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RESEARCH

A Modified MultiSite Gateway Cloning Strategy for Consolidation of Genes in Plants

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Abstract The genome information is offering opportunities to manipulate genes, polygenic characters and multiple traits in plants. Although a number of approaches have been developed to manipulate traits in plants, technical hurdles make the process difficult. Gene cloning vectors that facilitate the fusion, overexpression or down regulation of genes in plant cells are being used with various degree of success. In this study, we modified gateway MultiSite cloning vectors and developed a hybrid cloning strategy which combines advantages of both traditional cloning and gateway recombination cloning. We developed Gateway entry (*pGATE*) vectors containing *attL* sites flanking multiple cloning sites and plant expression vector (*pKM12GW*) with specific recombination sites carrying different plant and bacterial selection markers. We constructed a plant expression vector carrying a reporter gene (*GUS*), two *Bt* cry genes in a predetermined pattern by a single round of LR recombination reaction after restriction endonuclease-mediated cloning of target genes into *pGATE* vectors. All the three transgenes were co-expressed in *Arabidopsis* as evidenced by gene expression, histochemical assay and insect bioassay. The pGATE vectors can be used as simple cloning vectors as there are rare restriction endonuclease sites inserted in the vector. The modified multisite vector system developed is ideal for stacking genes and pathway engineering in plants.

Keywords Gateway vectors · Gene stacking · β-Glucuronidase (*GUS*) reporter gene · *Cry* genes · Insect bioassay

Introduction

Genetic engineering is emerging as a promising technology in modern agriculture. One of the major outcomes until now in crop biotechnology is yield security by improving resistance against pests and herbicide tolerance. In 2007, worldwide, genetically modified crops were grown over 120 million hectares [1]. Second and third generation genetically modified crops are being developed to improve crop performance under field conditions. Although manipulation of single gene has been successful to improve a certain plant characters, alteration of complex interactive metabolic pathways and important quantitative traits require coexpression of multiple genes. The major bottleneck in manipulating complex traits has been the technical limitations to introduce multiple genes into plants [2–5].

For the introduction of multiple genes into plant genome, a number of approaches such as sexual crossing between two transgenic plants [6], co-transformation with single plasmid carrying several linked transgenes [7] and multigene assembly vector system have been developed [3]. The approaches used for the development of transgenic plants carrying multiple transgenes such as retransformation [8, 9], stacking of several transgenes by successive delivery of single genes into transgenic plants [10], combined delivery of several transgenes in a single transformation experiment

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and sexual crosses between transgenic plants carrying different transgenes at a time [6, 11] is difficult and cumbersome. Although 12 and 11 gene constructs were delivered by particle bombardment into soybean embryogenic suspension culture [12] and rice, respectively [13], the issues related to the stability and independent segregation of transgenes that integrated at different genomic locations were considered as major limitations. Golden rice was developed by expressing four different transgenes using two independent gene constructs by co-transformation method [14]. Similarly, six transgenes were delivered into *Arabidopsis* to manipulate metabolic pathway for poly(3-hydroxybutyrate-co-3-hydroxyvalerate) [15]. Each approach has specific merits and limitations. For example, co-transformation with different plasmids decreases the efficiency with increasing number of plasmids, whilst sequential transformation and genetic crossing between transgenic lines is cumbersome and time consuming [10, 16]. Gene stacking using polycistronic strategy was reported to be useful for plastid genetic engineering where entire bacterial operons can be easily introduced and expressed [17]. In this approach, a part of the spacer region of a metallothionein-like protein was used to form a polyprotein. The approach has a limitation as the availability and expression level of endogenous protease is completely unknown for a specific plant [18].

Recombination-based MultiSite Gateway technology has given an option to introduce multiple genes in single T-DNA, and different Gateway-based plant binary vectors have been developed and evaluated [19]. The common Gateway approach involves amplification of target gene flanked by specific *attB* sites (*attB1-B4*, *attB4r-B3r*, *attB3-B2*) and homologous recombination-based mobilization to donor vectors (*P1-P4*, *P4r-P3r*, *P3-P2*) to generate entry clones (*L1-L4*, *R4-R3*, *L3-L2*) [20]. These entry clones with destination vector are used together for LR recombination reaction to create MultiSite Gateway expression clone. Many Gateway-based binary destination vectors are available for the expression of two or three genes in plants under the control of different promoters [19]. With the intention of delivering different transgenes simultaneously into plants, Gateway multi-round system was developed, which allows series of LR recombination reactions with the help of two different entry vectors [21]. This strategy is based on two unique Gateway entry vectors which have a few restriction endonuclease sites flanked by *attR* and *attL* sites. In this method, LR reaction has to be performed each time and selection markers play a crucial role. The availability of destination vectors compatible for specific entry vectors in terms of *att* recombination sites and resistance (R) gene is considered as a major limitation.

Development of stable expression vectors using existing sets of binary vectors for the assembly of multiple genes by traditional cloning methods would be useful. In this report, we describe the development and validation of a vector system for gene stacking in plants. The overall approach involves both conventional cloning and final one step recombination (LR) mediated reaction. We strategized and generated entry vectors with multiple cloning sites (MCS) to facilitate cloning of genes by conventional approach, which are compatible for subsequent LR reaction. The Gateway destination vector developed is compatible with entry vectors with specific plant selection marker and different "R" selection for bacteria. We validated the vector system by expressing three gene cassettes (a reporter gene, *cry1Aabc* and *cry1Ec* genes) in *Arabidopsis*. The vector system has broad applications for manipulating different proteins and biochemical pathways, and might be useful for functional genomic approaches involving protein–protein interactions.

Methods

Construction of *pGATE* Vectors

In Gateway cloning reactions, DNA sequences are flanked by *attB* sites which assist in recombination mediated by *BP clonase* enzyme. MCSs flanked with standard *attB* sites (518 bp) were custom synthesized (Piscataway, NJ08854, USA, www.genescript.com) and obtained in *pUC57* cloning vector. *pUC57* vector carrying MCS with *attB* sites (*attB1–MCS–attB4–attB4r–MCS–attB3r–attB3–MCS–attB2*) was restrict digested with *Not*I, *Bgl*II and *Pst*I to release 162, 155 and 158 bp of *attB1–MCS–attB4*, *attB4r–MCS–attB3r* and *attB3–MCS–attB2* fragments, respectively. The individual fragments were subjected to recombination reaction with *pDONOR P1-P4*, *P4r-P3r* and *P3-P2* (Invitrogen, USA, Catalog No. 12537-102, 12537-103, 12537-104, 12537-100) respectively, in a 20-µl reaction containing digested fragment (2–4 µl), 1.5 µl (200 ng μl^{-1}) of respective *pDONOR* vector and 2 µl of *BP clonase* enzyme (as per manufacture's protocol, Invitrogen, USA). The reaction was performed at 25°C overnight and terminated by *proteinase K* (1 µl) (Invitrogen, USA). The reaction mix was used to transform chemically competent *Escherichia coli* (DH5α) and transformed colonies were selected on LB agar plates containing kanamycin (50 mg l^{-1}). The modified vectors were designated as *pGATE*.

Construction of Destination Vector

The *pKM43GW* vector obtained from Plant Systems Biology (www.psb.ugent.be) was digested with *Hin*dIII and *Sma*I to release the fragment of 1.8-kb *attR3–ccdb–cmr–attR4*. *pMDC99* obtained from Addgene (www.addgene.org) was digested with *Hin*dIII and *Ecl*136II to

release 1.8-kb fragment of *attR1–ccdb–cmr–attR2*. The vector backbone of *pKM43GW* was ligated with 1.8-kb fragment of *attR1–ccdb–cmr–attR2* to generate *pKM12GW*, which carries *nptII* as selection marker. The ligated product was used to transform *E. coli* (DB3.1) and selected on spectinomycin (50 mg l^{-1}).

LR Recombination Reaction to Construct Binary Vector

Three genes, *β*-glucuronidase (*GUS*) with intron, *cry1Aabc* and *cry1Ec* were sub-cloned along with promoter (*CaMV35S*) and terminator sequences into the entry vectors at *Hin*dIII and *Eco*RI restriction sites of *pGATE L1-L4*, *pGATE R4-R3* and *pGATE L3-L2*, respectively. The resulting recombinant entry vectors (*pGATE L*1:35S::*GUS:L4*, *pGATE R4*:35S::c*ry1Aab*c:*R3* and *pGATE* L3:35S::*cry1Ec*:*L2*) were subjected to recombination reaction with modified destination vector *pKM12GW* in the ratio of 1:1:1:1.5(v/v/v/v) in a 15-µl reaction mix containing 2 µl of *LR clonase II plus* enzyme mix (Invitrogen, USA). The reaction was performed at 25°C overnight and the reaction was terminated with *Proteinase K* (1 µl). The recombined product was used to transform *E. coli* (XL1-Blue) and recombinant clones were selected on spectinomycin (50 mg l^{-1}).

Transformation of *Agrobacterium*

Recombinant binary vector (*pKM12GW*) carrying three gene cassettes (*CaMV35S*::*GUS-Tnos*, *CaMV35S*::*cry1Aabc-Tnos* and *CaMV35S*::*cry1Ec-Tnos*) was used for transformation of *Agrobacterium tumefaciens* (strain *EHA105*) by electroporation [22]. For electroporation, the binary vector (200 ng) was mixed with 100 µl (OD_{590} 0.66) competent cells and exposed to electric pulse of 1,440 V cm^{-1}, duration of ~5 ms (Eppendorf, Hamburg, Germany) and pulse was repeated after 15–20 s twice. The recombinant *Agrobacterium* was selected on spectinomycin (50 mg l^{-1}) and rifampicin (10 mg l^{-1}) and positive colonies were screened by PCR using gene-specific primers.

Development of Transgenic Plants

Arabidopsis thaliana (ecotype Columbia) wild-type plants were grown at 24°C under long day conditions (light intensity 250 µmol $m^{-2}s^{-1}$). Healthy plants were transformed with recombinant *Agrobacterium* using floral dip method [23] and treated plants were allowed to set seeds under controlled growth conditions. The T_1 seeds were subjected to stratification at 4°C for 2 days and were germinated on a selective medium containing kanamycin (100 mg l^{-1}) to select putative transformants. The selected lines were transplanted to pots filled with potting mixture for normal growth under controlled conditions.

Expression Analysis

Total RNA was extracted according to the protocol described by Datta et al. [24]. The first strand cDNA was synthesized by oligo (dT) primers using Molony Murine Leukaemia Virus reverse transcriptase (*MMLV-RT*; MBI Fermentas, Hanover, MD, USA). The cDNA pool was used as a template for RT-PCR analysis. The expression analysis of transgenes (*GUS*, *cry1Abc* and *cry1EC*) was carried out under standardized PCR conditions using gene-specific primers (Table 1).

β-Glucuronidase (*GUS*) Assay

The transgenic and wild-type plants were subjected to in situ *GUS* assay [25] using specific staining solution (50 mM sodium phosphate buffer, pH 7.0, 1 mM EDTA, 0.5 mg ml^{-1} 5-bromo-4-chloro-3-indolyl *β*-D glucuronic acid [*X-Gluc*], 0.4% Triton X-100, 100 mg ml^{-1} chloramphenicol and 5 mM each of potassium ferri/ferrocyanide). The reaction was carried out at 37°C for 24 h.

Insect Bioassay

Diamond back moth (*DBM*) is a major pest which feeds on *Arabidopsis* plants. DBM larvae were used for insect bioassay by culturing the first instar larvae under controlled conditions. For insect bioassay, ten larvae were released on 20- to 25-days-old individual plants grown under controlled condition [26]. Damage caused by larvae and larval mortality was monitored for 48 h.

Results

We designed a strategy to convert the three *pDONOR* vectors to ENTRY vectors (designated as *pGATE*) using BP reaction to replace the *ccdb* and *cmr* with unique MCS flanked by *attB* recombination sites. The common restriction endonuclease sites which are generally used in conventional cloning were used in MCS. We selected 18 rare cutting enzyme sites which include 2, 2, 1, and 13 enzymes of 8, 13, 7, and 6 nucleotide cutters, respectively in MCS, for constructing novel *pGATE* vectors. The MCS sequence was synthesised such that the region was flanked by respective *attB* sites compatible with the *attP* sites of *pDONOR* vectors. We utilised six gateway-based specific recombination sites (*att* sites) with 6-bp overlapping region and the adjacent sequences. The *pUC57* vector harbouring

Table 1 Primers used for constructing vector and gene expression studies

S. no	Primer name	Nucleotide length (bases)	5′–3′ Sequence
1	attb1	31	GGGGACAAGTTTGTACAAAAAAGCAGGCTGC
2	attb4	30	GGGGACAACTTTGTATAGAAAAGTTGGGTG
3	attb4r	28	GGGGACAACTTTTCTATACAAAGTTGGC
4	attb3r	27	GGGGACAACTTTATTATACAAAGTTGT
5	attb3	28	GGGGACAACTTTGTATAATAAAGTTGGC
6	attb2	27	GGGGACCACTTTGTACAAGAAAGCTGGGTA
7	M13 forward	16	GTAAAACGACGGCCAG
8	M13 reverse	17	CAGGAAACAGCTATGAC
9	Gus forward	22	CAGCGCCGTCGTCGGTGAACAG
10	Gus reverse	22	CATTGTTTGCCTCCCTGCTGCG
11	cry1Aabc forward	21	AACCCAAACATCAACGAGTGC
12	cry1Aabc reverse	19	TTATGCTGTTCAAGATGTC
13	cry1Ec forward	24	GGAACCAGGCAATTAGCCGTCTCG
14	cry1Ec reverse	23	GGAGCAATGGTACGTCTTGGCTC

MCS region flanked by *attB* sites was digested individually and released fragments were subjected for recombination reaction with *pDONOR* vectors (Fig. 1). Analysis of the recombinant plasmids through restriction endonuclease digestion and also by PCR analysis confirmed the integration of MCS fragment. The identity of recombinant vectors carrying *attL1–MCS–attL4*, *attR4–MCS–attR3* and *attL3–MCS–attL2* was confirmed by sequencing the region of interest. We generated vector maps for modified vectors using Vector NTI software (Invitrogen, USA) and renamed as *pGATEL1-L4*; *pGATER4-R3; pGATEL3-L2* to distinguish them from that of existing gateway cloning vectors (Fig. 1). The sequences of the *pGATE* vectors are given in the Figs. S1, S2, S3 in the Supplementary Material.

Modification of Destination Vector

The *pGATE* vectors created have recombination sites which are specific for reaction with destination vector having *attR1–attR2* recombination sites. We noticed that only *pMDC99*, *pMDC100* (ABRC clones—www.biosci.ohio-state.edu) vectors are compatible for recombination reaction. As these vectors have similar bacterial selection marker as that of *pGATE*, we modified the destination vector *pKM43GW* which has spectinomycin as bacterial selection by cloning the *attR1–attR2* site of *pMDC99* to replace *attR4–attR3*. *attR1–ccdb–cmr–attR2* fragment was released from *pMDC99* and ligated to *pKM43GW* backbone. Transformation of bacteria (*E. coli* DB3.1, as ccdB gene product is toxic to DH5α) with the modified vector yielded sufficient number of colonies and analysis of modified plasmid by restriction endonuclease digestion indicated integration of *attR1–attR2* fragment (Fig. S4 in Supplementary Material). The modified destination vector was designated as *pKM12GW* (Fig. 2, sequence given in Fig. S5 in Supplementary Material).

Development of Multigene Construct

To validate the *pKM12GW* vector we stacked three genes namely, *GUS* with intron, *cry1Aabc* and *cry1Ec* and expressed in *Arabidopsis*. All three genes were cloned into *pGATE* vectors through conventional approach and subsequently subjected for LR reaction with *pKM12GW* (Fig. 3a). Initial PCR amplification and subsequent sequencing of targeted regions showed integration of transgenes in the binary vector. To improve the efficiency of *Agrobacterium* transformation using the vector having multiple genes assembled along with promoter and terminator, we modified the protocol by increasing the number of electric pulses during electroporation and period of incubation (6–8 h) after electroporation. The modification improved efficiency of transformation substantially (Fig. 3b).

Transgene Expression in *Arabidopsis*

The kanamycin-resistant *Arabidopsis* transgenic lines were analysed for the presence of three genes by genomic PCR analysis. The expression levels of multiple transgenes were analysed by RT-PCR assays (Fig. 4). *GUS* histochemical assay in representative plants showed expression in leaves, inflorescence and silique of transgenic lines (Fig. 5a–d). Stable expression of the reporter gene was observed in all parts of the plants in T_3 generation.

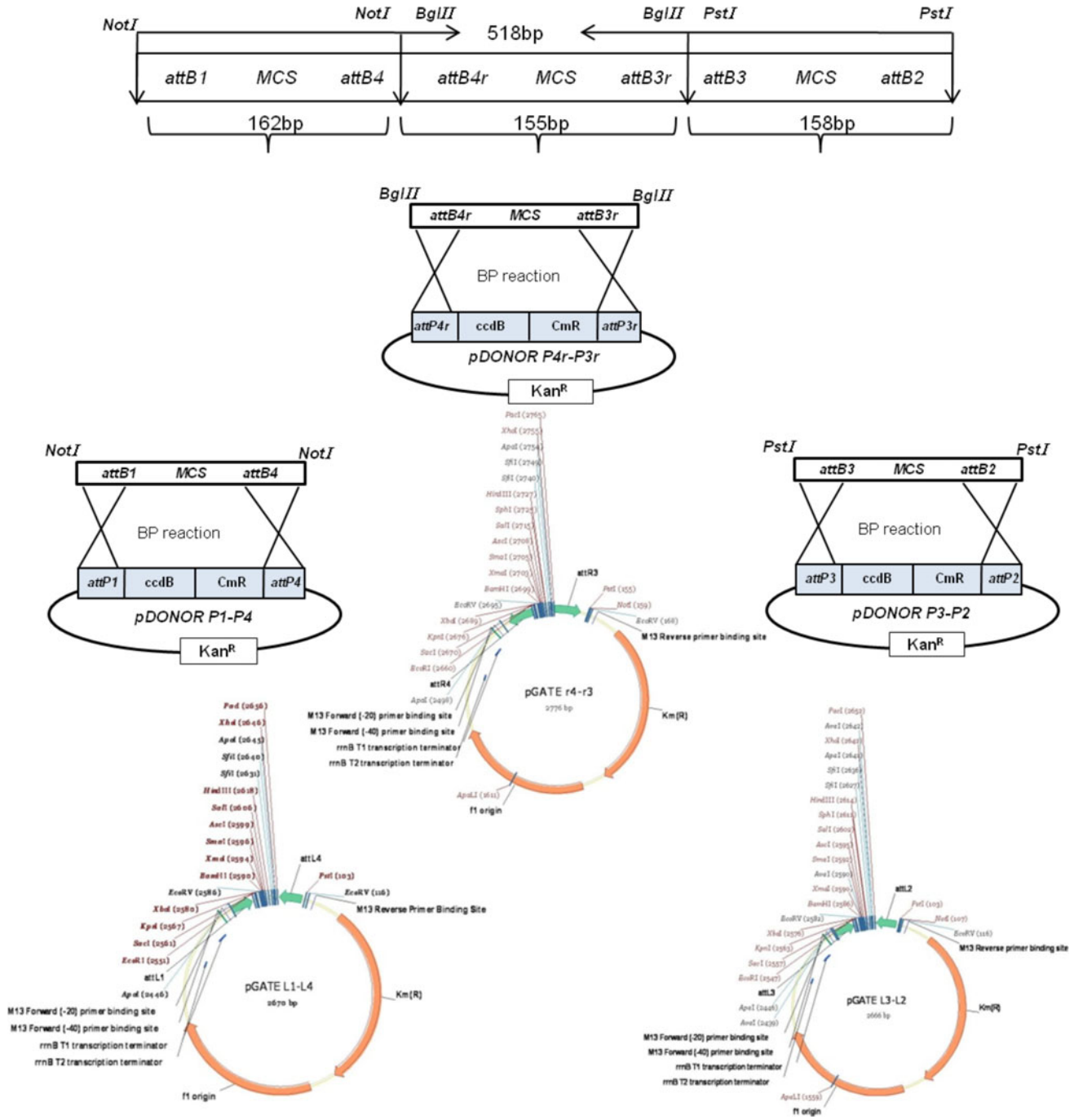

Fig. 1 Strategy followed to construct the three entry vectors. The synthesized fragments were digested using *Not*I, *Bgl*II and *Pst*I restriction sites to release *attB1:MCS:attB4*, *attB4r:MCS:attB3r* and *attB3:MCS:attB2*, respectively, and further subjected to BP clonase reaction with pDONOR221 P1-P4, P4r-P3r and P3-P2, respectively

Insect Bioassay

To examine the expression of the crystal protein in transgenic plants, bioassay was carried out using first instar *DBM* larvae. The damage of leaf and stem caused by the larvae was less in transgenics compared to the damage in wild-type plants (Fig. 6a–c). After 24–48 h, 80–100% mortality was recorded in the larvae fed with transgenic plants whereas larval growth was not inhibited and mortality was not observed in wild-type plants (Fig. 6d).

Discussion

The introduction of multiple transgenes for manipulating traits in plants has been attempted in model system and

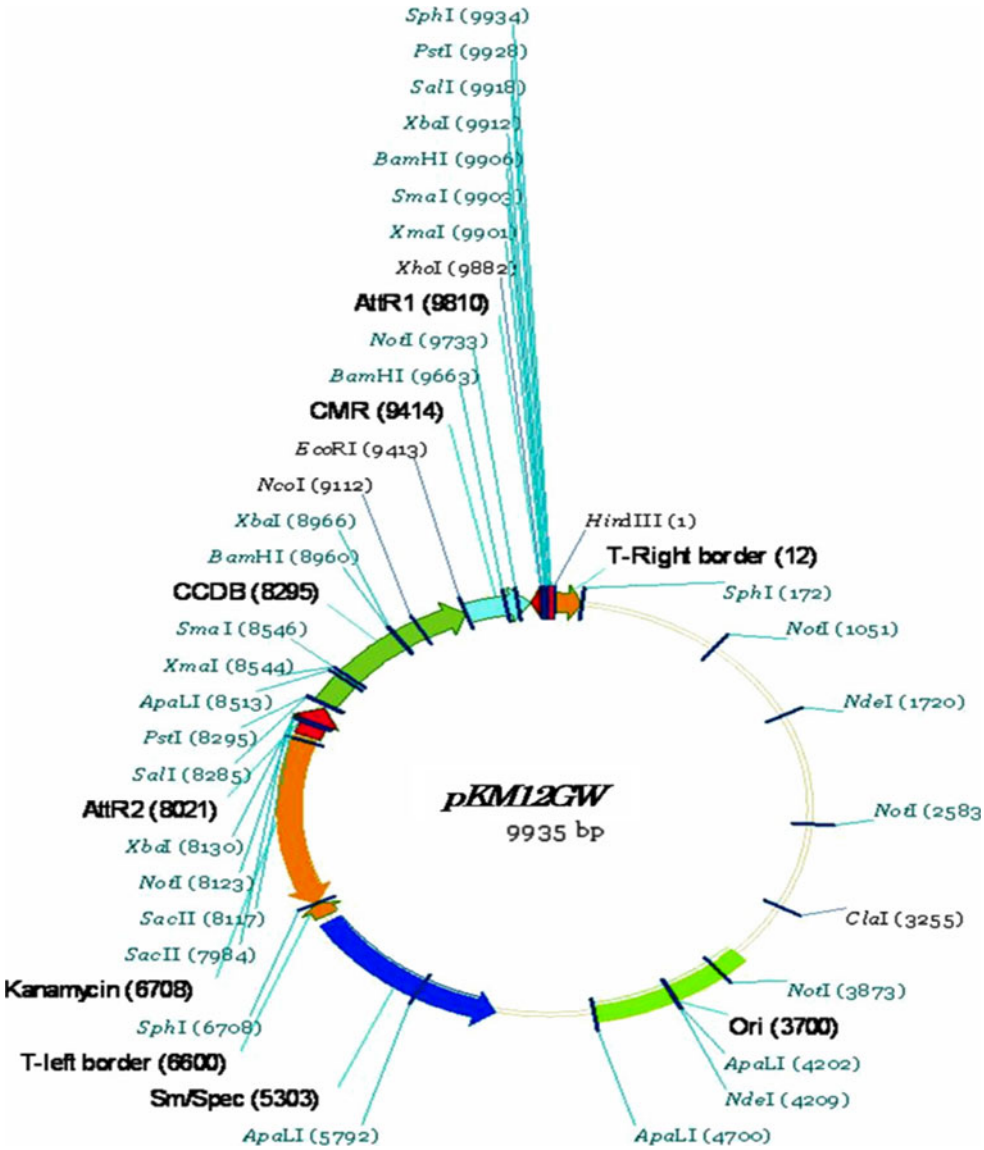

Fig. 2 Construction of pKM12GW. Vector map drawn using VNTI tool (Invitrogen, USA)

crop plants [5]. Most binary vectors and families of plasmids that exist today are limited to the expression of a single gene. Many agronomical traits are polygenic in nature; therefore, use of such plasmids for the coordinated manipulation of multiple traits presents a unique challenge. In the next generation plant biotechnology, research focus will be shifted towards analyzing complex metabolic pathways and exploitation of multigene traits [4]. Several approaches can be considered whilst using single gene vectors for the delivery of multiple genes into plant cells [17, 27]. Simultaneous manipulation of multiple genes might result in significant reduction in cloning efficiencies. Assembly of multigene cassette in a single T-DNA can be one of the ideal approaches for delivery of multigenes into plant cells. However, limitations of type II restriction endonuclease-mediated cloning in many binary-vector systems hinder the ability to assemble several genes as a single transformable unit. Gateway compatible plant transformation vectors available are not designed for stacking of different cassettes in a single expression vector [28–31].

Modified vectors developed in this study allow cloning of whole gene expression cassettes into the entry vectors and assembling multiple DNA segments into *attR1–attR2* plant binary vector. In the vector system developed, there are neither redundant recombination sites nor vector backbones derived from recombination, which avoids the steps used to remove byproducts. The MultiSite destination vector *pKM12GW* (kanamycin as plant selection) can be used for multigene stacking with two different kinds of entry vector systems namely, (i) *pDONOR221* vectors with *pDONORP1-P4*, *pDONOR P4r-P3r*, *pDONOR P3-P2* system and (ii) *pGATE* vectors *pGATE L1-L4*, *pGATE R4-R3* and *pGATE L3-L2* system, which we modified. The

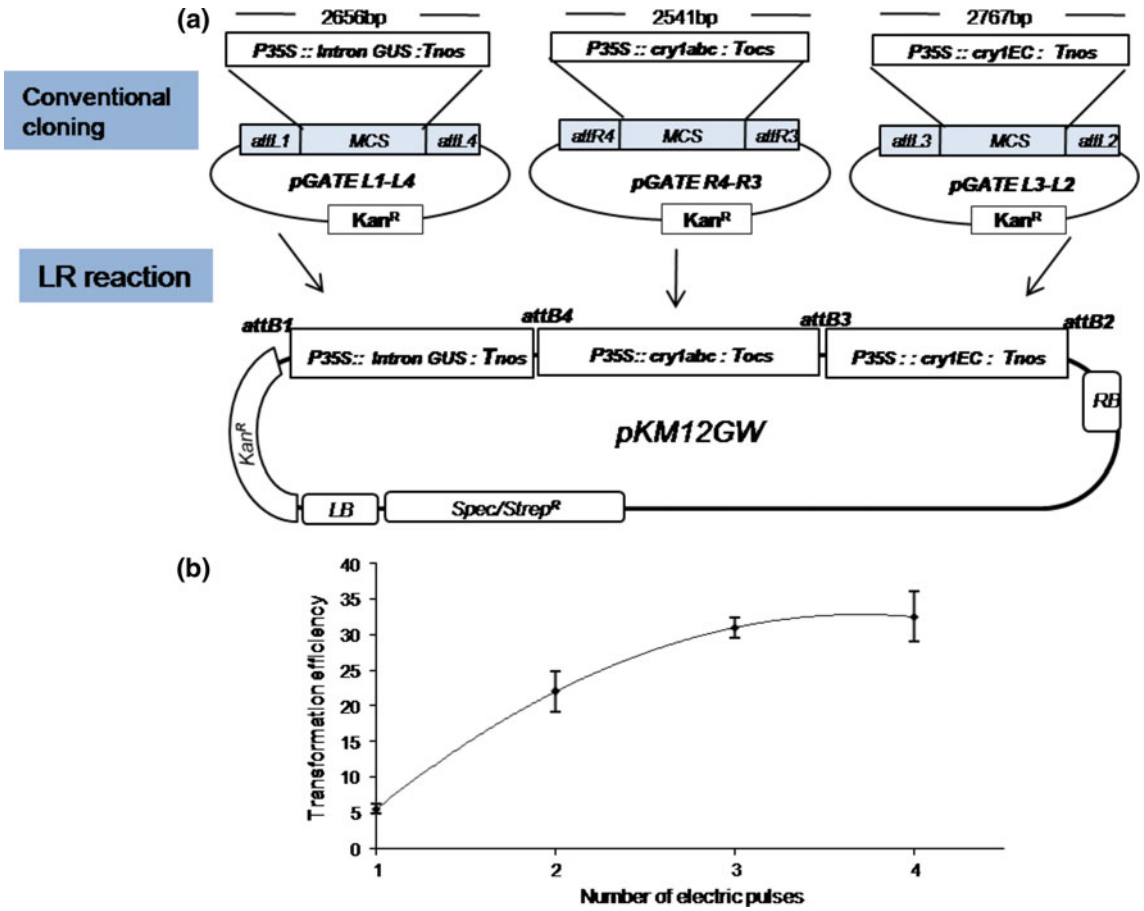

Fig. 3 Overview of multigene expression cassette development using modified vectors. **a** Development of overexpression cassette with *GUS:cry1abc:cry1EC* genes using modified entry and destination vectors, **b** transformation efficiency (calculated as total number of colonies per unit amount of DNA plated) of *Agrobacterium*

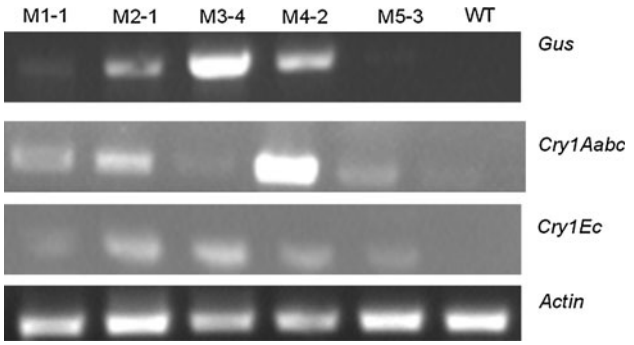

Fig. 4 Expression analysis of transgenes by semi-quantitative RT-PCR. M1-1, M2-1, M3-4, M4-2, M5-3 are different transgenic lines, *WT* wild type

pGATE vector system enables to clone two genes in a single entry vector simultaneously which can be assembled in the gateway destination vector (Fig. 7a). The binary vector having *attR1–attR2* sites when subjected to LR reaction with *pGATE* vectors having *attL* sites yields expression vector with respective *attB* sites (Fig. 7b). The *pGATE* vectors can also be used as any other cloning vectors as there are rare restriction endonuclease enzyme sites, such as SfiI, PacI and AscI which are not commonly present in most cloning vectors. These *pGATE* vectors can serve as basic cloning vectors independently and can also be used for gateway cloning in any organism. As the vectors have been validated for relatively small sized gene cassettes (~11 kb), the capacity of binary vector to hold large gene cassettes (>25 kb) needs to be analysed further.

To validate the modified vectors, we used *GUS* reporter gene along with two *Bt* crystal protein genes *cry1Aabc* and *cry1Ec*. The transgenic *Arabidopsis* showed integration of all the three target genes in the *Arabidopsis* genome. Expression analysis by RT-PCR, *GUS* histochemical assay and insect bioassay indicated efficient expression of the transgenes in *Arabidopsis*. Variation in expression of *cry1Aabc* was noticed between the transgenic lines and there are reports on the similar pattern of transgene expression in different transgenic lines [10, 29, 32]. As *DBM* is one of the pests attacking on *Arabidopsis*, several

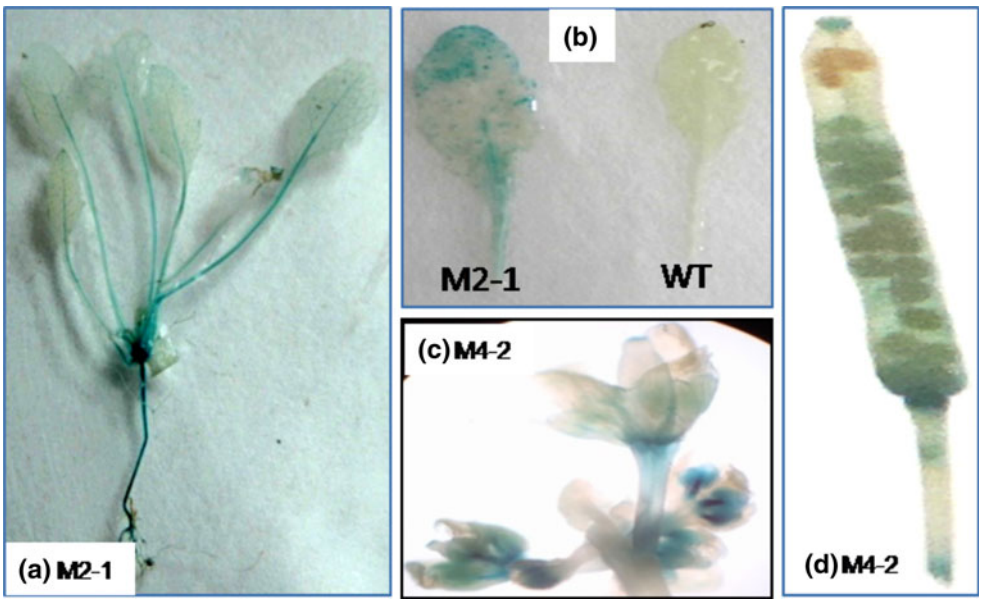

Fig. 5 GUS histochemical assay. Whole plant staining (**a**), 3-week-old transgenic and wild-type leaves (**b**), microscopic view of inflorescence (**c**), siliques (**d**), *M2-1, M4-2* different transgenic lines; *WT* wild type

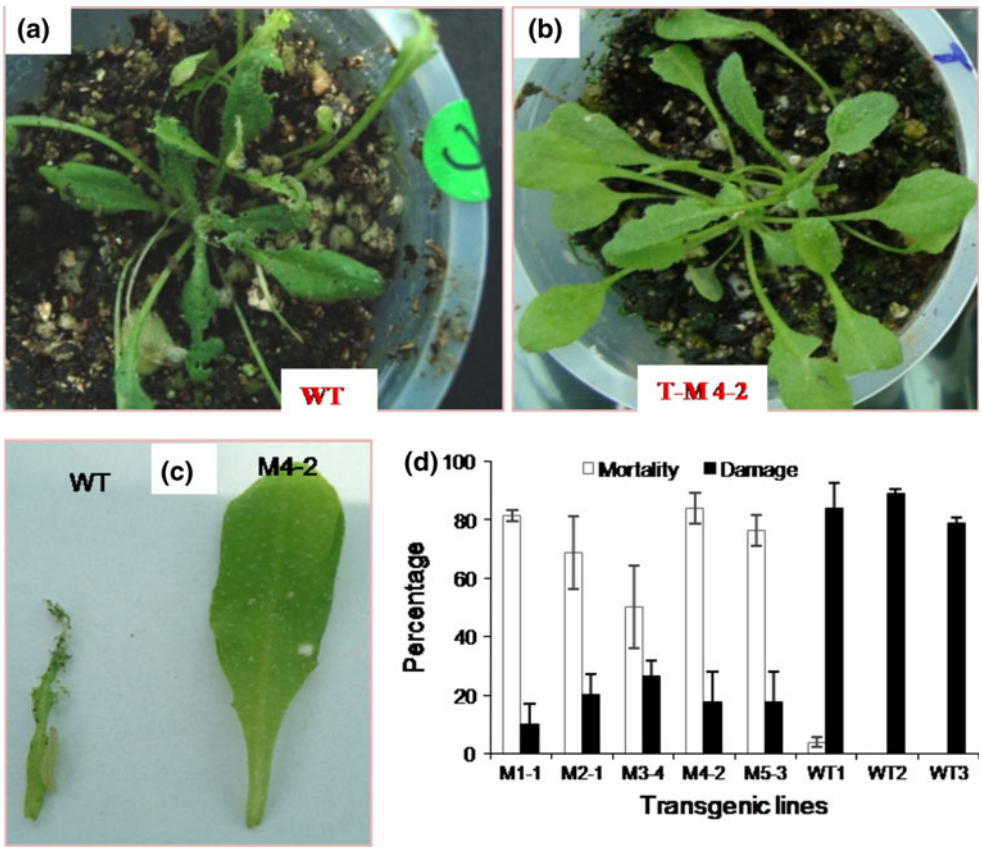

Fig. 6 Insect bioassay using DBM larvae. Representative picture of wild type (**a**) and transgenic plants (**b**) fed by DBM larvae. Photographs were taken 48 h after releasing the larvae. (**c**) indicates the extent of insect damage in wild type (WT) and transgenic line (M4-2), (**d**) represents mortality and damage (in percent) in different transgenic lines (M1-1, M2-1, M3-4, M4-2, M5-3) and wild type plants (WT1, WT2, WT3) caused by DBM larvae

cry genes have been tested for *DBM* insect bioassays in *Arabidopsis* transgenics. In one of the studies, combination of *cry1Ac* and *cry1F* exhibited interactive effect, *cry1Ac* toxin is 100 times more toxic than *cry1F*; the combination of 1:1 showed 26 times higher synergistic effect [33]. The stacking of two *Bt* crystal protein genes attempted in this study might have produced synergistic effect and co-expression of different resistant genes can be attempted by this approach to obtain better resistance against diverse pests. The MultiSite cassette carrying vector system developed is ideal for pathway engineering and stacking genes in plants as demonstrated in this study.

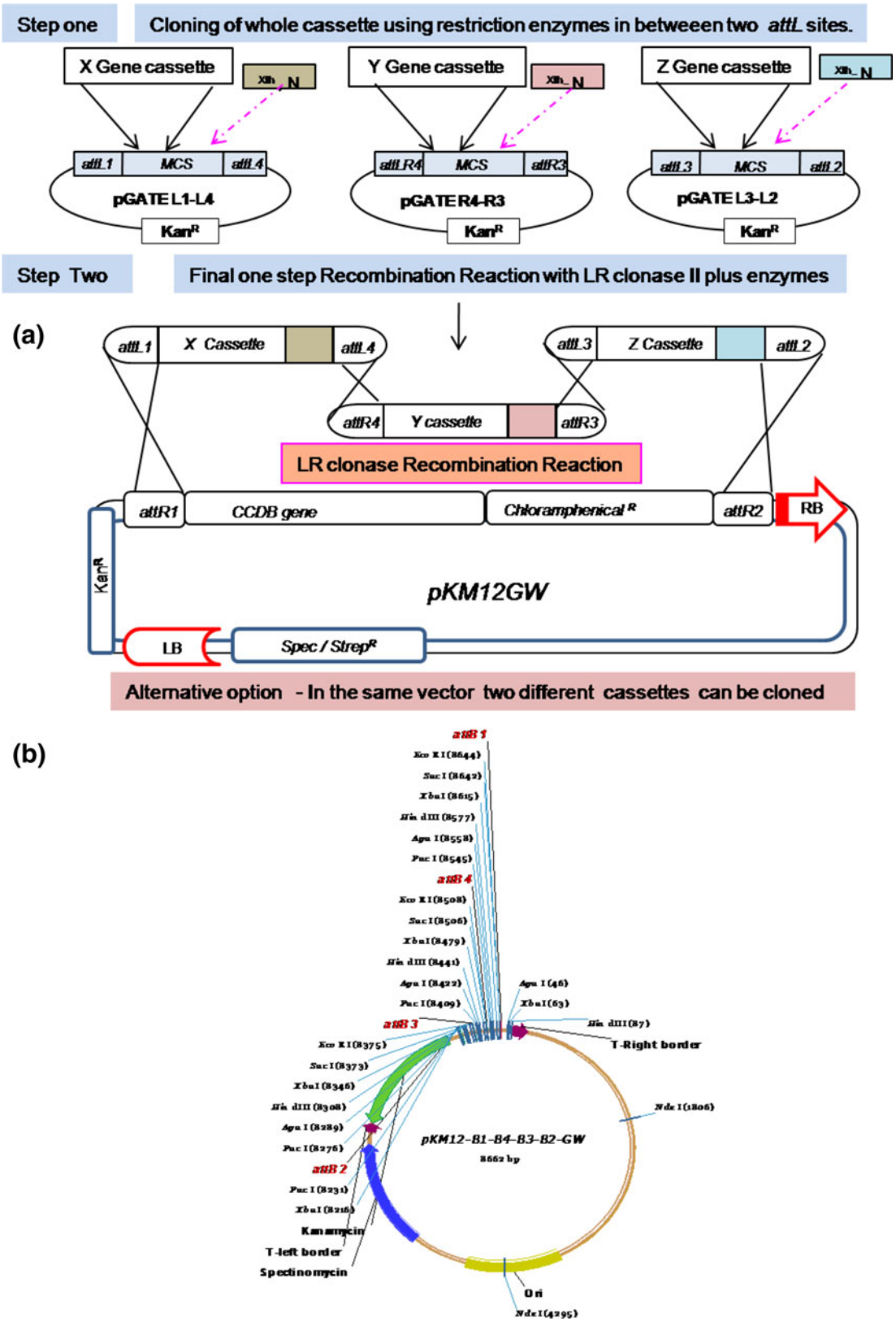

Fig. 7 Overview of gene stacking method described in this study. **a** Sequential events in modified entry vector system. As first step the target gene/genes can be cloned with entire cassette using conventional cloning method to the entry vectors and subsequently in single LR recombination reaction all three genes can be combined together. Alternatively, in the entry vectors two different cassettes can be cloned to co-express more than three genes. **b** Final vector map after LR recombination reaction. The map was drawn using Vector NTI software

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