Thermal Damage to Chloroplast Envelope Membranes

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ABSTRACT

Nuclear magnetic resonance was used to detect thermal injury to chloroplasts in vivo. A lesion occurs in the chloroplast envelope membrane at temperatures between 53°C and 57°C, depending on species, leaf condition, and heating rate. The injury is associated with a sudden loss of water from the chloroplast.

Plant leaves may be injured by brief exposure to elevated temperatures (2). The onset of damage occurs over a narrow temperature range, and more than one type of lesion is involved. In soybeans (5), for example, no injury occurs when leaves are dipped for 1 min into water at temperatures no higher than 53°C. If the temperature is 54°C, the treatment causes chlorosis, and when the water is 55°C or more, leaf necrosis is observed.

Fluorescence has been used to study thermal injury in leaves (16, 17) and isolated chloroplasts. The intensity of Chl fluorescence increases abruptly at the onset of damage (often between 50 and 55°C) to components of the thylakoid membrane. Differential scanning calorimetry has been used to observe thermal transitions in chloroplasts and thylakoids; some of the transitions have been assigned to denaturation of specific thylakoid proteins (18, 19). Microscopy also has been used to study thermally induced morphological changes in chloroplasts (1).

NMR offers a new way to study thermal damage. Signal intensity in the 1H NMR spectrum of a leaf arises almost entirely from 1H2O. However, a leaf spectrum is more complex than that of pure water because internal structures in the leaf distort the applied magnetic field (10). In some species, the NMR peak from chloroplast water is displaced from that of water in other leaf compartments (12); this provides an opportunity for studying chloroplast water in vivo (8, 11). In this paper, we report the use of NMR to measure thermally induced changes in the permeability of chloroplast envelope membranes. The NMR results are compared with thermal changes monitored using fluorescence induction kinetics.

MATERIALS AND METHODS

Mature leaves were harvested during August and early September from Norway maple (Acer platanoides L. cv Em-

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signals in a leaf; however, the ppm scale may be used to measure relative peak positions.

NMR spectra (Fig. 1a) showed no significant change as the sample was warmed from 44 to 52°C. However, at 53°C, the chloroplast peak shifted slightly upfield; this effect was much more pronounced at 54°C when the peak shifted 0.1 ppm to the right of its position in the preceding trace. Smaller upfield-shifts continued from 55 through 62°C. The nonchloroplast peak remained stationary throughout the series, although it broadened somewhat at higher temperatures.

Figure 1b presents a series of difference spectra which are especially useful for detecting small changes in the NMR signal intensity. In these difference spectra, an upward deviation indicates an increase in NMR signal intensity as temperature increases. Many of the traces show the signature of a peak-shift (a downward deflection adjacent to an upward deflection) at the position of the chloroplast peak. The trace that represents the difference between spectra obtained at 53 and 54°C (i.e. the one labeled 54°C) exhibited the largest peak-shift; it also showed a sudden increase in the intensity of the nonchloroplast peak.

NMR results similar to those in Figure 1 were obtained from 28 different maple leaf samples. In each case we observed a transition (an abrupt upfield shift in the chloroplast peak and a simultaneous increase in intensity at the nonchloroplast peak) that occurred over a narrow temperature range. The transitions were irreversible; when sample temperatures were raised and subsequently lowered, the spectra did not return to their original appearance. When experimental conditions were exactly reproduced, the transition temperature (i.e. the temperature at which the effect was strongest) was remarkably consistent (within ±1°C) from one sample to another, but it was somewhat dependent on heating rate; average maple leaf transition temperatures were 53, 54, and 56°C at heating rates of 0.25, 0.5, and 1.0°C/min, respectively. Comparing spectra recorded just before a transition with those obtained immediately after, we found that peak splittings (i.e. the distance between the tops of the two resolved peaks) increased by 9 ± 1% and that integrated chloroplast-peak intensities decreased by 8 ± 1% during the transition.

No significant difference was found between results from sun leaves and shade leaves. We used shade leaves for most of the samples because they have larger chloroplast water peaks (11).

Samples that were heated rapidly to temperatures much higher than 60°C or that were held above 60°C for long periods of time ultimately produced broad, unresolved spectra. Except for these general trends, results from temperatures above 60°C were not very reproducible.

Spectra from the other species studied also displayed two distinct NMR peaks from chloroplast and nonchloroplast water; however, the peaks were not as well resolved as those from maple (12). Transitions similar to those observed in maple were recorded at 55°C for birch, 54°C for linden, and 57°C for hickory, using 1°C/min heating rates. No transition was detected in tulip poplar leaf samples. Transitions in senescent maple leaves were weaker and broader than those in younger maple leaves, and they occurred at slightly lower temperatures; at heating rates of 1°C/min, the transition in senescent maple leaves was recorded at 53 ± 2°C.

Maple leaves were immersed in warm water for 20 s while still attached to the tree and then used immediately as NMR samples. Leaves exposed to 51, 53, 55, and 57°C showed normal 56°C NMR transitions (at heating rates of 1°C/min), while samples taken from leaves treated at 59, 60, 62, and 63°C showed no transitions (i.e. the transitions already had occurred before the NMR series was obtained). One day after immersion, leaves that had been exposed to 55°C or less showed normal 56°C NMR transitions, while leaves exposed to 57°C gave broad, weak NMR transitions at 54°C that resembled those of senescent leaves. Leaves that had been exposed to 55°C or less had a normal, healthy appearance 2 weeks after treatment, and they displayed a normal senescence at the same time as unexposed leaves. Leaves treated at 57, 59, and 60°C showed chlorosis within 1 week and necrosis within 2 weeks after treatment. Leaves exposed to 62 and
63°C displayed immediate necrosis; they were dry (but still green) within an hour after treatment.

Typical Chl a fluorescence induction kinetics were observed (Fig. 2) in Norway maple leaves that had been heat-treated for 20 s at temperatures ranging from 35 to 50°C. Deviations from typical kinetics began with the abrupt disappearance of the M phase at 53°C. The rate and amount of P quenching also began to decrease at 53°C and continued to decrease as the temperature increased. Fluorescence quenching disappeared entirely at 65°C when the recorder trace became a square wave.

**DISCUSSION**

According to theory (10), three conditions must be met simultaneously to produce NMR spectra that are resolved into chloroplast and nonchloroplast peaks: (a) chloroplast water must not mix with nonchloroplast water over short intervals of time (i.e. the chloroplast envelope membrane must be intact); (b) the thylakoid membranes must be intact and they must be oriented with respect to the leaf surface; (c) a large quantity of 'reserve' manganese must be bound to outer surface of the thylakoid membrane. Reserve Mn is not the same as Mn at the active site for water-splitting in PSII. Reserve Mn is much less effective as a relaxation agent than aqueous Mn²⁺ because it is relatively inaccessible to water. The leaves of most species do not satisfy all three conditions (usually because the third condition is not met); consequently, the NMR spectra of most leaves do not display well resolved chloroplast peaks (12).

Experimental results allow us to draw the following conclusions about the nature of the NMR transition:

1. The chloroplast envelope membrane, the thylakoid membrane, and the proteins that bind reserve manganese remain intact at temperatures beyond the NMR transition (i.e. to at least 60°C). This is shown by the persistence of the two resolved NMR peaks; if the chloroplast envelope membrane ruptured, if the thylakoids were seriously disrupted or disordered, or if manganese were released from the thylakoid, the two peaks would merge (as they do eventually at higher temperatures). This conclusion does not require that all membrane components must be undamaged, but only that the membranes retain their general integrity. Other studies also have shown that thylakoid and chloroplast envelope membranes persist intact to temperatures above 60°C (1, 2, 7).

2. The transition can be described as a sudden loss of water from the chloroplast. Two different observations both point to this conclusion. The nonchloroplast peak increases in intensity suddenly at the transition; the nonchloroplast peak intensity is proportional to water content of the cytoplasm and vacuole. The chloroplast peak moves suddenly at the transition to increase the peak splitting; peak splittings are inversely proportional to the quantity of water in the chloroplast (for constant amounts of reserve manganese). The similar relative magnitudes of the two effects (8 and 9%, respectively) and the fact that the chloroplast and nonchloroplast water compartments contain approximately equal quantities of water (11) demonstrate that the nonchloroplast water compartments gain about as much water as the chloroplasts lose.

Experimental NMR transition temperatures were somewhat higher when faster heating rates were used. The shift in transition temperature was not caused by thermal inertia (i.e. sample temperatures that lagged behind the temperature-control sensor) because we used a calibration procedure that corrected for inertia. Instead, we believe the effect was caused by a somewhat sluggish transition. This explanation can account for the differences between maple leaf NMR transitions observed at 53°C using the lowest heating rate, 56°C using the fastest heating rate, or between 57 and 59°C on the tree (where leaves were heated for only 20 s). Experimental differences in transition temperature from one species to another probably are real, as are the changes caused by senescence.

Our experiments with leaves attached to the tree suggest that the NMR transition is produced by a different lesion from those that cause chlorosis or immediate necrosis. On the living tree, the NMR transition occurred when leaves were treated at 59°C (or higher), but not at 57°C. Chlorosis without immediate necrosis was observed in leaves treated at 57, 59, and 60°C, but not at 53°C. Immediate necrosis was produced by treatment at temperatures of 62°C or higher. However, these results do not prove that chlorosis and the NMR transition were caused by different lesions. The transition begins at 53°C; but it is sluggish, and the damage occurs at a faster rate at higher temperatures. Therefore, treatment for a short time at 57°C would produce a small amount of damage. If chlorosis could be caused by a small amount of damage, but if a larger amount were needed for an observable NMR

![Figure 2](https://www.plantphysiol.org/)

**Figure 2.** Kinetics of Chl a fluorescence induction in maple leaves that were heat treated for 20 s at the temperature indicated.
transition, then the same lesion could be responsible for both
effects even though they appear at different temperatures.

Our results also suggest that the lesion responsible for the
NMR transition results in a loss of semipermeability in the
chloroplast envelope membrane. The sample holder (8, 12)
prevents rapid loss of water during the course of an experi-
ment; but when a leaf sample is heated, some water escapes,
and the leaf is somewhat water-stressed when the transition
temperature is reached. Previous NMR studies have shown
that water is lost primarily from nonchloroplast compart-
ments during the initial stages of leaf dehydration (8, 12); this
study suggests that upon heating, the chloroplast suddenly
loses water as the NMR transition occurs. Similar observa-
tions have been made using light microscopy; in 1937 Sheib-
mair (15) found that moss chloroplasts suddenly contract as
they are heated. NMR has been used previously to measure
the water permeability of chloroplast envelope membranes
(9).

No major changes were observed in fluorescence induction
below 53°C, a temperature that is higher than that associated
with thermal damage in isolated chloroplasts. This difference
is probably due to the brief heat treatments used in this study
and the fact that chloroplasts are more heat stable in vivo than
when isolated (2). Injury to the oxygen-evolving complex (4,
23), loss of metal ions (22), and loss of Hill activity (4) are
known to occur in isolated chloroplasts at temperatures as
low as 37°C, and irreversible grana destacking takes place in
isolated chloroplasts below 45°C (6). The disappearance of M
(at 53°C) indicates the loss of carbon assimilation (21), due
probably to damage within chloroplast thylakoids (7, 17).
Progressive changes in the level of P and suppression of P
quenching (from 53–65°C) indicate damage affecting the
proton gradient, the high energy state of the chloroplasts and
acceptors (3, 13, 14).

Based on a comparison of transition temperatures, none of
the changes detected using fluorescence induction appear to
be related to the changes observed using NMR. Fluorescence
changes presumably reflect alteration in the local environ-
ment of Chl molecules, i.e. in the thylakoid membrane (6, 16, 17).
Although the fluorescence work provides negative evidence, it
does substantiate our conclusion that the NMR transition is
cau ted by a change in the permeability of the chloroplast envelope membrane rather than by some change
that occurs in the thylakoid.

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