RepB C-terminus mutation of a pRi-repABC binary vector affects plasmid copy number in Agrobacterium and transgene copy number in plants

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Abstract

A native repABC replication origin from pRiA4b was previously reported as a single copy plasmid in Agrobacterium tumefaciens and can improve the production of transgenic plants with a single copy insertion of transgenes when it is used in binary vectors for Agrobacterium-mediated transformation. A high copy pRi-repABC variant plasmid, pTF::Ri, which does not improve the frequency of single copy transgenic plants, has been reported in the literature. Sequencing the high copy pTF::Ri repABC operon revealed the presence of two mutations: one silent mutation and one missense mutation that changes a tyrosine to a histidine (Y299H) in a highly conserved area of the C-terminus of the RepB protein (RepBY299H).

Reproducing these mutations in the wild-type pRi-repABC binary vector showed that Agrobacterium cells with the RepBY299H mutation grow faster on both solidified and in liquid medium, and have higher plasmid copy number as determined by ddPCR. In order to investigate the impact of the RepBY299H mutation on transformation and quality plant production, the RepBY299H mutated pRi-repABC binary vector was compared with the original wild-type pRi-repABC binary vector and a multi-copy oriV binary vector in canola transformation. Molecular analyses of the canola transgenic plants demonstrated that the multi-copy pRi-repABC with the RepBY299H mutation provides no advantage in generating high frequency single copy, backbone-free transgenic plants in comparison with the single copy wild-type pRi-repABC binary vector.

Introduction

Single copy, vector backbone-free transgene events are required by government regulatory agencies for commercial transgenic product deployment, and are preferred for many other biotechnology applications. This is because most vector backbones contain antibiotic genes...
and other not fully characterized sequences, and that multi-copy transgenes may have reduced or silenced gene expression [1–3]. Efforts have been made to increase single copy transgene frequency, and to eliminate the presence of vector backbone sequences in transgenic plants using particle bombardment or Agrobacterium-mediated transformation [4–9]. Lowering DNA loading in particle bombardment [4–8], or using a single copy binary vector rather than multi-copy vectors in Agrobacterium transformation [9] can significantly increase single copy transgenic plant production.

Agrobacterium-mediated plant transformation often uses a binary vector to maintain the plasmid in Escherichia coli for gene cloning manipulation and in Agrobacterium cells to deliver the T-strand into plant cells. Different origins of replication control the binary vector copy number in Agrobacterium which can further affect T-strand processing and integration into the plant genome. The broad host range plasmid RK2-derived origin of replication (RK2 oriV, incPα group, GenBank accession #J01780) replicates to approximately 10 copies/Agrobacterium chromosome [10], whereas the pRi-repABC, a plasmid with the origin of replication from the Agrobacterium rhizogenes plasmid pRiA4b, (hereafter denoted oriRi, GenBank accession #X04833), replicates the plasmid as 1 copy/Agrobacterium chromosome [11–13]. The oriRi plasmid contains the repABC operon encoding the RepA and RepB proteins for plasmid partitioning, and the replication initiator protein RepC for DNA synthesis [14,15]. A small RNA, which binds to the region of repABC mRNA between repB and repC, tightly regulates repC expression to control plasmid copy number and determines plasmid incompatibility [16,17]. RepB proteins bind specifically to parS-like sequences, and are required for proper segregation of replicated plasmids to daughter cells. RepB can form dimers and oligomers in solution. The central part of RepB is responsible for parS binding and the C-terminus determines RepB dimerization/oligomerization [18]. The putative parS binding site identified in the plasmid pRiA4b is 28 bp downstream from the end of the repC ORF [19, 20].

Native oriRi-based binary vectors produce a higher percentage of single copy, backbone-free transgenic plants across multiple crops than do multi-copy binary vectors [9]. A new variant of the pRi-repABC replicon based vector, named pTF::Ri, has been reported with an estimated copy number 15–20 per Agrobacterium cell. This binary plasmid provided no advantage in single copy transgenic plant production [21].

To address the discrepancy between the results obtained with the native oriRi base vector [9] and the pTF::Ri [21] multi-copy vector, we sequenced the high copy plasmid pTF::Ri. Sequence analysis of the entire plasmid resulted in the identification of two point mutations in the repB gene of the pTF::Ri plasmid. In this manuscript, we characterize these two mutations in the repB gene of the pTF::Ri plasmid for their effect on plasmid copy number in Agrobacterium, and their impact on the frequency of single copy, backbone-free transgenic canola plant production.

**Materials and methods**

**Plasmid sequence and construction**

The high copy oriRi plasmid pTF::Ri [21] was sequenced and assembled by the Monsanto sequence center. This new sequence was aligned with the published pRiA4b sequence (GenBank accession #X04833) using with Needleman-Wunsch algorithm [22].

The oriRi plasmid pMON83937 [9], which contains 4114 bp of the repABC sequence from pRiA4b (GenBank X04833, between 7–4120 bp), was used for all site-directed mutagenesis experiments using the QuickChange II protocol (Agilent Cat # 200521). The primers 5’ GATCATGTGCCAGCTGAGTCAATGACCGCTACGCATG 3’ (forward) and 5’ CAGCGTGGTACGCTTGATGACACGGCTGGGACATGATC 3’ (reverse) were used to...
generate the RepB\textsuperscript{Y299H} mutation, whereas the primers 5’ GCAGTTTTCTCGAGAGATcGTCATCGCCGCGATGTC 3’ (forward) and 5’ CGACATCGCGGCGATGACgATCTCTCGAGAAAACTGC 3’ (reverse) were used to produce the repB\textsuperscript{T486C} silent mutation. Ten ng of pMON83937 was used in a 50 μl reaction volume and amplified for 25 cycles with 5 min elongation time. The reaction mixture was incubated with Dpn\textsc{I} to remove the template and transfected into E. coli DH10B competent cells to recover the plasmids. The resultant plasmids were extracted using a Qiagen maxiprep kit and verified by full plasmid sequencing. The plasmids used for this study are summarized in Table 1.

**Agrobacterium DNA extraction and plasmid copy number determination**

*Agrobacterium* total DNA was prepared according to Estrella et al. [23] with modifications. Briefly, *Agrobacterium* containing the corresponding plasmids was grown in LB medium at 30˚C with 50 mg/L spectinomycin selection. When the A\textsubscript{600} reached approximately 1.0, 1 ml of *Agrobacterium* cells was harvested into an Eppendorf tube and centrifuged at 15000 rpm for 5 min. The pellet was suspended in 300 μl P1 buffer (from Qiagen Maxiprep kit with RNaseA), 100 μl N-lauroylsarcosine (5% solution in TE) and mixed well, followed by addition of 100 μl Pronase (Calbiochem, 2.5 mg/ml solution in TE buffer). The solution was incubated at 37˚C for ~2 hours. The lysate was sheared by passing through a 1 ml pipet tip ~10 times, extracted twice with an equal volume of phenol/chloroform (pH 8), and then twice with an equal volume of chloroform. Total DNA from the aqueous phase was precipitated by addition of an equal volume of isopropanol at room temperature, followed by centrifugation at 15000 rpm for 4˚C for 10 min. The DNA pellet was washed with 70% ethanol, air-dried, suspended in 100 μl water, and quantified by a Thermo Fisher Nanodrop instrument.

Plasmid copy number was determined using droplet digital PCR (ddPCR) according to Jahn et al. [24] with minor modifications. Primers and MGB (minor groove binding) probes were designed using Primer Express 3.0 (Thermo Fisher Scientific, www.thermofisher.com). The plasmid-specific Taqman assay was designed to target a β-glucuronidase (GUS) expression cassette included in all plasmids. The native *Agrobacterium* gene lipA (GenBank AE007869.2) was used as a chromosomal reference template. The gusA and lipA probes were labeled with FAM and VIC fluorophores, respectively. The primers 5’ AGCCCTTCCACGCCTTCTC 3’ (forward) and 5’ CGGCTTTTCCCGACGATgATCTCTCGAGAAAACTGC 3’ (reverse) were used to amplify a lipA short sequence, and the MGB Taqman probe VIC-5’ CCACCTTGGCAGATTG 3’-MGB was used for real time lipA amplification. The primers 5’ ACCGAATAAC

| Plasmid | Description of repABC | Estimated copy in Agrobacterium* | Reference |
|---------|-----------------------|---------------------------------|-----------|
| pTF:Ri  | PCR amplified from pRiA4b, RepB\textsuperscript{Y299H} + repB\textsuperscript{T486C} | 15–20 | Oltmanns et al. (2010) |
| pMON83937 | 4114 bp native repABC, same as GenBank X04833 between 7–4120 bp | 1 | Ye et al. (2011) |
| pMON83937-RepB\textsuperscript{Y299H} | Site-directed RepB\textsuperscript{Y299H} mutation from pMON83937 | 9–10 | This study |
| pMON83937-repB\textsuperscript{T486C} | Site-directed repB\textsuperscript{T486C} mutation from pMON83937 | 1 | This study |
| pMON83937 RepB\textsuperscript{Y299H} + repB\textsuperscript{T486C} | Site-directed double mutations from pMON83937 | 9–10 | This study |
| pMON67438 | Non-repABC control, incPα group, RK2 oriV plasmid origin of replication | 12 | Ye et al. (2011) |

* The plasmid copy number is estimated by ddPCR in this study except for the pTF::Ri from the original DNA blot.

https://doi.org/10.1371/journal.pone.0200972.t001
GGCGTGGATAC 3’ (forward) and 5’ TCCAGCCATGCACACTGATAC 3’ (reverse) were used to amplify a gusA short sequence, and the MGB Taqman probe 6FAM–5’ TGTAC ACCGACATGTGG 3’–MGB was used for real time gusA amplification. Duplex PCR reactions were performed following the manufacturer’s recommendations [25]. The 20 μl reaction volume included 0.24 pg/μl total template DNA. The concentrations of each primer and probe were 0.9 μM and 0.25 μM, respectively. Following an initial denaturation step at 95°C, a two-step amplification cycle (94°C and 59°C) was repeated 40 times. Four technical replicates were taken for each DNA sample. Template concentrations were calculated by the Quantasoft 1.4 software (Bio-Rad, www.bio-rad.com) and were given as copy/μl values. Plasmid copy numbers were calculated by normalizing the gusA template concentrations to the lipA template concentrations.

Canola transformation

The pMON83937 RepB<sup>Y299H</sup> mutation plasmid (designated as pMON138207), the original oriRi binary vector pMON83937, and the RK2 oriV binary vector pMON67438 were transferred into <i>Agrobacterium tumefaciens</i> ABI (a disarmed nopaline-type strain with the C58 chromosomal background) in a 1 mm gap cuvette by electroporation using a Bio-Rad MicroPulser at 2 kV, and the plasmid-containing bacteria were selected using 50 mg/L spectinomycin and 50 mg/L kanamycin [9]. Canola (<i>Brassica napus</i> L, cultivar Ebony) transformation, CP4 transgene copy number determination, and statistical analysis have been described previously [9].

Statistical analysis

All experiments were carried out under identical conditions and repeated at least three times. Canola explants were randomized before <i>Agrobacterium</i> inoculation. Data are presented as averages of the mean of three independent experiments with standard deviation. Significant differences among the results were assessed by SAS with PRO GLIMMIX procedure for plasmid copy number, Analysis of Variance (ANOVA), and/or a Generalized Linear Model for bacterial growth and canola transformation.

Results and discussion

pTF::Ri RepB protein sequence has a mutation in a highly conserved residue at the C-terminus

Alignment of the pTF::Ri sequence with that of pRi-<i>repABC</i> (GenBank # X04833) indicates that there are two T to C point mutations in the repB gene of the oriRi: one a T to C silent mutation at 486 bp of the repB coding region (<i>repB</i> T486C), and the other a T to C point mutation at 895 bp of the repB gene leading to a Y299H mutation (RepB<sup>Y299H</sup>; see the <i>repB</i> alignment data in S1 Fig). The RepB<sup>Y299H</sup> mutation results in a change from a hydrophobic to a hydrophilic amino acid in the C-terminal region (Fig 1).

We aligned 26 RepB C-termini from GenBank (Fig 2). The oriRi RepB Y299 is conserved in the majority of <i>repABC</i> replicons, indicating that it may play an important role in RepB function.

The RepB<sup>Y299H</sup> mutation increases plasmid copy number in <i>Agrobacterium</i> and enhances culture growth

The <i>repABC</i> sequence in the oriRi replicon of pMON83937 used in a prior study [9] is identical to that of the oriRi GenBank reference sequence. To address the question of whether the
presence of either or both mutations leads to the increase in plasmid copy number of the pTF::Ri plasmid in *Agrobacterium* [21], we carried out site-directed mutagenesis to recapitulate the T to C base changes at 486 bp and 895 bp in pMON83937. These two plasmids were designated pMON83937-
repB

T486C

and pMON83937-RepB

Y299H

, respectively. Both plasmids and the control pMON83937 were separately introduced into the nopaline-type strain *Agrobacterium tumefaciens* ABI [9] by electroporation, and the cultures were spread on LB medium containing 50 mg/L spectinomycin and 10 mg/L gentamicin. After 72 hours, colonies harboring pMON83937-RepB

Y299H

were much larger than those containing the control plasmid or the pMON83937-
repB

T486C

silent mutation (Fig 3A). The faster growth could result from a gene dose effect of the elevated plasmid copy number, providing a growth advantage from the multiple copies of the *aadA* gene as increased plasmid copy number [26], which is consistent with our observation that replacing the original *aadA* promoter with a strong prokaryotic promoter can drastically increase the spectinomycin resistance in *Agrobacterium* with the native oriRi binary vector backbone [27]. To investigate this phenomenon further, at least two individual colonies from two sequence confirmed plasmids per mutation were suspended into 5 ml LB medium with the requisite antibiotics at A

600

= 0.5, an equal volume of culture was inoculated into 50 ml medium, and the ODs recorded after overnight growth (21 hrs). Over three repetitions of this experiment, the cultures with the pMON83937-RepB

Y299H

mutation consistently showed a 20–25% higher culture density (A

600

) than did the control, whereas the A

600

of the pMON83937-RepB

T486C

cultures showed an approximate 10% growth reduction compared to the control (Fig 3B). Cultures containing the RepB

Y299H

mutation reached A

600

= 1.0 about 3 hours earlier than did the control culture.

These observations may indicate an increase in plasmid copy number resulting from the pMON83937-RepB

Y299H

mutation which confers a growth advantage, and inspired us further to estimate the plasmid copy number against a chromosome reference marker.

### Table

| Gene    | Sequence                  | Length |
|---------|---------------------------|--------|
| repB    | TTTTCTGAGATAGGCTGCTGCTG   | 2115   |
| repB    | TTTTCTGAGATAGGCTGCTGCTG   | 2515   |
| repB    | TTTTCTGAGATAGGCTGCTGCTG   | 2850   |
| repB    | TTTTCTGAGATAGGCTGCTGCTG   | 2850   |
| repB    | TTTTCTGAGATAGGCTGCTGCTG   | 2850   |

*Fig 1. Sequence alignment between part of the genes encoding wild-type repB and repB from the pTF::Ri binary vector.* The T to C changes are highlighted. The repB stop codon is marked in red. Upper strand: wild-type repB from GenBank X04833; lower strand: pTF::Ri sequence.

https://doi.org/10.1371/journal.pone.0200972.g001
RepB mutation affects plasmid and transgene copy numbers

![Diagram](https://doi.org/10.1371/journal.pone.0200972.g002)

Fig 2. Alignment of the oriRi RepB C-terminus with other RepB C-termini. Arrow indicates the Y299H point mutation. The number at right indicates the last amino acid of each RepB protein. (GenBank accession: oriRi, # X04833; pRL7, *Rhizobium leguminosarum* bv. *viciae* plasmid # AM236081; C58linear, *Agrobacterium tumefaciens* str. C58 linear chromosome # AE007870.2; pRL12, *Rhizobium leguminosarum* bv. *viciae* plasmid pRL12 # AM236086; pRT TA1d, *Rhizobium leguminosarum* bv. trifoli strain TA1 plasmid # FJ592234; pNGR234b, *Sinorhizobium fredii* NGR234 plasmid pNGR234b # CP000874; p42b, *Rhizobium etli* CN 42 plasmid p42b # NC_007763; pRL204, *Rhizobium leguminosarum* bv. * trifolii* WSM2304 plasmid pRL204 # NC_011371; pR132504, *Rhizobium leguminosarum* bv. * trifolii* WSM1325 plasmid pR132504; pRL9, *Rhizobium leguminosarum* bv. *viciae* plasmid pRL9 # NC_008379; pRL201, *Rhizobium leguminosarum* bv. * trifolii* WSM2304 plasmid pRL201; pNLG234a, *Sinorhizobium fredii* NGR234 plasmid pNLG234a # U00090; pRL12, *Rhizobium leguminosarum* bv. * trifolii* WSM1325 plasmid pRL12 # NC_013250; pRT TA1d, *Rhizobium leguminosarum* bv. trifoli strain TA1 plasmid pRT TA1d # FJ592235; pRL10, *Rhizobium leguminosarum* bv. *viciae* plasmid pRL10 # NC_008381; pRLG203, *Rhizobium leguminosarum* bv. * trifolii* WSM2304 plasmid pRLG203 # NC_011370). Yellow, red, and blue shading indicate generally conserved amino acids.

https://doi.org/10.1371/journal.pone.0200972.g002
To determine plasmid copy number in *Agrobacterium*, a DNA-based Taqman assay was employed using the single copy *Agrobacterium* chromosomal gene *lipA* as an internal copy number control. Atu0972 (lipA) was chosen as the internal control for comparison to the gusA sequence in the binary vectors because *lipA* copy number does not vary upon acetosyringone treatment [28]. A third repB double mutation plasmid, pMON83937 RepBY299H + repBT486C, was produced for copy number determination. As shown in Fig 4, the plasmid copy numbers varied between 1 (pMON83937, native oriRi) and 12 (pMON67438, multi-copy oriV binary vector) per cell. The oriRi RepBY299H mutant and the RepBY299H and repBT486C double mutants were estimated to contain 9–10 copies/cell (Fig 4D and 4F), whereas the oriRi repBT486C silent mutation alone showed approximately 1 copy/cell (Fig 4E).

The trans-acting RepA and RepB proteins of the repABC plasmid resemble ParA and ParB partitioning proteins. The RepB proteins bind the cis-acting centromere-like parS, and all three elements are essential for repABC plasmid partitioning [18, 19, 20, 29]. In the ParAB system, ParB proteins bind parS to form a partition complex which recruits the ParA ATPase to form a segrosome and activate the ATPase for DNA segregation [30]. RepB proteins can form dimers-multimers, and the dimerization domain is located at the C-terminus of RepB from *Rhizobium leguminosarum* [18], which is conserved among the repABC family (Fig 2). The RepB with a C-terminus deletion was not able to bind DNA in a gel shift assay, suggesting that lack of dimerization prevents DNA binding of RepB [18]. The increased copy number of the oriRi with the RepBY299H mutation in *Agrobacterium* may result from decreased RepBY299H binding affinity to the the parS site, which prevents forming a partition complex and may lead to the appearance of plasmid multimers. It is believed that plasmid multimers may be formed during low copy plasmid replication, requiring action of a multimer resolution system to...
convert them into monomers through site-specific recombination [14]. Transcription of repABC genes is also regulated by the RepA autorepressor, and RepB enhances RepA binding to the P4 promoter motif [31]. It is also possible that the RepB Y299H mutation in the oriRi plasmid reduces RepA-RepB cooperative binding to the repABC operon promoter due to diminished RepB Y299H dimerization, leading to disturbances in transcriptional autorepression exerted by RepAB. The plasmid copy number increase by the inducer is not fully understood yet [31].

The stability of the repABC replicon with the RepB Y299H mutation was not determined because our plant transformation experiments were carried out with antibiotic selection, as was the case in previous experiments by Oltmanns et al. [21]. It is possible that the mutation affects dimerization/oligomerization, which partially blocks plasmid partitioning and leads to

Fig 4. *Agrobacterium* plasmid copy number determination by ddPCR. A) *A. tumefaciens* no binary plasmid control; B) pMON67438 oriV replicon; C) pMON83937 oriRi native repB; D) pMON83937-RepBY299H; E) pMON83937-RepBY299H + repBY486C double mutations; F) pMON83937-RepBY299H + repBY486C (T486C) silent mutation. Error bars are shown in the diagram. * B, D and F are significant higher copies than C (native oriRi) and E (T486C silent mutation) at P = 0.05. B (RK2 oriV) is also significantly higher than D and F (the oriRi mutants). Small letters a, b, c and d above the bars indicate statistically significantly different groups (data and statistical analysis in S1 Table).

https://doi.org/10.1371/journal.pone.0200972.g004
the accumulation of higher plasmid copy numbers in cells. Plasmid-free Agrobacterium cells may be avoided or minimized because the Agrobacterium used for plant transformation was grown using antibiotic selection.

The difference in plasmid copy number between Oltmanns et al. [21] and this study may result from the assay method, Agrobacterium strain, or growth time difference. Plasmid copy numbers are determined primarily by their replication origins, which show significant cell-to-cell variation within every population [32]. Oltmanns et al. [21] reported 15–20 copies/cell with pTF::Ri carrying RepB\textsuperscript{Y299H} and repB\textsuperscript{T486C} mutations in A. tumefaciens EHA105 using a DNA blot assay, whereas in this study we estimated that both the single and the double mutations in a nopaline-type ABI strain have 9–10 copies/cell, using a ddPCR assay. The DNA blot method likely has less resolution. The control vector RK2 oriV vector was estimated to be 7–10 copies/cell by DNA blot [21] and 12 copy/cell by ddPCR in this study. In previous work we estimated that the copy number of the control RK2 oriV vector in ABI strain was 4–8 times higher than that of the native oriRi based vector using DNA blot analysis [9]. The RK2 oriV copy number determined by ddPCR is close to the RK2 oriV-containing plasmid pSoup which was determined by a real time PCR method [10].

The wild-type oriRi outperforms the RepB\textsuperscript{Y299H} mutated oriRi for generating single copy, backbone-free transgenic events in canola transformation

Due to the contradictory observations in single copy, backbone-free plant production between the two versions of oriRi binary vectors [9,21], we speculated that the higher copy number of pTF::Ri with the RepB\textsuperscript{Y299H} mutation abolished the advantage of the native oriRi binary vector for production of single copy transgenic plants. To test this hypothesis, we compared the wild-type oriRi binary vector (pMON83937) and the RepB\textsuperscript{Y299H} mutant (pMON138207), as well as the multi-copy RK2 oriV based vector (pMON67438), in canola transformation. In total, 1262 transgenic canola events were generated for all three constructs, and all three constructs showed statistically equivalent transformation frequencies, which is defined as the number of independent transformed plants produced per number of explants used (Fig 5). Both the single copy and the single copy, backbone-free transgenic plant frequencies were equivalent between the multi-copy RK2 oriV based vector and the oriRi vector with RepB\textsuperscript{Y299H} high copy mutant, whereas the native single copy oriRi vector showed a significant improvement in the frequency of plants with a single copy of the transgene and absence of vector backbone sequences. The frequency of plants containing vector backbone sequences was decreased three-fold using the native oriRi vector compared to the RK2 oriV and the high copy oriRi mutant vectors.

This canola transformation result is consistent with the earlier observation that the high copy pTF::Ri resulted in a similar frequency in single copy, backbone-free plants to the multi-copy RK2 oriV binary vectors [21]. Our results support the hypothesis that T-DNA border processing involves a stoichiometric reaction between the endonuclease VirD2 and the border elements, and an excess of borders may titrate-out VirD2, resulting in incomplete processing [9, 33]. This hypothesis is also supported by experiments in which a T-DNA was integrated as a single copy into an Agrobacterium chromosome, which drastically decreased Agrobacterium chromosome integration frequency in transgenic plants compared to other multi-copy binary vectors [21].

Conclusion

Our data revealed that the RepB C-terminus is critical for copy number control in a repABC replicon, which may be related to plasmid partitioning [18]. A single nucleotide mutation at
RepB\textsuperscript{Y299H} in the pRi\textendash repABC derived plant transformation binary vectors is responsible for an increase in plasmid copy number in \textit{Agrobacterium}, and a concomitant decrease in relative frequency of single copy, backbone-free transgenic plants produced. This result also cautions biotechnological researchers that sequence confirmation is required for critical experiments and applications, because a single nucleotide mutation could lead to a very different conclusion.

### Supporting information

S1 Fig. RepB nucleic acid, protein sequences and mutations.
(ODCX)

S1 Table. Plasmid copy number determination by ddPCR and statistical analysis.
(XLSX)

S2 Table. OriRi mutant canola transformation raw data and statistical analysis.
(XLSX)

### Acknowledgments

We thank our colleagues at the Monsanto Genome Sequencing Center for sequencing the pRi::Ti plasmid and all repB mutants, Drs. David Somers and Doug Boyes for supporting this
research, Dr. Charles Armstrong for critical review of the manuscript, and Mr. Edwards Williams, Ms. Beiyan Zeng and Dr. Krishna Sridharan for statistical analyses.

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