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Operation of an efficient site-specific recombination system of *Zygosaccharomyces rouxii* in tobacco cells

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ABSTRACT

Recombinase encoded by the *R* gene of pSR1 of *Zygosaccharomyces rouxii* mediates reciprocal recombination between two specific recombination sites (RSs) to induce excision or inversion of the DNA segment that is flanked by the RSs. We report here that site-specific recombination mediated by this system takes place efficiently in tobacco cells. To monitor the recombination events in tobacco cells, we have constructed two types of cryptic β-glucuronidase reporter gene in such a way that recombination such as inversion of the construct or excision of the intervening sequence results in their expression. When these cryptic reporter constructs were transiently introduced together with the *R* gene by electroporation into protoplasts of tobacco cells, β-glucuronidase activity was detected. The cryptic reporter genes, when stably resident in the chromosome of tobacco cells, were also activated by the *R* gene. Structural analyses of the genomic DNA isolated from these tobacco cells showed that the R protein did in fact catalyze precise recombination between two copies of RSs in tobacco cells, with resultant activation of the cryptic reporter genes. This observation provides the basis for development of a DNA technology whereby large regions of DNA can be manipulated in plant chromosomes. Potential uses of this recombination system are discussed.

INTRODUCTION

Development of recombinant DNA technology for manipulation of large segments of DNA is of importance for structural and functional analyses of eukaryotic chromosomes. The advent of the molecular cloning of yeast centromeres, replication origins and telomeres has allowed the construction of artificial chromosomes (1,2), which make it possible to clone DNA fragments of more than 100 kilobase pairs (kbp) (3). The DNA technology for structurally modifying such large regions of DNA remains, however, to be developed for a wide variety of organisms, although generation of abbreviated chromosomes of yeast by insertion of a telomere has been described (4,5).

It has been suggested that procedures for introducing large-scale rearrangement of DNA of an eukaryotic chromosome can be developed by using a bacteriophage P1-derived *loxP*-Cre site-specific recombination system (6). In addition, two site-specific recombination systems of yeasts, which are similar to one another in terms of mechanism, appear to be potentially available for development of such technologies, namely, the FLP recombination system derived from the 2-μm plasmid of *Saccharomyces cerevisiae* (7) and the pSR1 system of *Zygosaccharomyces rouxii* (8). Use of these site-specific recombination systems for chromosomal rearrangements in heterologous organisms seems to offer several advantages: the recombination takes place only between specific sequences which are usually several dozen base pairs (bp) in length (high specificity); recombination is catalyzed by a single recombinase protein and no other protein is required (simple mechanism); and the frequency is remarkably high (high efficiency) (6,7,8,9) unlike that of homologous recombination which is usually rare in somatic cells of higher eukaryotes (10,11).

The *loxP*-Cre system was the first example that indicated the occurrence of site-specific recombination in mammalian cells (12). Recently, it was reported also to be functional in a transgenic tobacco plant (13). The FLP recombination system was shown to catalyze specific excision of DNA segments of several thousand base pairs (bp) that had been flanked by two recombination targets (FRTs), both in transgenic Drosophila (14) and in transformed mammalian cells (15).

Occurrence of excision, inversion and translocation of a large chromosomal segment was first demonstrated by use of a pSR1 system from *Z.rouxii* (16). pSR1 is a circular plasmid, with a structure similar to that of the 2-μm plasmid of *S.cerevisiae*, although the nucleotide sequence of pSR1 exhibits no similarity to that of the 2-μm plasmid. The pSR1 molecule has a pair of inverted repeat sequences of 959 bp which contain the recombination sites (RS; 58 bp at most) for intramolecular recombination. Experiments *in vitro* with this recombination

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system indicated that it requires only the R protein, *i.e.*, the recombinase, that is encoded by the *R* gene of pSR1 itself (17). Recently, Matsuzaki et al. examined whether the pSR1 system can induce rearrangement of large segments of chromosomal DNA in *S. cerevisiae* cells (16). When two RSs were inserted into the *RAS1* and *HIS3* loci on chromosome XV of *S. cerevisiae*, respectively, which are separated by approximately 180 kb, the R protein efficiently catalyzed the excision or inversion of the chromosomal region between these loci, depending on the relative orientations of the RSs, and it also catalyzed chromosome translocation when two RSs were present on two nonhomologous chromosomes. These results suggest that the pSR1 recombination system is useful for generating chromosomal rearrangements in heterologous eukaryotes.

To develop a technology by which plant chromosomes can be manipulated, we have examined whether or not the pSR1 system functions in tobacco cells. Here we report that *R* gene-mediated excision and inversion are inducible in tobacco cultured cells even when the *R* gene is introduced only transiently.

MATERIALS AND METHODS

Plant cells and bacterial strains

Suspension cultures of the cell line BY-2 of tobacco (*Nicotiana tabacum* L. cv. Bright Yellow 2) (18), *Escherichia coli* JM109 (19) and *Agrobacterium tumefaciens* EHA101 (20) have been described elsewhere. The bacteria were used as the host for cloning and for transformation of BY-2 cells.

Enzymes, chemicals and plasmids

Restriction and modifying enzymes were purchased from Takara Syuzo Co. Ltd. (Japan) or Toyobo Co. Ltd. (Japan). Cellulase Onozuka RS and Pectolyase Y-23 for preparation of protoplasts were purchased from Yakult Co. Ltd. (Japan) and Seisin Pharmaceutical Co. Ltd. (Japan), respectively. 4-methylumbelliferyl β-D-glucuronide (MUG) and 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-gluc), which were used as substrates for GUS (β-glucuronidase), were obtained from Sigma (USA) and Clontech Laboratories, Inc. (USA), respectively. A Protein Assay Kit (Bio-Rad, USA) was used for determination of protein concentrations. Plasmids pUC18, pBluescript KS(+) and pBI221 (21) were purchased from Toyobo Co. Ltd., Stratagene (USA) and Clontech Laboratories, Inc., respectively.

Construction of plasmids

Plasmids used and their relevant characteristics are listed in Table 1. All the manipulations used for construction of plasmids were performed by standard recombinant DNA techniques (19). Structures of reporter plasmids were shown schematically in Fig. 1. To construct plasmid pGI, the 58-bp DNA fragment containing RS (22) was isolated from pREC58 after digestion with *Sal*I and inserted into the cleavage site for *Sal*I of pBluescript KS(+). A DNA fragment containing RS was isolated from this Bluescript construct by use of suitable restriction enzymes. The 2.1-kbp DNA fragment containing the promoter-less gene for GUS and the 0.8-kbp fragment containing the promoter of 35S RNA from cauliflower mosaic virus (P35S) were isolated from pBI221 (21). The RS fragments, the 2.1-kbp GUS fragment and 0.8-kbp promoter fragment were ligated and inserted into suitable cloning sites of pBluescript KS(+) to generate plasmids pGI and pRGR (Fig. 1). pCATG was constructed by inserting the P35S fragment, the RS fragment, the 2.3-kbp fragment containing the gene for chloramphenicol acetyltransferase (CAT) from pCaMVCAT (23), and the RS-linked GUS fragment into the multiple cloning sites of pBluescript KS(+). Plasmid pdP35R carrying the *R* gene and plasmid pdP35ΔR carrying the mutant *R* gene were constructed as follows. A 0.35-kbp *Sal*I-*Eco*RV fragment containing the enhancer region of P35S from pCaMVCAT was inserted into the *Sma*I site in the 5′-upstream region of P35S to duplicate the enhancer region of P35S. The fragment containing the modified 35S promoter and the 1.9-kbp fragment containing the coding sequence of the *R* gene from pHM153 (16) were inserted into the multiple cloning site of pUC18 to yield pdP35R. Then, a 1.3-kbp *Bgl*II-*Mlu*I fragment in the *R* gene was removed from pdP35R to yield pdP35ΔR.

To transform BY-2 cells, the binary vector plasmid pGAH was constructed by inserting the gene for hygromycin phosphotransferase as the second selectable marker into pGA492 which carries the gene for neomycin phosphotransferase II (24). We inserted a 3.4-kbp fragment that contained the IGUS construct from pGI into the multiple cloning site of pGAH to generate pGAHG. pBICATG was constructed by replacing the GUS gene of pIG221 (25) with the CAT/GUS construct. The 2.7-kbp fragment from pdP35R and the 1.4-kbp deleted fragment from pdP35ΔR were inserted into the multiple cloning site of pGAH

Table 1. Plasmids used in this study

Plasmid	Relevant characteristics	Reference
pGI	Derivative of pBluescript KS(+) containing the GUS construct in the anti-sense orientation (IGUS)	This study (Fig. 1)
pCATG	Derivative of pBluescript KS(+) containing the CAT/GUS construct	This study (Fig.1)
pRGR	Derivative of pBluescript KS(+) containing the GUS construct in the sense orientation and used for a positive control for GUS activity	This study (Fig. 1)
pdP35R	Derivative of pUC18 containing the *R* gene under control of the modified 35S promoter	This study
pdP35ΔR	Derivative of pUC18 containing the mutant *R* gene under control of the modified 35S promoter	This study
pGAHGI	Derivative of pGAH (binary vector plasmid) containing the IGUS construct	This study
pBIHCATG	Derivative of pBI121 (binary vector plasmid) containing the CAT/GUS construct	This study
pGAHR	Derivative of pGAH containing the *R* gene under control of the modified 35S promoter	This study
pGAHΔR	Derivative of pGAH containing the mutant *R* gene, with the C-terminal deletion, under control of the modified 35S promoter	This study
pIG221	Derivative of pBI121 (26) containing a GUS gene with an intron in the 5′ proximal region of the GUS sequence	(25)
pBI221	Derivative of pUC19 containing the GUS gene under control of P35S	(21)
pCaMVCAT	Derivative of pBR322 containing the CAT gene under control of P35S	(23)
pREC58	Shuttle vector plasmid containing the 58-bp RS sequence flanked by the *Sal*I linkers	This study
pHM153	Shuttle vector plasmid containing the *R* gene	(16)
pGA492	Binary vector plasmid containing the neomycin phosphotransferase II gene and a multiple cloning site	(24)
pGAH	Derivative of pGA492 containing a multiple cloning site between the neomycin phosphotransferase II gene and the hygromycin phosphotransferase gene	This study

to yield pGAHR and pGAHΔR, respectively. Plasmids pGAHGI, pBICATG, pGAHR and pGAHΔR were introduced separately into *Agrobacterium* by electroporation (26). Expanded details of the procedures for construction of the various plasmids will be sent on request.

Isolation of protoplasts and electroporation

Protoplasts were isolated from BY-2 cells by the method of Nagata *et al.* (27). Protoplasts (3×10^6) were suspended in 800 μl of precooled buffer that contained 70 mM KCl, 5 mM MES (pH 5.8), 0.3 M mannitol, 1% PEG and plasmid DNA (3 pmoles each). The protoplast-DNA mixture was transferred to a precooled cuvette. Electroporation was carried out with a Gene Pulser (Bio-Rad) using a pulse generated by a capacitor of 125 μF at 750 V/cm. After protoplasts had been incubated for 10 min at 0°C, they were transferred to 12 ml of Linsmaier and Skoog (LS) liquid medium (28) supplemented with 1% sucrose, 0.4 M mannitol and 0.2 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D), and cultured for 20 h at 26°C in the dark.

Transformation of BY-2 cells and transient introduction of the *R* gene by *Agrobacterium*

To transform tobacco BY-2 cells with the cryptic reporter GUS constructs, BY-2 cells were co-cultured with *Agrobacterium tumefaciens* strain EHA101 that harboured the binary vector pGAHGI or pBICATG, as described by An (29). Stable transformants were selected for resistance to kanamycin and hygromycin and maintained in LS liquid medium which was supplemented with 0.2 mg/l 2,4-D, 200 mg/l kanamycin and 50 mg/l hygromycin. The same co-culture method was used for transient introduction of the *R* gene into the transformed BY-2 cells with the following modifications. *Agrobacterium* cells carrying pGAHR or pGAHΔR were cultured until the absorbance at 600 nm was 1.5 and the culture was concentrated four-fold. 100 μl of this culture was mixed with 4 ml of a suspension culture of transformed BY-2 cells and the mixture was incubated with LS medium with 0.2 mg/l 2,4-D at 26°C in the dark for 48 h, 72 h or 96 h.

Assays for GUS activity

Both fluorometric and histochemical assays for GUS activity were performed by the procedures of Jefferson et al. (21). The electroporated protoplasts were collected by centrifugation after culture for 20 h and lysed by sonication for 30 sec in 200 μl of GUS extraction buffer (21). In the case of co-cultured tobacco cells, tobacco cells were collected 48 h, 72 h or 96 h after co-culturing and lysed by sonication (three 30-sec pulses). After removal of cell debris by centrifugation, the supernatants were recovered and used for determination of protein concentration and GUS activity by a fluorometric assay. For detection *in situ* of tobacco cells that expressed GUS, the tobacco cells that had been co-cultured with *Agrobacterium* cells that harboured pGAHΔR, pGAHR or pIG221 for 4 days were treated with cellulase and pectolyase (23); then they were incubated overnight at 37°C in a solution of 1 mg/ml X-gluc, 50 mM sodium phosphate (pH 7.0) and 0.35 M mannitol.

Extraction of DNA, PCR analysis and nucleotide sequencing

After co-culturing for 96 h, tobacco cells were washed twice with Linsmaier and Skoog medium that contained 0.2 mg/l 2,4-D to remove any excess *Agrobacterium* cells. DNA was extracted from the cells by the cetyltrimethylammonium bromide methods, as previously described (30), and used as a template for amplification by the polymerase chain reaction (PCR). PCR was carried out in a DNA Thermal Cycler (Perkin-Elmer Cetus, USA) using 0.5 μg of genomic DNA in a total volume of 100 μl. The reaction mixture contained 20 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 25 mM KCl, 100 μg/ml gelatin, 50 μM of each dNTP, 2 units of Taq DNA polymerase (Perkin-Elmer Cetus) and 1 mM of each primer. Reaction mixtures were overlaid with 50 μl of mineral oil. The amplification occurred during 27 cycles of 1-min denaturation at 94°C, 2-min annealing at 60°C and 3-min elongation at 72°C. The reaction mixture was analyzed on a 4% polyacrylamide gel. The sequences of the primers were 5′-CAGTATGGACGATTCAAG GC-3′ (primer 1), 5′-CAACGCTGATCAATTCCACA-3′ (primer 2), 5′-ACGCTGGACTGGCATGAACT-3′ (primer 3) and 5′-GTTCTTTACGATGCCATTG-3′ (primer 4). The products amplified by PCR were blunted

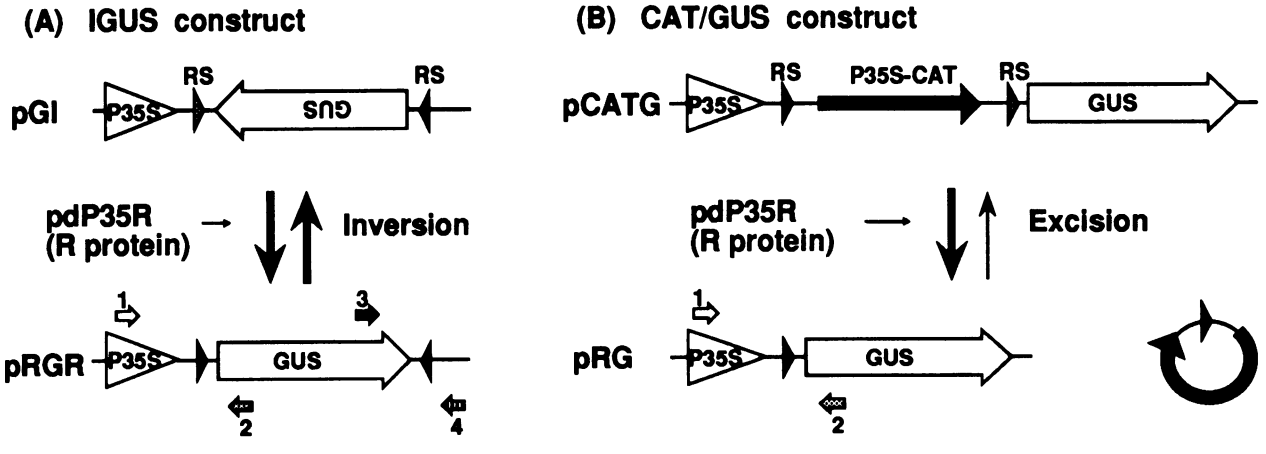

Figure 1. Strategy for detection of site-specific recombination mediated by the *R* gene in tobacco cells. (A) Schematic diagram of the IGUS construct in pGI used for detection of inversion. pRGR has the same structural organization as pGI except for the orientation of GUS. (B) Schematic diagram of the CAT/GUS construct in pCATG used for detection of excision. The heavy line represents the CAT gene expression unit (23). P35S, the promoter for 35S RNA from cauliflower mosaic virus 35S RNA; GUS, the coding sequence of β-glucuronidase; RS, recombination site recognized by R protein. The large open arrow represents the orientation of the GUS coding sequence. Arrowheads indicate the orientations of RSs. The pdP35R plasmid contains the *R* gene which is driven by the modified P35S (see text). Small arrows with numbers represent positions of oligonucleotide primers for PCR analysis.

Table 2. GUS activities of BY-2 cells after co-transfection with plasmids that carried the cryptic reporter and the *R* gene by electroporation.

Reporter carrier	*R* gene carrier	GUS activity (pmole/min/mg protein)
pGI	no plasmid	0.85 ± 0.17
	pdP35ΔR	1.12 ± 0.10
	pdP35R	21.3 ± 1.8
pCATG	no plasmid	1.13 ± 0.26
	pdP35ΔR	1.26 ± 0.11
	pdP35R	30.3 ± 10.0
pRGR	no plasmid	208 ± 28
	pdP35ΔR	161 ± 33
	pdP35R	125 ± 16

Table 3. GUS activity of By-2 cells transformed with the cryptic reporter constructs after co-culture with *Agrobacterium* cells that carried the *R* gene

Cryptic GUS in transformed line	Binary vector	GUS activity (pmoles/min/mg protein) after co-culturing for 48 hr	72 hr	96 hr
CAT/GUS (cell line 1)[a]	pGAHΔR	14.2 ± 2.3	9.1 ± 0.7	11.5 ± 1.4
	pGAHR	81.5 ± 17.6	163 ± 2.7	252 ± 4.4
CAT/GUS (cell line 2)[a]	pGAHΔR	23.3 ± 0.7	23.9 ± 0.8	19.3 ± 0.7
	pGAHR	36.4 ± 4.0	94.4 ± 2.3	124 ± 5
IGUS	pGAHΔR	16.2 ± 2.6	15.0 ± 1.1	15.6 ± 1.4
	pGAHR	18.8 ± 3.1	52.9 ± 7.5	90.5 ± 12.4

[a] Independently-isolated cell lines

with T4 DNA polymerase, cloned into the cleavage sites for suitable restriction enzymes in pUC18 and sequenced by the method of Sanger *et al.* (31).

RESULTS

A system for detecting recombinational events mediated by the *R* gene protein

To examine recombinational events induced by the recombinase (*R*) gene in tobacco cells, we constructed two types of reporter plasmid that were substrates for recombination and a plasmid that carried the *R* gene to supply the R protein (Figure 1).

pGI, which is used for detecting inversions, contains the coding sequence for the GUS gene, flanked by two copies of recombination sites (RSs) in the inverted orientation. This GUS construct with two RSs was inserted, in the anti-sense orientation, downstream from the promoter for 35S RNA from cauliflower mosaic virus (P35S). The GUS coding sequence is not transcribed until the inversion between two RSs is induced by the product of the *R* gene (we refer to this construct in pGI as IGUS) [Fig. 1(A)]. The inversion event can generate a pRGR plasmid that carries the GUS coding region in the sense orientation. Since R protein catalyzes forward and backward reactions [Fig. 1(A)], both pGI and pRGR plasmids must exist in the presence of the R protein in cells, although the proportion of each type of plasmid at the equilibrium state is not known. We also constructed the pRGR plasmid and used it as a positive control. To monitor the *R* gene-mediated deletion, pCATG was constructed [Fig. 1(B)]. This plasmid contained the chloramphenicol acetyltransferase (CAT) transcription unit between P35S and the GUS coding sequence (CAT/GUS). Since the CAT unit is bounded by two RS sequences in the direct orientation, precise *R* gene-mediated recombination can be expected to excise the CAT cassette to generate the pRG plasmid, from which the GUS gene can be transcribed [Fig. 1(B). Upon excision, the CAT cassette is thought to be circularized (16). Although R protein can catalyze the backward (integration) reaction, the relative rate of this reaction is expected to be much lower than that of the excision reaction. In the present study, we refer to these reporter constructs (IGUS and CAT/GUS) as 'cryptic' reporter genes because they cannot be expressed without the *R* gene.

To drive the *R* gene, the modified 35S promoter, in which the enhancer region of P35S, was duplicated was joined to the *R* gene to generate pdP35R. As a negative control, we constructed pdP35ΔR which contained a mutant *R* gene with an internal deletion [nucleotide positions, from the initiation codon, 144 to 1400 (8)].

R gene-mediated recombination of extrachromosomal DNA co-transfected transiently into tobacco protoplasts by electroporation

A co-transfection assay by electroporation was used to detect *R* gene-mediated recombination of extrachromosomal DNA in tobacco protoplasts. Tobacco BY-2 cultured cells at logarithmic phase were harvested and treated with cellulase and pectolyase for preparation of protoplasts. DNAs of plasmids carrying the cryptic reporter (pGI) and the *R* gene (pdP35R) were introduced into these protoplasts by electroporation as described in Materials and Methods. After incubation at 26°C for 20 h (the maximum activity was obtained during this period), soluble proteins were extracted from the protoplasts and GUS activity was assayed. Table 2 shows the results of three experiments. When pGI was co-transfected with pdP35R, a significant level of GUS activity was detected (3rd line in Table 2), although the level was even lower than that of GUS activity when the pRGR plasmid with the GUS coding sequence in the sense orientation was introduced (7th to 9th lines). In contrast, when pGI was introduced without pdP35R (1st line) or with pdP35ΔR (2nd line), no activity was observed. Thus, GUS activity was detected only in the presence of the *R* gene. These results indicate that the observed enzymatic activity was due to inversion of the GUS cassette in pGI, which was induced by the co-transfected *R* gene, although the GUS cassette was not inverted in all the cells that took up the *R* gene. The result of the experiment with pCATG also indicates that the excision of the CAT cassette between RSs was induced by the co-transfected *R* gene in protoplasts (4th to 6th lines).

We assayed GUS activity at various times after co-transfection with cryptic reporter and plasmid pdP35R. The results showed that the level of activity increased for about 20 h then diminished (data not shown). Therefore, the recombinase protein may have been transiently synthesized from the *R* gene on extrachromosomal pdP35R plasmid and acted on the co-transfected cryptic reporter genes, which may also have been present extrachromosomally.

Recombination between two RSs in tobacco nuclear chromosomes

To examine whether recombinational events take place between RSs that are present on the chromosomes of tobacco cells, we transformed BY-2 cells with each of the cryptic GUS genes, as depicted in Figure 1, using the Ti plasmid vector, and we isolated transformed cells that carried each of the cryptic reporters [BY-2(CAT/GUS) and BY-2(IGUS)]. To introduce the *R* gene transiently into these transformed BY-2 cells, each of the cell

Table 4. Histochemical staining of protoplasts of CAT/GUS-transformed BY-2 cells co-cultured with *Agrobacterium* cells that carried the *R* gene in the binary vector.

Binary vector	Number of blue cells	Total cells examined	Percentage of blue cells
pGAHΔR	0	1542	0
pGAHR	43	2153	2.0
pIG221[a)]	70	1525	4.6

[a)] pGI221 contained the GUS gene with an intron in the 5′ non-coding region in such a way that it can be expressed only in plant cells.

lines was co-cultured with *Agrobacterium* cells that carried the binary vector plasmid (pGAHR) which included the *R* gene and pTiEHA101 [disarmed super-virulent Ti plasmid (20)]. It has been shown that a T-DNA region of a binary vector plasmid in the super-virulent strain of *Agrobacterium* is approximately twenty-fold more efficiently transferred into BY-2 cells by the co-culture procedure than by the standard electroporation procedure (Matsuoka and Nakamura, unpublished; 32,33). GUS activity was measured at various times after co-culturing. As shown in Table 3, significant levels of activity were detected in both BY-2(CAT/GUS) and BY-2(IGUS) cell lines 48 h after co-culturing, and activity increased as co-culturing was prolonged. When cells were co-cultured with *Agrobacterium* that harboured pGAHΔR with the mutant *R* gene, GUS activities were at background levels. These results indicate that R protein mediates the site-specific recombination between RSs that are resident on tobacco nuclear chromosomes.

When we transfected pdP35R plasmid DNA into protoplasts of BY-2(CAT/GUS) cells by electroporation and examined GUS activity as described above, no enzymatic activity of GUS was detected (data not shown). This result may have been due to the presence of only a single copy of the reporter CAT/GUS construct in the transformed BY-2 cells.

We next measured the proportion of tobacco cells in which DNA excision, mediated by R protein, took place under our conditions. To do that, the BY-2(CAT/GUS) cells that had been co-cultured with the *Agrobacterium* cells that harboured the pGAHR were incubated with the histochemical substrate used for detection of tobacco cells that express GUS activity. Since BY-2 cells are usually clustered, before histochemical staining we separated the clustered cells by treating them with wall-digesting enzymes to rule out diffusion of the reacted substrate from cells that expressed GUS activity to those that did not. As shown in Table 4, 2% of the co-cultured cells showed clear blue staining. When we co-cultured BY-2(CAT/GUS) cells with *Agrobacterium* cells that carries the GUS reporter gene instead of the *R* gene in the binary vector (pIG221 in Table 3), 4.6% of the cells were stained. Therefore, it is likely that excision of the CAT cassette took place in approximately 40% of the plant cells into which the *R* gene had been incorporated.

Structural analysis of the DNA product of *R* gene-mediated recombination

To examine structures of GUS constructs in BY-2(CAT/GUS) and BY-2(IGUS) cells that had been co-cultured with *Agrobacterium*, total chromosomal DNAs were purified from these tobacco cells and subjected to analysis by PCR with appropriate oligonucleotide primers (see Fig. 1). We used primers 1 and 2 for analyzing the excision product from the

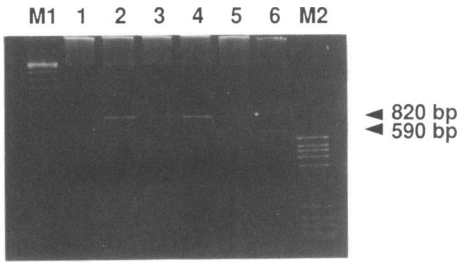

Figure 2. PCR analysis of DNA products of *R* gene-mediated recombination. Template DNA was extracted from BY-2(CAT/GUS) cells co-cultured with *Agrobacterium* cells that carried pGAHΔR (lane 1) or pGAHR (lane 2); BY-2(IGUS) cells co-cultured with *Agrobacterium* cells that carried pGAHΔR (lane 3 and 5) or pGAHR (lane 4 and 6). PCR was performed as described in Materials and Methods using primers 1 and 2 (lanes 1–4) or primers 3 and 4 (lanes 5 and 6) which are depicted in Figure 1. Amplified DNA products were fractionated on a 4% polyacrylamide gel. *Sty*I-digests of λ phage DNA (lane M1) and *Hae*III digests of pBR322 DNA (lane M2) were also fractionated as a size marker.

CAT/GUS construct and, in addition to these primers, we used primers 3 and 4 for analyzing inversion products of the IGUS construct. As shown in Figure 2, only when the *R* gene was introduced into BY-2(CAT/GUS) cells, the 820-bp DNA fragment was amplified (lane 2). This result is to be expected if recombination takes place between two RSs in the CAT/GUS construct. When we analyzed the inversion product, the 820-bp fragment and the 590-bp fragment were amplified when primers 1 plus 2 (lane 4) and primers 3 plus 4 (lane 6), respectively, were used. Polynucleotides of these lengths are to be expected if inversion of the IGUS cassette occurs. Analysis of the nucleotide sequences of the amplified DNA fragments showed that recombination took place precisely within the two RSs of each construct (data not shown).

DISCUSSION

We have demonstrated here that the R protein of *Z. rouxii* catalyzes recombination between RSs that are either present extrachromosomally in tobacco cells or stably resident in the tobacco chromosome. Co-transfection experiments provide a rough estimate of the efficiency of recombination in tobacco cells. Co-transfections with pGI plus pdP35R and with pCATG plus pdP35R generated 17% and 24%, respectively, of the GUS activity seen after the control transfection (pRGR plus pdP35R) (Table 2). If efficiencies of transfer of the reporter plasmids are in a similar level, these percentages may provide an indication of the proportions of cells in which inversion and excision occurred. These comparisons, however, probably underestimate the efficiencies of recombinations because production of β-glucuronidase from the cryptic reporter constructs must have occurred only after recombinations induced by R protein, while the GUS gene in the positive control was available for expression immediately after transfection. Histochemical analysis of BY-2(CAT/GUS) cells co-cultured with *Agrobacterium* cells also provides an estimate of the recombination frequency. Just 2% of the cells co-cultured with *Agrobacterium* that carried the *R* gene were GUS$^+$, while after co-culturing with *Agrobacterium* cells that carried the GUS gene (positive control) 4.6% of the cells were GUS$^+$ (Table 4). Assuming similar efficiencies of gene transfer, a comparison of the relative numbers of GUS$^+$

cells suggests that approximately 40% of the tobacco cells that took up the *R* gene were converted to a GUS$^+$ phenotype. This percentage may also be an underestimate of the frequency of excision for the same reason as that given above. In the present study, recombination was induced by R protein which must have been only transiently synthesized in protoplasts or suspension-cultured cells. Since the R protein synthesized in this way induced a detectable level of recombination, this recombination system is efficiently operative in tobacco cells. In addition, structural analysis of recombinational junctions showed that it is highly specific (Fig. 2, and unpublished data).

Site-specific recombination systems, such as the pSR1 system, the *loxP*-Cre system of bacteriophage P1 and the FLP-FRT system of the 2-μm plasmid of *S. cerevisiae* are believed to have many potential applications for regulation of gene expression (13), for investigations of cell lineage (14,15) and for manipulation of genomes of higher eukaryotes (12,14,16). In particular, it is worth pointing out here that the pSR1 system has been shown to introduce rearrangements of a large chromosomal segment of over 100 kbp (16). The present recombination system will undoubtedly be useful for introducing the deletion of a large chromosomal region and for isolating specific segments of chromosomal DNA such as the centromeric region and the origin of DNA replication, if it proves possible to introduce two RSs with the direct orientation into two desired sites, respectively, on a single chromosome. The present results provide the basis for development of such a DNA technology in plants by use of the pSR1 system.

It is crucial to such future studies that we develop procedures for introducing RSs into chromosomal sites that are of biologically interest, since such procedures are yet to be available in plants. One of possible approaches to this goal may involve introducing RSs into a specific chromosomal sites by exploiting sequence homologies, as in gene targeting. Some recent observations of DNA transfer into plant cells mediated by the *Agrobacterium* encoded function suggest that development of such a procedure is possible. When *Agrobacterium* T-DNA (a DNA segment that can be transferred into plant cells and eventually integrated into a nuclear chromosome) with a certain DNA segment was transferred into plant cells, it was actually integrated into the cognate DNA sequence in a nuclear chromosome, but the frequency of integration was much lower than that of random integration of T-DNA (34,35). In addition, it has ben reported that there are short incomplete homologies between DNA regions around breakpoints of T-DNA and plant pre-integration targets (36,37,38). These observations seem to promise success in the development, in plants, of a gene-targeting technique.

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