An ion beam–induced *Arabidopsis* mutant with marked chromosomal rearrangement

Ayako N. Sakamoto¹,*,†, Vo Thi Thuong Lan¹,2,†, Satoru Fujimoto³,†, Sachihiro Matsunaga³ and Atsushi Tanaka¹

¹Department of Radiation—Applied Biology Research, National Institutes for Quantum and Radiological Science and Technology, 1233 Watanuki-machi, Takasaki, Gunma 370-1292, Japan

²Faculty of Biology, Hanoi University of Science-Vietnam National University, 334 Nguyen Trai Street, Thanh Xuan, Hanoi, Vietnam

³Department of Applied Biological Science, Faculty of Science and Technology, Tokyo University of Science, 2641 Yamazaki, Noda, Chiba 278-8510, Japan

*Corresponding author. Department of Radiation—Applied Biology Research, National Institutes for Quantum and Radiological Science and Technology, 1233 Watanuki-machi, Takasaki, Gunma 370-1292, Japan. Tel: +81-27-346-9537; Fax: +81-27-346-9688; Email: sakamoto.ayako@qst.go.jp

†These authors contributed equally to this research.

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**ABSTRACT**

Ion beams have been used as an effective tool in mutation breeding for the creation of crops with novel characteristics. Recent analyses have revealed that ion beams induce large chromosomal alterations, in addition to small mutations comprising base changes or frameshifts. In an effort to understand the potential capability of ion beams, we analyzed an *Arabidopsis* mutant possessing an abnormal genetic trait. The *Arabidopsis* mutant *uvh3-2* is hypersensitive to UVB radiation when photoreactivation is unavailable. *uvh3-2* plants grow normally and produce seeds by self-pollination. SSLP and CAPS analyses of F2 plants showed abnormal recombination frequency on chromosomes 2 and 3. PCR-based analysis and sequencing revealed that one-third of chromosome 3 was translocated to chromosome 2 in *uvh3-2*. FISH analysis using a 180 bp centromeric repeat and 45S ribosomal DNA (rDNA) as probes showed that the 45S rDNA signal was positioned away from that of the 180 bp centromeric repeat in *uvh3-2*, suggesting the insertion of a large chromosome fragment into the chromosome with 45S rDNA clusters. F1 plants derived from a cross between *uvh3-2* and wild-type showed reduced fertility. PCR-based analysis of F2 plants suggested that reproductive cells carrying normal chromosome 2 and *uvh3-2*-derived chromosome 3 are unable to survive and therefore produce zygote. These results showed that ion beams could induce marked genomic alterations, and could possibly lead to the generation of novel plant species and crop strains.

**KEYWORDS:** ion beams, chromosomal rearrangement, *Arabidopsis*, FISH, NHEJ

**INTRODUCTION**

Ion beams have been applied to various farm and horticultural crops for the creation of novel plant characteristics [1]. The practical effectiveness of ion beams is based on their higher biological effect compared with other mutagens, such as γ-rays or chemical agents. Ion beams deposit energy in a high linear energy transfer (LET) manner, providing the bulk of energy around the ion tracks. Therefore, ion beams are more effective at inducing lethal damage such as double-strand breaks (DSBs) compared with other reagents [2–4]. Moreover, recent studies revealed that ion beams induce clustered damage on DNA [5–7], the repair of which is difficult to achieve with cellular repair systems [8]. Consequently, ion beam radiation kills cells more efficiently than the same dose of low-LET radiation [9–11].

Decades of analysis using the model plant *Arabidopsis thaliana* revealed that ion beams induce large DNA alterations, in addition to small mutations comprising base changes or frameshifts [12–14]. Furthermore, employing ion beams with higher LET for the irradiation results in larger deletions within the *Arabidopsis* genome [15, 16]. Analyses of mutations suggested that ion beam-induced damage was mainly repaired by non-homologous end joining (NHEJ), which sometimes rejoins the incorrect DSB ends [12, 15]. Thus, ion beams have the ability to induce marked mutations comprising large chromosome alterations, such as deletions, inversions, translocations,
etc., thereby resulting in the production of new mutants or new varieties of crops.

Here we describe the creation of a new ion beam–induced mutant which possesses a large chromosomal alteration with novel character. The mutant grows normally, but has reduced fertility when crossed with wild-type plant, which is probably due to abnormal chromosome pairing at meiosis and loss of a chromosomal fragment. The results obtained here support the further use of ion beam breeding for the creation of novel plant species by means of chromosome engineering techniques.

**MATERIALS AND METHODS**

**Plant material and mutagenesis**

*A. thaliana* ecotype Columbia was the wild-type plant used in this study. For mutagenesis, wild-type seeds were irradiated with 220-MeV carbon ions from an azimuthally varying field (AVF) cyclotron (TARRI, QST, Takasaki, Japan) at a dose of 150 Gy, as previously described [17]. Approximately 750 M1 seeds were grown and self-pollinated to obtain M2. The offspring seeds derived from each M2 were pooled to establish ~3000 M2 lines.

**Isolation of UVB-sensitive lines**

For the isolation of UVB-sensitive mutants, M2 lines were screened using the root-bending assay under non-photoreactivating conditions, as previously described [17]. Seven to ten seeds per each M2 line were sown on a nutritive agar plate (2% sucrose and 0.1% [v/v] commercial nutrient; Hyponex, Osaka, Japan). The plate was placed vertically in a growth chamber (LH-200-RD; NK System, Osaka, Japan) at 23°C, 180 μmol m⁻² s⁻¹ of continuous white light (~40 μmol m⁻² s⁻¹) for 3 days. The seedlings were exposed to UVB (0.5 kJ m⁻²) of 310–400 nm, and incubated in the dark or under continuous white light for 3 days. The length of root growth after UVB irradiation was measured using the root-bending assay under non-photoreactivating conditions, DNA was extracted from the leaf disc corresponding to homozygous UVB–sensitive lines. Polymorphism of chromosomes was detected using simple sequence length polymorphism (SSLP) [19] or cleaved amplified polymorphic sequence (CAPS) [20]. The sequence and location of markers are listed in the TAIR database (http://www.arabidopsis.org/).

**TAIL-PCR and analysis of chromosomal rearrangements**

TAIL-PCR was performed as previously described [21] with AD primers [AD1, 5′-GTGCTGA(A/T)GANA(A/T)GAA-3′; AD2, 5′-GTGCTGA(G/C)(A/T)GANA(A/T)GAA-3′; AD3, 5′-GTGCTGA(G/C)(A/T)CAN(A/T)GTT-3′ and SP primers listed in Supplemental Table S1. The obtained sequence was compared with entries in the TAIR 10 database (http://www.arabidopsis.org/) to detect mutations in *uvw3*-2.

**Fluorescence in situ hybridization**

Young flower buds were used for the cyrogenetic analysis. Fluorescence in situ hybridization (FISH) was performed essentially as previously described [22], with some modifications. Centromeric 180 bp repeats and 45S rDNA were amplified from the genomic DNA with sets of primers (180 bp-F: 5′-GTGCTGA(A/T)GANA(A/T)GAA-3′; 180 bp-R: GTGCTGA(G/C)(A/T)GANA(A/T)GAA-3′; AD3, 5′-GTGCTGA(G/C)(A/T)CAN(A/T)GTT-3′ and SP primers listed in Supplemental Table S1). Amplified 180 bp repeats and 45S rDNA were labeled by nick translation with biotin-16-dUTP (Roche, Basel, Switzerland) and digoxigenin-11-dUTP (Roche), respectively. Streptavidin-Alexa 488 (Invitrogen, Carlsbad, CA) was used for detection of dig-labeled probe. Slides were counter-stained using 0.2 μg/ml DAPI and observed using fluorescent microscopy (BX55, Olympus, Tokyo, Japan) with ×100 objective (UPLSAPO ×100, Olympus). Images were captured using a CCD camera (DOC CAM U3-50S5M-C, Molecular Devices, Sunnyvale, CA) controlled with MetaVue (Molecular Devices). The distances between 45S rDNA and 180 bp signals were measured using Fiji software [23].

**Segregation test**

Segregation of the UVB-sensitive trait was examined by χ² test using the following equation:

\[ χ^2 = \sum_{i=1}^{k} \frac{(O_i - E_i)^2}{E_i} \]

where \( O_i \) is the observed frequency count and \( E_i \) is the expected frequency count.

**Analysis of meiotic recombination**

F2 plants from a cross between *uvw3*-2 and Landsberg erecta (*Ler*) were grown, and a leaf disc from each plant was obtained and stored for DNA preparation. Plants were allowed to self-pollinate to prepare F2 line seeds, which were screened for UVB sensitivity by the root-bending assay. DNA was extracted from the leaf disc corresponding to homozygous UVB–sensitive lines. Polymorphism of chromosomes was detected using simple sequence length polymorphism (SSLP) [19] or cleaved amplified polymorphic sequence (CAPS) [20]. The sequence and location of markers are listed in the TAIR database (http://www.arabidopsis.org/).

**Analysis of fertility**

*uwh3*-2 plants were crossed with Columbia to generate *uvw3*-2/+. Columbia, *uvw3*-2 and *uvw3*-2/+ plants were grown on soil and allowed to self-pollinate. Fertility was scored as previously described [24], with slight modifications. In brief, the 10–12th siliques on the main stem were dissected at about 7–10 days after pollination, and the number of normal seeds (green colour), abnormal seeds (brown
or white) and aborted ovules within one side of the septum was counted under a stereomicroscope.

Analysis of chromosome segregation
In an effort to analyze chromosome segregation in the offspring of heterozygous plants, *uvh3-2* plants were crossed with *gl1-5* plants in a Columbia background [12]. The F1 plant was self-pollinated to produce F2 seeds. Eighty-nine F2 plants were grown on soil, and DNA was extracted. Each plant was examined to determine whether it carried *gl*-derived chromosome 3 (3) and/or *uvh3-2*–derived chromosome 2 (2') using the primer pairs 3-Top F/3-Top R and 3-Top R/2-Top R, respectively. Plants carrying both chromosomes (3 and 2') were further examined to determine the presence of *gl*-derived chromosome 2 (2) and/or *uvh3-2*–derived chromosome 3 (3') using the primer pairs 2-Top F/2-Top R and 3-Top F/3-Bot R, respectively (Supplemental Figure S1A). The sequences of the primers are shown in Supplemental Table S1.

RESULTS
Isolation of a UVB-sensitive mutant with an abnormal recombination pattern
In order to isolate UVB-sensitive mutants, *A. thaliana* Columbia seeds (M1) were irradiated using carbon ion beams with a dose of 150 Gy. M2 plants derived from M1 by self-pollination were
were totally abnormal (Fig. 1A). In addition to a few recombinations detected by markers on chromosome 2, many markers located at the upper arm of chromosome 3 detected no recombination. Since the mutant phenotype, **suv4** was crossed with Landsberg erecta (Ler) by the usual method, and the resultant F2 plants were grown. A part of the leaf of each F2 plant was sampled for subsequent DNA extraction (see below), and each plant was self-pollinated to prepare a seed pool (an F2 line). When the F2 lines were examined for UVB sensitivity, they were segregated as UVB-resistant (UVR) lines and UVB-sensitive (UVS) lines (Table 1). We first hypothesized that **suv4** is a single recessive mutation and expected the segregation with ratio of 3 UVR: 1 UVS. However, the ratio of UVS lines to total lines was less than 25%, and the $\chi^2$ test revealed that the observed count was significantly different from the expected count ($\chi^2 (1, n = 283) = 8.11, p < 0.01$).

The DNA from UVS F2 plants was used to detect meiotic recombinations using SSLP and CAPs markers. In general, the recombination frequencies at sites near a given locus (for example, a recessive mutation locus) would be the lowest and as low as 0, although the frequencies must be $\sim 0.5$ at sites unlinked to the given locus. The results confirmed that the recombination frequencies in **suv4** were totally abnormal (Fig. 2). In addition to a few recombinations detected by markers on chromosome 2, many markers located at the upper arm of chromosome 3 detected no recombination. From these results, we assumed a possibility that the **suv4** genome comprises quite a large chromosomal rearrangement.

**Detection of chromosomal rearrangements by PCR**

It is a quite common and well-known phenomenon that inversions prevent recombination [26–28]. Supporting this, we have previously observed that meiotic recombinations were prevented when a large inversion occurred, which had disrupted a gene at the endpoint(s) of the inversion to cause the mutation [12, 17, 29]. Based on the abnormal recombination frequency, we wondered whether a similar event had happened, and thus examined whether any disrupted genes were present that might be responsible for the **suv4** mutation at around the top or centre of chromosome 3.

**suv4** showed a UVB-hypersensitive phenotype under dark conditions much more clearly than under light conditions (Fig. 1A), which is reminiscent of mutants of ‘dark repair’ pathways such as nucleotide excision repair (NER) or base excision repair (BER). Since the *A. thaliana* XPG (*AtXPG*) gene encodes a component of NER, located at the centre of chromosome 3 [30], we hypothesized that a disruption of this gene could be responsible for the **suv4** phenotype. To examine this hypothesis, we prepared five sets of primers covering the open reading frame of *AtXPG*. Our results showed that two of the primer pairs failed to amplify the DNA from **suv4** (Fig. 3A), suggesting that the chromosome is discontinuous at a region between primers X-2F and X-3R. Therefore, we concluded that *AtXPG* gene is responsible for the majority of (if not all) the phenotype of **suv4**, and hereafter we renamed the **suv4** as *uvh3-2* after the reported *AtXPG* mutant *uvh3* [30].

In an effort to identify the nucleotide changes in *uvh3-2*, we then tried to amplify the boundaries of the break points at the bottom of chromosome 3 by TAIL-PCR. The specific primers X-Sp7, 8 and 9 were designed based on the sequence from the 10th intron to the 11th exon of the *AtXPG* gene, reading from the right side of the possible break point (BP-I). The primers X-Sp16, 17 and 18 were designed based on the sequence from the 8th exon to the 8th intron, reading from the left side of BP-I. As a result, we found that the

| Phenotype       | Number of lines |
|-----------------|-----------------|
| UVB-sensitive   | 50              |
| UVB-resistant   | 133             |
| Total           | 283             |

Fig. 2. Abnormal recombination pattern in a cross between **suv4** and *Ler*. The **suv4** mutant was crossed with the Landsberg erecta (*Ler*) plant. F2 plants from this cross were screened for UVB sensitivity. The recombination events (presented by numbers of *Ler* genotype/numbers of analyzed chromosomes) in UVB-sensitive F2 were detected by the SSLP/CAPs method. Markers and the distance from the top (centimorgan; cM) are shown on the left side of chromosomes 2 (black bar) and 3 (gray bar), and recombination events are shown on the right side of the columns. White ovals indicate approximate positions of centromeres. *m246 is reported at 11.0 cM at TAIR, but is actually closer to the centromere than to RNS1.

Table 1. Segregation of UVB sensitivity in F2 lines

| Phenotype       | Number of lines |
|-----------------|-----------------|
| UVB-sensitive   | 50              |
| UVB-resistant   | 133             |
| Total           | 283             |
AtXPG is directly connected to the top of chromosome 3 (Fig. 3C, top), showing that there are two break points, at the centre (BP-I) and top (BP-II) of chromosome 3 (Fig. 3B, top). On the other hand, TAIL-PCR with X-SP16, 17 and 18 showed that the 3′-half of AtXPG is connected to the sequence present in chromosome 2, implying that there is a third break point (BP-III) on chromosome 2 (Fig. 3B, bottom). To confirm this idea, we repeated the TAIL-PCR assay using the specific primers F21O3-Sp1-6, based on both sides of predicted BP-II, and primers T3P4–Sp4-15, based on both sides of BP-III. Taking all of the PCR results into account, it was revealed that a large fragment (~8 Mbp) of chromosome 3 was translocated to chromosome 2 with an inverted direction (Fig. 3C, bottom). Moreover, additional detailed PCR and sequencing analysis revealed that at least five break points are present within two chromosomes of *uvh3-2*, accompanied by a number of alterations such as deletion, insertion, duplication and inversion (Fig. 4).

Visual detection of chromosomal rearrangements

To confirm the chromosomal rearrangement in *uvh3-2*, we performed FISH analysis using centromeric 180 bp repeats and 45S rDNA as probes. The centromeric 180 bp repeats are present at the centromere of all chromosomes of *A. thaliana* and form a cluster of 2.7–3 Mb [22]. The 45S rDNA, which also forms a cluster, is present at the...
subtelomeric regions of the short arms of chromosomes 2 and 4 [31]. When mitotic cells in wild-type plants were hybridized with both probes, 45S rDNA signals were always detected at close proximity to those of the 180 bp repeats (Fig. 5A). By contrast, mitotic cells from

uvh3-2 showed a different pattern, in that some 45S rDNA signals were detached from those of the 180 bp repeats (Fig. 5B, arrowheads). Similar patterns were observed with interphase cells of wild-type and uvh3-2 plants. To quantify the separation, the distances between both signals were measured and plotted (Fig. 5C). The results suggested that the 45S rDNA and centromeric 180 bp repeats were separated by a large chromosomal fragment in uvh3-2, which is consistent with the results of the sequencing analysis.

Rearranged chromosomes reduced the fertility of uvh3-2/+ plants

The marked changes in chromosome structures suggested that abnormal chromosomes affect chromosome segregation in meiosis. To examine this possibility, we crossed uvh3-2/+ plants with Columbia and obtained uvh3-2/+ seeds. Columbia, uvh3-2 and uvh3-2/+ plants were then grown on soil and self-pollinated, after which time normal seeds, abnormal seeds and aborted ovules in siliques were analyzed. In contrast to uvh3-2 siliques which look normal and are indistinguishable from those of wild-type, the siliques of uvh3-2/+ plants were slightly shorter in length (data not shown). The average number of normal seeds in Columbia and uvh3-2 was almost identical, while that in uvh3-2/+ plants was significantly lower (Fig. 6). The number of aborted ovules in uvh3-2/+ plants was significantly higher than that in Columbia or uvh3-2. These data suggested that uvh3-2/+ plants possess reduced fertility, probably due to a failure in ovule development.

To examine the reason for this reduced fertility in uvh3-2/+ plants, we analyzed chromosome segregation in the offspring of heterozygous plants. To recognize the segregation visually, we crossed uvh3-2 with gl1-5 mutant, which develops no leaf hairs, in a Columbia background. About 90 of the F2 plants derived from the cross were analyzed by phenotype and/or PCR with primers specific to normal chromosomes 2 and 3, and to uvh3-2–derived chromosomes 2’ and 3’ (Figure S1). Based on the combination of chromosomes, F2 plants were classified into five groups (Table 2), comprising gl1 type (2, 2, 3, 3), uvh3-2 type (2’, 2’, 3’, 3’), heterozygote I (2, 2’, 3, 3’), heterozygote II (2, 2’, 3, 3) and heterozygote III (2’, 2’, 3, 3’). No other types of heterozygotes, such as (2, 2, 3, 3) for example, were detected, suggesting that reproductive cells carrying normal chromosome 2 and uvh3-2–derived chromosome 3’ were unable to survive and thereby produce zygotes. Moreover, the smaller number of heterozygotes II and III compared with heterozygote I suggested that reproductive cells carrying uvh3-2–derived chromosome 2’ and normal chromosome 3 also affected development of the zygote, probably due to abnormal crossover on meiosis.

**DISCUSSION**

Ion beams have been utilized in mutation breeding since it was empirically determined that ion beams induce novel characters more effectively than other mutagens such as γ-rays or chemicals.
Using model plants such as Arabidopsis and/or rice, the mechanisms responsible for the marked biological effects of ion beams have been investigated, and the results suggested that ion beams (i) deposit the bulk of their energy in small areas, and (ii) induce DSBs or clustered damage, the repair of which is negligible by cellular repair systems. To deal with DSBs, plant cells predominantly employ NHEJ pathways, which involve the risk of rejoining the incorrect break ends. Therefore, it is predicted that ion beams induce marked mutations, including deletions, insertions, inversions, duplications and/or translocations. Supporting this idea, several mutant plants carrying an inversion [12, 17, 29] or deletion [32, 33] within a given chromosome have been reported. Moreover, cytological analysis using tobacco cultured cells has shown that abnormal chromosomes such as minichromosomes or bridge structures are induced by ion beams [34, 35], suggesting that ion beams induced aberrations involving plural chromosomes. However, it is assumed that most of the cells or plants carrying a marked change could not survive or became sterile, thereby preventing further investigations in general. In this study, we have reported for the first time detailed analysis of a marked mutation involving two chromosomes. The uvh3-2 mutant involves a translocation of an ~8 Mbp fragment from chromosome 3 to chromosome 2. This suggested that ion beam irradiation induced plural DSBs on the two chromosomes, and that the break ends were rejoined incorrectly during the repair process. Sequence analysis showed that uvh3-2 also involved several deletions and insertions, suggesting that multiple fragments were rejoined randomly. The presence of inverted repeats and duplication near the rejoined sites suggested that some DSBs were rejoined using a short homologous sequence. Furthermore, the insertion of a short filler fragment is a typical characteristic of ion beam–induced mutations [12, 13]. These results suggested that the NHEJ pathway was predominantly employed in the process of uvh3-2 mutation. The marked and complicated changes in uvh3-2 indicated that plant cells are far more adaptable than predicted.

Fig. 5. Detection of centromeric 180 bp repeats and 45S rDNA. (A) Columbia (B) uvh3-2. The upper and lower images show mitotic chromosomes and interphase nuclei, respectively. Arrowheads indicate 45S rDNA signals not associated with 180 bp signals. Scale bars, 2 μm. (C) Dot-density plot of distance between 45S rDNA and the nearest 180 bp repeat signals from interphase nuclei of Columbia (n = 100) and uvh3-2 (n = 100). A significant difference was detected between Columbia and uvh3-2 using Welch’s t-test (P < 0.01).
uvh3-2 plants possessed normal fertility when self-pollinated (Fig. 6), suggesting that disruption of AtXPG hardly affected the process of reproduction under normal growth conditions. In contrast, F1 plants derived from an outcross between uvh3-2 and wild-type plants possessed reduced fertility (Fig. 6). The PCR analysis of the F2 population showed uneven segregation of chromosomes (Table 2), probably due to some chromosome combinations being lethal and prohibiting the development of gametophytes. For example, a gametophyte cell, termed gametophyte ii (see Supplemental Figure 1B), carrying a normal chromosome 2 and a uvh3-2-derived chromosome 3', has lost a large chromosome arm containing numerous genes. Therefore, gametophyte ii seems to have been almost lethal, which accounts for the fact that zygotes carrying (2, 2, 3') were not obtained. Moreover, the translocated fragment in chromosome 2 may have induced abnormal crossovers during meiosis. If a crossover between normal chromosome 3 and chromosome 2' occurred, this would produce a dicentric or acentric chromosome and prohibit the development of gametophytes. Thus, the number of gametophyte iii (Supplemental Figure 1B) seems to have been less than that of other gametophytes. In summary, uvh3-2/+ showed low fertility due to (i) a partial loss of chromosome segments, and (ii) abnormal meiotic recombinations caused by translocated chromosomes.

Chromosomes with multiple inversions have been used as 'balancer chromosomes' in Drosophila, which are useful in screening analyses and in maintaining heterozygous mutations [36]. Similar chromosomal techniques are utilized in mammals [37] and worms [38]. Chromosome engineering techniques are becoming increasingly popular in plant breeding. For example, a chromosome carrying pericentric inversion avoids crossover with the homologous normal chromosome, thereby preventing segregation in F2 and maintaining heterozygous offspring. Additionally, due to the presence of a chromosome with inversion, plural alleles on the opposite chromosome inherit together, which is useful in maintaining a multigenic trait.

At present, a large amount of F1 hybrid crops is being cultivated in the world, which utilizes 'hybrid vigour' to effect high yields or useful characters. Maize breeding programs exploiting heterosis began in the USA in the 1930s, and subsequent development of a double-cross hybrid scheme has successfully produced a large amount of hybrid seed corns [39]. In China, breeding systems were established in the 1970s, and hybrid rice has occupied >50% of the total rice area since the 1990s [40]. However, the process of creating hybrids often requires pollination control, such as the use of a male sterile strain or detasseling. Maintaining heterozygotes by means of engineered chromosomes would cut costs and therefore result in widespread use in the seed market. Interchromosome translocations, found in uvh3-2, affected the independence of chromosomes 2 and 3. This suggested that insertion of a homologous sequence into an ectopic site could regulate the distribution of plural chromosomes during meiosis. Such chromosome engineering techniques should continue to develop with the help of genome editing tools such as CRISPR/CAS.

The partial sterility of the F1 derived from uvh3-2 and Columbia is reminiscent of reproductive isolation observed in the wild. Noor and colleagues suggested that chromosome inversion caused reproductive isolation between two related Drosophila species [41]. It is believed that 17 translocations and 9 inversions have occurred over 10 million years since the divergence of Brassica oleracea and A. thaliana from a common ancestor [42]. The generation of uvh3-2 may have mimicked, at least in part, the creation of a new species by evolution.

**SUPPLEMENTARY DATA**

Supplementary data are available at Journal of Radiation Research online.

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CONFLICT OF INTEREST
The authors declare that there are no conflicts of interest.

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