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Molecules and Cells

An Insertional Mutagenesis System for Analyzing the Chinese Cabbage Genome Using *Agrobacterium* T-DNA

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In this study, we applied insertional mutagenesis using *Agrobacterium* transfer DNA to functionally characterize the gene of *Brassica rapa* L. ssp. *pekinensis*. The specific objectives were to: (i) develop and apply a gene tagging system using plasmid rescue and inverse PCR, (ii) select and analyze mutant lines, and (iii) analyze the phenotypic characteristics of mutants. A total of 3,400 insertional mutant lines were obtained from the Chinese cabbage cultivar, 'Seoul', using optimized condition. Plasmid rescue was performed successfully for transgenic plants with multiple T-DNA insertions, and inverse PCR was performed for plants with a single copy. The isolated flanking DNA sequences were blasted against the NCBI database and mapped to a linkage map. We determined the genetic loci in *B. rapa* with two methods: RFLP using the rescue clones themselves and sequence homology analysis to the *B. rapa* sequence database by queries of rescued clones sequences. Compared to wild type, the T_1 progenies of mutant lines showed variable phenotypes, including hairless and wrinkled leaves, rosette-type leaves, and chlorosis symptoms. T-DNA inserted mutant lines were the first population that we developed and will be very useful for functional genomics studies of Chinese cabbage.

INTRODUCTION

Functional genomics is the field of molecular biology that attempts to make use of the vast wealth of data produced by genomic projects to describe gene functions and interactions. Functional genomics focuses on dynamic aspects of the genome such as gene transcription, translation, and protein-protein interactions, as opposed to the static aspects of genomic information such as DNA sequence or structure. Functional genomics assigns a biological function to a particular gene sequence and has entered the high-throughput stage following the completion of the sequencing of the genome of two model plants: *Arabidopsis* (The *Arabidopsis* Genome Initiative, 2000) and rice (Goff et al., 2002; Lee et al., 2006). Different functional genomic methodologies have been developed including transcriptomics [expressed sequence tags (ESTs) and DNA microarrays], gene knockout by RNA interference or T-DNA insertion, proteomics, and metabolomics. A combination of these methodologies is more likely to elucidate gene function than use of a single technique (Ge et al., 2003; Holtorf et al., 2002).

The genus *Brassica* contains more important agricultural and horticultural crops than any other genus. Chinese cabbage (*B. rapa*) is widely cultivated in Asia, especially in China, Japan, and Korea. Interspecies hybridization in the genus *Brassica* was attempted as early as the nineteenth century when the chromosome number in the genus was still unknown (Tsuchiya and Gupta, 1991). The chromosome number of *B. rapa* is now known to be 2n = 20. Chinese cabbage is in the same taxonomic family as *Arabidopsis thaliana* and shares more than 80% of its genome with *Arabidopsis* (Lee et al., 2004a); therefore, genetic tools used in *Arabidopsis* can potentially be used in Chinese cabbage.

Agrobacterium tumefaciens-mediated transformation is one of the most widely genetic used engineering techniques and has great potential to improve established cultivars by introducing genes of interest while maintaining commercially desirable phenotypes (Jin et al., 2002; Lee et al., 2004a; Ryu et al., 1998). T-DNA as a mutagen has also been used to tag genes in *Arabidopsis* (Babichuk et al., 1997; Krysan et al., 1999). However, an analysis of T-DNA insertion sites showed unpredictable patterns of integration between the T-DNA border repeats and the flanking DNA (Kim et al., 2003; Van Der Graaff et al., 1996). *Agrobacterium*-mediated transformation often results in insertions of more than one copy of foreign DNA and binary vector backbone sequences (Denis et al., 1995; Kononov et al., 1997; Martineau et al., 1994; Ramanathan and Veluthambi, 1995; Wenck et al., 1997). Several studies have been performed to determine the mechanism of integration of two or more copies at the same site (De Buck et al., 1999; De Neve et al., 1997; Krizkova and Hrouda, 1998) and binary vector backbone transfer (De Buck et al., 2000; Martineau et al., 1994; Ramanathan and Veluthambi, 1995).

Plasmid rescue, one of several strategies used to determine the function of a target gene, is a simple method that can be used together with sequencing analysis of plant genomic DNA and

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isolation of T-DNA tagged chromosomal DNA segments (Yanofsky et al., 1990) to elucidate gene function. Integration patterns of T-DNA into a plant genome can be identified by the sequencing of rescued plasmids, even though the location of the T-DNA insertion site is very difficult to predict (Gustavo et al., 1998; Kim et al., 2003). Another strategy that has been developed to identify gene positions is inverse PCR (Tsuei et al., 1994). While this requires primers complementary to both termini of the target DNA, PCR can be carried out even if only one sequence is available from which primers may be designed. Compared with rescue cloning which requires multiple copies of T-DNA for mutagenesis, inverse PCR requires only a single copy for mutagenesis.

To screen for mutants, several traits including plant size, leaf morphology, leaf color, flowering, flower morphology, flower color, sterility, disease resistance, and stress tolerance were recorded (Butaye et al., 2004).

MATERIALS AND METHODS

Plant material and T-DNA tagging vector

Chinese cabbage 'Seoul' (Dong-bu Seed, Korea) was used for transformation. Seeds were sterilized in 70% ethanol for 1 min and 1% sodium hypochlorite (NaClO) for 20 min with vigorous shaking. After sterilization, they were rinsed five times with deionized water. We used the pRCV2 vector as described by Lee et al. (2003) (constructed by ligating pCAMBIA 1301 with pBluescript II KS(+)) for transformation of Chinese cabbage with *A. tumefaciens*.

Chinese cabbage transformation

The Chinese cabbage transformation procedure was similar to the method reported by Kim et al. (2006b). Surface-sterilized seeds were placed on sowing medium (MS basal medium, 3% sucrose, and 0.8% plant agar) and incubated in a culture room with a 16 h photoperiod at 22°C for 7-9 d. Hypocotyls were cut into 1 cm segments and placed on pre-culture medium (MS basal medium, 1 mg/L NAA, 4 mg/L BA, 4 mg/L $AgNO_3$, 10 mg/L acetosyringone, 200 mg/L cefotaxime, and 0.8% plant agar). The pre-cultured hypocotyls were inoculated with *Agrobacterium* suspension in MS liquid medium for 15-20 min. The inoculated hypocotyls were then placed on filter paper over cocultivation medium (MS basal medium, 3% sucrose, 0.8% plant agar, 1 mg/L NAA, 4 mg/L BA, 4 mg/L $AgNO_3$, and 10 mg/L acetosyringone at pH 5.8) and incubated for 3 d in the dark at 22°C. To remove the *Agrobacterium*, the explants were washed five times in liquid cocultivation medium supplemented with 200 mg/L cefotaxime and transferred to selective medium (MS basal medium, 3% sucrose, 1.6% bacto-agar, 1 mg/L NAA, 4 mg/L BA, 4 mg/L $AgNO_3$, 10 mg/L acetosyringone, 200 mg/L cefotaxime, and 5 mg/L hygromycin at pH 5.8). Calli that formed on the hypocotyls were subcultured on fresh selective medium that was replaced every 2 weeks until shoots regenerated. The regenerated shoots were transferred to rooting medium (1/2 MS medium, 3% sucrose, 0.8% plant agar, and 200 mg/L cefotaxime at pH 5.8) to induce root formation. The transformed plants were grown in a greenhouse after acclimatization. Then, acclimatized transformed plants were transferred to a cold room to induce flowering. In the cold room, the transformed plants were grown under long-day conditions (24 h photoperiod) at 4°C and 50 ± 10% humidity for 50 d. After cold treatment, the transformed plants were transplanted to a greenhouse.

Plant genomic DNA isolation and PCR confirmation for transformants

Leaves were harvested from all of the T_1 and T_2-progeny for PCR confirmation and subsequent DNA sequence analysis. Genomic DNA was isolated as described by Lee et al. (2004b). Chinese cabbage leaf tissue (1 mg) was ground in liquid nitrogen in a chilled mortar and pestle. Then, the fine powder was mixed with extraction buffer (0.5 M NaCl, 0.1 M Tris-HCl, 50 mM EDTA, and 1.25% SDS) and maintained at 65°C for 30 min. The mixture was extracted with phenol and chloroform and precipitated in isopropyl alcohol. The extracted genomic DNA was washed in 70% ethanol and dried. PCR was performed in a 20 μl reaction mixture with Maxime PCR PreMix kit (iNtRON biotechnology, Korea) with 35 cycles of 30 s of denaturation at 95°C, 30 s of annealing at 61°C, and 4 min of extension at 72°C. The primer sequences were 5′-TTT CCA CTA TCG GCG AGT AC-3′ and 5′-TGT CGA GAA GTT TCT GAT CGA-3′.

Total RNA extraction and real-time PCR

The leaf tissue (300 mg) was ground in liquid nitrogen using a pestle. The powder was mixed with RNA extraction buffer and acidic phenol. The solutions were separated into aqueous and organic layers by centrifugation. The aqueous layer was purified with 600 μl of phenol/chloroform. After centrifugation, one-third volume of 8 M LiCl was added and the mixture was kept at -20°C for 1 h. The pellets obtained after centrifugation were resuspended in DEPC-treated water, treated with one-tenth volume ammonium acetate, and then two volumes of ethanol was added. The precipitated RNA pellets were washed with 1 ml 70% ethanol, dried, and dissolved in RNase-free water. Real-time PCR was performed using a Rotor-Gene 6000 (Corbett, Australia) and a Mix One-Step Kit (Quantace, USA). The following reagents were combined for a 50 μl reaction mixture: 2× SensiMix one-step, 50× SYBR green solution, RNase inhibitor, 5 μM forward and reverse primers, and 500 ng total RNA. The PCR mixture was initially incubated at 42°C for 30 min followed by a 10 min enzyme activation step at 95°C. This was followed by 35 cycles of 10 s of denaturation at 95°C, 15 s of annealing at 60°C, and 20 s of extension 72°C. Fluorescence data were analyzed using the instrumentation software. The relative concentrations were calculated relative to reference using the $\Delta\Delta C_T$ values (Livak and Schmittgen, 2001).

Strategy for rescue cloning and/or inverse PCR

pBluescript II KS(+) contains a bacterial origin of replication (ori) and an ampicillin resistance gene to allow for the selection of antibiotic-resistant colonies during rescue cloning; this vector also contains an independent multiple cloning site. pCAMBIA 1301 contains a hygromycin resistance gene and a pUC18 multiple cloning site inside the T-DNA. To allow for recombination of these two vectors, they were double digested with *Bam*HI and *Hin*dIII and ligated (Fig. 1). For rescue cloning, if the gDNA was digested with *Bam*HI and ligated, the rescue plasmid would contain the ori and ampicillin resistance gene from pBluescript II KS(+), the β-glucuronidase (GUS) reporter gene, and the right border. If the gDNA was digested with *Hin*dIII, the rescue plasmid would contain the ampicillin resistance gene, ori, hygromycin resistance gene, and the left border. For inverse PCR, if *Bam*HI or *Eco*RI was used, the hygromycin resistance gene and left border would be obtained, while if *Hin*dIII, was used, the GUS reporter gene and the right border would be obtained (Fig. 2). Plasmid rescue was performed for transgenic plants with multiple T-DNA insertions and inverse PCR for single copy insertions. Overall, 74% of the flanking DNA was obtained by rescue cloning and 26% by inverse PCR.

Rescue cloning and inverse PCR

In each experiment, 10 μg of genomic DNA was digested with

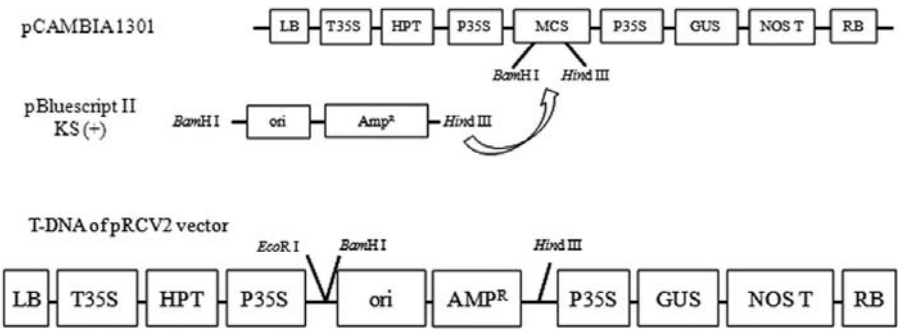

Fig. 1. Schematic map of pRCV2. LB, left border; T35S, 3′ signal of CaMV 35S; HPT, hygromycin resistance gene; P35S, CaMV 35S promoter; ori, pBR322 origin of replication; AMP^R, ampicillin resistance gene active in *E. coli*; GUS, β-glucuronidase gene; NOST, 3′ signal of nopaline synthase; RB, right border.

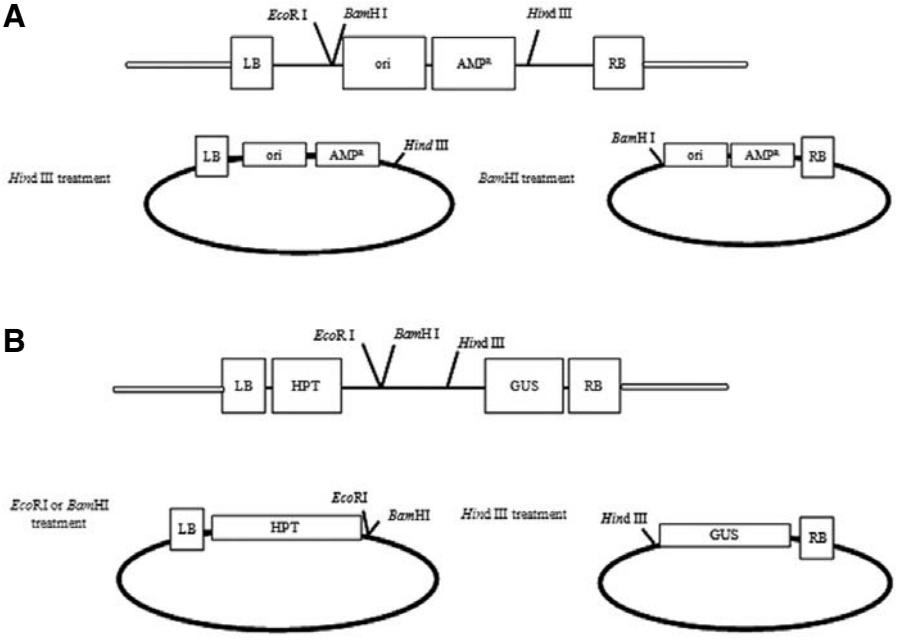

Fig. 2. Strategy for plasmid rescue and/or inverse PCR. After Southern blot analysis, plasmid rescue was performed for transgenic plants with multiple T-DNA insertions, and inverse PCR was performed for transgenic plants single insertions.

*Bam*HI (or *Eco*RI) or *Hin*dIII for 18 h. The digested DNA was then purified using a phenol-chloroform-isoamyl alcohol (25:24:1) solution, and the DNA pellet was dried in a vacuum. For rescue cloning, the dried DNA pellet was dissolved in deionzed water and then self-ligation was performed. Ligated DNA (50 ng) was transformed into SURE electroporation-competent *E. coli* cells (Stratagene, USA) following the manufacturer's instructions. To confirm that the rescue clones included the T-DNA region, they were digested with *Bam*HI (or *Eco*RI) and *Hin*dIII to confirm pBluescript II KS(+) (2960 bp). For inverse PCR, all amplifications were performed in a 50 μl reaction volume using a thermocycler (Biometra, USA). The reaction mixture comprised 5 units of *Ex-Taq* polymerase (Takara, Japan), 2.5 mM dNTPs, 10× *Taq* polymerase buffer containing $MgCl_2$, 10 pmol of each primer, and 50 ng gDNA. The samples were denatured initially at 95°C for 5 min, followed by 35 cycles of 30 s of denaturation at 95°C, 30 s of annealing at 58°C, and a 4 min extension step at 72°C. This was followed by a final extension step at 72°C for 10 min. The rescue clones and inverse PCR products were analyzed on a 1.0% agarose gel and were cloned into the pGEM-T Easy Vector (Promega, USA).

Sequencing analysis

Purified plasmids from the rescue clones were sequenced using adjacent plant flanking DNA primers. The inverse PCR products that were ligated into the pGEM-T Easy Vector were sequenced by Macrogen (Korea) using the SP6 and/or T7 primers. The sequencing results obtained were BLAST searched against the National Center for Biotechnology Information (NCBI) database (www.ncbi.nlm.nih.gov).

Construction of a genetic map by rescue clone RFLP

A JWF_3p and parent are used for genetic mapping. $F_{2:3}$ families (40 F_3 seedling) of 134 F_2 lines ("JWF_3p") were developed from Chinese cabbage F_1 cultivar Jangwon (*B. rapa* ssp. *pekinensis*). These two biennial inbred parent lines were made available courtesy of the former "Seoul Seed" company in Korea (Kim et al., 2006a). Parent polymorphism was screened using a blot with seven restriction enzymes (*Bam*HI, *Dra*I, *Eco*RI, *Hin*dIII, *Eco*RV, *Xba*I, and *Sca*I) and ^{32}P-dCTP-labeled rescue clone. Hybridization and RFLP analysis was followed the previous publication (Park et al., 2005). The linkage analysis and map construction were carried out using JoinMap 3.0 (Van Ooijen and Voorrips, 2001), as described previously (Kim et al., 2006a). This mapping information is shown in Table 1. Table 1 includes locus name, and the international chromosome number of *B. rapa* (http://www. brassica.info/resource/maps/lg-assignments. php), and location. The restriction enzyme used for the JWF_3p

Table 1. Construction of a genetic map of *B. rapa* by T-DNA rescue clones

Loci name	Chromosome number of *B. rapa*	Position	Restriction enzyme of a JWF3p blot	Subject ID	Putative function	Bit score	E-value
f841a	A1	57.9	*Eco*RI	At3g18810	Protein kinase family	280	2.00E-74
f613	A2	69.8	*Sca*I	At5g25610.1	Eukaryotic translation initiation factor 2	85.7	2.00E-16
f531	A2	109	*Eco*RV	At5g25610.1	Dehydration-responsive protein (RD22)	345	3.00E-94
f588	A3	7.2	*Eco*RI	No homology			
f841b	A3	52.5	*Eco*RI	At3g18810.1	Protein kinase family protein	280	2.00E-74
f888	A3	109	*Bam*HI	At2g37060.3	Similar to CCAAT-box binding transcription factor	65.9	4.00E-10
f608	A3	136.2	*Bam*HI	At3g05300.1	Cytidine/deoxycytidylate deaminase	93.7	2.00E-18
f874	A4	37.4	*Eco*RI	At2g22790.1	Expressed protein	184	6.00E-46
f621a	A4	91.4	*Sca*I	At2g43000.1	No apical meristem (NAM) family	309	2.00E-84
f1574b	A4	115.9	*Eco*RI	At1g25350.1	Glutamine-tRNA ligase	67.9	8.00E-11
f621b	A5	21.3	*Sca*I	At2g43000.1	No apical meristem (NAM) family	309	2.00E-84
f251a	A5	93.4	*Bam*HI	At1g51690.2	Serine/threonine protein phosphatase 2A	170	9.00E-42
f546	A7	61.5	*Eco*RI	At1g78895.1	Expressed protein	208	5.00E-53
f1155	A8	79	*Eco*RI	At4g25010.1	Nodulin MtN3 family protein	109	4.00E-23
f251b	A8	88.8	*Bam*HI	At1g51690.2	Serine/threonine protein phosphatase 2A	170	9.00E-42
f271	A9	45.3	*Eco*RV	At1g09020.1	SNF4, protein kinase	274	1.00E-72
f504	A9	53.4	*Bam*HI	At1g15750.4	Similar to WD-40 repeat family protein	236	1.00E-72
f1574a	A9	107.6	*Eco*RI	At1g25350.1	Glutamine-tRNA ligase	67.9	8.00E-11
f744	A10	42	*Eco*RI	At5g16910.1	Cellulose synthase family protein	222	3.00E-57

blot and subject ID, putative function, bit score, and e-value for rescue clone are also listed in Table 1.

In silico mapping by rescue clone sequences

Extensive sequence comparison with the rescue clone sequences to the *B. rapa* bacteria artificial chromosome (BAC) sequence database (the last total BAC 01/12/2009 updated in the internal site of NIAB, Korea; unpublished data) was performed. BLASTs were performed using whole rescue clone sequences, and the results were summarized in an Excel spreadsheet. These sequence homology results were pared to an e-value less than -20 and the highest homology result per query to the corresponding BAC and then sorted by each chromosome and mapping locus. The query ID represents the name of the rescue clone and the subject ID is the contig number of each BAC sequence of *B. rapa*. These comparisons are shown by the percent identity, alignment length, and mismatches in the comparison region, as well as the sequence comparison region identified by q. start, q. end, s. start, and s. end. The e-value and bit score per comparison are described in Supplementary Table S1. The genetic mapping information was annotated using the BAC anchoring information based on JWF_3p.

RESULTS AND DISCUSSION

BLAST search of flanking DNA

For the plasmid rescue and inverse PCR experiments, 10 μg of genomic DNA was digested with *Bam*HI or *Hin*dIII to rescue the sequences adjacent to the right T-DNA border or the left T-DNA border, respectively. The T-DNA of the pRCV2 vector only has one *Bam*HI or *Hin*dIII cleavage site, and the ampicillin resistance gene is not cleaved by these restriction enzymes. The rescued clones and inverse PCR products were sequenced with sequencing primers adjacent to the near flanking DNA and T-DNA, respectively. The obtained nucleotide sequences were analyzed by BLASTN homology searches of the NCBI database. The results of the BLASTN searches are provided in Table 2. Although 9 of 13 flanking DNA sequences did not have significant similarity to other sequences in the NCBI database, M8, M9, M11, and M17 had significant matches to the Chinese cabbage EST contigs 'KHCT-23H02', 'KFPC-010G11', 'KFFB-099E12', and 'KBFS-073A07', respectively (Table 3). Because the Chinese cabbage EST database is not yet completed, the remaining nine flanking DNAs are expected to be T-DNA that has been inserted into the genome. We also anticipate that the remaining nine flanking DNA sequences are most likely derived from ESTs that have not been fully characterized. The gDNA deleted by insertion of T-DNA was also classified according to cellular component, molecular function, and biological process categories using the gene ontology (GO) tool at TAIR (www.arabidopsis.org). The flanking DNA was classified into "other intracellular components" and "plasma membrane" in the cellular component category, "transferase activity", "protein binding", and "kinase activity" in the molecular process category, and "other cellular processes" and "other metabolic processes" in the biological process category (Fig. 3).

T-DNA integration and transcription level of T-DNA inserted genes

To determine the copy number of pRCV2 T-DNA inserted into the mutant lines, Southern blots were conducted. Overall, 43% of the lines had one copy inserted, 21% had two copies inserted, 17% had three copies inserted, and 19% had more than three copies inserted. This demonstrates that *Agrobacterium*-mediated transformation can be used for low copy-number T-DNA insertion into Chinese cabbage (Fig. 4A). In addition, the frequency of T-DNA integration in the exonic and intronic regions of the *B. rapa* genome was 40% and 23%, respectively

Table 2. BLAST search results of flanking DNA of mutant lines

No.	Method	Sequence primer	gi No.	Gene
gM1	Rescue	sM1H-IP19R	gi|7209734	*Arabidopsis thaliana* genomic DNA, chromosome 3, BAC clone:F5N5
gM5	IPCR	sM5B-T7	gi|7657870	*Brassica oleracea* S-12 SRK gene for S-locus receptor kinase, complete cds
gM6	Rescue	sM6H-IP19R	gi|5302794	*Arabidopsis thaliana* DNA chromosome 4, ESSA I FCA contig fragment No. 6
gM7	Rescue	sM7H-IP19R	gi|38196008	*Brassica oleracea* clone BAC Bo37N21, partial sequence
gM8	Rescue	sM8B-Gusi2	gi|20197505	*Arabidopsis thaliana* chromosome 2 clone F3N11 map B68, complete sequence
gM9	Rescue	sM9H-IP18	gi|7270470	*Arabidopsis thaliana* DNA chromosome 4, contig fragment No. 83
gM11	Rescue	sM11H-IP18	gi|21405806	*Arabidopsis thaliana* RHA3B; ubiquitin-protein ligase/ zinc ion binding (RHA3B) mRNA, complete cds
gM13	Rescue	sM13H-IP19R		Unknown
gM17	Rescue	sM17H-IP18	gi|5281015	*Arabidopsis thaliana* DNA chromosome 4, ESSA I FCA contig fragment No. 4
gM18	Rescue	sM18H-IP19R	gi|395073	*B.napus* DNA for myrosinase
gM19	Rescue	sM19H-IP19R	gi|395073	*Brassica rapa* subsp. *pekinensis* clone KBrH080C09, complete sequence
gM20	Rescue	sM20-IP19R		*Oryza sativa* (japonica cultivar-group) genomic DNA, chromosome 2, complete sequence
gM22	Rescue	sM22B-M2	gi|6782244	*Arabidopsis thaliana* DNA chromosome 3, BAC clone T3A5

Table 3. Comparison of flanking DNA of mutant lines with Chinese cabbage ESTs

No.	Matched EST contig	E-value	Library name	Bit score	% identity	Matched length	Compared length
gM8	KHCT-23H02	2.00E-52	Cotyledon, in greening stage (5′)	206	100%	104	104
gM9	KFPC-010G11	0.0	Leaf (3-weeks-old) (5′)	593	97%	640	657
gM11	KFFB-099E12	0.0	Floral bud & open flower (5′)	567	98%	591	599
gM17	KBFS-073A07	E-150	Floral bud, < 2mm in size (5′)	269	100%	269	269

(Fig. 4B). The T-DNA insertion rate in exons and introns of the rice genome has been reported to be 17.4% and 40.7%, respectively (Chen et al., 2003), and the T-DNA insertion rate in exons and introns of the *Arabidopsis* genome has been reported to be 22% and 13.4%, respectively (Szabados et al., 2002). Our results suggest that the T-DNA inserted by *Agrobacterium*-mediated transformation is integrated into the genome at random and is not concentrated in any particular region. A Total of flanking DNA sequences obtained from the rescued clones and inverse PCR products showed high homology to *A. thaliana, B. rapa*, *B. napus*, *B. oleracea*, *Oryza sativa*, and *B. juncea*, in order of sequence homology (Fig. 4C). Although a large percentage of the flanking DNA that we obtained had no significant homology to any sequences in the NCBI database, we anticipate being able to identify the sequences in the near future as more genome sequences become available. The expression level of the deleted genes was analyzed by real-time PCR in the mutant and non-transgenic lines. Actin was used as the housekeeping gene, as expression of the actin gene was found to be uniform between the lines. The expression level of three mutant lines is shown in Table 4. As expected, the expression of the disrupted gene in the three mutant lines was decreased compared to the WT. While the expression of not all of the disrupted genes was decreased, we hypothesize that other copies of the disrupted gene may have compensated for the disrupted gene (data not shown).

The ambiguous phenotype though happen of > 60% single insertion events in genome redundancy of *B. rapa*

The *Brassica* genome was triplicated approximately 15 to 18 million years ago and the triplicate *Brassica* chromosomes have maintained sequence-level co-linearity with the homologous *Arabidopsis* chromosome region, with concerted deletions (about 50% of triplicated genes) and a few insertions (about 9% of genes) in the diploidized *B. rapa* genome (Yang et al., 2006). The previous JWF_{3p} genetic map of *B. rapa* (Kim et al., 2006a) reported an average of 1.31 loci per probe (520/396) and mainly determined in triplication of inter chromosomal duplication comparison with the same DNA markers loci in *B. rapa* genome. In our study, base on the Southern analysis using the hygromycin gene probe, approximately 43% of the T-DNA insertions were the result of a single copy insertion into the *B. rapa* genome. Although single insertions occur at a high rate, phenotype variants could not be identified as a result of some gene knockouts. Because of gene redundancy due to the genome triplication events in *Brassicecae*, the chance of a single insertion happening in a single gene locus resulting in a distinguishable phenotype variant in the greenhouse and field is very rare.

Extensive genome characterization of T-DNA insertion mutagenesis by genetic mapping of *B. rapa*

The Multinational *Brassica* Genome Project (MBGP) and *B. rapa* Genome Sequencing project (BrGSP) aim to completely sequence the genome of *B. rapa* (www.brassica.info). Korea sequenced the biggest two chromosomes of cytogenetic chromosome 1 (A9) and 2 (A3) in 2004, which were mostly sequenced by shot-gun sequencing with 85-95% coverage (www.brassica-rapa.org). Approximately 1,800 BAC clone sequences are available on this internal and external website. Extensive

Table 4. Real-time PCR analysis of mutant and wild type (WT) lines by $\Delta\Delta C_T$ analysis

Line		GOI C_T[a]	Norm. C_T[b]	Delta C_T	Delta-delta C_T	Relative concentration
g267	WT	14.91 ± 0.07	11.43 ± 0.14	3.50 ± 0.21	0.00 ± 0.00	1.00 ± 0.00
	mutant	16.57 ± 0.08	10.93 ± 0.15	5.64 ± 0.23	2.15 ± 0.02	0.23 ± 0.01
g899	WT	17.55 ± 0.21	19.66 ± 0.14	-2.11 ± 0.36	0.00 ± 0.00	1.00 ± 0.00
	mutant	18.67 ± 0.05	19.96 ± 0.15	-1.29 ± 0.21	0.81 ± 0.15	0.57 ± 0.06
gM18	WT	7.63 ± 0.00	11.43 ± 0.14	-3.80 ± 0.16	0 ± 0.00	1.00 ± 0.00
	mutant	13.55 ± 0.01	11.74 ± 0.18	1.81 ± 0.18	5.61 ± 0.03	0.02 ± 0.00

[a]Gene of interest analysis
[b]Normalizer gene

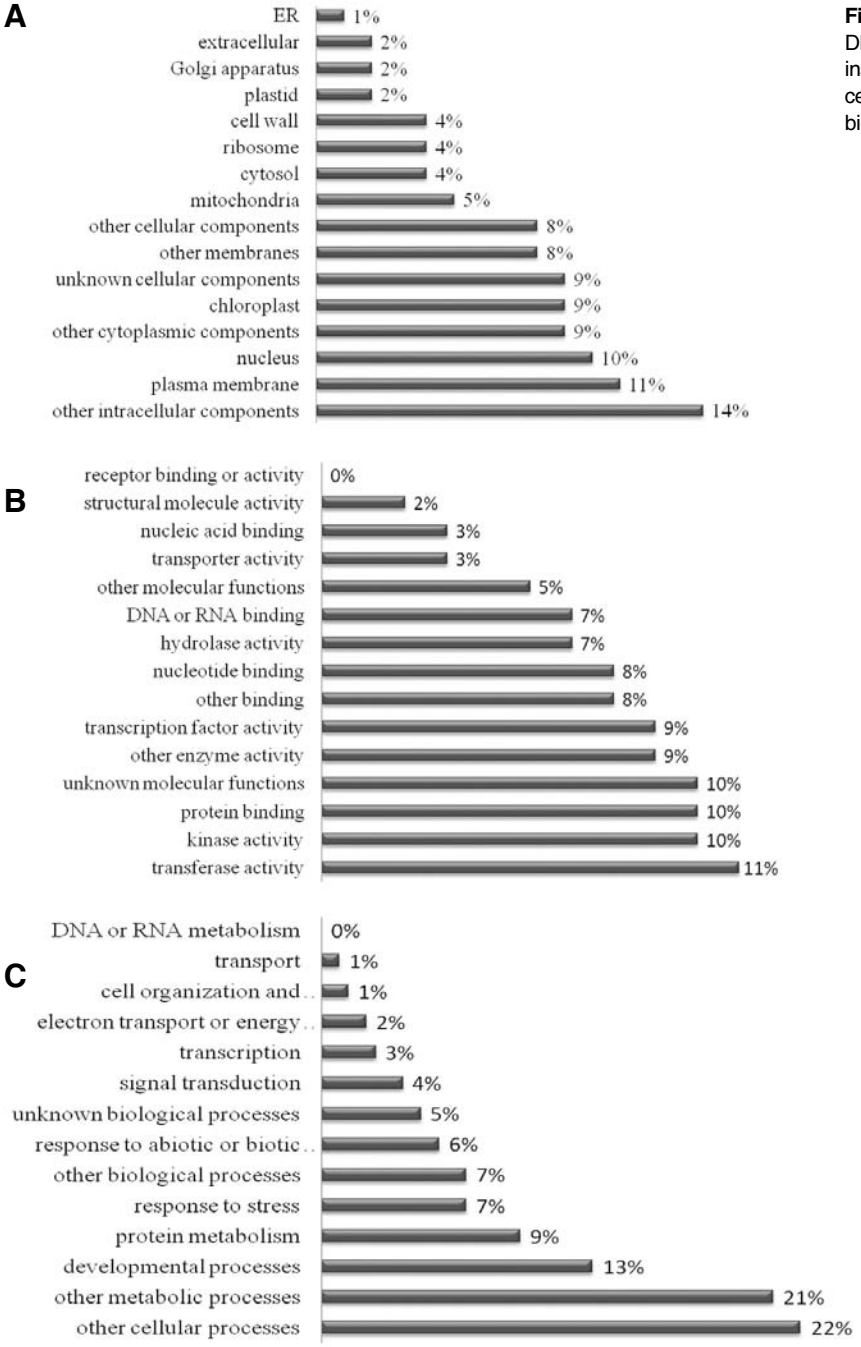

Fig. 3. Gene ontology analysis with flanking DNA sequences at TAIR. The gDNA deleted by insertion of T-DNA was classified according to cellular component, molecular function, and biological process categories.

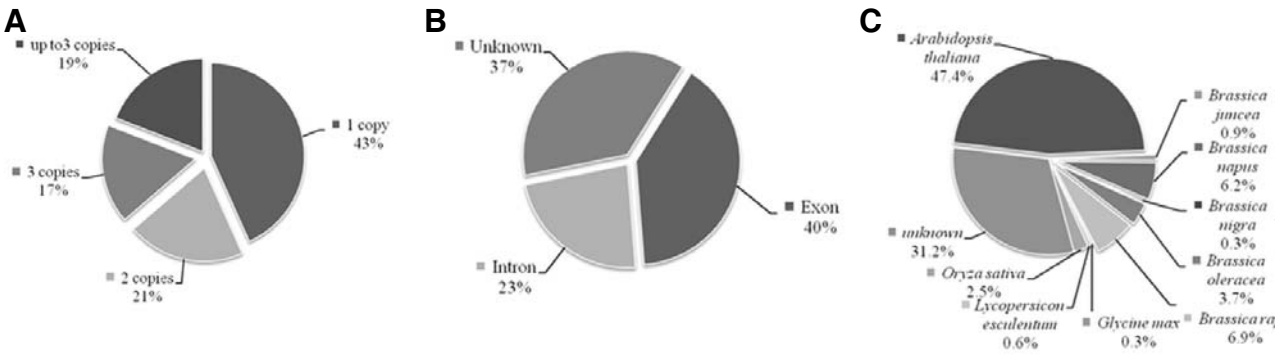

Fig. 4. T-DNA copy number in the *B. rapa* genome and homology search using flanking DNAs. (A) Analysis of T-DNA copy number inserted into Chinese cabbage. (B) Distribution of T-DNA insertions in the genome. (C) Homology analysis of flanking DNA sequences.

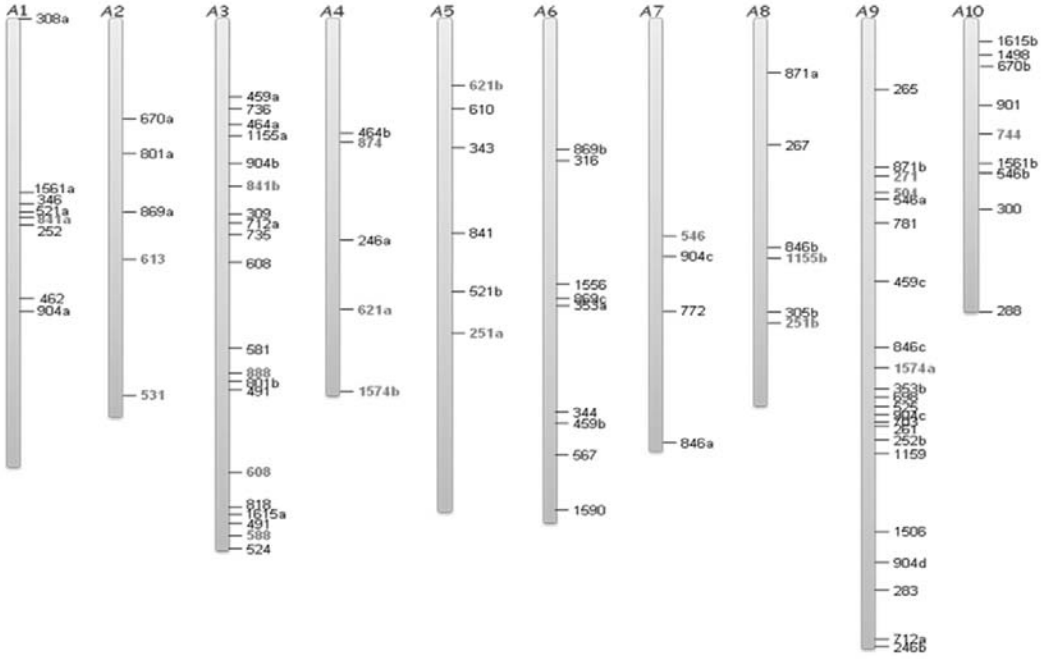

Fig. 5. RFLP and in silico genetic mapping of loci with rescue clones and sequences from *B. rapa* by T-DNA tagging mutagenesis. A1 to A10 correspond to each chromosome of *B. rapa*, A3 and A9 are the biggest chromosomes and are assigned as cytogenetic chromosome 2 and 1, respectively. The red loci are the JWF_3p segregating genotype ratio by Southern analysis and the black loci are the sequences homologous to the *B. rapa* BAC clone sequence database (www.brassica-rapa.org) with e < -20.

genetic mapping with the rescue clones was carried out by Southern analysis with the rescue clones themselves or by comparisons of the rescue clones sequences with the *B. rapa* BAC clone sequence database (www.brassica-rapa.org). As a result of genetic mapping, the T-DNA tagging mutagenesis in *B. rapa* was evenly conducted to 10 chromosomes, according to the chromosome size of *B. rapa* (Kim et al., 2006a; Lim et al., 2005; 2007). The A3 and A9 chromosomes have been sequenced by our group, and there are 20 loci in A3, 22 loci in A9, and 63 loci in the remaining 8 chromosomes (Fig. 5). We determined the genetic loci in the *B. rapa* mutants using two methods: RFLP of the rescue clones themselves and sequence homology analysis to the *B. rapa* sequence database (www.brassica-rapa.org). There is a lack of complete genome sequence information for *B. rapa*, with the information available representing 1,548 BAC sequences with an average size of a 126 kb (37% coverage) (Park et al., 2005) when the *B. rapa* genome size is supposed to be 529Mb (Johnston et al., 2005). The in silico mapping of rescue clone 871, which mainly hybridized as three copies, identified two genetic loci on A8 and A9 (Fig. 6B). The rescue clones, 736 and 744 showed onto two copies and they were mapped to one locus each. These in silico mapping result was described in the Supplemental Table S1, and the putative exon and intron regions of *B. rapa* for each comparison are shown. The T-DNA insertion mutants generated here will aid in the investigations of particular genomic regions of *B. rapa*.

Phenotype screening of mutant lines

The T_1 progenies were sown from ten seeds of each mutant line, selected by PCR, and transplanted to a greenhouse. Control untransformed plants were grown at the same time. After 3 months of growth in the greenhouse, a phenotypic analysis was conducted. Flower types of the 'Seoul' wild type have four petals,

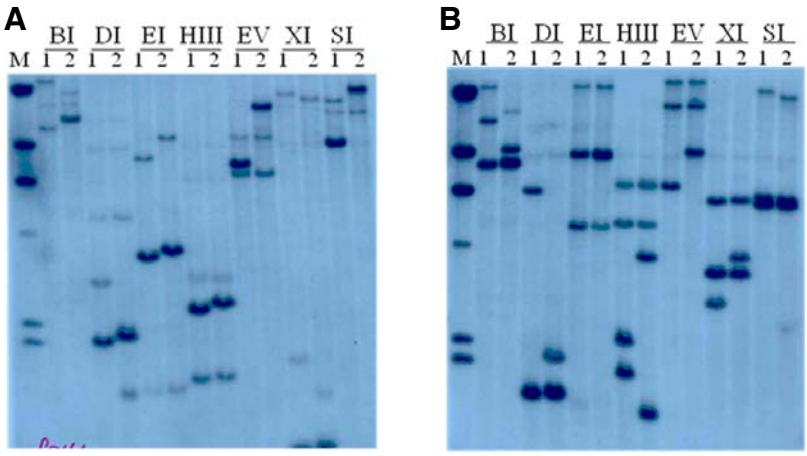

Fig. 6. Southern blot analysis with rescue clones 841 and 871. The DNA of two parental *B. rapa* were digested with *Bam*HI (BI), *Dra*I (DI), *Eco*RI (EI), *Hin*dIII (HIII), *Eco*RV (EV), *Xba*I (XI), or *Sca*I (SI); M, λ *Hin*dIII DNA marker; Lane 1, one parent of *B. rapa*; lane 2, the other parent of *B. rapa*. (A) A blot hybridized with rescue clone 841 detected two polymorphisms in a *Sca*I digest, and this polymorphism was mapped to A1 and A3 by segregating a population hybridization blot with the *Sca*I digest. (B) A blot was hybridized onto two or three copies and this 871 rescue clone was mapped to A8 and A9 by in silico mapping.

Fig. 7. Floral characteristics of mutant lines. (A) Wild type. (B) Five-petaled flower. (C) Dragonfly wing–shaped flower. (D) Dark yellow and wrinkled petals. (E) Warped crossed flower.

Fig. 8. Various phenotypes of mutant lines. (A) Wild type. (B) Deeply wrinkled leaves. (C) Long leaves. (D) Small leaves (rosette type). (E) Small and round leaves.

six stamens, are yellow, and have a cross-like and flat flower shape. The mutant lines demonstrated various phenotypes including: (i) a five-petaled flower, (ii) a dragonfly wing-shaped flower, (iii) dark yellow and wrinkled petals, and (iv) warped crossed flowers (Fig. 7). In addition, 30 T_2 progeny lines that demonstrated abnormal flower phenotypes in the T_1 generation were vernalized, and progeny tests were performed. Most, but not all T_2 plants retained their phenotypic characteristics when flowering. During the ripening stage, several abnormal forms were also observed: (i) deeply wrinkled leaves, (ii) long leaves, (iii) small leaves (rosette type), and (iv) small and round leaves (Fig. 8). Ten seeds from all T_1 lines were sown to propagate the T_1 lines, and the confirmed transformants were then transplanted in the greenhouse for phenotype screening. Some of the transgenic plants showed partial chlorosis or albino symptoms, were hairless, and had narrow leaves; these weak plants with yellowish-green outer leaves withered and later died. The remaining mutants had undulatory, rounded, sinus-like, lustrous, and uneven leaves.

Note: Supplementary information is available on the Molecules and Cells website (www.molcells.org).

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