

The Cre-*loxP* recombination-based reporter system for plant transcriptional expression studies[★]

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Abstract

To facilitate the characterization of plant genes, the Cre-*loxP* site-specific recombination system was adapted to make reporter vectors for plant expression studies. This system allows promoter fragments to be cloned into a small vector (univector) and subsequently recombined *in vitro* with binary vectors containing different reporter genes precisely at near-perfect efficiency. We have constructed univector-adapted vectors with three reporters, β -glucuronidase, luciferase, and green fluorescent protein, and a BASTA-resistance gene for selection of plant transformants. Expression in plants using the new system was validated by comparison to conventional reporter vectors. These new vectors are efficient and economical alternatives to the other plant reporter vectors currently available. The royalty-free Cre-*loxP* system serves as a platform for the future expansion of recombination-based cloning vectors for plant research.

Introduction

The availability of plant genome sequences allows the large-scale analysis of gene function, including gene expression patterns. Microarray analysis has revolutionized the ability to study gene expression on a genome-wide scale. However, the production of stable transgenic lines carrying promoter-reporter or promoter-whole gene-reporter constructs remains a powerful method to determine expression patterns that are regulated developmentally and/or in a cell- or tissue-specific manner.

Many reporter genes are currently used to study gene expression and regulation in plant biology, though the vectors pose several limitations. Some of the popular reporter genes include β -glucuronidase (GUS), luciferase (LUC), and the green fluorescent protein (GFP) and its derivatives. The first GUS vectors were the pBI series (Jefferson (1989)). Since then, a variety of GUS vectors have been developed, such as pRLT2-GUS (Restrepo *et al.*, 1990), and the CAMBIA series available from the Center for the Application of Molecular Biology to International Agriculture (CAMBIA, Canberra, Australia). For LUC expression studies, pPZP series vectors are widely used (Hajdukiewicz *et al.*, 1994). Although each of these vectors is suitable for assessing gene

[★] GenBank accession number: pUNI100, AY741665, pHP100-GUS, AY860533, pHP100-LUC, AY860534, pHP100-GFP, AY860535.

Table 1. Primers used to amplify promoter fragments.

NHD1-pF1: 5'-ATTAGGTCA <u>FGGCCAAATCGGCCAGGGGAAAGAATTGCAGCGTTGCAGCAGTTGGTTTCTC</u> -3'
NHD1-pR1: 5'-GAAGAGCTCCTTATGGCCCTTATGGCCCTCACGAGAACACCATGTTTGGATAACCTCGTC-3'
SUC2-F-GUS: 5'-AATAAAAGCTT <u>GTTTTCATATTAATTTT</u> CAC-3' (<i>Hind</i> III)
SUC2-R-GUS: 5'-CTCCCGGGATGGCTGACCAGATTTGAC-3' (<i>Sma</i> I)
SUC2forward: 5'-CCAGCTGGCCAAATCGGCCTACCAGATTTCCGGTAAATTGG-3'
SUC2promoterrev: 5'-GCACCTTGGCCCTTATGGCCCTGGCTGACCATATTTGACAAAAC-3'
CAX7-F: 5'-GAATTCGGCCAAATCGGCCAAAGATGGCGGTCCAAAATTTTATC-3'
CAX7-R: 5'-GAATTCGGCCCTTATGGCCAATAATGAGGTGTGTTAGGTTTTC-3'

Underlined sequences are *Sfi*I sites unless otherwise indicated.

52 expression, they are large and have limited restric-
53 tion sites. Thus it becomes relatively labor-inten-
54 sive to subclone each promoter and to generate
55 multiple reporter constructs.

56 Here we have adapted the widely used *Cre-loxP*
57 site-specific recombination (Liu *et al.*, 1998) to
58 make a set of vectors for the expression of
59 promoter-reporter constructs in plants. The clon-
60 ing enzyme Cre can be made inexpensively and
61 easily (Liu *et al.*, 1998, 2000). In our system, the
62 initial cloning of a promoter is performed in a
63 small vector (univector), and then is easily recom-
64 bined into binary vectors with a reporter gene
65 (host vectors) *in vitro* using the Cre recombinase
66 (Liu *et al.*, 1998, 2000). We have made the GUS,
67 luciferase, and enhanced green fluorescent protein
68 (EGFP, Yang *et al.*, 1996) versions of the host
69 vector, and the same univector clone can be easily
70 transferred to any one or all of the host vectors.
71 *Arabidopsis* plants expressing the promoter-GUS
72 fusion are indistinguishable from plants expressing
73 traditional reporter constructs. One key advantage
74 of this method over a series of Gateway vectors
75 (Karimi *et al.*, 2002; Curtis and Grossniklaus,
76 2003) recently available to the public is that no
77 commercial enzyme mixture is required for recom-
78 bination. The *Cre-loxP* technology allows all plant
79 laboratories the access to high-throughput cloning
80 into an array of publicly available reporter vectors.

81 Materials and methods

82 PCR amplification and cloning of promoters

83 *Arabidopsis* Col-0 genomic DNA was used as
84 template for PCR using the primers listed in
85 Table 1. The 2.1 kb NHD1 (At3g19490, a putative
86 Na⁺/H⁺ antiporter in *Arabidopsis*) and 2.0 kb

SUC2 (At1g22710, sucrose/H⁺ symporter in 87
Arabidopsis) promoter fragments were digested 88
with *Sfi*I and cloned into the pUNI100 univector. 89
All univector constructs were propagated in an *E.* 90
coli BW23474. Using Cre recombinase, these 91
vectors were recombined with the binary vector 92
pHP100-GUS to yield translational fusions of the 93
first 60 amino acids of NHD1 and the first 3 amino 94
acids of SUC2 to GUS, respectively. The products 95
of recombination were propagated in *E. coli* strain 96
DH5 α to select against non-recombined univector. 97

98 For making the traditional SUC2 promoter-
99 GUS construct, the promoter was cloned into the
100 *Hind*III/*Sma*I sites of pGPTV-HPT (Becker *et al.*,
101 1992) to generate a transcriptional fusion. This
102 SUC2 promoter construct was previously shown
103 to drive GUS expression in companion cells
104 (Schulze *et al.*, 2003).

105 *Arabidopsis* CAX7 (At5g17860) promoter frag-
106 ment (1 kb upstream of the start codon) was first
107 cloned into pCRII-TOPO vector (Invitrogen,
108 Carlsbad, CA). The *Sfi*I fragment was then cloned
109 into pUNI100 univector and *Cre-loxP* recombi-
110 nation was performed.

111 Expression and purification of the GST-Cre 112 fusion protein

113 The GST-Cre fusion protein (referred to as Cre
114 protein in this paper) was expressed and purified
115 according to Liu *et al.* (1998). GST-Cre retains
116 high recombinase activity in the *in vitro* recombi-
117 nation reaction (Liu *et al.*, 1998).

118 Recombination reaction

119 The recombination reaction between a univector
120 clone and a host vector was carried out according

121 to Liu *et al.* (1998). Briefly, a reaction mixture
 122 consisting of 1 μ l (100 ng) of a univector plasmid,
 123 1 μ l (100 ng) of a host vector plasmid, 2 μ l of
 124 10 \times univector plasmid-fusion system (UPS) buffer
 125 (1 \times buffer is 50 mM Tris-HCl, pH 7.5, 10 mM
 126 MgCl₂, 30 mM NaCl, and 0.1 mg/ml BSA.), 15 μ l
 127 of water, and 1 μ l (0.1–1.0 μ g) of Cre protein was
 128 incubated at 37 °C for 20 min. Five microliters of
 129 the reaction was used to transform *E. coli*, and
 130 selected on media containing ampicillin (100 μ g/
 131 ml) and kanamycin (50 μ g/ml).

132 *Analysis of recombinants*

133 The host vectors and the univector pUNI100 both
 134 have a single *EcoRI* site. Therefore, when the host
 135 vector is recombined with a univector, it gains one
 136 *EcoRI* fragment after the restriction digestion.
 137 Internal *EcoRI* sites within the promoter fragment
 138 will result in additional bands.

139 *Arabidopsis transformation*

140 The binary vectors made by the *Cre-loxP* or the
 141 traditional 'cut-and-paste' method were trans-
 142 formed into *Agrobacterium* strain C58C1 or
 143 GV3101 by the freeze and thaw method (An
 144 *et al.*, 1988) or by electroporation. Transformants
 145 were selected on YM medium (0.04% yeast extract,
 146 1% mannitol, 1.7 mM sodium chloride, 0.8 mM
 147 magnesium sulfate, and 2.2 mM dipotassium
 148 phosphate, pH 7.0, 1.5% agar) containing kana-
 149 mycin (50 μ g/ml) and gentamycin (10 μ g/ml).
 150 *Arabidopsis* (Col-0) plants were transformed by
 151 *Agrobacterium* using the floral dip method
 152 (Clough and Bent, 1998) and T1 transformants
 153 were selected by seedling resistance to 0.01%
 154 BASTA.

155 *GUS staining*

156 GUS activity in transgenic *Arabidopsis* plants were
 157 detected by vacuum infiltration of 1mM X-Gluc in
 158 50 mM sodium phosphate pH 7.2 containing 0.5%
 159 Triton X-100. Plants were incubated overnight at
 160 37 °C and cleared in 70% ethanol at 37 °C.

161 *Luciferase imaging*

162 LUC activity in transgenic *Arabidopsis* plants was
 163 captured with a high-performance CCD camera

(Roper Scientific, Trenton, NJ). Transgenic plants
 164 grown in half-strength Murashige–Skoog agar
 165 media (Murashige and Skoog, 1962) were sprayed
 166 uniformly with 100 mM luciferin (dissolved in
 167 0.1% Triton X-100) and kept in the dark for 5 min.
 168 The seedlings were subsequently transferred to a
 169 dark chamber equipped with a CCD camera and
 170 the signals were acquired for 5 min. The images
 171 were analyzed using the software 'MetaVue6.0'
 172 (Universal Imaging Corporation, Downingtown,
 173 PA). 174

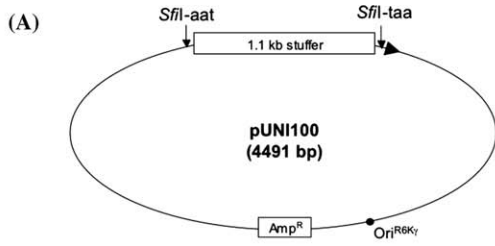
175 **Results**

Cre-loxP recombination system 176

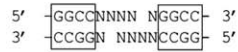
The *Cre-loxP* recombination is efficient and pre-
 177 cise (Liu *et al.*, 1998). The *Cre-loxP* recombination
 178 occurs naturally in bacteriophage P1 between two
 179 34 bp *loxP* sequences in the presence of the Cre
 180 recombinase protein, and is involved in the reso-
 181 lution of P1 dimers generated by replication of
 182 circular lysogens (Sternberg *et al.*, 1981). To place
 183 a reporter under the control of a promoter of one's
 184 choice, the promoter fragment is first cloned into a
 185 small vector with an R6K γ replication origin
 186 (univector), which can multiply only in a compat-
 187 ible *E. coli* strain. The stuffer fragment in the
 188 vector is replaced by the promoter fragment using
 189 two *Sfi* I sites. The *Sfi* I sites were chosen because
 190 of its infrequent occurrence in the *Arabidopsis*
 191 (every 460 kb) and possibly in other plant
 192 genomes. However, the absence of *Sfi* I sites in
 193 the insert is not a prerequisite, because the
 194 digestion with *Sfi* I produces unique overhangs,
 195 and the digested fragments can only religate with
 196 their original partners (Shigaki and Hirschi, 2002). 197

Univector construction 198

The univector for the cloning of promoter frag-
 199 ments was made by inserting a stuffer fragment in
 200 the *KpnI* site of pUNI(Amp)-GFP (Liu *et al.*,
 201 2000; GenBank accession number AF149264). The
 202 *KpnI* site is located immediately upstream of *loxP*.
 203 The stuffer fragment could be any sequence, and
 204 we used a 1.1 kb fragment from the coding
 205 sequence of *Arabidopsis* cation exchanger *CAX2*
 206 (At3g13320) which was amplified by PCR with
 207 *KpnI* and *SfiI* tags at both ends. The new vector 208

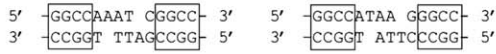


SfiI recognition sequence

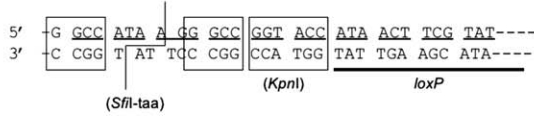


SfiI-aat

SfiI-taa



(B)



(C)

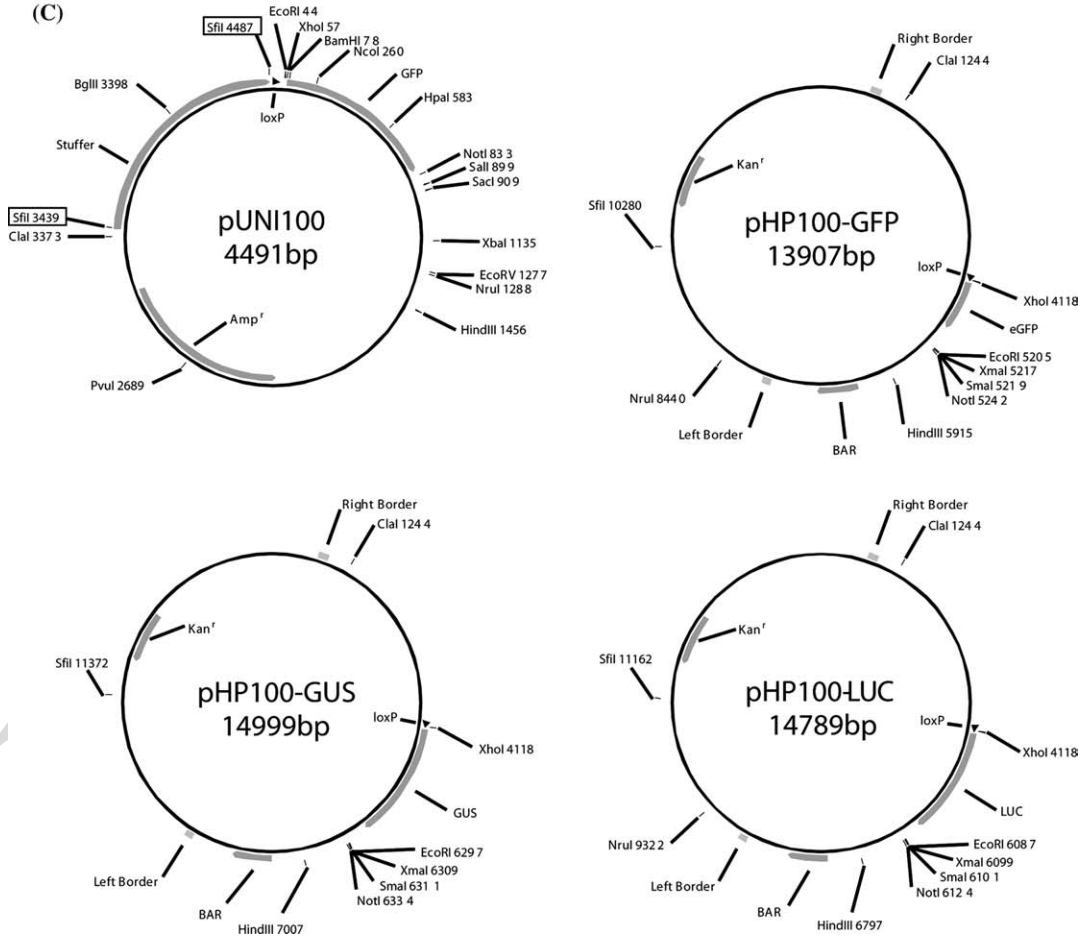


Figure 1. The univector pUNI100 and host vectors used for the plant reporter gene expressions. (A) A promoter fragment is cloned directionally between the two *Sfi*I sites of the univector pUNI100. Note that the *Sfi*I sites create unique overhangs. (B) To make a translational fusion with a reporter, the coding sequence of the gene must be in frame. Each codon is underlined to indicate the reading frame. (C) The univector clone is recombined with host vectors pHP-100-GUS, pHP-100-LUC, or pHP-100-GFP.

209 was termed pUNI100 (alias p713–909; Figure 1A).
 210 *Sfi*I restriction endonuclease requires at least two
 211 recognition sites on the plasmid for digestion
 212 (Wentzell *et al.*, 1995). The two *Sfi*I sites of
 213 pUNI100 produce unique overhangs upon diges-
 214 tion and a promoter fragment with the same *Sfi*I
 215 tags can be cloned directionally. The *Sfi*I tag
 216 sequence distal to the *loxP* is 5'-GGCCA
 217 AATCGGCC-3' (*Sfi*I-aat), and the *Sfi*I tag se-
 218 quence proximal to the *loxP* is 5'-GGCC
 219 ATAAGGCC-5' (*Sfi*I-taa). To amplify the
 220 DNA fragment to be cloned in this vector, the
 221 tags must have the same sequences as the vector.

222 The univector has a conditional replication
 223 origin γ of *E. coli* plasmid R6K, and requires pi
 224 protein encoded by the *pir* gene. Therefore the
 225 vector must be propagated in an *E. coli* strain
 226 expressing pi (e. g., BW23474). The vector is
 227 unable to replicate in commonly used *E. coli*
 228 strains such as DH5 α or TOP10. However, after
 229 recombination DH5 α or TOP10 cells were used to
 230 isolate the recombinant product and select against
 231 non-recombined univector. When a translational
 232 fusion with a reporter is desired, the coding region
 233 must be in frame with the *loxP* sequence indicated
 234 in Figure 1B. If the DNA fragment is cloned in
 235 frame with *loxP*, it will be automatically in frame
 236 with the GUS coding sequence after the recombi-
 237 nation reaction.

238 Host vector construction

239 The template used for the host vector was pBE-
 240 NEE-blue (LeClere, 2002). A GUS gene sequence
 241 along with the nos terminator region was inserted
 242 between *Xho*I and *Eco*RI sites of pBENEE-blue. A
 243 *loxP* sequence was added to the 5'-end of the GUS
 244 gene. This plasmid contains an ampicillin selection
 245 marker for bacterial selection, along with the
 246 kanamycin resistance gene. To prevent the

replication of unrecombined host vector, the
 247 ampicillin resistance gene was destroyed by insert-
 248 ing a blunt-ended 750 bp *Msc*I fragment of the
 249 *Arabidopsis* cation/proton antiporter gene *CAX3*
 250 (At3g51860) into the *Sca*I site. The new vector has
 251 been termed pHP100-GUS (alias p713–905). A
 252 similar strategy was used to make luciferase and
 253 EGFP versions of the host vector. The new vectors
 254 have been designated pHP100-LUC (alias p713–
 255 947) and pHP100-GFP (alias p713–1511), respec-
 256 tively (Figure 1C). 257

258 Recombination efficiency

259 We have tested several promoters cloned in the
 260 univector pUNI100 for recombination efficiency
 261 with pHP100-GUS, pHP100-LUC, and pHP100-
 262 GFP vectors. Out of 54 randomly selected colo-
 263 nies, 52 (96.3%) contained correctly recombined
 264 plasmid based on the restriction digestion pattern.
 265 Sequencing confirmed the correct fusion of all of
 266 the clones selected.

267 Promoter::GUS expression of NHD1 and SUC2

268 We first examined whether the recombination
 269 constructs were expressed efficiently in plants.
 270 The 2.1 kb NHD1 (*Arabidopsis* Na⁺/H⁺ antiporter,
 271 At3g19490) promoter–GUS fusion constructed
 272 by the Cre-*loxP* recombination was successfully
 273 expressed in *Arabidopsis*. The expression was
 274 predominantly observed in leaf veins, the pedicel,
 275 and the stigma tip of the developing seed pod
 276 (Figure 2). *Arabidopsis* plants expressing a recom-
 277 binant of empty vector pUNI100 and the host
 278 vector pHP100-GUS showed no background GUS
 279 staining (results not shown). 279

280 Next, we compared the expression levels of
 281 reporters made by the traditional and the recombi-
 282 nation methods. The 2.0 kb SUC2 (*Arabidopsis*
 283 sucrose/H⁺ symporter, At1g22710) promoter was
 284 fused with GUS using the Cre-*loxP* system and the
 285 traditional cloning based pGPTV-HPT vector (Bec-
 286 ker *et al.*, 1992). In both systems, the GUS expres-
 287 sion was observed in the root and leaf vasculature.
 288 No significant difference was observed in expression
 289 patterns or intensity of GUS staining when the two
 290 methods were compared (Figure 3). 290

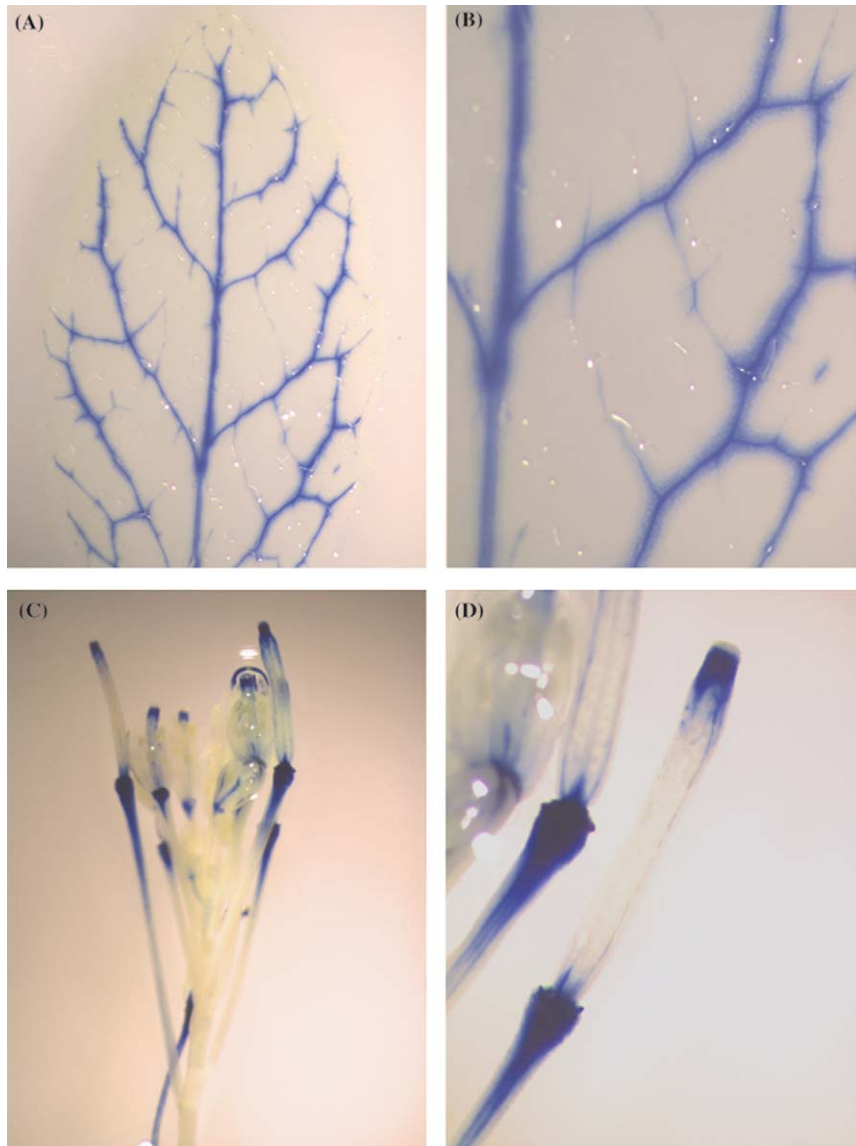


Figure 2. NHD1 promoter-driven GUS expression by Cre-loxP recombination. *AtNHD1* (At3g19490) expression in leaves and fruit in *Arabidopsis*. The 2.1 kb *AtNHD1* promoter-GUS fusion was constructed using the Cre-loxP recombination cloning system. (A) and (B) GUS expression in major veins of a cauline leaf. (C) and (D) GUS expression at the base of siliques and at the stigma following fertilization.

291 *Luciferase expression by Cre-loxP recombinant*
 292 *CAX7-LUC fusion*

293 Unlike GUS, the luciferase reporter system pro-
 294 vides non-destructive means to quantify the tran-
 295 scriptional expression over time. Preliminary GUS
 296 experiments suggested that CAX7 (a putative
 297 Ca²⁺/cation antiporter in *Arabidopsis*) is
 298 expressed in leaves at relatively high levels. There-

fore, we tested the efficiency of the Cre-loxP-based
 luciferase reporter construct using the CAX7
 promoter. Three-week old *Arabidopsis* plants
 grown on agar plates were used in the experiment.
 A plate of plants was sprayed with luciferin and
 activity of luciferase was detected in leaves of
 many plants after 5 min exposure time (Figure 4).

Several promoter- or promoter-entire coding
 region-EGFP reporter constructs were also made

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308 using the Cre-*loxP* recombination and signals were
 309 detected at levels consistent with results using
 310 other reporter constructs (results not shown).
 311 Thus, the EGFP reporter system is suitable not
 312 only for studying tissue-specific expression, but
 313 also for intracellular localization of the fusion
 314 protein.

315 Discussion

316 A few years ago, we independently wanted to
 317 establish an efficient and economical way to make
 318 reporter gene constructs by using the Cre-*loxP*
 319 recombination system of bacteriophage P1 (Abremski
 320 *et al.*, 1983) has been utilized to create ligation-free
 321 plasmid fusions for gene expressions in bacterial,
 322 yeast, insect, and mammalian cells. This ligation-free
 323 system is especially advantageous for two
 324 reasons: (1) promoters are cloned into a small
 325 univector rather than a large binary vector, and (2)
 326 once the univector construct is made, recombina-
 327 tion with a variety of host vectors containing
 328

different reporters can be conducted easily. The
 plant vector system is a new addition to the widely
 used Cre-*loxP*-based vectors.

We tested transgenic plants expressing the Cre-*loxP* based vectors for GUS expression under the control of several different promoters of transporter genes. The expression was indistinguishable when compared with methods based on traditional vector systems (Figure 3). Therefore, our vectors can be used in place of traditional restriction digestion based cloning vectors without loss of detection efficiency. The recombination step is highly accurate and efficient, minimizing the number of colonies that must be screened for a correct clone.

The LUC and GFP vectors were similarly efficient in monitoring gene expression. An advantage of this system is that the same univector clone can be quickly used for fusion with different reporter genes containing the appropriate *loxP* site. The GFP host vector can also be used to monitor the cellular localization of a protein when an entire coding sequence is cloned into the univector. It must be noted that the initial cloning of the promoter is still dependent on the restriction

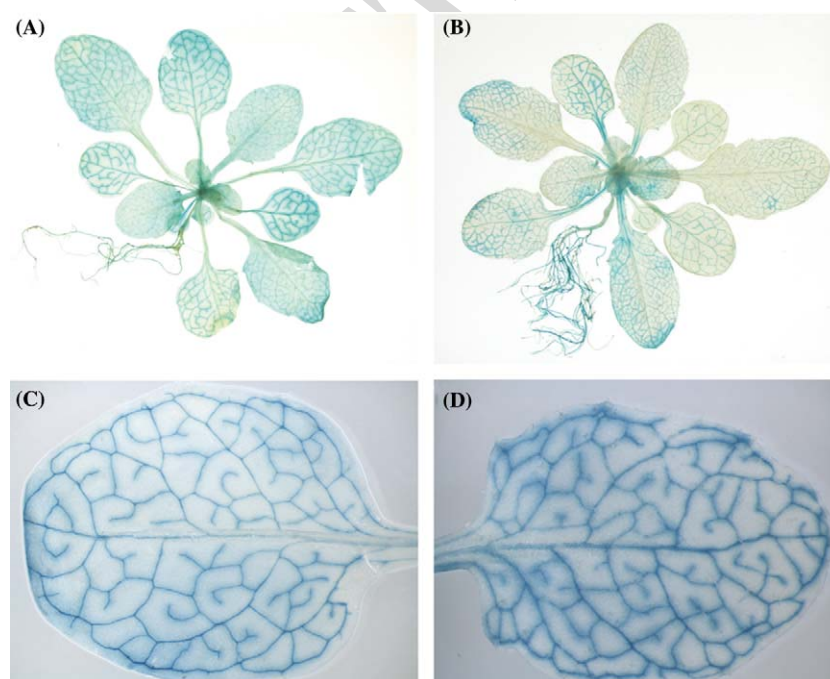


Figure 3. Comparison of recombination and traditional cloning methods to determine *AtSUC2* (*At1g22710*) promoter-GUS constructed using traditional and Cre-*loxP* cloning systems. (A) and (C) A 2.0 kb *SUC2* promoter was amplified from Col-0 DNA, subcloned into pUNI100 and recombined with binary vector pHP100-GUS to yield a translational fusion with GUS. (B) and (D) A 2.1 kb *SUC2* promoter transcriptional fusion to GUS was constructed in pGPTV-HPT (Becker *et al.*, 1992). In A through D *Arabidopsis* plants were stained overnight in X-Gluc solution and cleared in 70% ethanol.

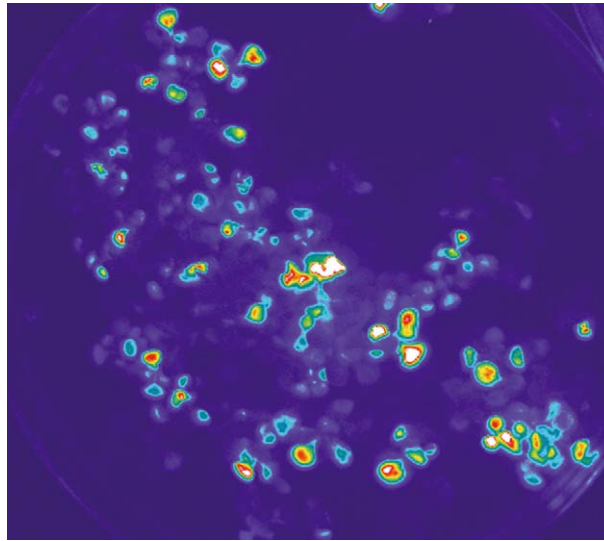


Figure 4. Luciferase expression by Cre-*loxP* recombination driven by CAX7 promoter. Agar-grown *Arabidopsis* plants expressing a 1.0 kb *AtCAX7* (At5g17860) promoter-luciferase fusion were made by Cre-*loxP* recombination. Plants were sprayed with luciferin and incubated in the dark for 5 min. The luminescence intensity is indicated in false colors ranging from blue (low) to white (high).

353 digestion and ligation. However, cloning into the
354 small univector is easier than cloning into a large
355 binary vector. Currently, the Cre-*loxP* system
356 lacks the multiple fragment cloning feature of the
357 Gateway system.

358 The presence of the *loxP* sequence in a plant
359 genome would possibly cause an erratic recombina-
360 tion *in vivo*, and compromise the usefulness of
361 the system. However, there is no *loxP* sequence in
362 *Arabidopsis*, rice, or any other vascular plant
363 genome in the public databases. There is, however,
364 an exact *loxP* sequence in the genome of the model
365 bryophyte *Physcomitrella patens*.

366 The Cre-*loxP* vectors can be used for both
367 transcriptional and translational fusions. Liu *et al.*
368 (1998) reported that the *loxP* sequence placed
369 between the *GALI* promoter and β -galactosidase
370 gene is inhibitory for expression in *E. coli* due to the
371 stem-loop structure that is inherent for *loxP*. One
372 *loxP* mutant termed *loxH* was used to increase the
373 transcription efficiency. In planta, however, we did
374 not observe reduced efficiency with *loxP* (data not
375 shown). In the report by Liu *et al.* (1998), the use of
376 *loxH* results in fourfold reduction in recombina-
377 tion efficiency in *E. coli*. Therefore, we decided to
378 use *loxP* rather than *loxH* in our vectors.

379 Another method to make expression vectors by
380 recombination is recently available from Invitro-
381 gen using a restriction digestion/ligation-free sys-
382 tem. The method is based on the *att* recombination

and public domain plant vectors are available
(Karimi *et al.*, 2002; Curtis and Grossniklaus,
2003). However, relatively costly proprietary
enzyme mixtures are required. In contrast, the
Cre recombinase protein can be easily produced in
any laboratory (Liu *et al.*, 2000), making this
approach an inexpensive alternative to the Invi-
trogen system. In conclusion, the Cre-*loxP* system
we developed is an efficient and economical
method to study the spatial and temporal expres-
sion of genes in plants. The Cre-*loxP* system serves
as a common platform for the future expansion of
recombination-based cloning vectors for plant
research.

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