Low polyphenol oxidase mutant induced by $^{12}C^{6+}$ ion beam irradiation to protoplasts of lettuce (*Lactuca sativa* L.)†

R. Sawada,[*1,*2] T. Hirano, [*3*4] K. Iimure,[*1] T. Abe,[*4] and Y. Ozaki[*5]

Lettuce is one of the most important vegetables in Japan and has been established as a designated vegetable by the Ministry of Agriculture, Forestry and Fisheries. Lettuce is often eaten raw in dishes such as salads, and the browning of the cut section is one of the causes of quality loss during storage. In the cut section, activated phenylalanine ammonia-lyase (PAL) produces cinnamic acid, and polyphenols are synthesized from the cinnamic acid. The browning is due to the accumulation of pigments derived from the oxidation of the polyphenols by polyphenol oxidase (PPO). Since PAL and PPO are key enzymes for the browning, the suppression of enzyme activity can prevent the browning.[1)] In the present study, the induction of *PPO* gene mutation by heavy-ion beam irradiation was attempted for the production of lettuce plants with a low browning property as a cut vegetable.

We used protoplasts as plant materials for the irradiation to prevent of chimera mutants. Protoplasts of *L. sativa* 'Round' were embedded at a density of 0.75×10^4/ml in modified Murashige and Skoog medium[3)] supplemented with 0.3% gellan gum and 100 mg/l cefotaxime sodium. The protoplasts were stored overnight at 4°C in the dark. Then, the protoplasts were transported at room temperature and cultured at 25°C in the dark until irradiation. The protoplasts were irradiated with $^{12}C^{6+}$ ions (135 MeV/nucleon, corresponding to 22.5 keV/μm linear energy transfer) at a dose of 0.5, 1.0, 2.0, and 5.0 Gy. The irradiated protoplasts were cultured according to the methods described by Nishio et al. (Ref. 2). Twenty three days after the irradiation, the number of colonies (> 0.1 mm in diameter) in each sample was counted, and colony formation rates were calculated as percentages relative to values in unirradiated protoplasts. In the calli derived from the protoplasts, mutation in the *PPO* gene was detected by the Targeting Induced Local Lesions IN Genomes (TILLING) method[3)] with Surveyor Mutation Detection kits (Transgenomic, Inc).

The colony formation rates decreased with increasing dose (Fig. 1); the colony formation rate was approximately 64% at 2.0 Gy and 26% at 5.0 Gy. A total of 869 calli formed from the colonies were used for mutation detection by TILLING, and two of them, 2Gy-3-36 from 2.0-Gy irradiation and 5Gy-2-27 from 5.0-Gy irradiation, showed positive fragments due to mutation in the *PPO* gene. The mutation frequencies for 2.0-Gy and 5.0-Gy irradiation were 0.41% and 1.10%, respectively.

We obtained regenerated M_1 plants from the callus of 2Gy-3-36, and their M_2 progenies were produced by self-pollination. When PPO activity in the leaves was measured according to the method of Dan et al. (Ref. 4) with modification, the M_1 plants show about half the PPO activity of the control. In the M_2 progenies, the segregation of individual genotypes was estimated by TILLING analysis and PPO activity; homozygous dominant plants showed no cleavage fragment in TILLING analysis and showed similar or higher PPO activity compared with the control, heterozygous plants showed the cleavage fragment and similar or lower PPO activity, and homozygous recessive plants showed no cleavage fragment and markedly lower PPO activity. These results suggest that mutation in the *PPO* gene is cause of the PPO activity decrease.

The reverse genetic approach combined with the carbon ion beam has been thought to be effective in *Arabidopsis thaliana*.[5)] It is revealed in the present study that this approach is also useful in horticultural crops. The homozygous recessive plants are promising as breeding materials for low-browning lettuce.

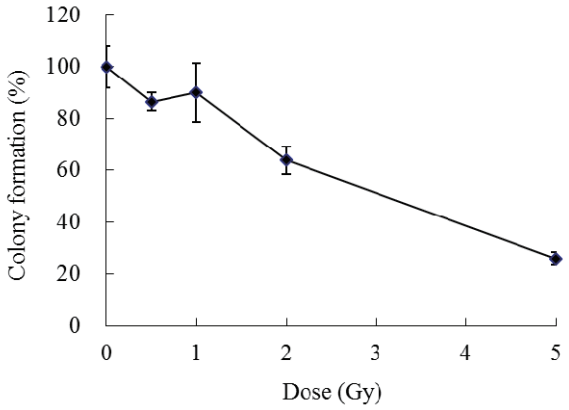

Fig. 1 Effect of carbon ion irradiation on colony formation. Data are represented as mean ± standard error of three individual experiments.

References

1) H. Hashimoto et al., Biosci. Biotechnol. Biochem. **65**, 1016 (2001).
2) T. Nishio et al., Japan. J. Breed. **38**, 165 (1988).
3) B. J. Till et al., Genome Res. **13**, 524 (2003).
4) K. Dan et al., Food Preser. Sci. **25**, 209 (1999).
5) Y. Kazama et al., BMC Plant Biol. **11**, 161 (2011).

† Condensed from the article in Hort. Res. (Japan) **15**, 347 (2016)

*1 Kumamoto Prefectural Agricultural Research Center.
*2 Graduate School of Bioresource and Bioenvironmental Sciences, Kyushu University
*3 Faculty of Agriculture, University of Miyazaki.
*4 RIKEN Nishina Center
*5 Faculty of Agriculture, Kyushu University