Nondestructive Real-Time Monitoring of the Redox Status in a Potted Plant by Using a Surface-Coil-Type ESR Resonator

Mika Tada, Takuo Shiraishi, Hidekatsu Yokoyama,*[†] Hiroaki Ohya,[†,††] Tateaki Ogata,[†††] and Hitoshi Kamada[†]
Regional Joint-Research Project of Yamagata Prefecture, Japan Science and Technology Corporation, 2-2-1 Matsuei, Yamagata 990-2473
[†]*Institute for Life Support Technology, Yamagata, 2-2-1 Matsuei, Yamagata 990-2473*
[††]*Graduate School of Science and Engineering, Yamagata University, 4-3-16 Jonan, Yonezawa 992-8510*
[†††]*Department of Chemistry and Chemical Engineering, Faculty of Engineering, Yamagata University, 4-3-16 Jonan, Yonezawa 992-8510*

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To perform nondestructive real-time monitoring of the redox state in plants against environmental stress, a 700-MHz surface-coil-type ESR resonator was employed and temporal changes in ESR signals of an exogenous nitroxide radical in the leaves of a potted tobacco plant were observed after chilling. ESR signals of the nitroxide radical were barely observed at room temperature, indicating that the nitroxide radical was reduced to the hydroxylamine by reductants in the leaves. Chilling significantly increased the signal-to-noise ratios (S/Ns) of the nitroxide radical in the leaves. Because hydroxylamines are oxidized to nitroxide radicals by oxidants, it is thought that the ESR S/Ns can be used as an index for oxidation in leaves.

Various environmental conditions cause oxidative damages to plants through the intracellular production of oxidants, such as reactive oxygen species (ROS).[1–3] UHF band-ESR analysis, using exogenous nitroxide radicals, has provided noninvasive and real-time information about reduction and/or oxidation in living animals.[4–8] However, this technique does not apply to nondestructive estimate of the effect of environmental stress on plants. Nitroxide radicals are reduced to ESR-silent compounds, hydroxylamines, by reductants such as ascorbic acid.[9] Hydroxylamines are oxidized to ESR-detectable compounds, nitroxide radicals, by oxidants such as ROS. Thus, ascorbic acid in plants may reduce a nitroxide radical that has been administered to them. A reduced form of the nitroxide radical (i.e., hydroxylamine) in these plants would be oxidized to ESR-detectable compounds if the environmental stress (e.g., chilling) produces oxidants (e.g., ROS) in them. Under these conditions, augmentation of the ESR signal can be used as an index for the generation of oxidants caused by this environmental stress. A surface-coil-type resonator (SCR),[10–12] one of the resonators that operate in the UHF band, is composed of a single-turn coil and a transmission line. Because the coil diameter of the SCR is very small compared to the wavelength of the operating RF, the radiation loss is negligible. Furthermore, because the transmission line is formed by a symmetrical structure, the SCR is barely affected by external perturbations. For these reasons, ESR measurements that employ an SCR do not require a shielded case. In an ESR spectrometer equipped with an SCR, the airgap in magnets that are used to generate an external magnetic field corresponds to a set space for a sample, which is adequate for holding of an entire potted plant. In this study, nondestructive real-time monitoring of the redox status in plants subjected to environmental stress was tried. For this purpose, a 700-MHz RF ESR spectrometer equipped with an SCR was used and temporal changes in the signal-to-noise ratio (S/N) of ESR spectra of a nitroxide radical in potted tobacco plants were investigated after chilling.

This ESR spectrometer, which was constructed in our laboratory (and has already been described in detail[6–9]), consisted of a main electromagnet, a pair of field scan coils, a pair of field modulation coils, an RF circuit for homodyne detection, and an SCR. The SCR consisted of a single-turn coil (10 mm in inner diameter) and transmission lines (semirigid coaxial cables with a 50-ohm characteristic impedance).[10–12] By using this resonator, one can observe free radicals in a 4-mm-thick area under the single-turn coil.[12] The SCR was connected to a VSWR bridge in the RF circuit. Conventional matching circuits were used to match the impedance of the SCR and input impedance (50 ohm) of the VSWR bridge at a resonant frequency of approximately 700 MHz.[12,13]

Before taking measurements, 3-carbamoyl-2,2,5,5-tetramethylpyrrolidin-1-yloxy (carbamoyl-PROXYL, Aldrich Chemical Co., U.S.A.) was administered to the tobacco leaves (Petit-Havana SR1)[14] via their surface. Filter papers cut to the size of the leaves were dipped into a 100 mmol dm^{-3} aqueous solution of carbamoyl-PROXYL. Each piece of the filter paper was allowed to adhere to the surface of a tobacco leaf[15] for 18 h. The carbamoyl-PROXYL administration employed in this procedure was seen to be harmless to the tobacco plants because the plants continued to grow normally for more than 40 days after the administration of carbamoyl-PROXYL. Subsequently, the entire potted tobacco plant was placed in the airgap of the electromagnets of the ESR spectrometer. A single-turn coil of the SCR was placed on a leaf that had received carbamoyl-PROXYL in the static magnetic field. Twenty minutes after the administration of carbamoyl-PROXYL, ESR

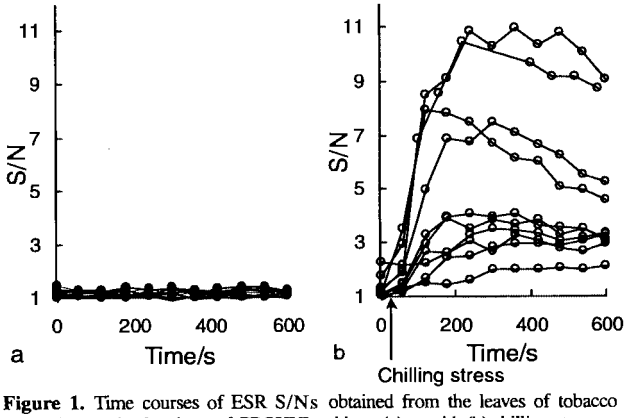

Figure 1. Time courses of ESR S/Ns obtained from the leaves of tobacco plants that received carbamoyl-PROXYL without (a) or with (b) chilling stress.

measurements were started, then repeated every 60 s with a field sweep width of 10 mT at room temperature (25 °C) and at an illuminance of 800 lx. One spectrum was obtained from an average of 48 accumulations of scans at a scan time of 1 s. The signal intensity was obtained from the peak-to-peak height of the lowest component in the triplet spectrum. The noise intensity was defined as the product of the standard deviation from the baseline of the spectrum multiplied by $2\sqrt{2}$. At room temperature, the ESR signal of carbamoyl-PROXYL was not detected (S/N<2) and temporal changes in the S/Ns were barely observable (Figure 1a). This suggested that carbamoyl-PROXYL was reduced to hydroxylamine by reductants in the leaf.

The leaves were chilled by using a cooling spray 20 s after the ESR measurements were started. The surface temperature of the leaf was lowered to –10 °C for 5 s. The chilling increased the S/Ns of all samples (Figure 1b). Because the peak-to-peak linewidth showed no temporal changes, each S/N means the carbamoyl-PROXYL concentration in a leaf. The rate of increase of the S/Ns, which was derived from the maximum S/N divided by the initial S/N, obtained from the leaf with or without the stress was 4.99 ± 2.38 or 1.29 ± 0.17 (mean ± SD), respectively. The values from the treated leaves were significantly larger than those without treatment (Student t test, $p < 0.001$; $n = 9$ with or $n = 8$ without treatment). These findings suggest that oxidants caused by chilling of the leaves oxidize hydroxylamine to carbamoyl-PROXYL. The S/N for most of the stress-treated samples reached the maximum, then decreased gradually. This shows that carbamoyl-PROXYL, which was formed from hydroxylamines by oxidants, was reduced again in the leaves. However, the S/Ns of a few samples kept increasing during the observation.

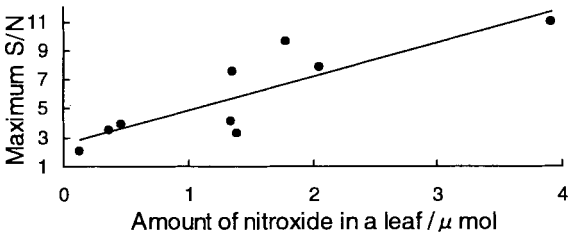

Figure 2. Relationship between the absorbed amount of carbamoyl-PROXYL in the leaf and the maximum S/N of carbamoyl-PROXYL after the chilling stress.

Although all samples responded to chilling, the maximum S/Ns showed large deviations. To investigate the relationship between the absorbed amounts of carbamoyl-PROXYL in the leaves and the maximum S/Ns of carbamoyl-PROXYL spectra, the former was measured as follows. The leaves were severed from the plant 5 min after the nondestructive ESR measurements and immediately frozen at –20 °C. The leaves were homogenized in a 20 mmol dm^{-3} sodium phosphate buffer (pH 7.4) and centrifuged at $10000 \times g$ for 10 min. The supernatants (final volume, 4.5 dm^{-3}) were boiled for 10 min to oxidize hydroxylamines, and then were mixed well.[16] The ESR spectra of each sample were recorded by using a conventional X-band ESR spectrometer (FR30, JOEL); and the amount of carbamoyl-PROXYL was estimated from the ESR signal intensity and a calibration curve. Figure 2 shows the relation between the absorbed amount of carbamoyl-PROXYL in a leaf[17] and the maximum S/N of carbamoyl-PROXYL after the chilling. The relationship was significantly correlated (coefficient, 0.8495; F-test, $p < 0.005$).

It was confirmed in this study that the redox balance in a leaf of a potted tobacco is likely to be in the oxidative state after the chilling stress; and that the S/N of the ESR spectra of carbamoyl-PROXYL within the leaf could be used as an index of oxidation. To our knowledge, this is the first study in which the redox balance in plants has been nondestructively monitored. The technique of measurement—in which carbamoyl-PROXYL was administered to a leaf and a 700-MHz ESR spectrometer equipped with an SCR was used—has made it possible. We think that our measuring method will be useful because it is applicable to other plant species, and other types of environmental stress can be accommodated.

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14 The authors are grateful to Ph. D. Hiroyasu Kitashiba, Regional Joint-Research Project of Yamagata Prefecture, Japan Science and Technology Corporation, for providing tobacco plants and useful advice.
15 Fifth leaves from the top of the longest branch of 8-month-old plants, which had two branches, were used for these experiments.
16 The addition of an oxidant such as potassium ferricyanide showed no augmentation of the ESR signal in the sample prepared in this procedure, confirming that the hydroxylamines in the sample have been completely oxidized to nitroxyl radicals during the preparation.
17 Variation in the size of the leaves used for this experiment was about 10 %.