CRISPR/Cas9-mediated genome editing of RsGL1a and RsGL1b in radish (Raphanus sativus L.)

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The clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9) is a powerful tool widely used for genome editing in various organisms, including plants. It introduces and facilitates the study of rare genetic mutations in a short time and is a potent tool to assist in plant molecular breeding. Radish (Raphanus sativus L.) is an important Brassicaceae vegetable cultivated and consumed worldwide. However, the application of the CRISPR/Cas9 system is limited by the absence of an efficient transformation system in radish. This study aimed to establish a CRISPR/Cas9 system in radish employing the Agrobacterium-mediated genetic transformation system reported recently. For this purpose, we performed genome editing using the CRISPR/Cas9 system targeting the GLABRA1 (GL1) orthologs, RsGL1a and RsGL1b, that induces leaf trichome formation in radish. A Cas9/single guide RNA (sgRNA) vector with a common sgRNA corresponding to RsGL1a and RsGL1b was transferred. A total of eight T0 plants were analyzed, of which six (editing efficiency 75%) had a mutated RsGL1a, five (62.5%) had a mutated RsGL1b, and five showed mutations in both RsGL1a and RsGL1b. Most mutations in T0 plants were short (<3 bp) deletions or insertions, causing frameshift mutations that might produce non-functional proteins. Chimeric mutations were detected in several T0 generation plants. In the T1 generation, the hairless phenotype was observed only in plants with knockout mutations in both RsGL1a and RsGL1b. The majority of mutant alleles in T0 plants, with the exception of the chimeric mutant plants detected, were stably inherited in the T1 generation. In conclusion, we successfully knocked out RsGL1a and RsGL1b using the CRISPR/Cas9 system and demonstrated that both RsGL1a and RsGL1b independently contribute to the induction of leaf trichome formation in radish. In this study, genome-edited plants without T-DNA, which are useful as breeding material, were obtained. The findings prove the feasibility of genome editing in radish using a CRISPR/Cas9 system that could accelerate its molecular breeding to improve agronomically desirable traits.

KEYWORDS
CRISPR/Cas9, single guide RNA (sgRNA), gene editing, Raphanus sativus L., RsGL1a, RsGL1b, trichome, molecular breeding
Introduction

Radish (Raphanus sativus L.), characterized by enlarged hypocotyls and taproots, belongs to the Brassicaceae family and is an important vegetable cultivated and consumed worldwide. The edible parts of radish including the roots, leaves, and sprouts, are of high nutritional value (Hara et al., 2011; Banhani, 2017). Therefore, the genetics and genomics of radish have been the subject of extensive research interest. Rapid advances in sequencing technologies have enabled the sequencing of whole genomes of five cultivated radish varieties and the development of an open-source genome database (Kitashiba et al., 2014; Mitsui et al., 2015; Xiaohui et al., 2015; Jeong et al., 2016; Shirasawa et al., 2020), which facilitates the genetic researches in radish. Mitsui et al. (2015) showed that the R. sativus originates from a divergence of the Brassica rapa and has a triplicated genome compared to Arabidopsis thaliana. Given its close relatedness with other Brassica crops (Chen et al., 2011) and A. thaliana, it is expected that the availability of genetic information and experimental techniques from these crops could be extended to the development of molecular breeding and gene function analysis in radish.

The clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9) system, derived from the immune system of bacteria and archaea (Gasiunas et al., 2012; Jinek et al., 2012), is composed of the Cas9 endonuclease and a synthetic single-guide RNA (sgRNA), which directs the Cas9 protein to the targeted genomic DNA (gDNA) sequence preceding the protospacer-associated motif (PAM). CRISPR/Cas9 has gained tremendous popularity for targeted mutagenesis in transformable eukaryotic species (Cardi et al., 2017). CRISPR/Cas9 recognizes and cleaves the 20-mer target sequence and the 5’-NGG-3’ (PAM) sequence and frequently introduces one or two base insertions or deletions (in/del) during the repair of these cleavages, resulting in frameshift mutations to produce gene knockouts (Ran et al., 2013). Genome editing with CRISPR/Cas9 has been widely applied to many plant species (Cardi et al., 2017; Mohd Saad et al., 2021; Tian et al., 2021; Liu et al., 2022). Furthermore, it has also been used to assess the gene function in many plant species owing to its simplicity and easy accessibility. However, in radish, although several gene functions and their effects on traits, such as root color (Lai et al., 2020) and root shape (Gancheva et al., 2016), have been speculated, most of the functions of the genes have not been validated because of the difficulty of producing gene knockouts. Moreover, genome editing by CRISPR/Cas9 in radish was limited by the unavailability of an efficient transformation system and the difficulties to introduce the CRISPR/Cas9 gene and sgRNA. Recently, we have successfully established a highly efficient method for Agrobacterium-mediated genetic transformation in radish (Muto et al., 2021). The method enabled the successful and efficient transformation of radish hypocotyl explants, suggesting its potential to accelerate genome editing and gene function analysis in radish.

The leaf hairs (trichomes) extend from the epidermis of aerial tissues of plants and serve as a physical barrier against biotic (Kariyat et al., 2018) and abiotic (Wu and Kao, 2009; Bickford, 2016; Escobar-Bravo et al., 2018) stress. In A. thaliana, the leaf trichome has been explored as an excellent model system to study plant differentiation at a single-cell level (Kryvych et al., 2008; Kryvych et al., 2011). Furthermore, trichomes can be visually assessed, and the presence/absence of the trichomes can be used to measure gene knockout efficiency, making it a suitable model to investigate the efficiency of genome editing (Stuttmann et al., 2021). It has also been reported that trichomes play an important role in imparting textures to vegetables (Kawakatsu et al., 2017); therefore, understanding the genetic mechanism of trichome development in radish can lead to the breeding of radishes with suitable edible leaves. GLABRA1 (GL1), a member of the R2R3 MYB transcription factor family (Oppenheimer et al., 1991), is known to control the presence of leaf hair in Brassicaceae plants (Bloomer et al., 2012; Li et al., 2013; Kawakatsu et al., 2017). GL1 and the bHLH transcription factor GLABRA3 (GL3) form a transcriptional activator complex that regulates the transcription of GLABRA2 (GL2) and induces leaf hair formation (Wang and Chen, 2008). In radish, two GL1 orthologs—RsGL1a and RsGL1b—have been predicted to induce leaf hair formation (Li et al., 2013). Therefore, we hypothesized that using these two genes as targets could provide insights into the efficiency of the CRISPR/Cas9 system in radish.

In this study, we aimed to explore the potential of the CRISPR/Cas9 system in radish. For this purpose, we performed the CRISPR/Cas9-mediated genome editing of RsGL1a and RsGL1b. To the best of our knowledge, this study, for the first time, demonstrates the knockout of RsGL1a and RsGL1b using the CRISPR/Cas9 system targeting the two genes simultaneously. It shows that the knockout genes obtained in the T0 generation were inherited and displayed the desired phenotype in the T1 generation. These results provide evidence for the potential of the CRISPR/Cas9 system to accelerate molecular breeding and gene function analysis in radish.

Materials and methods

Target sequences selection and sgRNA design

The workflow of transformation and genome editing in radish is shown in Figure 1A. gDNA of the radish cultivar ‘Pirabikku’ (R. sativus L. var. sativus) was extracted using the NucleoSpin Plant II kit (MACHEREY-NAGEL GmbH & Co. KG, Germany). Sequence information for RsGL1a (Accession Number AB747346.1) and RsGL1b (Accession Number
AB747347.1) were obtained from NCBI (https://www.ncbi.nlm.nih.gov/) to design PCR primers (Supplementary Table 1). To amplify RsGL1a and RsGL1b, PCR was performed using Blend Taq (TOYOBO, Japan) and the following PCR conditions: 95°C for 2 min, followed by 35 cycles of 30 s at 95°C, 30 s at 60°C, and 30 s at 72°C. The PCR products obtained were cloned into the Target Clone vector (TOYOBO, Japan). The vector and PCR products were ligated using the DNA Ligation Kit Ver.1 (TaKaRa, Japan). The ligated plasmid was transferred into Escherichia coli DH5α cells, amplified, and purified using QIAprep Spin Miniprep Kit (QIAGEN, Netherlands). To determine the nucleotide sequences of RsGL1a and RsGL1b, the purified plasmid was Sanger sequenced using the universal primer M13 (5'-GAGCGGATAACATTTCAACAGG -3'). The protein sequences of RsGL1a and RsGL1b were predicted, and protein domain analysis was performed using PROSITE (https://prosite.expasy.org/) (Figure 1B).

**Vector construction**

A binary vector was constructed using restriction enzymes and ligation following the method described in the previous studies (Ito et al., 2015; Mikami et al., 2015; Nakajima et al., 2017). The single guide RNA (sgRNA) cloning vector (pUC19_AtU6_ccdB_oligo) and the binary vector (pZH_gYSA_FFCas9) were obtained from Dr. Makasi Endo from The National Agriculture and Food Research...
Detection of plant transformation and restorer of fertility gene

The *Raphanus* transformation was carried out following the procedure described in our previous study (Muto et al., 2021). Transformed plants were selected with 10 mg/L hygromycin. To verify the introduction of T-DNA, gDNA of the regenerated plants was extracted, and PCR was performed using Hpt_F and Hpt_R primers (Supplementary Table 1) derived from the sequence of hpt using the Quick Taq HS DyeMix (TOYOBO, Japan). The PCR conditions were as follows: 95°C for 2 min, followed by 25 cycles of 30 s at 95°C, 30 s at 60°C, and 30 s at 72°C. The mitochondrial male sterility gene, orf138, and the nuclear restorer of fertility gene (Rf gene) orf687 are present in radish (Yasuamoto et al., 2008). The radish cv. ‘Pirabikku’ with Rf-type orf687 produces pollen (Muto et al., 2021); therefore, we screened for Rf-type orf687 in this study using the PCR-Restriction Fragment Length Polymorphism (RFLP) assay. The PCR was performed using the orf687_F and orf687_R primers (Supplementary Table 1), and the PCR products were digested with SspI. The T0 plants with T-DNA containing the Cas9 gene and Rf-type orf687 were used for further experiments. The flowering and pollen formation in these selected transgenic plants were confirmed to ensure fertility.

**Confirmation of CRISPR/Cas9-mediated mutation in T0 plants**

A total of eight transgenic T0 plants were analyzed to confirm the CRISPR/Cas9-mediated mutations. Reportedly, in *Brassica napus*, the mutant gene from the regenerating plants are maintained in only a small portion of the mature plants (Okuzaki et al., 2018). To confirm the mutation of RsGL1a and RsGL1b in mature plants, we extracted gDNA from the upper leaf of the flowering plant and performed PCR using RsGL1a_PCR_F, RsGL1a_PCR_R, RsGL1b_PCR_F, and RsGL1b_PCR_R primers (Supplementary Table 1). The PCR conditions were as follows: 95°C for 2 min, followed by 35 cycles of 30 s at 95°C, 30 s at 60°C, and 30 s at 72°C. The PCR products were then used for direct sequencing using the RsGL1a_seq and RsGL1b_seq primers (Supplementary Table 1). Chromatograms and nucleotide sequences (text data) were obtained. The chromatograms with a single peak and the nucleotide sequence identical to the wild-type allele were defined as “Wild-type” (no mutation). The multi-peaks near the target sequence in the chromatograms were defined as “Heterogeneous”. The chromatograms with a single peak and a nucleotide sequence other than the wild-type allele were defined as “Homogeneous” (Table 1). The exact allele sequences of the five plants with mutations in both RsGL1a and RsGL1b were obtained. The PCR products of the RsGL1a and RsGL1b were cloned into DH5α and sequenced according to the method previously described. The nucleotide sequences were aligned using NCBI BLAST (Table 2). Subsequently, the self-pollinated seeds of plants with mutations in both RsGL1a and RsGL1b were collected.

**Off-target analysis**

The sgRNA (5'-GAATACAAGAAAGGGTTA-3') corresponding to *RsGL1a* and *RsGL1b* were analyzed using CRISPR RGEN Tools (http://www.rgenome.net/cas-offinder/).
Off-target analysis was performed on *Raphanus sativus* (Rs1.0, genome assembly from cultivar WK10039) as a reference, and a gene with a single nucleotide mismatch (5’-GAATACAAGAAAGGTTTATGG-3’) was detected. This gene was a *Raphanus* ortholog gene of *Arabidopsis* AtMYB23 (Kirik et al., 2001; Kirik et al., 2005). To evaluate the possibility of this gene being modified with the CRISPR/Cas9, primers were designed to amplify the region around an off-target sequence of this RsMYB23-like gene (RsMYB23_F and RsMYB23_R; Supplementary Table 1). The PCR products of the eight transgenic T0 plants were obtained. Nucleotide sequences were obtained from the PCR products by direct sequencing to confirm the mutations using sequence primer (RsMYB23_SEQ; Supplementary Table 1).

**Confirmation of CRISPR/Cas9-mediated mutation in T1 plants**

Leaf trichomes of T1 plants were visually observed, and the number of hairy and hairless plants was counted. (Table 3). Two progenies with hairy and hairless phenotypes were randomly selected at T1 generation, and gDNA was extracted from each progeny. The PCR of the target region was performed as in the T0 analysis. Homogeneous/Heterogeneous mutation was detected in the chromatograms with direct Sanger sequencing of the PCR products of either *RsGL1a* and *RsGL1b*, and inheritance of the mutations observed at T0 generation to T1 generation was verified by the basecalls. If there were multiple

| T0 plant No. | Mutation | RsGL1a Clones/total | RsGL1b Clones/total | Double mutations |
|--------------|----------|---------------------|---------------------|-----------------|
| #1           | Wild type| Wild type           |                     |                 |
| #2           | Heterogeneous | Heterogeneous    |                     | Yes             |
| #3           | Homogeneous | Heterogeneous    |                     | Yes             |
| #4           | Heterogeneous | Heterogeneous    |                     | Yes             |
| #5           | Heterogeneous | Heterogeneous    |                     | Yes             |
| #6           | Homogeneous | Heterogeneous    |                     | Yes             |
| #7           | Heterogeneous | Wild type       |                     |                 |
| #8           | Wild type | Wild type         |                     |                 |
| Editing efficiency | 6/8 (75%) | 5/8 (62.5%) | 5/8 (62.5%) |

**TABLE 2** Alignment analysis of *RsGL1a* and *RsGL1b* in T0 generation plants.

| T0 plant No. | Mutation | RsGL1a | Clones/total | RsGL1b | Clones/total |
|--------------|----------|--------|--------------|--------|--------------|
| #2           | AT in    | T in   | 7/8          | TGGACA | 7/8          |
| #3           | 3 del    | C in   | 1/8          | TGGACA | 1/8          |
| #4           | A in     | T in   | 1/8          | TGGACA | 1/8          |
| #5           | 5 del    | A in   | 1/8          | TGGACA | 1/8          |
| #6           | A in     | WT     | 5/8          | TGGACA | 5/8          |
| #7           | A in     | GG del | 3/8          | TGGACA | 2/8          |
| #8           | TT del   | G del  | 3/8          | TGGACA | 2/8          |
| #9           | 2 del, 1 sub | 18 del, 22 in | TGGACA | 4/8          |
| #10          | T in     | TGGACA | 1/8          | TGGACA | 1/8          |
| #11          | A in     | TGGACA | 8/8          | TGGACA | 1/8          |
| #12          | T in     | TGGACA | 1/8          | TGGACA | 1/8          |

WT, Wild Type; in, insertion; del, deletion; sub, substitution. PAM sequences are underlined. Insertions are shown in red font. Deletions are shown using hyphens. Substitutions are shown in bold font. In/del greater than 3-bp are shown using angle brackets and hyphens.
peaks near the target sequence, each DNA molecule was cloned into *E. coli* and an individual sequence was identified. Furthermore, as T1 plants are considered to be anchored to two genes (*RsGL1a* and *RsGL1b*), a minimum of four clones were analyzed to detect alleles from both genes (Tables 4A, B).

**Evaluation of trichome structure**

The plants evaluated for trichomes were grown in a growth chamber, under the same conditions as those for the transformation, at 25°C under long-day conditions (16 h Light/8 h Dark) at a light intensity of 60 μmol m⁻² s⁻¹. The trichomes on the frontside and backside of the leaves of the T1 plants were observed visually. Leaves of plants with different densities of trichomes were photographed under a fluorescent stereomicroscope (Leica M165 FC) (Figure 2).

**Results**

**Sequencing of target genes and determination of target sequences**

The nucleotide sequences of the *RsGL1a* and *RsGL1b* are shown with the deduced protein sequences in Supplementary Figure 1. These gene sequences were submitted in DDBJ (https://www.ddbj.nig.ac.jp/index-e.html) for *RsGL1a* (Accession Number LC719984) and *RsGL1b* (Accession Number LC719985).

The identity of the ‘Pirabikku’ nucleotide sequences *RsGL1a* and *RsGL1b* with that of Ra38 DH line source, another *Raphanus* variety, was 1216/1307 (93%) and 1174/1311 (90%), respectively. The protein domain analysis identified two Myb DNA-binding domains for both *RsGL1a* and *RsGL1b*, suggesting the association of both genes in trichome

**TABLE 3** Observation of trichome formation in T1 generation plants.

| T0 plant No. originated | Hairy | Hairless | Total |
|------------------------|-------|----------|-------|
| #3                     | 17    | 2        | 19    |
| #4                     | 10    | 4        | 14    |
| #5                     | 0     | 17       | 17    |
| #6                     | 19    | 4        | 23    |

**TABLE 4A** Alignment analysis of *RsGL1a* in hairless and hairy phenotypes of T1 generation plants.

| T1 plant No. | Phenotype | Mutation | *RsGL1a* | Clones/Total | Detected in T0 | T-DNA |
|--------------|-----------|----------|----------|-------------|----------------|-------|
| #3-4         | Hairless  | 14 del   | AGGAATACAA-------CA       | 3/5          | No             | +     |
|              |           | 33 del   | AGGAATACAAG<------->CA     | 2/5          | No             |       |
| #3-16        | Hairless  | 7 del    | AGGAATACAAGAGAGAC-----ACA | 3/4          | No             | +     |
|              |           | T del    | AGGAATACAAGAAAAGTTATGGACA | 1/4          | No             |       |
| #3-3         | Hairy     | WT       | AGGAATACAAGAAAAGTTATGGACA | 1/7          | No             | +     |
|              |           | T in     | AGGAATACAAGAAAAGTTATGGACA | 4/7          | Yes            |       |
|              |           | G in     | AGGAATACAAGAAAAGTTATGGACA | 1/7          | No             |       |
|              |           | 2 del, 32 in | AGGAATACAAGAAAGTTATGGACA | 1/7          | No             |       |
| #3-18        | Hairy     | A in     | AGGAATACAAGAAAAGTTATGGACA | –            | Yes            | +     |
| #4-6         | Hairless  | A in     | AGGAATACAAGAAAAGTTATGGACA | –            | Yes            |       |
| #4-7         | Hairless  | A in     | AGGAATACAAGAAAAGTTATGGACA | 3/4          | Yes            | +     |
|              |           | TT del   | AGGAATACAAGAAAAGTTATGGACA | 1/4          | No             |       |
| #4-4         | Hairy     | A in     | AGGAATACAAGAAAAGTTATGGACA | 3/4          | Yes            | –     |
|              |           | GG del   | AGGAATACAAGAAAGTTATGGACA | 1/4          | Yes            |       |
| #4-9         | Hairy     | A in     | AGGAATACAAGAAAAGTTATGGACA | –            | Yes            | +     |
| #5-1         | Hairless  | TT del   | AGGAATACAAGAAAAGTTATGGACA | 3/4          | Yes            | +     |
|              |           | 2 del, 1 sub | AGGAATACGAGAAAAGTTATGGACA | 1/4          | Yes            |       |
| #5-2         | Hairless  | 2 del, 1 sub | AGGAATACGAGAAAAGTTATGGACA | 3/5          | Yes            | +     |
|              |           | A in     | AGGAATACGAGAAAAGTTATGGACA | 2/5          | Yes            |       |
| #6-1         | Hairless  | A in     | AGGAATACAAGAAAAGTTATGGACA | –            | Yes            | +     |
| #6-2         | Hairless  | A in     | AGGAATACAAGAAAAGTTATGGACA | –            | Yes            | +     |
| #6-3         | Hairy     | A in     | AGGAATACAAGAAAAGTTATGGACA | –            | Yes            | +     |
| #6-4         | Hairy     | A in     | AGGAATACAAGAAAAGTTATGGACA | –            | Yes            | +     |

WT, Wild Type; in, insertion; del, deletion; sub, substitution. PAM sequences are underlined. Insertions are shown in red font. Deletions are shown using hyphens. Substitutions are shown in bold font. In/del greater than 3-bp are shown using angle brackets and hyphens. 5′Splice mutation.
### Alignment analysis of RsGL1b in hairless and hairy phenotypes of T1 generation plants.

| T1 plant No. | Phenotype | Mutation | RsGL1b | Clones/Total | Detected in T0 | T-DNA |
|--------------|-----------|----------|---------|-------------|----------------|-------|
| #3-4         | Hairless  | T in     | AAGAATACAAGAAAGGGTTATGGACA | 2/4 | Yes | + |
|              |           | T del    | AAGAATACAAGAAAGGGTTATGGACA | 2/4 | No |   |
| #3-16        | Hairless  | T in     | AAGAATACAAGAAAGGGTTATGGACA |   | Yes | + |
| #3-3         | Hairy     | WT       | AAGAATACAAGAAAGGGTTATGGACA | 3/4 | Yes | + |
|              |           | A in     | AAGAATACAAGAAAGGGTTATGGACA | 1/4 | Yes |   |
| #3-18        | Hairy     | WT       | AAGAATACAAGAAAGGGTTATGGACA | 2/4 | Yes | + |
|              |           | T in     | AAGAATACAAGAAAGGGTTATGGACA | 1/4 | Yes |   |
|              |           | TT del   | AAGAATACAAGAAAGGGTTATGGACA |   | No |   |
| #4-6         | Hairless  | G del    | AAGAATACAAGAAAGGGTTATGGACA |   | Yes | - |
| #4-7         | Hairless  | G del    | AAGAATACAAGAAAGGGTTATGGACA |   | Yes | + |
| #4-4         | Hairy     | WT       | AAGAATACAAGAAAGGGTTATGGACA | 3/6 | Yes |   |
|              |           | G del    | AAGAATACAAGAAAGGGTTATGGACA | 3/6 | Yes |   |
| #4-9         | Hairy     | WT       | AAGAATACAAGAAAGGGTTATGGACA | 1/4 | Yes | + |
|              |           | G del    | AAGAATACAAGAAAGGGTTATGGACA | 3/4 | Yes |   |
| #5-1         | Hairless  | A in     | AAGAATACAAGAAAGGGTTATGGACA | 3/6 | Yes | + |
|              |           | 18 del, 22in | <---------TTATGGACA | 3/6 | Yes | - |
| #5-2         | Hairless  | A in     | AAGAATACAAGAAAGGGTTATGGACA |   | Yes | + |
| #6-1         | Hairless  | T in     | AAGAATACAAGAAAGGGTTATGGACA | 3/7 | Yes | + |
|              |           | 14 del   | AA---------TTATGGACA | 4/7 | No |   |
| #6-2         | Hairless  | T in     | AAGAATACAAGAAAGGGTTATGGACA | 3/4 | Yes | + |
|              |           | 14 del   | AA---------TTATGGACA | 1/4 | No |   |
| #6-3         | Hairy     | T sub    | AAGAATACAAGAAAGGGTTATGGACA |   | No | + |
| #6-4         | Hairy     | T sub    | AAGAATACAAGAAAGGGTTATGGACA |   | No | + |

**WT**: Wild Type; **in**: insertion; **del**: deletion; **sub**: substitution. PAM sequences are underlined. Insertions are shown in red font. Deletions are shown using hyphens. Substitutions are shown in bold font. In/del greater than 3-bp are shown using angle brackets and hyphens.

### FIGURE 2
Phenotypes of trichome formation in T1 generation plants. (A–D) hairy plants. (E–H) hairless plants. (A, E) The adaxial surface of leaves. (B, F) petioles, and meristems. (C, G) Enlarged views of adaxial surface of leaves. (D, H) Enlarged views of abaxial surface of leaves. The scale bars in (A, B, E, F) are 10 mm, and in (C, D, G, H) 1 mm are shown.
formation as transcription factors. The 18-mer + PAM (5’-GAATACAGAAAGGGTTA TGG -3’) sequence (21-mer) common to both genes located upstream of the Myb DNA-binding domain in the first exon of both genes were selected as target sequences (Figure 1B).

Production of RsGL1 edited T0 plants

In the eight T0 plants, the chromatograms of RsGL1a and RsGL1b sequences, obtained through direct sequencing, were analyzed (Table 2). Six of these plants had mutations in RsGL1a (editing efficiency 75%), two of which were homogeneous, whereas five plants had mutations in RsGL1b (editing efficiency 62.5%), and none were homogeneous. Double mutations were detected in five of the eight plants (editing efficiency 62.5%). The PCR products of RsGL1a and RsGL1b of the T0 plants with double mutations were cloned into E. coli and eight colonies from each PCR product were sequenced (Table 2). All mutations occurred at positions two or three bases upstream of the PAM sequence. Analysis of RsGL1a detected no wild-type alleles in any T0 plants. Most in/del were short (<3 bp) and caused frameshift mutations that might abolish protein function. The only deletion of 3 bp in plant #3 did not cause a frameshift mutation, indicating this deletion might not affect the gene function. We expected the following genotypes: homogenous (two alleles having the same mutation); heterogenous (only one allele is mutated); biallelic (two alleles having different mutations); chimeric (two or more different alleles are present in the sequence chromatogram), or wild-type. In T0 plants, the RsGL1a in #5, RsGL1b in #6, and both RsGL1a and RsGL1b in #3 were identified to have three or more different alleles, indicating that they have chimeric mutations. The RsGL1a of T0 plant #6 was identified to harbor a homogenous mutation both by direct sequencing and clone sequencing However, the RsGL1a of T0 plant #3, which was shown to have a homogeneous mutation by direct sequencing, had three different alleles. These cloning results were further confirmed by detecting the PCR products for heterogeneous double-stranded DNA using T7 endonuclease I (T7EI; New England Biolabs, Ipswich, MA, USA) assay (Supplementary Figure 2). These results indicate that direct sequencing might miss minor alleles. Direct sequencing of T0 plants demonstrated that the editing efficiency did not differ significantly between RsGL1a and RsGL1b (75% vs. 62.5%), recognized by the same target sequence to induce genome editing. “The wild-type RsGL1a was not detected in any plant upon the analysis of the cloned sequences of individual alleles; however, the wild-type RsGL1b was detected in plants #3, #4, and #6.” This result showed that the ability of CRISPR/Cas9 to edit wild-type alleles differs even when targeting the same target sequences of paralogous genes. Off-target analysis on eight transgenic plants did not detect off-target mutations by direct sequencing (Supplementary Figure 3).

Analysis of T1 generation plants with double mutation of RsGL1a and RsGL1b

The self-pollinated seeds from four T0 plants with double mutations (one with <10 self-pollinated seeds were not used) were collected for phenotypic evaluation. The leaf trichome formation in the T1 generation plants was observed visually, and the numbers of hairless and hairy plants were counted (Table 3). Hairless phenotypes were observed in the progenies of all four T0 plants, wherein the hairy phenotype was not observed in plants #4 and #5. T-DNA negatives (null-segregant) were obtained for each T1 plant (Supplementary Table 2), and T1 plant #5 was both hairless and T-DNA negative. Visual and microscopic observation revealed considerable differences in trichome formation between hairless and hairy plants. The trichome density on the adaxial and abaxial surfaces of the leaves also showed marked differences (Figure 2). Two hairless and hairy progenies of each T1 plant (except for #5) were randomly selected. Allele analysis of RsGL1a and RsGL1b in hairless and hairy phenotypes of T1 plant generations is shown in Tables 4A, B. In hairless plants, all RsGL1a and RsGL1b were mutated, causing frameshifts, and there were no functional RsGL1 genes. In contrast, hairy plants had either RsGL1a, RsGL1b, or both with functional wild-type allele(s). Among the 14 T1 generation plants, 5 plants (#4–6, #4–4, #4–9, #5–1, and #5–2) had the allelic composition of RsGL1a and RsGL1b as detected in T0 plants. The T1 progenies of plant #5 inherited both RsGL1a and RsGL1b, #4 inherited RsGL1b, and #6 inherited RsGL1a detected in their respective T0 plants (Tables 4A, B). The progenies of T0 plant #3 had a significantly lower inheritance of alleles (Tables 4A, B).

Discussion

Mutations detected in this study were predominantly short (<3 bp) in/del, which might lead to frameshift mutations affecting the amino acid sequence downstream of the target sequence. In contrast, base substitutions were very few compared to in/del induced by the CRISPR/Cas9 system in radish. Concordant with our findings, several other studies conducted on other dicotyledonous plants, such as Brassica napus (Yang et al., 2017), Arabidopsis (Feng et al., 2014), and tomato (Zhang et al., 2020), have shown that most mutations induced by the CRISPR/cas9 system are insertion, deletion, or complex type which might cause frameshift mutations.
The majority of the mutant alleles in RsGL1a and RsGL1b in T1 plants were also detected in T0 plants (Tables 2 and Tables 4A, B), suggesting that the mutant alleles in T0 plants were stably inherited in T1 plants. However, the mutant alleles in RsGL1a and RsGL1b in T0 plant #3 were not stably inherited. This result could be attributed to the chimera mutation in T0 generation, as observed in some Brassica species such as B. napus (10–100%, Yang et al., 2017) and B. campestris (56%, Xiong et al., 2019) and B. oleracea (Lawrenson et al., 2015), suggesting that the novel editing might have occurred during plant regeneration, which uses hypocotyl or cotyledonary petiole as explants that undergo Agrobacterium infection during the transformation (Cao et al., 2006; Dun et al., 2011; Muto et al., 2021). Chimeric mutations in the T0 generation might obstruct the prediction of the original alleles to be inherited; however, it may increase the diversity of mutant alleles in the T1 generation. In summary, we concluded that the mutation pattern introduced by CRISPR/Cas9 in radish is similar to that in other previously reported Brassica plants.

As shown in Tables 4A, B, T1 plants #3–3 and #3–18 with three or more alleles in RsGL1a or RsGL1b had the wild-type allele and retained T-DNA. This result suggests that Cas9 was active during the reproduction stage from T0 to T1 generations and mutated the wild-type allele to generate new alleles in the T1 generation. Interestingly, the binary vector used in this study has the promoter::Cas9::terminator and promoter::sgRNA suitable for the floral dip method in A. thaliana (Fauser et al., 2014), which might have mutated the wild-type allele in the next generation of propagation as reported in A. thaliana.

Li et al. (2013) reported that the expression of RsGL1a in radish contributes to trichome formation. In this study, we obtained hairy T1 plants in which RsGL1a was knocked out and RsGL1b was functional (#3–18, #4–4, #4–9, #6–3, and #6–4). These results showed that a single knockout of either RsGL1a or RsGL1b did not affect trichome formation; however, double knockout plants produced hairless leaves, suggesting that these two alleles contribute to trichome formation independently. Furthermore, the identification of “double knockout” T1 plants indicates that neither RsGL1a nor RsGL1b is essential for the basic growth performance of the plant (Figure 2F). Therefore, these plants can be used for breeding hairless radishes. However, further investigations are needed to validate the effects of the “double knockout” on the tolerance to several stresses, yield, and edible quality of radish.

In conclusion, we successfully performed genome editing using the CRISPR/Cas9 system targeting the genes associated with leaf trichome formation in radish. We showed that although the radish genome has two to three redundant paralogs, efficient genome editing can be achieved using a single sgRNA specific for the modification of multiple paralogs simultaneously. This study showed that genome-edited radish without T-DNA (null-segregant) could be obtained. This radish is useful as a novel breeding material because it might not be treated as a transgenic plant. The findings demonstrated that the CRISPR/Cas9 system is a highly efficient tool for genome editing in radish. However, limited varieties of radishes can be genetically transformed (Muto et al., 2021). Moreover, whether the wild-type allele in F1 plants can be edited by crossing the mutated ‘Pirabikkü’ plants obtained in this study with other radish varieties should be investigated in the future. We believe the CRISPR/Cas9 mediated targeted gene mutagenesis can contribute to the efficient elucidation of gene function mechanisms and assist in the acceleration of molecular breeding of novel radish varieties.

Data availability statement

The original contributions presented in the study are publicly available. This data can be found here: DDBJ, LC719984 & LC719985.

Author contributions

NM performed all the experiments, analyzed the data, created the Figures and Tables, and wrote the manuscript. TM conceived the study, participated in its design, and helped to draft the manuscript. All authors have read and approved the final manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2022.951660/full#supplementary-material
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