s at $–25^\circ$C, but that of the short fraction was about 10$\mu$s at $–25^\circ$C, and the relative value of signal intensity of the long fraction decreased to about 0.2 (Fig. 7A Phase3). The $T_2$ value below 100$\mu$s is thought to be associated with water tightly bound to macromolecules in cells (Hills and Remigereau, 1997). These results indicate that vacuolar water in leaf tissues disappeared at $–25^\circ$C. In root tissues, the component of the long $T_2$ fraction indicating over 300ms vanished and the $T_2$ values of both fractions was about 1ms at $–10^\circ$C equilibrium (Fig. 7B Phase3). The relative values of the signal intensity in the long fraction decreased from about 0.5 to 0.03 at $–10^\circ$C and the value did not change at $–15^\circ$C (Fig. 7B Phase3). In parenchyma tissue of apple, a peak of $T_2$ corresponding to the vacuole vanished at $–5^\circ$C showing that the vacuolar compartment had frozen, but the two peaks corresponding to the cytoplasm and cell wall compartments were observed indicating that these compartments remain unfrozen (Hills and Remigereau, 1997). The activity of 2,3,5-triphenyltetrazolium chloride (TTC) reduction in ryegrass leaves and roots which reflected the viability of the tissues vanished at $–25^\circ$C and $–15^\circ$C, respectively (data not shown). Furthermore, the leakage of electrolytes indicated that both leaves and roots exposed to subzero temperatures were severely damaged (Table 2). The shortening of $T_1$ has been attributed to a decline in membrane permeability under freezing injury in wheat crowns (Chen and Gusta, 1978). Therefore, the abrupt shortening of $T_2$ suggested that two water components in leaves froze at $–20$ to $–25^\circ$C and those in roots froze at $–10^\circ$C. The $T_2$ of cellular water is known to be dependent on plasmalemma and tonoplast permeability (Van As, 1992). Thus, a drastic decrease of the relative value of signal intensity as well as that of $T_2$ values in the two water components reflected the freezing of vacuoles and cytoplasmic water in the leaves and roots of perennial ryegrass cv. 'Friend'.

3. **Influences of heat stress on $T_i$ and $T_2$ in organs**

Arrhenius plots of $T_i$s in leaves exposed to a rise in temperature from 20 to 50$^\circ$C are presented in Fig. 3A. $T_i$s in leaves were almost constant or slightly decreased when the temperature was raised from 20 to 35$^\circ$C and they markedly decreased above 40$^\circ$C, whereas $T_2$ values in roots decreased linearly with the increase in temperature (Fig. 3B). On the other hand, $T_2$ values in leaves and roots gradually decreased with an increase in temperature (Fig. 4). Thus, $T_i$ and $T_2$ in both organs exposed to heat stress decreased in temperature from 20 to 50$^\circ$C. The water content of plant tissues is a main factor affecting water status of the tissues (Burke et al., 1974). Water contents of both fresh ryegrass leaves and roots were 8.3 g H$_2$O g$^{-1}$ dry wt, but after the heat stress the water contents of leaves and roots were 5.8 and 5.1g H$_2$O g$^{-1}$, respectively (Table 2). Therefore, the decrease in $T_i$ and $T_2$ of both organs exposed to the heat stress is partly ascribed to decrease in the water content.

Electrolytes leakage in leaves exposed to heat stress suggested the absence of severe damage (Table 2). Abass and Rajashekar (1991) reported that $T_2$ of the major fraction in grape leaves decreases steadily with an increase in temperature and sharply decreased at 48 and 45$^\circ$C, which correspond to the lethal temperature measured by both electrolyte leakage and TTC reduction tests. $T_i$s in leaves markedly decreased above 40$^\circ$C, whereas those in roots decreased linearly with the increase when the temperature was raised from 20 to 50$^\circ$C (Fig. 3). Heat shock treatment (40 ºC for 4h) lowered the $T_i$ values in hypocotyls of mung bean and it induced 71, 73 and 76kD proteins (Iwaya-Inoue et al., 1993). In the seedlings of the same species, members of heat shock protein HSP70 family are induced by heat shock at 41ºC for 4h (Kawata and Yoshida, 1988). The HSP70 family members in yeast cells might stabilize denatured proteins and might play a role as a "surfactant" in cells (Komatsu et al., 1990).

4. **Differences in the response to temperature stresses between leaves and roots**

Although there is a little information on the supercooling ability of herbaceous plants, $T_2$ determination revealed that perennial ryegrass 'Friend' has supercooling ability at $–20$ to $–25^\circ$C in leaves and at $–10^\circ$C in roots. Furthermore, the temperature dependency of $T_1$ at 40ºC in leaf tissues might reflect a primary response against heat stress because the level of electrolyte leakage was low even at 50ºC. Heat shock has been reported to induce HSP family in leaves of maize (Ristic et al., 1999) and pea (Dudley et
the ryegrass organs cannot decide (Table 1). This means that the water content alone of (Table 2), difference in water contents between leaves and roots of the leaves and roots. Although there was no dynamic states of water reflected the thermo-tolerance attended technical support. grateful to Mr. S. Nagai of JEOL Datum Ltd. for his provided by Yukijirushi Shubyo Co. Ltd. We are also advice. Seeds of ryegrass cv. 'Friend' were kindly Ntnl. Food Res. Inst. for giving M. I. I. their valuable both cold and heat stresses. and non-invasive way for evaluating tissue response to both cold and heat stresses.

These results show the importance of selecting temperature resistant perennial ryegrass organs for adaptation to cool and warm climates. Arrhenius plots of NMR relaxation times ($T_1$, $T_2$) in the leaves and roots of perennial ryegrass cultivar provided sensitive and non-invasive way for evaluating tissue response to cold and heat stresses.

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