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Effect of heavy ion-beam irradiation on plant growth and mutation induction in *Nicotiana tabacum*

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Abstract Ion-beam mutagenesis is a highly effective way to rapidly create new cultivars. To optimize conditions for heavy ion mutagenesis, we irradiated tobacco (*Nicotiana tabacum* L.) tissues at various developmental stages with heavy ion beams of various doses and examined the effects of irradiation by monitoring plant growth and mutation induction. The effects differed among irradiated tissues. Sensitivity to heavy ion-beam irradiation increased in the following order: dry seeds, imbibed seeds, and culture tissues. We isolated three white flower mutants. One, *BWF1*, was found to be a novel mutant, in which the synthesis of proanthocyanidin was up-regulated. The others may have a mutation in some regulatory genes involved in the flavonoid biosynthetic pathway. These results suggest that the developmental state of plant tissues is critical for efficient plant mutagenesis, and that the broad spectrum of mutations may be induced by heavy ion-beam irradiation at molecular level.

Key words: Heavy-ion beam, *Nicotiana tabacum*, RIBF, white-flower mutant.

The RIBF (RI-Beam Factory) is not only for physics but pursuing multidisciplinary research utilizing energetic ion beams up to 135 MeV per nucleon (about 50% of speed of light). Research into cancer therapy was fostered at RIBF by the collaboration of radiation oncologists, physicists, and biologists starting in 1986. The experience of these researches then encouraged plant scientists to use RIBF for radiation biology research starting in 1988. Eventually, we started our trials in plant breeding about 15 years ago. Initially, we found that the ion beam is highly effective in inducing mutagenesis of seed embryos at a particular stage during fertilization without damage to other plant tissues (Abe et al. 1995). We isolated many types of mutants in tobacco including albino, periclinal chimera, sectorial chimera, herbicide-tolerant and salt-tolerant phenotypes (Abe et al. 2000a). The new color *Dahlia* "World" was developed through joint research with the Hiroshima City Agriculture, Forestry and Fisheries Promotion Center and launched in the fall of 2001 for pilot marketing in Hiroshima City (Hamatani et al. 2001). The sterile *Vervena* mutant, "Temari Bright Pink" was the first new variety of plant created by heavy-ion beam irradiation in the world to become commercially available in the spring of 2002 (Kanaya et al. in this issue). The developmental period of these new cultivars were only three years. Similar successful cases were demonstrated by the new sterile *Verbena* "Temari Sakura Pink" (2003) and "Temari Momo" (2006), the new color *Petunia* "Surfinia Rose Veined" (2003) and the new color *Torenia* "Summer Wave Pink" (2007). Thus, we conclude that the ion beam is an excellent tool for mutation breeding to improve horticultural and agricultural crops with high efficiency.

To improve the method of heavy ion-beam mutagenesis, both physical and biological factors must be opti-

Abbreviations: ANR, anthocyanidin reductase; DFR, dihidroflavonol reductase; DMACA, dimethylaminocinnamaldehyde; HPLC, high performance liquid chromatography; LET, linear energy transfer; MAPs, morphologically abnormal plants; MS, Murashige-Skoog; PA, proanthocyanidin; RIBF, RI-Beam Factory.

The article can be found at htttp://www.jspcmb.jp/

mized. The former factors include dose, linear energy transfer (LET), and ion species (Kazama et al. in this issue). The biological factors include the developmental stage of the plant tissues. As a first step toward determining irradiation effects, we investigated the survival rates of several plant tissues after heavy ion-beam irradiation in tobacco (*Nicotiana tabacum* L.), and concluded that survival increased in the following order: mixiotrophic calli, embryos during the fertilization cycle, imbibed seeds, and dry seeds (Abe et al. 2000b).

Here we examined the effects of heavy ion beams on plant growth and mutation induction in seeds and in *in vitro* cultured cells in anther culture and leaf cultures. Sensitivities to heavy ion-beam irradiation differed markedly between tissues. In addition, we isolated three white flower mutants that were defective in either structural or regulatory genes involved in the flavonoid biosynthetic pathway. Characterization of these three mutants revealed that each might have an independent defect in the flavonoid biosynthetic pathway, indicating that mutations induced by heavy ion-beam irradiation have a broad mutation spectrum. These detailed characterizations can be valuable in optimizing the appropriate conditions for heavy ion-beam mutagenesis.

Materials and methods

Plant material

Nicotiana tabacum L. cvs. Xanthi and BY-4 were used throughout this study. Plant seeds were surface-sterilized and sown on soil or on Murashige–Skoog (MS) medium containing 1.5% (w/v) sucrose and 0.7% agar. The germinated seedlings were cultivated in pots in a controlled environment chamber at 25°C under a 16 h/8 h light/dark cycle. To prepare imbibed seeds, dry seeds were incubated on half-strength agar medium plates for 1 to 2 days before irradiation. Anthers from flower buds (10–14 mm) were cultured on Nakata agar medium containing 2 mg L^{-1} IAA for BY-4 or 1 mg L^{-1} NAA for Xanthi, and 3% sucrose under continuous light for 1 to 10 days before irradiation. For leaf cultures, leaf disks (5–10 mm) were prepared from *in vitro* grown 2-week-old seedlings and cultured in MS medium containing 1 mg L^{-1} benzyladenine (BA), 3% sucrose, and 0.2% gellan gum under continuous light for 1 or 14 days before irradiation.

Heavy ion-beam irradiation and screening of mutants

Dry seeds, imbibed seeds, cultured anthers, and cultured leaves (Xanthi) were irradiated with $^{12}C^{6+}$ ion beams (1.62 GeV) accelerated by RRC (RIKEN ring cyclotron) at a dose range of 5–300 Gy. The LET of ions corresponded to 23 keV μm^{-1}. BY-4 cultured anthers were irradiated with $^{14}N^{7+}$ ion beams (1.89 GeV, 30 keV μm^{-1}) at a dose range of 5–100 Gy. After irradiation, seeds were incubated in half-strength MS agar medium for observation of germination. Germinated seeds grown until they produced new leaves were scored as surviving plants. Leaf explants were transferred to the same fresh medium to regenerate adventitious shoots. All plants were grown in a greenhouse at 25°C under a 16 h/8 h light/dark cycle. The frequency of albino plants in 20 M_2 seeds from each line was measured 2 weeks after sowing in half-strength MS agar medium. Five M_2 seedlings obtained from each irradiation treatment were grown in a greenhouse. M_1 and M_2 progenies were screened for morphologically abnormal plants (MAPs).

Plantlets derived from the anthers were transferred to MS medium and subcultured three times at intervals of 2 months. Plants were acclimatized in a greenhouse, and flow-cytometric analysis was conducted with the surviving plants after acclimatization. Assessment of characters of foliage and flowers of plants derived from anthers treated with the heavy ion beam were conducted during the flowering period. The selected mutated plants were maintained, and mutated characters were consecutively evaluated for 4 years. To measure viability, pollen grains in triplicate plants were collected on the day of flowering; they were then stained with aceto-carmine and observed under a microscope. Dimethylaminocinnamaldehyde (DMACA) staining was performed as described previously (Xie et al. 2006). Before observation, the stained flowers were dissected using scissors and forceps. High-performance liquid chromatography (HPLC) analysis was performed as described previously (Murakami et al. 2004). Two hundred milligrams of corolla limb samples from six flowers were harvested, mixed, and used for the HPLC analysis.

Isolation of flavonoid biosynthesis-related genes

Total RNA was isolated from the petals of tobacco plants using an RNeasy Plant Mini Kit (Qiagen, Hilden, Germany). cDNA was synthesized from 3 μg of total RNA using Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA, USA). A fraction of the cDNA preparation was then subjected to 30 cycles of PCR amplification (94°C for 1 min, 50°C for 1 min, and 70°C for 1 min) with primers designed for amplification of the *ANS* gene (ANS-2F, 5′-GAR TGG GAR GAY TAY TTY TT-3′ and ANS-2R, 5′-GGY TCR CAR AAN CAN GCC CA-3′), the *ANR* gene (ANRf1, 5′-GAT CCH GAG AAH GAC ATG AT-3′and ANRr1, 5′-CCA GAA GYW GWT TCT TTC TC-3′), and the *LAR* gene (LARf4, 5′-CCK TCR GAR TTY GGR CAT GA-3′and LARr5, 5′-CAT DGT GAA YTT YCC DAT GTC-3′). Primers used for the amplifications of the *DFR* and *CHI* genes were designed from identified sequences of these genes (Nishihara et al. 2005; Nakatsuka et al. 2007): DFR-F, 5′-GGC TCT TGG CTT GTC ATG AG-3′and DFR-R, 5′-CCC CTT TAT ACA TAT CCT CC-3′ for *DFR*, and NtCHIf1, 5′-GGC GTC TAC TTG GAA GAG AG-3′and NtCHIr1, 5′-CTC AAT CAC TGG ATC GGA GG-3′ for *CHI*. The PCR products were then subcloned into the pCR 2.1 TOPO vector (Invitrogen). To determine the sequence of full-length cDNAs of isolated genes, both 5′ and 3′ Rapid Amplification of cDNA Ends (RACE) was performed using GeneRacer (Invitrogen).

Southern blot analysis

Genomic DNA was extracted from leaves of the wild type and *BWF1* using the Nucleon PhytoPure kit (Amersham Biosciences). Fifteen micrograms of digested DNA were subjected to Southern blot analysis. Hybridization and detection were carried out as previously described (Kazama et al. 2006).

Results

Seeds

Seed germination was not affected by any irradiation dose (Table 1). A decrease in the survival rate was observed only in the plants from 300-Gy irradiated dry seeds. While the percentage of flowering plants did not show a significant difference, irrespective of the irradiation dose, the plant heights slightly decreased in plants from 250-Gy irradiated dry seeds and in 20-Gy irradiated imbibed seeds. In addition, seed fertility decreased at a dose of 250 Gy. However, no decreases in the rate of germination of M_2 seeds was observed at any dose (data not shown). MAPs were observed in the M_1 progeny of 200- to 250-Gy irradiated plants. However, morphological abnormalities of these plants were unstable. In addition, most M_2 seeds germinated and most seedlings developed normal true leaves. Thus, no albino plants were obtained in any of the 171 M_2 lines after seed irradiation. Among the M_2 progenies derived from 20-Gy irradiated seeds, a white flower mutant of Xanthi (*XWF1*) was segregated (Figure 1A).

Leaf cultures

A decrease in the number of adventitious shoots regenerated and the frequency of rooting of adventitious shoots was observed with increasing irradiation dose (Table 2). Differences between 1- and 14-day precultured explants were observed in the effects of C-ion beams on seed fertility and frequency of MAPs (Figure 1C). The seed fertility rates were 34% and 76% at a dose of 10 Gy in the 1-day and 14-day precultures, respectively. The highest rate of MAPs was 30% in 1-day precultured explants at a dose of 10 Gy. Most MAPs showed chimeras and unstable morphological abnormalities. These results indicate that sensitivity to the heavy ion-beam irradiation in the 1-day preculture is higher than in the 14-day preculture. Most M_2 seeds germinated and most seedlings developed normal leaves. Thus, no albino plants were obtained in any of the 237 M_2 lines after cultured leaf irradiation. We found another white flower mutant, named *XWF2*, from the M_2 progeny of 5-Gy irradiated leaves that were precultured for 1 day before irradiation. MAPs only produced seeds in 11 plants, and M_2 plants of these MAPs grew normally.

Anther culture

A high dose of irradiation caused a decrease in the regeneration of plants from pollen grains (Table 3). The regeneration rate was not affected by N-ion irradiation at doses lower than 10 Gy in BY-4; however, in Xanthi, the regeneration rate decreased by 50% at a dose of 10 Gy with C-ion irradiation. This result indicates that sensitivity to the heavy ion-beam irradiation in Xanthi is higher than in BY-4. Three semi-dwarf mutants and a white flower mutant of BY-4 (*BWF1*) were selected from plants regenerated after irradiation at a dose of 5 Gy. Plant height was 115.4±17.3 cm in control plants and 39.7±8.5 cm in semi-dwarf plants. Flow cytometric

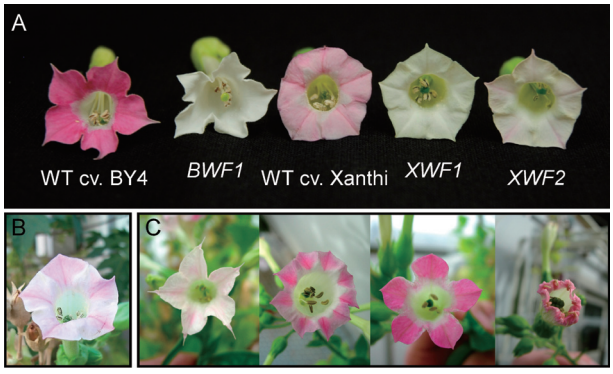

Figure 1. Flowers of heavy ion-beam irradiated tobacco plants. (A) Flowers of white flower mutants isolated among M_2 plants. (B) A flower of wild-type cv. Xanthi. (C) Flowers of M_1 morphologically abnormal plants (MAPs).

Table 1. Effects of C-ion beam irradiation on germination, growth, and mutation induction in dry and imbibed seeds[a].

Days of imbibition	Irradiation dose (Gy)	No. of seeds irradiated	No. of seeds germinated (%)	No. of plants surviving (%)	No. of plants flowering (%)	Plant height at flowering (cm)	No. of plants producing seeds (%)	No. of MAPs in M_1 (%)	No. of M_2 mutant line	
									albino (%)	white flower (%)
0[b]	0	60	60 (100)	60 (100)	60 (100)	150.6±1.8	59 (98.3)	0	0	0
	200	40	39 (97.5)	39 (97.5)	39 (97.5)	152.1±3.4	37 (92.5)	3 (7.7)	0	0
	250	40	39 (97.5)	39 (97.5)	39 (97.5)	143.8±4.2	31 (77.5)	3 (7.7)	0	0
	300	40	38 (95.0)	24 (60.0)	—	—	—	—		
1	10	40	40 (100)	40 (100)	—	—	—	—		
	15	40	40 (100)	40 (100)	—	—	—	—		
	20	40	39 (97.5)	39 (97.5)	39 (97.5)	136.7±2.6	36 (90.0)	0	0	0
2	10	40	40 (100)	40 (100)		—	—	—		
	15	40	40 (100)	40 (100)	40 (100)	152.0±1.3	34 (85.0)	0	0	0
	20	40	40 (100)	40 (100)	40 (100)	148.6±1.7	33 (82.5)	0	0	1 (3.0)

[a] Irradiation-derived plants were grown in a greenhouse during summer.
[b] Irradiation of dry seeds.

Table 2. Effects of C-ion beam irradiation on adventitious shoot regeneration, shoot growth, and mutation induction in *in vitro* leaf cultures[a].

Days of culture before irradiation	Irradiation dose (Gy)	No. of leaf explants irradiated	No. of shoots regenerated per explant	% of shoots rooted	No. of shoots planted	No. of plants flowering (%)	Plant height at flowering (cm)	No. of plants producing seeds (%)	No. of MAPs in M_1 (%)	No. of M_2 mutant line	
										albino (%)	white flower (%)
1	0	50	22.2±1.3	82.9	24	21 (85.7)	144.1±4.6	20 (83.3)	0	0	0
	5	50	15.1±1.0	42.6	100	100 (100)	132.0±3.0	75 (75.0)	11 (11.0)	0	1 (1.3)
	10	50	6.9±0.7	45.2	50	50 (100)	104.3±3.3	17 (34.0)	15 (30.0)	0	0
	15	50	1.8±0.4	39.1	—						
	20	50	0	—	—						
14	0	50	15.8±1.5	91.9	14	14 (100)	146.8±5.2	14 (100)	0	0	0
	5	50	11.6±1.3	68.4	95	91 (95.8)	131.9±1.8	80 (84.2)	3 (3.2)	0	0
	10	50	10.5±0.1	47.1	50	49 (98.0)	134.7±2.9	38 (76.0)	1 (2.0)	0	0
	15	50	8.0±0.7	34.8	45	45 (100)	112.4±3.9	27 (60.0)	5 (11.1)	0	0
	20	50	4.7±2.0	25.7	—	—					

[a] Irradiation-derived plants were grown in a greenhouse during winter.

Table 3. Effects of ion beam irradiation on shoot regeneration and mutation induction anther cultures.

Cultivar	Irradiation dose (Gy)	No. of anther irradiated	No. of shoots regenerated anther (%)	No. of shoots planted	No. of MAPs (%)	No. of white-flower (%)
BY-4	0	14	4 (28.6)	—		
	5	173	42 (24.3)	32	0	1 (3.1)
	10	168	48 (28.6)	61	0	0
	20	154	2 (1.3)	2	0	0
	50	58	0	—		
Xanthi	0	60	25 (41.7)	25	0	0
	5	60	19 (31.7)	32	3 (9.4)	0
	10	60	12 (20.0)	16	0	0
	15	60	12 (20.0)	18	0	0
	20	60	1 (1.7)	—		

analysis indicated that *BWF1* was haploid. The white color of the corolla of *BWF1* was stable for 4 years, and no pigmentation was observed in any corolla. *BWF1* did not produce any fertile eggs or pollen because of the haploidy, and did not set any fruit in the first 3 years. However, in the fourth year of cultivation in the greenhouse, one inflorescence of a *BWF1* plant set fruit. Each capsule contained more than 500 seeds. Flow cytometric analysis demonstrated that the leaves and corolla that had been sampled from the scion with a fertile florescence were chimeric with respect to ploidy level, with c and 2c peaks. More than 80% of pollen grains sampled from the flowers in the fertile scion were stained with aceto-carmine and were viable. Flow cytometric analysis of these pollen grains demonstrated a c peak of the vegetative nucleus and 2c in the sperm nucleus. Thus, the pollen grains sampled from the scion that fruited were normal and fertile.

Analysis of white flower mutants

We successfully isolated three white flower mutants. Two (*BWF1* and *XWF1*) had completely white petals, while the other (*XWF2*) produced white petals that looked slightly pink (Figure 1). We analyzed the accumulation of flavonoid compounds in petals of the latter plant. Flavonoid pigments were extracted from petals and subjected to HPLC analysis (Figure 2). This analysis showed that wild-type flowers accumulated cyanidin in the petals, whereas *BWF1* accumulated cyanidin at a substantially lower level, as predicted from its flower color. Dihydroflavonols and flavonols, precursors of anthocyanidins and metabolites of dihydroflavonols, respectively, were also analyzed. *BWF1* accumulated dihydrokaempferol (DHK), while wild-type cv. BY4 did not accumulate this compound. Conversely, the amount of kaempferol in *BWF1* was lower than that in wild-type cv. BY4. To explore this difference, we carried out DMACA staining of the white flower mutants. Blue staining with the DMACA reagent is often used as a diagnostic test for proanthocyanidins (PAs, Li et al., 1996). Three independent experiments were performed, and same results were observed among them. The petals of *BWF1* stained blue with the DMACA reagent (Figure 3), while petals of neither of the other two white mutants or the wild type were stained blue. This indicates accumulation of PAs in the petals of *BWF1*, and suggests that *BWF1* harbors a different mutation from the other white flower mutants.

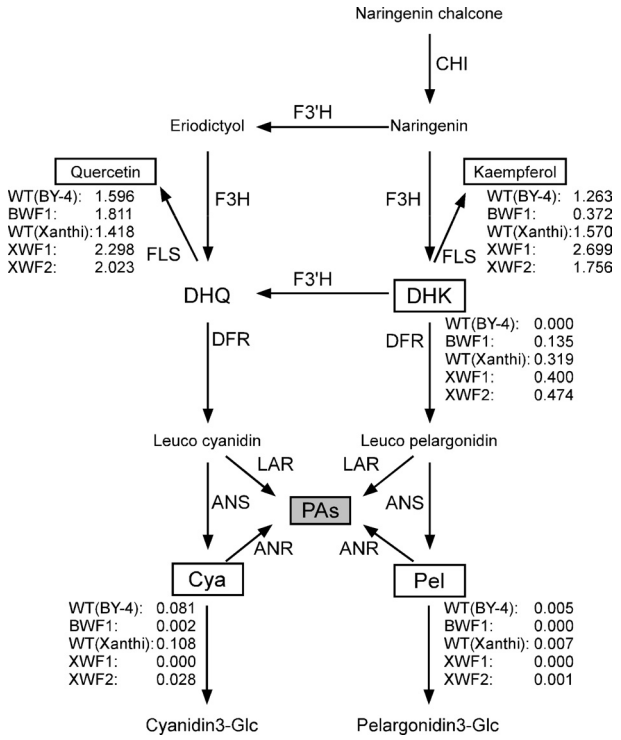

Figure 2. Flavonoid biosynthetic pathway of anthocyanins and proanthocyanidins (PAs). Kaempferol, quercetin, dihydrokaempferol (DHK), cyanidin (Cya), and pelargonidin (Pel) were analyzed by HPLC (white box). The results are shown under the white boxes (mg/g petal). PAs were analyzed by DMACA staining (gray box; shown in Figure 3). DHQ, dihydroquercetin; CHI, chalcone isomerase; F3H, flavanone-3-hydrogenase; FLS, flavonol synthase; DFR, dihydroflavonol 4-reductase; ANS, anthocyanidin synthase; LAR, leucoanthocyanidin reductase; ANR, anthocyanidin reductase; 3GT, 3-*O*-glucosyl-transferase.

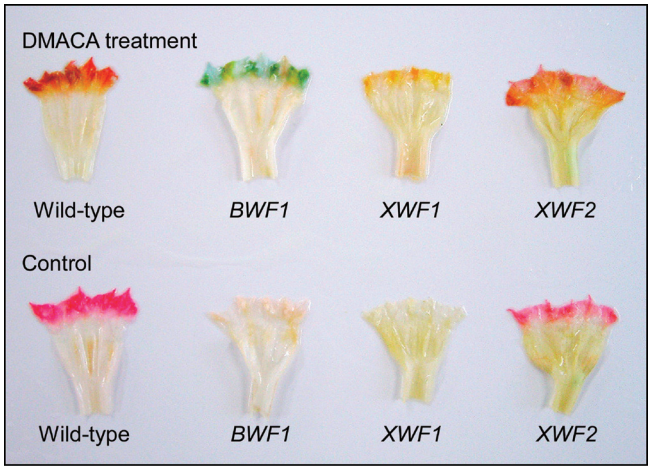

Figure 3. DMACA staining of petals. Flowers were harvested, placed directly into DMACA reagent (2% [w/v] DMACA in 1.5 M HCl/50% [w/v] methanol) for 2 h, and then rinsed three times with 70% (w/v) ethanol. For the control, the same samples were placed into the reagent without DMACA (1.5 M HCl/50% [w/v] methanol). *BWF1* was stained with DMACA, showing accumulation of proanthocyanidins.

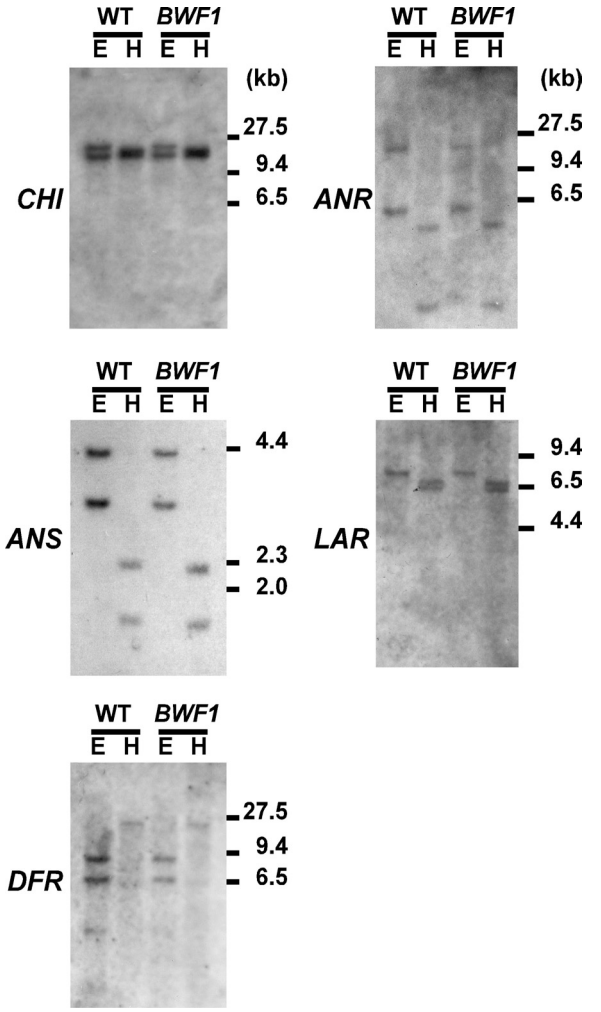

Figure 4. Southern blot analyses of the *BWF1* mutant. Fifteen micrograms of genomic DNA were digested by *Hin*dIII or *Eco*RI restriction enzymes, separated, and hybridized with the *CHI*, *ANS*, *DFR*, *ANR*, or *LAR* probes. No differences were detected in signal patterns between wild-type plants and the *BWF1* mutant.

To examine whether any genes related to the anthocyanin biosynthetic pathway are defective in *BWF1*, we isolated anthocyanidin biosynthetic genes from tobacco using degenerated PCR. An 894-bp fragment for *DFR*, a 509-bp fragment for *ANS*, a 494-bp fragment for *ANR*, and a 270-bp fragment for LAR were successfully obtained and then sequenced. BLAST searches revealed that the putative amino acid sequences encoded by these amplicons were significantly similar to *DFR*, *ANS*, *ANR*, and *LAR* sequences, respectively, in other organisms. The partial sequence of the *CHI* gene was also obtained by PCR amplification using a primer set designed to identify *CHI* sequences (Nishihara et al. 2005). These partial DNA sequences were used as probes for Southern blot analysis. Southern blot analysis showed no difference in band patterns between the wild type and *BWF1* (Figure 4). In addition, we sequenced the full-length cDNAs of these genes in the wild type and *BWF1* using RACE. No

mutations were found in these genes (data not shown). These results indicate that the genes have no mutations in the *BWF1* mutant. Molecular analyses of the other mutants are in progress.

Discussion

Tissue differences

We examined the biological effects of heavy ion-beam irradiation using several different tissues from *N. tabacum*. The results show that sensitivity to the heavy ion beam is markedly different between developmental stages of the plant tissues. The sensitivity decreased in the following order: anther culture, leaf culture, imbibed seeds, and dry seeds. In addition, the preculture period of leaf explants affected sensitivity to the heavy ion beam: 1-day-precultured samples were more sensitive to heavy ion-beam irradiation than 14-day-precultured samples. Plants regenerated from leaf culture had higher rates of MAPs than those from seed embryos (Abe et al. 2000); however, the morphological abnormalities were unstable. Our current experiment indicates that sterility can also be induced in *N. tabacum*, and that a 10-Gy irradiation of 1-day-old leaf cultures is most effective for inducing sterility. The sterile phenotype is very important for many ornamental plants, because sterility increases the number of flowers and extends the flowering period. Sterile mutants have been successfully isolated by heavy ion-beam irradiation of *Verbena hybrida* (Kanaya et al. in this issue).

Characterization of white flower mutants

We isolated three white flower mutants (Figure 1A). A fertile scion of *BWF1* was chimeric, with original haploid cell layers and diploid cell layers, which were the result of spontaneous chromosome doubling in the inner layer comprised of pollen grains and eggs. Then *BWF1* produced only white flower plants in the progeny. *BWF1* and *XWF1* had completely white petals without accumulation of cyanidin, while *XWF2* had slightly pink petals with low accumulation of cyanidin. DMACA analysis showed that the petals of *BWF1* could produce PAs, while those of *XWF1* and *XWF2* could not. These observations raise the possibility that the different genes involved in the flavonoid biosynthetic pathway had mutated in individual mutants. A broad mutation spectrum of flower color has been observed in irradiated *Torenia* (Miyazaki et al. 2006; Sasaki et al. in this issue). Although more detailed characterization is needed to determine the mutations in the white flower mutants isolated, our current results show the broad spectrum of mutations induced by heavy ion-beam irradiation at the molecular level.

The biosynthetic pathway of anthocyanins is shared with PAs for production from phenylalanine to leucoanthocyanidin (flavan-3,4-diol; Springob et al. 2003). The petals of wild-type tobacco do not produce PAs (Xie et al. 2003, 2006). When PAs were overproduced in flowers of tobacco, a reduction in anthocyanin pigmentation was observed and white flowers were produced (Xie et al. 2003). Therefore, it is possible that overproduction of PAs is induced by some change in the anthocyanidin biosynthetic pathway causing the white flower mutant.

White flower mutations are generally the result of defects in either structural or regulatory genes involved in the flavonoid biosynthetic pathway (Martin et al. 1991; Mato et al. 2000; Spelt et al. 2002; Quattrocchio et al. 1999). All structural genes have been cloned from many plant species (Tanaka et al. 2005). Some of these genes have been used to produce white flowers from colored flowers by transgenic methods (Nakamura et al. 2006). However, the regulatory genes involved are not fully characterized. In addition, a branch pathway of the flavonoid biosynthetic pathway, which leads to production of PAs, is also not fully characterized. The biosynthetic pathway of anthocyanins is shared with PAs for production from phenylalanine to leucoanthocyanidin (flavan-3,4-diol; Springob et al. 2003). The petals of wild-type tobacco do not produce PAs (Xie et al. 2003). When PAs were overproduced in flowers of tobacco by overexpression of the anthocyanidin reductase (ANR) gene, a reduction in anthocyanin pigmentation was observed and white flowers were produced (Xie et al. 2003). In our experiment, the petals of *BWF1* stained blue with DMACA reagent (Figure 3). A similar phenotype was observed when the anthocyanidin reductase (ANR) gene was ectopically expressed in *N. tabacum* (Xie et al. 2003). HPLC analysis revealed no accumulation of cyanidin in *BWF1* (Figure 2). From these results, it is expected that anthocyanidin synthase (ANS) was defective in *BWF1*. However, in *BWF1*, no mutation was found in the ANS gene; therefore, we postulated that *BWF1* may be a gain-of-function mutant, in which the synthesis activity of PAs is up-regulated. As the mechanism of the PA synthesis pathway remains largely unknown, further study of the *BWF1* mutant at the molecular level may shed more light on the PA synthesis pathway.

In conclusion, we propose that appropriate doses of heavy ion-beam irradiation to dry seeds, imbibed seeds, leaf culture, or anther culture of tobacco should be 250 Gy, 20 Gy, 5–10 Gy, and 5 Gy, respectively. We successfully isolated white flower mutants from 5-Gy irradiated leaves and anthers and from 20-Gy irradiated imbibed seeds. Although sensitivities to heavy ion beams are slightly different between plant species, the irradiation conditions obtained in this study can be used for effective mutagenesis of other plants.

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