CRISPR/Cas-induced targeted mutagenesis with Agrobacterium mediated protein delivery

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Chapter 2

CRISPR/Cas mediated curing of RP4 and mini Ti plasmids in *Agrobacterium*

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Abstract

The genome editing toolkit has been expanded with the RNA guided Cas9 endonuclease from the type II CRISPR/Cas system from *Streptococcus pyogenus*. Compared to its rapid adaptation in eukaryotes as a genome editing tool, applications of CRISPR/Cas systems in bacteria have progressed relatively slowly. Nonetheless CRISPR/Cas systems have been used to edit bacterial genomes and to control gene expression in a range of prokaryotes. Here we developed an engineered CRISPR/Cas system for use in the plant pathogen *Agrobacterium tumefaciens*. We show that this system can be used to cure *Agrobacterium* from the promiscuous plasmid RP4 and from vectors with the replication unit of the octopine Ti plasmid. Curing of complete Ti plasmids was not successful, possibly because of the presence of a toxin anti-toxin system.

Introduction

CRISPR (clustered regularly interspaced short palindromic repeats) and Cas (CRISPR associated) nucleases are found in many different prokaryotes, where they function as adaptive immune systems that act against bacteriophages and other invading nucleic acids [1]. The prototypical CRISPR/Cas endonuclease from the type II bacterial CRISPR/Cas system found in *Streptococcus pyogenus* can be programmed to create targeted double strand breaks (DSBs) by an engineered single guide RNA (sgRNA) [2]. The Cas9 endonuclease is directed to a desired sequence by specifying a 20 basepair sequence of the sgRNA which directs the Cas9 nuclease to a 20 nucleotide complementary sequence [2,3]. Selection of target sequences is limited by the requirement of a so called protospacer-associated motif (PAM) which is a requisite for Cas9 activity [4,5].

DSBs introduced in the genome with the CRISPR/Cas system can either be repaired via non-homologous end joining (NHEJ) or by homologous recombination (HR) and thus the system can be exploited for targeted mutagenesis or gene replacement. Because most bacteria lack an efficient NHEJ repair mechanism, DSBs are lethal if repair cannot occur by HR. Repair via HR can however be used to replace or correct existing genes by providing an artificial repair template with homology to the target sequence.

DSBs induced by an engineered CRISPR/Cas system from *Streptococcus* have been used for genome editing via HR in *Streptococcus* [6], *E. coli* [6–10], multiple *Streptomyces* species [11–13], *Lactobacillus reuteri* [14] and two different *Clostridium* species [15–17]. Furthermore the CRISPR/Cas system has also been used to selectively repress transcription of target genes using a mutated Cas9 protein without nuclease activity [18–21].

In this chapter we describe development of an engineered CRISPR/Cas system in the gram-negative soil dwelling bacterium *Agrobacterium*. We show that Cas9 is active in *Agrobacterium* and can be used for the curing of the promiscuous plasmid RP4 and small derivatives of the octopine Ti plasmid. Furthermore we show that our version of the Cas9 protein with a C-terminal translocation tag for translocation by the *Agrobacterium* VirB/D4 T4SS is functional and therefore can be used in the following chapters as an effective nuclease.

Results

Design of a Cas9/sgRNA expression system for Agrobacterium

In order to induce DSBs at specific sites in the *Agrobacterium* genome we expressed Cas9 and sgRNAs under the control of the acetosyringone inducible *virF* promoter that has been used previously for the expression of various heterologous proteins [22,23]. The Cas9 protein used in our experiments was modified to contain the C-terminal transport signal that is recognized by the *Agrobacterium* VirB/D4 T4SS secretion channel, which is required for the experiments in Chapters 3 and 4.

Initially a vector was designed for expressing a sgRNA directed against the *sacB* marker (pNCas9FsacB) and introduced into an *Agrobacterium* strain with an pTiAch5 into which a small plasmid conferring kanamycin resistance and containing the *sacB* gene had integrated by HR via a single crossover. This plasmid can be lost again by the reverse HR reaction. This will occur spontaneously at a low frequency, but these deletion events can be selected on sucrose plates. A DSB within the integrated small plasmid can efficiently be repaired by this reverse HR reaction. In order to test CRISPR/Cas activity in *Agrobacterium* we therefore directed the system for induction of DSBs in the *sacB* gene. Efficient DSB induction would therefore lead to loss of kanamycin resistance in the survivors.

Single colonies obtained after electroporation with the Cas9 and sgRNA expression vector were re-streaked on medium with kanamycin and all were sensitive (Fig. 1a) indicating that recombination between the flanking repeats had been induced by the DSB and that the integrated small plasmid had been lost from pTiAch5. To ensure that loss of the plasmid was not the result of spontaneous recombination events that had occurred without DSB induction by the engineered CRISPR/Cas system, a control experiment was performed with a vector expressing the modified NCas9F protein in the absence of the sgRNA. In absence of the sgRNA all of the colonies obtained after electroporation had remained resistant to kanamycin (Fig. 1b).

These results combined indicate that the *Agrobacterium* CRISPR/Cas expression cassette is functional and is capable of efficiently inducing DSBs that promote the recombination of direct repeats flanking the DSB.

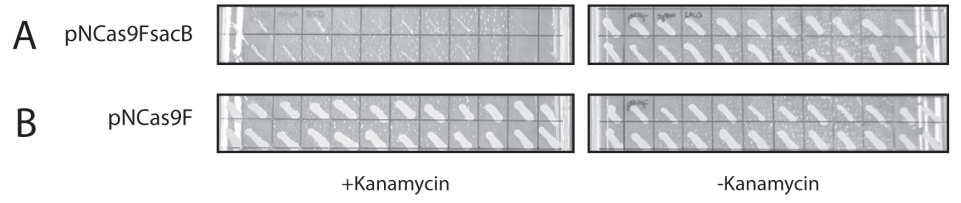

Figure 1. Screening for homologous recombination of repeats surrounding the *SacB* and Km marker present on the modified pTiAch5 identified by the loss of Km marker. LBA4001/pSDM3684 transformed with pNCas9FsacB (**A**) or pNCas9F (**B**) were grown in the absence (control) or presence of kanamycin.

Curing of RP4

As the previous experiments showed that the CRISPR/Cas system was active in *Agrobacterium,* we tested if it could be used to cure plasmids by inducing DSBs. As a candidate for curing we chose the promiscuous plasmid RP4 which is maintained extremely stable under non-selective conditions and has a copy number of about four to seven [24]. The CRISPR/Cas system described in the previous experiments was modified to target a region upstream of the replication initiation gene *trfA* (pNCas9FtrfA) and upstream of the replication origin region (*oriV*) (pNCas9ForiV) both with a protospacer containing a GG motif at the 3' end that enhances DSB break induction [25]. Cells were plated on medium with gentamicin selecting for the presence of pNCas9FtrfA and pNCas9ForiV after electroporation. After *vir* induction these colonies were checked for sensitivity to kanamycin, tetracyclin and carbenicillin as curing of RP4 would result in loss of the *nptII*, *tetA* and *bla* genes found on RP4. Induced *Agrobacterium* cells expressing only the NCas9F protein were all still resistant to kanamycin (Fig. 2b), thereby excluding that the curing of RP4 could occur due to the restrictive induction conditions. However, after expression of the NCas9F protein combined with an sgRNA targeting the region upstream of *trfA* 100% (36/36) of the colonies had become sensitive to kanamycin (Fig. 2a), tetracyclin and carbenicillin. After expression of the NCas9F protein combined with an sgRNA targeting upstream of the oriV about ~69% (25/36) of the tested colonies had become sensitive to kanamycin, tetracyclin and carbenicillin. PCR on the *trfA* locus confirmed the loss of RP4 in these colonies (Fig. 2c). Similar curing efficiencies were obtained after expression of the NCas9F protein and the sgRNA targeting the region upstream of *trfA* under control of the coliphage T5 promoter.

These results showed that DSB induction in the RP4 plasmid is an effective method for the curing of RP4 from *Agrobacterium*.

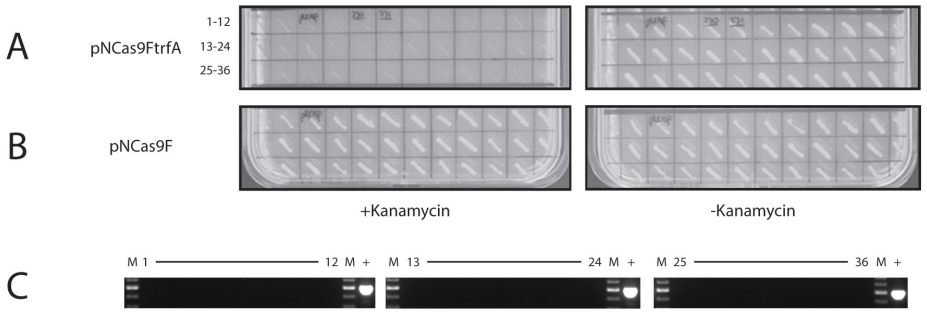

Figure 2. Screening of *Agrobacterium* for the loss of plasmid RP4. Loss is detected by kanamycin sensitivity. LBA1100(RP4) transformed with pNCas9FtrfA (**A**) or pNCas9F (**B**) were grown in the absense (control) or presence of kanamycin. PCR on the *trfA* locus using kanamycin sensitive colonies (**C**). M: DNA marker, +: positive control

Stability of the octopine Ti plasmid

As the previous experiments showed that *Agrobacterium* can be cured of RP4 we performed an experiment to test if *Agrobacterium* can be similarly cured of its octopine Ti plasmid. Because *vir* induction of *Agrobacterium* cells also increases the copy number of the Ti plasmid and its stability [26] an expression system was used in which Cas9 and the sgRNA were brought under the control of the T5 coliphage promoter [27]. With this system curing of RP4 was effective (see previous paragraph). Two new plasmids were created with the T5 expression system targeting the *virD4* (pT5NCasFvirD4) and *agaA* (pT5NCasFagaG) locus on the Ti plasmid and introduced into the *Agrobacterium* strains LBA1010, LBA1100 and LBA288. LBA1010 contains the complete Ti plasmid pTiB6 [28], LBA1100 contains the T-region-deleted helper plasmid [29] and LBA288 does not contain a Ti plasmid [30]. The pT5NCasFvirD4 or pT5NCasFagaG plasmids were introduced by electroporation. Cells were plated on medium with gentamicin selecting for the presence of the plasmids. Selected colonies were analyzed for the presence of the Ti plasmid by PCR. This analysis showed that the Ti plasmid was still present in all the LBA1010 and LBA1100 transformants. Sequence analysis of the segment encompassing the DSB site in the regions of the *virD4* and *agaA* gene, respectively, did not reveal any mutations introduced as the result of error prone repair via a NHEJ mechanism.

The transformation of both pT5NCasFvirD4 and pT5NCasFagaA to LBA1010 resulted in low numbers of transformants. To determine if this was a direct result of DSB induction we introduced a control plasmid that targets the *trfA* locus of RP4 (pT5NCas9FtrfA) and thus introduces no DSBs in the Ti plasmid of LBA1010 and LBA1100. The high numbers of transformants obtained after electroporation with this control plasmid were used to estimate the relative electroporation frequencies of the plasmids targeting the *virD4* and *agaA* locus. These results suggests that only 3% (pT5NCas9FagaA) up to 20% (pT5NCasFvirD4) of the transformants receiving a CRISPR/Cas plasmid inducing a DSB survive.

The construct targeting the *agaA* locus did not cause a strong decrease in the transformation frequency in LBA288 (no Ti plasmid) and LBA1100 (disabled Ti plasmid without the *agaA* locus) (Fig. 3). After transformation of pT5NCas9FvirD4, there was also a decrease in the relative transformation efficiency seen in LBA288 (Ti plasmid less), but presence of *virD4* in LBA1010 and LBA1100 led to a further strong decrease in relative transformation efficiency. These results combined indicate that DSBs induction in the Ti plasmid negatively affects the number of transformants.

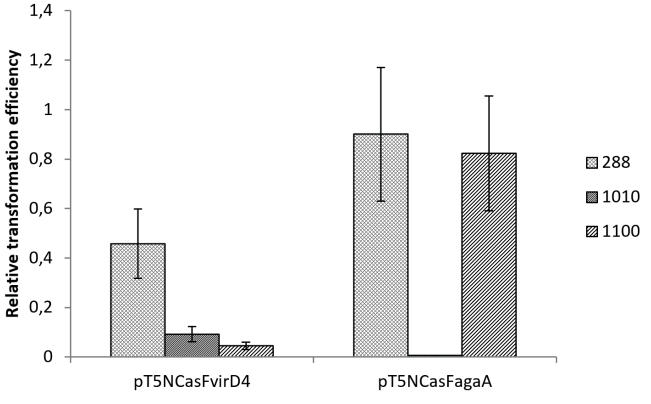

Figure 3. Transformation efficiencies of different *Agrobacterium* strains electroporated with pT5NCasFvirD4 and pT5NcasFagaA, targeting the *virD4* and *agaA* locus respectively. Relative transformation efficiencies are determined by dividing colony count of pT5NCas9FvirD4 and pT5NCas9FagaA, respectively, by the colony count of transformation with pT5NCas9trfa. Error bars indicate the SEM (N=3).

To test if the presence of the Ti plasmid is indeed involved in the reduction of the transformation efficiency a crossing was set up between LBA657, which can transfer its octopine Ti plasmid without requiring induction, and LBA288 derivatives expressing either pT5NCasFvirD4 or pT5NCasFtrfA. The transconjugant frequency decreased ~65 fold with the strain containing pT5NCasFvirD4 in the recipient compared to the transconjugant frequency with pT5NCasFtrfA (Fig. 4). This shows that presence of pT5NCas9virD4 in the recipient can prevent establishment of the Ti plasmid and indicates that a DSB is efficiently formed at the *virD4* locus in the Ti plasmid.

These results combined with the strong decrease in number of transformants seen after electroporation indicate that DSB induction in an established octopine Ti plasmid and its subsequent degradation is lethal to *Agrobacterium*.

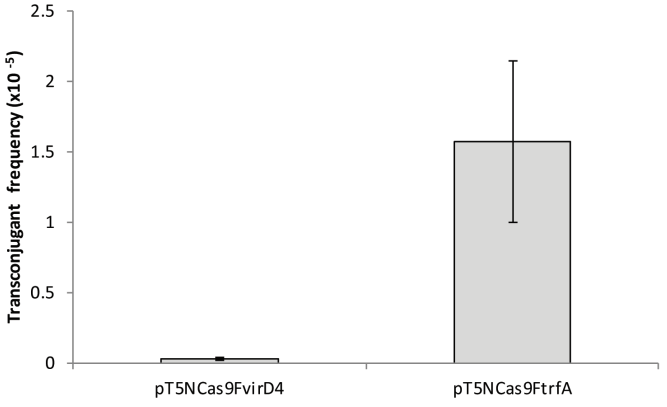

Figure 4. Transconjugant frequency after mating between LBA657 and LBA288 pT5NCas9FvirD4 or LBA288 pT5NCas9F-trfA, respectively. Error bars indicate the SEM (N=3).

Curing of mini Ti plasmids

As curing of the octopine Ti plasmid was not successful and DSB formation in the Ti plasmid led to lethality instead, this suggested the presence of one or more toxin anti-toxin systems on the Ti plasmid preventing its loss from the host. In order to exclude that the Ti *repABC* replicator had special properties preventing its loss, we tested whether small mini Ti plasmids that lack such putative toxin anti-toxin system could be cured by DBS induction in these mini Ti plasmids. Three different *Agrobacterium* strains were used, each carrying a different octopine Ti plasmid derivative all of which have a Tn1 insertion and therefore provide carbenicillin resistance (LBA2803, LBA2811 and LBA2821) [30]. A CRISPR/Cas construct targeting the *bla* gene encoding a β-lactamase present in the Tn1 insertion on these plasmids was tested for its ability to cure the mini octopine Ti plasmid derivatives (pNCas9TN1). The introduction of a vector only encoding the NCas9F protein into LBA2803, LBA2811 and LBA2821 did not result in colonies that had become sensitive to carbenicillin. DSB induction on the small Ti plasmid derivatives with the pNCas9FTN1 vector did however result in high curing efficiencies of 77% (LBA2803) (Fig. 5a), 97% (LBA2811) (Fig. 5b) and 100% (LBA2821) (Fig. 5c) based on sensitivity to carbenicillin. PCR on the *repC* locus confirmed the loss of the mini Ti plasmids in all the carbenicillin sensitive colonies in LBA2811 and LBA2821 expressing Cas9 and the sgRNA. PCR on the LBA2803 colonies expressing NCas9F and the sgRNA that had remained resistant to carbenicillin also showed no PCR product (*repC* locus), indicating that the mini Ti plasmid had been lost also in these colonies and that carbenicillin resistance

may have been maintained by spontaneous mutations or by transposition of Tn1 to another site in the genome. As a control we also introduced pNCas9TN1 in LBA657 (containing a Ti plasmid with an Tn1 insertion) which resulted in a low transformation efficiency but no curing.

These results show that DSB induction in mini octopine Ti plasmids is an effective tool for curing of these mini Ti plasmids. These results also provide a further indication of the presence of toxin anti-toxin systems elsewhere in the Ti plasmid that prevents their curing.

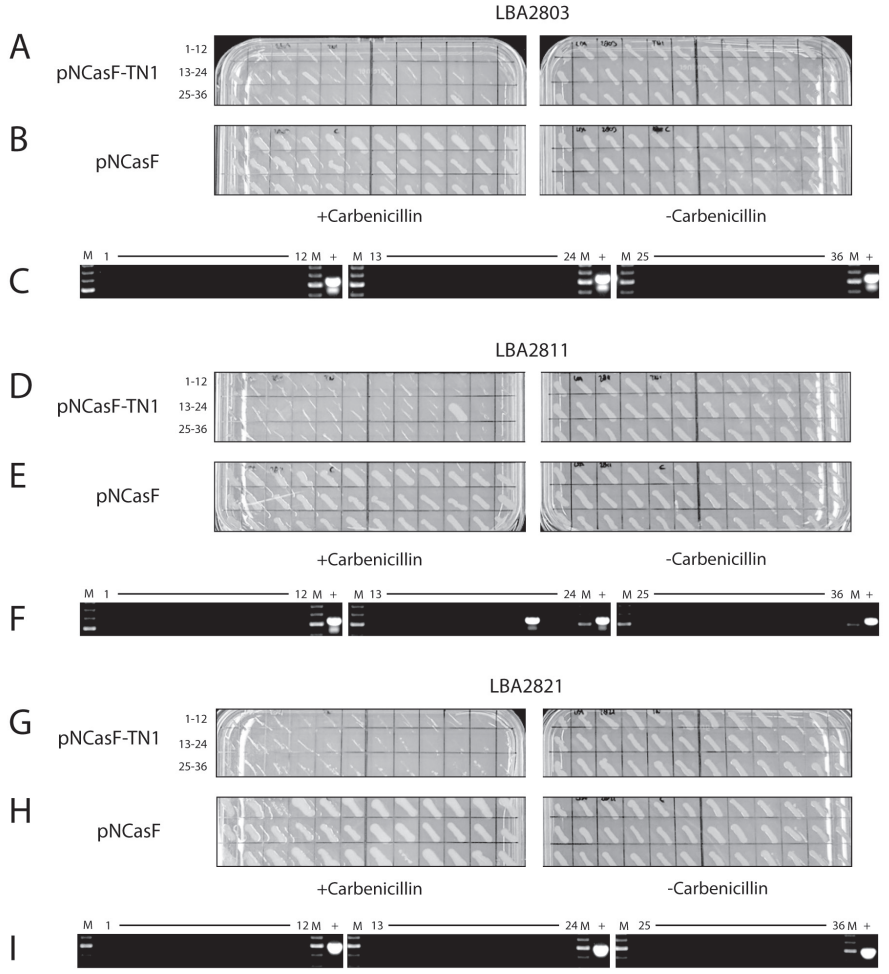

Figure 5. Screen for the loss of mini Ti plasmids. *Agrobacterium* strains expressing both the NCas9F protein and sgRNA targeting the *bla* gene encoding β-lactamase (pNCasF-TN1) were compared to *Agrobacterium* strains only expressing the NCas9F protein. Loss of the mini Ti plasmid results in loss of carbenicillin resistance. LBA2803 (**A**-**C**), LBA2811 (**D**-**F**) and LBA2821 (**G**-**I**) each harboring a different mini Ti plasmid were transformed with pNCasF-TN1 (**A**, **D**, **G**) or pNCasF (**B**, **E**, **H**) and grown in presence or absense (control) of carbenicillin. PCR on the *repC* locus of the mini-Ti plasmids using carbenicillin sensitive colonies of LBA2803, LBA2811 and LBA2821 (**C**, **F**, **I**). M: DNA marker, +: positive control

Discussion

In this study we have shown that the CRISPR/Cas9 based endonuclease system can be expressed in *Agrobacterium* and can be used to induce DBSs. Such DSBs could be used to increase the recombination between flanking direct repeats to excise a previously integrated non-replicating plasmid and can be used therefore to increase the efficiency of obtaining gene deletion events which until now required the screening of vast numbers of colonies. Furthermore the CRISPR/Cas9 system could be used to cure *Agrobacterium* cells of the promiscuous IncPα RP4 plasmid and mini Ti plasmids.

The Cas9 protein used in our experiments has been fused with a hydrophilic C-terminal secretion signal with a net positive charge [31] used previously to translocate several heterologous proteins through the type IV secretion system of *Agrobacterium* [23,31,32]. We show that the addition of this secretion signal does not negate the ability of Cas9 to induce DBSs.

The loss of the integrated non-replicating plasmid through DSB induction occurred at low basal expression levels of Cas9 and the sgRNA without requiring induction of the virF promoter. Curing of RP4 did however require higher expression levels of Cas9 and the sgRNA either through induction of the *virF* promoter or by constitutive expression under control of the T5 promoter. This suggests that with low expression levels of Cas9 and the sgRNA DSB induction does not occur on all copies of RP4 simultaneously and therefore not all copies are lost and cut RP4 molecules can be repaired via HR using still intact copies of RP4 as a template.

DSB induction in the octopine Ti plasmid negatively correlated with the survival of *Agrobacterium* cells harboring the Ti plasmid. Also a very low transconjugant frequency was observed when the Ti plasmid was transferred via mating to a recipient *Agrobacterium* expressing a CRISPR/Cas targeting the Ti plasmid. This shows that Ti plasmids have difficulty in establishing in a host expressing the CRISPR/Cas system which in line with its original biological function in defense. However, removal of an already established Ti plasmid by introduction of CRISPR/Cas turned out to be impossible. This suggests that one or more toxin anti-toxin systems are encoded by the Ti plasmid in which a long-lived toxin is neutralized by a short lived anti-toxin. Evidence for the presence of such a system has already been found for stability of the nopaline pTiC58 plasmid [33]. Attempts to identify the toxin anti-toxin system present on the octopine Ti plasmid using bioinformatics tools for the prediction of toxin anti-toxin systems was however not successful. This could be because the bioinformatics tools used can only identify type II toxin anti-toxin.

In summary, we have created a CRISPR/Cas system that is active in *Agrobacterium* and can be used to promote homologous recombination on DNA repeats and to cure *Agrobacterium* of RP4 and mini octopine Ti plasmids.

Material & Methods

Agrobacterium strains and media

Agrobacterium strains used in this study are listed in Table 1. All *Agrobacterium* strain were grown in LC (LB medium with 5g/l NaCl) at 290C with the appropriate antibiotics at the following concentrations: gentamicin 40 μg/ml; carbenicillin 75 μg/ml; kanamycin 100 μg/ml; tetracyclin 2 μg/ml. Plasmids were electroporated into *Agrobacterium* as described in den Dulk-Ras and Hooykaas [34].

Plasmid construction

The HindIII/NotI fragment with the *virF* promoter and the gene encoding a translation fusion between a nuclear localization signal, the Cre-recombinase and the last 37 amino acids of the *Agrobacterium* virulence protein VirF (pvirFpromoter-NLS::Cre::VirF37c) [32] from pSDM3155 was inserted into the HindIII/NotI sites of pUC18 to create pSDM2131. The Cre-recombinase fragment was removed by digestion of pSDM2131 with SalI and BglII and was replaced by a small linker containing a BglII site (annealed oligos DS061 and DS062). The Cas9 BamHI fragment from plasmid pMJ920 (Addgene plasmid #42234) [35] was inserted into the BglII site of pvirF-NLS::BglII Linker::VirF37c creating a translational fusion between a nuclear localization signal, Cas9 and the C-terminal 37 amino acids of VirF (NLS::Cas9::VirF37c) under the control of the *virF* promoter. The HindIII/NotI fragment with pvirF-NLS::Cas9::VirF37c was inserted into the HindII/NotI sites of pBBR6 (see Table 1) creating plasmid pNCas9F.

For the construction of the sgRNA construct under the control of the *virF* promoter a segment of DNA was synthesized at BaseClear and was provided in the pUC57Kan backbone (pDualsgRNAcassette). This synthesized DNA fragment contains two sgRNA expression cassettes in direct repeat with a BbsI and BsaI restriction site respectively in which annealed oligo's with the protospacer sequence were cloned into the BbsI site. Table 3 shows an overview of the oligo's used to create the different protospacers. The entire fragment was excised with NotI and cloned into the NotI site of pNCasF.

pT5NCasF was created by amplifying the NLS::Cas9::VirF37c fragment from pNCas9F with DS356 and DS359. This fragment was cloned into the HindIII and NotI sites of pBBR6. Into this construct the T5 promoter amplified with DS360 and DS361 was cloned into BamHI and NheI site 5' of the NLS::Cas9::VirF37c resulting in pT5NCas9F. To create the sgRNA expression cassette under control of the T5 promoter and the *virF* 3' terminator the T5 promoter fragment was amplified from pQE-30 with DS349 and DS350 and the structural part of the sgRNA together with the *virF* 3' terminator was amplified from pDualsgRNAcassette with DS351 and DS352. These PCR products were combined and used as template with DS349 and DS352 and the resulting PCR product was cloned into NotI digested pDualsgRNAcassette backbone (pT5sgRNA). To insert the protospacer sequence, annealed and phosphorylated oligo's were inserted into BbsI digested pT5sgRNA. After insertion of the protospacer sequence the T5sgRNA cassettes were cloned into the NotI site of pT5NCas9F.

The pTi Ach5 plasmid with cointegrated pSDM3684 conferring kanamycin resistance and containing the *sacB* gene was created by electroporation of pSDM3684 into wildtype *Agrobacterium* strain Ach5 (LBA4001). Single crossover events of pSDM3684 with pTiAch5 were detected by selection on kanamycin. Plasmid pSDM3684 is a derivative of pIN61 containing fragments homologous to the pTiAch5. As pSDM3684 cannot replicate in *Agrobacterium* maintenance is only possible after cointegration with the Ti plasmid by HR.

Recombination and curing experiments

For the recombination experiments the pNCas9FsacB and pNCas9F plasmids were introduced via electroporation into *Agrobacterium* strain Ach5 with the modified cointegrated Ti-Ach5:: pSDM3684 (described above). Cells were plated on medium with gentamycin selecting for the presence of the plasmids. Single colonies were re-streaked on medium with and without kanamycin (100 μg/ml) to detect loss of the integrated pSDM3684 plasmid. No induction

was required as un-induced levels of expression were sufficient for DSB induction and recombination of direct repeats flanking the DSB.

For the RP4 curing experiments pNCas9FtrfA and pNCas9F were electroporated into LBA1100 (RP4). Colonies were grown overnight at 29°C in LC with appropriate antibiotics. Induction of the *virF* promoter was performed at 28°C at OD600 = 0.25 for 16 hours in induction medium containing 200μM acetosyringone (Sigma-Aldrich Co.) . Dilution series were plated to obtain single colonies which were re-streaked on plates containing tetracycline, carbenicillin or kanamycin and on plates without antibiotics. Loss of RP4 was confirmed by PCR with primers DS347 and DS348 (*trfA* locus).

For Ti plasmid and mini Ti plasmid curing experiments, the pT5NCas9virD4, pT5NCas9FagaA, pT5NCas9FtrfA and pT5NCas9TN1 plasmids were electoporated into LBA288, LBA1010 and LBA1100. Loss of the Ti plasmid was checked via PCR using primers DS317 and DS318 (*repC* locus). Loss of the mini Ti plasmids from LBA2803, LBA2811 and LBA2821 were checked by re-streaking single colonies on medium with and without carbenicillin (75 μg/ml) and was further confirmed by PCR using primers DS317 and DS318 (*repC* locus). The relative transformation efficiencies depicted in Figure 3 were calculated by dividing the colony count after electroporation with pT5NCas9FvirD4 and pT5NCas9FagaA by the colony count after electroporation with pT5NCas9FtrfA.

Table 1. Plasmids used in this study

Plasmid	Marker	Properties	Source
pIN61	Km	sacB vector	Vergunst (unpublished)
pDSM2131	Cb	Cloning vector with NLS-Cre-VirF42n fragment	Vergunst (unpublished)
pDSM3155	Gm	Cre::virFΔ42N	[35]
pSDM3684	Km	Vector containing octopine T-DNA flanks, sacB gene and Km marker	Den Dulk-Ras (unpublished)
pNCas9F	Gm	vir inducible NCas9F expression vector	This study
pQE-30	Cb	Cloning vector for expression under control of the T5 promoter	Qiagen
pDualsgRNAcassette	Km	sgNRA cloning vector, virF promoter	This study
pNCas9FsacB	Gm	vir inducible NCas9F and sgRNA targeting sacB	This study
pT5NCas9	Gm	NCas9F expression vector under control of the T5 promoter	This study
pNCas9FtrfA	Gm	NCas9F and sgRNA targeting upstream of trfA under control of the virF promoter	This study
pNCas9ForiV	Gm	NCas9F and sgRNA targeting upstream of oriV under control of the virF promoter	This study
pT5NCasFT5virD4	Gm	NCas9F and sgRNA targeting virD4 under control of the T5 promoter	This study
pT5sgRNA	Gm	sgNRA cloning vector, T5 promoter	This study
pT5NCasFT5trfA	Gm	NCas9F and sgRNA targeting trfA under control of the T5 promoter	This study
pT5NCasFT5agaG	Gm	NCas9F and sgRNA targeting agaG under control of the T5 promoter	This study
pT5NCasFT5Tn1	Gm	NCas9F and sgRNA targeting Tn1 under control of the T5 promoter	This study
pBBR6	Gm	Derrivative of the broad host-range plasmid pRL662	[22]
pMJ920	Cb	Vector for mammalian expression of Cas9	[35]

Table 2. Overview of *Agrobacterium* strains used in this study

Strain	Chromosomal Background	Properties	Source
LBA288	C58	Cured of Ti plasmid	[36]
LBA657	C58	pTiB6::Tn1	[36]
LBA1010	C58	pTiB6	[28]
LBA1100	C58	pTiB6 disarmed	[29]
LBA2803	C58	mini Ti plasmid	[30]
LBA2811	C58	mini Ti plasmid	[30]
LBA2821	C58	mini Ti plasmid	[30]
LBA1100 RP4	C58	pTiB6 disarmed + RP4	Wittleben (Unpublished)
LBA4001/pSDM3684	Ach5	pTi-Ach5::pSDM3684	den Dulk-Ras, Hooykaas (Unpublished)

Table 3. Overview of primers used in this study

Primer name	Sequence
DS060 Linker	GATCTACTAGTGCTGCACGG
DS061 Linker	GATCCCGTGCAGCACTAGTA
DS256 SacB guideRNA NotI FW	ATATGCGGCCGCAGCTCCTATGATAGTCGATA
DS257 SacB guideRNA NotI RV	ATATGCGGCCGCGGGACCAGCACACTTAGATA
DS270 trfA protospacer	ATTGCTGGACACCAAGGCACCAGG
DS271 trfA protospacer	AAACCCTGGTGCCTTGGTGTCCAG
DS272 OriV protospacer	ATTGTGCCACCCGCGTCGCCGAGG
DS273 OriV protospacer	AAACCCTCGGCGACGCGGGTGGCA
DS317 repCfw	TGCCTAACAAAGGTACAAGG
DS318 repCrv	GTCAAGAACCTGATCAATCGC
DS339 virD4 target fw	ATTGACCTCTGTGCTGGGCGACGG
DS340 virD4 target rv	AAACCCGTCGCCCAGCACAGAGGT
DS345 agaA fw	ATTGCGCGGAGTCTCGGATCGAGG
DS346 agaA rv	AAACCCTCGATCCGAGACTCCGCG
DS349 FW T5 promoter	GATCGCGGCCGCCTCGAGAAATCATAAAAAATTT
DS350 RV T5 promoter	TTCTTCACGAAGACCCCAATCTGTGTGAAATTGTTATCCG
DS351 FW guideRNA + virF 3′	ATTGGGGTCTTCGTGAAGAA
DS352 RV guideRNA + virF 3′	CGATGCGGCCGCGGGACCAGCACACTTAGATA
DS356 RV Cas9F	TCGAGCGGCCGCTCATAGACCGCGCGTTGATC
DS359 NCas9F HindIII BamHI NheI	CTAGAAGCTTGGATCCCTACGGCTAGCATGGATAAAGCG GAATAATTCC
DS360 T5 FW BamHI	CTAGGGATCCCTCGAGAAATCATA
DS361 T5 RV NheI	GGTAGCTAGCAGTTAATTTCTCCT
DS376 TN1 fw1	ATTGTTACTTCTGACAACGATCGG
DS377 TN1 rev1	AAACCCGATCGTTGTCAGAAGTAA

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