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Research Note

Construction of a Derivative of *Agrobacterium tumefaciens* C58 That Does Not Mutate to Tetracycline Resistance

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Agrobacterium tumefaciens **C58 mutates to tetracycline resistance at high frequency, complicating the use of many broad-host-range cloning and binary vectors that code for resistance to this antibiotic as the selection marker. Such mutations are associated with a resistant gene unit, *tet*$_{C58}$, that is present in the genome of this strain. By deleting the *tet*$_{C58}$ locus, we constructed NTL4, a derivative of C58 that no longer mutates to tetracycline resistance. The deletion had no detectable effect on genetic or physiological traits of NTL4 or on the ability of this strain to transform plants.**

Additional keywords: genetic engineering.

Agrobacterium tumefaciens strain C58 and its derivatives such as Ti plasmid-cured strains NT1 (Waston et al. 1975), A136 (Waston et al. 1975), and C58C1 (Van Larebeke et al. 1974) are used in many genetic and molecular analyses of *Agrobacterium* spp. and their plasmids. Such emphasis is largely a result of several useful traits or properties of this biovar 1 strain, including transformability (Cangelosi et al. 1991; Holsters et al. 1978), the availability of auxotrophic mutants, genetic (Hooykaas et al. 1982; Miller et al. 1986) and physical maps (Goodner et al. 1999) of the chromosomes (Allardet-Servent et al. 1993), a well-characterized *recA* mutant (Farrand et al. 1989), and cosmid clone banks (Farrand et al. 1989; Luo and Farrand 1999). Furthermore, some properties of the C58 chromosomal background may condition virulence on certain plant species that are recalcitrant to other *A. tumefaciens* strains. For example, the Ti plasmid pTiBo542 (Hood et al. 1986; Komari et al. 1986) in its cognate chromosomal background confers moderate virulence. However, this plasmid confers hypervirulence on many plants, including important crops such as soybean, when present in the C58 chromosomal background (Hood et al. 1986). Because of this enhanced virulence, derivatives of C58 containing pEHA101 (Hood et al. 1986) and pEHA105 (Hood et al. 1993), disarmed *vir* helper plasmids derived from pTiBo542, are widely used as binary plant transformation agents.

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C58 and its derivatives give rise to tetracycline-resistant mutants at a high frequency (Luo and Farrand 1999). Such mutants arise because of a latent tetracycline resistance determinant, *tet*$_{C58}$, which is located on one of the chromosomes of C58 and its derivatives (Luo and Farrand 1999). This resistance unit consists of two genes, *tetA*$_{C58}$, which apparently codes for an efflux pump that confers the resistance, and *tetR*$_{C58}$, which encodes a repressor that inhibits the expression of *tetA*$_{C58}$ (Luo and Farrand 1999). Mutations in the repressor (which is not responsive to the antibiotic) can result in strains that express constitutive resistance to high levels of tetracycline. Such mutations pose problems because many broad-host-range vectors used in the genetic and molecular analysis of *Agrobacterium* spp. utilize tetracycline resistance as the selection marker. Such selections must be performed with caution, and using this antibiotic to maintain the plasmid entails a high risk of selecting for tetracycline-resistant mutants that can lose the plasmid of interest.

We report here the construction of a C58 derivative with a precise internal deletion of a portion of *tet*$_{C58}$. We used a two-step deletion–allele exchange procedure in which the resultant strain has not acquired additional resistance markers and does not mutate to tetracycline resistance. We also show that deleting *tet*$_{C58}$ has no other detectable physiological or genetic effects on this bacterium and does not affect the ability of the strain to transform plants.

To construct an internal deletion in the *tet*$_{C58}$ locus, we first cloned an approximately 8.0-kb *Eco*RI fragment (corrected from 8.5) (Luo and Farrand 1999) that contains the wild-type *tet*$_{C58}$ region from pZLE8.5 into pSW213 (Table 1) to generate pSWE8.5. Because the multiple cloning site (MCS) region of the vector is flanked by two *Eco*RI sites, the cloning removed all of the other recognition sites in the polylinker, allowing us to delete an internal portion of the *tet*$_{C58}$ locus by restriction enzyme digestion. The *tet*$_{C58}$ locus was disrupted by digesting pSWE8.5 with *Hin*dIII and *Kpn*I that cut at the 3′ end of *tetR*$_{C58}$ and within the 5′ region of *tetA*$_{C58}$, respectively (Luo and Farrand 1999). Digested DNA was blunted with mung bean nuclease and religated, and the clones were recovered by $CaCl_2$-mediated transformation (Sambrook et al. 1989) into *Escherichia coli* DH5α (Table 1). In the resultant clone,

pSWE7, the treatment removed almost all of $tetR_{C58}$, the 5′ one-fifth of $tetA_{C58}$, and the entire promoter control region located between the two divergently expressed genes. As predicted from the DNA sequence of the tet_{C58} locus (Luo and Farrand 1999), after digestion with *Eco*RI, the insert size in pSWE7 is about 1 kb shorter than that of the wild type (data not shown). In addition, each side of the Δtet_{C58} locus is flanked by about 3 kb of C58 genomic DNA (Fig. 1), which is sufficient to allow allele replacement by homologous recombination.

The vector component of pSWE7, pSW213, codes for resistance to tetracycline (Table 1), which necessitates the recloning of the Δtet_{C58} fragment to a vector suitable for further experiments. The IncP1α vector, pRK415K (Table 1), contains three *Sma*I sites, one in the MCS region and two in its own tet_{RP4} gene unit (Cook and Farrand 1992; Keen et al. 1988). Purified pRK415K, which also contains the *lacZ*α gene, was partially digested with *Sma*I, religated, and transformed into *E. coli* DH5α. Blue colonies on agar plates containing 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside and kanamycin were picked and screened for sensitivity to tetracycline. One such colony containing pRK415KTc$^-$ was retained for further analysis. The removal of the *Sma*I fragment containing the tet_{RP4} gene unit from the vector was confirmed by restriction endonuclease analysis of this plasmid (data not shown). The 7.0-kb *Eco*RI fragment containing Δtet_{C58} from pSWE7 was cloned into pRK415KTc$^-$ to give pZLE7. This plasmid and pZLE8.5, which carries the intact tet_{C58} locus, were independently introduced into *E. coli* DH5α. Neither plasmid conferred tetracycline resistance on this enteric host. However, while DH5α(pZLE8.5) gave rise to tetracycline-resistant variants, the strain harboring pZLE7 failed to produce such mutants at a detectable level ($<10^{-8}$) (data not shown).

Plasmid pZLE7 was used in a two-step strategy to introduce the deleted tet_{C58} locus into the chromosome of *A. tumefaciens* NT1. The method is similar to that used by Jones et al. (1988) to construct a *tra*-deletion derivative of the agrocin 84 plasmid, pAgK84. First, pZLE7 was cointegrated by homologous recombination into the chromosome of NT1 (Table 1 and Fig. 1). This was achieved by mobilizing pZLE7 from *E. coli* S17-1(pZLE7) into NT1TcR1, a spontaneous tetracycline-resistant mutant of NT1 (Table 1) (Luo and Farrand 1999), producing NT1TcR1(pZLE7). To select for derivatives of NT1TcR1 that contain the cointegrated form of pZLE7, another IncP plasmid, pPH1JI (Hirsch and Beringer 1984), encoding a gentamicin resistance marker, was mated into NT1TcR1(pZLE7) from *E. coli* 2174. Colonies resistant to kanamycin, tetracycline, and gentamicin were selected, and one such transconjugant, NT1TcR1KG (Table 1), was retained for further use. To allow the second recombination event that resolves the cointegrate by deleting the tet_{C58} locus (Fig. 1), NT1TcR1KG was grown in Luria-Bertani (LB) broth (Sambrook et al. 1989) with gentamicin as the only selection. After four subcultures, a portion of the culture was plated onto nutrient agar (NA) (Difco, Detroit, MI, U.S.A.) at a dilution giving about 150 to 200 colo-

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s)[a]	Source and reference
Escherichia coli		
DH5α	F$^-$ ϕ80d*lacZ*ΔM15 *endA1 recA1 hsdR17*($r_K^- m_K^+$) *supE44 thi-1 gyrA96* Δ(*lacZYA-argF*) U169	Sambrook et al. 1989
S17-1	Pro$^-$Res$^-$ Mod$^+$ *recA*; integrated RP4-Tet::Mu-Kan::Tn*7*, Mob$^+$	Simon et al. 1983
2174(pPH1JI)	Met Pro Gm^r Sp^r used to evict IncP plasmids	Hirsch and Beringer 1984
Agrobacterium tumefaciens		
NT1	pTiC58-cured derivative of strain C58	Waston et al. 1975
NT1TcR1	Randomly chosen tetracycline-resistant mutant of NT1; $tetR_{C58}$::IS*426*, Tc^r	Luo and Farrand 1999
NT1TcR1GK	Derivative of NT1TcR1 into which pZLE7 is cointegrated at the tet_{C58} locus; Tc^r, Gm^r, Km^r	This study
NTL4	Derivative of NT1 with an internal deletion of the tet_{C58} locus, Δtet_{C58}, Tc^s	This study
Plasmids		
pRK415	Broad-host-range cloning vector; IncP1α, *lacZ*α, Tc^r	Keen et al. 1988
pRK415K	Kanamycin-resistant derivative of pRK415; IncP1α, *lacZ*α, Tc^r, Km^r	Cook et al. 1993
pRK415KTc$^-$	Derivative of pRK415K with an internal deletion of the tetracycline resistance cassette; Km^r	This study
pSW213	Broad-host-range cloning vector; IncP, Tc^r	Chen et al. 1994
pPH1JI	IncP1 R plasmid used to evict IncP vectors; Gm^r	Hirsch and Beringer 1984
pZLE8.5	8.0-kb *Eco*RI fragment containing the wild-type tet_{C58} locus cloned into pBluescript SK(+), Ap^r	Luo and Farrand 1999
pZLOP1	66-bp tet_{C58} intergenic region cloned into promoter reporter vector pRG970b; IncP/ColE1, Ap^r, Sm^r/Sp^r	Luo and Farrand 1999
pDLB4	Broad-host-range expression vector containing *araC* and the P_{BAD} promoter; unknown Inc group, Km^r	Luo and Farrand 1999
pDLB4-tetR	$tetR_{C58}$ cloned into pDLB4 as a 2.7-kb *Nco*I fragment; expression of $tetR_{C58}$ from P_{BAD} is controlled by AraC and arabinose	Luo and Farrand 1999
pSWE8.5	8.0-kb *Eco*RI fragment containing wild-type tet_{C58} from pZLE8.5 cloned into pSW213	This study
pSWE7	Derivative of pSWE8.5 with an approximately 1-kb deletion within tet_{C58}	This study
pZLE7	7.0-kb EcoRI DNA fragment containing Δtet_{C58} from pSWE7 cloned into pRK415KTc$^-$	This study
pZP212	Binary vector encoding *nptII* driven by the 35S promoter of *Cauliflower mosaic virus*; IncP/ColE1, Sm^r/Sp^r, Cm^r	Hajdukiewicz et al. 1994
pEHA105	*vir* helper plasmid derived from pTiBo542	Hood et al. 1993

[a] Ap^r = ampicillin resistant, Cm^r = chloramphenicol resistant, Gm^r = gentamicin resistant, Km^r = kanamycin resistant, Sm^r/Sp^r = streptomycin/spectinomycin resistant, Tc^r = tetracycline resistant, and Tc^s = tetracycline sensitive.

nies per plate. The resultant colonies were patched individually onto NA and NA containing 2 µg of tetracycline per milliliter. A single tetracycline-sensitive clone was identified among the first 300 colonies screened. This isolate was cured of pPH1JI by growing the strain for several subcultures in L broth without antibiotic selection. One colony that was susceptible to tetracycline and gentamicin was recovered from this culture regimen and named *A. tumefaciens* NTL4.

We confirmed the deletion–mutation by Southern analysis. The approximately 8.0-kb *Eco*RI fragment containing tet_{C58} was used to probe *Eco*RI-digested chromosomal DNA of NT1, NT1TcR1, two colonies of NTL4, and preparations of pSWE8.5 and pSWE7. The probe hybridized with an approximately 8.0-kb fragment in the digest of NT1, which is consistent with the size of the fragment carried on pSWE8.5 (Fig. 2). Furthermore, two fragments, one about 4.5 kb and the other about 4.9 kb, were detected in the digest of NT1TcR1 (Fig. 2). These results are in agreement with our DNA sequence analysis of the tet_{C58} locus in this mutant (Luo and Farrand 1999). The $tetR_{C58}$ gene of this isolate was disrupted by IS*426*, which introduced an *Eco*RI site into the repressor gene and divided this locus into two *Eco*RI fragments. Both of these fragments were absent in genomic DNA from two colonies of NTL4, being replaced by a single band of approximately 7.0 kb that matched the size of the insert carrying the deletion allele present on pSWE7 (Fig. 2).

We examined the response of this new strain to tetracycline and several other antibiotics. Approximately 5×10^9 cells from saturated cultures of NT1 and NTL4 in LB broth were inoculated onto plates of NA and ABM agar minimal medium (Chilton et al. 1974) containing 1, 2, 5, and 10 µg of tetracycline per milliliter. Similar volumes of NTL4 were plated onto media containing kanamycin or gentamicin. While wild-type NT1 yielded several thousand colonies after 72 h on plates containing all concentrations of tetracycline, NTL4 gave no colonies on any of these plates, even after 120 h of incubation at 28°C (Fig. 3 and data not shown). Furthermore, as with the wild-type strain, NTL4 is susceptible to kanamycin and gentamicin (data not shown). Analysis of plasmid DNA from this strain by a mini-prep procedure (Hayman and Farrand 1988) did not detect any plasmids less than 450 kb (data not shown), indicating that the strain was cured of the eviction plasmid pPH1JI. NTL4 retains pAtC58, the 450-kb santhopine-catabolic plasmid present in C58 (Vaudequin-Dransart et al.

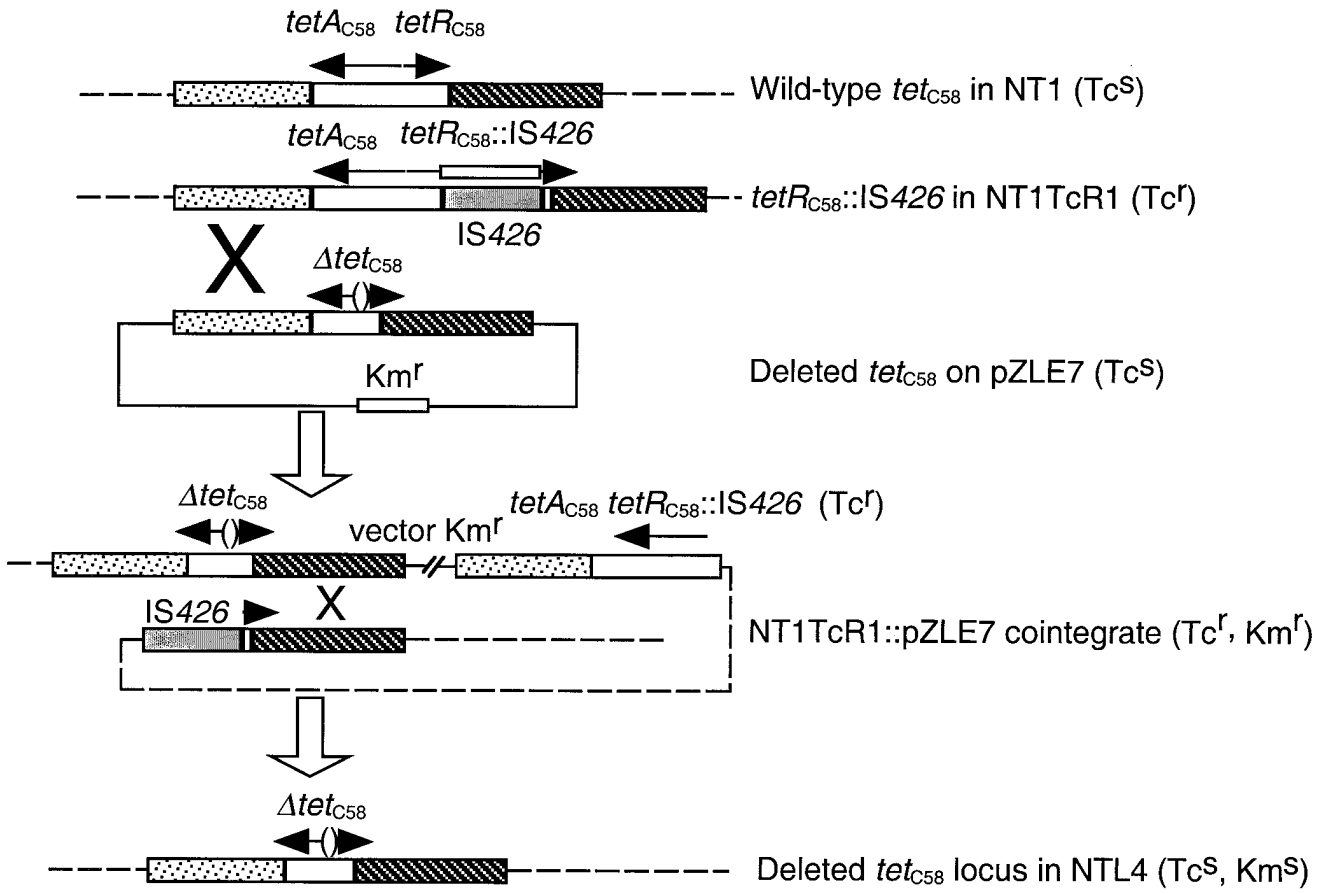

Fig. 1. Construction of *Agrobacterium tumefaciens* strain NTL4. Structures of the tet_{C58} locus in strains NT1, NT1TcR1, NT1TcR1::ZLE7, and NTL4 are shown. The first crossover (large X) between pZLE7 and the tet_{C58} locus in NT1TcR1 led to the duplication of the flanking sequences of the tet_{C58} locus and generation of strain NT1TcR1::ZLE7, which is resistant to tetracycline and kanamycin. The second recombination event occurring between one of the duplicated regions (small X) resulted in the resolution of the cointegrate by excision of the vector region of pZLE7 and deletion of the tet_{C58}::IS*426* locus. Recombination events leading to the deletion are shown occurring first on the left side of tet_{C58} to form the cointegrate and then on the right side to resolve the cointegrate, but they could have occurred in reverse order. Flanking sequences of C58 genomic DNA on both sides of tet_{C58} (open box) and IS*426* (gray box) are shown as stippled and cross-hatched boxes. Vector DNA of pZLE7 and the chromosomal region far beyond tet_{C58} are solid lines and dashed lines, respectively. Arrows = $tetA_{C58}$, $tetR_{C58}$, or truncated $tetR_{C58}$. Arrows separated by parenthesis = deleted form of the tet_{C58} locus.

1998). We conclude from these results that NTL4 lacks a functional $tetA_{C58}$ gene.

Given the size and location of the deletion, NTL4 should not encode an active $TetR_{C58}$ protein. To test this hypothesis, we introduced into the new strain pZLOP1 a construct that contains the bidirectional promoter region of tet_{C58}, driving the expression of two divergently oriented reporter genes, *uidA* and *lacZ* (Table 1) (Luo and Farrand 1999). Expression of the reporter fusions was determined by measuring the activities of β-galactosidase (*tetA*$_{C58}$::*lacZ*) and β-glucuronidase (*tetR*$_{C58}$::*uidA*), as described previously (Luo and Farrand 1999). Expression levels of both reporters in NTL4 were significantly higher than those observed in NT1(pZLOP1), which is $tetR_{C58}^{+}$, and approached the levels observed in the *tetR*$_{C58}$::IS*426* strain, NT1TcR1(pZLOP1) (Table 2). Furthermore, when a functional $tetR_{C58}$ gene coded for by pDLB4-tetR (Table 1) was supplied in trans in NTL4(pZLOP1), the expression of the two reporter fusions was repressed (Table 2). These results indicate that the product of the disrupted $tetR_{C58}$ gene in NTL4 no longer represses the tet_{C58} operator.

As assessed by an increase in culture turbidity (Piper and Farrand 2000), NTL4 and NT1 grow with indistinguishable kinetics and to the same final population sizes in LB medium and ABM minimal medium (data not shown). Similarly, the time of development, size, morphology, consistency, and color of the colonies of the two strains are indistinguishable on NA and ABM agar medium (data not shown). Furthermore, plasmid DNA can be transformed, electroporated, or mated into NTL4 with the same efficiency as NT1 (data not shown).

To assess whether disruption of tet_{C58} affects plant transformation activity of NTL4, we introduced the *vir* helper

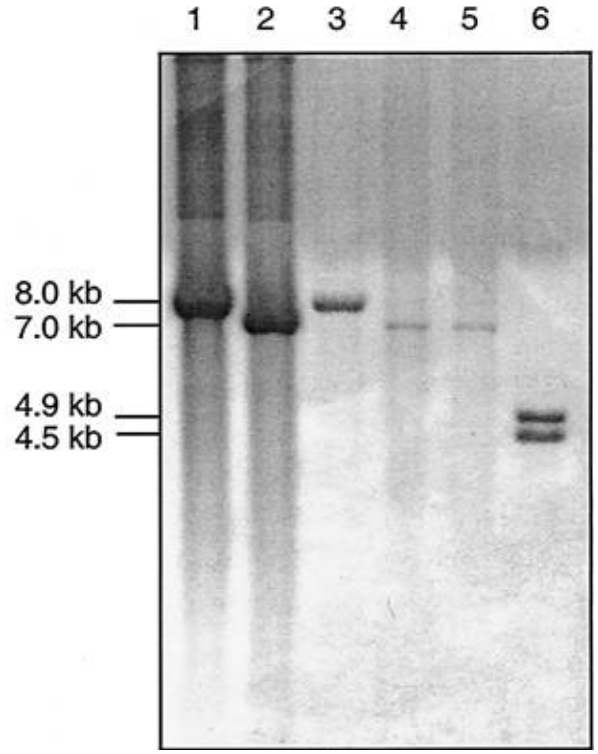

Fig. 2. Southern analysis of the tet_{C58} locus of NTL4. Plasmid DNA and total genomic DNA were digested with *Eco*RI, and the fragments were separated by electrophoresis in an 0.8% agarose gel and transferred onto a nitrocellulose membrane. The 8.0-kb *Eco*RI fragment containing the tet_{C58} locus labeled with dioxygenin was used as the probe. Hybridization, washing, and detection were conducted as previously described (Luo and Farrand 1999). Lanes: 1, pSWE8.5; 2, pSWE7, and digested genomic DNA from lanes 3, NT1; 4, NTL4 colony 1; 5, NTL4 colony 2; and 6, NT1TcR1.

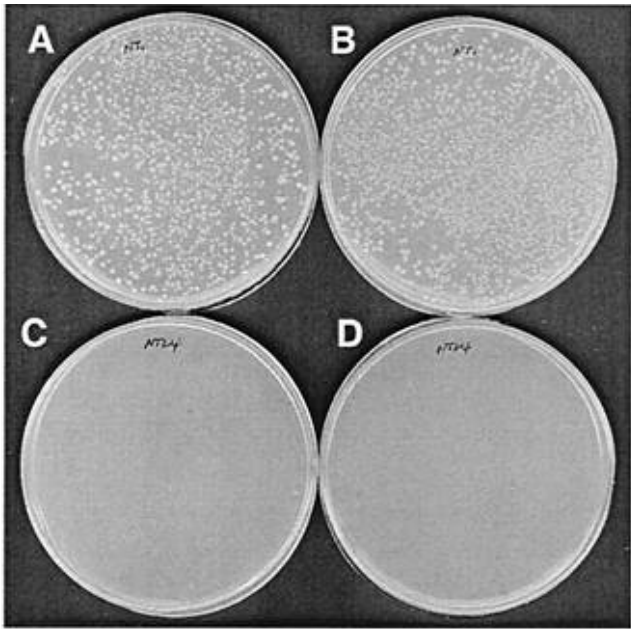

Fig. 3. NTL4 no longer mutates to tetracycline resistance. **A and B,** Strains NT1; **C and D,** NTL4 were grown to saturation in Luria-Bertani broth. Volumes of 200 µl of the cultures (approximately 10^9 CFU) were plated onto nutrient agar containing tetracycline at concentrations of 2 (**A**) and 5 (**B and D**) µg/ml. Emergence of mutants resistant to the antibiotic was judged by the appearance of colonies on the plates after incubation at 28°C for 72 h. No colonies appeared on plates inoculated with NTL4, even after 120 h of incubation.

Table 2. The genome of NTL4 does not contain a functional $tetR_{C58}$

		β-Galactosidase activity (*tetA*::*lacZ*)[a]		β-Glucuronidase activity (*tetR*::*uidA*)	
Strain	***tetR*$_{C58}$ source or allele**	**–Ara[b]**	**+Ara[c]**	**–Ara**	**+Ara**
NT1(pZLOP1, pDLB4)	Chromosome	19	14	14	20
NT1(pZLOP1, pDLB4-tetR)	Chromosome, pDLB4-tetR	25	2	14	7
NT1TcR1(pZLOP1, pDLB4)	*tetR*::IS*426*	77	75	53	45
NTL4(pZLOP1, pDLB4)	None	74	73	50	45
NTL4(pZLOP1, pDLB4-tetR)	pDLB4-tetR	63	2	17	5

[a] β-Galactosidase and β-glucuronidase activities are expressed as units per 10^9 CFU as previously described (Luo and Farrand 1999). The experiment was repeated twice with similar patterns of activities.
[b] Results without arabinose (Ara).
[c] Ara was added at a final concentration of 0.4% to induce expression of $tetR_{C58}$ from pDLB4-tetR.

plasmid pEHA105 (Hood et al. 1993) into this strain as well as NT1 by electroporation (Cangelosi et al. 1991), selecting for utilization of mannopine. A binary vector, pZP212 (Hajdukiewicz et al. 1994), which codes for a plant-expressing *nptII* gene, was introduced into these NTL4 constructs, and the resultant strains were used to transform *Nicotiana tabacum* cv. Xanthi (TC533, North Carolina State University, Raleigh, U.S.A.) with a modified leaf-dip protocol (Horsch et al. 1985). Following cocultivation with the bacteria, plant leaf segments were transferred to regeneration medium composed of Murashige and Skoogs medium with B5 vitamins (MS–B5) (Manandhar and Gresshoff 1980) supplemented, per milliliter, with 1 µg of 1,2-bis(2-amino-5-bromophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid (BAP), 0.1 µg of naphthalene acetic acid (NAA), and 150 µg of kanamycin. The explants were subcultured on the same medium every 2 weeks. The percentages of leaf disks with differentiating kanamycin-resistant shoots that arose following infection by the three clones of NTL4(pEHA105, pZP212) were indistinguishable from the percentages of explants giving shoots following infection with NT1(pEHA105, pZP212) (data not shown). Uninoculated explants cultured on the same medium were completely bleached after 4 weeks in culture (data not shown). A selected subset of kanamycin-tolerant shoots were excised after 6 to 8 weeks in culture and were rooted on an MS–B5-based medium supplemented, per milliliter, with 0.1 µg of NAA and 50 µg of kanamycin. The shoots induced by NTL4 (pEHA105, pZP212) regenerated roots with frequencies indistinguishable to those of shoots induced by NT1 that harbored the same binary system (data not shown). A subset of transgenic plants transformed with each strain were established in soil, and plants were assayed for NptII protein content with a commercially available enzyme-linked immunosorbent assay kit (5′ to 3′, Boulder, CO, U.S.A.), following the manufacturer's protocol. As shown in Table 3, all of the transgenic plants contained comparable levels of the NptII protein.

We also compared NTL4(pEHA105, pZP212) with its parent for using their ability to transform *Arabidopsis thaliana* with an in planta transformation protocol (Bechtold et al. 1993). The two strains yielded essentially the same numbers of antibiotic-resistant germinants when seeds from infected plants were plated onto selection medium containing kanamycin (data not shown). The results from these two sets of experiments indicate that the deletion in tet_{C58} has no detectable effect on the plant transformation properties of this *A. tumefaciens* strain.

In summary, we have constructed a derivative of NT1 that contains an internal deletion of the tet_{C58} locus. The strain, which no longer mutates to resistance to this antibiotic ($<10^9$), simplifies the use of tetracycline resistance as a selection marker in the C58 genomic background. The two-step deletion–allele exchange strategy did not introduce any additional markers, and NTL4 remains susceptible to all antibiotics to which NT1 is sensitive. Moreover, because no tetracycline-resistant mutants arise, and it is entirely effective at low concentrations, we routinely use 2 µg of this antibiotic per milliliter in rich and minimal media when required for genetic selection. In our use of this strain over the past 2 years, we have not observed any physiological alterations attendant to this mutation. Furthermore, the ability of NTL4 to transform the two plant species tested is indistinguishable from that of its parent strain, NT1.

Table 3. Plants transformed by NTL4 exhibit NptII activity

Transforming strain	N[a]	NptII[b]
NT1(pEHA105, pZP212)	6	37.9 ± 17.6
NTL4(pEHA105, pZP212)-1	5	35.0 ± 1.03
NTL4(pEHA105, pZP212)-2	3	33.2 ± 12.4
NTL4(pEHA105, pZP212)-3	5	16.7 ± 15.5

[a] The number of regenerated transgenic plants assayed.
[b] The mean of NptII expressed as picograms of protein per milligram of total protein per plant ± standard deviation.

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