RecQ helicase enhances homologous recombination in plants

Hong-Qing Li[a,*], Mei-Ru Li[a,b]

[a]Guangdong Key Lab of Biotechnology for Plant Development, South China Normal University, Guangzhou 510631, China
[b]South China Institute of Botany, Chinese Academy of Sciences, Guangzhou 510650, China

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Abstract RecQ helicase is a key component in the RecF pathway of *Escherichia coli* for initiation of homologous recombination. Here, we demonstrate that transient expression of *RecQ* gene in rice embryogenic cell increases the homologous recombination efficiency as much as 4-fold. Further experiments reveal that this effect is influenced by the RecQ dosage. Stable expression of *RecQ* in rice dramatically increases the homologous recombination events 20- to 40-fold in leaf tissue from different transgenic lines. This is the first evidence indicating that overexpression of *RecQ* gene can stimulate homologous recombination in plants.

Keywords: RecQ helicase; Homologous recombination; Rice

1. Introduction

Gene targeting is a powerful reverse genetic method for in situ modification of genes. It has been successfully applied to *Escherichia coli*, yeast and even mammalian cells, and led to establishment of different loss-of-function phenotypes, which facilitated the elucidation of the function of numerous genes [1]. In contrast, gene targeting in plants is still very inefficient. Different strategies, including extending the homology length of targeting vectors [2] and using negative selection [3] to enrich the targeted events, had been tested, but none of them gave a promising result. Although the highest reported gene targeting frequencies in higher plants range in the order of one in 750 [4] and one in 2580 events [5], most experiments yielded 10^{-4} to 10^{-5} or even less [2,3]. The recent successful gene targeting in rice revealed that the absolute targeting events were still in the range of 10^{-4} to 10^{-5} [6].

To apply gene targeting in plants, one of the important aspects is to improve the homologous recombination ability of plant cell. For this purpose, overexpression of *E. coli* recombination-related genes *RecA* and *RuvC* in tobacco was conducted and showed improvement of extra- and intrachromosomal recombination frequencies in one order or above [7,8]. These two genes are involved in the "latter steps" of homologous recombination, responsible for homologous pairing and resolution of the recombination intermediates, respectively [9]. As former experiments indicated that plant cell has the basic machinery for processing of homologous recombination [10], the initiation step might have much more potential to increase the recombination frequency by providing more suitable substrates.

Generally, the initiation of homologous recombination needs single-stranded DNA tails for homologous pairing, which can be mediated by a DNA helicase, a double-stranded DNA exonuclease or a combination of these two activities [9]. RecQ helicase is an initiator of homologous recombination in the RecF pathway in *E. coli recBCsbcBC* mutant [11]. Comparing with RecBCD, the helicase/nuclease responsible for initiation of the majority of recombination events in wild-type *E. coli*, RecQ helicase can act on a wider variety of double-stranded DNA substrates, including nicks and gaps present in the donner DNA [12]. It is functional not only in the initiation step for producing single-stranded substrates, but also in the latter steps for moving the Holliday junction and resolving the recombined molecules. It has been proved by an in vitro experiment that RecQ together with RecA and single strand binding protein (SSB) can initiate homologous recombination [13].

In plants, six RecQ homologs have been characterized in *Arabidopsis* [14] and one of the RecQ homolog RecQsim can suppress the MMS hypersensitivity of the yeast *Sgs1* mutant [15]. But the function of this protein in plant is still unknown. In this report, we studied the influence of transient and stable expression of *E. coli RecQ* gene on homologous recombination in rice by using the transient GUS recombination assay system, together with the delivery of recombination substrates by particle bombardment. We determined that both transient and stable expression of *E. coli RecQ* gene could enhance homologous recombination in rice.

2. Materials and methods

2.1. Plasmids

Plasmid pAHC27 carries the β-glucuronidase (*GUS*) reporter gene driven by the maize *Ubiquitin* promoter with *Ubiquitin* intron [16]. The recombination partner pHQIN1L with 3′ of *GUS* deleted was from restriction of pAHC27 with both *Eco*RV and *Hin*dIII, another partner pHQIN2L with promoter deleted was obtained by digestion of pAHC27 with *Bam*HI (Fig. 1). For construction of pHQRecQ, the *RecQ* ORF was obtained by digestion of pSQ211 (a gift from Dr. Hiroshi Iwasaki, Osaka University) with *Nco*I. A 1.9 kb fragment was subcloned into the *Nco*I site between the maize *Adh1* (alcohol dehydrogenase) promoter with *Adh1* intron and the *CaMV 35S* terminator by using a linker. pHQRecQ$^-$ was constructed by a connection of the maize *Adh1* promoter with *Adh1* intron to *RecQ* in an opposite

* Corresponding author. Fax: +86-20-85212078.
E-mail address: hqli@scnu.edu.cn (H.-Q. Li).

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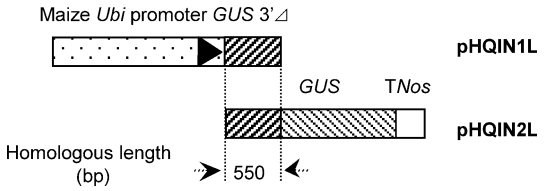

Fig. 1. Schematic map of the homologous region of two partners used for recombination analysis. pHQIN1L is a derivative of pAHC27 with the 3′ of GUS coding region deleted; pHQIN2L is a derivative of pAHC27 with the promoter deleted. *GUS* expression can be recovered by homologous recombination between the two linear molecules through the 550 bp homologous region.

direction. pHQRecA was constructed by connecting the maize *Ubiquitin* promoter and *Ubiquitin* intron to the nt*RecA* [17].

2.2. Transient recombination assay by bombardment

Mature seeds of rice cultivar "Murasaki-ine" (*Oryza sativa* L.) were dihusked, surface sterilized and cultured on a callus induction medium 2N6 (N6 salts [18], 2,4-D 2 mg/l, and 3% sucrose). The embryogenic calli were obtained after 6 weeks of cultivation. These calli were transferred to the new medium with the same components and continuously cultured for additional 2 weeks. Three grams of embryogenic calli, including about 450 small calli, was spread on the central area of each petri dish for high osmotic treatment. This amount of calli can cover an area of about 6 cm in diameter and is sufficient to capture at least 95% of the particles from the bombardment. The medium for osmotic treatment contains N6 medium supplemented with 3% sucrose and 5% mannitol. After at least 4 h, these calli were biolistically transformed with different plasmids. The plasmids for bombardment were purified using the QIAquick Gel Extraction Kit (QIAGEN, Cat. No. 28704) before binding to gold particles. The particles were delivered into rice calli through biolistic bombardment, using the helium-driven PDS-1000/He system (Bio-Rad), according to the manufacturer's instructions. 12 h later, the bombarded materials were transferred onto the 2N6 medium for recovery of 3 days. For leaf tissue bombardment, the young leaves were taken from transgenic plants grown about one month in greenhouse, surface sterilized and cut into pieces of about 0.5 cm in length. The small pieces were placed on osmotic treatment medium occupying the central area of about 6 cm in diameter. They were bombarded in the same way as for the embryogenic calli. GUS activity was determined by histochemical staining of bombarded materials with X-Gluc solution as described previously [19]. Homologous recombination events were calculated by counting the number of blue spots in each shot.

2.3. Transformation and regeneration of transgenic plants

Transformation of embryogenic calli by bombardment was as described above. The plasmids are pHQRecQ and a hygromycin selectable marker pHQ6, in which the *HPT* gene is driven by rice actin1 promoter. The bombarded calli were transferred to 2N6 medium supplemented with 50 mg/l hygromycin for selection of resistant calli. After 30 days, the hygromycin resistant calli were transferred to regeneration medium MSRE (MS salts, NAA 1 mg/l, BAP 2 mg/l, sucrose 30 g/l and hygromycin 30 mg/l). The regenerated shoots were finally rooted on 1/2 MS medium and transferred to greenhouse.

2.4. Molecular analysis of transgenic plants

Total DNA was extracted from the leaves of wild type and transgenic plants. Ten microgram of DNA from each sample was digested with *Eco*RI and *Hin*dIII, separated on a 0.8% agarose gel, and transferred to a nylon membrane. The membrane was hybridized with an 893 bp RecQ probe labeled with digoxigenin using PCR method (Boehringer Mannheim Biochemica). The primers used for amplification of the fragment are RECQ2FW 5′-gccgcgattgcctcgtcgtcatg-3′ and RECQ2RE 5′-gcatcgcttccgcgggcaggcca-3′. For Northern blot hybridization, total RNA was isolated from the leaves of transgenic lines. Ten microgram of RNA from each sample was separated by electrophoresis and blotted to a nylon membrane, and hybridized with the 1.9 kb *RecQ* fragment labeled with ^{32}P as a probe [20].

3. Results

3.1. Extrachromosomal recombination system

To quantify the homologous recombination events in plant cell, we used a transient *GUS* recombination system. We prepared recombination substrates of pHQIN1L and pHQIN2L by enzyme restriction of pAHC27, each of which harbored an incomplete GUS expression cassette and have about 550 bp overlap sequence between them (Fig. 1). Examination of the recombination system indicated that either pHQIN1L or pHQIN2L alone does not show any GUS activity in rice cell, and also the circular molecules are inert to recombine. Only when the linearized form of both substrates is delivered together into rice cell, the GUS activity can be restored. This indicates that homologous recombination occurred between the two molecules. To reduce the possibility of unstable results brought by particle bombardment transformation method, the following measures were taken:

1. The positive control with pAHC27 was included in each experiment.
2. The materials used in each bombardment are enough to cover the whole shooting area and receive more than 95% of the particles from the gene gun.
3. Each treatment was repeated five times for embryogenic calli and three times for leaf tissue in independent experiments. Under these conditions, we repeatedly obtained an average number of blue spots of about 3100 with pAHC27 at 1 μg per shot, with a variation in the range between 2800 and 3500.

3.2. Transient expression of RecQ enhances homologous recombination

To study the effect of RecQ helicase on homologous recombination, we made the following constructs (Fig. 2A). pHQRecQ, the *RecQ* expression was driven by the maize *Adh* promoter. PHQRecQ$^-$, with the promoter in an opposite direction, was used as a negative control. *E. coli RecA* gene, which was reported to improve intrachromosomal recombination in tobacco [13], was also included in this experiment (pHQRecA). We examined the effects of RecQ helicase on extrachromosomal recombination by co-bombardment of the above vectors and recombination substrates pHQIN1L and pHQIN2L into rice embryogenic calli. In the absence of pHQRecQ, the average number of blue spots, generated after delivering the two linear recombination substrates, was 312 (Fig. 2B, lane 2), which was about 1/10 of that in the positive control (Fig. 2B, lane 1). Delivering the recombination substrates with either pHQRecQ$^-$ or pHQRecA showed little effect for improving the recombination frequency (Fig. 2B, lanes 3 and 4). When delivering the recombination substrates with pHQRecQ, the number of blue spots increased dramatically to 1320/shot, it was 4-folds of that in the absence of pHQRecQ (Fig. 2B, lane 5). Interestingly, after co-delivering the recombination substrates with both pHQRecQ and pHQRecA, the number of blue spots increased compared with the negative control, but less significantly than that in delivering pHQRecQ only (Fig. 2B, lane 6). These results indicate that the *E. coli*-originated RecQ helicase has the capacity to increase the homologous recombination frequency in rice embryogenic cell. However, the results obtained from combined application of *RecA* and *RecQ* are somehow different from some former results, in which RecQ together with

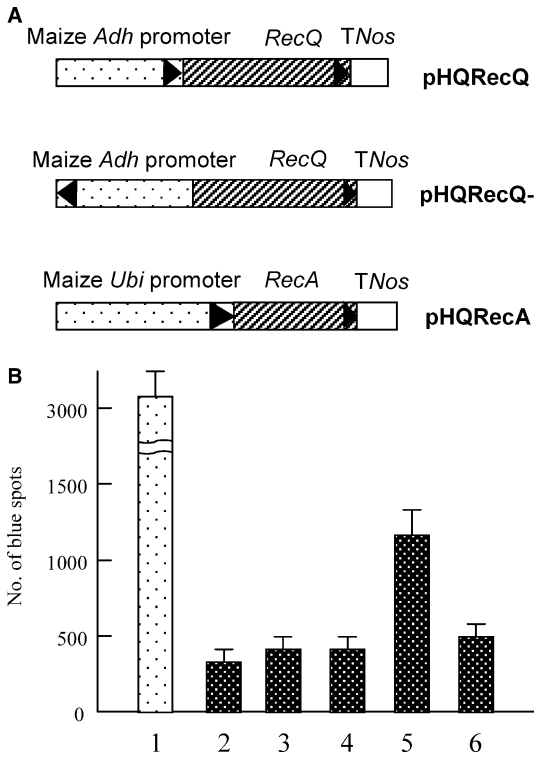

Fig. 2. Effects of RecQ on extrachromosomal recombination. (A) *RecQ* and *RecA* expression vectors. pHQRecQ which contains the maize *Adh*1 (alcohol dehydrogenase) promoter and its first intron, *RecQ* coding region and a *NOS* terminator; pHQRecQ$^-$, the same as pHQRecQ except the promoter in an opposite direction; pHQRecA which contains the maize *Ubiquitin* promoter and its first intron, *RecA* coding sequence and a *NOS* terminator. (B) Effects of RecQ on extrachromosomal recombination. The combinations of plasmids used for bombardment are: lane 1, pAHC27; lane 2, pHQIN1L and pHQIN2L; lane 3, pHQRecQ$^-$, pHQIN1L and pHQIN2L; lane 4, pHQRecA, pHQIN1L and pHQIN2L; lane 5, pHQRecQ, pHQIN1L and pHQIN2L; lane 6, pHQRecQ, pHQRecA, pHQIN1L and pHQIN2L. The amount of each components used for bombardment was 1 μg/shot. Mean values and S.D. from five shots are shown.

RecA has synergistic effects for increasing homologous recombination [13].

3.3. Influence of the amount of RecQ on homologous recombination

In a previous in vitro study, it was found that RecQ helicase could unwind double-stranded DNA and initiate homologous recombination at a concentration of 100 nM, while it dissociated the joint molecules at a concentration of 500 nM [13]. To test whether this effect exists inside a plant cell, we analyzed the influence of the *RecQ* expression level on homologous recombination. Because the particle bombardment method is used for delivering the *RecQ* expression construct and recombination substrates into rice cell, the *RecQ* expression level can be adjusted simply by binding different amount of plasmids onto the particles. The average number of blue spots was 321/shot when bombardment with only the recombination substrates pHQIN1L and pHQIN2L was at 1 μg/shot each (Fig. 3). The recombination frequency was slightly increased under the co-bombardment of pHQRecQ at 0.5 μg/shot (548 spots/shot). The remarkable increase of recombination frequency to 1476 blue spots/shot was observed when 1 μg of

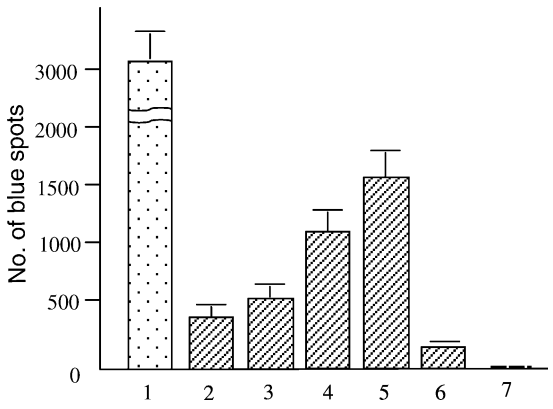

Fig. 3. Influence of the amount of pHQRecQ on homologous recombination. Lane 1: pAHC27; lanes: 2,3,4,5,6,7: the amount of pHQRecQ at 0, 0.5, 1.0, 2.0, 3.0, 4.0 μg/shot, respectively. 1 μg of each recombination substrates of pHQIN1L and pHQIN2L was used for one shot. Values and S.D. from five shots are shown.

pHQRecQ was bombarded. Further increase of the recombination events to 1680 blue spots/shot was observed at the shot of pHQRecQ at 2 μg/shot. However, when pHQRecQ was at 3 μg/shot, the recombination events dropped to 1/3 of that in the absence of pHQRecQ. Furthermore, the recombination events were almost reduced to zero when pHQRecQ was at 4 μg/shot. These results suggested that the extrachromosomal recombination frequency can be enhanced dramatically in the presence of proper amount of RecQ helicase, and excess amount of RecQ would prevent homologous recombination. To make sure that the decreased recombination events are related to the high level of *RecQ* expression, we performed the same treatment using pHQRecQ$^-$ instead of pHQRecQ. We found that the recombination events are similar to only shooting with recombination substrates, which indicates that pHQRecQ$^-$ at different amount showed little effects on the recombination events (data not shown). The representative GUS staining results were shown in Fig. 4. Bombardment with recombination substrates pHQIN1L, pHQIN2L together with 1 μg/shot (Fig. 4B) or 2 μg/shot of pHQRecQ (Fig. 4C) produced more blue spots than without pHQRecQ (Fig. 4A). In accordance with the above results, the transient expression of *RecQ* also increased the intensity of the blue spots. The increased intensity of the blue spots could mean an increase of recombined molecules in a single cell. These results suggested that the extrachromosomal recombination frequency could be enhanced dramatically in the presence of RecQ helicase. However, the further increase of the amount of pHQRecQ to 3 μg/shot showed a negative result and the recombination events were greatly suppressed (Fig. 4D). When pHQRecQ increased to 4 μg/shot, the recombination events dropped to very few.

3.4. Stable expression of RecQ enhances homologous recombination

To study the effects of RecQ on homologous recombination as well as for obtaining the *RecQ* expression material for gene targeting, we transformed the *RecQ* expression vector pHQRecQ (Fig. 5A) into rice by co-bombarding it together with a hygromycin selection marker pHQ6. In total, we generated 33 hygromycin resistant plants, among which 20 had the expected RecQ fragment integrated in the genome, Southern blot

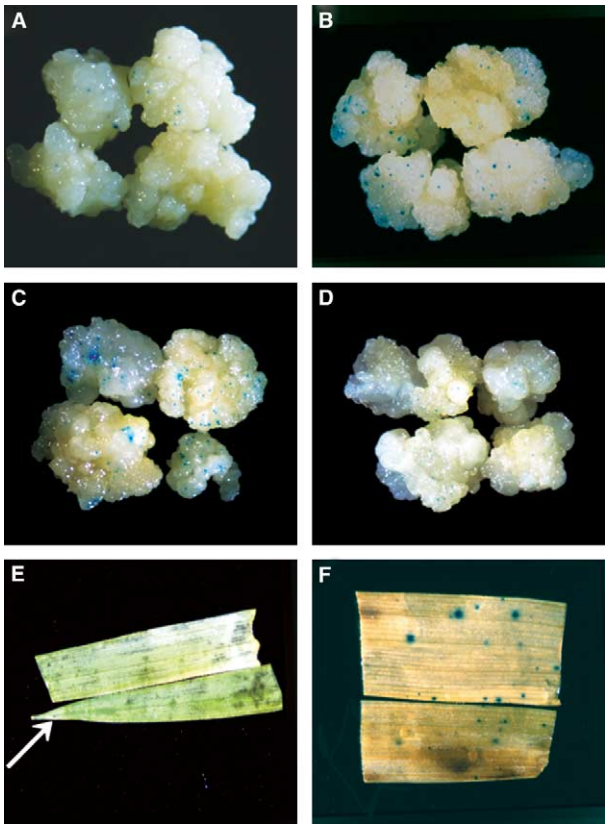

Fig. 4. Representative GUS staining of the recombination events. (A) Bombardment of embryogenic calli with recombination substrates pHQIN1L and pHQIN2L; (B, C, D) bombardment of embryogenic calli with recombination substrates pHQIN1-1, pHQIN2-2 together with pHQRecQ at 1, 2, 3 μg/shot, respectively. (E, F) Bombardment of leaf pieces from wild-type and transgenic plant #3-11 with recombination substrates pHQIN1-1and pHQIN2-2, respectively. Arrow indicates the single spot found on leaf piece of wild-type plant. The amount of pHQIN1L and pHQIN2L was 1 μg/shot each.

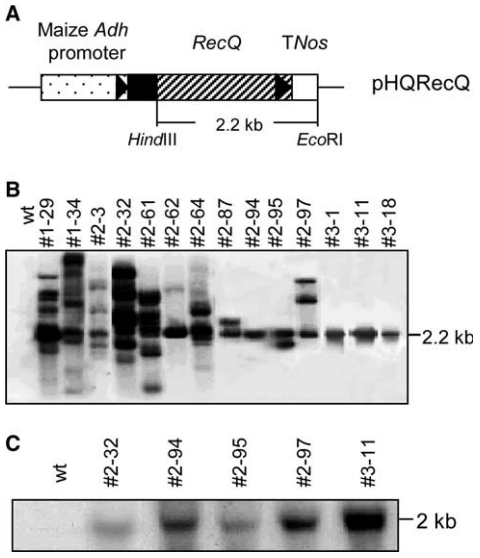

Fig. 5. *RecQ* integration and expression in transgenic rice plants. (A) Restriction map of pHQRecQ shows the 2.2 kb fragment after restriction with *Eco*RI and *Hin*dIII. (B) Southern blot analysis of transgenic rice plants. A 2.2 kb specific band including *RecQ* and nos terminator was detected in different transgenic lines as indicated using a *RecQ* fragment as probe. (C) Northern blot analysis of transgenic plants. A 2.0 kb specific transcript of *RecQ* was detected in different transgenic lines as indicated using a *RecQ* fragment as probe.

Table 1
Stable expression of *RecQ* enhances homologous recombination in leaf cell

RecQ expression lines	No. of blue spots/shot	Average enhancement
WT	3 ± 2	1
#2-94	74 ± 23	24
#2-95	62 ± 31	20
#3-11	122 ± 45	40

Mean values and S.D.from three shots are shown.

hybridization showed the patterns of some of the transgenic lines (Fig. 5B). Five of them showed *RecQ* expression by Northern blot analysis (Fig. 5C). By using the same transient recombination GUS assay, we bombarded the linear recombination substrates pHQIN1L and pHQIN2L into the leaf pieces from the *RecQ* expression lines. The GUS staining results showed that the recombination events were enhanced about 20- to 40-folds in the leaf tissue of three independent *RecQ* expressing lines, respectively (Table 1 and Fig. 4F). While within the leaf pieces from wild-type, few blue spot was observed (Fig. 4E). In addition, we also observed that *RecQ* expression level in the stable transgenic plants has a positive effect in enhancing homologous recombination. (Fig. 5C and Table 1).

4. Discussion

This study shows that overexpression of *RecQ* can enhance homologous recombination in plants by using the transient GUS recombination assay. The effect was observed for both transient expression of *RecQ* in rice embryogenic calli (4-fold increase) and stable expression of *RecQ* in leaf cell (20- to 40-fold increase). To our knowledge, this is the first evidence indicating that overexpression of *RecQ* stimulates homologous recombination in plants.

In wild type *E. coli*, the RecBCD enzyme is essential for 99% of the recombination events. When RecBCD enzyme is rendered non-functional, recombination can proceed at nearly wild-type levels in strains with *sbcB* (exonuclease 1) and *sbcC* (or D) mutations. Approximately 75% of the events require RecQ function. Like the RecBCD enzyme, RecQ protein is a recombination specific helicase, without nuclease activity, and can unwind plasmid-sized DNA in the presence of SSB protein [21]. Here, we show that transient and stable expression of *RecQ* in rice cell can stimulate homologous recombination, the effects are in accordance with the function of RecQ in homologous recombination. We reasonably suppose that RecQ acts in the similar way as in *E. coli* for initiation of homologous recombination by providing more recombination substrates, as well as for resolving recombination intermediates. However, some interesting points were found in our experiments. First, *E. coli* RecQ was originally shown to initiate homologous recombination and function together with RecA and SSB in the recombination pathway [9], while the results from our experiment indicate that overexpressing of *RecA*

suppresses the effect of RecQ on improving homologous recombination. The reason might be the incompatibility of RecA and RecQ in plant cell system. Second, RecQ shows a dose effect on homologous recombination. Although the amount of plasmids used for binding particles during bombardment might not reflect exactly the protein level inside the target cell, it is clear that the increase of the plasmids will increase the gene expression level, and also the increase of the amount of plasmid for bombardment will not cause the suppression of gene expression, especially in the case of transient expression. The suppression of recombination events under excess amount of RecQ (when pHQRecQ at 3 or 4 μg/shot) is probably due to the high RecQ protein level. As RecQ is free to act on a wide range of substrates, its ability on unwinding double-stranded DNA for initiation of recombination and disrupting the recombined intermediates possibly is similar. The processing of the recombined products and finally formation of end products possibly depends on the other components inside the cell. Finally, our results showed that stable expression of *RecQ* in rice under different expression levels can increase homologous recombination in leaf cell, and no suppressive effect on recombination was observed. As discussed before, the suppressive effects of *RecQ* on homologous recombination could only be observed under extremely high *RecQ* expression levels, the stable expression of *RecQ* in transgenic plants possibly does not reach the levels that could reduce the recombined molecules.

So far, two recombination-related genes from *E.coli* have been expressed in plant cell. *RecA*, which is responsible for homologous pairing, showed some effects in improving intrachromosomal recombination and rendering the plant cell resistant to mitomycin [7]. It facilitates obtaining precise targeting events while has no effect for enhancing targeting frequency [22]. *RuvC*, which is involved in the resolution of recombination intermediate, showed effects in improving homologous recombination [8], its effects on gene targeting has not been reported. Compared with the above two genes, *RecQ* gene has its unique features for improving gene targeting in plants. First, it acts in the initiation of homologous recombination by providing more suitable substrate of single-stranded DNA tail for later steps, which is supplementary to the plant recombination system. This effect is proved by our results that transient expression of *RecQ* gene can dramatically increase the homologous recombination frequency. While in the case of *RecA* and *RuvC*, the increasing of homologous recombination can only be observed in the stable transformants [7,8]. Second, in plant gene targeting experiments, most of the events are through one-sided invasion, leading to "imperfect targeting" which includes homologous recombination in one side and illegitimate recombination in the other side [23]. RecQ helicase might function at the break sites to make single-stranded tail and facilitate two-sided homologous recombination. Finally, RecQ helicase as a suppressor of illegitimate recombination could possibly change the ratio of illegitimate recombination to homologous recombination events, and thus to concentrate the targeting events. With the recent success in gene targeting in rice [6], we are now carrying out series of experiments to examine the effects of transient and stable expression of *RecQ* gene on rice gene targeting.

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