

RESEARCH ARTICLE

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PhiC31 recombination system demonstrates heritable germinal transmission of site-specific excision from the *Arabidopsis* genome

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Abstract

Background: The large serine recombinase phiC31 from broad host range *Streptomyces* temperate phage, catalyzes the site-specific recombination of two recognition sites that differ in sequence, typically known as attachment sites *attB* and *attP*. Previously, we characterized the phiC31 catalytic activity and modes of action in the fission yeast *Schizosaccharomyces pombe*.

Results: In this work, the *phiC31* recombinase gene was placed under the control of the *Arabidopsis* *OXS3* promoter and introduced into *Arabidopsis* harboring a chromosomally integrated *attB* and *attP*-flanked target sequence. The phiC31 recombinase excised the *attB* and *attP*-flanked DNA, and the excision event was detected in subsequent generations in the absence of the *phiC31* gene, indicating germinal transmission was possible. We further verified that the genomic excision was conservative and that introduction of a functional recombinase can be achieved through secondary transformation as well as manual crossing.

Conclusion: The phiC31 system performs site-specific recombination in germinal tissue, a prerequisite for generating stable lines with unwanted DNA removed. The precise site-specific deletion by phiC31 *in planta* demonstrates that the recombinase can be used to remove selectable markers or other introduced transgenes that are no longer desired and therefore can be a useful tool for genome engineering in plants.

Background

Plant biotechnology has a role in addressing global needs for food, fiber and fuel, by developing new crop varieties with increased pest resistance, biofortification, and abiotic stress tolerance. Publicly acceptable forms of biotechnology offer an avenue for meeting these demands [1]. Recombinase-mediated genetic engineering provides a favorable direction for enhancing the precision of biotechnological approaches. Concerns over the presence of antibiotic resistance genes in the food supply and their escape into the environment [2] can be relieved through the use of recombinase technology to excise unwanted DNA from the genome of genetically engineered (GE) crops prior to marketing or release [3,4]. A study by Chawla and colleagues [5] documented how site-specific integration in rice exhibited stable gene expression over multiple generations. The research

also demonstrated that rice with multicopy transgene inserts, initially silenced for expression, recovered expression when resolved by recombinase technology to a single genomic copy. Such studies demonstrate other potential uses for recombinase technology in the development of plant biotechnology.

Genomic engineering took a large step forward with the discovery that site-specific recombinases, a group of enzymes that are capable of precise DNA cleavage and ligation without the gain or loss of nucleotides, could facilitate conservative DNA manipulation in a heterologous host [6]. The recombinase super family is split into two fundamental groups, the tyrosine and serine enzymes. This grouping is based on the active amino acid (Y or S) within the catalytic domain of each enzyme family. The best known tyrosine recombinases are Cre, Flp and R [7]. Tyrosine recombinases utilize identical recognition sites and perform a bi-directional mode of recombination. They have been shown to be effective for excision of unwanted DNA from the

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genome of the host but require complex schemes for integration.

The serine enzyme group includes the phiC31, TP901-1 and Bxb1 recombinases among others [8,9]. Members of this group recognize two non-identical recognition sites (*attB* and *attP*) and perform a uni-directional mode of recombination. While less research has been conducted on this group, it appears that the serine enzymes are well suited for precise genomic recombination due to their uni-directional catalytic activity that prevents the reversion of recombination products.

In previous studies, we identified a number of prokaryotic site-specific recombination systems that function in the eukaryote *Schizosaccharomyces pombe* [8,10]. Among those, the phiC31 uni-directional recombinase was highly efficient. The system has been successfully shown capable of recombinase mediated excision, inversion and integration reactions. The phiC31-*att* system is derived from the broad host range *Streptomyces* temperate phage phiC31 [11]. The 613 amino acid phiC31 protein acts on recognition sites *attB* and *attP* that are minimally 34 bp and 39 bp, respectively [12]. Published evidence has demonstrated that the phiC31 system is functional for excision and transmission of marker-free plastids in the seed of tobacco and in the genome of *Arabidopsis* and wheat [13-17] but has yet to be demonstrated capable of germinal transmission of nuclear DNA *in planta*.

In this research, we tested the phiC31 recombination system for the capacity to germinally transmit a target sequence that has undergone site-specific excision from within the *Arabidopsis* genome to a subsequent generation in the absence of the recombinase gene. Plants transgenic for an *attB* and *attP* flanked target sequence were introduced with a second construct that contained the recombinase gene. The phiC31 recombinase performed excision of the target sequence from three independent plant lines (i.e. genomic locations) and generated stably excised progeny plants that carry only the recombined target DNA of interest in the absence of the recombinase gene. This demonstrates that the phiC31 recombination system is suitable for the generation of stable marker-free, recombinase-free transgenic plants.

Results

Experimental design

To test for site-specific recombination, we initially sought to use a gain-of-function strategy whereby excision of a transgene would lead to promoter fusion with a previously distal marker [18]. Hence, pN3-phiC31 was configured with a CaMV 35S promoter (35S) proximal to a 760 bp non-coding stuffer region followed by a distal *gusA* coding region (Fig. 1a). The stuffer region is

flanked in direct orientation by the 54 bp *attB* and 57 bp *attP* phiC31 attachment sites (Fig. 1d) derived from pPB-phiC31 [8] located in the binary vector pCambia 1301 <http://www.cambia.org/daisy/cambia>. The expectation was that prior to site-specific recombination, 35S would not drive expression of *gusA* due the presence of the stuffer region. After recombination, the non-coding stuffer would be removed and activate expression of *gusA* (Fig. 1c). In this strategy, we first introduced the recombination target (pN3-phiC31) into the *Arabidopsis* genome via *Agrobacterium* transformation. The target construct contains *hptII* (*hygromycin phosphotransferase II*) for selection of transgenic plants and was intentionally placed outside of the recombination recognition sites (and thus is not excised by phiC31) to aid the tracking of excised plants. These target lines, or 'TA' lines, were then transformed with the second construct, pCOXS3-phiC31 (Fig. 1b) that expresses the recombinase gene to produce the 'TR' lines. Upon site-specific excision of the recognition site-flanked DNA, the TR₁ plants were backcrossed to wild type plants and the BC₁ progeny screened for segregants that retain the excision event but lack the recombinase gene (Fig. 2).

Target lines for phiC31 recombination

The target construct pN3-phiC31 was introduced into *Arabidopsis* and 23 hygromycin resistant lines were confirmed by PCR detection of a 1.26 kb product that spans the recognition site-flanked non-coding stuffer region (data not shown). Of those, 13 pN3-phiC31 lines were propagated to the TA₂ generation and examined by Southern blot for single copy T-DNA integration. *EcoRI* or *BamHI* each cuts once within the target T-DNA (Fig. 1a). Hybridization with a *gusA* probe of *EcoRI* or *BamHI* cleaved genomic DNA should reveal a band size >4.17 kb, the length of the cleaved T-DNA. A hybridizing band <4.17 kb would indicate integration of a truncated T-DNA. From this analysis, three of the 13 pN3-phiC31 plants were determined to contain a single copy of a likely complete T-DNA (data not shown) and designated TA₂-phiC31.22, 31, and 34. The 1.26 kb PCR product from each of these lines was sequenced to confirm the presence of intact *attB* and *attP* sites (Fig. 1d).

Arabidopsis OXS3 promoter for expression of phiC31

As previous research has demonstrated successful germline tissue expression of the *parA* and *cre* recombinase genes [19], we chose the 1.5 kb promoter fragment of the *Arabidopsis Oxidative Stress 3* gene (*OXS3*) (AGI At5g56550) for *phiC31* gene expression and termed the plasmid pCOXS3-phiC31 (Fig. 1b). Independent research, through the use of tiling microarrays, has also confirmed that the *OXS3* gene is constitutively expressed in most *Arabidopsis* tissues [20,21].

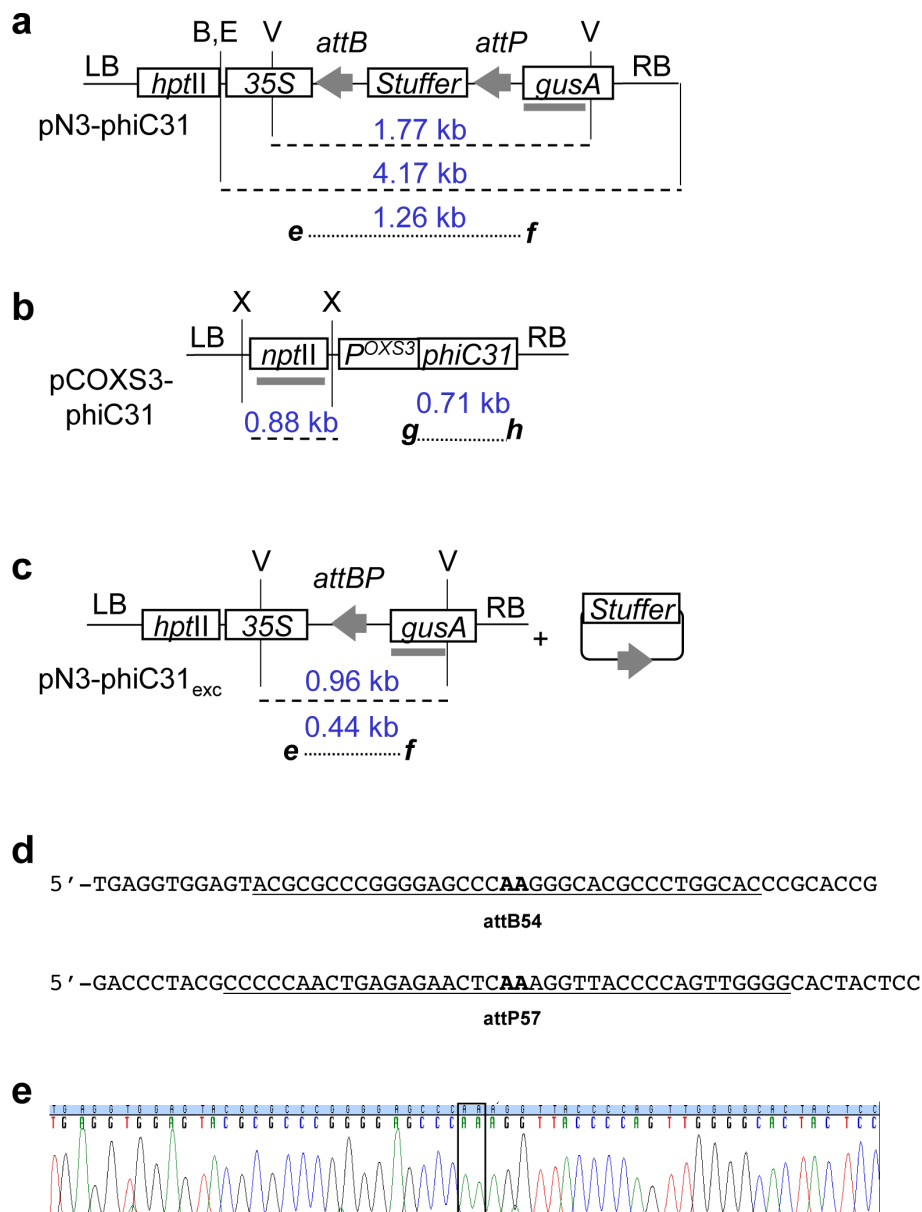


Figure 1 T-DNA structures. (not to scale) from a) pN3-phiC31; b) pCOXS3-phiC31; and c) predicted single copy T-DNA structures after excision of stuffer by phiC31-att recombination. PCR primers shown as **e**, **f**, **g**, **h**; att sites as grey arrowheads; hybridization probes as grey rectangles. Abbreviations: B, *Bam*HI; E, *Eco*RI; V, *Eco*RV; X, *Xho*I; RB, T-DNA right border; LB, T-DNA left border. Length in kb of PCR products (dotted lines) and DNA fragments (dashed lines). d) Sequence of the 54 bp *attB* and 57 bp *attP* phiC31 recognition sites, where the minimal required sequence is underlined and the 2 nucleotide 'AA' core region of crossover is in bold. e) sequence of a PCR product detecting a conservative site-specific excision event. Not shown are gene terminators and promoters for *hptII* (*hygromycin phosphotransferase II*) and *nptII* (*neomycin phosphotransferase II*) and the gene terminator for *gusA* (β -glucuronidase).

Secondary transformation of TA target lines

The TA₃ generation of phiC31.22, 31, 34 plant lines were transformed with *Agrobacterium* harboring the pCOXS3-phiC31 vector. Kanamycin resistant transformants that exhibited wild type appearance and growth rate were identified and grown in the greenhouse. Three-week old TR₁ transformants were tested for the presence of the *phiC31* gene. PCR amplification by

primers **g** and **h** (Fig. 1b) showed that a majority of the plants harbor the recombinase gene (Fig. 3). The groups of plants that harbor the *phiC31* gene were designated TR₁-phiC31.22, 31 and 34 (Table 1).

The TR₁-phiC31 lines were examined using histochemical staining to detect *gusA* encoded β -glucuronidase activity. GUS expression in the TR₁-phiC31 lines, however, showed variable levels of β -glucuronidase

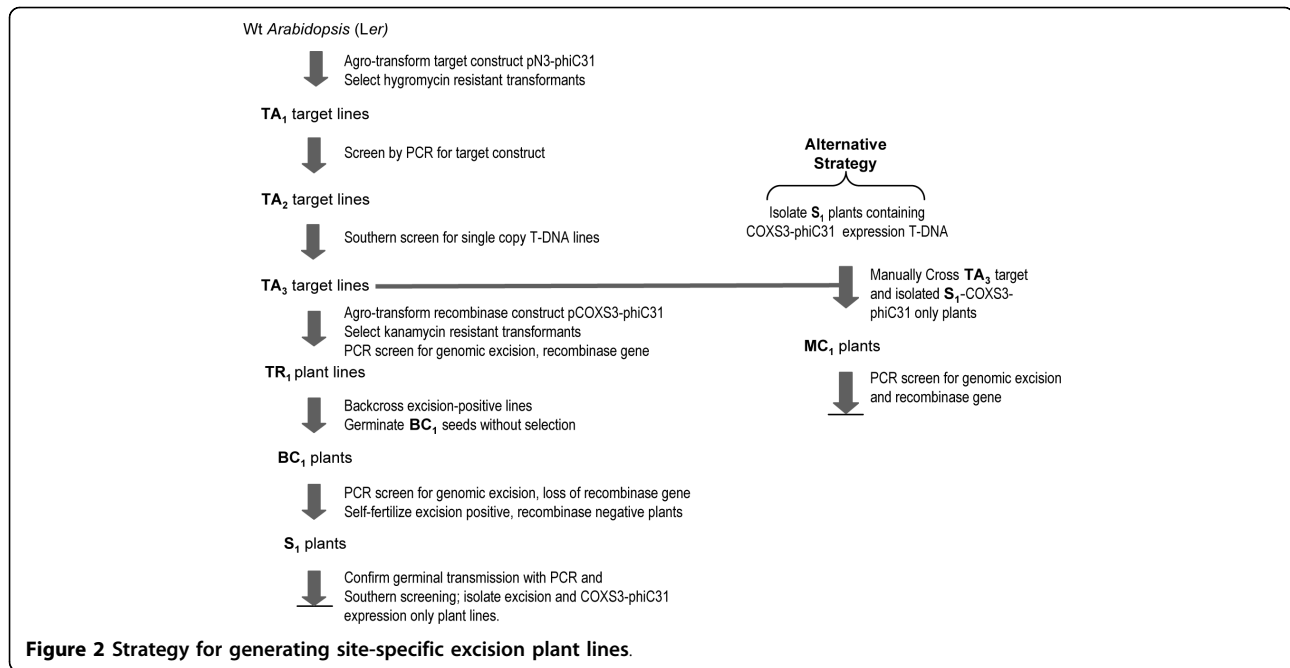


Figure 2 Strategy for generating site-specific excision plant lines.

activity. Initially we attributed this reduced activity to lower levels of *phiC31*-mediated excision, but PCR analysis of lines where GUS activity was weak or undetectable were positive for excision of the target DNA. Given that the screening for GUS activity was not a reliable indicator of *phiC31* site-specific recombination, we subsequently utilized PCR to screen for site-specific excision.

With the 65 TR₁-*phiC31*.22, 31 TR₁-*phiC31*.31 and 19 TR₁-*phiC31*.34 individuals, PCR with primers *e* and *f* (Fig. 1c) detected a 0.44 kb product expected for site-specific excision (Fig. 3a). However, the 1.26 kb product representing the parental configuration was also detected in some individuals, which indicates the presence of unexcised target DNA. As each individual harbors an independent COXS3-*phiC31* T-DNA integration at a different genomic location, with perhaps a different copy number or structural arrangements, the incomplete excision in some individuals may be due to variability in recombinease gene expression.

Removal of the *phiC31* gene by segregation

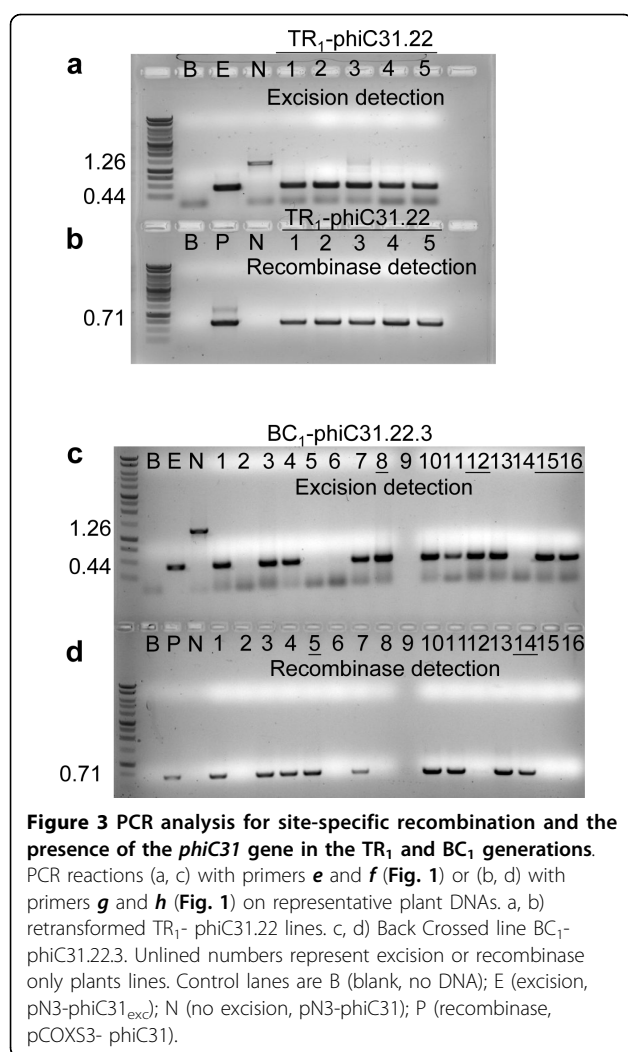
To determine if the genomic excision event occurred in the germline tissue, we examined whether the excised target was heritably transmitted to the progeny lacking the *phiC31* gene. This analysis further resolved whether or not the excision reaction was generated *de novo* in each generation. We chose 5 individuals (Table 2) from each of the TR₁-*phiC31*.22, TR₁-*phiC31*.31 and TR₁-*phiC31*.34 families to pollinate wild type recipients. The backcross progenies (BC₁) were grown without selection

and then screened by PCR for the target locus (primers *e* and *f*) and the recombinease gene (primers *g* and *h*), which reveals whether excision occurred (0.44 kb band) or not (1.26 kb band) and if *phiC31* was present or absent (Fig. 3c, d). With the TR₁-*phiC31*.22, TR₁-*phiC31*.31 and TR₁-*phiC31*.34, 59% (115 of 194), 78% (178 of 227) and 55% (118 of 214) of the BC₁ plants harbored the target DNA, respectively.

For the five TR₁-*phiC31*.22 plants that were backcrossed, 93% of the plants (107 of 115) that harbor the target locus showed excision of the *attB* and *attP*-flanked DNA, with 48% (51 of 107) lacking the recombinease gene (Table 2). Of the TR₁-*phiC31*.31 plants, 80% (142 of 178) of target plants showed excision of the *attB* and *attP*-flanked target, and 43% (61 of 142) lack the recombinease gene (Table 2). A total of 87% of the TR₁-*phiC31*.34 plants (103 of 118) harbored the target locus with excision of the *attB* and *attP*-flanked DNA, 1% (1 of 103) lacked the recombinease gene (Table 2). The genomic excision 0.44 kb PCR product from two representative individuals from each family was sequenced and examined for conservative recombination. All of the *phiC31*-mediated excision PCR products sequenced were conservative and site specific (GenBank accession No. GU564447, Fig. 1e).

BC₁ progeny for molecular confirmation

BC₁ plants that showed excision but lacked the recombinease gene were self-fertilized to yield progeny designated S₁-*phiC31*. PCR analysis on these plants again confirmed excision in the absence of the *phiC31*



recombinase gene (Fig. 4a, b), which indicates germinal transmission of the excision event. For further confirmation, Southern blot hybridization was conducted on some of these S₁ individuals. The genomic DNA was isolated and cleaved with *EcoRV*, which is expected to liberate either a 1.77 kb or a 0.96 kb fragment from the non-recombined or recombined structure, respectively (Fig. 1a, c). The GUS1350 probe detected the 1.77 kb

band in the parental lines but not in the S₁ plants (Fig. 5a, lanes 1-6). Instead, only the 0.96 kb band was observed for S₁ plants from the TR₁-*phiC31* lineage. Genomic DNA was also cleaved with *XhoI*, which should liberate a 0.88 kb fragment if the genome were to harbor a COXS3-*phiC31* T-DNA. Hybridization with the NPT690 probe detected the *nptII* gene fragment in the parental controls but not in the S₁ plants determined to be excision positive and *phiC31* negative (Fig. 5b, lanes 1-5) with the exception of a non-segregated S₁-*phiC31.34.9* plant line that contains both the excision product and the recombinase expression cassette (Fig. 5b, lane 6).

We further isolated, by segregation, *phiC31* recombinase expression lines for the purpose of crossing to the original TA₃ target lines to determine if a genomic excision event could be facilitated using this alternative approach (Fig. 2). Two independent lines from each of the three (TR₁-*phiC31.22*, 31 and 34) secondary transformation events were isolated via PCR and designated lines S₁-COXS3-22.3, 22.15; S₁-COXS3-31.40, 31.83 and S₁-COXS3-34.9, 34.20. Each of these six independently isolated lines has been crossed to the original target line TA₃-*phiC31.22*. As the lines S₁-COXS3-22.3 and S₁-COXS3-22.15 were derived from the original TR₁-*phiC31.22* secondary transformation; crossing them back to the TA₃-*phiC31.22* target line was performed as a positive control for this line of investigation. The manually crossed progenies (MC₁) were grown without selection and screened by PCR for the target locus (primers **e** and **f**) and the recombinase gene (primers **g** and **h**; Fig. 6). Of the MC₁-*phiC31.22* plants that carried both the target locus and *phiC31* gene, 61% (19 of 31) of the tested individuals displayed the 0.44 kb excision band in the absence of the unexcised 1.26 kb target band when screened using PCR (Table 3; Fig. 6, lanes 1, 2). Of the MC₁-*phiC31.31* plants with both the target and *phiC31*, 100% (25 of 25) generated only the 0.44 kb excision PCR product (Table 3; Fig. 6, lanes 3, 4). While 92% (34 of 37) of the MC₁-*phiC31.34* individuals generated only the 0.44 kb PCR product derived from an excised genomic target (Table 3; Fig. 6, lanes 5, 6).

Table 1 PCR analysis of TR₁ plants

TA Parent line	Plants tested	Positive for recombinase gene ^a and target locus ^b	Positive for excision ^c	Positive for excision and negative for unexcised product ^d
<i>phiC31.22</i>	88	65	47	35
<i>phiC31.31</i>	67	31	21	14
<i>phiC31.34</i>	43	19	17	2

^a Primers **g** and **h** yielded the 0.71 kb *phiC31* fragment.

^b Primers **e** and **f** yielded the 1.26 and/or 0.44 kb fragment.

^c Primers **e** and **f** yielded the 0.44 kb excision fragment.

^d Primers **e** and **f** failed to detect a 1.26 kb target fragment.

Table 2 PCR analysis of BC₁ and S₁ plants

TR ₁ -Parent line	Plants tested	Positive for target locus ^a	Positive for excision ^b	Positive for excision and negative for recombinase gene ^c	Positive for recombinase gene and negative for target locus ^d
phiC31.22.3	42	23	23	21	0
phiC31.22.15	17	16	16	2	0
phiC31.22.23	68	29	21	3	1
phiC31.22.29	61	44	44	24	1
phiC31.22.87	6	3	3	1	1
phiC31.31.1	59	55	28	14	1
phiC31.31.13	44	29	22	10	14
phiC31.31.23	14	8	8	2	4
phiC31.31.29	62	59	59	22	0
phiC31.31.36	48	27	25	13	5
phiC31.34.2	43	18	18	1	0
phiC31.34.5	44	23	8	0	2
phiC31.34.9	44	13	13	0	9
phiC31.34.20	42	26	26	0	1
phiC31.34.24	41	38	38	0	0

^a Primers *e* and *f* yielded the 1.26 and/or 0.44 kb target fragment.

^b Primers *e* and *f* yielded the 0.44 kb excision fragment.

^c Primers *e* and *f* yielded the 0.44 kb excision fragment while primers *g* and *h* failed to detect the 0.71 kb *phiC31* fragment.

^d Primers *g* and *h* yielded the 0.71 kb *phiC31* fragment while primers *e* and *f* failed to detect the 1.26 and/or 0.44 kb target fragment.

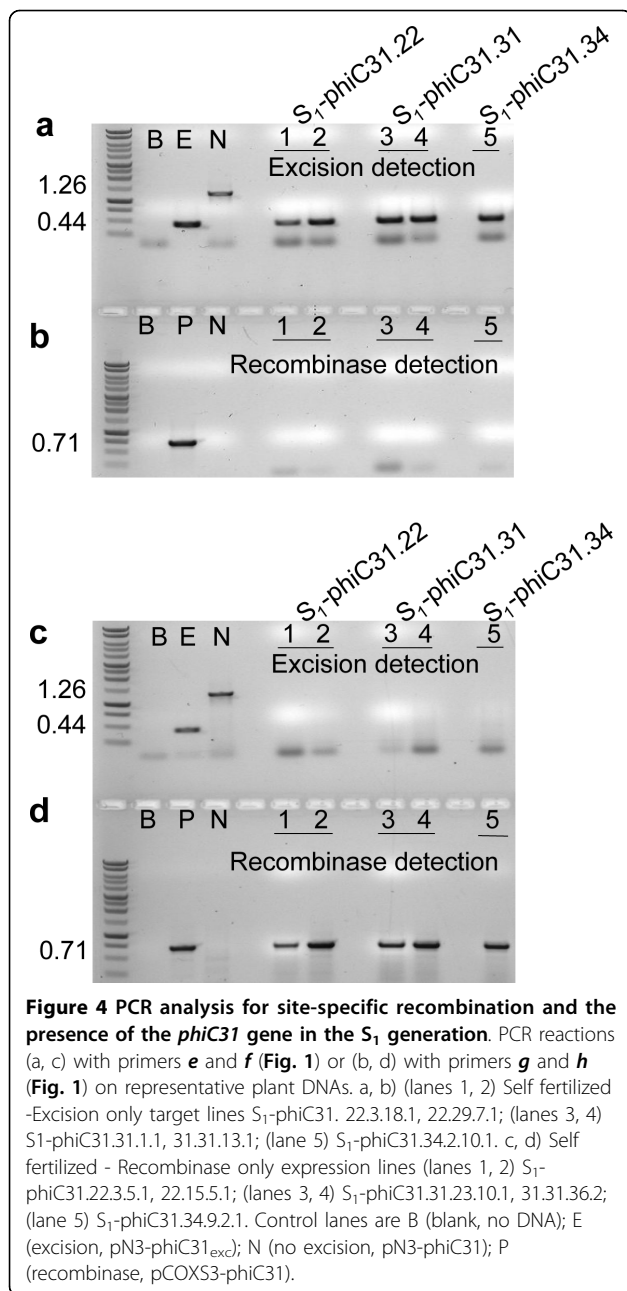
Discussion

Our interest in site-specific recombination lies in its ability to facilitate crop improvement through controlled engineering of the plant genome. Recently transgenic corn has been deregulated for the production of high lysine, a consumer directed product [22,23]. Further, this transgenic crop was engineered with the assistance of the site-specific recombinase technology for marker removal. Deregulation in this case required extensive studies to ensure that the recombinase mediated excision event was heritably transmitted to subsequent generations in the absence of the recombinase gene [23]. Such agricultural requirements, while obviously necessary, have elicited few detailed studies on the transmission of recombined chromosome transmission to progeny plants. The recombinase systems *Cre/lox*, *Flp/FRT*, *R/RS*, β /*six* and *ParA/MRS* have all been shown capable of germinal transmission *in planta* [19,24-30]. Therefore, our research investigated the publicly available *phiC31* recombination system as a potential tool for the precise removal of plant transgenes. In order to demonstrate its utility for crop genome engineering and increase public acceptance of transgenic technology, the potential for predefined nuclear excision events and their germinal transmission was investigated. An advantage of *phiC31* over existing recombinase systems is its unidirectional recombination activity, which prevents

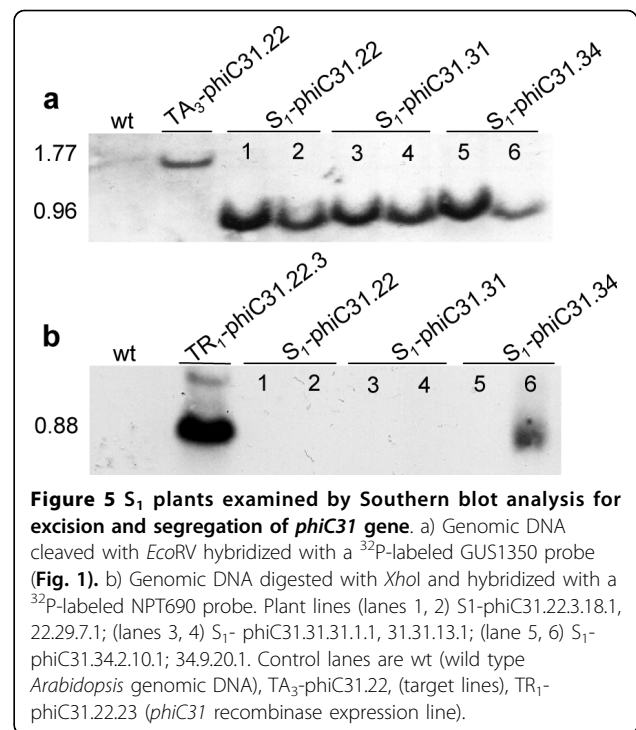
the re-insertion of the excision product into the genome. In addition, *phiC31* has the ability to site-specifically integrate DNA into the host genome [8,13] making this a versatile enzyme.

Our strategy began with the assumption that we could use *gusA* expression as a reporter for site-specific recombination. The pattern of GUS enzyme activity would reveal genomic excision of the target sequence and any tissue specificity in recombination. This strategy, however, failed to perform as expected with initial excised plants being either weak or completely devoid of GUS activity. Subsequent analysis of the original TR₁-*phiC31* progeny confirmed that use of reporter enzyme activity was an unreliable indicator of excision. We had also observed this phenomenon with other constructs used in both *Arabidopsis* and *S. pombe* [8,19]. It is possible that the 54 bp *attB/P* hybrid sequence present within the transcript leader sequence of the *gusA* gene may cause poor expression due to methylation or by some other mechanism that inhibits gene expression. Due to this circumstance, the analysis and scoring of site-specific excision was performed using PCR.

Site-specific excision was detected in all TR₁-*phiC31.22*, TR₁-*phiC31.31* and TR₁-*phiC31.34* plants. The majority (72%) of the TR₁-*phiC31.22* and TR₁-*phiC31.31* plants that demonstrated the presence of the excision product did not yield the PCR amplified



unexcised target band. This indicates that the *phiC31*-mediated genomic excision reaction was complete, or nearly so, within many of these TR_1 plants. The exception was line TR_1 -*phiC31*.34. Only 12% of the TR_1 -*phiC31*.34 plants were positive for the 0.44 kb excision band in the absence of the 1.26 kb unexcised target band. This may be due to unfavorable placement of the target construct within the *Arabidopsis* genome. Indeed, although the TR -*phiC31*.34 lines generated lower levels of recombinase-mediated excision than either the TR -*phiC31*.22 or TR -*phiC31*.31 lines, when segregants (derived from TR -*phiC31*.34) containing only the



phiC31 expression cassette were manually crossed with TA -*phiC31*.22 target plants, 92% of the progeny generated only the 0.44 kb excised target PCR product. This indicates that *phiC31* functions well in these plants, despite performing less efficiently on the TA -*phiC31*.34 target. The simplest explanation is that the TA -*phiC31*.34 genomic location or structure was unfavorable to recombination in the germinal tissue.

From analysis of the BC_1 plants, 85.6% (352 of 411) of those derived from the three TR_1 -*phiC31* lines showed excision, while in a previous line of research 77.3% and 99.6% of the BC_1 plants of the TR_1 -ParA and TR_1 -Cre lines exhibited excision, respectively [19]. By this measure, it appears that the *phiC31* recombinase mediated excision efficiency is more effective than ParA and approaching that of the Cre-*lox* system. Although, the majority of the BC_1 lines displayed excised genomic target, it is difficult to give a precise quantitative assessment of the *phiC31* activity since only a modest number of different target locations were thoroughly characterized. Variability in copy number and chromosome locations of the *phiC31* gene can affect the amount of recombinase protein produced and thus impact the efficiency of the excision reaction observed, making a direct comparison difficult. Other excision strategies for the *phiC31* recombinase are being investigated. These include the use of inducible or tissue specific promoters for controllable expression [31] use of self-deleting designs [32] and use of viral inoculation or

Table 3 PCR analysis of MC₁ plants

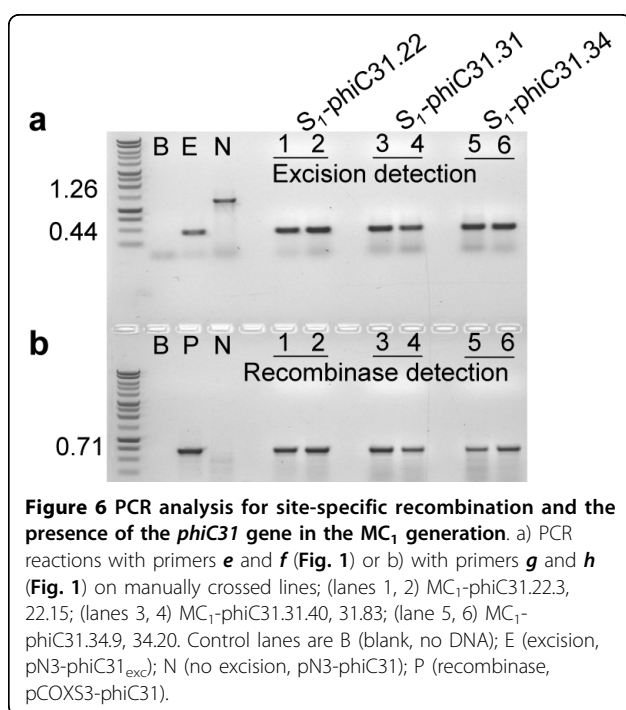
MC ₁ -Parent line	Plants tested	Positive for target locus ^a	Positive for recombinase gene ^b	Positive for excision and recombinase gene ^c	Positive for excision and negative for unexcised product ^d
phiC31.22.3	11	10	11	8	8
phiC31.22.15	34	31	32	23	11
phiC31.31.40	17	16	16	8	8
phiC31.31.83	17	17	17	17	17
phiC31.34.9	68	44	23	15	15
phiC31.34.20	44	33	40	22	19

^a Primers *e* and *f* yielded the 1.26 and/or 0.44 kb target fragment.

^b Primers *g* and *h* failed to detect the 0.71 kb *phiC31* fragment.

^c Primers *e* and *f* yielded the 0.44 kb excision fragment and primers *g* and *h* detect the 0.71 kb *phiC31* fragment.

^d Primers *e* and *f* yielded the 0.44 kb excision fragment and failed to detect a 1.26 kb target fragment.



Agrobacterium-infiltration for immediate but transient expression [33,34].

As an alternative method of recombinase introduction into the plant target lines, our lab tested hand pollination between *phiC31* recombinase expressing plants and pN3-*phiC31* target plants. PCR analysis of the manually crossed MC₁ progeny demonstrated that this is a viable method for the generation of individuals with genomic target excision (Fig. 6). However, it was observed that like secondary *Agrobacterium* transformation with the recombinase expression cassette, the genomic excision results varied between lines (Table 3). Use of a demonstrated recombinase expression line such as phiC31.31.83

(Table 3) enabled sufficient recombinase mediated excision events to fully excise all target DNA when crossed together. It was also observed that segregation of the secondary *Agrobacterium* transformed TR₁ lines, without benefit of backcrossing, produced excised target and recombinase expression-only T-DNA lines in the TR₂ and TR₃ generations (data not shown). This indicates that the *phiC31* expression T-DNA in these lines was at a single locus or a low number of loci within the genome and that expression was sufficient to facilitate recombination allowing segregation by self-pollination.

Since PCR assays of genomic DNA from leaf tissue only indicates that excision has occurred in somatic cells, we utilized Southern blot analysis to ascertain whether target sequence removal had occurred in the germline. As long as *phiC31* DNA was present in the genome, or the *phiC31* protein was present in the germline cells, the possibility that recombination was generated *de novo* could not be ruled out. Hence, BC₁ plants were screened by PCR for the absence of the *phiC31* recombinase gene, and the following generation (S₁ plants) was confirmed by Southern blot hybridization. As is clearly shown in Fig. 5 lanes #1 - 5, germinal transmission of the genomic excision event in the absence of the *phiC31* recombinase gene occurred, illustrating that the production of stable lines with the unwanted DNA removed can be achieved.

Controlled targeted integration with recombinase technology allows the application of more sophisticated recombinase strategies [35]. This technology enables the production of precisely engineered transgenic plants through genome specific transgene integration and has been reported to function in *Arabidopsis*, tobacco and rice [5,36-44] with Cre, FLP and R recombinase systems. The *phiC31* recombinase with its uni-directional catalytic activity presents a novel way to facilitate stable site-specific integration events without the elaborate

strategies required by the bi-directional systems. Peer-reviewed literature reported that phiC31 is capable of mammalian genome targeting [45,46] and targeted integration into the plastid genome of tobacco [13]. Utilization of phiC31 for genome modification has been facilitated in mammalian species through the identification of cryptic *attB* or *attP* sites as potential locations for transgene introduction [46]. To this end our lab investigated, *in silico*, the presence of sequences similar to the phiC31 *att* sites within the *Arabidopsis thaliana* genome. We used a BLASTn search to investigate whether the *Arabidopsis* genome contains sequences similar to the minimal 34 bp *attB* and 39 bp *attP* sites [12]. The genomic sequences with the highest similarity to the *att* sites exhibited >60% overall nucleotide identity. A total of seven sequences had 21-23 (61.8-67.7%) of the 34 nucleotides conserved with the minimal *attB* sequence, while 14 native sequences had 24-27 (61.5-69.2%) nucleotides in common with the 39 bp *attP* sequence (Fig. 7). While most of the sequences including the best matches for *attP* did contain the conserved core domain presumably essential for phiC31-mediated recombination, only three of the *attB*-like sequences contained the core sequence (Fig. 1d; Fig. 7). It is possible that some of these *att*-like sequences could potentially be used as a native target site for phiC31 mediated integration in *Arabidopsis*. Pseudo phiC31 *attP* sequences in the mouse, bovine and human genomes have been reported and some of them have been shown suitable for integration of introduced DNA [47-49].

Although unlikely, the potential for genomic excision, inversion and translocation mediated by these cryptic *att* sequences in *Arabidopsis* is possible. For excision, *Arabidopsis* chromosomes 3 and 5 carry both *attB* and *attP*-like sequences in direct orientation (Fig. 7). The closest correctly oriented sites are located >500 kb apart on chromosome 3, but the cryptic *attB* does not contain a conserved core domain. Although it is theoretically possible that genomic recombination could occur via endogenous *att*-like sequences, the OXS3 promoter-*phiC31* plants did not exhibit compromised viability, morphological or growth defects. This differs from earlier observations using a 35S-*phiC31* construct where *Arabidopsis* plants with crinkled leaves were common [C. Day and D.W. Ow, unpublished data]. Hence, this underscores the importance in controlling expression of the recombinase gene through appropriate use of promoters.

Conclusion

The purpose of the research was to provide proof-of-concept that the phiC31 recombinase can mediate site-specific genome modification in the plant germline tissue without affecting fecundity. The research established

that the excision event was passed to subsequent generations in the absence of phiC31 and that the excision of *attB* and *attP*-flanked DNA from the plant genome was a conservative site-specific event. In a majority of the phiC31 lines examined (11 out of 15), at least one BC₁ segregant was recovered that contained a germinally transmitted excision event lacking the *phiC31* gene. These results were validated with Southern blot hybridization and demonstrate that the secondary transformation strategy used in this study is feasible for the production of marker-free transgenic plants. This approach may prove particularly useful in those species where cross pollination is not possible or undesirable. We further demonstrate that an alternative approach to marker removal where the recombinase is introduced into the excision test target plants with cross pollination is also a viable strategy. Molecular analysis confirmed that the genomic excision was site-specific and conservative. Therefore, taken together the results clearly establish that the phiC31 system performs genomic excision, generating stable transgenic recombinase-free *Arabidopsis* plants with unwanted DNA removed.

Methods

DNA constructs

pN3-phiC31 (GenBank accession No. GU564446), (Fig. 1a): An *NheI*-*attB*-stuffer-*attP*-*AscI* fragment was retrieved from pPB-phiC31 [8] and inserted into binary vector pCambia-1301 <http://www.cambia.org/daisy/cambia> in which the *NcoI* site between 35S and *gusA* had been changed to *SpeI* and *AscI*. The vector contains *hptII* (*hygromycin phosphotransferase II*) for selection in plants outside the region of site-specific excision to allow for progeny tracking. The pN3-phiC31_{exc} vector for control lanes (Fig. 3, 4 and 6, lane E) was generated by removal of the non-coding stuffer region by recombinase-mediated excision in bacteria.

pCOXS3-phiC31 (GenBank accession No. GU564445), (Fig. 1b): The *phiC31* ORF was Phusion (NEB, New England Biolabs) PCR amplified with a 5' *AscI* and 3' *SpeI* sites (underlined) and inserted into pCOXS3-ParA [19] to generate the final construct. Primers used were 5'-AGTCGCGCGCCATGACACAAGGGGTTGT-GAC-3' and 5'-AGTCACTAGTCTACGCCGCTACGTCTTC-3'. The 1.5 kb fragment promoter of the OXS3 gene (AGI At5g56550) from *Arabidopsis thaliana* (ecotype: *Ler*) was used to express the *phiC31* ORF, as previously described [19,20]. The pCAMBIA 2300 <http://www.cambia.org/daisy/cambia>, binary vector with *nptII* (*neomycin phosphotransferase II*) for plant selection was used as the backbone for plant transformation.

Agrobacterium tumefaciens GV3101 was used for transformation of *Arabidopsis* (ecotype: *Ler*) by the floral dip method [50] modified by adding 0.01% Silwet

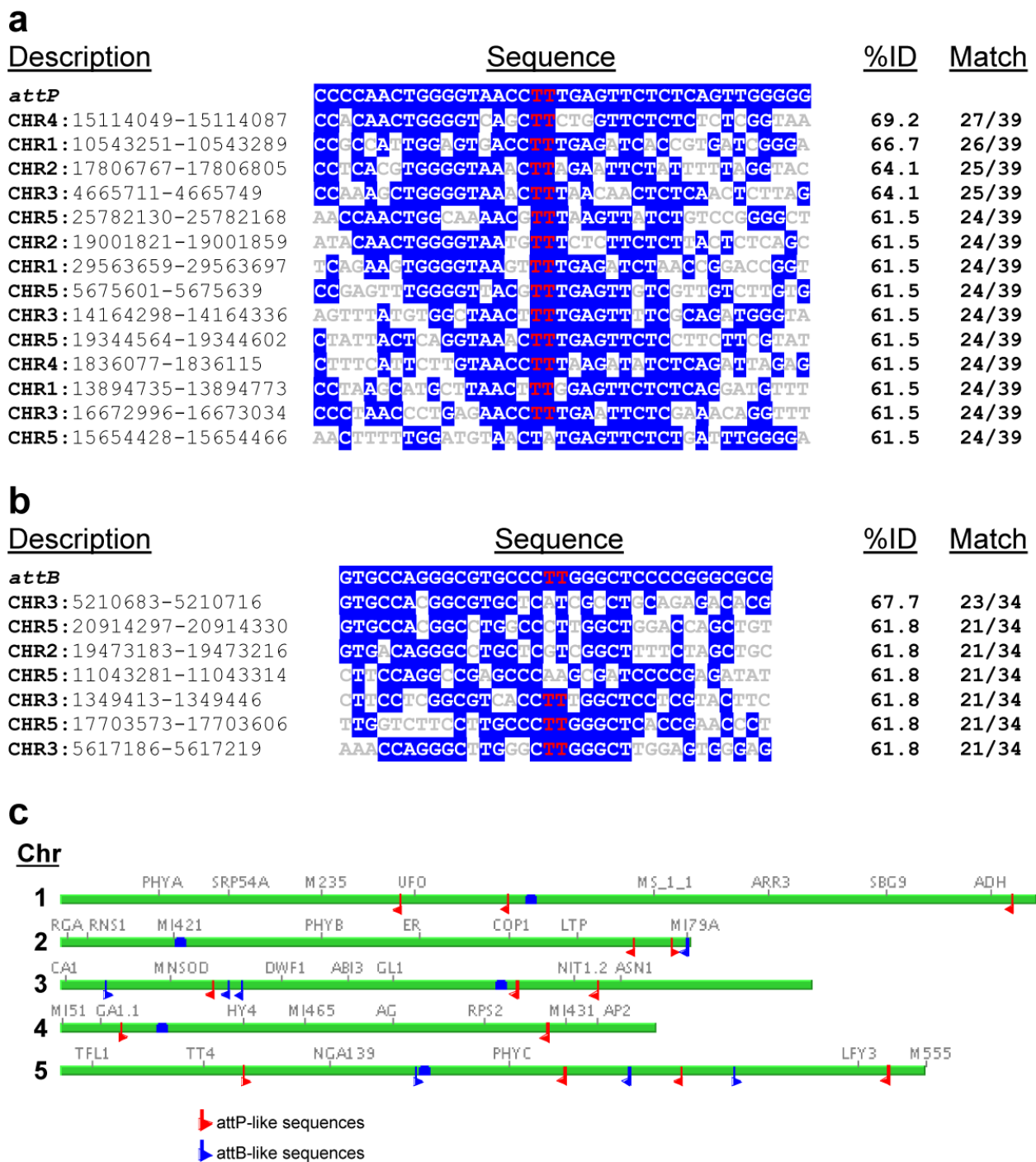


Figure 7 Arabidopsis genomic DNA sequences with >60% similarity to phiC31 attP and attB sites. a) Alignment of the 39 bp attP site with 14 sequences from the Arabidopsis genome. b) Alignment of the 34 bp attB site with seven sequences from the Arabidopsis genome. Nucleotides identical to the att site are highlighted with white text and blue backshading. A conserved core domain is highlighted in red text. The chromosomal location coordinates of each sequence are shown on the left, the percent identity and nucleotide match is shown on the right. c) The position and orientation of the 21 att-like sequences are displayed on a diagram of the five Arabidopsis chromosomes.

L-77 (Lehle Seeds, Round Rock, TX) to the infiltration medium. Primary transformants were selected on 1× MS medium (Sigma), 1% sucrose, 0.7% agar with 20 µg/ml hygromycin or 50 µg/ml kanamycin as needed for 10 days prior to cultivation in soil.

PCR analysis

Genomic DNA was extracted by grinding a single leaf in 400 µl of buffer (200 mM Tris HCl pH 7.8, 250 mM NaCl, 25 mM EDTA, 0.5% SDS). After centrifugation, the isopropanol precipitated pellet was washed with 70% ethanol and resuspended in 50 µl of H₂O. Two µl of genomic DNA in 25 µl volume was used per PCR reaction. Primers were (Fig. 1): *e* (5'-ATATCTCCACT-GACGTAAGG-3'), *f* (5'-ATCATCATCATAGACA-CACG-3' for N3-phiC31); *g* (5'-AGTCGCGCGCCAT-GACACAAGGGTTGTGAC-3'), *h* (5'-GTGCGTCTT-GATCTCACG-3' for *phiC31*). Gel images were digitized with a resolution of 200 dpi in black on white background TIF format.

Southern blot analysis

Genomic DNA was extracted from plant aerial portions using a modified cetyl-trimethyl-ammonium bromide method as described [51]. The 0.79 kb GUS1350 and 0.69 kb NPT690 ³²P-labeled probes were produced by *Taq*[™] polymerase (Promega) using primers 5'-CAA-GACCCTTCCTCTATATAAG-3' and 5'-CGAGTTCA-TAGAGATAACCTTC-3' for GUS1350 and primers 5'-GATTGAACAAGATGGATTGCACGC-3' and 5'-CCA-CAGTCGATGAATCCAGAAAAGC-3' for NPT690.

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Authors' contributions

JT designed the approach, constructed the plasmids, collected data, interpreted the research results, drafted and edited the manuscript. JT supervised RC, prepared and submitted the manuscript. RC provided technical assistance with plant maintenance, DNA preparation, PCR data collection and analysis. Participated with manuscript preparation and editing. RT provided bioinformatics research on the cryptic *attB* and *attP* sites and performed Southern blot hybridization and analysis. Participated in the drafting and editing of the manuscript. YY provided assistance on background studies, data interpretation and manuscript editing. DO provided data interpretation and manuscript editing. All authors have read and approved the final manuscript.

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