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ORIGINAL ARTICLE

Development of a highly effective T-DNA inserted mutant screening method in a Chinese cabbage (*Brassica rapa* L. spp. *pekinensis*) reverse genetics system

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Abstract We present a highly effective T-DNA inserted gene screening method as part of a reverse genetics model system using the Chinese cabbage (*Brassica rapa* L. spp. *pekinensis*). Three-step two-dimensional (2D) matrix strategies are potentially accurate and useful for the identification of specific T-DNA inserted mutants from a large population. To construct our Chinese cabbage model, we utilized a forward genetics screening approach for the abnormal phenotypes that were obtained from transgenic plants of *Brassica rapa* generated with *Agrobacteria tumefaciens* containing the pRCV2 vector. From one transgenic plant with an abnormal phenotype, we observed that the *st1* gene (which is related to senescence-associated process proteins) contained a T-DNA fragment, and that its expression level was decreased. This T-DNA insert was then used as a control to construct an effective screening pool. As a result, the optimum template concentration was found to be 0.1–1 ng in our PCR strategy. For other conditions, positive changes to the Gibbs free energy prevented the formation of oligo dimers and hairpin loop structures, and autosegment extension gave better results for long fragment amplification. Using this effective reverse genetics screening method, only 23 PCR reactions were necessary to select a target gene from a pool of 100 individual DNAs. Finally, we also confirmed that the sequence we obtained from the above method was identical to the flanking sequence isolated by rescue cloning.

Keywords Chinese cabbage · DNA pool · Functional genomics · Insertional mutagenesis

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Introduction

Reverse genetics is one of the approaches used to elucidate gene function, and is readily performed in conjunction with DNA sequencing protocols. *Arabidopsis thaliana* is one of the best organisms to use in such functional genetics studies, and a number of key genes have indeed been identified by constructing insertion mutants in *Arabidopsis*. An alternative organism is *Caenorhabditis elegans*, which is conducive to random DNA deletions in order to screen for genes of interest. Moreover, deletions have been created in every nonessential gene in the yeast genome. Another reverse genetics technique involves the application of RNA interference, which has been used to interfere with the expression of most genes in *C. elegans*. Reverse genetics strategies also include the creation of transgenic organisms that overexpress a gene of interest, which can reveal its function. Recently, genome information has been used more widely in gene function studies, but this presents considerable research challenges.

The Chinese cabbage (*Brassica rapa* L. spp. *pekinensis*) is one of the most important vegetables in the *Brassicaceae* family and is widely cultivated in Asia, particularly in China, Japan, and Korea. The multinational *Brassica* Genome Project (MBGP) and *Brassica rapa* Genome Sequencing Project (BrGSP) are ongoing and are expected to develop effective transformation systems for the large-scale production of *Agrobacterium tumefaciens*-mediated T-DNA transformations (Lee et al. 2004; Yang et al. 2005). As a result, ourselves and others are seeking to develop new and effective reverse genetics strategies. Effective PCR design is essential in order to properly screen a DNA pool in order to individual transgenic plants. In our current study, we focused on the Gibbs free energy (ΔG) to optimize our PCR method and produce larger fragments. This in turn will

optimize the calculation of the oligonucleotide nearest-neighbor (NN) thermodynamics and facilitate the application of autosegment extension in the PCR program. The autosegment extension strategy was one of the seminal strategies used to enable long PCR analysis (Mukai and Nakagawa 1996; Ohler and Rose 1992).

The sulfurtransferases (STs) are a group of enzymes widely added to plants, animals, and bacteria that catalyze the transfer of a sulfur atom from a donor molecule to a thiophilic acceptor (Alphey et al. 2003). Members of the ST family include thiosulfate:cyanide ST (rhodanese, EC2.8.1.1), 3-mercaptopyruvate:(di)thiol ST (EC 2.8.1.2), thiosulfate:thiol ST (EC 2.8.1.3), and thiosulfate:dithiol ST (EC 2.8.1.5). These enzymes are closely related, both in terms of the type of reaction catalyzed and in their primary amino acid sequences. Several physiological roles have been proposed for the STs, including cyanide detoxification, management of the cytotoxicity of reactive oxygen species in aerobic tissues, sulfur metabolism, and mobilization of sulfur for Fe–S cluster biosynthesis or repair. Because it has also been shown that the expression and enzyme activity of STs increases with increasing age of the *Arabidopsis* plant, one could postulate that STs play a role in mobilizing sulfur from senescent leaves into growing leaves, fruits, and seeds (Meyer et al. 2003).

We herein present an optimized PCR strategy through which we have devised a highly effective T-DNA insertion mutant screening method using a *Brassica rapa* reverse genetics model system.

Materials and methods

Binary vectors, plant materials, and forward screening

The transgenic Chinese cabbage *Brassica rapa* L. subsp. *pekinensis* var. Seoul (Dong-bu Seed, Korea), the *Agrobacterium tumefaciens* (LBA4404)-mediated transformation method, and the large-scale production of transgenic commercial F_1 hybrids were used in this study. The T-DNA tagging vector, pRCV2 (Kim et al. 2003), generated from a ligation of pCAMBIA 1301 and pBluescript II KS(+) (Stratagene, La Jolla, CA, USA) is 14,730 bp in size and contains a *hpt* (hygromycin resistance gene), ampicillin resistance gene, and GUS (beta-glucuronidase reporter gene) cassette, which were used to generate approximately 5,000 transgenic plants (Lee et al. 2004). Seeds that produced T_1 transgenic generations via the RCV2 tagging method were sown into a plug tray in a greenhouse. Phenotype screening of the plants was performed at seven weeks of age.

Plant genomic DNA isolation and adjustment of concentration

Plant genomic DNA (gDNA) was isolated as described previously by McCouch et al. (1998). Briefly, approximately 0.5 g of Chinese cabbage leaf tissues, harvested at six weeks of age, were ground with liquid nitrogen in a chilled mortar and pestle to create a purer plant gDNA preparation. The ground fine power was then mixed with extraction buffer (0.5 M NaCl, 0.1 M Tris–Cl/pH 8.0, 50 mM EDTA and 1.25% SDS) and kept at 65°C for 20 min. The mixtures were extracted with phenol:chloroform:isoamyl alcohol, and gDNA was precipitated using isopropanol. The gDNA was then washed in 70% ethanol and dried at room temperature for 18 h. Finally, the pellets were dissolved in TE buffer (pH 8.0) and adjusted to concentrations of 100, 25, and 5 ng for subsequent systemic, three-step two-dimensional (2D) matrix strategies (screening from DNA pools of *Brassica rapa*), using a NanoDrop® spectrophotometer (Thermo Scientific, Wilmington, DE, USA).

Rescue cloning and Southern blotting

An examination of mutant phenotypes was performed to confirm the T-DNA insertion sites within the genome. *Bam*HI and *Hin*dIII have one recognized site within the T-DNA of pRCV2, but do not cleave the ampicillin resistance gene. Accordingly, the gDNA of transgenic Chinese cabbage was digested, self-ligated at 16°C for 12–16 h, transformed by electroporation, and sequenced (Macrogene, Seoul, Korea). *E. coli* SURE® Electroporation-Competent Cells (Stratagene) were used, and the sequencing results obtained were subjected to a BLAST search using the National Center for Biotechnology Information (NCBI) database. To confirm integration of the T-DNA into the plant genome, Southern blot analysis was conducted using the modified Southern neutral transfer method (Sambrook and Russel 2001; Southern 1975).

Total RNA extraction and real-time PCR

Total RNA was extracted from the leaf tissues of transgenic plants. Briefly, 200 mg of leaf tissue were ground with liquid nitrogen and extracted using the RNeasy® Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Real-time PCR quantification was then performed on a Rotor-Gene™ 6000 (Qiagen, Doncaster, Australia), and PCR was performed using the SensiMix One-Step kit (Quantance SYBR green). The primers used were 5′-GTT TCT GTT GAC TGG CTC CAT-3′ and 5′-TCT GAA CAT CCA CCA TAC ACG-3′ for the *st1* gene and 5′-GGT ATT GTA AGC AAC TG G GAT-3′ and

5′-TCA CCA GAG TCA AGC ACA ATA-3′ for the housekeeping gene from Chinese cabbage. The samples were incubated initially at 49°C for 30 min and then for a further 10 min at 95°C to inactivate the RNase, followed by a 15 s annealing step at 59°C, and a 20 s extension step at 72°C. Melting analysis was performed for each PCR product at the rate of 0.5°C/s at 45–95°C. Fluorescence intensity data were collected at the end of each cycle and analyzed using the instrument's software. The delta–delta C_T values were calculated relative to the reference PCR values (Livak and Schmittgen 2001).

Optimization of PCR strategy

The control reaction for the *st1* (mercaptopyruvate sulfurtransferase 1) mutant line harboring a T-DNA insertion in a *Brassica rapa* subsp. *pekinensis* clone KBrB007M04

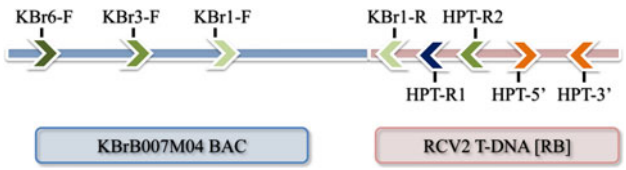

Fig. 1 Primer design strategy for PCR-based large-scale screening using *Brassica rapa* subsp. *pekinensis* clone KBrB007M04. 1-kb Fragment experiment I: KBr1-F and KBr1-R; 1-kb fragment experiment II: KBr1-F and HPT-R1; 1-kb fragment experiment III: HPT-5′ and HPT-3′; 3-kb fragment: KBr3-F and HPT-R2; 6-kb fragment: KBr6-F and HPT-R2

background was analyzed using the BrGP database (*Brassica rapa* Genome Project, http://www.brassica-rapa.org), and was used as a control line for the optimized PCR strategy. To optimize the PCR method for *Brassica rapa*, we employed a gDNA pool with a T-DNA left border and used three kinds of gene-specific primers that were designed to identify 1, 3, and 6 kb flanking DNA fragments (Fig. 1). To maximize the reaction effectiveness, the PCR primer design incorporated a calculation of the Gibbs free energy (ΔG) for dimer hybridization and hairpin formation during the annealing step. The PCR primers were then analyzed using the Vector NTI oligo-calculator (VectorNTI Advance™; Invitrogen, Carlsbad, CA, USA) (Table 1). To confirm the changes in the Gibbs free energy and to determine the efficacy and stability of the result, three different Gibbs free energy (ΔG) primer pairs were examined. The experiments were designed to retain constitutive pairs of primers by adopting $\Delta G < 0$ in experiment I, but using $\Delta G \geq 0$ conditions in experiment II with reverse primers. The control reaction involved the use of positive ΔG primer sets to detect the hygromycin resistance gene in the pRCV2 plasmid.

PCR was performed with 10, 1, 0.1, and 0.01 ng of gDNA and the following primer pairs: 1-kb fragment, experiment I: KBr1-F, 5′-GGGAATAAGAA-GCGGTCATATC-3′, KBr1-R, 5′-TCCTATAGGGTTTCGCTCATGT-3′; 1-kb fragment, experiment II: KBr1-F, 5′-GGGAATAAGAAGCGGTCATATC-3′, HPT-R1, 5′-CGTCCG-AGGGCAAAGAAATAG-3′; 1-kb fragment, experiment III:

Table 1 Primers used in this study and their energetic properties

Size (kb)	Primer	Sequence	ΔG	ΔH	ΔS	Dimer ΔG	Hairpin ΔG
1	KBr1-F[c]	5′-GGGAATAAGAAGCG GTCATATC-3′	−35.9[a]	−171.4	−448.4	2.6	2.2
	KBr1-R[b]	5′-TCCTATAGGGTTTCG CTCATGT-3′	−35.8	−168.2	−438.0	−7.7	–
	HPT-R1	5′-CGTCCGAGGGCAAA GAAATAG-3′	−37.6	−171.4	−442.8	–	–
	HPT-5′	5′-TTTCCACTATCGGCG AGTAC-3′	−32.7	−153.4	−398.9	2.1	1.2
	HPT-3′	5′-TGTCGAGAAGTTTC TGATCGA-3′	−31.9	−146.9	−379.6	−0.1	−2.2
3	KBr3-F	5′-CATCGGTTTGGTCA TCACATTTGAACAC-3′	−45.6	−200.7	−514.3	1.5	2.6
6	KBr6-F	5′-GGGAAGCTTCTTTT CTGTTGCAATGACA-3′	−47.6	−212.1	−545.6	1.7	3.9
3 6	HPT-R2	5′-AGCACTCGTCCGAG GGCAAAGAAATA-3′	−47.2	−208.2	−534.0	−1.8	–

[a] kcal/mol

[b] Reverse primer

[c] Forward primer

HPT-5′, 5′-TTTCCACTATCG-GCGAGTAC-3′, HPT-3′, 5′-TGTCGAGAAGTTTCTGATCGA-3′; 3-kb fragment: KBr3-F, 5′-CATCGGTTTGGTCATCACATTTGAACA C-3′, HPT-R2, 5′-AGCACTCGTCCGAGG-GCAAAGA AATA-3′; 6-kb fragment: KBr6-F, 5′-GGGAAGCTTC TTTTCTGTTGCAATG-ACA-3′, HPT-R2, 5′-AGCACTC GTCCGAGGGCAAAGAAATA-3′. Amplifications were performed in a reaction mixture (final volume: 20 µl) with Takara EX *Taq* (Takara, Shiga, Japan) and a thermocycler (Biometra, Goettingen, Germany). The 1-kb fragment experiment I, II, and III samples were initially denatured at 94°C for 2 min, followed by 40 cycles of a 20 s denaturation at 95°C, 40 s of primer annealing at 59°C, 1.5 min of extension at 72°C; and a final extension at 72°C for 5 min. In addition, long PCR reactions (for the 3- and 6-kb fragments) were compared for efficacy using autosegment extension and a normal PCR program. The autosegment extension PCR program was performed for 40 cycles. The initial denaturing step was set at 94°C for 2 min; during cycles 1–24, denaturation was at 95°C for 20 s, with 15 min of primer annealing and synthesis at 71°C, using the auto-extended cycle; after the 25th cycle, the extension time was increased by 15 s for each cycle, with a final extension at 72°C for 15 min. The normal PCR program for denaturation was initially 94°C for 2 min, followed by 40 cycles of 95°C for 15 s for denaturation and 71°C for 15 min for annealing and extension, with a final extension at 72°C for 15 min. The PCR products were analyzed on an ethidium bromide-stained agarose gel (1.0%) (Mukai and Nakagawa 1996; Ohler and Rose 1992).

Construction of a DNA pool for reverse genetics

The DNA pool constructed in these experiments used a three-step two-dimensional (2D) matrix strategy that potentially allows for the accurate and useful identification of individual transgenic Chinese cabbages from a large population. In the *Brassica rapa* reverse genetics system model, the DNA pool consisted of 98 mutant lines, a positive control (a transgenic line with the pRCV2 plasmid), and a negative non-transgenic control line. The three-step two-dimensional (2D) matrix strategy was as follows: (1) hyperpool screening by combining a DNA sample from each of the 100 mutants in a single tube at 100 ng/µl; (2) screening of a superpool of DNA from 25 mutants in a single tube at 25 ng/µl; and (3) screening of a subpool of DNA from five mutants in one tube at 5 ng/µl (Fig. 2).

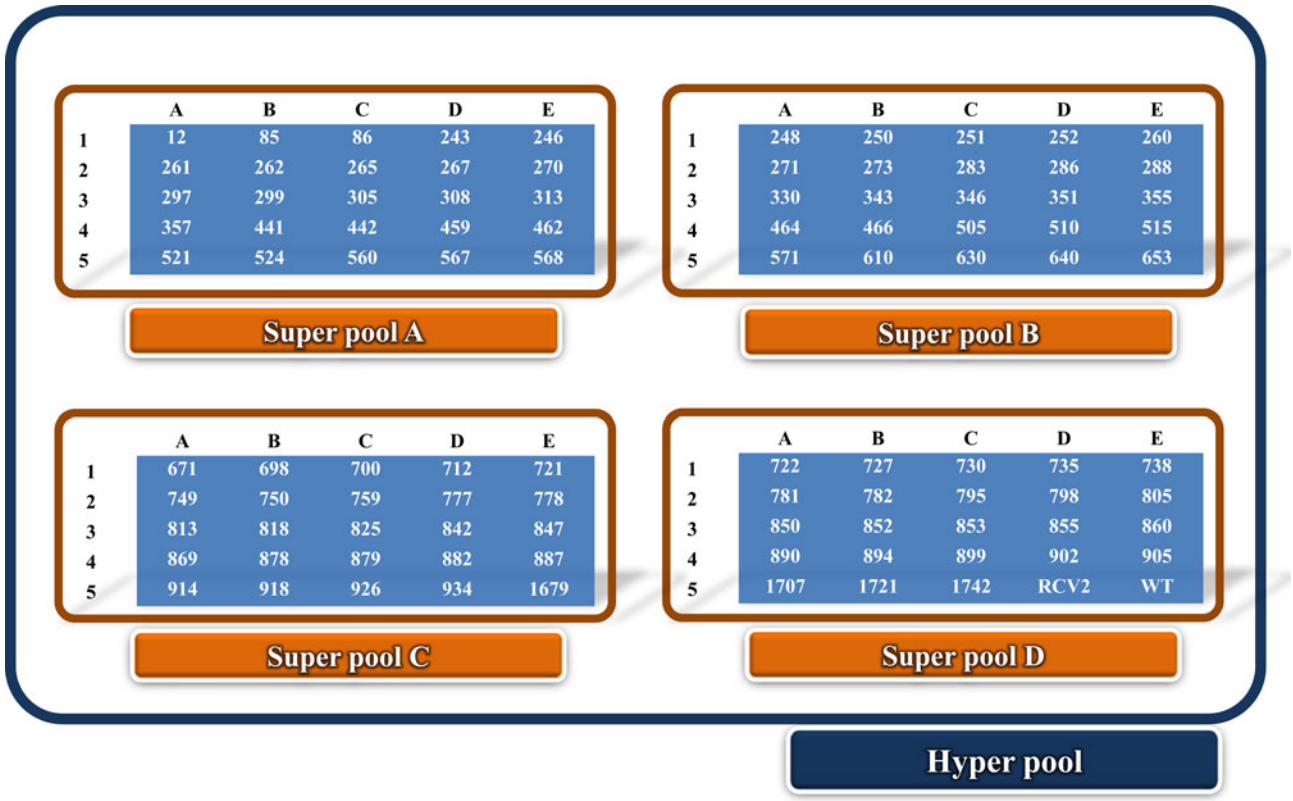

Fig. 2 Construction of the RCV2 T-DNA insertion mutant pool used in this study

Control reactions in the DNA pool screening

The identification of individual transgenic plants from a DNA pool was conducted using the aforementioned PCR strategy. This was performed with hyper-, super-, and su-pools and using the optimized forward primer KBr1-F (22mer) and reverse primer HPT-R1 (21mer), which amplify an 1,187-bp fragment. PCR was performed in a 20-μl reaction mixture, and each pool was initially denatured at 94°C for 2 min, followed by 40 cycles of 20 s at 95°C, 40 s at 59°C, and 1.5 min at 72°C, with a final extension at 72°C for 5 min. The first step used the hyperpool of 100 individual gDNA preparations. The PCR screening configuration for the hyperpool used only one tube and one PCR reaction to identify the *st1* mutant. The second step involved the use of four tubes to screen a superpool of 25 individual DNAs, while the third step screened the subpool which makes use of the confirmed superpool.

Sequencing analysis of PCR products

To analyze PCR products from the DNA pool screening, specific fragments were eluted onto an agarose gel, purified with an extraction kit (HiYield^{TM} Gel/PCR DNA extraction Kit, Real Biotech Corporation, Taiwan) and sequenced at Macrogene Co. (Seoul, Korea) using an automated system. Two of the obtained sequences and the rescue cloning sequence were aligned using BLAST searches of the NCBI database.

Results and discussion

Forward genetics screen

In our current study, we performed a phenotype screen of *Brassica rapa* plants transfected with the pRCV2 vector via infection with *A. tumefaciens* (Lee et al. 2004). After seven weeks of growth in a greenhouse, phenotypic variations were observed. For example, mutant line 899 showed a stunted phenotype with thick, curled, and asymmetric leaves, a rough surface, and early senescence in its leaves compared with the wild type. It is believed that there were considerable effects on plant growth in this line, as regulated by phytohormones (auxin, cytokinin, gibberellins, abscisic acid, or ethylene) and certain essential enzymes (Fig. 3).

To confirm the T-DNA insertion site, rescue cloning was performed using the gDNA of mutant line 899. For plasmid rescue cloning, 10 μg of gDNA were digested with *Bam*HI or *Hin*dIII to rescue sequences adjacent to the right and left T-DNA borders. *Bam*HI or *Hin*dIII enzymes have only one

Fig. 3 A phenotype comparison of wild-type Chinese cabbage (*Brassica rapa* subsp. *pekinensis*) (*left*) and its mutant line 899 (*right*). Compared with the wild type, mutant line 899 yielded stunted plants with thick, curled, and asymmetric leaves, a rough surface, and early-senescing leaves

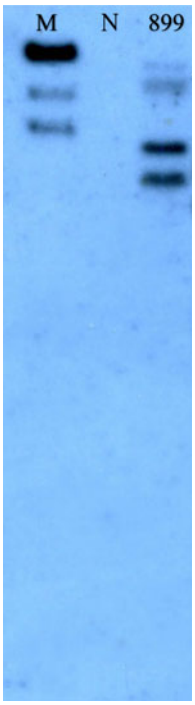

Fig. 4 Southern blot analysis of gDNA from transformants of Chinese cabbage RCV2 mutant line 899. Genomic DNA was digested with *Hin*dIII, and probed with a [^{32}P]-labeled 700 bp *Xho*I fragment of the hygromycin resistance gene (*HPT*) from pRCV2 plasmid DNA. *M*, λ *Hin*dIII molecular markers; *N*, wild-type Chinese cabbage (*Brassica rapa* L. subsp. *pekinensis* var. Seoul); *899*, RCV2 mutant line 899

recognized site within the T-DNA of the pRCV2 vector, but do not cleave the ampicillin resistance gene. From this analysis, we deduced a method of rescue cloning that should be compatible with blue/white color screening and standard ampicillin selection on IPTG/X-Gal plates. The two rescued clones were sequenced using primers that recognize the regions adjacent to the flanking DNA. In the case of *Bam*HI treatment, the BR primer (5′-ACGG GGAAAGCCGGCGGA-3′) was used as the sequencing

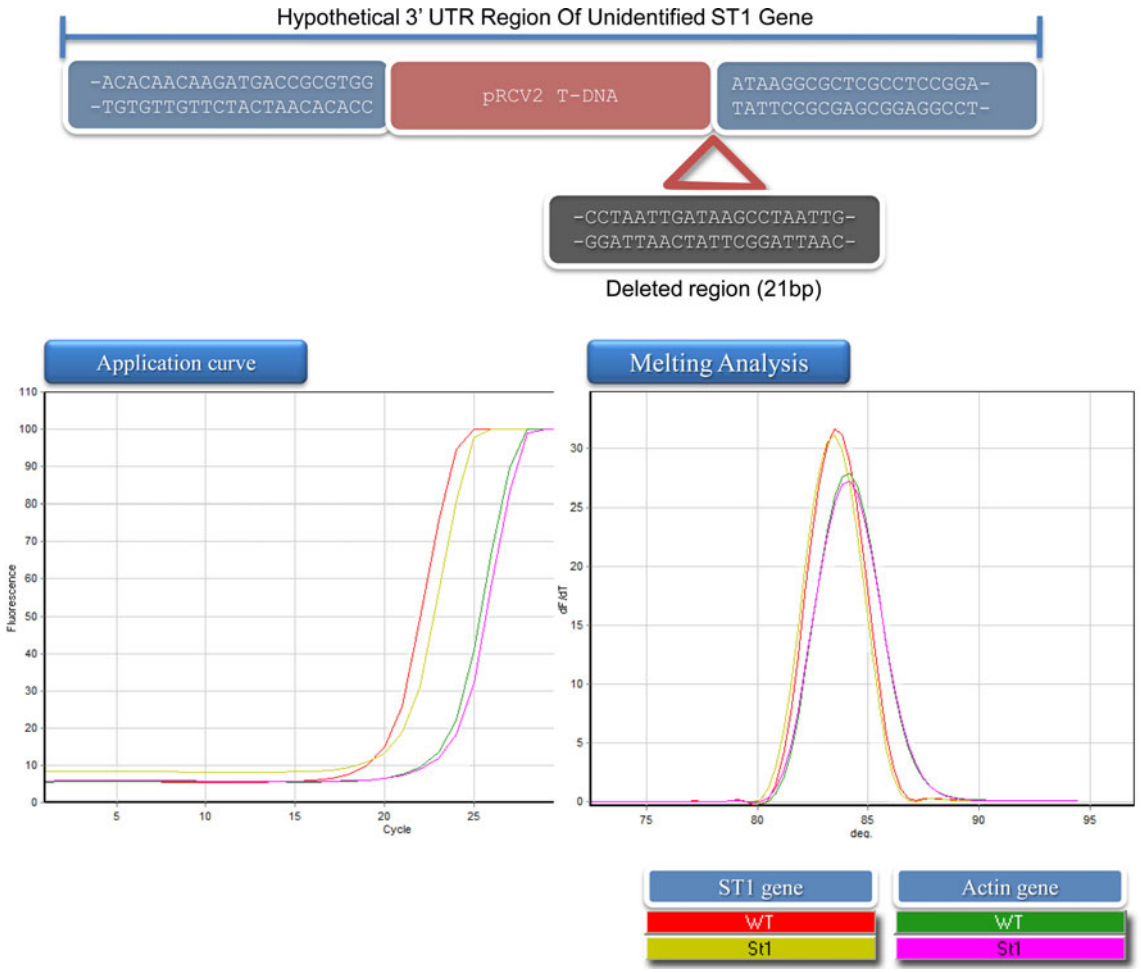

Fig. 5 T-DNA insertion site and quantitative real-time PCR analysis of mutant line 899 and wild-type Chinese cabbage (*Brassica rapa* subsp. *pekinensis*). The delta–delta C_T values were calculated relative to the reference PCR values and using melting analysis. Interference with the 3′ UTR region induced strong pleiotropic phenotypes such as the altered organization of plant organs

primer. For the *Hin*d III treatment, the IP-19R primer (5′-GCTGTGTAGAAGTAC-TCGCCG-3′) was used.

The results of subsequent BLAST analysis confirmed that the flanking DNA sequences obtained from rescued clones had homology with the *Arabidopsis thaliana* ST1 (mercaptopyruvate sulfurtransferase 1) gene from *Brassica rapa* subsp. *pekinensis* BAC clone KBrB007M04. In addition, Southern blot analysis showed two copies of the T-DNA in mutant line 899 (*st1*) (Fig. 4). The T-DNA insertion site in this line was the hypothetical 3′ UTR region of the unidentified ST1 gene in *Brassica rapa* subsp. *pekinensis*. Interference with the 3′ UTR region induces strong pleiotropic phenotypes such as the altered organization of plant organs (Kandasamy et al. 2005). Accordingly, suppression of *st1* gene expression results in abnormal phenotypes. Recently, sequence similarities between the STs and senescence-associated proteins were identified. In particular, STs may play a role in senescence by acting to detoxify the cyanide produced during ethylene synthesis, in which one molecule of cyanide is produced for each molecule of ethylene by the oxidation of 1-aminocyclopropane-1-carboxylic acid (ACC) in the presence of iron and oxygen (Kende 1993; Yang and Hoffman 1984). Cyanide is highly toxic to cells, especially due to its inhibition of metalloenzymes, and therefore must be detoxified immediately. In plants, ST proteins have been proposed to act in the detoxification process, thereby catalyzing the formation of a less toxic thiocyanate (Meyer et al. 2003). However, the *st1 Arabidopsis* mutant cannot remove the cyanide produced during ethylene biosynthesis. This suggests that the plant cells in this mutant are severely damaged and would be expected to show decreased transpiration because of the accumulated cyanide (Larsen et al. 2005). Hence, we performed quantified analysis of ST1 mRNA accumulation in wild-type Chinese cabbage (WT) and the T-DNA inserted mutant line 899 (*st1*) using real-time PCR. The T-DNA insertion-mutant plant had a significantly decreased level of ST1 transcript accumulation

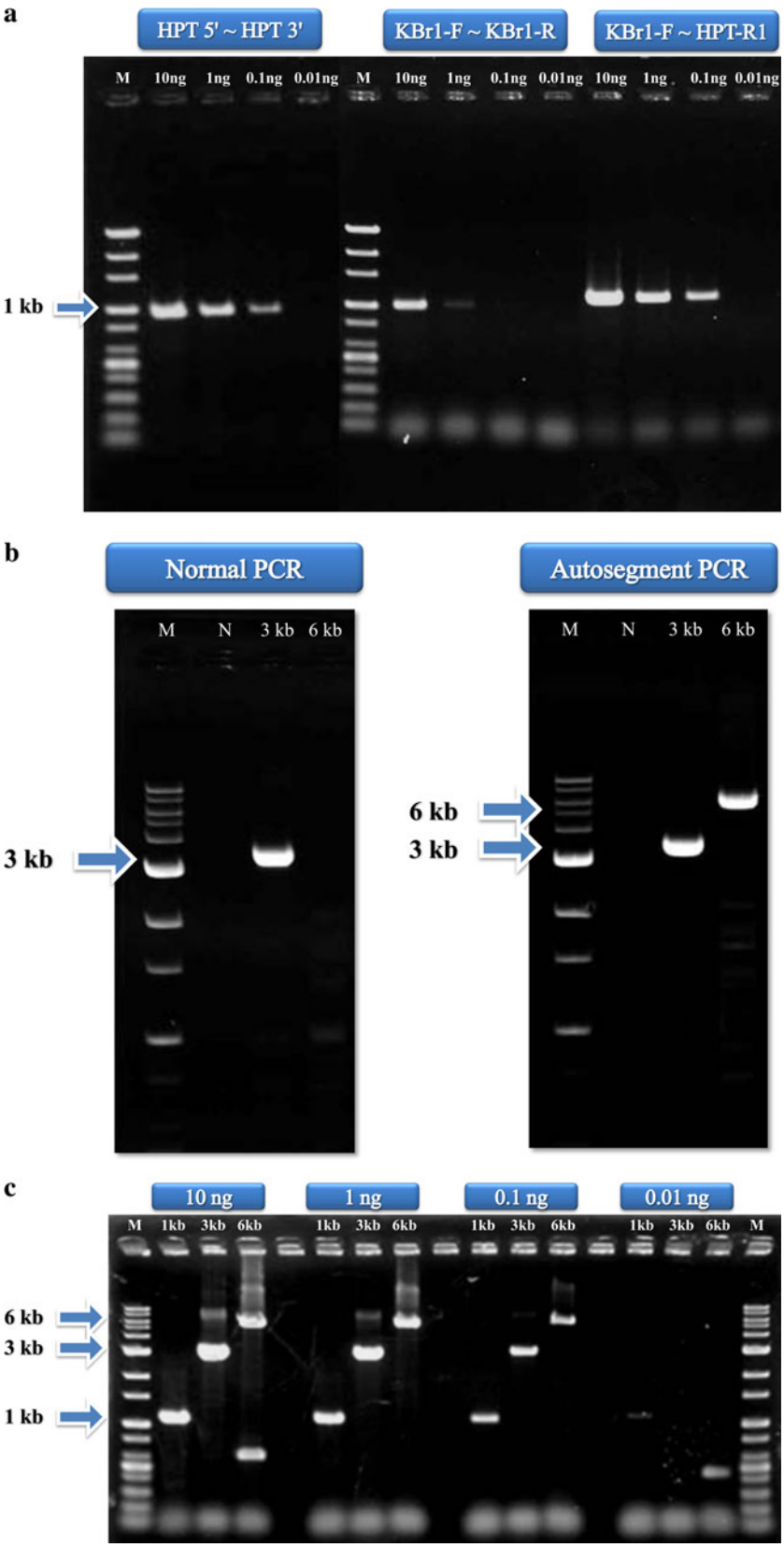

Fig. 6 Optimization of the PCR strategy used for large-scale T-DNA gene screening in a *Brassica rapa* reverse genetics system model. **a** Relationship between the DNA concentration and changes in the Gibbs free energy of the PCR primers. **b** Application of autosegment extension to long PCR. **c** Optimization of the PCR strategy using positive changes to the Gibbs free energy of the primers and an autosegment extension strategy [*M*, 1 kb DNA ladder; *N*, wild-type Chinese cabbage (*Brassica rapa* L. subsp. *pekinensis* var. Seoul)]

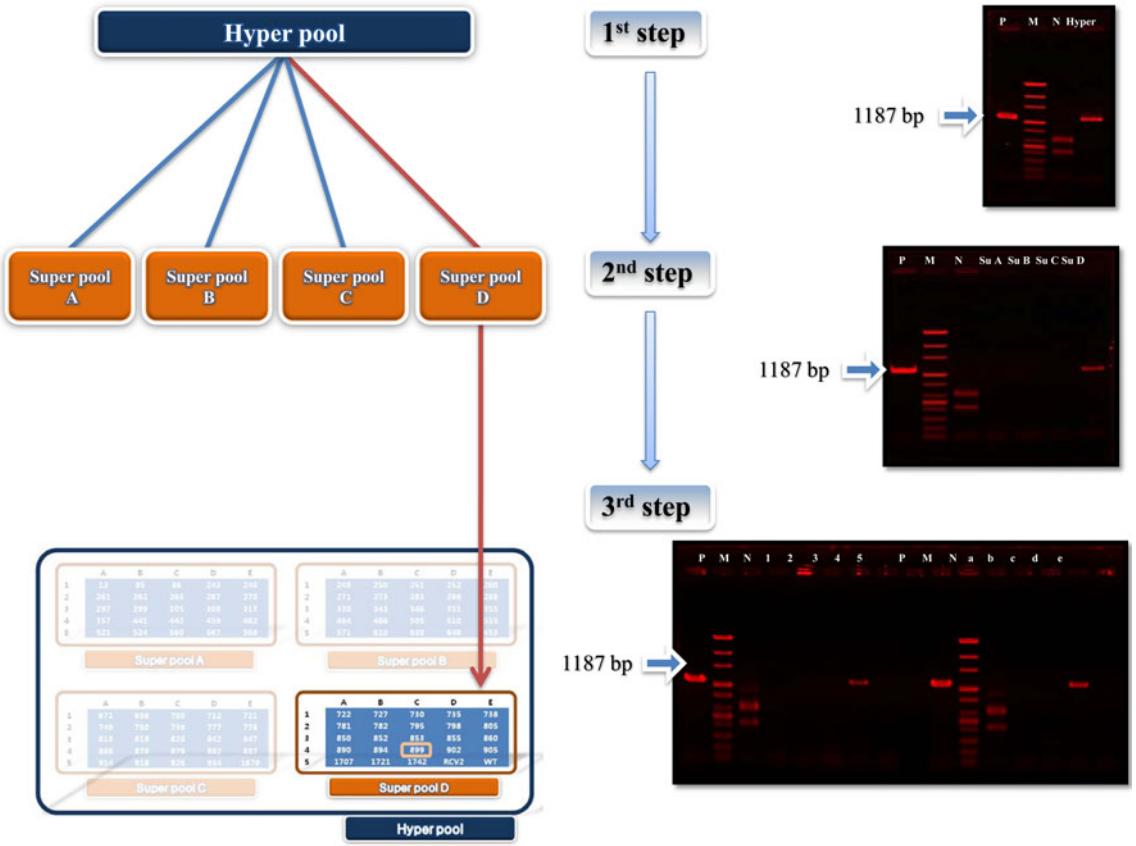

Fig. 7 Analysis of *Brassica rapa* mutant 899 via the DNA pool method and the three-step two-dimensional (2D) matrix strategy. The mutant line was screened with gene specific primer (KBr1-F) and an inner primer specific for the T-DNA that was located at the end of the border (HPT-R1). Using this effective reverse genetic screening method, only 23 PCR reactions were needed to select the target gene from a pool of 100 individual DNAs

compared with the wild type. A fluorescence graph from our real-time PCR analysis indicates that these levels were 0.44 ± 0.13 compared with the controls, signifying a 2.29-fold reduction based on the reference gene (actin). The Chinese cabbage mutant line 899 was thus found to be T-DNA tagged within the *st1* gene (Fig. 5).

Optimization of the PCR strategy used for large-scale screening

Our PCR strategy is a convenient and easy method of screening target mutant lines, and has the advantage of being able to minimize possible errors in the results when repeat experiments are performed. This methodology also works well as a reverse genetics system, thereby offering a more effective screening technique for large populations. The optimization of our PCR strategy was designed to minimize the template concentration, thus allowing the detection of specific fragments from a DNA pool. The first experiment was designed to produce effective PCR, taking into account factors such as the primer sequences and the $MgCl_2$, dNTP, PCR buffer, and *Taq* polymerase contents. Additional parameters such as dimethylsulfoxide (DMSO), bovine serum albumin (BSA), and glycerol levels have been used previously to improve the effectiveness of PCR methods (Yukihiro et al. 2003). In our present PCR strategy, effective primer design was critical, and we needed to consider primer length, melting temperature, GC content, 3′ bases, the possibility of dimer formation and false priming (which can cause misleading results), specificity, sequence complementarity, and the Gibbs free energy of the oligonucleotides using the nearest-neighbor model (Breslauer et al. 1986; Abd-Elsalam 2003; Rahmann and Gräfe 2004).

We analyzed the efficacy and stability of our PCR results by changing the Gibbs free energy (ΔG) of the primers in the system, given as $\Delta G = \Delta H - T\Delta S$, where T is the temperature in kelvins and ΔH and ΔS are the parameters for enthalpy and entropy, respectively, in this state (with the same temperature and pressure). There is a direct correlation between the state and the total entropy change ratio. The secondary structure of an oligonucleotide can be anticipated from a calculation of the Gibbs free energy value. When the primer is made, consideration of

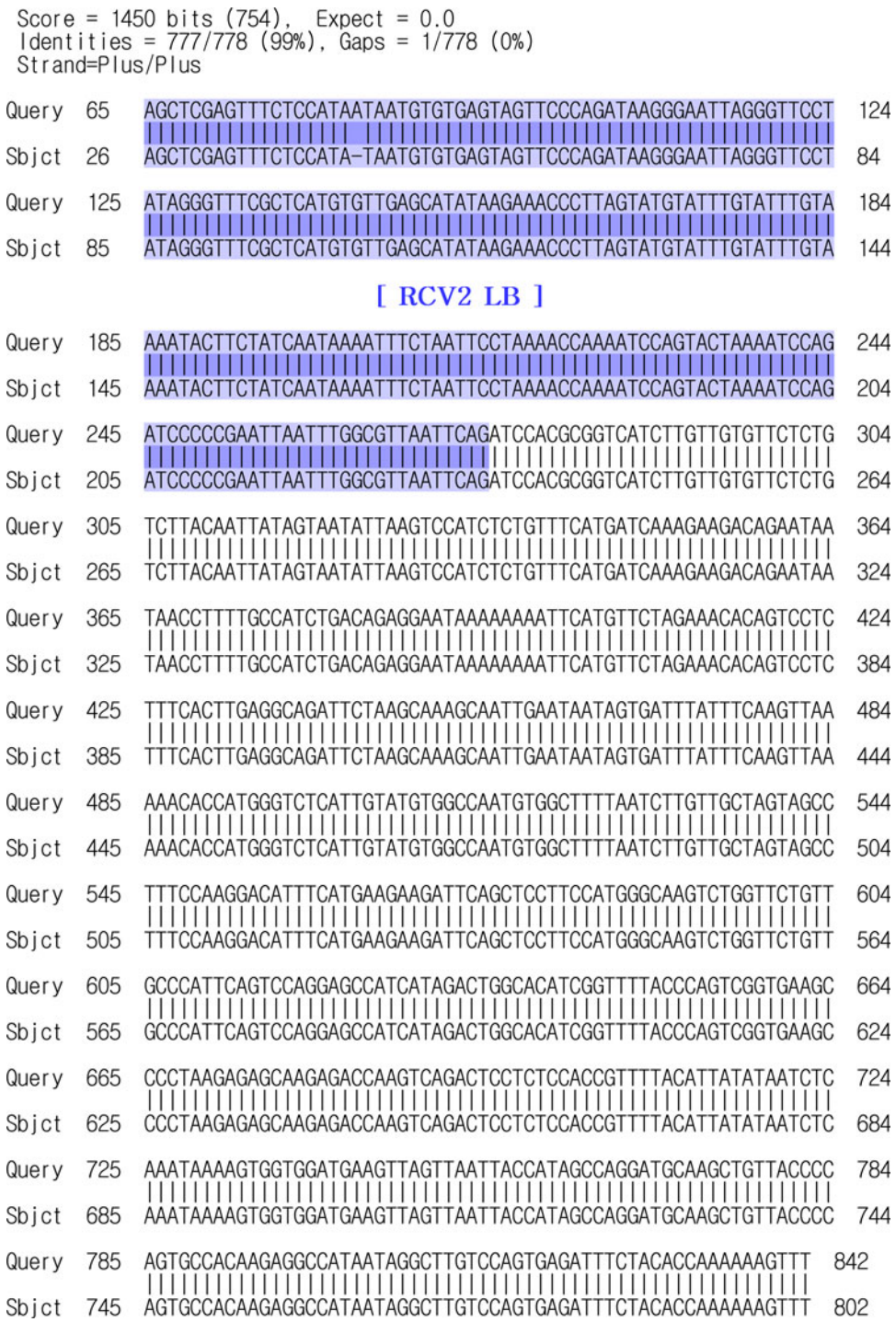

Fig. 8 Alignment search with the flanking DNA sequence of the *st1* mutant and that of the PCR product from the *B. rapa* reverse genetics system using NCBI Blast. *Query* denotes the flanking DNA sequence and *Subject* denotes the PCR product sequence of ST1

the ΔG value of the oligonucleotide hairpin loop will enable the combination efficiency to be increases, and will result in a more effective result. When designing primers, the most suitable primer pair combination is at least -4 kcal/mol. By analyzing the variation in the Gibbs free energy, more optimal primers can be generated to produce a more efficient PCR (Han and Kim 2002; Sahdev et al. 2007; SantaLucia 1998). Accordingly, we generated three types of Gibbs free energy primer pairs in our present study. These were $\Delta G < 0$ (1-kb fragment, experiment I), a low-amplified target fragment in relation to $\Delta G \geq 0$ (1-kb fragment, experiment II) and a control reaction (1-kb fragment, experiment III) which caused the formation of dimer and hairpin loop structures. Thus, a highly effective screening of a DNA pool by PCR required a positive ΔG so that the primers are prevented from forming dimers and

hairpin loops (Fig. 6a). The second experiment was conducted to determine the optimal conditions for long PCR, and involved autosegment extension.

In comparison to the normal PCR program, the use of autosegment extension produces effective results for 6-kb fragments (Fig. 6b). The autosegment extension strategy automatically increased the extension time of the PCR cycle, which provided sufficient time for polymerase activity (Mukai and Nakagawa 1996; Ohler and Rose 1992). When applying these conditions in the experiments, we obtained satisfactory results with minimal concentrations of the template and detected a greater number of longer fragments in the DNA pool screening. The 1-kb fragment was detected from a 0.01 ng template concentration, but with low efficiency. Additionally, the 3- and 6-kb fragments were successfully detected using the same 0.1 ng template concentration. However, at a 10-ng template concentration, nonspecific products were produced. As a result, we propose that the optimal DNA pool concentration is 0.1–1 ng/μl of template. By obtaining primers that change the Gibbs free energy in the positive direction, and with the application of autosegment extension, long PCR analyses are possible (Fig. 6c).

Reverse genetics screening

Our *Brassica rapa* reverse genetics system model uses a DNA pool method with three-step two-dimensional (2D) matrix strategies. This 10 × 10 DNA pool contained 100 individual DNAs, and we successfully identified our target mutant line using a 100-PCR reaction. Using this strategy, only a 23-PCR reaction was required. The target gene mutant line was screened using a gene-specific primer and an inner primer specific for the T-DNA that was located at the end of the border. The first step in the DNA pool screening (the hyperpool) was to identify the *st1* mutants of line 899. The second step used four superpools, A–D, and we identified one or more mutants from superpool D. Finally, the third-step involved subpools 1–5 and a–b, which were included in superpool D. Consequently, the *st1* mutant was identified at coordinates (C, 1) and (C, 4) on a pool matrix that expressed the *Brassica rapa* mutant line 899 (Fig. 7). To verify the PCR product, gel elution and sequence analyses were conducted. We confirmed that the sequence obtained from the above method was identical to the flanking sequence isolated by rescue cloning (Fig. 8).

In conclusion, we have presented a method for highly effective T-DNA gene screening in a *Brassica rapa* reverse genetics system model which can yield meaningful results from a large T-DNA mutant pool.

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