Introduction

The leaf photosynthetic rate is an important potential breeding target for improving crop yields (Long et al. 2006, Raines 2011). There are large variations in photosynthetic capacity among plant genotypes and even within a single species (Driever et al. 2014, Flood et al. 2011), implying that photosynthetic productivity can be manipulated using genetic and molecular biology techniques. Photosynthetic capacity is potentially limited by various physical and biochemical parameters. First, upregulation of the amount and activity of photosynthetic proteins, such as electron transport components and Calvin cycle enzymes, may increase photosynthetic capacity (Farquhar et al. 1980). Second, limiting CO₂ diffusion by altering various parameters, such as the size and density of stomata and mesophyll conduc-

tance, is an important way to increase photosynthetic capacity because the photosynthetic rate strongly depends on the CO₂ concentration at the carboxylation site (Farquhar and Sharkey 1982). Third, altering carbon utilization in sink organs (sink strength) also influences the photosynthetic rate; the accumulation of carbohydrates such as sugars imposes negative feedback regulation on photosynthesis (Krapp and Stitt 1995, Sheen 1994). Such downregulation of photosynthesis is often observed in plants grown under elevated CO₂ levels (Ainsworth and Rogers 2007).

Radish (Raphanus sativus L. var. sativus), a widely cultivated root vegetable crop, possesses a large sink organ (the root), implying that photosynthetic activity in radish can be enhanced by altering both the source and sink capacity of the plant. However, since radish is a self-incompatible plant, improved mutation-breeding strategies are needed for this crop. TILLING (Targeting Induced Local Lesions IN Genomes) is a powerful method used for reverse genetics. In this study, we developed a new TILLING strategy involving a two-step mutant selection process for mutagenized radish plants: the first selection is performed to identify a BC₁M₁ line, that is, progenies of M₁ plants crossed with wild-type, and the second step is performed to identify BC₁M₁ individuals with mutations. We focused on Rubisco as a target, since Rubisco is the most abundant plant protein and a key photosynthetic enzyme. We found that the radish genome contains six RBCS genes and one pseudogene encoding small Rubisco subunits. We screened 955 EMS-induced BC₁M₁ lines using our newly developed TILLING strategy and obtained six mutant lines for the six RsRBCS genes, encoding proteins with four different types of amino acid substitutions. Finally, we selected a homozygous mutant and subjected it to physiological measurements.

Key Words: reverse genetics, TILLING, radish, Raphanus sativus, self-incompatibility, sink capacity, Rubisco small subunit.
sink-source balance on photosynthesis and growth (Sugiura et al. 2015, Usuda and Shimogawara 1998). Establishing a reverse genetics method to produce mutant radish plants would facilitate investigations of the molecular mechanisms underlying source-sink balance and the breeding of high-yielding plants.

Artificial genetic modification is a useful strategy for breeding plants with improved traits. The recently developed genome editing tools CRISPR/Cas9 and TALEN are powerful methods that can be used in reverse genetics studies in any organism (Mao et al. 2017, Nemudryi et al. 2014, Rani et al. 2016, Zhu et al. 2017). However, the altered genomes resulting from the use of these tools are not completely welcome in vegetables and other crops grown for human use. Furthermore, genetic transformation techniques have not yet been established for radish. TILLING (Targeting Induced Local Lesions IN Genomes) is a powerful tool for reverse genetics to detect mismatched sequences induced via point mutations (with EMS) by the specific nuclease, CEL 1, which does not require the use of genetic transformation (Wang et al. 2012). TILLING is a particularly efficient tool for mutation breeding in plants. The use of this method has dramatically expanded since its initial use in the model plant Arabidopsis thaliana (McCallum et al. 2000a, 2000b). Over the past 15 years, TILLING has been adapted for use in major model plants, such as rice, wheat, and barley (Acevedo-Garcia et al. 2017, Hwang et al. 2016, Lai et al. 2012), as well as major vegetable crops including tomato, soybean, pumpkin, canola, cucumber, melon, and oilseed rape (Anai 2012, Boualem et al. 2014, Okabe et al. 2011, Rashid et al. 2011, Stephenson et al. 2010). Although this method continues to be developed (Colbert et al. 2001), to date, a TILLING platform has not been established for the self-incompatible plant radish.

In the present study, we developed a TILLING strategy for use with the self-incompatible plant radish. We chose ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco) as the primary target because it is the most abundant plant protein and the most important enzyme, catalyzing the first step of CO₂ fixation (carboxylation) during photosynthesis at the chloroplast stroma (Evans 1989). Rubisco is composed of two types of subunits, i.e., large and small octamers formed by 55 kDa and 15 kDa proteins, respectively, in all photosynthetic organisms (Spriteritz 2003). The small subunit (RBBCS) is encoded by multiple genes in the nuclear genome and is post-translationally imported and processed by the chloroplast. In Arabidopsis thaliana, a major model Brassica plant, the RBBCS gene family consists of four members divided into two subfamilies based on sequence similarity. Type B includes three genes (RBBCS 1B, 2B, and 3B) that are tandemly arranged at a single locus on chromosome 5. Type A includes the fourth gene (RBBCS 1A) on chromosome 1, which is shorter than type B genes (Krebbers et al. 1988). The expression of RBBCS genes is differentially regulated by light quality and condition (Dedonder et al. 1993). The five RBBCS genes in tomato are also differentially expressed in each tissue (Wanner and Gruissem 1991). Brassica rapa, which is closely related to radish, contains six RBBCS genes, including four and two on chromosomes 2 and 4, respectively, among the 10 chromosomes of this species (Anisimov et al. 2007). While RBBCS genes have been analyzed in some plant species, radish RBBCS (RsRBBCS) genes remain to be identified.

To obtain a more accurate estimate of mutation density from TILLING, the target genes should occupy as large a genomic region as possible. The multiple RBBCS genes provide an optimum target for testing the quality of TILLING methods. Here, we identified RBBCS genes in radish and developed a TILLING strategy to obtain homozygous mutant plants with amino acid substitutions in RBBCS proteins.

Materials and Methods

Plant materials and EMS mutagenesis
Open pollinated radish (Raphanus sativus L. var. sativus cv. ‘Comet’) seeds were purchased from Takii Seed Co., Ltd. (Kyoto, Japan). The seeds were vernalized by soaking in water at 4°C in the dark for 2 weeks prior to EMS treatment. The seeds were incubated in solutions containing various concentrations of EMS (0.015–1.0%) for 15 h in a 100 mL volume (~330 seeds) at room temperature, followed by washing in a detergent solution comprising 0.1% Triton X-100. After rinsing the seeds with H₂O, the treated seeds were sown in pots containing commercial soil with nutrients (Nippi Engei Baido 1; Nihon Hiryo Co., Tokyo, Japan). The treatments were independently performed four times (total of 4300 seeds) in the spring and fall for a 2-year period. EMS-treated seeds (M₁) were sown in a square container (56 cm × 17 cm × 17 cm) and transferred to a greenhouse. After 1 month of growth, the plants were thinned to eight plants per container.

DNA extraction and pooling
The mutagenized 1283 M₁ plants were grown and open-pollinated with wild-type (‘Comet’) pollen by hand at the mature stage. Seeds were successfully harvested from 955 M₁ individuals (Fig. 3). These seeds, which were the progeny of M₁ plants (♂) backcrossed with wild-type radish (‘Comet’) plants (♀️), were termed BC₁ M₁ in this study. To obtain bulked DNA for the TILLING assay, eight plants were grown for each BC₁ M₁ line, and genomic DNA was extracted from the bulked leaf samples using a Chloropure Kit (Beckman Coulter, CA, USA). The bulked DNA samples from the 955 BC₁ M₁ individuals were stored at –20°C.

Two-step TILLING, PCR amplification, and detection of mismatched DNA
High-throughput screening was performed via gel-based screening of heteroduplex PCR products as described in Till et al. (2003). Genomic DNA samples were independently extracted from eight plants per BC₁ M₁ line. In most cases, two BC₁ M₁ DNA samples were combined and used as
template DNA for PCR. To screen the 955 BC1M1 lines, approximately 477 combined DNA samples were subjected to TILLING analysis. Forward primer labeled with 700 nm infrared fluorescent dye (IRD) label and reverse primer labeled with 800 nm IRD label attached to their 5' ends were designed using the Tm Calculator program (Applied Biosystems; http://www6.appliedbiosystems.com/support/techtools/calc/) to identify the best amplicons for TILLING, with the aim of obtaining a predicted primer with Tm of 70°C and >30 bp, with PCR products 1000–1300 bp long. After the target genes were amplified using labeled and unlabeled primers, nucleotide mismatches of these PCR products were specifically digested with CEL1 prepared as described in Till et al. (2003). The reactions were stopped by adding 0.15 M EDTA, pH 8.0. The CEL1-digested PCR products were purified using a Fast Gene Gel/PCR Extraction Kit (Nippon Genetics Co., Ltd., Tokyo, Japan), loaded onto a 6.5% acrylamide gel with 7 M urea, separated by electrophoresis, and detected using a LI-COR DNA Analyzer (CA, USA). For size standards, 806 bp and 379 bp PCR products amplified from wild-type genomic DNA using primer sets IRDye700RsRBCS3A-22F (AGAGAAAGAAGAAGAT TAGTC)/IRDye800RsRBCS3A784R (CGATGAAACTGA TGCACTGC) and IRDye700RsRBCS4A313F (ATTCA CCACTGGAATGCG)/IRDye800RsRBCS4A691R (ACTTCTTCAACATCGAGC), respectively, were used. When the PCR products were digested with CEL1, each genomic DNA sample was independently amplified and digested with CEL1 to identify the BC1M1 population with a mutation. The position of the mutation was confirmed by direct sequencing using a Genome Lab DTCS Quick Start Kit and Genome Lab GeXP (Beckman Coulter, CA, USA). A second TILLING was performed to isolate individual BC1M1 plants from the heterozygous populations to obtain more precise TILLING results.

Confirmation of polymorphisms in wild-type (‘Comet’)

A total of 180 wild-type radish (‘Comet’) seeds were grown in soil in small pots, and genomic DNA was extracted from the bulked four plants as described above. Each genomic DNA sample was mixed with control wild-type (‘Comet’) genomic DNA and used as template DNA to amplify the PCR products of six target DNAs with specific primers (Supplemental Table 1). After digesting the PCR products with CEL1, DNA fragments were visualized using a LI-COR DNA Analyzer.

Measurement of photosynthetic parameters

The plants were grown in a greenhouse in an experimental garden at Tohoku University. BC1M2 seeds, i.e., the progenies of self-crossing of BC1M1 plants using the bud pollination technique, were sown in a 4-L pot filled with commercial soil with nutrients (Nippi Engei Baido 1). Homozygous mutants were selected based on the presence of single mutated sequences in individual BC1M2 lines. At 6 weeks after germination, the photosynthetic parameters of the leaves were determined using a LI-6400 portable gas exchange system (LI-COR Inc., NE, USA). The CO2-dependent photosynthesis rates (A) were measured at 35°C, saturating light of 2000 μmol photons m⁻² s⁻¹ under CO2 concentrations (Ca) of 100, 200, 300, 400, 700, 1000, and 1500 μmol mol⁻¹. The net photosynthesis rate (A) was plotted against intercellular CO2 concentration (Ci), and RuBP carboxylation capacity was evaluated based on the initial slope of the A-Ci curve (IS) obtained with the rates measured at Ca of 100, 200, and 300 μmol mol⁻¹ CO2.

Results

Radish plants and bud pollination

Plants from EMS-mutagenized radish seeds that were grown in soil in the greenhouse exhibited swollen bulbs at 5 weeks, started bolting at 3 months, and reached the flowering stage at 4 months after sowing (Fig. 1A, 1B, 1F). In general, M1 plants obtained from mutagenized seeds were self-crossed to obtain homozygous mutants (as M2 plants). However, it is difficult to obtain M2 radish plants by self-crossing because radish is a self-incompatible plant. Therefore, the M1 plants were crossed with wild-type (‘Comet’) pollen by open pollination (by hand). Among the 1283 M1 plants that were backcrossed with the wild type (‘Comet’), 955 M1 plants successfully produced BC1M1 seeds.

The fertility of the M1 plants depended on the concentration of EMS used for mutagenesis (Supplemental Fig. 1). At concentrations greater than 0.75%, the M1 plants became increasingly less fertile. Fig. 1D and 1E show a fruit that was successfully produced and a pistil that failed to be fertilized, respectively. To obtain BC1M2 seeds, flowers with petals that slightly protruded from sepals were used for bud pollination (Yamaji and Ohsawa 2015). Petals, sepals, and stamens were removed from the flowers with tweezers, and stamens with pollen from the same mutated flower were

![Fig. 1. Radish plant morphology. Five-week-old plant (A) and 3-month-old plant that had initiated bolting (B). Flower development is shown in (C). A flower used for bud pollination is indicated by an arrow, and a flower in which the carpels were artificially opened is shown in the inset photograph. A seedpod that was successfully pollinated (D) and a pistil that failed to be pollinated (E) are shown. A 4-month-old radish plant that flowered is shown in (F).](image)
attached to carpels to produce self-crossed BC1M2 plants (Fig. 1C arrow and inset). After TILLING selection, it took 1–1.5 years to obtain BC1M3 because the lifecycle of radish is long compared to the lifecycles of model plants such as *A. thaliana* and efficient fruit production depends on the season.

**Identification of RsRBCS genes**

The *RBCS* gene family consists of two subfamilies, type A and B, in *A. thaliana*. Six *RBCS* genes have been found in *Brassica rapa*, which correspond to type B genes in *A. thaliana*, whereas type A genes like those found in *A. thaliana* are absent in *B. rapa*. To identify radish *RBCS* (*RsRBCS*) genes, we used the sequences of four *AtRBCS* genes in *A. thaliana* (*At5G38410, At5G38420, At5G38430, and At1G67090*) from TAIR (The Arabidopsis Information Resource; https://www.arabidopsis.org/) as queries to search for homologous regions in the two available radish genomes (Michigan State University (MSU), http://radish.plantbiology.msu.edu; and Kazusa DNA Research Institute (Kazusa), http://radish.kazusa.or.jp/) with TBLASTN software (e-value < 1e−10 and coverage > 70%) (Boratyn et al. 2013) (Supplemental Table 1). We manually removed redundant contigs in the two radish genomes and identified 10 regions homologous to *RsRBCS* genes (Supplemental Table 1). We designed 10 sets of primer pairs for the homologous regions to identify *RsRBCS* genes in ‘Comet’ (Supplemental Table 1). Only seven *RsRBCS* genes were amplified by PCR among the 10 *RBCS* genes predicted in the databases, indicating that *R. sativus* has seven *RsRBCS* genes. **Supplemental Table 2** shows the putative chromosome positions of the *RsRBCS* genes on the nine radish chromosomes. Since genome analysis in radish has not yet been finished, the chromosome positions were estimated using the homologous regions in *R. sativus*.

![Fig. 2. Genomic structures of radish Rubisco small subunit (*RsRBCS*) genes. The three exons are indicated by white boxes. Forward and reverse primers labeled with dye (indicated by black arrows) were designed to amplify approximately 1000–1300 bp PCR products for TILLING analysis. The locations of the detected mutations induced by EMS treatment are indicated by black arrowheads. Asterisks above the black arrowheads indicate effective missense mutations inducing amino acid substitutions. White arrowheads indicate nucleotide insertions or deletions, which are likely natural mutations. Gray arrowheads indicate problematic conserved mutations in the radish (‘Comet’) and *R. sativus* family, e.g., ‘Daikon’. *RsRBCS1B* is a pseudogene with a specific frame-shift site and stop codon.](image)

![Fig. 3. The Radish-TILLING platform. In this TILLING system, radish seeds were mutagenized with the chemical mutagen EMS to produce M1 plants. The M1 plants were crossed with wild-type plants because radish is self-incompatible, producing 955 BC1M1 lines. DNA was extracted from eight bulked BC1M1 plants per line for analysis. The target DNA was amplified using a forward primer labeled with 700 nm dye and a reverse primer labeled with 800 nm dye. The amplified PCR products were digested with CEL1, and the resulting DNA fragments were separated and detected in the 700 and 800 dye channels of a LI-COR DNA Analyzer. Since the BC1M1 population (in bulk) was predicted to exhibit a mutation ratio of 1:3, a peak sequence signal corresponding to a mutation would have one-fourth the signal intensity of wild type. Therefore, it would be difficult to detect the mutated nucleotide by sequencing DNA from the BC1M1 population. To facilitate mutant identification, an individual BC1M1 plant was grown and analyzed again using TILLING gel analysis. A peak sequence signal corresponding to a mutation in the selected BC1M1 individual should have the same intensity as that of wild type. Selected BC1M1 individuals with heterozygous mutations were self-crossed by bud pollination. The homozygous mutant was selected based on the presence of a single peak in the sequencing signal representing a mutation. The population was then harvested as BC1M2 seeds from BC1M2 plants subjected to bud pollination.](image)
been completed, we estimated the locations of these genes on the radish chromosomes based on high-resolution linkage maps between *B. rapa* and *R. sativus* ‘Daikon’ (Kitashiba et al. 2014a, 2014b). Seven *RsRBCS* genes were predicted to be distributed on LG1/LG4 and LG3. These genes contain three exons and two introns, a highly conserved structure throughout photosynthetic organisms (Fig. 2). The genome and amino acid sequences are also highly conserved among *RsRBCS* genes (Supplemental Figs. 2, 3). Furthermore, these genes were categorized into two types, *RsRBCS1A–5A* and *RsRBCS1B/2B*, based on differences in intronic sequences.

Six loci are likely to be functional *RBCS* genes, but one gene, *RsRBCS1B*, was predicted to be a pseudogene because a conserved donor site in the first intron was mutated and a nonsense mutation was also found in the first exon (Fig. 2, Supplemental Fig. 2). We confirmed this prediction by analyzing the expression patterns of the genes using gel electrophoresis analysis of RT-PCR products and quantitative RT-PCR of *RsRBCS1B* (Supplemental Fig. 4). The seven *RsRBCS* genes resemble each other (Supplemental Fig. 2), with *RsRBCS1B* and *RsRBCS2B* sharing the closest homology (87%). We compared the expression level of *RsRBCS2B* in the wild type (‘Comet’) with that of *RsRBCS2B* using specific primers (Supplemental Fig. 4). As shown in Supplemental Fig. 4C and 4D, *RsRBCS1B* was not expressed, whereas *RsRBCS2B* was expressed, indicating that *RsRBCS1B* is indeed a pseudogene. We therefore subjected the six standard *RsRBCS* genes to TILLING.

**Mutant screening by two-step TILLING targeting the key photosynthetic factor *RBCS***

We pollinated EMS-treated radish plants with wild-type radish (‘Comet’) pollen because it was difficult to self-cross *M1* plants, even at the bud stage (Fig. 3). Of these *M1* plants, 955 successfully produced *BC1 M1* seeds. Eight individual *BC1 M1* seedlings, with an expected wild type: heterozygous ratio of 1:1, were harvested for DNA extraction. In general, approximately 1200 bp PCR products were amplified using the specific primer set IRD 700 (labeled forward primer) and IRD 800 (labeled reverse primer) for TILLING (Supplemental Table 1). Since we used DNA samples extracted from eight bulked *BC1 M1* seedlings, the bulked DNA samples contained wild-type sequences: mutated sequences at a ratio of 3:1. Therefore, we did not need to add non-mutated (wild-type) DNA in order to form hetero-double-stranded DNA between wild-type sequences and mutated sequences, which should be digested by CEL 1 endonuclease. After CEL 1 digestion, fluorescently-labeled DNA fragments were detected using a LI-COR DNA Analyzer (Till et al. 2003) (Supplemental Fig. 5). The putative position of the mutation was confirmed by direct sequencing, but the results were unclear because the ratio of the mutated gene to the genes examined (the mutated gene: wild-type gene) in the eight bulked *BC1 M1* samples was 1:3 or less. To obtain more accurate results, a second TILLING was performed to isolate the heterozygous mutant from the *BC1 M1* populations.

We attempted to isolate radish mutants with nucleotide transitions in all six *RsRBCS* genes, but not the pseudogene (*RsRBCS1B*), using our TILLING strategy. Supplemental Fig. 5 shows an image of a gel in which genomic DNA from *RsRBCS2B* was amplified by PCR, digested with CEL 1, and separated by gel electrophoresis. The results indicate that the *RsRBCS2B* DNA fragments from six *BC1 M1* lines (lines 21, 27, 37, 50, and 52) were digested by CEL 1. In the second TILLING step, *BC1 M1* seeds from six TILLING-positive lines were re-sown and individually subjected to CEL 1 digestion. The mutations were confirmed by direct sequencing, resulting in the identification of mutations in lines 27, 37, and 52 from EMS-treated seeds, with a GC to AT nucleotide transition (Supplemental Table 3). A mutation was not found in line 43, and lines 21 and 50 had identical single base-pair deletions (Fig. 2).

Table 1. Mutation frequencies for six *RsRBCS* genes in the radish mutant population revealed by two-step TILLING

<table>
<thead>
<tr>
<th>Gene ID (EMS concentration)</th>
<th>PCR product screened (bp)</th>
<th>Primer length (bp)</th>
<th>No. of screened lines</th>
<th>Total screened length (kb)</th>
<th>No. of TILLING positive mutant (kbp)</th>
<th>No. of mutations obtained</th>
<th>Mutation density (kb-1)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>RsRBCS1A</em></td>
<td>1224</td>
<td>57</td>
<td>955</td>
<td>1114</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><em>RsRBCS2A</em></td>
<td>1082</td>
<td>62</td>
<td>955</td>
<td>974</td>
<td>1</td>
<td>1</td>
<td>0/974</td>
</tr>
<tr>
<td><em>RsRBCS4A</em></td>
<td>1180</td>
<td>62</td>
<td>955</td>
<td>1067</td>
<td>0</td>
<td>0</td>
<td>0/1067</td>
</tr>
<tr>
<td><em>RsRBCS5A</em></td>
<td>1166</td>
<td>70</td>
<td>955</td>
<td>1046</td>
<td>2</td>
<td>0</td>
<td>0/1046</td>
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<tr>
<td><em>RsRBCS3A</em></td>
<td>1172</td>
<td>72</td>
<td>955</td>
<td>1050</td>
<td>4</td>
<td>0</td>
<td>0/1050</td>
</tr>
<tr>
<td><em>RsRBCS2B</em></td>
<td>1105</td>
<td>72</td>
<td>955</td>
<td>986</td>
<td>5</td>
<td>3</td>
<td>0/986</td>
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<td>(0.25% EMS)</td>
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<tr>
<td><em>RsRBCS1A</em></td>
<td>6929</td>
<td>395</td>
<td>222</td>
<td>1450</td>
<td>–</td>
<td>1</td>
<td>0/1450</td>
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<tr>
<td><em>RsRBCS2A</em></td>
<td>6929</td>
<td>395</td>
<td>270</td>
<td>1764</td>
<td>–</td>
<td>3</td>
<td>0/1764</td>
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<td><em>RsRBCS4A</em></td>
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<td><em>RsRBCS5A</em></td>
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<td><em>RsRBCS3A</em></td>
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<tr>
<td><em>RsRBCS2B</em></td>
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</tr>
<tr>
<td>Total</td>
<td>6929</td>
<td>395</td>
<td>5370</td>
<td>6239</td>
<td>13</td>
<td>4</td>
<td>7/1039</td>
</tr>
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</table>

The mutation frequency was calculated as [total number of identified mutations]/[(PCR product size screened – total primer size) × (total number of screened lines)] (Lai et al. 2012). The average mutation frequency was estimated to be one mutation per 1039 kb. *A nucleotide was substituted out of an exon region.*
Two-step TILLING in self-incompatible radish

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digested with CEL 1 from among the 6237 kb of DNA screened, not including the primer region. However, only six PCR products contained nucleotide transitions induced by EMS, i.e., the frequency of nucleotide transitions in TILLING-positive lines was 46%. Based on the six target genes screened, the average mutation density in the Radish-TILLING collection was estimated to be 1/1039 kb⁻¹ (Table 1). In this mutagenesis, six mutants were successfully obtained from BC₁M₁ lines derived from M₁ plants treated with 0.25% or 0.5% EMS (Supplemental Table 3). We estimated the mutation density obtained for each EMS concentration, finding that the efficiency of 0.25% and 0.5% EMS was 1/483 kb⁻¹ and 1/588 kb⁻¹, respectively (Table 1).

In this TILLING assay, 13 TILLING-positive lines were obtained, including four missense mutations and two intronic mutations (Table 1). Among the seven remaining TILLING-positive lines, four and two lines had an identical mutation in RsRBCS3A and RsRBCS2B, respectively. Since it is unlikely that these identical mutations arose independently, we attempted to identify a polymorphism in six RsRBCS genes from the wild type (‘Comet’). First, we independently isolated genomic DNA from 20 wild-type (‘Comet’) plants, mixed the DNA with reference wild-type genomic DNA, amplified DNA fragments from six RsRBCS genes, and treated this DNA with CEL 1. CEL 1-digested genomic DNA, amplified DNA fragments from six RsRBCS genes were not detected. Furthermore, we examined a polymorphism in RsRBCS3A and RsRBCS2B using 160 additional wild-type radish plants. One polymorphism in RsRBCS3A (G580A, R101Q in the third exon) was found in one of the 160 plants, suggesting that G580A (R101Q) in RsRBCS3A is a natural variation. The same nucleotide mutation was found in four BC₁M₁ lines via TILLING analysis. However, the same deletion in RsRBCS2B was not detected in the 160 wild-type radish plants.

In this study, we developed an effective TILLING strategy for radish (Fig. 3). It was possible to perform direct sequencing after the first gel analysis, but the sequencing signal of the mutation harboring a nucleotide transition was not clear because the bulked sample from BC₁M₁ plants had a wild-type: mutated sequence ratio of 3:1 (or less) (Fig. 4A, 4B). By contrast, it is easier to detect the mutation in an individual heterozygous mutant, because the mutation can theoretically be present at a 1:1 ratio. BC₁M₁ seeds from the candidate lines selected after performing the first TILLING step were separately planted and subjected to a second TILLING step, yielding heterozygous mutants: wild type at a ratio of 1:1 (or less). The peak sequencing signal of the heterozygous mutated nucleotides was similar in intensity to that of the wild type (Fig. 4C). The homozygous mutant lines were subsequently segregated from BC₁M₁ individual plants by sequencing after self-crossing by bud pollination (Fig. 3).

In this TILLING, mutants with four different amino acid substitutions were obtained: i.e., RsRBCS2\textsubscript{2A}\textsuperscript{G104R}, RsRBCS\textsubscript{2B}\textsuperscript{S45F}, RsRBCS\textsubscript{2B}\textsuperscript{S45F}, and RsRBCS\textsubscript{2B}\textsuperscript{D118N} (Supplemental Table 3). We estimated the photosynthetic activity of one of these mutants, the homozygous mutant RsRBCS\textsubscript{2B}\textsuperscript{S45F}, by performing A-Ci curve analysis (A; CO\textsubscript{2} assimilation, Ci; internal CO\textsubscript{2}) of plants grown in the field (Supplemental Fig. 6). The homozygous RsRBCS\textsubscript{2B}\textsuperscript{S45F} and non-mutated control plants (homozygous RsRBCS\textsubscript{2B}\textsuperscript{WT}) were segregated in the BC₁M₁ plant population and identified by sequencing. The each values for the A-Ci curve were collected at 100, 200, 300, 400, 500, 700, 1000, and 1500 μmol mol⁻¹ CO\textsubscript{2} concentrations under a light intensity of 2000 μmol photons m⁻² s⁻¹. The maximum CO\textsubscript{2} assimilation rate under 1000 μmol mol⁻¹ CO\textsubscript{2} in the control and the homozygous mutant was 39.83 ± 2.60 and 40.70 ± 5.46, respectively (Supplemental Fig. 6A). We calculated the RuBP carboxylation rates based on the initial slopes of the A-Ci curve (under 100, 200, and 300 μmol mol⁻¹ CO\textsubscript{2}), finding that the photosynthetic activity of RsRBCS\textsubscript{2B}\textsuperscript{S45F} was similar to that of the control (Supplemental Fig. 6B), suggesting that one amino acid substitution in this highly redundant protein has no effect on photosynthetic parameters.

**Discussion**

In the present study, we established a new two-step TILLING platform for radish (Radish-TILLING). Since
Radish is a self-incompatible plant, we used an unconventional technique for pollination to obtain progeny. Fortunately, the crossing problem in this plant family can be overcome using bud pollination, but the fertility ratio is extremely low. Instead of self-crossing the mutagenized M₁ plants, they were backcrossed with wild-type plants ('Comet'), as we needed to collect as many populations of the next generation and as many seeds as possible for TILLING. As a result, the BC₁M₁ populations included wild-type and heterozygous-mutant plants at a ratio of approximately 1:1. Meanwhile, the use of backcrossing makes it difficult to identify mutations via direct sequencing of bulked samples of BC₁M₁ DNA, which include wild-type and mutated sequences at a ratio of 3:1 (Fig. 4). If the mutated chromosome migrates to all megaspore mother cells, half of the egg cells would contain the mutated chromosome. However, the actual ratio of mutation: wild-type is thought to be 1:3 or less because the germline in M₁ plants is a chimera of wild type and heterozygous mutants and because not all female megaspore mother cells have mutations. This estimation is consistent with our results, as heterozygous mutants were usually found in 10–50% of BC₁M₁ plants in our study. This mutation rate in the BC₁M₁ population reduces the strength of the sequencing signal from the mutated nucleotide when using DNA from this population. To facilitate the identification of a mutation by sequencing, we used an improved TILLING strategy in which the BC₁M₁ pool was individually re-planted and individually subjected once again to gel electrophoresis (second TILLING step) to identify a heterozygous BC₁M₁ mutant individual. As a result, the intensity of the sequencing-signal peak of the mutated nucleotide in DNA from the heterozygous BC₁M₁ mutant individual became similar to that of the wild type.

In this study, we obtained 13 TILLING-positive lines from a total of 6239 bp of DNA, with mutations in six target genes, including six nucleotide transitions, four natural variations, and three deletions (Table 1). Analysis of polymorphisms using 180 wild-type plants revealed that the nucleotide transition (G580A) in RsRBCS3A found in four BC₁M₁ lines is also present in wild-type radish ('Comet'), suggesting that it represents a natural variation. By contrast, a deletion of the nucleotide A at position +13 from ATG in RsRBCS2B that was found in two BC₁M₁ lines, but not in the 180 wild-type ('Comet') plants, implying a possibility that this deletion does not represent a polymorphism in wild-type plants. EMS predominantly induces nucleotide transitions from GC to AT. However, Shirasawa et al. (2016) showed that among mutations detected in EMS-treated tomato plants, 1.3% were INDELS (insertions/deletions) of nucleotides whereas 98.7% were single nucleotide transitions. Therefore, the possibility that these INDELS detected in the current study were induced by EMS mutagenesis cannot be ruled out. On the other hand, the six nucleotide transitions were likely induced by EMS, as they were GC/AT transitions and we were not able to find two or more BC₁M₁ lines that had the same GC/AT nucleotide transition in our screening. Since the mutation density in TILLING analysis is generally estimated by counting only GC/AT transitions, we calculated this rate in our radish-TILLING using six nucleotide transitions. As a result, the mutation density under 0.25% and 0.5% EMS treatment in this study was estimated to be 1/483 kb and 1/588 kb, respectively (Table 1).

According to a recent report, tetraploid plants are more tolerant to EMS than diploid plants, suggesting that polyploidy affects the mutation efficiency of EMS treatment (Tsai et al. 2013). In previous TILLING analyses of various crops, the mutant densities of diploid plants were usually estimated to be between 1/100 kb and 1/700 kb (Rashid et al. 2011). The mutation density estimated in the current study is similar to that obtained in diploids using other TILLING approaches because radish is a diploid plant.

Four amino acid substitution mutants and two mutants with no effect (in introns) were selected from approximately 1000 BC₁M₂ populations, but no nonsense mutants were obtained. RBCS genes are highly redundant, which explains why an amino acid substitution in a single protein had no effect on photosynthesis activity (Supplemental Fig. 6). There are at least two possible explanations for this: perhaps the mutation had no effect on Rubisco activity, or perhaps other redundant RBCS proteins complement the activity of the mutant enzyme. Indeed, single knock-out T-DNA mutants of RBCS1A or RBCS3B in A. thaliana have the same plant mass and maximum quantum yield in Photosystem II as the wild type, whereas the double mutant has drastic phenotypes (Izumi et al. 2012). To reduce the amounts and activities of a highly redundant component, multiple mutants would be required. It might be possible to produce double mutants from some of our RsRBCS mutants because RsRBCS2A and RsRBCS1A are likely located on chromosomes LG3 and LG1/LG4, respectively (Supplemental Table 2).

Radish can be used as a model plant to investigate the effects of source-sink balance on photosynthesis and growth (Sugiura et al. 2015, Usuda and Shimogawara 1998). Rubisco, which contains 20–40% of total leaf nitrogen, functions in a rate-limiting step of photosynthesis at ambient and lower CO₂ concentrations under high light (von Caemmerer and Farquhar 1981), but this protein is present in excess under elevated CO₂ conditions because RuBP regeneration and triose phosphate utilization limit photosynthesis (Sage 1990). Reducing Rubisco contents may improve nitrogen use efficiency during plant growth at elevated CO₂ levels (Hikosaka and Hirose 1998, Medlyn 1996, Sage 1994). The accumulation of carbohydrates (e.g., sugar or starch) in the chloroplast also affects photosynthetic activity and plant growth (Abramson et al. 2016). Forward genetics or genome analyses in model plants such as A. thaliana should increase our understanding of the factors that limit photosynthesis. The Radish-TILLING platform developed in this study could facilitate such analyses, as well as plant breeding research.
Two-step TILLING in self-incompatible radish

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Literature Cited


