Research Note

Novel Constructions to Enable the Integration of Genes into the *Agrobacterium tumefaciens* C58 Chromosome

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We constructed several versatile sets of vectors that can be used to introduce any gene into the *pgl/picA* locus of the *Agrobacterium tumefaciens* C58 chromosome without affecting T-DNA transfer. One set contains a fragment containing the *lacI^q* and *lacZ* genes and a multiple cloning site from pBluescriptII SK(+) inserted into a *PstI* site between the *pgl* and *picA* genes on an *incP α* plasmid. The resulting plasmid contains eight unique restriction endonuclease sites and the ability to use blue–white screening for the presence of an insert. A second plasmid also contains a β -lactamase gene within this locus and provides a convenient ampicillin–carbenicillin resistance marker for the selection of genes integrated into the chromosome following double homologous recombination (homogenotization). A third plasmid contains, in addition to the *lacZ*, *lacI^q*, and β -lactamase genes within the *pgl/picA* locus, a *sacRB* gene cassette within the vector to counterselect against the presence of the vector within *A. tumefaciens*. To test this system, we introduced a wild-type *virD2* gene into the *A. tumefaciens* chromosome at the *pgl/picA* locus. When a Ti plasmid harboring a deletion of *virD2* was in this strain, the integrated *virD2* gene complemented the *virD2* deletion and the resulting transformation phenotype was identical to that resulting from *A. tumefaciens* strains harboring a wild-type *virD2* gene located on a replicating plasmid.

In many genetic studies it is common for investigators to introduce several different genes into a bacterial cell. Frequently, these genes are located on different plasmids. The introduction of multiple different plasmids into a single cell can be problematic, however, because of difficulties with plasmid incompatibility, plasmid stability and copy number, and the lack of markers required for the selection and maintenance of all plasmids. Some of these problems can be resolved by introducing the genes into the bacterial chromosome. Subsequently, the genes can be maintained stably at a low-copy number.

The introduction of genetic elements into the chromosome of *Agrobacterium tumefaciens* has been used frequently to

cause targeted disruptions (Garfinkel et al. 1981). There are, however, few reports of genes or genetic elements such as T-DNA that have been placed deliberately into the chromosome (Hoekema 1984; Miranda et al. 1992). In most studies, the insertion site must not affect either bacterial growth or T-DNA transfer–tumorigenesis by the bacterium. We previously identified a chromosomal gene, *picA*, which is neither required for bacterial growth nor virulence under laboratory conditions (Rong et al. 1990; Rong et al. 1991). The *picA* gene of the *A. tumefaciens* C58 chromosome lies entirely within a 3.15-kbp *EcoRI* fragment. This fragment also contains the *pgl* gene and a *PstI* site between the two genes (Rong et al. 1991). We chose this *PstI* site to insert a *lacZ* α peptide expression cassette containing a multiple cloning site (mcs) and an antibiotic resistance gene. Any gene of interest, along with a selection marker, can be cloned into the mcs and introduced subsequently into the *A. tumefaciens* chromosome.

Figure 1 shows the various vectors we constructed. (Details of the processes used to construct these plasmids can be obtained from the corresponding author.) Plasmids pE1770 and pE1773 contain eight unique restriction enzyme sites (*KpnI*, *HindIII*, *EcoRI*, *PstI*, *BamHI*, *SpeI*, *XbaI*, and *SacI*) within a *lacZ* α -complementation fragment that can be used for cloning a gene and a selectable marker with blue–white selection between the *pgl* and *picA* genes. Alternatively, one can clone a gene of interest first into a plasmid such as pUC or pBluescript, subsequently linearize this recombinant plasmid, and ligate this entire plasmid (containing a β -lactamase gene) into pE1770 or pE1773.

To eliminate the need to cointegrate pE1770 or pE1773 with another plasmid in order to insert a selectable marker, we constructed the plasmid pE1931, which contains a multiple cloning site, *lacZ* and *lacI^q* genes, and a β -lactamase gene between the *pgl* and *picA* genes. The *pgl/picA* region of pE1931, pE1770, and pE1773 can be recombined with the C58 chromosome of *A. tumefaciens* to insert a gene of interest (cloned into the mcs) into the *A. tumefaciens* chromosome.

In order to counterselect against the presence of the vector sequences from pE1770 or pE1931 in bacteria, we inserted blunted *PstI* fragment from pUM24Cm containing the *sacR* and *sacB* genes from *Bacillus subtilis* (Ried and Collmer 1987) into the *BglII* sites of pE1770 and pE1931, resulting in plasmids pE1962 and pE1963, respectively. Bacteria contain-

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Fig. 1. Maps of the various plasmids described in the text. The plasmids are not necessarily drawn to scale. Restriction endonuclease sites in parentheses are not unique. Restriction endonuclease sites in brackets were eliminated from the plasmids. B, *Bam*HI; Bg, *Bgl*II; Bx, *Bst*XI; Cl, *Cla*I; D, *Dra*I; H, *Hind*III; K, *Kpn*I; N, *Not*I; P, *Pst*I; RI, *Eco*RI; RV, *Eco*RV; SI, *Sac*I; SII, *Sac*II; SIII, *Sac*III; Sm, *Sma*I; Sp, *Spe*I; Xb, *Xba*I; Xh, *Xho*I.

ing the plasmid backbone will die when grown on sucrose. Bacteria lacking this plasmid backbone (resulting from double-homologous crossover of the cloning cassette region into the *pgl/picA* region of the C58 chromosome) will survive growth on sucrose.

To test the efficacy of the *pgl/picA* locus as a site for recombination, we linearized a pUC-based plasmid derived from pWR1753 containing wild-type *virD1* and *virD2* genes under the control of the *virD* promoter (Shurvinton et al. 1992) at the *Eco*RI site and combined this plasmid with *Eco*RI-digested pE1770, thus generating pE1781 (which contained a tetracycline-resistance gene in the vector backbone). *A. tumefaciens* WR1715 contains a large nonpolar deletion within *virD2* on the Ti-plasmid (Shurvinton et al. 1992). We electroporated pE1781 into *A. tumefaciens* WR1715 and selected carbenicillin-resistant transformants. We also introduced pPH1JI (carrying a gentamicin resistance gene) into one transformant, selected carbenicillin- and gentamicin-resistant

colonies, and identified double-homologous recombinants of pE1781 into the *A. tumefaciens* chromosome WR1715 by screening for tetracycline sensitivity (Garfinkel et al. 1981). Of the 122 colonies tested, 38 (31%) were tetracycline sensitive. Recombination of the *virD2* gene into the *pgl/picA* region of the chromosome was confirmed by DNA blot hybridization with two diagnostic restriction endonucleases (*Eco*RI or *Eco*RV) and the 3.15-kbp *Eco*RI fragment containing the *pgl/picA* loci as a hybridization probe (data not shown). Of the 35 random colonies tested, 32 (86%) were correct. One correct colony, *A. tumefaciens* At1168, was used in subsequent experiments.

We infected *N. tabacum* W38 leaf disks with *A. tumefaciens* WR1715 (lacking a functional *virD2* gene) (Shurvinton et al. 1992), *A. tumefaciens* WR1753 (a derivative of *A. tumefaciens* WR1715 containing the wild-type *virD2* gene on a replicating plasmid) (Shurvinton et al. 1992), or *A. tumefaciens* At1168 (containing the wild-type *virD2* gene integrated into the C58

Fig. 2. Tobacco W38 leaf disc infections by various *Agrobacterium tumefaciens* strains. Discs were infected as described in Table 1; representative discs were photographed 15 days later. **A**, *A. tumefaciens* WR1715 (lacking a functional *virD2* gene). **B**, *A. tumefaciens* WR1753 (containing a wild-type *virD2* gene on a replicating plasmid). **C**, *A. tumefaciens* At1168 (containing a wild-type *virD2* gene integrated into the bacterial chromosome at the *pgl/picA* locus).

Table 1. Infection of tobacco W38 leaf discs with *Agrobacterium tumefaciens* strains

<i>A. tumefaciens</i> strain	Location of <i>virD2</i> gene	Number of leaf discs infected ^a	Average number of tumors per disc ^b
WR1715	None	83	0.0 ± 0.0
WR1753	On a replicating plasmid	151	5.2 ± 0.3
At1168	Inserted into the <i>pgl/picA</i> locus of the C58 chromosome	129	5.4 ± 0.7

^a *A. tumefaciens* strains were grown in yeast extract-peptone medium plus the appropriate antibiotics until they reached a density of 100 (10⁹ cells per ml), as measured by a Klett-Somerson spectrophotometer. Bacteria were washed in 0.9% NaCl and resuspended in 0.9% NaCl at a Klett of 100. Tobacco leaf discs were dipped into bacterial suspension for 10 min and placed on solidified MS medium lacking phytohormones, as previously described (Gray et al. 1992). Two days after inoculation, the discs were transferred to solidified MS medium lacking phytohormones but containing 100 µg of timentin per ml, and incubation was continued at 25°C.

^b Tumors were scored 15 days after infection.

chromosome). Figure 2 and Table 1 show that, although *A. tumefaciens* WR1715 was avirulent, strain At1168 was as virulent as WR1753. Thus, *virD2* expressed from the *pgl/picA* chromosomal locus was as effective in inciting crown gall tumors as the same gene expressed from a plasmid replicating in *A. tumefaciens*.

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