Radial expansion of root cells and elongation of root hairs were induced within 3 d of a massive dose (3 kGy) of gamma irradiation to *Arabidopsis thaliana*. Because treatment with the antioxidant *n*-propyl gallate before irradiation suppressed these changes, gamma irradiation partially rescued the *rh2* mutant (defective in NADPH oxidase); the superoxide-generating reagent paraquat induced similar root morphogenesis. These responses appeared to be induced by the active oxygen species (AOS) generated by water radiolysis. Ethylene production was induced immediately after gamma irradiation and reached a steady level after about 2 h. Addition of the ethylene precursor 1-aminocyclopropane-1-carboxylic acid partly induced a similar expansion of root cells and elongation of root hairs. Addition of an inhibitor of ethylene biosynthesis, aminoethoxyvinylglycine, before gamma irradiation completely suppressed the formation of abnormal structures. These results suggest that the AOS is involved in the root morphological changes through the ethylene biosynthesis induced by gamma irradiation in *Arabidopsis*.

**Keywords:** Active oxygen species — Ethylene — Gamma irradiation — Root hair.

**Abbreviations:** ACC, 1-aminocyclopropane-1-carboxylic acid; AOS, active oxygen species; AVG, aminoethoxyvinylglycine; MAP-KKK, mitogen-activated protein kinase kinase kinase; *n*-PG, *n*-propyl gallate.

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**Introduction**

Massive doses (1–3 kGy) of gamma irradiation produce severe abiotic stress in *Arabidopsis thaliana*. The plants respond by accumulating anthocyanin and ascorbic acid and forming new trichomes (Nagata et al. 1999, Nagata et al. 2003). In the present study, we focused on the morphological changes, namely, radial expansion of root cells and elongation of root hairs, observed in the tip regions of roots. *Arabidopsis* roots consist of single layers of epidermis, cortex, endodermis and pericycle surrounding a vascular bundle (Dolan et al. 1993, Benfey and Scheres 2000). Roots of abnormal shape or size have been genetically screened to identify genes that regulate root morphogenesis. Cell expansion and root hair differentiation are controlled by active oxygen species (AOS) generated by RHD2 NADPH oxidase (Foreman et al. 2003). The AOS are synthesized by NADPH oxidase at the elongation site of differentiating root cells. After many steps, they cause calcium ions to flood into the cells by activating the calcium channel on the plasma membrane, and induce cell elongation and root hair differentiation. Therefore, the generation of a large amount of AOS may induce many physiological changes in root tissues. We have been studying the regulation and location of AOS in living plants in order to gain an understanding of the signal transduction process.

The phytohormone ethylene plays a key role in the radial swelling of roots; root swelling has been used as a sensitive bioassay to test for contamination of soils with household gas, thereby indicating a gas leak (Harvey and Rose 1915). Ethylene regulates root elongation, as well as root hair initiation and expansion (Cao et al. 1999, Cho and Cosgrove 2002, Ma et al. 2003). Ethylene production can be induced by gamma irradiation of plants (Young 1965, Abdel-Kader et al. 1968, Lee et al. 1968, Akamine and Goo 1971, Romani 1984, Larrigaudiere et al. 1990). Identification of the ethylene biosynthetic pathway therefore provides a basis for investigating the action of gamma irradiation. However, little is known about the molecular mechanism of ethylene induction by gamma irradiation. In the present report, through biochemical and genetic analyses, we show that root morphological changes induced by gamma irradiation are mediated by ethylene production and that ethylene production is mediated by AOS generated by water radiolysis.

**Results**

*Gamma irradiation induces radial expansion of root cells and elongation of root hairs in Arabidopsis*

Photomicrographs of root morphological changes after gamma irradiation (3 kGy) are shown in Fig. 1. These changes appear to consist of termination of root extension, swelling of the root and change of the outgrowth direction of root hairs. These changes appeared 12 h after irradiation (Fig. 1A-2, B-2) and were complete by about 72 h after irradiation. Even 500 Gy could induce these alterations (Fig. 1A-4, B-4). These changes were induced more easily in the tip region of young and short lateral roots than in long and mature roots. Scanning...
The root of *Arabidopsis* is made up of organized cell layers. To see which cells or cell layers were changed after gamma irradiation, we made transverse and longitudinal sections of roots (Fig. 3). The cells in the epidermal and cortical cell layers expanded radially. The pericycle cell layer also expanded slightly, and the meristematic region was reduced (Fig. 3A-2, B-2). Furthermore, the diameter of the irradiated plant’s root (240±23 µm) was twice that of the control plant’s root (110±8 µm). Root hairs also were initiated and elongated in the swollen root region.

**Ethylene production attributed to the gamma-radiation-induced morphological changes in roots**

Because similar root morphological changes appear to be mediated by the phytohormone ethylene, we treated *Arabidopsis* plants with the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC) and the ethylene biosynthesis inhibitor aminoethoxyvinylglycine (AVG), then observed the root structure by means of scanning electron microscopy. ACC solution (100 µl, 5 mM) was dropped onto the base of plants. The plants were grown for 2 d, then the root structure was observed (Fig. 4). Moderate morphological changes were observed (Fig. 4B): a slight increase in root diameter, slight elongation of root hairs and conspicuous ectopic root hair outgrowth in the distal region of the root (Fig. 4C). In comparison, treatment with 1 mM AVG for 3 d before gamma irradiation completely suppressed the root morphological changes (Fig. 4D). These data suggest that ethylene may play an important role in root morphological changes.

We also examined the contribution of AOS to the root morphological changes. Two experiments were performed, one to test the inhibitory effect of the antioxidant *n*-propyl gallate (*n*-PG), and the other to test the additive effect of the radical-generating reagents paraquat and menadione (Fig. 5). Treatment with the antioxidant completely inhibited the radiation-induced root morphological changes (Fig. 5E), and treatment with paraquat or menadione slightly induced the radiation-associated radial expansion of root epidermal cells and outgrowth of root-hair-like structures (Fig. 5C, D). In these structures, the cell file rule appears to be maintained. These observations suggest that root morphological changes are mediated by AOS.

**Gamma irradiation of Arabidopsis induces ethylene production**

Many external agents—including wounding, anaerobiosis, viral infection, auxin treatment, chilling, injury, drought, and Cd²⁺ and Li⁺—induce ethylene production (Yang and Hoffman 1984, Abeles et al. 1992). Gamma irradiation also induces ethylene production in tomato (Abdel-Kader et al. 1968) and avocado (Young 1965). Ethylene production was measured by means of gas chromatography. Every hour after irradiation, 1 ml of air in the growth chamber (about 9 cm × 9 cm × 0.5 cm) was taken by syringe, then injected into the gas chromatograph. The kinetics of ethylene production after
gamma irradiation were measured first. Unirradiated plants did not produce ethylene, whereas ethylene production was induced within 1 h after gamma irradiation and continued for at least 3 h (Fig. 6).

Are AOS involved in gamma-radiation-induced ethylene production? To answer this question, we treated plants with an antioxidant (n-PG) and paraquat. We used 5-week-old plants that had already bolted and flowered. About 1.5 kGy of gamma rays was applied. Three hours after irradiation, ethylene production was measured. Then we treated plants with n-PG and the ethylene biosynthesis inhibitor AVG for 3 d before irradiation, as described in Materials and Methods. Gamma irradiation increased ethylene production to 4–5 times that of the control (Table 1). Addition of n-PG in the absence of irradiation gave no change in the basal level of ethylene biosynthesis. However, treatment with n-PG before gamma irradiation suppressed ethylene production. AVG suppressed basal ethylene production and gamma-radiation-induced ethylene overproduction. The addition of paraquat (10 µM) caused ethylene production to double. These data suggest that gamma-radiation-induced ethylene production is suppressed by pretreatment with antioxidant and that ethylene production is inducible by the addition of paraquat. Therefore, gamma-radiation-induced ethylene production may be mediated by AOS generated by water radiolysis.

\[ n\text{-PG} + \text{Ethylene} \rightarrow \text{AOS} \]

\[ \text{AOS} + \text{Paraquat} \rightarrow \text{Productos Inhibidores} \]

\[ \text{Ethylene} \rightarrow \text{AOS} \rightarrow \text{Productos Inhibidores} \]

Gamma-irradiation-induced morphological changes in mutant backgrounds

To identify which genes contribute to gamma-radiation-induced changes in root morphology, we examined root structural alteration in several mutants. RHD1, RHD2, RHD3, RHD4 and RHD6 all play roles in root hair initiation and elongation (Schiefelbein and Somerville 1990, Masucci and Schiefelbein 1994, Wang H et al. 2002). We tested the mutant strains rhd1-1, rhd2-1, rhd3-1 and rhd4-1. The rhd mutants showed immature outgrowth of root hairs (Fig. 7A, C, E, G). However, gamma irradiation induced a slight extension of the root hairs (Fig. 7B, D, F, H), especially in rhd2-1 and rhd4-1. Most plants showed radial expansion of root cells. The rhd2-1 phenotype was partially rescued by gamma irradiation: that is, gamma irradiation induced new trichome formation on the adaxial surface of the glabra rosette leaves of rhd2-1, as we reported previously (Nagata et al. 1999).

We isolated 15 ethylene-responsive mutants by triple-response screening and characterized them. The results revealed that ETR1 and EIN4 act before CTR1 as negative regulators. ETR1 encodes an ethylene receptor, and EIN4 might be a ’second component’ (Chang et al. 1993, Schaller and Bleecker 1995, Bleecker et al. 1998). CTR1 is a central component in the ethylene signal-transduction pathway; it acts as a negative regulator of EIN2, EIN3, EIN5, EIN6, EIN7, EIR1 and HLS1. In addition, CTR1 shows amino acid similarity to the Raf family.
of protein kinases; its structure is similar to that of mitogen-activated protein kinase kinase kinase (MAPKKK; Kieber et al. 1993). The *ctr1* mutant exhibits multiple changes in the root epidermis, including a modest increase in the proportion of root hair cells and a significant reduction in epidermal cell length (Masucci and Schiefelbein 1996). The effects of gamma irradiation on the roots of these mutants are shown in Fig. 8. The *etr1* and *ein4* mutants showed a modest increase in root diameter after gamma irradiation. Ectopic root hair initiation and elongation of root hairs were suppressed (Fig. 8D, F). The *ctr1* mutant showed an excessive increase in root diameter and a slight extension of root hairs (Fig. 8H). The *ein2*, *ein3* and *ein5* mutants showed a slight increase in root diameter, but elongation of root hairs was suppressed (Fig. 8J, L, N).

Auxin enhances ethylene production in roots (Abeles et al. 1992). *AXR2* represents an important component of the hormone response pathway that affects root hair initiation (Masucci and Schiefelbein 1996). The *axr1* mutant is auxin insensitive (Estelle and Somerville 1987, Lincoln et al. 1990) and slightly ethylene insensitive (Timpte et al. 1995); AXR1 is related to ubiquitin-conjugating enzymes (Leyser et al. 1993). Mutant *aux1* is auxin and ethylene insensitive in the roots (Pickett et al. 1990). The *axr4* mutant is auxin resistant (Hobbie and Estelle 1995). We tested mutants *aux1-7*, *axr2* and

Fig. 3 Transverse and longitudinal sections of roots. Transverse (A) and longitudinal (B) sections of normal (1) and irradiated (2) roots at the same magnification. Staining, solidification and sectioning procedures are described in Materials and Methods. Bar = 50 µm.

Fig. 4 Effects of an ethylene precursor (ACC) or an ethylene biosynthesis inhibitor (AVG) on root morphological changes. (A) Unirradiated root. (B) Gamma-irradiated (72 h) root. (C) Root treated with ACC. (D) Root treated with AVG and irradiated. The procedures for treatment with ACC and AVG are described in Materials and Methods. Root tissues were fixed at 72 h after irradiation or treatment. Bar = 150 µm.
Enlargement of Arabidopsis root hairs by irradiation

As shown in Fig. 9, aux1-7 and axr4-2 showed radial expansion of root cells like that of the wild type, but no elongation of root hairs (Fig. 9B, F). In comparison, axr2 showed little radial expansion of root cells but noticeable elongation of root hairs (Fig. 9D).

These results indicate that the ETR1 ethylene-responsive cascade (Ecker 1995) plays an important role in gamma-radiation-induced root morphological changes in Arabidopsis. Two other ethylene-responsive cascades have been reported: the AUX1 cascade and the AXR1 cascade (Timpte et al. 1995, Masucci and Schiefelbein 1996). The slight increase in root diameter in the etr1 and ein4 mutants may be due to the action

![Fig. 5](image) Effects of treatment with paraquat, menadione or an antioxidant (n-PG) on root morphological changes. (A) Normal root. (B) Gamma-irradiated (24 h) root. (C) Paraquat-treated root. (D) Menadione-treated root. (E) Root treated with n-PG and gamma irradiated. The procedures for treatment with paraquat, menadione and n-PG are described in Materials and Methods. All root tissues except (A) and (B) were fixed at 72 h after treatment or irradiation. Bar = 150 µm.

![Fig. 6](image) Kinetics of ethylene production after gamma irradiation. Twenty plates containing 36 plants grown on MS-agar medium for 34 d were irradiated. Every hour after irradiation, ethylene production during that hour was measured. One milliliter of air was sampled, and the amount of ethylene was measured by means of gas chromatography.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Concentration (nl ml⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.3890 ± 0.0197</td>
</tr>
<tr>
<td>Gamma irradiation (1.5 kGy)</td>
<td>1.9141 ± 0.1068</td>
</tr>
<tr>
<td>n-PG</td>
<td>0.4408 ± 0.0182</td>
</tr>
<tr>
<td>n-PG + gamma irradiation</td>
<td>0.4998 ± 0.0421</td>
</tr>
<tr>
<td>AVG</td>
<td>0.0614 ± 0.0023</td>
</tr>
<tr>
<td>AVG + gamma irradiation</td>
<td>0.3821 ± 0.0123</td>
</tr>
<tr>
<td>Paraquat</td>
<td>1.0338 ± 0.0436</td>
</tr>
</tbody>
</table>

*Mean ± 1 standard deviation of three independent experiments.*
of these cascades. Not only ethylene but also auxin appears to play an important role in the root morphological changes induced by gamma irradiation of *Arabidopsis*.

**Discussion**

*Gamma irradiation induces physiological and morphological changes in developing Arabidopsis roots*

The responses of *Arabidopsis* roots after gamma irradiation represent growth regulation by AOS. Therefore, the formation of root hairs and expansion of epidermal cells are controlled by AOS signal-transduction systems. Root hair differentiation and expansion are thought to be controlled by AOS generated by NADPH oxidase (*RHD2*) under normal conditions (Foreman et al. 2003). Our results support this hypothesis and suggest that AOS regulate the expansion of epidermal cells. Gamma-irradiation-associated control of differentiation of epidermal cells also occurred in leaves (Nagata et al. 1999). Trichomes on the adaxial surface of the leaves increased in number a few days after gamma irradiation. Therefore, there seem to exist common systems that control both root and leaf epidermal cells (Schellmann et al. 2002). The differentiation of root hairs and trichomes is regulated by genes common to both root and leaf epidermal cells ([*TTG1* (WD40 protein), [*GL2* (homeodomain protein), [*GL3* (bHLH), [*CPC* (MYB), [*TRY* (MYB)])] and by those that are similar between the two types of cell [WER (MYB) $\rightarrow$ root hair differentiation, GLI (MYB) $\rightarrow$ trichome differentiation]. *TTG* and *GL2* negatively regulate the differentiation of root hair cells (Walker et al. 1999, Ohashi et al. 2003). We gamma-irradiated the mutants *ttg1-1* and *gl2* and

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**Fig. 8** Gamma-radiation-induced root morphological changes in ethylene-responsive mutants. (A) Unirradiated root of wild type. (B) Gamma-irradiated root of wild type. (C) Unirradiated root of *etr1-1*. (D) Irradiated root of *etr1-1*. (E) Unirradiated root of *ein4*. (F) Irradiated root of *ein4*. (G) Unirradiated root of *ctr1-1*. (H) Irradiated root of *ctr1-1*. (I) Unirradiated root of *ein2-1*. (J) Irradiated root of *ein2-1*. (K) Unirradiated root of *ein3-1*. (L) Irradiated root of *ein3-1*. (M) Unirradiated root of *ein5-1*. (N) Irradiated root of *ein5-1*. All irradiated root tissues were fixed at 72 h after gamma irradiation (3 kGy). (A, B) Bar = 100 µm. (C–N) Bar = 150 µm.
Enlargement of Arabidopsis root hairs by irradiation

We observed that the root hair pattern of these mutants was altered. However, the radial expansion of root cells and extension of root hairs were similar to those of the wild type (data not shown). Although the root and trichome differ at the histological level, both tissues seem to have a common cell differentiation system. Epidermal cells of both are induced to differentiate exserted tissues; therefore, the same regulation systems may be conserved between root and leaf. In addition, TTG1 regulates root hair formation, trichome formation and anthocyanin synthesis. Therefore, transcription systems regulated by AOS may use the common hierarchical regulatory system of WD40 $\rightarrow$ MYB $\rightarrow$ homeodomain protein.

**Contribution of ethylene production to gamma-radiation-induced morphological changes**

Massive doses (1–3 kGy) of gamma irradiation induce marked responses in A. thaliana. We previously characterized these responses genetically and analyzed them physiologically. We also analyzed anthocyanin and ascorbic acid biosynthesis and trichome formation and reported the contribution of AOS to the signal-transduction pathways, the biological significance of these responses and the genetic components that contribute to these responses (Nagata et al. 1999, Nagata et al. 2003). In the present study, we have shown that ethylene production influences these responses by means of four independent experiments: ethylene production after gamma irradiation, treatment with an ethylene precursor (ACC), treatment with an inhibitor of ethylene biosynthesis (AVG), and analysis with ethylene- and auxin-responsive mutants.

The results of these experiments support the hypothesis that ethylene production participates in gamma-radiation-induced root morphological changes and is mediated by AOS. Ethylene is a gaseous phytohormone that influences many aspects of plant growth and development (Abeles et al. 1992). It promotes germination of seeds and senescence and abscission of flowers and leaves. The contribution of ethylene to root cellular organization and especially to root hair formation has been reported recently (Masucci and Schiefelbein 1996, Zhang et al. 2003). Ethylene has also been implicated in the response to a wide variety of stresses, including pathogen attack, wounding, anaerobiosis, heat shock, cold treatment, UV irradiation and ionizing radiation (Wang K.L. et al. 2002). Stress-induced ethylene has two effects. One is the induction of senescence of damaged tissue, and the other is the induction of the expression of self-protective genes. The ethylene biosynthetic pathway has been elucidated (Yang and Hoffman 1984). The two key enzymes are ACC synthase and ACC oxidase. ACC synthase is the rate-limiting step in ethylene biosynthesis. This enzyme’s activity is highly regulated and closely parallels the level of ethylene biosynthesis. In Arabidopsis, there are at least five ACC synthase genes that show distinct expression patterns in different tissues and that respond unequally to various inducers. This pattern suggests that the different ACC enzymes are functionally distinct. Yang and Hoffman (1984) were unable to elucidate which gene products are involved in the two types of ethylene production (developmental and stress-induced) because of the relatively low abundance of the gene products. Although the results of our antioxidant treatment before gamma irradiation and of our treatment with paraquat and menadione imply the possible contribution of AOS to some gamma-radiation-induced responses, we have shown that gamma-radiation-induced ethylene production is mediated by AOS.

Microarray analysis has revealed that the expression of ethylene response genes, development control genes and redox genes was dramatically changed after gamma irradiation (Nagata et al. 2003). Therefore, the responses to AOS-induced stress have been shown at the molecular level. As a next step we will analyze those genes to reveal the AOS signal-transduction systems.

**Materials and Methods**

*Arabidopsis strains and growth conditions*

The following genetic stocks (with associated stock numbers) were obtained from the Arabidopsis Biological Resource Center (Ohio State University, Columbus, OH, U.S.A.): ttg1-1 (CS89), gl2-1 (CS65), rhd1-1 (CS2257), rhd2-1 (CS2259), rhd3-1 (CS2260), rhd4-1 (CS2261).
solution was added to the surface of the solidified medium once per day. For 3 d before gamma irradiation, 100 TBq; Nordion Intl., Ontario, Canada). After irradiation, plants were washed in distilled water, tissues were dehydrated in ethanol and glutaraldehyde in 50 mM sodium phosphate buffer (pH 7.2). After two washes in distilled water and solidified in 1% agarose (Funakoshi, Tokyo, Japan) and photographed with an FX35DX camera (Nikon) using Fuji-blade. Sections were mounted on Microphoto-FXA (Nikon, Tokyo, Japan). Sections (30–50 µm) were made similarly. The tissues were dried with a critical point sputter source (E-1010; Hitachi). Tissues were observed with a TMS-50E (CS2261), ein4 (CS8053), ctrl-1 (CS8057), ein2-1 (CS3071), ein3-1 (CS8052), ein5-1 (CS8054), ein6 (CS8055), ein7 (CS8056), ein1-1 (CS8058), his1-1 (CS3073), aux-1 (CS3074), axr2 (CS3077) and axr4-2 (CS8019). Arabidopsis thaliana (ecotype Columbia) seeds were sown on agar-solidified Murashige and Skoog medium (Navagta et al. 1999). After germination, plants were cultured for 2–3 weeks under a 16-h light (4,000 lux)/8-h dark cycle at 22°C. For the observation of root structure, 12 seedlings were grown in a 9 cm × 9 cm Petri dish. For the assay of ethylene production, 36 seedlings were grown in the same type of dish.

**Gamma irradiation**

Three-week-old plants on agar medium were irradiated with gamma rays from cobalt-60 in a gamma cell (3.0 × 10^9 Gy h⁻¹, 1.3 × 10^2 TBq; Nordion Intl., Ontario, Canada). For the observation of root structure, 12 seedlings were grown in a 9 cm × 9 cm Petri dish. For the assay of ethylene production, 36 seedlings were grown in the same type of dish.

**Treatment with reagents**

n-PG (Sigma-Aldrich, Tokyo, Japan) was used as the antioxidant. For 3 d before gamma irradiation, 100 µL of a 1-mM aqueous solution was added to the surface of the solidified medium once per day. About 16 h after the last treatment with antioxidant, gamma irradiation was started. Paraquat (2 µM), menadione (1 mM), ACC (1 mM) and AVG (1 mM) (all Sigma products) were added similarly.

**Microscopy**

Root tissues were stained with 0.05% toluidine blue, washed with distilled water and solidified in 1% agarose (Funakoshi, Tokyo, Japan). Sections (30–50 µm) were made with a Microslicer DK-1000 microscope (Do-han EM, Kyoto, Japan) fitted with a disposable steel blade. Sections were mounted on Microphoto-FXA (Nikon, Tokyo, Japan) and photographed with an FX35DX camera (Nikon) using Fuji-chrome Sensia-100 film.

**Scanning electron microscopy**

Roots were fixed for 4 h with 3% paraformaldehyde and 0.25% glutaraldehyde in 50 mM sodium phosphate buffer (pH 7.2). After two washes in distilled water, tissues were dehydrated in ethanol and embedded in 3-methylbutyl acetate. The tissues were dried with a critical point dryer (Hitachi, Tokyo, Japan) and ion-coated with an ion sputter source (E-1010; Hitachi). Tissues were observed with a scanning electron microscope (S-2040N; Hitachi).

**Measurement of ethylene production**

After gamma irradiation or treatment with reagents, 1 ml of the air in the culture vessel was collected into a syringe through a hole made previously. The air was transferred to a gas chromatograph (GC-7AG, C-R5A; Shimadzu, Kyoto, Japan), and the peak area corresponding to ethylene was measured.

**References**


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