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Levels of Active Oxygen Species Are Controlled by Ascorbic Acid and Anthocyanin in *Arabidopsis*

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Stabilization of the levels of active oxygen species (AOS) is important to the survival of organisms. To clarify the system controlling levels of AOS in plants, this study used an electron spin resonance (ESR) method to directly measure superoxide radical ($O_2^{\bullet-}$) scavenging activities in the wild-type *Arabidopsis thaliana* (Col and L*er* ecotypes), two anthocyanin mutants (*tt3* and *ttg1*), and an ascorbic acid mutant (*vtc1*). Under ordinary growth conditions, *Arabidopsis* contained superoxide-scavenging activity (SOSA) of ∼300−500 SOD units/g of fresh weight. The ESR pattern indicated that most (40−50%) of this activity was due to ascorbic acid. For the analysis of SOSA under conditions of oxidative stress, synthesis of AOS was induced by γ-irradiation. The radical scavenging activity in irradiated plants increased ∼10-fold following an associated increase in the accumulation of ascorbic acid and anthocyanin. The accumulation of ascorbic acid and anthocyanin was suppressed by treatment with an antioxidant before irradiation and was induced by treatment with a radical-generating reagent. The contributions of ascorbic acid and anthocyanin to the total superoxide radical scavenging activity differed among ecotypes. In the L*er* ecotype, ascorbic acid accumulated at twice the level of that in the Col ecotype, and induction of anthocyanin was half that in Col. To confirm the activity of ascorbic acid and anthocyanin against AOS stress, the viability of the wild type and mutants (*tt2*, *tt3*, *tt5*, *ttg1*, and *vtc1*) was examined after γ-irradiation. Only the plants in which ascorbic acid and anthocyanin were induced had the ability to grow and flower.

KEYWORDS: Active oxygen species; ascorbic acid; anthocyanin; electron spin resonance

INTRODUCTION

Active oxygen species (AOS) ($O_2^{\bullet-}$, H_2O_2, OH, LOO$^{\bullet}$, L$^{\bullet}$, LOOH, LO$^{\bullet}$, etc.) are generated by autoxidation, enzymatic oxidation, photosensitization, and the reaction of metalloenzymes with oxygen, ozone, water, unsaturated fatty acids, and many other materials in vivo. The highly reactive AOS exert various effects on organisms by oxidizing biological materials. In animals, AOS have been shown to cause aging, cancer, Alzheimer's disease, cataracts, and blood circulation disorders (*1*−*5*). AOS are induced by stress (UV, radioactive irradiation, etc.) and produced by the electron transport system of mitochondria and can damage cells and tissues. There are two kinds of antioxidative systems: (1) systems such as Mn-SOD, Cu/Zn-SOD, Fe-SOD, glutathione peroxidase, and catalase and (2) systems that produce antioxidants (ascorbic acid, ubiquinone, cysteine, β-carotene, α-tocopherol, etc.) (*6*−*10*). Although these systems exist to eliminate AOS, organisms also have AOS-generating systems. Active oxygen-evolving systems include the enzymes NADH dehydrogenase, NADPH-cytochrome *c* reductase P-450, and NADPH oxidase, which are, respectively, used as a transmitter in signal transduction, an oxidative substance for energy production, and a bactericidal substance in defenses against pathogens (*11*, *12*). Therefore, controlling the levels of AOS is important to maintain homeostasis. In plants, various defense systems are also needed to eliminate the AOS generated in chloroplasts and tissues by the photosynthesis process and by environmental stresses. Because it is impossible for plants to escape from an environmental stress, they have developed ways to synthesize and store various antioxidants to adjust the concentrations of AOS (in particular, to remove excessive AOS). Various stresses (strong light, drought, wounding, UV irradiation, radioactive irradiation, disease, etc.) (*13*−*16*) induce the synthesis of flavonoids that act as scavengers of the AOS induced by these stresses (*17*−

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22). In addition to inducing flavonoids, factors such as germination, flowering, UV irradiation, and ozone stress induce the accumulation of ascorbic acid, which also acts as a radical scavenger (*23*).

In this paper, we analyze the mechanisms regulating the concentration of AOS ($O_2^{\bullet -}$) in *Arabidopsis*. We used electron spin resonance (ESR) spectroscopy to quantify the superoxide radical ($O_2^{\bullet -}$) scavenging activity (SOSA) of the plants of ordinary and oxidative stress conditions. To analyze the SOSA, plants were subjected to γ-irradiation to produce AOS. The radical scavenging activity in irradiated plants increased to ~10 times the usual level with a concurrent increase in the storage of ascorbic acid and anthocyanin, indicating that AOS also activated the synthesis of ascorbic acid and anthocyanin.

MATERIALS AND METHODS

***Arabidopsis* Strains and Growth Conditions.** The wild-type *Arabidopsis* strains that we used were the Columbia (Col) and Landsberg *erecta* (L*er*) ecotypes. The Arabidopsis Research Center (The Ohio State University, Columbus, OH) provided seeds of the mutants *tt3*, *tt4*, *tt5*, *ttg1*, and *vtc1*. Sterilized seeds were sown on a 1% agar medium containing 1/2× Murashige and Skoog basal salts (Sigma), 2% sucrose, 100 mg L^{-1} inositol, 1 mg L^{-1} thiamin, 0.5 mg L^{-1} nicotinic acid, and 0.5 mg L^{-1} pyridoxine and were grown under continuous illumination (400−500 μW m^{-2} s^{-1}) at 21 °C.

ESR Measurements. We used a free radical monitor (JES-FR30; JEOL, Tokyo, Japan). Manganese oxide provided a constant signal to which all peak heights were compared: a sample peak height was divided by the MnO peak height to give relative peak height.

ESR measurements were conducted under the following conditions: magnetic field, 335.6 ± 5 mT; power, 4 mW; microwave frequency, 9.41 GHz; modulation amplitude, 100 kHz; 0.63 × 0.1 mT; response time, 0.1 s; amplitude, 1 × 250; and sweep time, 2 min. ESR spectra were measured at room temperature. Data from the ESR spectrometer were analyzed with a computer program (version 5.2 for JES-FR30).

Whole bodies of plants were crushed in a mortar with liquid nitrogen, freeze-dried, and dissolved in a 0.1 M potassium phosphate buffer (pH 7.4). After centrifugation at 6000*g* for 10 min, solutions were diluted for appropriate concentrations and used as sample solutions.

Spin-trapping methods for superoxide radicals were based on the methods of Rosen et al. (*24*). Superoxide anions were generated by a hypoxanthine (HPX)−xanthine oxidase (XOD) system. A solution of 50 μL of 4 mM HPX, 50 μL of sample solution, 20 μL of 4.5 M 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO; Labotec, Tokyo, Japan) as a spin-trapping agent, and 50 μL of XOD (Boehringer-Mannheim) (0.4 unit mL^{-1}) was prepared in a 0.1 M potassium phosphate buffer (pH 7.4). This was then transferred to a 130-μL-capacity flat cell (LC-12), and the ESR spectra of the DMPO−$OO^{\bullet -}$ spin adduct were analyzed. SOSA was expressed as SOD equivalent units per milligram.

Extraction and Quantification of L-Ascorbic Acid. Extraction and measurement of L-ascorbic acid was performed according to the methods of Beutler et al. using an L-ascorbic acid analysis kit (Boehringer Mannheim). L-Ascorbic acid and oxidized ascorbic acid were extracted from whole plants with an extraction buffer containing the tetrazolium salt 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT). The fresh weight and number of plants were measured, and the material was crushed in a mortar with an aliquot of the extraction buffer. L-Ascorbic acid was reduced by MTT in the presence of the electron carrier 5-methylphenazinium methosulfate (PMS) at pH 3.5 to formazan. The reducing substances were measured in the cuvette.

$$\text{L-ascorbate } (x\text{-}H_2) + MTT^+ \xrightarrow{PMS} \text{dehydroascorbate } (x) + \text{MTT−formazan} + H^+$$

Adding ascorbate oxidase (AAO) to the sample blank oxidatively removes only the L-ascorbate. The dehydroascorbate formed does not react with MTT or PMS.

$$\text{L-ascorbate} + {}^1/_2\, O_2 \xrightarrow{AAO} \text{dehydroascorbate} + H_2O$$

The optical density (OD) of the sample minus the OD of the sample blank is equivalent to the quantity of L-ascorbate in the sample. The concentration of MTT−formazan is the measured parameter and is determined by light absorbance at 578 nm.

The volume of oxidative ascorbate acid was also measured with the same system after dehydration. We measured the OD at 578 nm after treating the extract with PMS, AAO, and DTT. The differences in values were normalized according to the weight of plants.

γ-Irradiation. Eighteen days after germination, when the fifth leaf had expanded fully, the plants growing on the agar medium were irradiated with γ-rays from a cobalt-60 source in a γ cell irradiator (3.0 kGy h^{-1}, 1300 TBq; Nordion, ON, Canada). After irradiation, the plants were grown alongside un-irradiated plants under the same conditions as before.

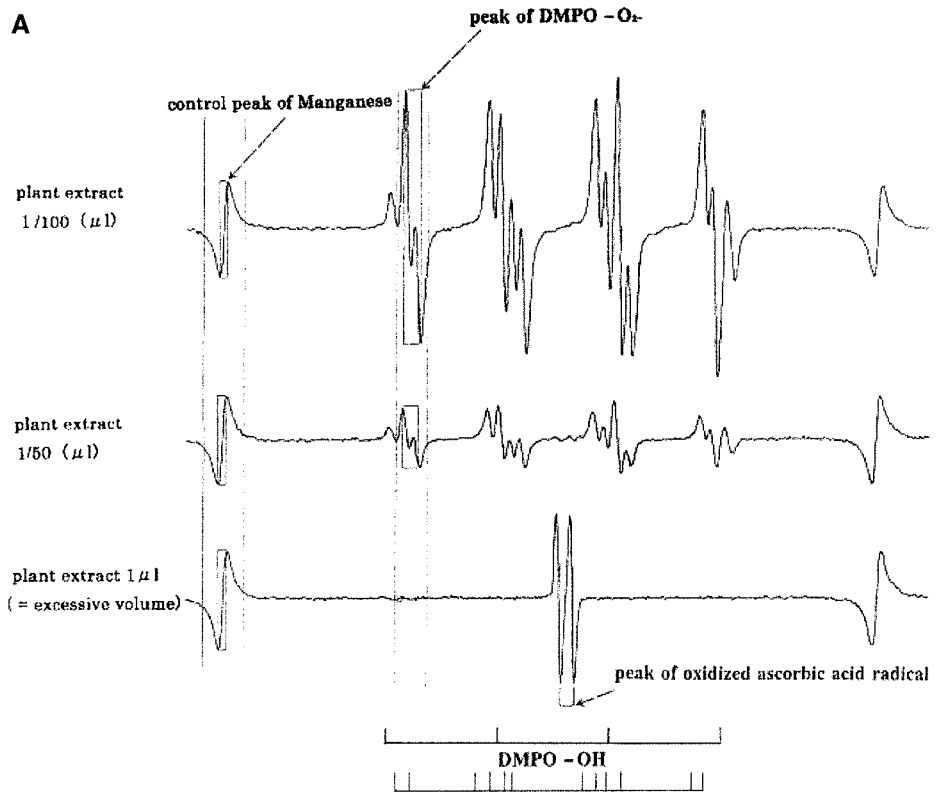
Treatment with Reagents. Nordihydroguaiaretic acid (1 mM; NDGA), 1 mM *n*-propyl gallate (*n*-PG), 10 mM L-ascorbic acid, and 1 mM pyrrolidine dithiocarbamate (PDTC) were used as antioxidants, and 2 μM methyl viologen (paraquat) was used as an active oxygen generator. The treatment was as follows: 100 μL of aqueous solution was spread on the surface of the culturing agar once a day for 3 days before γ-irradiation. Treated plants were grown and irradiated with γ-rays under the same conditions as for the untreated plants. After γ-irradiation, both types of plants were grown under the same conditions.

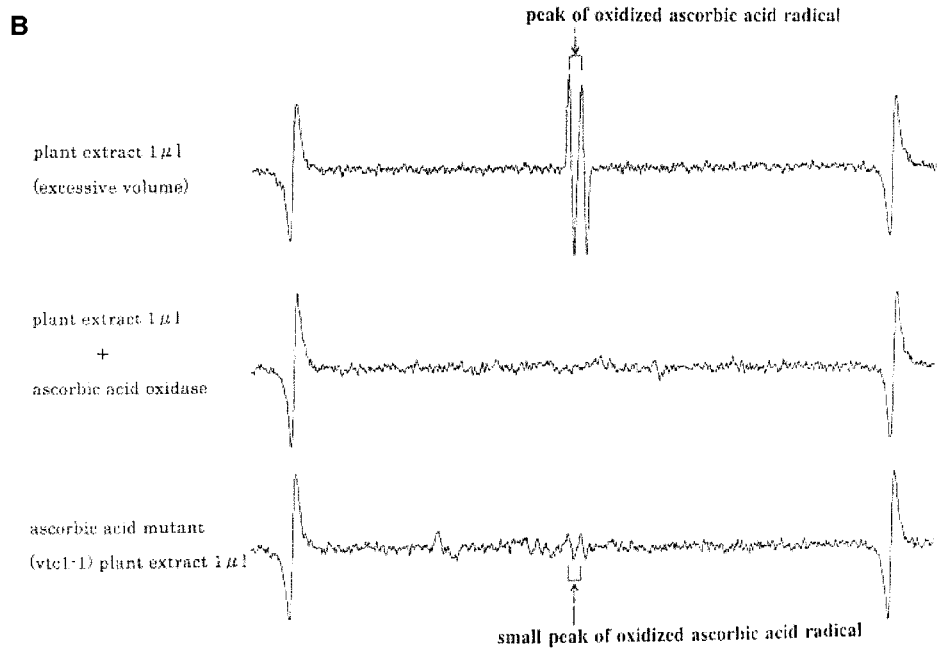
Extraction and Measurement of Anthocyanin. Anthocyanin was extracted from the whole plant body with an extraction buffer containing propanol/HCl/H_2O (18:1:81). The plants, once the fresh weight had been measured, were crushed in a mortar with an aliquot of the extraction buffer. The extract was boiled for 3 min and incubated overnight at 25 °C. The ODs at 535 and 650 nm were measured. The difference between these two values was normalized according to the fresh weight.

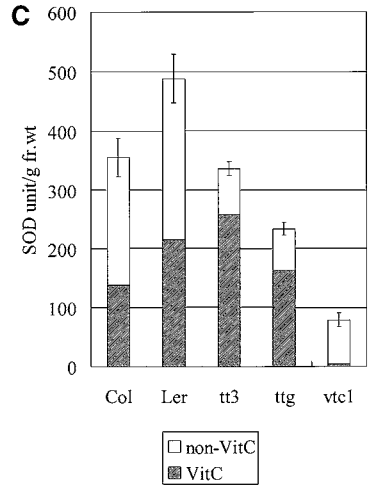
Northern Blot Analysis. Total RNA was extracted from the whole plant by Isogen (Nippongene). PolyA^+RNA was purified by Oligotex-dT (Super) (Daiichi Chemical) and electrophoretically separated on 1.4% agarose−2.2 M formaldehyde gels in a MOPS buffer. RNA was blotted onto a nylon filter with 20× SSC for 12−16 h and fixed by heating at 80 °C for 2 h. Northern hybridization was done with ^{32}P-labeled probe DNA, and the filter was washed in 0.1× SSC at 65 °C. chalcone synthase (CHS) and phenylalanine ammonia-lyase (PAL) probes were synthesized by PCR amplification of genomic DNA. The sequence (2317−3309) corresponding to the second exon of CHS (Accession no. M20308; Feinbaum and Ausbel, 1988) and the sequence corresponding to the second exon of PAL (Accession no. L33677) were amplified and labeled with α-^{32}P-dCTP using a Megaprime labeling kit (Takara Shuzo). Autoradiograms were analyzed with an image analyzer (BAS-2000, Fuji).

RESULTS AND DISCUSSION

Radical Scavenging Activities of Normally Grown Wild Types and Mutants. To examine the AOS control system in vivo, we measured SOSA by ESR spectroscopy. ESR has an advantage over the conventionally used chemiluminescence method in that it can detect superoxide-scavenging activity by directly measuring the amount of radicals. In the ESR spectra, the peaks of the DMPO adduct compounded with a superoxide anion radical (DMPO−$OO^{\bullet -}$) decreased as addition of plant extract increased (**Figure 1A**). This pattern of reduction can be quantified in terms of SOD units by using a standard SOD concentration-measuring curve. The spectra in **Figure 1A** were obtained from the spectra of two types of spin adduct: the hfcc values for one of the spin adducts were $a_N = 1.41$ mT, $a_H^{\beta} = 1.14$ mT, and $a_H^{\gamma} = 0.13$ mT; the hfcc values of the other spin adduct were $a_N = 1.49$ mT and $a_H^{\beta} = 1.49$ mT. With the addition of an excess volume of plant extract, the superoxide peaks disappeared and new peaks appeared (indicated by







Ratio (%) of ascorbic acid and non-ascorbic acid

Strain	ascorbic acid	non-ascorbic acid
Col	41	59
L*er*	44	56
tt3	77	23
ttg	72	28
vtc1	15	85

Figure 1. Superoxide radical scavenging activity in *Arabidopsis*: (A) ESR pattern of the radical scavenging reaction of the *Arabidopsis* extract; (B) ESR pattern of the scavenging reaction of plant extracts treated with ascorbic acid oxidase (an arrow indicates the peak for ascorbic acid); (C) radical scavenging activity in *Arabidopsis* wild-type plants (Col, L*er*) and mutants (*ttg1*, *tt3*, and *vtc1*). The shaded boxes indicate the radical scavenging activity due to ascorbic acid and the open boxes indicate other radical scavenging activity.

arrows). The pattern and value of the peaks are identical to those of ascorbic acid (*25*−*28*). These peaks were not observed when the plant extract was pretreated with ascorbic acid oxidase, and only a small peak was observed when the extract was prepared from the *vtc1* (low at the ascorbic acid synthesizing ability) mutant (**Figure 1B**). The *g* value and hyperfine coupling of the double peaks in the spectra were 2.0058 and 0.170 mM, respectively, suggesting that these peaks can be assigned to the ascorbic acid radical (DMPO−ascorbic acid). Comparing the radical scavenging activities of ascorbic acid oxidase-treated and untreated plant extracts can clarify the contribution of ascorbic acid to the total radical scavenging activity.

The total radical scavenging activities of the two wild types, the two anthocyanin mutants, and the ascorbic acid mutant under normal growth conditions are compared in **Figure 1C**; the contribution of ascorbic acid to total radical scavenging activity is also indicated. Col and L*er* showed a difference in the amount of total activity, but the contributions of ascorbic acid were

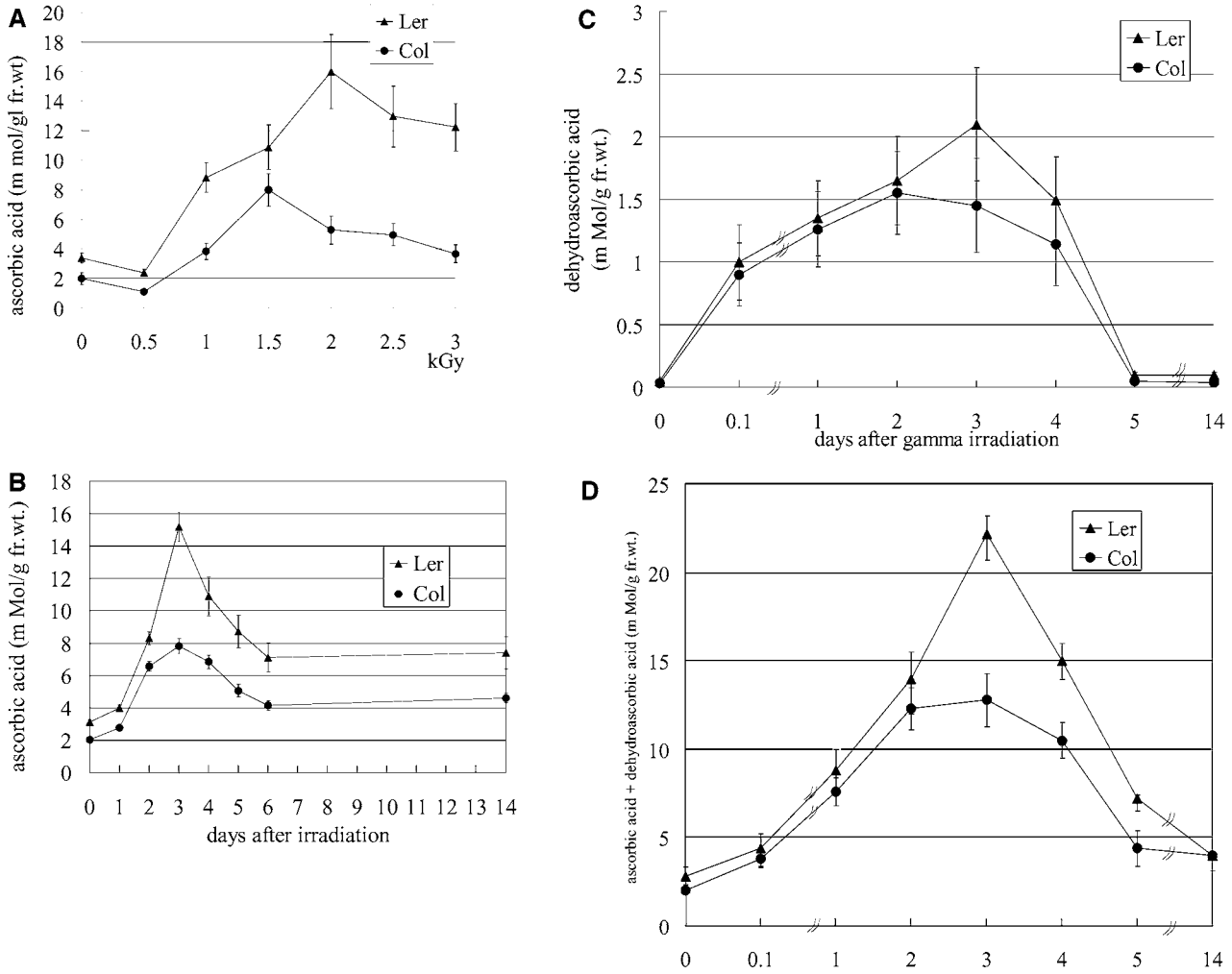


Figure 2. Accumulation of ascorbic acid induced by γ-irradiation: (A) dose-dependent accumulation of ascorbic acid (amount of ascorbic acid 4 days after irradiation is plotted against γ-irradiation dosage); (B) temporal pattern of ascorbic acid accumulation; (C) temporal pattern of accumulation of oxidized ascorbic acid; (D) temporal pattern of total ascorbic acid accumulation [accumulated amount of ascorbic acid is plotted against the period after irradiation (2.0 kGy)].

almost the same. In the *tt3* and *ttg1* mutants, the contribution of ascorbic acid increased, but in *vtc1* the contribution of ascorbic acid was very low. Therefore, under ordinary conditions, most (40−50%) of the SOSA (300−500 SOD units/g of fresh weight) was due to ascorbic acid.

Oxidative Burst by γ-Irradiation Increases the Ascorbic Acid Level. To analyze the mechanism controlling the concentrations of AOS, we administered a massive dose of γ-rays (1−3 kGy) to the plants and then analyzed biochemical phenomena and changes in radical scavenging activities. γ-Irradiation causes the formation of AOS by water radiolysis, but because γ-irradiation is an abiotic stress in nature, plants might not have a specific signal transduction system to defend against it. **Figure 2A** shows the dose dependency of ascorbic acid accumulation on the fourth day after irradiation. When plants were irradiated with >1 kGy, ascorbic acid accumulated to a level of several times that under normal conditions. The accumulation of ascorbic acid increased with γ-irradiation, up to dosages of 2 kGy in L*er* and 1.5 kGy in Col. At this dosage, the amount of ascorbic acid was ∼5−10 times that in unirradiated plants. At 2.5 kGy, the amounts of ascorbic acid began to drop, possibly due to the induction of senescence in the plants. The temporal pattern of ascorbic acid accumulation (**Figure 2B**) shows that after 2 kGy irradiation, ascorbic acid began accumulating within 2 days and, on the fourth day after irradiation, peaked at ∼4−6 times the amount at day 0; it had dropped to almost half the peak amount by days 5 and 6, retaining this level for >2 weeks. The amounts of ascorbic acid accumulated in L*er* were about twice those in Col.

We also analyzed the amount of oxidized ascorbic acid. It increased markedly (∼10−20-fold) immediately after γ-irradiation, and it decreased markedly by the fifth day (**Figure 2C**). Under normal conditions, the ratio of oxidized ascorbic acid to ascorbic acid ranges from <1% to ∼10%; after γ-irradiation, the ratio increased to about 1:1 before dropping to normal levels within 5 days. The increase in oxidized ascorbic acid indicates direct oxidation by the AOS.

A difference in the degree of accumulation of oxidized ascorbic acid was observed between Col and L*er* (**Figure 2C**). On the first day after γ-irradiation, there was almost the same volume of oxidized ascorbic acid in Col and L*er*, but on the third day, the amount of oxidized ascorbic acid in L*er* was ∼1.5 times higher than in Col. Because the two plant ecotypes have almost the same shape and size, the volume and species of the generated AOS should be almost identical. Therefore, it seems reasonable that, after the radiolysis reaction, the amounts of AOS generated and reacted with ascorbic acid were at first the same. The difference in the amount of oxidized ascorbic acid between the two ecotypes a few days after irradiation suggests that different systems are involved in the scavenging of long-life

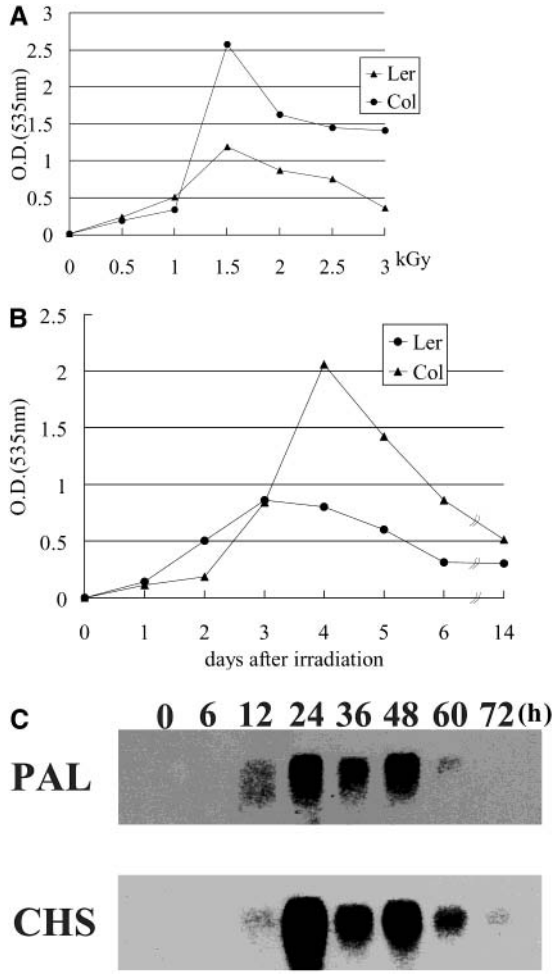


Figure 3. Accumulation of anthocyanin induced by γ-irradiation; (A) dose-dependent accumulation of anthocyanin (amount of anthocyanin 4 days after irradiation is plotted against γ-irradiation dosage); (B) temporal pattern of anthocyanin accumulation [accumulated amount of anthocyanin is plotted against the period after irradiation (1.5 kGy)]; (C) Northern blot analysis of PAL and CHS in γ-irradiated *Arabidopsis* [at the indicated time points after irradiation (1.5 kGy), 10 μg of polyA$^+$RNA from whole plants was extracted, electrophoretically separated, blotted onto a nylon membrane, and hybridized with ^{32}P-labeled DNA probes, which were prepared by PCR amplification according to information from the DNA database].

AOS. The total (oxidized plus nonoxidized) ascorbic acid increase was ~5-fold (**Figure 2D**). Here we can see that L*er* contains and is induced to synthesize more ascorbic acid than Col. Thus, in terms of ascorbic acid synthesis, L*er* is more active than Col.

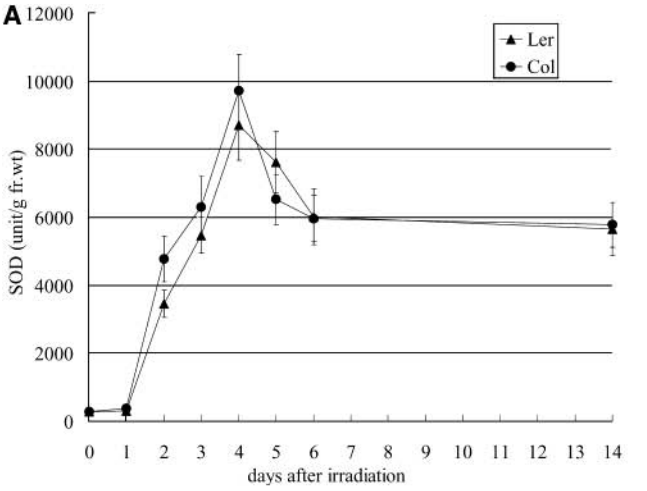
Anthocyanin Also Acts as an Antioxidant. γ-Irradiation induces another radical scavenging system. We observed the accumulation of a purple pigment in leaves and petioles 2−3 days after irradiation and, on the basis of the absorbency spectrum at OD 535 nm and the results of flavonoid mutant analysis, concluded that the purple pigment was an anthocyanin. The dose dependency of anthocyanin accumulation on the fourth day after irradiation is shown in **Figure 3A**. The accumulation of anthocyanin increased with γ-irradiation up to a dosage of 1.5 kGy. At this dosage, the amount of anthocyanin was ~10−20 times that in un-irradiated plants. Anthocyanin amounts began to drop at 2.0 kGy, possibly due to the induction of senescence in the plants. The temporal pattern of accumulation is shown in **Figure 3B** (1.5 kGy irradiation). The accumulation began within 2 days of irradiation and peaked on the fourth day. The peak amount of anthocyanin was ~10−20 times that on day zero and dropped to almost half the peak amount by days 5 and 6, remaining at this level for >2 weeks. The difference between Col and L*er* in the degree of accumulation of anthocyanin is clear; the amount of anthocyanin in Col is about twice that in L*er*. To examine the transcriptional regulation of the genes related to anthocyanin biosynthesis, we conducted a Northern blot analysis of the key enzymes PAL and CHS (**Figure 3C**). Both transcripts were first detected 12 h after irradiation, with a peak at 24 h, continuing until 60 h, thereby revealing that the anthocyanin was induced via the ordinary synthesis pathway. We thus showed that, although the accumulation of anthocyanin remains at half the peak level for >2 weeks, it is not maintained by continuous induction of synthesis but rather by the accumulated anthocyanin not decomposing. The mRNA levels of these enzymes were undetectably low during normal growth conditions, and it took 12 h for the transcriptional induction of these genes after irradiation. These findings are consistent with those of other stress treatments, such as exposure to high-intensity light and low temperature. We found that anthocyanin is induced more in Col than in L*er*; thus, contrary to the ascorbic acid induction, Col is more active than L*er* in anthocyanin synthesis.

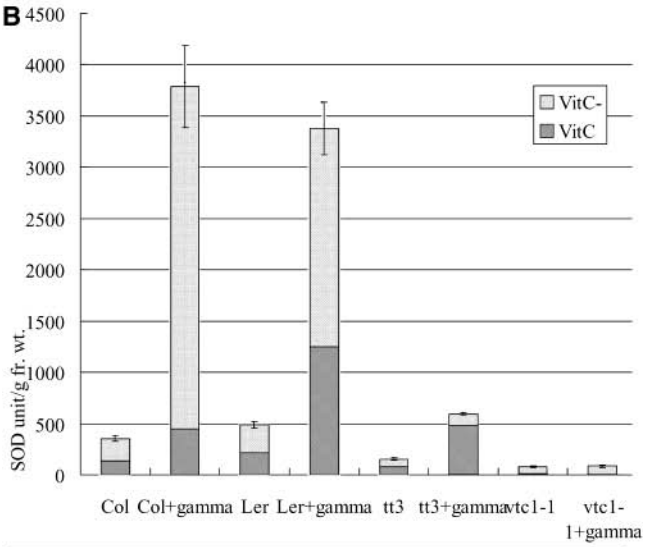
Table 1. Effect of the Antioxidant and Paraquat Treatment to the Ecotype Col on the Accumulation of Anthocyanin Irradiation by γ-Ray (2 kGy)

	anthocyanin O at 535 nm	ratio (%)
control (Col)	0.124 ± 0.012	
γ-irradiation (2.0 kGy)	2.456 ± 0.524	100
n-PG + γ-irradiation	0.880 ± 0.360	36
NDGA + γ-irradiation	1.032 ± 0.123	42
PDTC + γ-irradiation	1.596 ± 0.160	65
ascorbic acid (50 mM) + γ-irradiation	1.000 ± 0.123	40
paraquat (2 μM)	2.020 ± 0.600	82

Anthocyanin Synthesis Is Induced by Generation of AOS. To examine whether the synthesis of anthocyanin is a response to the generation of AOS, we treated plants with antioxidants prior to the γ-irradiation or with AOS-generating reagents (**Table 1**). We treated the plants with ascorbic acid and three antioxidant reagents—nordihydroguaiaretic acid (NDGA), pyrrolidine dithiocarbamate (PDTC), and *n*-propyl gallate (*n*-PG). An aqueous solution of each was added to the basal region of the plants once a day for 3 days before irradiation. As indicated in **Table 1**, treatment with NDGA and *n*-PG reduced the accumulations of anthocyanin after irradiation to 65 and 36% of that in untreated plants, respectively. We also examined the response of plants to methyl viologen (paraquat), an AOS-generating reagent. After absorption by the plant, paraquat accumulates in the chloroplasts. Under illumination, one electron is captured by paraquat and transferred to an oxygen molecule to form a superoxide radical. As indicated in **Table 1**, the treatment with paraquat induced an accumulation of anthocyanin that was 82% the level in γ-irradiated plants. These findings suggest that the induction of anthocyanin biosynthesis following γ-irradiation is a response to the generation of AOS.

Ascorbic Acid and Anthocyanin Are the Main Radical Scavengers in the Plants. To examine the effects of ascorbic acid and anthocyanin, we used ESR to measure the SOSA of the γ-irradiated plants. The temporal patterns of the scavenging activities (**Figure 4A**) are almost identical to the patterns of ascorbic acid and anthocyanin accumulation after γ-irradiation (**Figures 2B** and **3B**). Flavonoids exhibit radical scavenging





Ratio(%) of ascorbic acid and non ascorbic acid

Strain	ascorbic acid	non-ascorbic acid
Col	41	59
Col+gamma	14	86
L*er*	44	56
L*er*+gamma	37	63
tt3	77	23
tt3 +gamma	81	19
vtc1	15	85
vtc1 +gamma	10	90

Figure 4. Superoxide radical scavenging activity in γ-irradiated *Arabidopsis*: (A) temporal pattern of the radical scavenging activity [after γ-irradiation (2.0 kGy), plants were harvested on the days indicated and prepared for measurement of radical scavenging activity by ESR]; (B) changes in radical scavenging activities in γ-irradiated *Arabidopsis* [on the fourth day after γ-irradiation (2.0 kGy), plants were harvested and prepared for measurement of radical scavenging activity; the open boxes indicate ascorbic acid activity, and the shaded boxes indicate anthocyanin and other radical scavenging activities].

activity because of their polyphenol structures and their ability to chelate iron. Ascorbic acid also has a structure that can accept an additional electron. The increased radical scavenging activity in the irradiated plants can be attributed to the increased accumulation of ascorbic acid and anthocyanin. The γ-ray-induced antioxidant activity in the wild-type plants was ~10−20 times higher than usual. In the anthocyanin mutants (*tt3* and *ttg*), antioxidant activity increased to twice the usual amount, owing to a 2-fold increase in the storage of anthocyanin. The ascorbic acid mutant (*vtc1*) also showed a small increase in the amount of radical scavenging activity. Therefore, both ascorbic acid and anthocyanin are effective in scavenging AOS. We also measured radical scavenging activities of plant extracts after treatment with ascorbic acid oxidase and calculated the ratio of ascorbic acid scavenging activity to anthocyanin scavenging activity (**Figure 4B**). The results showed that the concentration of AOS in plants was adjusted mainly by the synthesis of ascorbic acid or of both ascorbic acid and anthocyanin in proportion to the volume of AOS generated. However, there was a difference between the L*er* and Col ecotypes in the levels of ascorbic acid and anthocyanin used as antioxidants. Although the total radical scavenging activities were almost the same, L*er* contained twice as much ascorbic acid as Col contained under normal conditions, and AOS stimulation induced the synthesis of only small quantities (half the amount) of anthocyanin. This shows that the ecotypes differ in their usage of the two substances as antioxidants.

Increase in Antioxidant Activity Makes Plants Resistant to Oxidative Stress. To examine whether the increase in radical scavenging activity is actually responsible for the elimination of AOS and whether it is advantageous for survival, the viabilities of the wild type, the anthocyanin mutants (*tt3*, *tt4*, *tt5*, and *ttg1*), and the ascorbic acid mutant (*vtc1*) were compared after γ-irradiation. The wild type formed more leaves than the mutants and continued to grow and develop flowers (**Table 2**). In comparison with the anthocyanin mutants (*tt3*, *tt4*, *tt5*, and *ttg1*) and the ascorbic acid mutant (*vtc1*), the antioxidant activity of the wild types appears to be increased by the synthesis of ascorbic acid and anthocyanin and to provide strong resistance to the active oxygen stress caused by long-life AOS. In addition, the plants that were intermittently administered antioxidant (*n*-PG) and ascorbic acid before γ-irradiation showed greater viability than the wild type, thus revealing the necessity of AOS elimination by radical scavenging activity for survival after irradiation. The ratios and quantities of antioxidant material under normal conditions and those induced by stress corresponded to the status and frequency of stress from AOS in the plant habitats, and the antioxidant materials were important for the survival of the organism.

In plants, AOS arise as a general physiological response. Although AOS are created to a large extent by stresses such as UV irradiation and strong light, systems such as the pathogen signal transduction system also generate AOS. Therefore, a system to stabilize the concentration of AOS is indispensable. A massive dose of γ-irradiation (1−3 kGy) to *Arabidopsis thaliana* induces disruption of the stamen, accumulation of anthocyanin, formation of trichomes on the adaxial surface of the leaves, radial expansion of the root epidermal and cortical cells, and extension of root hairs (*29*). These changes are all mediated by the AOS generated by water radiolysis. Therefore, for the normal generation and function of these organs, signal transduction of AOS at a moderate concentration is necessary, and plants have developed sophisticated systems to synthesize and accumulate radical scavenging materials to control AOS. In this experiment, we analyzed the total radical scavenging activity of plants by ESR. Under ordinary conditions, *Arabidopsis* contained 500−600 SOD units/g of fresh weight of radical scavenging activity throughout the whole plant. γ-Irradiation experiments revealed that AOS stress increased radical scavenging activity by 10−20 times. Plants control radical scavenging activity with two compounds—ascorbic acid and

Table 2. Viability after γ-Irradiation (2 kGy)

strain	weight (g)	no. of formed leaves							flowering (%)
		<5	6	7	8	9	10	>11	
Col	0.126 ± 0.051	0	0	0	0	0	23	77	95
L*er*	0.150 ± 0.095	0	7	9	0	21	15	48	95
Col + γ	0.114 ± 0.050	0	0	3	29	43	13	12	92
L*er* + γ	0.118 ± 0.091	0	5	26	26	16	22	5	95
L*er* + *n*-PG (1 mM) + γ	0.130 ± 0.050	0	0	6	10	36	40	8	95
L*er* + ascorbic acid (1 mM) + γ	0.127 ± 0.080	0	5	5	12	30	28	20	91
tt3 + γ	0.021 ± 0.012	33	19	33	10	5	0	0	5
tt4 + γ	0.029 ± 0.018	17	30	17	30	6	0	0	0
tt5 + γ	0.033 ± 0.022	29	21	15	18	0	7	10	0
ttg1 + γ	0.022 ± 0.028	57	13	9	4	13	4	0	0
vtc1	0.138 ± 0.100	0	0	0	4	0	10	86	88
vtc1 + γ	0.016 ± 0.012	71	18	11	0	0	0	0	0

anthocyanin. The AOS were usually controlled by ascorbic acid alone, but when the plants were faced with a large amount of AOS, both ascorbic acid and anthocyanin were utilized. Of the total radical scavenging activity, ascorbic acid accounted for 40−50% under normal conditions and 20−30% under conditions of γ-irradiation. In the radical scavenging reaction, ascorbic acid reacts with and quenched the radical (*30*−*32*). Ascorbic acid is widely used as a radical scavenger in both animals and plants. In animals, a deficiency of ascorbic acid is correlated with cancer and other diseases (*2*, *33*, *34*). In plants, ascorbic acid is used not only for oxidative stress but also during germination and rooting (*23*, *35*). Because ascorbic acid is recycled by a dehydrate reaction with tocopherol, the amount of this vitamin is easily controlled (*36*). Plants accumulate several times the volume of ascorbic acid that animals accumulate. Because ascorbic acid does not absorb light energy, it does not interfere with photosynthesis. Its solubility in water and high chemical reactivity are other explanations of why ascorbic acid is widely used as a radical scavenger in both animals and plants.

Anthocyanin was also detected as a major radical scavenger. Ordinarily it accumulates at a low level, but γ-irradiation induced a 10−20-fold increase. In γ-irradiated plants, anthocyanin accounted for 40−50% of the total radical scavenging activity. Anthocyanin synthesis is induced by various stresses, including UV irradiation, drought, strong light, and pathogen attack. This capacity to be induced by multiple factors indicates that anthocyanin is used as a radical scavenger for many oxidative burst phenomena. Although anthocyanin is a strong scavenger of $O_2^{\bullet -}$, the pathways for its dehydration and recycling are not as effective as those for ascorbic acid. Anthocyanin is stored in vacuoles, and it interferes with light absorption when it accumulates in photosynthesis organs such as leaves. Although anthocyanin helps to protect leaf tissues from the effects of UV irradiation, plants produce anthocyanin for scavenging AOS only in an emergency. Plants use ascorbic acid and anthocyanin depending on the species and the amount of AOS. Because induction and accumulation of ascorbic acid and anthocyanin takes 2−3 days, both systems are effective against long-lived radicals, but not against the first attack of short-lived radicals, which on average last <1 s. Nevertheless, the accumulation of both ascorbic acid and anthocyanin is usually effective for elimination of short-lived radicals, because stresses usually continue to attack the plant body. Under normal conditions, the degree of radical scavenging activity shown by ascorbic acid directly reflects the plant's sensitivity and resistance to AOS stress induced by environmental conditions. The induction of ascorbic acid and anthocyanin synthesis probably provides maximum resistance to oxidative stress. Either too much or too little sensitivity to oxidative stress can be life threatening. Thus, the maximum level and the threshold of synthesis and induction of anthocyanin and ascorbic acid are regulated according to the occurrence and strength of the oxidative stresses, which in turn depend on environmental conditions.

This study also revealed a difference in the radical scavenging systems of the ecotypes L*er* and Col. These ecotypes both contained approximately the same amount of total radical scavenging activity, but the rate at which ascorbic acid and anthocyanin increased under ordinary conditions and after γ-irradiation differed between the types. The total amounts of anthocyanin and ascorbic acid and the ratios between them also differed with ecotype. Because the habitats of the two ecotypes seem the same, we speculate that the differences originated from their different characteristics. These two ecotypes have several unique characteristics (e.g., life cycle and body plan). Trichome formation is observed after the increase in AOS caused by γ-irradiation (*30*). It may be relevant that L*er*, which contains more ascorbic acid than Col, has fewer trichomes (fewer than one-third the number in Col) and a higher threshold for γ-ray induction of trichome formation. We also suggest that these differences in phenotype between the two ecotypes are caused by both anthocyanin synthesis and trichome formation being controlled by the same gene (*ttg*) that is involved in signal transduction. This may be the key to understanding the control of the radical scavenging system in the two ecotypes. The genome of *Arabidopsis* has been completely sequenced. Therefore, comparing the differences in genomic sequence between the two ecotypes may provide information on the regulatory mechanism behind the environmental response. Studying these basic mechanisms may supply useful knowledge about both basic and pragmatic aspects. Currently, we are analyzing a signal transduction system for the stimulation of AOS.

LITERATURE CITED

(1) Beckman, K. B.; Ames, B. N. The free radical theory of aging matures. *Physiol. Rev.* **1998**, *78*, 547−581.

(2) Guyton, K. Z.; Kensler, T. W. Oxidative mechanisms in carcinogenesis. *Br. Med. Bull.* **1993**, *49*, 523−544.

(3) Cutler, R. G. Antioxidants, aging, and longevity. *Free Radicals in Biology*; Pryor, W. A., Ed.; Academic Press: Orlando, FL, 1984; pp 371−423.

(4) Vaziri, N. D.; Wang, X. Q.; Oveisi, F.; Rad, B. Induction of oxidative stress by glutathione depletion causes severe hypertension in normal rats. *Hypertension* **2000**, *36*, 142−146.

(5) Cutler, R. G. Genetic stability and oxidative stress. *Free Radicals and Aging*; Emerit, I., Chance, B., Eds.; Birkhouser Verlag: Basel, Switzerland, 1992; pp 31−46.

(6) Gupta, A. S.; Heinen, J. L.; Holaday, A. S.; Burke, J.; Allen, R. D. Increased resistance to oxidative stress in transgenic plants that overexpress chloroplastic Cu/Zn superoxide dismutase. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 1629−1633.
(7) Casano, L. M.; Gomez, L. D.; Lascano, H. R.; Gonzalez, C. A.; Trippi, V. S. Inactivation and degradation of CuZn-SOD by active oxygen species in wheat chloroplasts exposed to photo oxidative stress. *Plant Cell Physiol*. **1997**, *38*, 433−440.
(8) Noctor, G.; Foyer, C. H. Ascorbate and glutathione: Keeping active oxygen under control. *Annu. Rev. Plant Physiol. Plant Mol.* **1998**, *49*, 249−279.
(9) Niki, E. Action of ascorbic acid as a scavenger of active and stable oxygen radicals. *Am. J. Clin. Nutr*. **1991**, *54*, 1119−1124.
(10) King, C. G. The biological synthesis of ascorbic acid. *World Rev. Nutr. Diet.* **1973**, *18*, 47−59.
(11) Kagan, V. E.; Serbinova, E. A.; Minin, A. A.; Savov, V. M.; Novikov, K. N. Study of the mechanism of initiation of enzymatic NADPH-dependent lipid peroxidation in membranes of the endoplasmic reticulum. *Biokhimiyia* **1985**, *50*, 986−991.
(12) Grant, J. J.; Yun, B. W.; Loake, G. J. Oxidative burst and cognate redox signaling reported by luciferase imaging: Identification of a signal network that functions independently of ethylene, SA and Me-JA but is dependent on MAPKK activity. *Plant J.* **2000**, *24*, 569−582.
(13) Logemann, E.; Tavernaro, A.; Schulz, W.; Somssich, I. E.; Hahlbrock, K. UV light selectively coinduces supply pathways from primary metabolism and flavonoid secondary product formation in parsley. *Proc. Natl. Acad. Sci. U.S.A.* **2000**, *97*, 1903−1907.
(14) Glassgen, W. E.; Rose, A.; Madlung, J.; Koch, W.; Gleitz, J.; Seitz, H. U. Regulation of enzymes involved in anthocyanin biosynthesis in carrot cell cultures in response to treatment with ultraviolet light and fungal elicitors. *Planta* **1998**, *204*, 490−498.
(15) Fuglevant, G.; Jackson, J. A.; Jenkins, G. L. UV-B, UV-A, and blue light signal transduction pathways interact synergistically to regulate chalcone synthase expression in *Arabidopsis. Plant Cell* **1996**, *8*, 2347−2357.
(16) Diallinas, G.; Kanellis, A. K. A phenylalanine ammonialyase gene from melon fruit: cDNA cloning, sequence and expression in response to development and wounding. *Plant Mol. Biol.* **1994**, *1*, 473−479.
(17) Landry, L. G.; Chapple, C. C.; Last, R. L. *Arabidopsis* mutants lacking phenolic sunscreens exhibit enhanced ultraviolet-B injury and oxidative damage. *Plant Physiol.* **1995**, *109*, 159−166.
(18) Sanz, M. J.; Ferrandiz, M. L.; Cejudo, M.; Terencio, M. C.; Gil, B.; Bustos, G.; Ubeda, A.; Gunasegaran, R.; Alcaraz, M. J. Influence of a series of natural flavonoids on free radical generating systems and oxidative stress. *Xenobiotica* **1994**, *24*, 689−699.
(19) Decharneux, T.; Dubois, F.; Beauloye, C.; Wattiaux, D.; Coninck, S.; Wattiaux, R. Effect of various flavonoids on lysosomes subjected to an oxidative or an osmotic stress. *Biochem. Pharmacol.* **1992**, *44*, 1243−1248.
(20) Rice-Evans, C. A.; Miller, N. J.; Paganga, G. Structure-antioxidant activity relationship of flavonoids and phenolic acids. *Free Radical Biol. Med.* **1996**, *20*, 933−956.
(21) Kandaswami, C.; Middleton, E., Jr. Free radical scavenging and antioxidant activity of plant flavonoids. *Free Radicals Diagn. Med.* **1994**, 351−376.
(22) Ko, F. N.; Cheng, Z. J.; Lin, C. N.; Teng, C. M. Scavenger and antioxidant properties of prenylflavones isolated from *Artocarpus heterophyllus*. *Free Radical Biol. Med.* **1998**, *25*, 160−168.
(23) Arrigoni, O. Ascorbate system in plant development. *J. Biomembr.* **1994**, *26*, 407−419.
(24) Rosen G. M.; Freeman, B. A. Detection of superoxide generated by endothelial cells. *Proc. Natl. Acad. Sci. U.S.A.* **1984**, *81*, 7629−7273.
(25) Kirino, Y.; Kwan, T. ESR studies of ascorbic acid free radicals. *Chem. Pharm. Bull.* **1972**, *20*, 2651−2660.
(26) Duke, P. S. Relation of melanoma homogenate and ascorbate solution electron paramagnetic resonance doublets. *Exp. Mol. Pathol.* **1968**, *8*, 112−122.
(27) Vaughan, W. M.; Henry, J. I.; Commoner, B. Radiosensitivity and the ascorbic acid electron spin resonance doublet. *Biochim. Biophys. Acta* **1973**, *329*, 159−162.
(28) Yamazaki, I.; Mason, H. S.; Piette, L. Identification, by electron paramagnetic resonance spectroscopy, of free radicals generated from substrates by peroxidase. *J. Biol. Chem*. **1960**, *235*, 2444−2449.
(29) Nagata, T.; Hayashi, T.; Shibata, Y.; Mori, M.; Kanegae, H.; Kikuchi, S. Gamma-radiation induces leaf trichome formation in *Arabidopsis*. *Plant Physiol.* **1999**, *120* (1), 113−120.
(30) Yamazaki, I.; Piette, L. H. Mechanism of free radical formation and disappearance during the ascorbic acid oxidase and peroxidase reactions. *Biochim. Biophys. Acta* **1961**, *50*, 62−69.
(31) Bierski, B. H. G. *Chemistry of Ascorbic Acid Radicals*; American Chemical Society: Washington, DC, 1982; pp 81−98.
(32) Coudert, P.; Leal, F.; Duroux, E.; Rubat, C.; Couquelet, J. Effect on free radical processes of some ascorbic acid analogues. *Biol. Pharm. Bull.* **1996**, *19*, 220−223.
(33) Ghosh, M. K.; Chattopadhyay, D. J.; Chatterjee, I. B. Vitamin C prevents oxidative damage. *Free Radical Res.* **1996**, *25*, 173−179.
(34) Robertson, J. M.; Donner, A. P.; Trevithick, J. R. A possible role for vitamins C and E in cataract prevention. *Am. J. Clin. Nutr.* **1991**, *53*, 346−351.
(35) Córdoba, F.; González-Reyes, J. A. Ascorbate and plant cell growth. *J. Bioenerg. Biomembr.* **1994**, *26*, 399−405.
(36) Nakano, K.; Suzuki, S. Stress-induced change in tissue levels of ascorbic acid and histamine in rats. *J. Nutr.* **1984**, *114*, 1602−1608.

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