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# Mutation Rate and Novel *tt* Mutants of *Arabidopsis thaliana* Induced by Carbon Ions

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ABSTRACT

Irradiation of *Arabidopsis thaliana* by carbon ions was carried out to investigate the mutational effect of ion particles in higher plants. Frequencies of embryonic lethals and chlorophyll-deficient mutants were found to be significantly higher after carbon-ion irradiation than after electron irradiation (11-fold and 7.8-fold per unit dose, respectively). To estimate the mutation rate of carbon ions, mutants with no pigments on leaves and stems (*tt*) and no trichomes on leaves (*gl*) were isolated at the M2 generation and subjected to analysis. Averaged segregation rate of the backcrossed mutants was 0.25, which suggested that large deletions reducing the viability of the gametophytes were not transmitted, if generated, in most cases. During the isolation of mutants, two new classes of flavonoid mutants (*tt18*, *tt19*) were isolated from carbon-ion-mutagenized M2 plants. From PCR and sequence analysis, two of the three *tt18* mutant alleles were found to have a small deletion within the *LDOX* gene and the other was revealed to contain a rearrangement. Using the segregation rates, the mutation rate of carbon ions was estimated to be 17-fold higher than that of electrons. The isolation of novel mutants and the high mutation rate suggest that ion particles can be used as a valuable mutagen for plant genetics.

MUTATION rates after low linear energy transfer (LET) radiation, such as X rays, γ-rays, and fast neutrons, have been extensively studied in plants (see Smith 1972 for review). These radiation-induced mutants are widely used as important resources in plant genetics and breeding and in molecular biology. High LET radiation, such as ion particles, causes more localized, dense ionization within cells than does low LET radiation (Kraft *et al.* 1992). On the basis of microdosimetric and radiobiological considerations, it is assumed that high LET radiation could produce double-strand breaks with damaged end groups whose reparability would be low (Hagen 1994; Goodhead 1995; Blakely and Kronenberg 1998; Nikjoo *et al.* 1998). Therefore, it seems plausible that high LET radiation would be able to generate mutations more frequently than low LET radiation. In addition, it seems likely that large structural alterations may be induced by high LET radiation more frequently than by low LET radiation. We have analyzed three carbon-ion-induced mutations in *Arabidopsis thaliana*—*gl1-3*, *tt4*(C1), and *ttg1-21*—at the sequence level and found that they indeed contain inversions and translocations (Shikazono *et al.* 1998, 2001).

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The mutagenic effect of ion particle irradiation, mainly on somatic mutations, has been investigated using various plant species (Smith 1972). The highest relative biological effectiveness (RBE) was found with LETs ~100–200 keV/μm. In contrast, few studies have been done on germline mutations. Mei *et al.* (1994) investigated germline mutations in rice and observed a high incidence of semidwarf mutants induced by argon and iron ions. In addition, they determined the mutation spectrum of argon ions by scoring the change of characters of panicles, spikelets, and grains, as well as changes in sheath color, plant size, and the timing of maturity. However, these previously identified germline mutations were mainly studied on mutated phenotypes for which the numbers of the corresponding genes were unknown.

For genetics and breeding, it is fundamentally important to know the germline mutation rate induced by a mutagen. Koornneef *et al.* (1982) studied the mutation rates of ionizing radiation in Arabidopsis and found that mutation rates per Gray for X rays and fast neutrons were within the range of $10^{-6}$–$10^{-7}$ and $10^{-5}$, respectively. However, there are no reports, to our knowledge, of the germline mutation rate of plants after ion particle irradiation. As a first step to evaluate the use of ion particle mutagenesis in higher plants, the frequency of germline mutations after carbon-ion irradiation (LET = 113 keV/μm) was measured by Müller's embryo test (Müller 1963), and further, the segregation and mutation rates for carbon-ion-induced Arabidopsis mutants

were estimated by isolating *transparent testa* (*tt*) and *glabrous* (*gl*) mutants.

## MATERIALS AND METHODS

**Plant material:** Plants of *A. thaliana* ecotype Columbia were grown on metro-mix (HYPONEX, Osaka, Japan) or on rock wools (Nichiasu, Tokyo) at a temperature of 25° ± 3° in an air-conditioned greenhouse and were subirrigated at 3- or 4-day intervals with 0.03% HYPONeX (HYPONEX). Seeds of the mutants used for the complementation test were provided from the Nottingham Arabidopsis Stock Center (stock nos. N64, N65, N66, N84, N85, N86, N87, N88, N89).

**Irradiation:** Irradiation of dry seeds was carried out as previously described (Tanaka *et al.* 1997a). The energy of carbon ions was 220 MeV and the mean LET within the seeds was calculated to be 113 keV/μm. The energy of electrons was 2 MeV and the mean LET was calculated to be 0.2 keV/μm. The dry seeds were irradiated with a dose of 150 Gy for carbon ions and with a dose of 750 Gy for electrons.

**Embryo test:** A total of 600 M1 plants were subjected to analysis for each mutagen treatment. Frequencies of embryonic lethals and of chlorophyll-deficient mutants were scored as previously described (Müller 1963; Mesken and Van Der Veen 1968; Dellaert 1980a). In brief, the fifth siliques on the main stem of M1 plants were opened up just before ripening and their seeds were scored under a stereoscopic microscope. We classified the seeds of green color as normal, seeds with seed coat turned prematurely brown as embryonic lethal, and seeds of white/pale green color as chlorophyll-deficient mutants. Only the seeds on one side of the septum were counted for ease of handling. Embryonic lethality was calculated as a percentage of embryonic lethals among fertilized ovules, and chlorophyll deficiency as a percentage of chlorophyll-deficient mutants among nonlethal embryos.

**Isolation of *tt* and *gl* mutants:** Irradiated M1 seeds were sown and selfed to obtain M2 seeds. M2 seeds from 100 M1 lines were pooled, and 400–500 seeds from each pool were sown and screened for mutants. A total of 262 M1 pools for carbon ions and 96 M1 pools for electrons were screened in the present study. *tt* and *gl* mutants were identified at the M2 population, which showed a lack of purple pigmentation (anthocyanins) on leaves and stems and lacked trichomes on leaves, respectively. For the complementation tests, *gl* mutant lines were crossed with *gl1-1*, *gl2-1*, *gl3-1*, and *ttg1-1* mutants (Koornneef *et al.* 1982). Since *tt3-1*, *tt4-1*, *tt5-1*, *tt6-1*, and *tt7-1* are the known mutants lacking flavonoid pigments in leaves and stems (Shirley *et al.* 1995), isolated *tt* mutant lines were crossed with these mutants. The segregation rates were scored as the frequency of mutants in the $F_2$ population of the cross between Columbia and the M3 plants. The average mutation rate was estimated using the method proposed by Gaul (1957), where the mutant frequency per locus (fraction of mutants in the M2 population divided by the total number of loci) was divided by the segregation rate. Position of loci (*tt18*, *tt19*) was mapped using the $F_2$ between Landsberg erecta and the mutant with cleaved amplified polymorphisms and/or simple sequence length polymorphism markers.

**DNA extraction and molecular analysis:** The genomic DNA was extracted from the M3 mutants following the procedure described by Konieczny and Ausubel (1993). The sequences of the primers for PCR analysis of the leucoanthocyanidin dioxygenase (*LDOX*) gene were primer 1, 5′-TCACGCACTTA CCTCACAACAA-3′; primer 2, 5′-TAGCCAATTTACTTCCAT AGCC-3′; primer 3, 5′-TGGAAGAGAAGGAGAAGTATGC-3′; primer 4, 5′-CAGGAGAGAAGAAGGAGATTGA-3′; primer 5, 5′-TGGGAAGGAACAAGAGGAAT-3′; and primer 6, 5′-TTG GATGTGGTAGATGGTTGTT-3′. In the present study, amplification was carried out at 94° for 10 min, followed by 50 cycles of 94° for 30 sec, 60° for 30 sec, and 72° for 1 min. At the end of the 50 cycles, the samples were incubated at 72° for another 7 min to complete extension. The amplified DNA fragments were analyzed by 1.5% agarose gel electrophoresis in TAE buffer and were visualized by ethidium bromide staining. Fragments that showed no apparent size alterations were further analyzed by direct sequencing. Sequences of both DNA strands of PCR fragments were determined by the dideoxynucleotide chain termination method (Sanger *et al.* 1977) with an automated sequencer (ABI Prism 310 genetic analyzer, Perkin-Elmer, Norwalk, CT). Analyses of the sequences were performed with the GENETYX MAC program (version 10.0, Software Development, Tokyo).

## RESULTS

Electrons used in the present study have a LET of 0.2 keV/μm. Since this value is the same as the LET of γ-rays, biological effects of electrons could be regarded as equivalent to those of γ-rays. The RBE of carbon-ion exposures against electron exposures on the survival of Arabidopsis is approximately five (Tanaka *et al.* 1997a). Little effect on survival was observed up to 200 Gy for carbon ions and 1000 Gy for electrons, but survival rates were significantly reduced beyond those doses for each radiation. The doses for mutagenesis were determined to be 150 and 750 Gy for carbon ions and electrons, respectively. These doses were irradiated to give little effect on survival and an identical amount of lethal damage, thus minimizing the effects of lethal damage to the plant. Mutation frequencies per Gray or mutation rates per Gray were compared on the basis of the finding that radiation-induced mutation rates increase linearly with increasing dose (Koornneef *et al.* 1982).

**Embryonic lethals and chlorophyll mutants:** To study the mutational effect of carbon ions, we first looked at the frequency of embryonic lethals and of chlorophyll-deficient mutants in M1 siliques (Müller's embryo test; Table 1). Embryonic lethals and chlorophyll mutants were found even without irradiation, although the frequencies were low (both <1%). The induced frequencies of embryonic lethals and chlorophyll-deficient mutants after irradiation were estimated by subtracting the percentage of unirradiated samples and were found to be 6.0 and 8.4% for carbon ions of 150 Gy and 2.6 and 5.4% for electrons of 750 Gy, respectively. That is, per unit dose, the frequencies of embryonic lethals and chlorophyll-deficient mutants induced by carbon ions were 11-fold and 7.8-fold higher, respectively, than those induced by electrons.

***tt18* and *tt19* mutants:** A total of 62 *tt* mutants and 26 *gl* mutants were isolated after carbon-ion irradiation (see Table 4). From the complementation test, one line of *tt3*, seven lines of *tt4*, two lines of *tt5*, three lines of *tt6*, five lines of previously unidentified *tt* mutants, six lines of *gl1*, one line of *gl2*, and four lines of *ttg1* were identified. On the other hand, 4 *tt* mutants and 7 *gl* mutants were isolated after electron irradiation. One line each of *tt3* and *tt4* and three lines of *gl2* were

**TABLE 1**

**Frequencies of embryonic lethals and chlorophyll-deficient mutants**

| Mutagen | No. of M1 plants | No. of normal seeds | Embryonic lethals | | | Chlorophyll mutants | | |
|---|---|---|---|---|---|---|---|---|
| | | | No. of seeds | Frequency ($\times 10^{-2}$)[a] | Frequency/ Gy ($\times 10^{-5}$)[b] | No. of seeds | Frequency ($\times 10^{-2}$)[c] | Frequency/ Gy ($\times 10^{-5}$)[b] |
| Unirradiated | 600 | 11,927 | 119 | 0.98 | | 96 | 0.80 | |
| Electrons (750 Gy) | 600 | 6,397 | 256 | 3.6 | 3.5 | 420 | 6.2 | 7.2 |
| Carbon ions (150 Gy) | 600 | 2,986 | 247 | 7.0 | 40 | 301 | 9.2 | 56 |

[a] Calculated as a percentage of embryonic lethals among fertilized ovules.
[b] Frequency/Gy = (Frequency after irradiation − Frequency without irradiation)/Irradiated dose (Gy).
[c] Calculated as a percentage of chlorophyll-deficient mutants among nonlethal embryos.

identified. However, five carbon-ion-induced *tt* mutant lines in the present study were found to complement all of the *tt3*, *tt4*, *tt5*, *tt6*, and *tt7* mutants, which indicated that mutations had occurred in other loci. These five mutant lines were crossed to each other and sorted into two complementation groups. Each of the two loci was named *tt18* and *tt19*.

The seeds of the three *tt18* mutants were yellowish-brown and the *tt18* locus was mapped at 1.9 cM (±1.0, standard error) from the AG marker (63.2 cM) on chromosome 4. It is known that LDOX is required for converting flavan-3,4-diols to 3-OH-anthocyanidins, which is one of the enzymatic steps involved in the anthocyanin biosynthetic pathway (Pelletier *et al.* 1997, 1999). Since *tt18* had reduced pigmentation on leaves and stems and the *LDOX* gene was on the bacterial artificial chromosome (BAC; ATCHRIV58, accession no. AL16-1558) that was located near the map position mentioned above, it was thought that the mutation may have been in this gene. The *LDOX* gene was amplified by PCR in these mutants to clarify whether the mutation of the *LDOX* gene is responsible for this mutated phenotype. It was found that the entire locus could not be amplified in one of the mutants (*tt18-3*), indicating that a rearrangement or a deletion took place at the *LDOX* gene in this mutant line (Figure 1). We further analyzed the *tt18-3* mutation in more detail by PCR and sequence analysis and found that (1) the downstream region from the middle of exon 2 of the *LDOX* gene was rejoined to the region on BAC T3F17, which locates at the bottom of chromosome 2, and (2) the upstream region >3.9 kb from the translation initiation site of the *LDOX* gene was present in the mutant (data not shown). We have quite extensively performed thermal asymmetric interlaced-PCR (Liu *et al.* 1995) and suppression PCR (Siebert *et al.* 1995; Miyao *et al.* 1998) to identify the rejoined region to this upstream fragment of the *LDOX* gene, but have been unable to obtain an amplified fragment. We speculate that the failure of cloning the rejoined fragment was due to the sequence context of the rejoined fragment. For instance, if a fragment, which has extremely high/low GC content or short tandem repeats resulting in low affinity to the primer and is resistant to restriction enzyme cleavage, were rejoined to the upstream region of the *LDOX* gene, this fragment would be refractory to amplification by these two PCR methods. Although we are still far from full characterization of the mutation of *tt18-3*, we can at least conclude from these results that the *tt18-3* mutation contains a rearrangement. A study to reveal the overall structural alteration in this mutant is now in progress. The other two lines (*tt18-1*, *tt18-2*) showed no apparent alterations of the amplified fragments with those from the wild type. We further carried out sequence analysis and demonstrated that there was a small deletion in each of these two mutant lines (Table 2; *tt18-1*, accession no. AB084467; *tt18-2*, accession no. AB084468). Both of these deletions seem to generate premature stop codons in the coding sequence, which would result in truncated proteins. From this PCR and sequence analysis, it was concluded that the mutation of the *LDOX* gene was responsible for the *tt* phenotype in these *tt18* mutants.

Two *tt19* mutants were also isolated. These showed a phenotype of no or very reduced level of pigmentation on leaves and stems. The seeds of *tt19* have a brown-yellowish color that is different from brown-seeded wild type. The *TT19* locus was mapped at 1.6 cM (±1.0, standard error) from *TT4* (29.5 cM) on chromosome 5. Although *TT4* and also *TT7* are located close to the map position of *TT19*, both *tt4* and *tt7* complemented *tt19*, indicating that neither was allelic to *TT19*. The flavonol synthase 1(*FLS1*) gene, which encodes an enzyme known to catalyze the conversion of dihydroflavonols to flavonols, also maps close to the *TT19* locus (Pelletier *et al.* 1997; Wisman *et al.* 1998). To determine whether a mutation in the *FLS1* gene is responsible for the *tt19* phenotype, we sequenced the genomic DNA encompassing the entire *FLS1* gene. (The gene was sequenced from 375 bp upstream of the translation initiation site to 577 bp downstream of the translation termination site.) However, no mutation was found in *FLS1* in either of the two *tt19* alleles. Furthermore, Wisman *et al.* (1998) reported that the seed color of a *fls1* null mutant is brown and is indistinguishable from that of the wild type. On the basis of these facts and the lack of other mutants defective in flavonoid biosynthesis

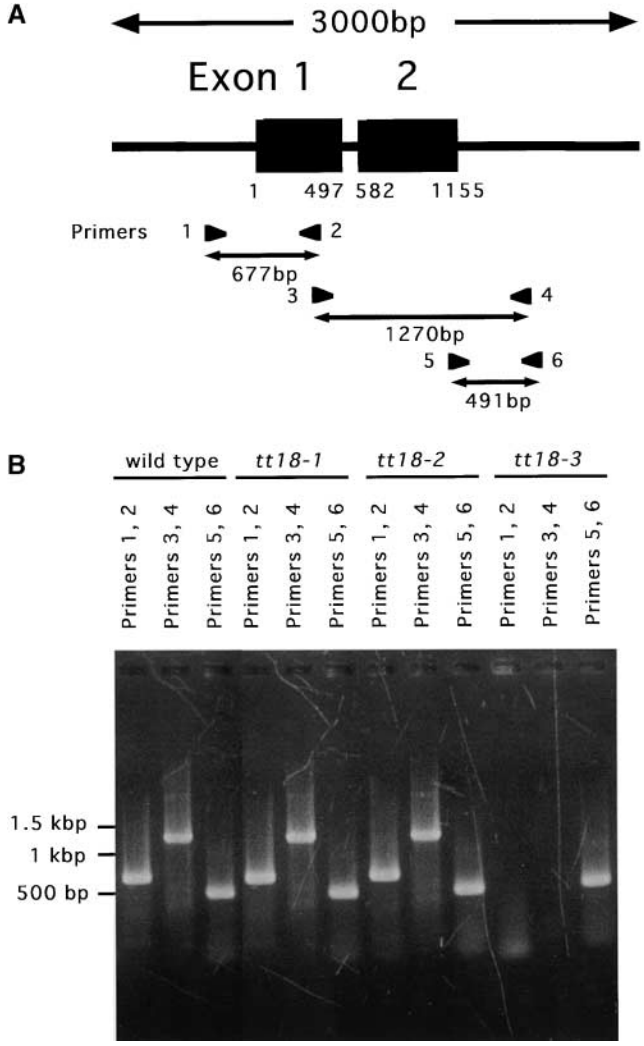


FIGURE 1.—PCR analysis of the *tt18* mutants. (A) Schematic representation of the *LDOX* locus. Putative exons are shown in solid boxes and the numbers below show the positions of the exons, where the translation initiation site is set as 1. The primers are shown as solid arrowheads. The size of the PCR products from the wild type is also shown. (B) Gel electrophoresis pattern of PCR-amplified fragments from the three *tt18* mutant lines. The primer sets used for amplification are shown above each lane.

mapped close to *tt19*, it is likely that *tt19*, generated by carbon-ion irradiation, is a novel mutant.

**Segregation rates:** To convert the observed mutant frequencies into mutation rates, the segregation rate had to be known. Therefore, we surveyed the segregation rate of the mutants in the $F_2$ population, which derived from the cross between parental Columbia plants and the radiation-induced M3 plants. The average segregation rates were found to be 0.250 (±0.008, standard error) and 0.256 (±0.012, standard error) for carbon-ion-induced and electron-induced mutants, respectively (Table 3).

**Mutation rates:** Due to the finding of *tt18* and *tt19* mutants, the number of loci responsible for the *tt* phenotype was considered to be 7. Recently, JOHNSON *et al.* (2002) reported on a novel mutant, *ttg2*, which would have been found as a *gl* mutant in the present screen. The total number of loci responsible for *tt* and *gl* phenotypes in this study, therefore, was regarded as 12. Using the average value obtained for segregation rate, average mutation rates per dose for carbon- and electron-induced mutants were estimated to be $1.9 \times 10^{-6}$ and $0.11 \times 10^{-6}$, respectively (Table 4). The present result demonstrates that the mutation rate of carbon ions was 17-fold higher than that of electrons. It should be noted that all mutants isolated in this study had no additional visible phenotypes.

## DISCUSSION

**Mutagenic effect of carbon ions:** It was demonstrated from the present study that the frequencies of embryonic lethals and chlorophyll-deficient mutants induced by carbon ions were 11-fold and 7.8-fold higher, respectively, than those induced by electrons (Table 1) and that the mutation rate per Gray of carbon ions ($1.9 \times 10^{-6}$/Gy) was 17-fold higher than that of electrons (Table 4). These values are comparable with the RBEs (12–35) estimated on somatic mutation in Arabidopsis after ion particle irradiation with similar LETs (74–230 keV/μm; FUJII *et al.* 1966, 1967; HIRONO *et al.* 1970). It is known that carbon ions have a LET ~500-fold higher than that of electrons. The high frequency of embryonic lethals and chlorophyll mutants and the high mutation rate after carbon-ion irradiation indicate that damage produced by a single carbon ion is more mutagenic than that produced by 500 tracks of electrons. Since all loci, except *tt7* and *gl3*, were found to be mutated by carbon-ion irradiation, it is likely that carbon ions could randomly mutate the genome (Table 4).

The mutation rate of electrons appeared to be approximately threefold lower than that of X rays (KOORNNEEF *et al.* 1982). It should be noted that, to obtain an averaged value, the mutation rate of X rays was recalculated under an assumption that a total of 80 loci corresponds to the phenotypes of the screened mutants and that the seeds were irradiated at a single dose of 273.13 Gy. We speculate this discrepancy is due to the irradiation conditions: they irradiated imbibed seeds, while we used dry seeds. In the case of carbon ions, the mutation rate per Gray also seemed to be three- to fourfold less than that of fast neutrons (KOORNNEEF *et al.* 1982). (The mutation rate of fast neutrons was also recalculated with a dose of 45.39 Gy, as mentioned above.) Considering the difference of irradiation conditions mentioned above, we estimate that the potential of carbon ions to induce germline mutations is similar to that of fast neutrons. The frequencies of embryonic lethals and chlorophyll-deficient mutants were not discussed in detail in this context, because the values differed signifi-

**TABLE 2**

**Altered sequences of the *LDOX* gene identified in carbon-ion-induced *tt18* mutations**

| Allele | Position of the mutation[a] | Altered nucleotide sequence | Predicted changes of amino acids[b] |
|---|---|---|---|
| *tt18-1*[c] | 1083–1084 | TT → deletion | Phe334Ala335Gln336His337 → Cys334Ser335Thr336Val337Stop |
| *tt18-2*[d] | 1052–1056 | TTGAG → deletion | Glu324Ser325 → Ser324Gly325Stop |

[a] The first nucleotide of the translation initiation codon was set as 1.
[b] Wild-type LDOX protein consists of 356 amino acids.
[c] Accession no. AB084467.
[d] Accession no. AB084468.

cantly between experiments (Mesken and Van Der Veen 1968; Dellaert 1980a). However, the RBEs of fast neutrons estimated with these endpoints (embryonic lethality and chlorophyll deficiency) were found to be fairly constant, ~6–7 (Timofeev-Resovskii *et al.* 1971; Dellaert 1980a). These results led us to suppose that the criteria of scoring mutants vary among different scorers but the ratio of the frequency itself in each experiment is accurate and meaningful. On the basis of this interpretation and provided that the frequency of mutation increases linearly with dose, similar values between fast neutrons (6–7) and carbon ions (7.8–11) support the conclusion that both types of radiation have a similar mutagenic effect on Arabidopsis seeds.

Koornneef *et al.* (1982) also looked at the mutagenic effect of EMS and isolated mutants with a frequency $\sim 0.20 \times 10^{-3}$ mutants/locus under an experimental condition of 10 mM for 24 hr. Our study showed that carbon ions had a frequency of $0.08 \times 10^{-3}$ mutants/locus. Thus, in Arabidopsis, carbon ions at a dose of 150 Gy seem to be severalfold less mutagenic than treatment with 10 mM EMS for 24 hr. Similarly, regarding the frequency of embryonic lethals, a 150 Gy of carbon-ion irradiation seems to be three times less mutagenic than a treatment of 8.3 mM EMS for 24 hr (Mesken and Van Der Veen 1968).

It is noteworthy that the high mutation rate by carbon-ion irradiation was observed at a relatively low dose (150 Gy) at which virtually all plants survive. This characteristic of ion particle mutagenesis is quite useful from the viewpoint of plant genetics and breeding.

**Segregation rate of carbon-ion-induced mutants:** Dellaert (1980b) concluded that any deviation from 25% in the segregation frequency of radiation-induced mutants could be ascribed to the reduced frequency of fertile gametes and not to chimerism or to the reduced viability of the M1 plants. On the basis of this evidence, the segregation rates in this study were estimated in the $F_2$ generation of a cross with Columbia (Table 3). The average segregation frequency calculated from eight

**TABLE 3**

**Segregation frequencies of the mutants induced by carbon ions and electrons**

| Mutagen | Mutant line | $F_2$ phenotype | | Total | Segregation frequency |
|---|---|---|---|---|---|
| | | Mutant | Wild type | | |
| Carbon ions | *tt4*(C1) | 54 | 170 | 224 | 0.241 |
| | *tt4*(C2) | 55 | 165 | 220 | 0.250 |
| | *tt6-2* | 44 | 176 | 220 | 0.200 |
| | *tt18-1* | 61 | 165 | 226 | 0.270 |
| | *tt18-2* | 95 | 277 | 372 | 0.255 |
| | *tt18-3* | 57 | 168 | 225 | 0.253 |
| | *gl1-3* | 60 | 165 | 225 | 0.267 |
| | *gl1-4* | 110 | 302 | 412 | 0.267 |
| | Average segregation frequency = 0.250 ± 0.008[a] | | | | |
| Electrons | *tt3-2* | 57 | 170 | 227 | 0.251 |
| | *gl2-4* | 65 | 160 | 225 | 0.289 |
| | *gl2-5* | 51 | 170 | 221 | 0.231 |
| | *gl2-6* | 56 | 166 | 222 | 0.252 |
| | Average segregation frequency = 0.256 ± 0.012[a] | | | | |

[a] Mean ± SE.

**TABLE 4**

**Mutation rates induced by carbon ions and electrons**

| Mutagen | No. of M1 plants | No. of M2 plants | Mutant group (loci) | No. of mutants in M2 | Average mutation rate/dose (Gy) ($\times 10^{-6}$)[a] |
|---|---|---|---|---|---|
| Carbon ions (150 Gy) | 26,200 | 104,088 | *tt* (*tt3*, *tt4*, *tt5*, *tt6*, *tt7*, *tt18*, *tt19*) | 62[b] | 1.9 |
| | | | *gl* (*gl1*, *gl2*, *gl3*, *ttg1*, *ttg2*) | 26[c] | |
| | | | | Total 88 | |
| Electrons (750 Gy) | 9,600 | 44,026 | *tt* (*tt3*, *tt4*, *tt5*, *tt6*, *tt7*, *tt18*, *tt19*) | 4[d] | 0.11 |
| | | | *gl* (*gl1*, *gl2*, *gl3*, *ttg1*, *ttg2*) | 7[e] | |
| | | | | Total 11 | |

[a] Segregation rates were 0.250 and 0.256 for carbon ions and electrons, respectively. Average mutation rate/dose (Gy) = Total no. of mutants in M2/(No. of M2 plants × 12 (no. of loci) × segregation rate × irradiated dose (Gy)).
[b] No. of independent mutant lines for each locus: *tt3* = 1, *tt4* = 7, *tt5* = 2, *tt6* = 3, *tt18* = 3, *tt19* = 2.
[c] No. of independent mutant lines for each locus: *gl1* = 6, *gl2* = 1, *ttg1* = 4.
[d] No. of independent mutant lines for each locus: *tt3* = 1, *tt4* = 1.
[e] No. of independent mutant lines for each locus: *gl2* = 3.

lines of carbon-ion-induced mutants was found to be quite close to the theoretical Mendelian value for a single recessive allele.

Vizir and Mulligan (1999) demonstrated, by analyzing diploid and triploid progeny obtained by pollinating γ-ray-irradiated haploid pollens to diploid or tetraploid multimarker lines of Arabidopsis, that 73% of the deletions were not transmitted to the diploid progeny. They concluded that large deletions could be rescued only in triploid progeny. Timpte *et al.* (1994) reported that a revertant of *axr2-1* of Arabidopsis induced by 5 krad of γ-rays had a deletion that spanned at least 2 cM. This deletion was transmitted to the progeny at a reduced frequency through only the male and was not transferred through the female gametophytes. Bruggemann *et al.* (1996) analyzed null *hy4* alleles that were lethal in homozygous Arabidopsis and found that they were rarely transmitted through male gametophytes. They showed that *hy4* alleles did not affect the viability of female gametophytes. All of these *hy4* alleles contained deletions >8 kb in size. These results imply that a large deletion in the gamete impairs the fertilization and thus reduces the segregation rate.

Considering the fact that the average segregation rate is around the expected Mendelian value (0.25), one may speculate that the size of deletions induced by carbon ions is relatively small under the present experimental conditions or that large deletions induced at M1 were infrequently transmitted through either or both gametophytes. In the latter case, those deletions could have rarely become homozygous in the progeny and thus evaded identification under our screening conditions. Consistent with this interpretation, we have not yet found large deletions but found two inversions, one reciprocal translocation (Shikazono *et al.* 2001), small (2- and 5-bp) deletions, and a rearrangement (present study) induced by carbon-ion irradiation. Further experiments identifying and analyzing heterozygotes of mutations, such as that of Bruggemann *et al.* (1996), would help to uncover whether large deletions are formed by carbon-ion irradiation in Arabidopsis.

**Novel mutations induced by carbon ions:** A collection of mutants of the flavonoid pathway have been identified in Arabidopsis and studies of these mutants have led to a detailed understanding of the enzymology, metabolic regulation, and physiological functions of the pathway (Shirley *et al.* 1995; Winkel-Shirley 2001).

In the present study, three *tt18* mutants, which complemented all the previously identified *tt* mutants that lack or have little pigment on leaves and stems, were isolated from the carbon-ion-mutagenized M2. Each of these mutants was found to have a mutation at the *LDOX* gene (Figure 1 and Table 2). Therefore, we concluded that disruption of the functional LDOX protein is responsible for the observed phenotype in these *tt18* mutants. LDOX converts flavan-3,4-diols to 3-OH-anthocyanidins and is one of the important enzymes required for constructing pigments (anthocyanin derivatives) from the precursor phenylalanine (Pelletier *et al.* 1997, 1999). The identification of mutations in the *LDOX* gene completes the series of mutations for the key enzymes involved in anthocyanin biosynthesis in Arabidopsis. A study to identify and characterize the *TT19* gene is now being undertaken. Further investigations of these novel Arabidopsis *tt18* and *tt19* mutants would provide a more detailed description of flavonoid metabolism.

In addition to *tt18* and *tt19*, the induction of additional novel mutations by ion particles has been reported in plants, such as *ast* mutation in Arabidopsis, which shows spotted anthocyanin pigmentation in testa (Tanaka *et al.* 1997b), UV-B-resistant mutants of Arabidopsis (Tanaka *et al.* 2002), *frl1* mutation in Arabidopsis resulting in altered shape of sepal and petal (Hase *et*

*al.* 2000), mutations showing a complex color in petals of Chrysanthemum (Nagatomi *et al.* 1995), and PVY-resistant mutations in tobacco (Hamada *et al.* 1999). Although one cannot rule out the possibility that these novel mutations have arisen merely because the mutations had not been saturated, the identification of these ion-particle-induced novel mutations may suggest that ion particles and widely used low LET radiation induce different mutation spectra. As a first step to verify this hypothesis, a comparison of the frequencies of point mutations, rearrangements, and deletions induced by carbon ions with those of electrons is now in progress.

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