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Direct observations of the redox states of frozen cherry buds by a unique in vivo ESR

Mika Tada,[a,*] Takuo Shiraishi,[a] Tadashi Takashina,[b] Hiroaki Ohya,[c,d] and Tateaki Ogata[e]

[a] *Japan Science and Technology Corporation, Joint-Research Project for Regional Intensive of Yamagata Prefecture, 2-2-1 Matsuei, Yamagata 990-2473, Japan*
[b] *Institute for Life Support Technology, Yamagata, Public Corporation for the Development of Industry, 2-2-1 Matsuei, Yamagata 990-2473, Japan*
[c] *Yamagata Horticultural Experiment Station, 423 Shiimaminami, Sagae 991-0043, Japan*
[d] *Graduate School of Science and Engineering, Yamagata University, 4-3-16 Jonan, Yonezawa 992-8510, Japan*
[e] *Department of Chemistry and Chemical Engineering, Faculty of Engineering, Yamagata University, 4-3-16 Jonan, Yonezawa 992-8510, Japan*

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Abstract

Low temperatures cause cellular damage in flower buds of the sweet cherry (*Prunus avium* L. cv. Satohnishiki). In this study, the redox states within the cherry buds suffering freezing damage were non-destructively observed by a unique in vivo electron spin resonance (ESR) technique with a spin probe such as carbamoyl-PROXYL. The ESR signals of carbamoyl-PROXYL-treated bud were continuously recorded under freezing and thawing condition, which was decreased to approximately −4 °C and maintained for 1.5 h, and then returned to room temperature. Most of the buds began to freeze at −2.5 to −3.9 °C. The peak areas of the ESR signals significantly increased during the period of temperature rise. These results show that the reduced carbamoyl-PROXYL within the frozen bud was re-oxidized and became ESR-detectable while the bud was thawing. Our in vivo ESR technique has confirmed the oxidative transition of the redox states within the buds during thawing.

Keywords: In vivo; ESR; Carbamoyl-PROXYL; Redox; Freezing damage; Cherry; Bud

The chilling and freezing of plants at low temperatures is a serious environmental factor, which suppresses growth and reduces the production yield. Flower buds of fruit trees are particularly susceptible to freezing damages at sub-zero temperatures [1–3]. The productivity of these fruit trees is considerably reduced by freezing damage to the tree buds [3,4]. Thus, investigation of the mechanics of this damage is necessary for agricultural practices. In order to acquire physiological information on the damaging process, non-destructive observations of physiological functions of living plants are necessary.

^{1}H nuclear magnetic resonance imaging (NMR) has been used to elucidate the relationships between freezing damage and fruit tissues, fruit tree buds or flower buds [5–7]. Water and sugar distribution in the fruit tissues of blueberries was mapped both before and after freeze/thaw using NMR imaging [5]. The freezing processes in the flower buds became clear through NMR images taken at various temperatures [6,7]. However, the use of NMR imaging in observing the physiological functions, biochemical reactions, and metabolisms of whole fruit buds or organs has until now been limited.

Oxidative stress has been implicated as a damaging factor in plants exposed to stressful temperature conditions [8–12]. The role of reactive oxygen spices (ROS), and free radicals involved with them, in causing oxidative damage during plant stress is well established. These ROS and free radicals are inevitable by-products of biological redox reactions [13]. Electron spin resonance (ESR) detection provides the most direct method for studying the role of free radicals in biological processes [11,13]. In vivo ESR technique using low

* Corresponding author. Fax: +81-23-647-3109.
E-mail address: mika@ckk.ymgt-techno.or.jp (M. Tada).

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frequency ESR and a spin probe technique using an exogenous nitroxyl radical are effective methods for the non-destructive observation the redox states of biological samples [14–17]. Low-molecular weight nitroxyl radicals have been used to observe the redox status (i.e., the balance of reductants and oxidants), which is a link for redox reaction of living animals. Nitroxyl radicals are reduced to ESR-silent compounds, hydroxylamines, by ascorbic acid [18–21]. The hydroxylamines are then oxidized to ESR-detectable compounds, nitroxyl radicals, by oxidants such as reactive oxygen species. Through the continuous detection of the ESR spectra of an exogenous nitroxyl radical, changes in the redox state of biological samples except animals might be observable using an in vivo ESR technique. The redox states of a non-destructed leaf of potted tobacco before and after chilling were previously observed by an in vivo ESR technique using a 700 MHz ESR spectrometer equipped with a surface-coil-type resonator (SCR) [22]. Chilling caused increased ESR signal intensity (peak-to-peak height) in the nitroxyl-treated leaf of tobacco. In the previous study, the nitroxyl radicals were reduced to ESR-silent hydroxylamines before chilling by reductants such as ascorbic acid in the tobacco leaves and the hydroxylamines were re-oxidized promptly after chilling.

Low temperatures below zero degree may affect the redox function of entire flower buds. In this study, the non-destructive real-time monitoring of the redox functions in fruit tree buds subjected to freezing damage was carried out using a 700-MHz microwave ESR spectrometer equipped with an SCR. A sample chiller was produced experimentally and used as the control temperature for living buds in order to observe and compare the redox status during freezing and thawing.

Materials and methods

The ESR spectrometer. A 700-MHz microwave ESR spectrometer, which was constructed in the laboratory [15–17,20,22,23], consisted of a main electromagnet (with air-core, water-cooled, and of a two-coil Helmholtz design), a pair of field scan coils, a pair of field modulation coils, an SCR, power supplies, a personal computer, and a 700-MHz microwave circuit for homodyne detection. Static magnetic field that is generated by the main electromagnet can be scanned by regulating the current through the field scan coils at a maximum scan rate of 15 mT/s. The magnetic field was modulated by the pair of modulation coils at a width of 0.2 mT with a frequency of 100 kHz. An airgap about 80 mm exists between the modulation coils attached to the surface of the field scan coils; wide enough to install living biological samples. The SCR consists of a single-turn coil (inner diameter, 10 mm Φ) and transmission lines formed by semirigid coaxial cables [24,25]. The single-turn coil must be aligned so that the microwave magnetic generated in the coil is perpendicular to the static magnetic field. This resonator can observe free radicals inner the single-turn coil and approximate 4-mm thick areas above and below the coil [17].

The sample chiller. The sample chiller manufactured for this report consists of a sample box, an airflow unit, and two cooling pipes. The sample box has an inner space of 36 mm (width) × 50 mm (height) × 60 mm (depth) and contains a pair of cooling plates attached to the inside surfaces. An airflow unit containing a fan to propel cold air is linked to this sample box (inner diameter, 20 mm Φ; length, 80 mm). The cooling plates and cooling pipes were made of macor, which is non-magnetic and very thermo-conductive. The cooling plates, the cooling pipes, and the airflow unit included tube spaces (inner diameters, 3–5 mm Φ) linked to each other. They are chilled by a temperature-controlled cooled coolant, which flows within the tube spaces using a re-circulating chiller (RTE 140 Bath Circulator, NESLAB Instruments, USA). The cold air circulates through the exclusive spaces in the sample chiller. The whole sample chiller is covered with thermal insulation. The sample chiller was able to decrease the air temperature inside the sample box from the 27 °C/h room temperature to −5 °C. The minimum temperature in the sample box was attained to be −8 °C. The sample box of the sample chiller was installed in an airgap between a pair of 100 kHz modulation coils in the ESR spectrometer. The single-turn coil of the SCR was inserted in the sample box and fixed in the center of the modulation coil.

In vivo ESR measurements. Flower buds of the sweet cherry tree (*Prunus avium* L. cv. Satohnishiki) were investigated. Twigs containing branches of flower buds were cut out; the length of these twigs was approximately 4 cm and the diameter of the buds was approximately 8 mm Φ. A nitroxyl radical, 3-carbamoyl-2,2,5,5-tetramethylprrolidine-1-yoxyl (carbamoyl-PROXYL, Aldrich Chemical, USA), was used as a spin probe reagent. The flower bud was fed with a carbamoyl-PROXYL aqueous solution of 100 mm concentration via the cut end of the twig for 5 h at room temperature. The carbamoyl-PROXYL-treated bud with the twig was placed in the sample box of the sample chiller and then supplied with water from outside of the sample box at room temperature. As shown in Fig. 1A, the single-turn coil of the SCR was fixed on the bud. T-type thermocouples with a thermo detection portion (diameter, 1 mM Φ; length, 5 mm) were used to measure the temperature of the bud.

The carbamoyl-PROXYL-treated buds were chilled for 2.5 h until the temperature around the buds reached below zero degree. The chilling was then stopped and the temperature inside the sample box was allowed to rise to room temperature. Meanwhile, the ESR spectra of the carbamoyl-PROXYL within the buds were continuously measured for 3.5 h. The ESR conditions were as follows: frequency power, 52 mW at 720 MHz; static magnetic field, 25.6 mT; filed scan width, 10 mT; field scan rate, 5 mT/s; time constant, 1 ms; accumulation number, 32; and field modulation, 0.2 mT at 100 kHz.

ESR spectra analysis. ESR spectra of nitroxyl radicals have three hyperfine structure lines, such as a triplet ESR spectrum ($M = +1, 0, -1$), due to N-14 nucleus ($I = 1$). Peak-to-peak linewidth of the $I_z(^{14}\mathrm{N}) = M_I$ hyperfine line of nitroxyl radicals is written as

$$W_{\mathrm{pp}}(M_I) = W_{\mathrm{pp}}(M_0) \times \sqrt{H_0/H_I}, \quad (1)$$

where $W_{\mathrm{pp}}(M_I)$ is the peak-to-peak linewidth and H_I is the peak-to-peak height of M_I hyperfine line of nitroxyl radicals. In this study, the peak-to-peak linewidth of carbamoyl-PROXYL spectra of flower buds was calculated according to Eq. (1).

Results and discussion

Figs. 1B and C show that typical curves of peak areas of ESR spectra of carbamoyl-PROXYL of a flower buds of sweet cherry (●) under temperature stressful condition. The peak areas can be used as a relative value of the radical quantity. Each black line shown in the figures indicates change in temperatures of

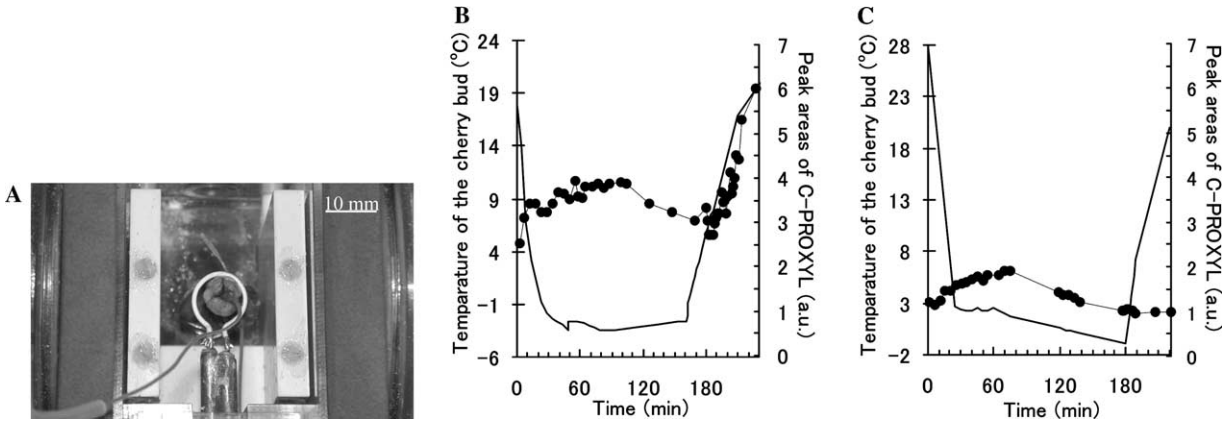

Fig. 1. The picture shows a flower bud of the sweet cherry with the SCR (A). Typical curves of peak areas of ESR spectra of carbamoyl-PROXYL-treated buds (●) that were frozen (B) and not frozen (C). Each black line shows change in the temperature of the bud.

the bud. As shown Fig. 1B, the temperature of the bud exhibited a rapid rise of about 1 °C at 50 min after cooling commenced. This temperature rise was believed to have derived from the generation of solidification heat, indicating that the bud began to freeze. Most of the temperatures of the buds exhibited a rapid rise at 30–60 min after cooling commenced. Temperatures when freezing started were below -2.5 ± 0.5 °C (values represent means ± SEM, $n = 6$). It was previously reported that flower buds of the sweet cherry began to freeze at temperature below −2 °C when stored for 1–2 h in a programmable deep freezer [4]. The peak area shown in Fig. 1B increased within 60 min after cooling commenced and then gradually decreased. The peak area began to increase immediately after cooling stopped. In the frozen buds investigated in this study, the time-dependent changes of the peak areas of carbamoyl-PROXYL spectra were similar to that shown in Fig. 1B. The change in the area of carbamoyl-PROXYL indicates that hydroxylamine, which was formed from carbamoyl-PROXYL by reductants before cooling commenced, was oxidized in the buds and then reduced again. In a preliminary experiment, we monitored a flower bud treated with a stable hydroxylamine, 1-hydroxy-2,2,5,5-tetramethyl-3-imidazoline 3-oxide (HTIO, Acros Organics, USA), under the same condition. Since HTIO is a ESR-silent compound, ESR spectra of HTIO-treated bud were not detected before cooling commenced. After cooling stopped, ESR spectra of HTIO-oxidizing form in the bud were detected clearly. Fig. 1C shows the typical curve of the peak areas of the carbamoyl-PROXYL-treated bud without freezing. Change in the peak area of the non-frozen bud under the cooling condition was similar to that shown in Fig. 1B and this peak area was changeless after the cooling stopped. Additionally, the color inside the frozen buds changed brown and that of the non-frozen bud did not change after the in vivo ESR measurements. The alteration of the bud color such as brown is one of the oxidative damages. These findings suggest that redox balance of the frozen buds shifted to an oxidative state as the bud-temperature rose.

Fig. 2 shows the ESR spectra of carbamoyl-PROXYL in the cherry bud corresponding to Fig. 1B. Each ESR spectrum was recorded during the following: (a) before cooling; (b) 50 min after cooling commenced during generation of solidification heat of the bud; and (c) 160 min after cooling commenced; (d, e, f, and g) 10, 20, 30, and 50 min after cooling stopped. Carbamoyl-PROXYL spectra of the buds after freezing (Figs. 2c and d) were broader than those of the buds

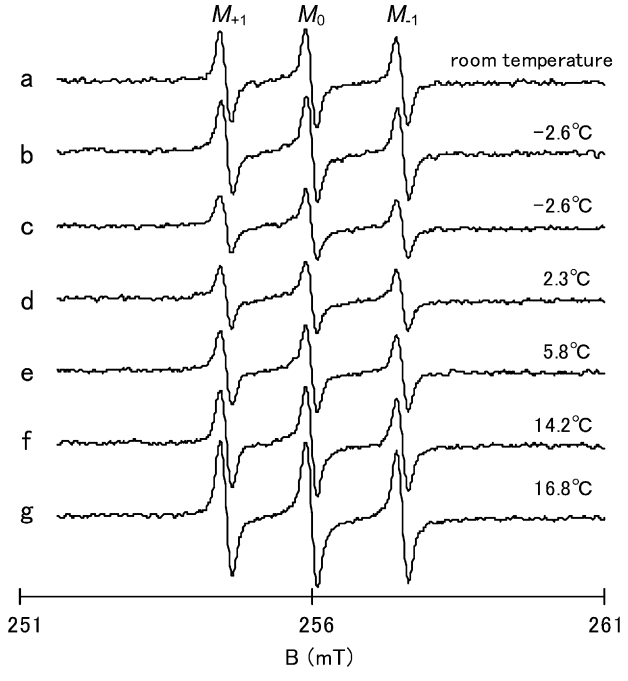

Fig. 2. ESR spectra of carbamoyl-PROXYL in a flower bud before cooling (a); 50 min or 160 min after cooling commenced (b, c); and 10, 20, 30, and 50 min after cooling stopped (d, e, f, and g), respectively. Each temperature shows the temperature of the bud at each point.

before cooling (Fig. 2a). The ESR spectra of nitroxyl radicals are known to change dramatically as the tumbling motion of the radicals slows, thus providing great sensitivity to fluidity in the neighborhood of the radicals [26,27]. Fukui et al. [27] reported that linewidth parameters of ESR spectra of nitroxyl radicals dissolved in several concentrations of sucrose solutions had been examined using a 700-MHz microwave ESR spectrometer. Although high concentration of sucrose (42 wt%) had reflected a 700-MHz ESR spectrum of a nitroxyl radical, 23 wt% of sucrose such as sucrose concentration in plant cells had not reflected the ESR spectrum very much [27]. The ESR spectrum of high sucrose concentration had the same asymmetric line broadening as carbamoyl-PROXYL spectrum of a frozen bud (Fig. 2c). Because sucrose is often a major solute constituent in cellular fluids (particularly in plants) and used in the plant cells for adjustment of osmotic pressure and protection from freezing damage [28], sucrose solution can be a good model for cellular fluids. The fluidity around carbamoyl-PROXYL in the cherry buds might be altered by freezing the buds. It is believed that the freezing of the buds was reflected in the shapes of the ESR spectra of carbamoyl-PROXYL within the buds.

The peak-to-peak heights of triplet ESR spectra of carbamoyl-PROXYL within flower buds were analyzed. Each peak-to-peak height of the lowest (M_{+1}), the middle (M_0), or highest (M_{-1}) component was 6.32 ± 1.18, 7.05 ± 1.32, or 6.17 ± 1.11 (before cooling), 8.10 ± 0.93, 8.97 ± 1.01, or 7.78 ± 0.88 (30–60 after cooling commenced as generation of solidification heat of the bud), 3.13 ± 0.57, 3.95 ± 0.71, or 2.95 ± 0.61 (160 min after cooling commenced), 4.75 ± 0.34, 5.68 ± 0.51, or 4.78 ± 0.36 (20 min after cooling stopped), and 6.03 ± 0.69, 6.82 ± 0.84, or 5.90 ± 0.65 a.u. (30 min after cooling stopped), respectively (values represent means ± SEM, $n = 5$–6). Each ratio of the peak-to-peak height of $M_{+1} : M_0 : M_{-1}$ was 0.9:1.0:0.9 (before cooling), 0.9:1.0:0.9 (30–60 after cooling commenced), 0.8:1.0:0.8 (160 min after cooling commenced), 0.8:1.0:0.8 (20 min after cooling stopped), and 0.9:1.0:0.9 (30 min after cooling stopped), respectively. In case of ESR spectra of nitroxyl radicals detected by low-frequency microwave (i.e., 700 MHz) ESR spectrometer, M_{+1} and M_{-1} hyperfine lines of the ESR spectra reflect change in the motion of the nitroxyl radicals [27]. These findings show that the frozen buds could be caused asymmetric line broadening of the carbamoyl-PROXYL spectra. In this study, the peak-to-peak linewidths of carbamoyl-PROXYL spectra of flower buds as shown in Fig. 3 were calculated according to Eq. (1) by using these peak-to-peak heights.

Fig. 3 shows changes in the peak areas of the ESR spectra of carbamoyl-PROXYL in the flower buds (values represent means ± SEM, $n = 5$–6). Each area was 2.08 ± 0.36 (a; before cooling), 2.79 ± 0.40 (b; the maximum of the area within 60 min after cooling commenced), 1.43 ± 0.44 (c; approximately 160 min after cooling commenced), 1.67 ± 0.39, 1.96 ± 0.44, 2.34 ± 0.53, or 3.03 ± 0.61 (d, e, f, and g; 10, 20, 30, or 50 min after cooling stopped), and 3.50 ± 0.71 (h; the maximum of the area within 90 min after cooling stopped). The peak areas reached a maximum within 60 min after cooling commenced. Meanwhile, the buds generated solidification heat at this time. In almost all the buds, the increase in peak areas stopped before freezing started. At approximately 160 min after cooling started, the peak areas decreased to below that before cooling. These findings suggest that redox balances in the buds shifted to an oxidative state by the lowering of the temperature and then returned to a steady state. After cooling of the buds stopped, the peak areas increased as the temperature around the buds rose. The peak areas reached a maximum and then leveled or decreased once again 60–90 min after cooling stopped. Since this decrease in the peak area after increase in the area due to the rise in the temperature of the buds shows that reoxidized carbamoyl-PROXYL in the bud was reduced once again, it is believed that carbamoyl-PROXYL reaction in the bud was reversible-like. The maximum of the areas within 90 min after cooling stopped was significantly larger than that approximately 160 min after cooling started (Student's t test, $p < 0.05$). These findings show that the oxidizing abilities of the buds increased during the rise in bud temperature. It is suggested that thawing of the frozen cherry buds caused oxidative damage to tissues and cells of the buds.

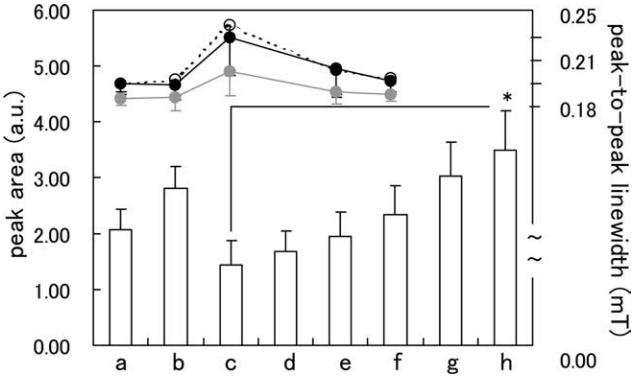

Fig. 3. Changes in the peak areas and peak-to-peak linewidths of carbamoyl-PROXYL in cherry flower buds. The values of the bars show the peak area before cooling (a); maxima of a peak area before freezing with cooling (b); 160–180 min after cooling commenced (c); 10, 20, 30, and 50 min after cooling stopped (d, e, f, and g); and the maximum peak area within 90 min after cooling stopped (h). The values represent means ± SEM ($n = 5$–6). *$p < 0.05$, Student's t test. The values of the circles show the peak-to-peak linewidth of M_{+1} (●), M_0 (◉), and M_{-1} (○) hyperfine lines at each time period. The values represent means ± SD ($n = 5$–6).

Fig. 3 also shows changes in the peak-to-peak linewithds of the ESR spectra of carbamoyl-PROXYL in

the flower buds (values represent means ± SD, $n = 5$–6). Each peak-to-peak linewidth of M_{+1}, M_0, or M_{-1} hyperfine lines was 0.20 ± 0.01, 0.18 ± 0.01, or 0.20 ± 0.01 (before cooling), 0.19 ± 0.01, 0.19 ± 0.01, or 0.20 ± 0.01 (30–60 after cooling commenced as generation of solidification heat of the bud), 0.23 ± 0.03, 0.21 ± 0.02, or 0.24 ± 0.04 (160 min after cooling commenced), 0.21 ± 0.02, 0.19 ± 0.01, or 0.21 ± 0.02 (20 min after cooling stopped), and 0.20 ± 0.01, 0.19 ± 0.01, or 0.20 ± 0.01 mT (30 min after cooling stopped), respectively. After cooling commenced, all of the peak-to-peak linewidths became broad. Twenty or thirty min after the cooling stopped, all of the peak-to-peak linewidths returned. Amounts of change in the linewidths of M_{+1} and M_{-1} were larger than that of M_0 This indicates that freezing of the flower buds might change the motion of carbamoyl-PROXYL in the buds. In a preliminary experiment, frozen carbamoyl-PROXYL solution of 1 mM, which was of near concentration in the bud, was measured by the ESR technique. We confirmed that all linewidths of triplet signal of the frozen carbamoyl-PROXYL were broad. Consequently, it was suggested that the linewidth broadening of carbamoyl-PROXYL in the bud was caused by two factors as follows: (1) direct freeze of carbamoyl-PROXYL solution in the buds, (2) change in biological conditions in the buds by freeze. We believe that real-time observation of ESR spectra of carbamoyl-PROXYL-treated buds can be confirmed by partially freezing of the buds.

Previously no techniques existed which simultaneously observe freezing behavior and changes in physiologically function in an entire flower bud. In this study, for using our 700-MHz ESR spectrometer equipped with the SCR, the non-destructive observation of redox balance in flower buds with cold damage was successfully observed. It was confirmed that the peak areas of the ESR spectra of carbamoyl-PROXYL within flower buds of the sweet cherry could be used as an index of oxidation. Furthermore, the freezing behavior of the flower buds was guessed from observations of changes in the ESR spectra of carbamoyl-PROXYL within the buds. It was shown that the point of freezing and the redox states inside the entire flower buds of the sweet cherry could be continuously observed with in vivo ESR measurements. This method is highly useful as it is also applicable to other plant species and environmental stresses. It is expected that such in vivo ESR experiment will become useful used in the understanding of various mechanisms of living plants.

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