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
The 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 unidirectional 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 unidirectional 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 recombinated 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 germ-line 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].
Secondary transformation of TA target lines

The TA$_3$ 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 TR$_1$ 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 TR$_1$-phiC31.22, 31 and 34 (Table 1).

The TR$_1$-phiC31 lines were examined using histochemical staining to detect gusA encoded β-glucuronidase activity. GUS expression in the TR$_1$-phiC31 lines, however, showed variable levels of β-glucuronidase activity.

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, BamHI; E, EcoRI; V, EcoRV; X, Xhol; 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).
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 recombinase 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 recombinase 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 recombinase 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 recombinase 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 recombinase 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).
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, 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).

![Figure 3](image-url) **Figure 3** PCR analysis for site-specific recombination and the presence of the phiC31 gene in the TR1 and BC1 generations

PCR reactions (a, c) with primers e and f (Fig. 1) or (b, d) with primers g and h (Fig. 1) on representative plant DNAs. a, b) retransformed TR1-phiC31.22 lines. c, d) Back Crossed line BC1-phiC31.22. Unlined numbers represent excision or recombinase only plants lines. Control lanes are B (blank, no DNA); E (excision, pN3-phiC31excl); N (no excision, pN3-phiC31); P (recombinase, pCOXS3-phiC31).

| 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 |
|----------------|---------------|---------------------------------------------------|------------------------|----------------------------------------------------------|
| phiC31.22      | 88            | 65                                                | 47                     | 35                                                       |
| phiC31.31      | 67            | 31                                                | 21                     | 14                                                       |
| phiC31.34      | 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.
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].

Thus, 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

| TR1-Parent line | Plants tested | Positive for target locus | Positive for excision | Positive for excision and negative for recombinase gene | Positive for recombinase gene and negative for target locus |
|-----------------|---------------|--------------------------|----------------------|-------------------------------------------------------|----------------------------------------------------------|
| 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                                                        |
| phiC31.1.1      | 59            | 55                       | 28                   | 14                                                    | 1                                                        |
| phiC31.1.13     | 44            | 29                       | 22                   | 10                                                    | 14                                                       |
| phiC31.1.23     | 14            | 8                        | 8                    | 2                                                     | 4                                                        |
| phiC31.1.29     | 62            | 59                       | 59                   | 22                                                    | 0                                                        |
| phiC31.1.36     | 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                                                        |

*Primers e and f yielded the 1.26 and/or 0.44 kb target fragment.
*Primers e and f yielded the 0.44 kb excision fragment.
*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.
*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.
unexcised target band. This indicates that the phiC31-mediated genomic excision reaction was complete, or nearly so, within many of these TR1 plants. The exception was line TR1-phiC31.34. Only 12% of the TR1-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 BC1 plants, 85.6% (352 of 411) of those derived from the three TR1-phiC31 lines showed excision, while in a previous line of research 77.3% and 99.6% of the BC1 plants of the TR1-ParA and TR1-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 BC1 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
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 germ-line 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, germinial transmission of the genomic excision event in the absence of the phiC31 recombinase gene, and the following generation (S1 plants) was confirmed by Southern blot hybridization.

Table 3 PCR analysis of MC1 plants

| MC1-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.

Figure 6 PCR analysis for site-specific recombination and the presence of the phiC31 gene in the MC1 generation. a) PCR reactions with primers e and f (Fig. 1) or b) with primers g and h (Fig. 1) on manually crossed lines; (lanes 1, 2) MC1-phiC31.22.3, 22.15; (lanes 3, 4) MC1-phiC31.31.40, 31.83, (lane 3) phiC31.34.9, 34.20. Control lanes are B (blank, no DNA); E (excision, pN3-phiC31exc); N (no excision, pN3-phiC31); P (recombinase, pCOXS3-phiC31).

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 cryptic 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 Nhel-attB-stuffer-attP- AscI fragment was retrieved from pHBl 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 Ascl. 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-phiC31vec 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′ Ascl and 3′ SpeI sites (underlined) and inserted into pCOXS3-ParA [19] to generate the final construct. Primers used were 5′-AGTCGCGCGCCATGACACAAGGG-3′ and 5′-AGTCCTATGCTCTAGCCGCG-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 1x 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′-ATCATCATCATAGACAGC-3′ for N3-phiC31); g (5′-AGTGGCAGCCGAC-GACAAGGGGTTGTGAC-3′), h (5′-GTGCGCTTGGATCTCAGC-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 cetyltrimethylammonium bromide method as described [51]. The 0.79 kb GUS1350 and 0.69 kb NPT69032P-labeled probes were produced by polymerase (Promega) using primers 5′-ATATCTCCACT-GACGTAAGG-3′ and 5′-ATCATCATCATAGACAGC-3′ for N3-phiC31 and primers 5′-GATGGCAGCCGAC-GACAAGGGGTTGTGAC-3′, h (5′-GTGCGCTTGGATCTCAGC-3′ for phiC31). Gel images were digitized with a resolution of 200 dpi in black on white background TIF format.

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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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