Note

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A Ti-plasmid-convertible λ Phage Vector System Harboring the Hygromycin B Phosphotransferase Gene

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Abstract

We constructed new λTI phage vectors, λTI3, λTI4, λTI5 and λTI6, which were automatically convertible into Ti-plasmid binary vectors by using the *Cre-lox* site-specific recombination system of bacteriophage P1. Hygromycin B phosphotransferase (HPT) gene was inserted in the T-DNA region of these λTI vectors as a plant selectable maker. Tobacco plants, which were transformed by *Agrobacterium* containing the Ti-plasmids resulted from the automatic conversion of the λTI vectors, could be tightly selected by hygromycin B. This λTI system will certainly provide a powerful tool for genetic complementation test of candidate genes obtained by positional cloning.

Positional cloning has been applied in order to isolate genes which have been defined by mutational analysis. The generation of long-distance contigs using yeast artificial chromosome (YAC) and bacterial artificial chromosome (BAC) libraries in *Arabidopsis thaliana* facilitates the identification of the YACs and BACs covering the region of a target gene [1, 2]. The isolation of target genes by positional cloning is accomplished by genetic complementation of mutant phenotypes. Subcloning of a number of genomic or cDNA clones, which have been identified by the direct probing with YACs and BACs, into plant transformation-competent vectors requires a great deal of time and labor. To facilitate the genetic complementation test, we previously constructed novel λ phage vectors, comprising λTI1 and λTI2 [3]. These vectors are automatically convertible into Ti-plasmid binary vectors using the *Cre-lox* site-specific recombination system of bacteriophage P1. The converted Ti-plasmid harbors neomycin phosphotransferase II (NPT II) gene and can be directly introduced into plants by *Agrobacterium*-mediated transformation. We herein report the construction of four new λTI phage vectors, λTI3, λTI4, λTI5, and λTI6, that contain hygromycin B phosphotransferase (HPT) gene as a selective marker.

These Ti-plasmid-convertible λ phage vectors were constructed using the precursor plasmids, pTI1 and pTI2, which are derivatives of pBI121 and contain directly repeated *lox* sites [3]. The 2-kb *Eco*R I fragment of pYL3 (Shibata, personal communication), consisting of the promoter sequence from the nopaline synthase (NOS) gene, the HPT gene and the NOS terminator, was cloned into the *Eco*R I site of the pTI1 to generate precursor plasmid pTI3. For construction of pTI4, this 2-kb *Eco*R I fragment was repaired using klenow fragments before being inserted into the *Ecl*136 II site of pTI2. The 2.5-kb fragment with the cauliflower mosaic virus (CaMV) 35S promoter, the HPT gene and the NOS terminator was generated by digestion of pLAN101MHYG (Shimamoto, personal communication) with *Sal* I and *Hind* III, and then made blunt by treatment with klenow fragments. For the construction of pTI5, this 2.5-kb fragment was ligated with pTI1 which had been digested with *Eco*R I and then blunted. pTI6 was created by insertion of this 2.5-kb fragment into the *Ecl*136 II site of pTI2. The phage vectors, λTI3, λTI4, λTI5, and λTI6, were constructed by ligation of the *Sal* I-*Xho* I fragments from pTI3, pTI4, pTI5, and pTI6, into the *Xho* I-cleaved λGEM11 arm (Promega).

The restriction maps of λTI3, λTI4, λTI5, and λTI6 and the outline of automatic subcloning are shown in **Fig. 1**. The linear λTI phages can be converted to the Ti-plasmid form by infection of the cre$^+$ *Escherichia coli* cells, NS5329, with these phages. The λTI3 and λTI5 vectors were designed for construction of the cDNA library and the converted Ti-plasmids are capable of expressing genes under the

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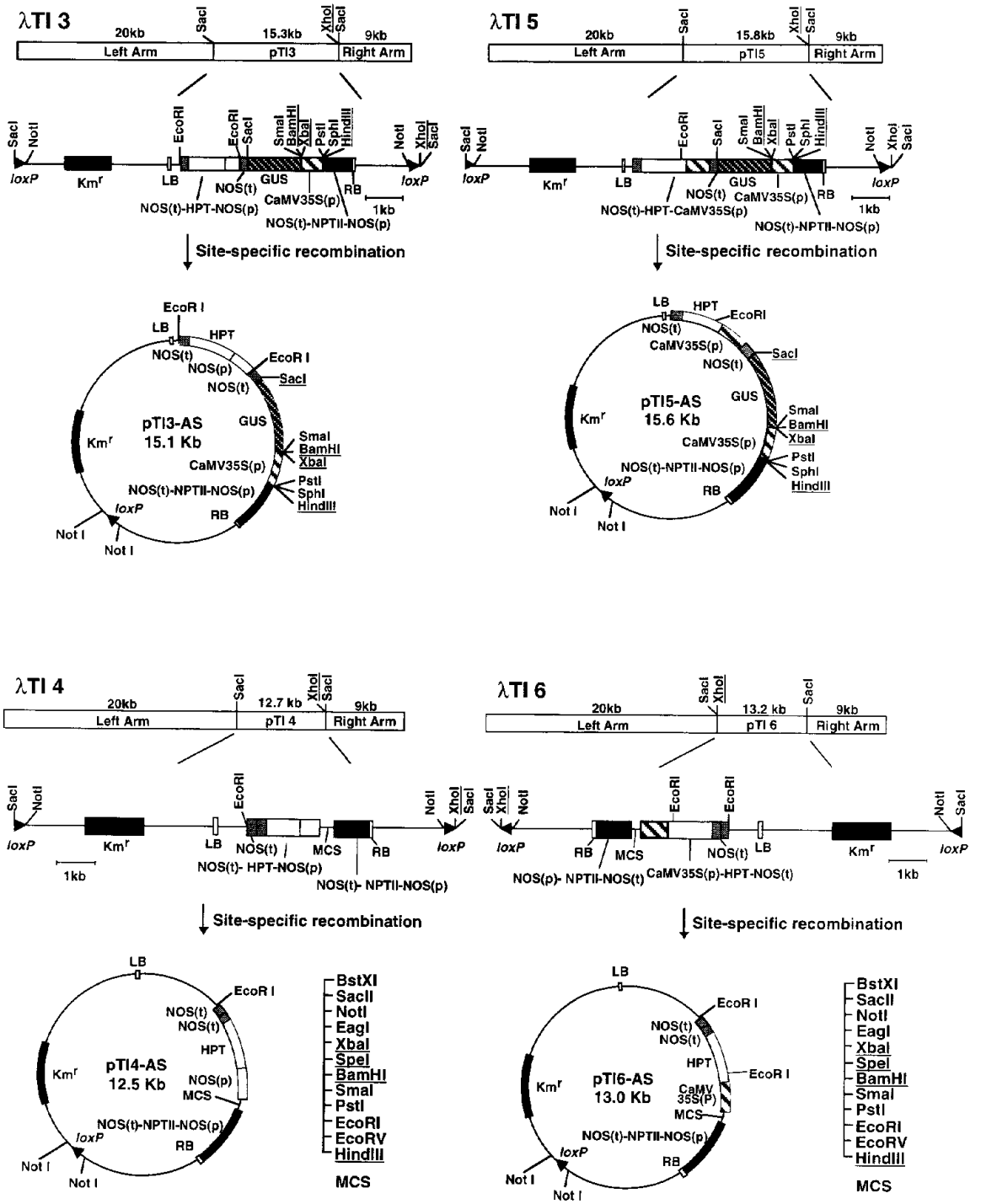

Fig. 1 Structure of the λTI vectors and schematic representation of *Cre-lox*-mediated automatic subcloning. The remaining unique cloning sites in the multiple cloning sites (MCS) of λTI4 and λTI6 are underlined. CaMV35S(p). the cauliflower mosaic virus 35S promoter; NOS(p). the promoter from the NOS gene; NOS (t). the terminator from the NOS gene; LB. the left T-DNA border; RB. the right T-DNA border; Km^r. the kanamycin resistance gene; GUS. the β-glucuronidase gene; HPT. the hygromycin B phosphotransferase gene; NPT II. the neomycin phosphotransferase II gene.

control of the CaMV 35S promoter in higher plants. The λTI4 and λTI6 vectors were designed for construction of the genomic library and have the insert capacity of 10.3 kb and 9.8 kb, respectively, within the packaging limit.

A genomic library was constructed using the λTI4 vector. The genomic DNA fragments from *A. thaliana* (Columbia ecotype) were digested partially with *Mbo* I and size-fractionated DNA fragments were cloned into the *Bam*H I site of the λTI4 vector. Approximately 300,000 primary clones were amplified and pooled. The background of non-recombinant plaques was less than 10% of pooled clones. **Figure 2** shows an example of restriction analysis of the Ti-

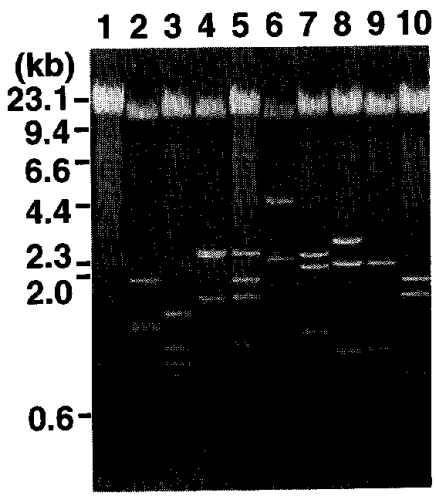

Fig. 2 Restriction analysis of the Ti-plasmids derived from λTI clones randomly chosen from the λTI4 genomic library.

Genomic DNA was prepared from the *A. thaliana* plants (Columbia ecotype) as described [9]. Partial digestion with *Mbo* I and size fractionation by sucrose density-gradient centrifugation were performed according to the standard procedures [10]. The phage vector λTI4 was automatically converted into the plasmid form by infection of *cre*$^+$ *E.coli* cells, NS5329 as described [3]. The resultant kanamycin-resistant cells were scraped, added to 10m*l* of LB medium [10] supplemented with kanamycin, and cultured at 37°C for 8 hours. The plasmid DNA was isolated from the culture as described [10], and then digested with *Hind* III and *Xba* I. The non-recombinant clone is shown in lane 1.

plasmids converted from the 10 randomly picked clones from the genomic λTI4 library. The average insert size was estimated to be about 6.8 kb. The Ti-plasmid shown in lane 6 carried the longest insert (about 8 kb) among the plasmids tested (**Fig. 2**).

A Ti-plasmid converted from the λTI3 vector was introduced into tobacco plants by *Agrobacterium*-mediated transformation. The resultant R_1 progeny showed hygromycin resistance (**Fig. 3**). The integration of the T-DNA in the transformed plants showing hygromycin resistance was confirmed by detecting the PCR-amplified fragments containing one section of the HPT gene (data not shown). These results indicated that the HPT gene in the λTI3 vector stably conferred resistance to hygromycin B when it was introduced into plant cells. Similar results were also observed when the Ti-plasmid converted from the λTI5 vector was introduced into tobacco plants (data not shown).

Hygromycin B inhibits protein synthesis in eukaryotes and has been used as an effective antibiotic in higher plants [4, 5]. In the λTI vectors reported

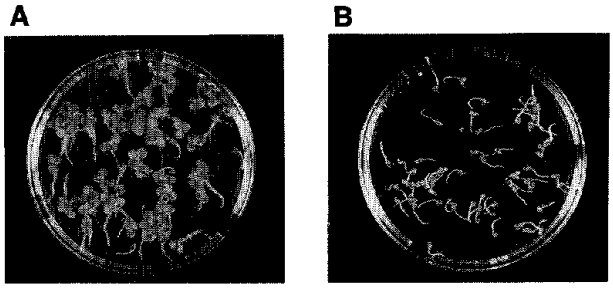

Fig. 3 Phenotypes of 2-week-old tobacco seedlings on hygromycin-containing medium.

Nicotiana tabacum cv. SR1 was transformed by the leaf disc method [11] using *Agrobacterium tumefaciens* LBA4404 containing the automatically converted plasmid, pTI3-AS. The R_1 seeds resulting from self-pollination of primary transformants (A) or seeds from the wild-type plants (B) were germinated on Murashige-Skoog medium [12] supplemented with 20μg/m*l* hygromycin B.

here, the HPT gene was inserted between the cloning sites and the left border of T-DNA. Since the right border of T-DNA is considered to be the first part to be integrated into the plant genome, and the region near the left border is considered to be the last [6], the hygromycin-resistant plants which have been transformed with Ti-plasmids converted from these new λTI phage vectors can be expected to carry the whole T-DNA region.

The HPT gene functions as a good selectable marker in several monocotyledonous plants, *e.g.* rice and wheat, which have been demonstrated to be susceptible to infection by *Agrobacterium* [7]. Therefore, these new λTI phage vectors can be used for transformation of such monocotyledonous plants. In several monocotyledonous plants, the NOS promoter confers considerably weak expression compared to the CaMV 35S promoter [8]. In such plant species, the λTI5 and λTI6 vectors, in which the HPT gene is placed under the control of the CaMV 35S promoter, should be useful for the efficient selection of transformed plants.

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