A novel gateway-compatible binary vector series (PC-GW) for flexible cloning of multiple genes for genetic transformation of plants

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Abstract

The rapidly advancing field of plant synthetic biology requires transforming plants with multiple genes. This has sparked a growing interest in flexible plant transformation vectors, which can be used for multi-gene transformations. We have developed a novel binary vector series, named the PC-GW series (GenBank: KP826769-KP826773), for *Agrobacterium*-mediated plant transformation. The PC-GW vectors use the pCAMBIA vector backbone, and contain *NPTII*, *hpt*, *bar*, *mCherry* or *egfp* genes as selectable markers for plant transformation. In a modified multiple cloning site (MCS) of the T-DNA region, we have placed the *attR1*, *attR2* and *ccdB* sequences for rapid cloning of one to four genes by GatewayTM-assisted recombination. In addition, we have introduced four meganuclease sites, and other restriction sites for multi-gene vector construction. Finally, we have placed a *CaMV* 35S promoter and a 35S terminator on the 5' and 3' ends of the MCS. The *CaMV* 35S promoter is flanked by *PstI* restriction sites that can be used to replace it with another promoter sequence if needed. The PC-GW vectors provide choices for selectable markers, cloning methods, and can accommodate up to eight gene constructs in a single T-DNA, thereby significantly reducing the number of transformations or crosses needed to generate multi-transgene expressing plants.

Keywords

Gateway, plant transformation, *Agrobacterium*, gene stacking

Abbreviations

PC-GW: pCAMBIA-Gateway

1. Introduction

Our increasing knowledge of promoters, gene functions and metabolic pathways has provided unprecedented opportunities to create novel pathways in plants to increase productivity (Naqvi et al., 2010). Instead of inserting one gene at a time, researchers need to express multiple genes in the same plant for crop improvement. Multi-gene engineering (gene stacking) has enabled expression of transgenic pathways for applications such as increasing photosynthetic efficiency (Kebeish et al., 2007) and improving synthesis of nutritionally valuable compounds such as polyunsaturated fatty acids (PUFAs) (Damude and Kinney, 2007; Kinney A, 2004; Wu et al., 2005), carotenoids (Fujisawa et al., 2009; Ravanello et al., 2003; Zhu et al., 2008) or vitamin E (Raclaru et al., 2006). Multi-gene expression is central to such pathway engineering approaches (Dafny-Yelin and Tzfira, 2007; Mansouri and Berger, 2014). To stack multiple genes, transgenic plants expressing a few of the desired genes can be crossed, sequentially transformed or co-transformed with one or two genes at a time (Datta et al., 2002). However, obtaining homozygous transgenic populations using these approaches is labor and time-intensive. Cloning multiple genes in the same T-DNA eliminates segregation analyses of the multi-gene transgenics. For that reason, in the last two decades a number of binary vectors have been developed to enable generation of multi-gene constructs (Lee and Gelvin, 2008).

There is a variety of binary vectors available for generating multi-gene constructs, each with its own advantages (Table 1) (Earley et al., 2006; Karimi et al., 2002; Lee and Gelvin, 2008; Nakagawa et al., 2007; Nelson et al., 2007; Traore and Zhao, 2011; Vemanna et al., 2013; Xiang et al., 1999). However, most of these vectors depend on either using only restriction sites-based cloning methods or only GatewayTM-based cloning strategies (Lee and Gelvin, 2008; Li et al., 2014). Binary vectors with a rich variety of unique restriction sites in the multiple cloning site

(MCS) allow cloning of gene constructs flanked by those restriction sites. This method does not limit how many gene elements (promoter, coding sequence, and terminator) can be added to the MCS. In this regard, the pCAMBIA series of binary vectors are popular because of the presence of many unique restriction sites in the MCS region, and higher plasmid copy number inside *Agrobacterium* (Lee and Gelvin, 2008). But with increasing numbers of gene elements, finding unique restriction sites can be difficult. Only a few available binary vectors contain additional meganuclease cutting sites which are not usually found in plant gene elements (Table 1) (Goderis et al., 2002; Vemanna et al., 2013). Cloning systems such as GB2.0 and MoClo are also gaining popularity as they depend on the use of type IIS restriction sites to easily combine large number of “modules” to form large inserts (Sarrion-Perdigones et al., 2014; Sarrion-Perdigones et al., 2013; Weber et al., 2011). But their use is also contingent on not having the desired restriction site within the sequence of the gene(s) of interest. To bypass this problem, gene elements may be artificially synthesized and conflicting restriction sites may be eliminated. This however can add to the cost of construct generation. Cloning gene elements using restriction sites also takes considerable time (proportionate to the number of constructs being cloned). An alternative to cloning using restriction sites is to use the GatewayTM cloning technology (Life Technologies, NY). This approach takes advantage of site-specific recombination properties of bacteriophage lambda (Landy, 1989). Plant genes flanked by *attL1* and *attL2* sites can be rapidly recombined (within 5 minutes) with *attR1* and *attR2* sites in a destination vector (Hartley et al., 2000). Gateway technology also allows for multi-site Gateway reactions, and up to four elements can be transformed into the Gateway sites as long as the first element has an *attL1* site at the 5’ end (with respect to the vector) and the fourth element has an *attL2* site at the 3’ end (with respect to the vector). Since this approach is fast and very efficient, it was rapidly adapted by plant

scientists to develop a variety of Gateway-compatible plant transformation vectors (Table 1) (Earley et al., 2006; Karimi et al., 2002; Traore and Zhao, 2011; Vemanna et al., 2013). However, most gateway compatible vectors are large and lack multiple cloning sites for restriction digestion-assisted cloning (Earley et al., 2006; Nakagawa et al., 2007). This makes it difficult to add more genes to the same vector at a later time. Binary vectors are often limited to about 50 kb of transgene material, after which deletions can occur within the sequence (Naqvi et al., 2010). For larger number of genes, artificial chromosomes may be developed with multiple genes of interest by using approaches such as the Gibson assembly (Gibson et al., 2009). However, for < 50kb of transgene material, four gene elements can be safely added to a Gateway region while still leaving room to add 3-4 more genes at a later time, depending on the sizes of individual gene elements.

We have generated the PC-GW vectors containing a unique combination of the pCAMBIA, meganuclease sites and Gateway technologies (Table 2). The PC-GW vectors contain unique restriction sites, rare-cutting meganuclease sites, and *attR1-attR2* sites for Gateway-assisted recombination on a pCAMBIA backbone to facilitate multiple gene construction and subsequent transformation. In addition, the PC-GW vectors offer a choice of multiple marker genes for selection of transgenic plants.

Materials and Methods

2.1 Generation of modified pCAMBIA vectors

The pCAMBIA2300 vector was obtained from CAMBIA (<http://www.cambia.org/daisy/cambia/585>). The *NPTII* gene was removed from pCAMBIA2300 using *XhoI* (New England BioLabs, Ipswich, MA) to be replaced with other selectable marker

genes. The *mCherry* sequence was amplified from the vector ER-RB (obtained from Dr. Marcela Rojas-Pierce, NCSU)(Nelson et al., 2007). The *egfp* sequence was amplified from the EGFP-N1 plasmid (Takara Clontech, Mountain View, CA). The *bar* sequence was amplified from the pEarleyGate100 plasmid ((Earley et al., 2006), obtained through ABRC). The coding sequences for mCherry, EGFP and BAR were amplified with primers that contained *XhoI* sites (for primer sequences, see Table 3). The *mCherry*, *egfp* and *bar* inserts were ligated into the pCAMBIA2300 vector, replacing the *NPTII* gene to generate the pCAMBIA-mCherry, pCAMBIA-EGFP and pCAMBIA-BAR plasmids (GenBank: KP795971- KP795973). Because the ligation used *XhoI* sites on both ends, plasmids with the insert in the correct orientation were identified using the gene-specific forward primer with a vector specific reverse primer (PC-RP).

2.2 Generation of PC-GW vectors

The MCS for the PC-GW vector was designed *in silico* using Vector NTI (Life Technologies, Grand Island, NY). The CaMV 35S promoter sequence was obtained from the PUGW5 sequence (GenBank: AB626665). The 35S terminator sequence was obtained from the cloning vector pSIM1 (GenBank: HM750245.1). The *attR1*, *attR2* and *ccdB* sequences were obtained as a cassette from the pEarleyGate 100 vector (Earley et al., 2006). The restriction sites and meganuclease sites were obtained through the New England BioLabs website (New England BioLabs). The newly designed MCS has an *EcoRI* site on the 5' end and a *HindIII* site on the 3' end. The MCS was synthesized and cloned in pUC57 plasmid by GenScript (GenScript, Piscataway, NJ). The original MCS in pCAMBIA2300, pCAMBIA1300, pCAMBIA-mCherry, pCAMBIA-EGFP and pCAMBIA-BAR was replaced by this new PC-GW MCS to generate the PC-GW-Kan, PC-GW-Hyg, PC-GW-mCherry, PC-GW-EGFP and PC-GW-BAR vectors respectively.

2.3 Gateway assisted recombination

Four gene constructs were individually synthesized for each construct and cloned in the plasmid pUC57 (GenScript). The four gene sequences were flanked by pairs of *attL* and *attR* sequences for multi-site Gateway recombination as follows: *attL1* and *attR5*, *attL5* and *attL4*, *attR4* and *attR3*, and *attL3* and *attL2*. The four plasmids and the PC-GW-mCherry or the PC-GW-EGFP plasmids were placed together in a reaction containing 2 µL of LR Clonase II Plus enzyme (Life Technologies). After sixteen hours of incubation at room temperature, 2 µL of the reactions were used to transform chemically competent One Shot^R Mach1TM T1^R *E. coli* cells (Life Technologies). The transformed cells were selected on kanamycin-containing LB plates. The plasmids were isolated and gene integration and direction of insertion were tested by restriction digestion and sequencing.

2.4 Generation of transgenic plants by stable transformation

The plasmids PC-GW-mCherry and PC-GW-EGFP containing four genes of interest each were then transformed into *Agrobacterium tumefaciens* strain GV3101. The *Agrobacterium* were used to stably transform seven-week-old *Camelina sativa* plants by floral dipping with a vacuum of 30 psi (Lu and Kang, 2008). To screen for mCherry expression, the T₁ seeds or seedlings were imaged at 560 nm excitation wavelength under a fluorescence dissection microscope (Nikon, ET-mCherry, set-ID 49008) to identify those plants transformed with the pCAMBIA-mCherry plasmid. To screen for plants transformed with the PC-GW-EGFP plasmid, plants were grown on petri plates containing ½ MS medium without sucrose at pH 5.7 for 6 days, and then imaged at 480 nm excitation wavelength using the Leica MZFLIII fluorescence stereomicroscope.

2.5 Transient transformation

The plasmid pCAMBIA-EGFP was used for transient transformation of tobacco cells (Bendahmane et al., 2000). *Agrobacterium tumefaciens* containing pCAMBIA-EGFP was inoculated into 3 ml LB medium supplemented with 50 µg/ml kanamycin and incubated at 28°C and 225 rpm for 16-24 hours. Two to three ml of the culture was then used to inoculate 250 ml LB medium supplemented with 50 µg/ml kanamycin and incubated under the same conditions for another 16-24 hours. The *Agrobacterium* cells were collected by centrifugation (2000 g for 30 minutes) and the pellet was re-suspended with the infiltration medium (10 mM MgCl₂, 10 mM MES, and 0.15 mM acetosyringone) to obtain an OD₆₀₀ reading of 0.3-0.4 in a final volume of 500 ml or 1 l infiltration medium (Bendahmane et al., 2000).

For transient transformation, a 2-3 mm wide strip was cut along the edge or at the tips of the leaves of well-watered tobacco plants before infiltration. The leaf strips were then submerged completely into the infiltration medium with the suspended *Agrobacterium* cells and vacuum of (-0.86) kPa was applied for 5 min. The vacuum was slowly released and the leaf strips were returned to a tray, covered with a dome (to maintain high humidity) and placed in the dark overnight. The transformed cells were visualized for EGFP expression using 480 nm excitation wavelength under Leica MZFLIII fluorescence stereomicroscope.

2. Results and discussion

3.1 Generation of the PC-GW MCS

To add the convenience of Gateway cloning to the pCAMBIA vectors, we replaced the MCS in pCAMBIA vectors with a custom-designed and synthesized PC-GW MCS sequence (Table 2).

In the new MCS, we have placed the *attR1* and *attR2* sites to enable directional Gateway-assisted recombination. A single gene or coding sequence containing the *attL1* and *attL2* borders, often provided by the gateway entry vectors, can be easily cloned in the vector. Using multisite Gateway technology, we have cloned up to four genes in the PC-GW vectors concurrently, as a result of a single cloning experiment. To further improve cloning efficiency, we have placed the *ccdB* gene between the *attR1* and *attR2* sites. Up to four genes can be added via Gateway cloning using the *attR1* and *attR2* sites. The selection marker for bacteria is kanamycin resistance. Therefore, either entry clones with different antibiotic resistance, or linearized entry clones may be readily used in LR recombination reactions to clone into the PC-GW vectors. Larger entry clones should be linearized to increase the efficiency of the LR recombination. The Gateway region, if undesired by a researcher, can be excised from the PC-GW vectors using the *ZraI* and *BamHI* sites. After, or instead of the gateway reactions, additional genes may be added at the 5' end using the unique restriction sites (*ZraI*, *SwaI*, *SpeI*, *AvrII*, *AflIII*, *XbaI*) and meganuclease sites (*I-SceI* and *I-CeuI*). Additional genes may be added on the 3' end using the meganuclease sites (*PI-PsPI* and *PI-SceI*) (Fig. 1b). Because of their rather large DNA recognition site for restriction digestion (~20 nt), the meganuclease sites occur rarely in the plant genome, so native genes could be cloned in between these restriction sites with minimum to no sequence optimization. Therefore, the newly designed MCS would enable researchers to put in a large number of gene constructs in the PC-GW vector.

To enable direct constitutive expression of a single cloned gene, we have placed a CaMV 35S promoter upstream of the PC-GW polylinker, and a CaMV 35S terminator downstream. To enable quick cloning for constitutive expression, the PC-GW vectors contain the *CaMV 35S* promoter on the 5' and a 35S terminator at the 3' end of the MCS (Fig. 1a). Since the choice of

the promoter will vary by the objective of the research, the *CaMV* 35S promoter is flanked by *Pst*I restriction sites that can be used to replace it with another promoter sequence. For example, if transgene expression in monocots is the goal, then the maize ubiquitin promoter may be a good choice (Christensen and Quail, 1996; Cornejo et al., 1993). Furthermore, use of same sequences repeatedly on the T-DNA could lead to homology based gene silencing. Since there are already two 35S promoter sequences on the T-DNA of the PC-GW vectors, care should be taken to avoid the 35S promoter for the other consecutive genes in the vector. For constitutive expression of genes, alternative promoters may be employed, such as tobacco *EntCUP* promoters (Malik et al., 2002) and the *Arabidopsis Actin2* promoter (An and Meagher, 2010).

3.2 Generation and validation of the PC-GW series vectors

The plasmid pCAMBIA2300 was digested with *Xho*I to excise the *NPTII* gene for kanamycin resistance in plants. In place of *NPTII*, *mCherry*, *EGFP* and *BAR* sequences were cloned between the *Xho*I sites on the pCAMBIA vector to generate three modified pCAMBIA vectors- pCAMBIA-mCherry (Fig. 2a) pCAMBIA-EGFP (Fig. 2c) and pCAMBIA-BAR (Table 2) (GenBank KP795971- KP795973). The vector pCAMBIA-mCherry was evaluated for transformation efficiency during stable transformation of *Camelina sativa* by floral dipping (Lu and Kang, 2008). The T₁ transgenic seedlings were identified using fluorescence microscopy (Fig. 2b). The transformation efficiency of camelina plants with pCAMBIA-mCherry vectors containing about 5kb of transgenic sequences was higher than 0.1%. To demonstrate the use of the modified pCAMBIA vectors in transient transformation, we used pCAMBIA-EGFP in transient transformation of tobacco leaves. The transformed cells were identified using fluorescence microscopy (Fig. 2d).

To generate the PC-GW vectors, we inserted the PC-GW MCS between the *EcoRI* and *HindIII* sites on the pCAMBIA1300, pCAMBIA2300 and three modified pCAMBIA vectors (Table 2). The PC-GW-mCherry and PC-GW-EGFP vectors were used for simultaneous transformation of four entry gene elements at a time using the multisite Gateway technology. Each gene element contained a promoter, coding sequence and a terminator, with a few exceptions (Fig. 3a, 4a). Because the first gene was cloned on the 3' end of the CaMV 35S promoter in the PC-GW vector, this construct was synthesized without a promoter. Similarly, because the fourth gene is cloned on the 5' end of the PC-GW 35 S terminator, this construct was synthesized without a terminator. Four different gene constructs were used to clone into the PC-GW-mCherry and PC-GW-EGFP vectors. All four inserts were cloned concurrently using Gateway technology with the binary vectors PC-GW-mCherry or PC-GW-EGFP. After sixteen hours of incubation with the enzyme LR Clonase II Plus (Life Technologies) and cloning, multiple bacterial colonies were isolated that had all the four gene constructs integrated into the modified binary vectors. Restriction digestion analysis was performed on the plasmids to confirm integration of different gene constructs (Fig. 3b, 4b). The integration of the gene constructs was also verified by gene sequencing (Eurofins MWG Operon, AL).

3.3 Transformation with PC-GW vectors

We used PC-GW-mCherry and PC-GW-EGFP vectors, each with four gene constructs to test stable transformation in *Camelina*. The transgenic plants cloned with PC-GW-mCherry could be easily screened at the seed stage using fluorescence microscopy (Fig. 3c). The T₁ seedlings transformed with the PC-GW-EGFP construct were identified using fluorescence microscopy as well (Fig. 4c). With about 15 kb of transgenic sequences in the MCS, the PC-GW plasmids had lower transformation efficiency (~0.75%) compared to the pCAMBIA vectors probably due to

the length of the T-DNA. However, once transformed, all the transgenics show strong expression for individual gene elements (data not shown).

3. Conclusions

Gateway recombination is a fast and efficient way to clone up to four genes in a binary vector. Yet most of the gateway vectors available for plant transformations are large and do not have polylinker regions to enable addition of more gene constructs into the same vector. The traditional binary vectors that contain polylinker regions for restriction digestion-mediated cloning require time consuming cloning reactions to sequentially add genes. Furthermore, lacking meganuclease sites available for cloning makes the introduction of additional gene constructs without codon alteration difficult to achieve. To combine the best features of Gateway recombination and restriction digestion-mediated cloning, we have generated five PC-GW vectors for convenient cloning and a wide selection of selectable markers for plant transformation. The plasmids can be used to easily clone in a large number of genes with little or no codon alteration for stable and transient transformations.

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335

Table 1.
Commonly used plant binary vectors

Vector series	Cloning method	R-gene in plants	Additional features	Reference
pCAMBIA	Restriction sites	Antibiotic selection markers	n/a	http://www.cambia.org/daisy/cambia/materials/vectors
pCB	Restriction sites	Herbicide and antibiotic selection markers	Shortened pBIN19 background, not self-mobilizable	Xiang et al., 1999
pPZP-RCS	restriction sites including rare cutting sites	Herbicide and antibiotic selection markers	Auxiliary vectors contain built-in promoters and terminators	Goderis et al., 2002
pMDC	Gateway	Herbicide and antibiotic selection markers	Built-in promoter, terminator	Curtis et al., 2003
pEarleyGate	Gateway	Herbicide and fluorescence selection markers	Built-in promoter, terminator	Earley et al. 2006
ImpGWBs	Gateway	Antibiotic, fluorescence selection markers	Built-in terminator	Nakagawa et al., 2007
pGATE	Gateway, rare cutting sites	Antibiotic selection marker	Intermediate gateway cloning vectors available	Vemanna et al. 2013
pCHF	Restriction sites	Same as pCAMBIA	Built-in promoter, terminator in pCAMBIA background	Li et al., 2014
PC-GW	Gateway, restriction sites including rare cutting sites	Herbicide, antibiotic and fluorescence selection markers	Built-in promoter, terminator in pCAMBIA background	Present report

Table 2.
The PC-GW series and modified pCAMBIA vectors

Plasmid name	Description	Selection/ screening gene plants	Selection gene bacteria	GenBank accession number
PC-GW-Kan	35S-Gateway-35S 3'	<i>NPTII</i>	<i>Kan</i>	KP826769.1
PC-GW-Hyg	35S-Gateway-35S 3'	<i>HPTII</i>	<i>Kan</i>	KP826770.1
PC-GW-mCherry	35S-Gateway-35S 3'	<i>mCherry</i>	<i>Kan</i>	KP826771.1
PC-GW-EGFP	35S-Gateway-35S 3'	<i>EGFP</i>	<i>Kan</i>	KP826772.1
PC-GW-BAR	35S-Gateway-35S 3'	<i>BAR</i>	<i>Kan</i>	KP826773.1
pCAMBIA-mCherry	pCAMBIA MCS	<i>mCherry</i>	<i>Kan</i>	KP795971.1
pCAMBIA-EGFP	pCAMBIA MCS	<i>EGFP</i>	<i>Kan</i>	KP795972.1
pCAMBIA-BAR	pCAMBIA MCS	<i>BAR</i>	<i>Kan</i>	KP795973.1

Table 3.

Primer sequences to amplify inserts for the modified pCAMBIA vectors

Primer name	Sequence
mCherry FP	5'-TGGAGAAACTCGAGCTTGTCGATCGACCTTGTACAGC TCGTCCAT -3'
mCherry RP	5'- TAAAGCATGGTTCTCGAGCTTTTCGCAGATATGGTGAGC AAGGGC-3'
EGFP FP	5'-ATCGGGATTCTCGAGATGGTGAGCAAGGGC-3'
EGFP RP	5'-GCTGAGTACTCGAGTTACTTGTACAGCTCGTC-3'
BAR FP	5'-CAATCCTCGAGGAATCGATGAGCCCAGAAC-3'
BAR RP	5'-GAATCCTCGAGTCAAATCTCGGTGACGGGCA-3'
PC-RP	5'-ACTGATGGGCTGCCTGTATC-3'

Figure

(a)

(b)

Fig. 1

Generation of the PC-GW MCS and the PC-GW cloning strategy. (a) A novel construct was designed to replace the MCS in the T-DNA of pCAMBIA vectors between the *EcoRI* and *HindIII* restriction sites. The new MCS has 35S promoter and 35S terminator sequences. The 35S promoter is flanked by *PstI* sites. In between, unique restriction sites (in red) have been placed. The *attR1*, *ccdB* and *attR2* sequences have been placed in the middle. Homing endonuclease sites *I-CeuI*, *I-SceI*, *PI-PspPI* and *PI-SceI* are placed, two on each end of the construct. (b) Using the PC-GW cassette, up to four genes can be cloned via gateway. Additional genes may be cloned via the unique restriction sites (for e.g. *ZraI*, *SwaI*, *XbaI* etc) or by using the homing endonuclease sites.

Fig. 2

Demonstration of fluorescent marker expression *in planta* after transformation of pCambia-mCherry and pCambia-EGFP vectors into *Camelina sativa*. The mCherry and EGFP coding sequences were cloned in between the *Xho*I sites to generate (a) pCambia-mCherry and (c) pCambia-EGFP. (b) The pCambia-mCherry plasmid was used for *Agrobacterium*-mediated transformation of *Camelina sativa* inflorescences. The transgenic seedlings were identified using fluorescence microscopy. (d) The pCambia-EGFP plasmid was used for transient transformation of tobacco leaves. The expressing cells were identified using fluorescence microscopy.

(a)

PC-GW-mCherry
(11,657 bp)

(b)

(c)

Fig. 3

Demonstration of the use of the PC-GW-mCherry vector to introduce four genes into *Camelina sativa*. The PC-GW MCS was cloned between the *EcoRI* and *HindIII* sites of the pCAMBIA-mCherry plasmid to generate PC-GW-mCherry. (a) Example of a four gene element-containing construct cloned into PC-GW-mCherry. Each gene element contains a promoter, coding sequence and terminator, except element 1 which lacks a promoter and element 4 which lacks a terminator sequence. (b) The PC-GW-mCherry was used to successfully clone the four gene elements simultaneously using Gateway™ technology. The resulting clones were confirmed for the presence of the four gene elements using restriction digestion. The *Bgl/II* digest resulted in bands (11.4kb, 4kb, 1.7kb, 1.6kb, 1.1kb) consistent with the sizes expected for cuts in gene elements 1, 2, and 4 indicating that these elements are present in the correct order. *SalI* cuts gene element 2 and the digest resulted in bands (14kb, 5.5kb) of the expected sizes, further confirming the results of the *Bgl/II* digest. *XhoI* cuts gene element 3, and the digest resulted in the expected bands (10kb and 9kb running together, 0.73bp). The constructs were also digested with *I-CeuI* and *PI-SceI* enzymes. The 11.6kb band represents the size of the PC-GW insert with the four genes. The 8.8kb band represents the remaining vector backbone. (c) The PC-GW-mCherry plasmid with the four gene elements was used to genetically transform *Camelina sativa* inflorescences by *Agrobacterium*-mediated transformation. Transgenic seeds were identified by fluorescence microscopy. TG= transgenic, NTG= non-transgenic

(a)

(c)

(b)

Fig. 4

Demonstration of the use of the PC-GW-EGFP vector to introduce four genes into *Camelina sativa*. The PC-GW MCS was cloned between the *EcoRI* and *HindIII* sites of the pCambia-EGFP plasmid to generate PC-GW-EGFP. (a) Example of a four gene element-containing construct cloned into PC-GW-EGFP. (b) The PC-GW-EGFP was used to successfully clone the four gene elements simultaneously using Gateway™ technology. The resulting clones were confirmed for the presence of the four gene elements using restriction digestion. *XhoI* cuts gene element 1 at three sites, and the resulting bands (14.8kb, 3.4kb, 0.73kb) indicate the presence and correct orientation of gene element 1. *SspI* cuts gene element 2 at two sites and the digest resulted in expected band sizes (11.8kb, 7kb). *XbaI* cuts gene element 3 at two places, and the digestion resulted in bands (13.1kb, 5.7kb) indicating correct insertion of gene element 3. *BglII* has four different cutting sites at gene elements 1 and 4. The digest results in bands (11.1kb, 4kb, 2.4kb, 1.3kb) of expected sizes, indicating that all genes were cloned in as expected. *BamHI* linearizes the vector and show correct total size of the plasmid (18.8kb). The constructs were also digested with *I-SceI* and *PI-PsPI* enzymes. The 9.9kb band represents the size of the PC-GW insert with the four genes. The 8.9kb band represents the remaining vector backbone. (c) The PC-GW-EGFP plasmid with the four gene elements was used to genetically transform *Camelina sativa* inflorescences by *Agrobacterium*-mediated transformation. Transgenic seedlings were identified by fluorescence microscopy. TG= transgenic, NTG= non-transgenic.

Fig. 1

Generation of the PC-GW MCS and the PC-GW cloning strategy. (a) A novel construct was designed to replace the MCS in the T-DNA of pCAMBIA vectors between the *EcoRI* and *HindIII* restriction sites. The new MCS has 35S promoter and 35S terminator sequences. The 35S promoter is flanked by *PstI* sites. In between, unique restriction sites (in red) have been placed. The *attR1*, *ccdB* and *attR2* sequences have been placed in the middle. Homing endonuclease sites *I-CeuI*, *I-SceI*, *PI-PspPI* and *PI-SceI* are placed, two on each end of the construct. (b) Using the PC-GW cassette, up to four genes can be cloned via gateway. Additional genes may be cloned via the unique restriction sites (for e.g. *ZraI*, *SwaI*, *XbaI* etc) or by using the homing endonuclease sites.

Fig. 2

Demonstration of fluorescent marker expression *in planta* after transformation of pCAMBIA-mCherry and pCAMBIA-EGFP vectors into *Camelina sativa*. The mCherry and EGFP coding sequences were cloned in between the *XhoI* sites to generate (a) pCAMBIA-mCherry and (c) pCAMBIA-EGFP. (b) The pCAMBIA-mCherry plasmid was used for *Agrobacterium*-mediated transformation of *Camelina sativa* inflorescences. The transgenic seedlings were identified using fluorescence microscopy. (d) The pCAMBIA-EGFP plasmid was used for transient transformation of tobacco leaves. The expressing cells were identified using fluorescence microscopy.

Fig. 3

Demonstration of the use of the PC-GW-mCherry vector to introduce four genes into *Camelina sativa*. The PC-GW MCS was cloned between the *EcoRI* and *HindIII* sites of the pCAMBIA-

mCherry plasmid to generate PC-GW-mCherry. (a) Example of a four gene element-containing construct cloned into PC-GW-mCherry. Each gene element contains a promoter, coding sequence and terminator, except element 1 which lacks a promoter and element 4 which lacks a terminator sequence. (b) The PC-GW-mCherry was used to successfully clone the four gene elements simultaneously using GatewayTM technology. The resulting clones were confirmed for the presence of the four gene elements using restriction digestion. The *Bgl*II digest resulted in bands (11.4kb, 4kb, 1.7kb, 1.6kb, 1.1kb) consistent with the sizes expected for cuts in gene elements 1, 2, and 4 indicating that these elements are present in the correct order. *Sal*I cuts gene element 2 and the digest resulted in bands (14kb, 5.5kb) of the expected sizes, further confirming the results of the *Bgl*II digest. *Xho*I cuts gene element 3, and the digest resulted in the expected bands (10kb and 9kb running together, 0.73bp). The constructs were also digested with *I-Ceu*I and *PI-Sce*I enzymes. The 11.6kb band represents the size of the PC-GW insert with the four genes. The 8.8kb band represents the remaining vector backbone. (c) The PC-GW-mCherry plasmid with the four gene elements was used to genetically transform *Camelina sativa* inflorescences by *Agrobacterium*-mediated transformation. Transgenic seeds were identified by fluorescence microscopy. TG= transgenic, NTG= non-transgenic

Fig. 4

Demonstration of the use of the PC-GW-EGFP vector to introduce four genes into *Camelina sativa*. The PC-GW MCS was cloned between the *Eco*RI and *Hind*III sites of the pCAMBIA-EGFP plasmid to generate PC-GW-EGFP. (a) Example of a four gene element-containing construct cloned into PC-GW-EGFP. (b) The PC-GW-EGFP was used to successfully clone the

four gene elements simultaneously using GatewayTM technology. The resulting clones were confirmed for the presence of the four gene elements using restriction digestion. *XhoI* cuts gene element 1 at three sites, and the resulting bands (14.8kb, 3.4kb, 0.73kb) indicate the presence and correct orientation of gene element 1. *SspI* cuts gene element 2 at two sites and the digest resulted in expected band sizes (11.8kb, 7kb). *XbaI* cuts gene element 3 at two places, and the digestion resulted in bands (13.1kb, 5.7kb) indicating correct insertion of gene element 3. *BglII* has four different cutting sites at gene elements 1 and 4. The digest results in bands (11.1kb, 4kb, 2.4kb, 1.3kb) of expected sizes, indicating that all genes were cloned in as expected. *BamHI* linearizes the vector and show correct total size of the plasmid (18.8kb). The constructs were also digested with *I-SceI* and *PI-PsPI* enzymes. The 9.9kb band represents the size of the PC-GW insert with the four genes. The 8.9kb band represents the remaining vector backbone. (c) The PC-GW-EGFP plasmid with the four gene elements was used to genetically transform *Camelina sativa* inflorescences by *Agrobacterium*-mediated transformation. Transgenic seedlings were identified by fluorescence microscopy. TG= transgenic, NTG= non-transgenic.

Wild-type plant

Multi-gene expressing plant

PC-GW vectors

Plant transformation: Gene stacking

Need Gateway™ ?

Yes	Clone up to four genes
No	Excise Gateway™ out

Need to clone additional genes? Yes Use restriction/meganuclease sites No Proceed with transformation

Need to switch selection marker?	Yes	Replace using <i>Xho</i> I sites
	No	Proceed with transformation

Need to switch 35S promoter for gene expression?

Yes	Replace using <i>Pst</i> I sites
No	Proceed with transformation