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ORIGINAL PAPER

Delivery of multiple transgenes to plant cells by an improved version of MultiRound Gateway technology

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Abstract At present, only few methods for the effective assembly of multigene constructs have been described. Here we present an improved version of the MultiRound Gateway technology, which facilitates plant multigene transformation. The system consists of two attL-flanked entry vectors, which contain an attR cassette, and a transformation-competent artificial chromosome based destination vector. By alternate use of the two entry vectors, multiple transgenes can be delivered sequentially into the Gateway-compatible destination vector. Multigene constructs that carried up to seven transgenes corresponding to more than 26 kb were assembled by seven rounds of LR recombination. The constructs were successfully transformed into tobacco plants and were stably inherited for at least two generations. Thus, our system represents a powerful, highly efficient tool for multigene plant transformation and may facilitate genetic engineering of agronomic traits or the assembly of genetic pathways for the production of biofuels, industrial or pharmaceutical compounds in plants.

Keywords MultiRound Gateway · Multigene plant transformation · Gateway vectors · Multigene vectors · Transformation-competent artificial chromosome

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Introduction

To date, the biotechnological improvement of plants has frequently been limited to the introduction or manipulation of single genes. However, most agronomic characteristics are polygenic in nature and rely on complex metabolic and regulatory pathways. Therefore, genetic modifications of traits or introduction of new pathways often require transfer of multiple genes into the plant genome (Lyznik and Dress 2008; Naqvi et al. 2010). Examples are the golden rice (*Oryza sativa*) from Ye et al. (2000) or the red corn (*Zea mays*) from Zhu et al. (2008). Several strategies like cotransformation (Zhu et al. 2008), retransformation (Li et al. 2003) and sexual crossing (Zhao et al. 2003) can be used for the introduction of multiple genes into plant cells. Through repeated transformation or crossing an essentially unlimited number of transgenes can be combined in a single plant. But these techniques are very time-consuming, labor-intensive and demand distinct selection markers for every round of transformation or crossing. Furthermore the multiple integration sites strongly impede the generation of homozygous lines and exacerbate commercial use because multiple integration sites are not compatible with current legal

requirements for the release of transgenic plants (Taverniers et al. 2008). Improvements provide the cotransformation via cobombardment of several plasmids. With this approach numerous genes can be simultaneously transformed using a single selection marker (Francois et al. 2002). Chen et al. (1998) demonstrated the integration of up to 13 different genes and plasmids, respectively, in rice. Surprisingly, cobombardment of several plasmids often results in transgenic plants that integrated all the transgenes at a single genomic locus (Agrawal et al. 2005; Zhu et al. 2008). However, cotransformation often leads to the integration of multiple copies and complex integration patterns (Pawlowski and Somers 1996; Birch 1997), again complicating commercial use. The delivery of the transgenes as a single multigene construct offers considerable benefits. Although multigene constructs were used successfully in a few studies (Zhong et al. 2007; Fujisawa et al. 2009), the assembly of such constructs remains challenging. With the conventional cloning methods it's nearly impossible (Dafny-Yelin and Tzfira 2007; Naqvi et al. 2010), because of the lack of unique restriction sites when multiple genes need to be linked.

The Gateway technology is based on phage lambda site-specific recombination (Hartley et al. 2000). It relies on the action of recombinases that are capable of sequence-specific recombining compatible DNA sequences. For example the LR clonase can recombine attL with attR sites in an irreversible way and is usually used for the delivery of DNA fragments from an entry clone into a destination vector (Walhout et al. 2000). Several Gateway-compatible plant transformation vectors were designed for diverse purposes like the functional analysis of genes *in planta* or protein localization studies (Curtis and Grossniklaus 2003; Earley et al. 2006). Originally the Gateway technology was not laid out for stacking multiple genes in a single vector. However, the introduction of Multisite Gateway vectors opened an alternative way for recombining several DNA fragments in a single recombination step (Cheo et al. 2004; Sasaki et al. 2004; Karimi et al. 2005; Magnani et al. 2006). However, this technique is limited by the availability of more attachment sites. Chen et al. (2006) presented another method, which is called MultiRound Gateway technology, to stack multiple DNA fragments into a single vector. The system is based on two different entry vectors which can be alternately used to deliver sequentially multiple DNA fragments into a Gateway-compatible destination vector.

Here we describe an improved version of the MultiRound Gateway technology. By the use of a transformation-competent artificial chromosome (TAC)-based destination vector and a recombination deficient strain of *Agrobacterium*, we could increase the stability of the multigene construct as well as the efficiency of the transformation process. The constructed Gateway-compatible TAC vector possesses a cloning capacity greater than 100 kb (Liu et al. 1999; Lin et al. 2003). Therefore the vector can accept numerous DNA fragments from the entry vectors. With our system we could introduce up to eight genes located on a single destination vector including a selection marker, five enzymes, two translocators, two Scaffold attachment regions (SAR) and three putative transcription blockers (Padidam and Cao 2001) into tobacco plants. By real-time PCR we could confirm the stable expression of all transgenes for at least two generations. The expression of four of the five enzymes was also verified by enzyme activity assays.

Materials and methods

Entry vector construction

The recombination sites (attL and attR), homing endonuclease sites (I-*Sce*I and PI-*Sce*I), *Pme*I and *Not*I restriction sites and Multiple cloning sites (MCSI and MCSII) were synthesized in four pBS SK(+) plasmids: pBS SK(+)_G325A (*Pme*I-I-*Sce*I-attL1-MCSI-attR3-PI-*Sce*I-*Not*I), pBS SK(+)_G325B (*Pme* I-I-*Sce*I-attL2-MCSII-attR4-PI-*Sce*I-*Not*I), pBS SK(+)_G324A (*Pme*I-I-*Sce*I-attL3-MCSI-attR1-PI-*Sce*I-*Not*I), pBS SK(+)_G324B (*Pme*I-I-*Sce*I-attL4-MCSII-attR2-PI-*Sce*I-*Not*I) (Euro-fins Medigenomix, Ebersberg). The entry vector pEntry1_SpecR (Fig. 1a) was obtained by the following cloning steps. First, the spectinomycine resistance gene *aadA* was amplified using pWBVec8 (Wang et al. 1998) as template and primers 5′-CC tct aga CCA AGA GCT TGT CGG GAA GAT TGA AC-3′ and 5′-GA gga tcc ATG CCA TCG CAA GTA CGA GGC TTA GAA C-3′, digested with *Xba*I and *Bam*HI and cloned into vector pBS SK(+)_G325A to make pBS SK(+)_G325A_SpecR. Second, the fragment *Pme*I-I-*Sce*I-attL2-MCSII-

attR4-PI-*Sce*I-*Not*I from vector pBS SK(+)_G325B was cloned into vector pBS SK(+)_G325A_SpecR by using *Bam*HI and *Xho*I to make pBS_G325_SpecR. Thirdly, the pUC origin and the *bla* gene was amplified using an empty pBS SK(+) vector as template and primers 5′-G TAC caa ttg CGA GCG GTA TCA GCT CAC TCA AAG G-3′ and 5′-C TGA ctg cag CGG GGA AAT GTG CGC GGA AC-3′ and cloned into vector pBS_G325_SpecR by using *Mun*I and *Pst*I to remove undesired restriction sites in the lacZ-fragment of the pBS SK(+) vector. The entry vector pEntry2_CmR (Fig. 1b) was obtained by the following cloning steps. First, the chloramphenicol resistance gene *cat* was amplified using pEG301 (Earley et al. 2006) as template and primers 5′-CC tct aga TTA GGC ACC CCA GGC TTT ACA C-3′ and 5′-GG tct aga TCA ATA AAC CGG GCG ACC TCA G-3′, digested with *Xba*I and cloned into vector pBS SK(+)_G324A to make pBS SK(+)_G324A_CmR. Second, the fragment *Pme*I-I-*Sce*I-attL4-MCSII-attR2-PI-*Sce*I-*Not*I was released from vector pBS SK(+)_G324B using *Bam*HI and *Xho*I and cloned after blunting with T4 DNA polymerase into the EcoRV restriction site of vector pBS SK(+)_G324A_CmR to make pBS SK(+)_G324_CmR. Thirdly, the backbone of the plasmid was changed accordingly to pEntry1_SpecR. The entry vectors pEntry1_SpecR_iMCS and pEntry2_CmR_iMCS were obtained by exchanging the MCSI site for an inverted version.

The entry vectors with target genes pEntry1_PPT, pEntry2_StPEPC_TB1, pEntry1_Oac1, pEntry2_PPDK_TB2, pEntry1_SbMDH, pEntry2_HvME_TB3, pEntry1_FbCA, pEntry2_PEPS_TB2, pEntry1_EcMDH, pEntry2_EcME_TB3, pEntry2_PCK_TB2 were obtained by cloning the respective expression cassettes into the MCSI or iMCSI of pEntry1_SpecR, pEntry2_CmR, pEntry1_SpecR_iMCS and pEntry2_CmR_iMCS. For details of the cloning processes and maps of the final constructs see supplementary material (Table 3, Fig. 5). A table describing the genes to be transformed and their expected functions can also be found in supplementary material (Table 4).

Destination vector construction

For destination vector pTRA_R12_CmR_ccdB (Fig. 2a) the gateway cassette attR1-CmR-ccdB-attR2 was amplified using pEG301 (Earley et al. 2006) as template and primers 5′-CA ggc gcg ccA CGT CTT GCG CAC TGA TTT G-3′ and 5′-GA ggc cgg ccA TCG TAT GGG TAC ACC ACT TTG-3′, digested with *Asc*I and *Fse*I and cloned into vector pTRA, a derivative of pPAM (gi13508478). For destination vector pYLTAC7_R12_CmR_ccdB (Fig. 2b) the gateway cassette attR1-CmR-ccdB-attR2 including the SARs was removed from pTRA_R12_CmR_ccdB using S*ap*I and P*me*I and cloned after blunting of the S*ap*I end with T4 DNA polymerase into the P*me*I site of vector pYLTAC7 (Liu et al. 1999).

LR recombinations

The LR recombinations were performed according to the manufacturer's instructions (Invitrogen, Carlsbad, USA) with equimolar amounts of entry and destination vectors. As negative control the LR recombinase was omitted. Aliquots of 2 µl of each reaction mixture were transformed into *Escherichia coli* TOP10 using electroporation. Using pTRA_R12_CmR_ccdB as destination vector, recombinants were selected on LB agar plates supplemented with 50 µg ml^{-1} ampicillin and 50 µg ml^{-1} spectinomycin after recombination with pEntry1_SpecR entry vectors and 34 µg ml^{-1} chloramphenicol after recombination with pEntry2_CmR entry vectors. Using pYLTAC7_R12_ CmR_ccdB as destination vector, recombinants were selected on LB agar plates supplemented with 50 µg ml^{-1} kanamycin and 15 µg ml^{-1} spectinomycin and 20 µg ml^{-1} chloramphenicol, respectively. Plasmids were prepared from 5 ml LB overnight cultures, supplemented with the appropriate antibiotics (same concentrations as before) according to Birnboim and Doly (1979). In case of using pYLTAC7_R12_CmR_ccdB as destination vector also 0.16 mM IPTG was added.

Plant transformation

Nicotiana tabacum cv. Petit Havana plants were grown in pots at 25 °C in a growth chamber under 16 h light:8 h dark cycle. All multigene constructs were transformed into *Agrobacterium* strain AGL1 (Lazo et al. 1991; kindly provided by Paul Hooykaas, Molecular and Developmental Genetics Department, Institute of Biology Leiden, Leiden University Leiden, Netherlands) and screened on LB plates containing

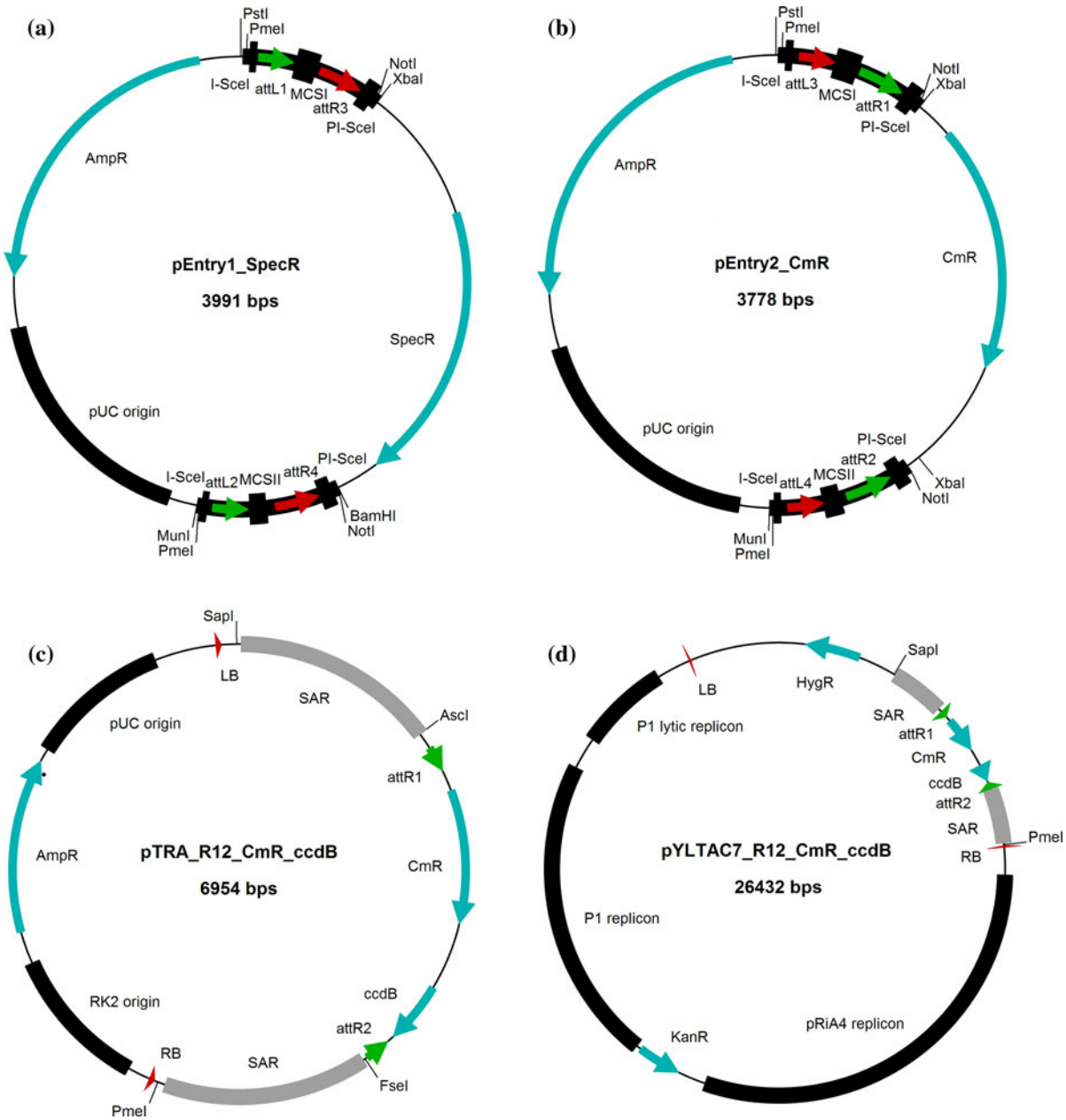


Fig. 1 Physical maps of the two entry vectors pEntry1_SpecR (**a**) and pEntry2_CmR (**b**) and the two destination vectors pTRA_R12_CmR_ccdB (**c**) and pYLTAC7_R12_CmR_ccdB (**d**), respectively. Important regions and restriction enzymes used in previous construction steps are indicated

15 μg ml^{-1} kanamycin and 50 μg ml^{-1} carbenicillin. For verification by PCR the following primers were used (Table 1): PPT: 3472 and 865; StPEPC: 4255 and 4225; Oac1: 3971 and 4100; PPDK: 4048 and 4052; SbMDH: 4281 and 4282; HvME: 3545 and 3546; FbCA: 4309 and 4310; PEPS: 3874 and 4284; EcMDH: 3978 and 3979; EcME: 4256 and 4223; PCK: 4233 and 4234.

Stable transformation of tobacco plants was done according to De Block (1988) and Dietze et al. (1995). Putative T0 transgenic plants were regenerated from the callus in the presence of 50 μg ml^{-1} hygromycin.

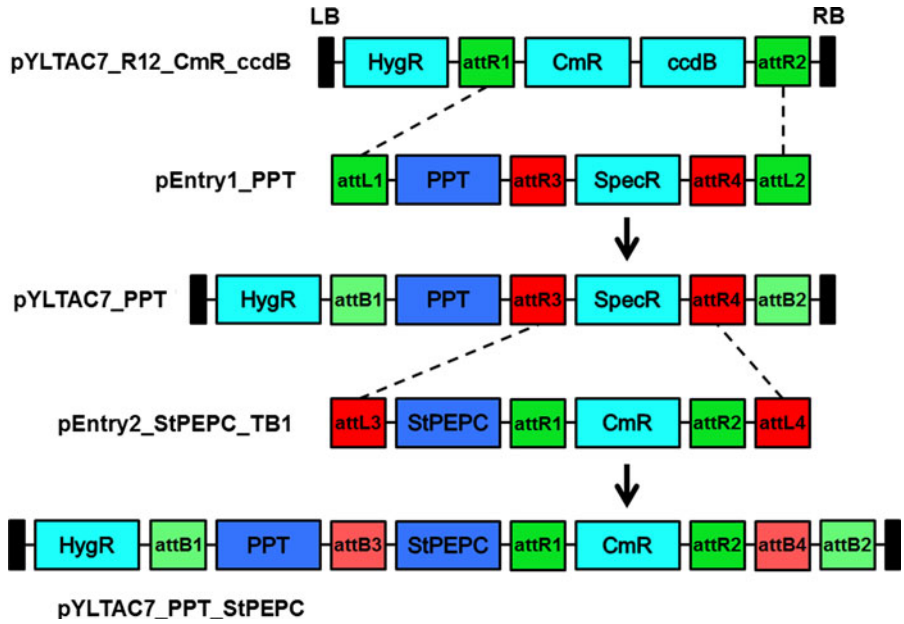


Fig. 2 Schematic diagram of the first two rounds of LR recombination. The first round of LR recombination between attL1 and attL2 of the entry vector pEntry1_PPT and attR1 and attR2 of the destination vector pYLTAC7_R12_CmR_ccdB exchange the chloramphenicol resistance (CmR) for the spectinomycin resistance (SpecR) and integrate the PPT expression cassette and R3 and R4 in the destination vector. The second round of LR recombination between attL3 and attL4 of the entry vector pEntry2_StPEPC_TB1 and the new destination vector pYLTAC7_PPT reexchange SpecR for CmR and integrate the StPEPC expression cassette and attR1 and attR2 in pYLTAC7_PPT to make pYLTAC7_PPT_StPEPC. By alternating use of the two entry vectors further genes can be integrated. *PPT* phosphoenolpyruvate/phosphate translocator, *StPEPC* modified phosphoenolpyruvate carboxylase from *Solanum tuberosum*

Real-time-PCR

Total RNA was prepared from 2 weeks old soil-grown plants by using a combined RNA-DNA extraction assay. Approximately 0.02 g leaf material were ground in liquid nitrogen and extracted with extraction buffer (0.05 M Tris–HCl, pH 7.6, 0.5 % SDS) and water saturated phenol. Reverse transcription was performed by using moloney murine leukemia virus reverse transcriptase (MMLV-RT, Promega, Madison, USA) with a random nonamer primer. Gene specific primers for quantitative RT-PCR were: Actin2: 584 and 585; PPT: 4506 and 4507; StPEPC: 4546 and 4547; Oac1: 4510 and 4511; PPDK: 4512 and 4513; SbMDH: 4514 and 4515; HvME: 4516 and 4517; PEPS: 4520 and 4521; EcMDH: 4522 and 4523; EcME: 4524 and 4525; PCK: 4526 and 4527; FbCA: 4571 and 4572 (sequences see Table 1). Actin2 was used as an internal standard (Igarashi et al. 2003). PCR amplification was done using the Platinum®SYBR® Green qPCR SuperMix-UDG with Rox (Invitrogen, Carlsbad, USA) according to the manufacturer's instructions with 40 cycles of 94 °C for 15 s and 60 °C for 60 s.

Extraction of leaf soluble protein and enzyme assays

Leaf discs of about 60 mg were harvested from the mid-section of the third leaf at noontime and immediately stored in ice cold extraction buffer. For determination of phosphoenolcarboxylase (PEPC), malic enzyme (ME) and malate dehydrogenase (MDH) activity, extraction buffer I (50 mM HEPES–NaOH, pH 8.0, 10 mM $MgCl_2$, 5 mM DTT, 1 mM EDTA, 20 % glycerin, 0.01 mg ml^{-1} chymostatin, 0,5 mM PefablocSC was used. Samples were ground in 250 µl extraction buffer I. After centrifugation (14,000*g*, 5 min, 4 °C) the supernatant was transferred into a new tube and stored on ice until used. For determination of pyruvate orthophosphate dikinase and phosphoenolpyruvate synthetase activity, extraction buffer IIA (50 mM Hepes–KOH, pH 8.0, 10 mM $MgSO_4$, 1 mM EDTA, 2.5 mM K_2HPO_4, 20 % Glycerin) and

Table 1 Primers used for PCR verification of transformed Agrobacteria and RT-PCR analysis of transgenic plants

Primer names	Sequences (5′→3′)	Product (bp)
3472_PPT_F	ACATGAGCCTCGGGAAAGTCT	1,019
865_pA35S_R	GCTCAACACATGAGCGAAACC	
4255_StPEPC_ F	GTCTGAACTCAGTGGCAAGAGAC	145
4225_StPEPC_ R	CCATGTGGCGCGCCTAACCGGTGTTCTGCATTCCAG	
3971_Oac1_R	CCATCAGTCTAGATTAATTATGGCCTAAAACTCTCGAC	1,000
4100_Oac1_ F	CCTGGAACTAGTATGTCATCTGACAACTCTAAACAAG	
4048_PPDK_F	GGACCAGAAGACCTCATGATGAGTTCGTTGTCTGTTGAAG	778
4052_PPDK_R	CAGCAGTGAGATCGGTGTCGAGATG	
4281_SbMDH_ F	GCTGATCCATGGGCCTCTCAACGGCTTAC	1,315
4282_SbMDH_ R	CCAGTTGGATCCCTAAACTACACTTCTCCCGGTAGC	
3545_HvME _F	GCTTGTTCGGCAGTGCATTC	1,192
3546_HvME _R	CAAAGGACTCGCCTTCACAG	
4309_FbCA_ F	GGAGTACCATGGGAAGTAAATCATATGATGAG	799
4310_FbCA_ R	CCTGATGGCGCGCCTTATGCCCGGGTAGTAGGTG	
3874_PEPS _F	GAAACCGTGCGCTCACGCGGTCAGGTCATGGAG	1,385
4284_PEPS_R	GGTCCAGGTCTCACGCGTTATTTCTTCAGTTCAGCCAGGCTTAACC	
3978_EcMDH_F	GCTGACACGCGTTAGGTGCATGAAAGTCGCAGTCCTC	970
3979_EcMDH_R	CCATCGTCTAGATTACTTATTAACGAACTCTTCGCCCAG	
4256_EcME _F	GATTGTGGTGACTGACGGTGAAC	1,264
4223_EcME _R	GCTCTGTCTAGATTAGATGGAGGTACGGCGGTAGTC	
4233_PCK_ F	CCTGGTGGTCTCACGCGTTAGGTGCATGGCATC	1,794
4234_PCK_R	GGTCCAGGTCTCACGCGTCAGAAGTTGGGGCCTGCGGCAAG	
584_Actin2_RT_F	GGTAACATTGTGCTCAGTGGTGG	113
585_Actin2_RT_R	GGTGCAACGACCTTAATCTTCAT	
4506_PPT_RT_R	GACCACCACACGCTTCACAC	101
4507_PPT_RT_F	CACTCTGCTTCCACGCATACC	
4546_StPEPC_RT_F	CCAGGCATTGCTGCATTGTTC	100
4547_StPEPC_RT_R	GGAGGCTCTTTGTCTCCTCATAC	
4510_Oac1_RT_R	AGCGGTTACACCTTCGATTC	128
4511_Oac1_RT_F	TAGGTGTTGCCGTCGTTATG	
4512_PPDK_RT_F	GTCGTGCAGCAAATCCTAGC	134
4513_PPDK_RT_R	CCTTGCGATAGGAACCCTAAATGG	
4514_SbMDH_RT_R	TGGGCAACGCATTTCTTCTCAG	111
4515_SbMDH_RT_F	GGGTGATGGTGATTACGAACTAGC	
4516_HvME_RT_F	CGGTCTTGGCCTTGTCATGTC	109
4517_HvME_RT_R	GCCCTTCTCGAAGTTCTCCTC	
4571_FbCA_RT_F	GACCCAGTCCTCAATGAAGTC	118
4572_FbCA_RT_R	GCATCTCAAGGTGGAGCAGATAG	
4520_PEPS_RT_F	ACTGCTGTCGATGGCTATCC	103
4521_PEPS_RT_R	CATCAACCATGCGGCAAAGTC	
4522_EcMDH_RT_R	GCCTTCAACGTAGGCACATTC	106
4523_EcMDH_RT_F	GTCTGCAACCCTGTCTATGG	
4524_EcME_RT_F	GCGTCACGTATCACCGATGAG	102
4525_EcME_RT_R	CAGTTCCGGCAGTACCATACC	

Table 1 continued

Primer names	Sequences (5′→3′)	Product (bp)
4526_PCK_RT_F	GATCATCGACGCCATCCACTC	140
4527_PCK_RT_R	CCTTGTCCGTCCAGGTGTTG	

IIB (Buffer IIA including 5 mM DTT, 5 mM pyruvate, 1 mM PefablocSC, 2 % PolyclarAT, 0.005 mg ml^{-1} chymostatin) was used. Samples were ground in 250 µl extraction buffer IIB and mixed with 950 µl extraction buffer IIA. After centrifugation (14,000*g*, 15 min, 4 °C) 800 µl of supernatant were transferred into a new tube. Protein was precipitated with 0.312 g ammonium sulfate and centrifugation (14,000*g*, 10 min, 4 °C). The pellet was resuspended in 100 µl extraction buffer IIA and stored on ice until use.

Enzyme activities were determined spectrophotometrically at 30 °C. The PEPC activity was assayed by the method of Rademacher et al. (2002). The NADP-ME activity was determined according to Hausler et al. (1987), the NAD-ME activity according to Bologna et al. (2007). The NADP-MDH activity was assayed by the method of Ashton and Hatch (1983), the NAD-MDH activity by the method of Sutherland and McAlister-Henn (1985). The PPDK and PEPS activity were determined according to Ashton et al. (1990). For PEPS measurement K_2HPO_4 was omitted.

Results

Construction of entry and destination vectors

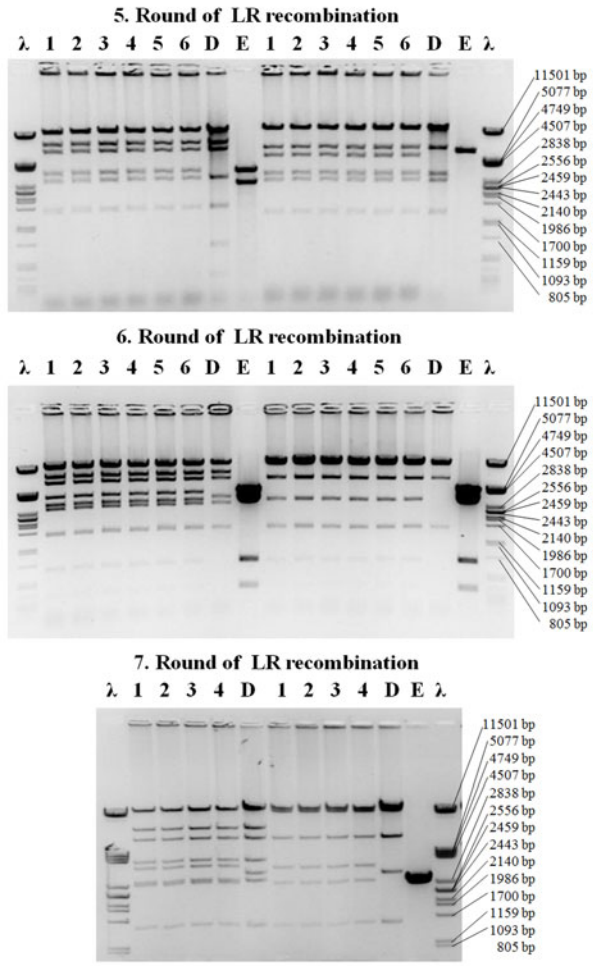
Four different entry vectors were designed: pEntry1_SpecR, pEntry2_CmR, pEntry1_SpecR_iMCS and pEntry2_CmR_iMCS. The sequences of the recombination sites attL1/2/3/4 and attR1/2/3/4, respectively, have been chosen according to Chen et al. (2006). A multiple cloning site (MCSI) between attL1 and attR3 serves as integration site for DNA fragments to stack into the destination vector, because integration by Gateway BP recombination is not possible. A second multiple cloning site (MCSII) allows integration of a further DNA fragment, which can be recombined simultaneously. Two I-*Sce*I and *Pme*I sites, respectively, flanking the attL sites allow linearization of entry vectors and removal of the vector backbones. This will eliminate cotransformation of the entry vectors whereby cloning efficiency would be reduced. Two PI-*Sce*I and *Not*I sites, respectively, flanking the selection marker allows linearization of destination vectors. It is assumed that this will increase recombination efficiency (Chen et al. 2006). Use of the homing endonuclease sites has the advantage, that these sites are extremely rare in natural sequences, whereas use of *Pme*I and *Not*I for linearization of the vectors will reduce costs.

As destination vectors two different plasmids were constructed. pTRA_R12_ CmR_ccdB is based on a pTRA vector, a derivative of pPAM (gi13508478), and contains a high copy origin (ColE1) for replication in *E. coli* and a broad host low copy origin (RK2) for replication in *Agrobacterium*. pYLTAC7_R12_CmR_ccdB is based on the TAC vector pYLTAC7 (Liu et al. 1999) and contains the single copy origin from the P1 phage for replication in *E. coli* and the single copy origin from *Agrobacterium rhizogenes* for replication in *Agrobacterium*. Additionally it contains the P1 lytic replicon under the control of the lac operon, which allows induction of higher copy numbers with IPTG. Both vectors possess the cis elements LB and RB for *Agrobacterium* mediated transfer into plant cells and two flanking SARs to reduce position effects (Grosveld et al. 1987; Stief et al. 1989; Bonifer et al. 1990). The gateway cassette consists of attR1 and attR2 recombination sites and a positive (chloramphenicol resistance, CmR) and a negative (*ccdB*) selection marker. CmR allows selection of the plasmid in *E. coli* and *Agrobacterium*, *ccdB* allows counter selection of non-recombined plasmids in *E. coli*.

Use of TAC vector allows better integration of transgenes

For MultiRound Gateway recombination the destination vector pTRA_R12_CmR_ccdB or pYLTAC7_R12_CmR_ccdB and the entry vectors with target genes were used. In experiments with the high copy vector pTRA_R12_CmR_ccdB only in the first

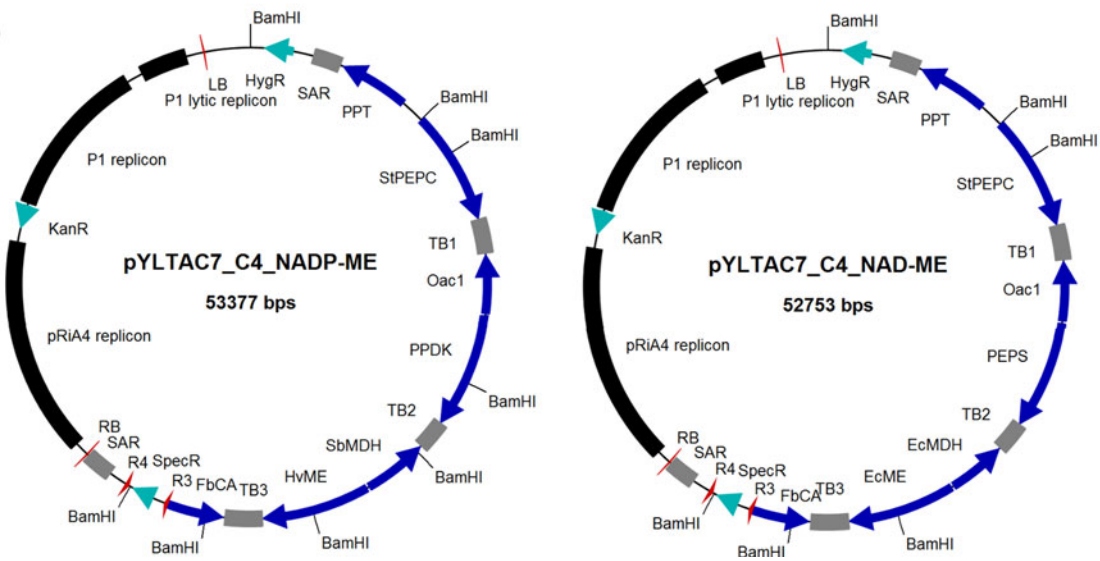
(a) pYLTAC7_C4_NADP-ME pYLTAC7_C4_NAD-ME



(b)

pYLTAC7_C4_HvMe recD	D	E	pYLTAC7_C4_EcMe recD	D	E
5. round of LR recombination					
digestion with *Bam*HI			digestion with *Acc*65I		
45742	43030	6305	45184	42719	6058
22011	21936	3663	25383	28309	6058
8621	8621	2642	6962	6962	
6821	6811		5391	3277	
6811	3058		3277		
3663	1597		2789		
3039	703		1382		
1597	304				
6. round of LR recombination					
digestion with *Bam*HI			digestion with *Bam*HI		
50970	45742	9031	50554	45184	8965
22119	22011	4578	22223	22011	4601
8621	8621	3446	15559	14765	3357
6811	6811	703	6811	6811	703
4320	3663	304	3357	1597	304
3446	3039		1597		
3039	1597		703		
1597			304		
703					
304					
7. round of LR recombination					
digestion with *Bam*HI			digestion with *Bam*HI		
53377	50970	6000	52857	50450	6000
22194	22119	3083	22194	22119	3083
8621	8621	2917	15559	15559	2917
6811	6811		6822	6811	
4330	4330		3779	3357	
3868	3446		2917	1597	
3039	3039		1597	703	
2917	1597			304	
1597	703				
	304				

(c)



◀ **Fig. 3** Restriction analysis of pYLTAC7_C4_NADP-ME and pYLTAC7_C4_NAD-ME. After the fifth, sixth and seventh round of LR recombination the respective entry vectors (E), destination vectors (D) and derived constructs (1–6 and 1–4, respectively) were digested with *Bam*HI or *Acc*65I and separated on 0.6 % agarose gels (**a**). As DNA size marker lambda DNA digested with *Pst*I was used (λ). The enzymes used and the resulting fragment sizes for the respective entry vectors, destination vectors and derived recombined destination vectors (recD) are indicated in the table aside (**b**). The physical maps of pYLTAC7_C4_NADP-ME and pYLTAC7_C4_NAD-ME after the last round of LR recombination are showed below (**c**). The different gene expression cassettes are indicated as *blue arrows*. Used Abbreviations are the same as before. (Color figure online)

two rounds of LR recombination, correct clones were obtained. For unknown reasons several attempts did not lead to correct recombinants in subsequent rounds (data not shown). In contrast, with pYLTAC7_R12_CmR_ccdB_SAR even in the seventh round of recombination nearly all clones were correct. Sporadically occurring false clones could be explained by cotransformation of entry and destination vectors. However, by previous linearization of the entry vectors, this number could be kept very low (<5 %), because linear DNA is poorly transformed in *E. coli* (Cohen et al. 1972; Conley and Saunders 1984; Conley et al. 1986).

With pYLTAC7_R12_CmR_ccdB as destination vector three different multigene constructs were successfully assembled: pYLTAC7_C4_NADP-ME (a) pYLTAC7_C4_NAD-ME (b) and pYLTAC7_C4_PCK (c). The corresponding entry vectors were recombined in the stated order:

a) pYLTAC7_C4_NADP-ME
   1) pEntry1_ PPT
   2) pEntry2_ StPEPC_TB1
   3) pEntry1_ Oac1
   4) pEntry2_ PPDK_TB2
   5) pEntry1_ SbMDH
   6) pEntry2_ HvME_TB3
   7) pEntry1_ FbCA

b) pYLTAC7_C4_NAD-ME
   1) pEntry1_PPT
   2) pEntry2_StPEPC_TB1
   3) pEntry1_Oac1
   4) pEntry2_PEPS_TB2
   5) pEntry1_EcMDH
   6) pEntry2_EcME_TB3
   7) pEntry1_FbCA

c) pYLTAC7_C4_PCK
   1) pEntry1_PPT
   2) pEntry2_StPEPC_TB1
   3) pEntry1_Oac1
   4) pEntry2_PCK_TB2
   5) pEntry1_FbCA

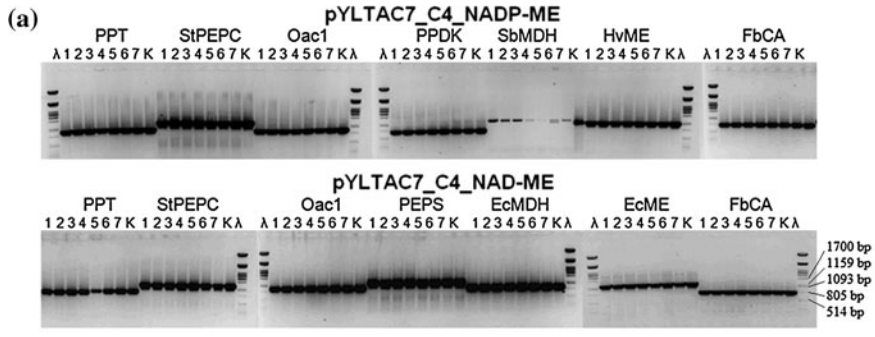
Figure 2 shows the first two rounds of LR recombination, which were equal for all three vectors. In the first round, recombination occurred between pEntry1_PPT and pYLTAC7_R12_CmR_ccdB to yield pYLTAC7_PPT. Successful recombinants were selected by kanamycin and spectinomycin treatment. The second round of recombination was conducted between pEntry2_StPEPC_TB1 and pYLTAC7_PPT to make pYLTAC7_PPT_StPEPC. Correct transformants were selected by kanamycin and chloramphenicol treatment.

By alternating use of the two entry vectors we performed seven rounds of LR recombination for pYLTAC7_C4_NADP-ME and pYLTAC7_C4_NAD-ME, respectively, and five rounds for pYLTAC7_C4_PCK. The orientation of the gene expression cassettes in the entry vectors was chosen in such a way, that after recombination two consecutives cassettes are arranged head to head or separated by a putative transcription blocker if arranged tail to tail (Fig. 6 of supplementary material). According to Padidam and Cao (2001) this will prevent silencing caused by transcriptional interference. The transcription blockers are AT-rich sequences from the bacteriophage lambda.

The multigene constructs were confirmed after every round of recombination by digestion with appropriate restriction enzymes. Figure 3a shows the results after the three last rounds of recombination for pYLTAC7_C4_NADP-ME and pYLTAC7_C4_NAD-ME. The expected bands produced by digestion are indicated in Fig. 3b. The physical maps of pYLTAC7_C4_NADP-ME and pYLTAC7_C4_NAD-ME after the last round of LR recombination are shown in Fig. 3c.

Multigene constructs are more stable in recombination deficient *Agrobacterium* strain

For transformation of the multigene constructs two different *Agrobacterium tumefaciens* strains were tested: GV2260 (McBride and Summerfelt 1990) and AGL1. Compared to GV2260, AGL1 possesses



(b)

gene	PPT	StPEPC	Oac1	PPDK	SbMDH	HvME	FbCA	PEPS	EcMDH	EcME
product (bp)	1019	1454	1000	778	1315	1192	799	1385	970	1264

Fig. 4 PCR check of multigene constructs pYLTAC7_C4_NADP-ME and pYLTAC7_C4_NAD-ME in *Agrobacterium* AGL1. Colonies were tested for presence of all seven genes: PPT, StPEPC, Oac1, PPDK, SbMDH, HvME, FbCA for pYLTAC7_C4_NADP-ME and PPT, StPEPC, Oac1, PEPS, EcMDH, EcME, FbCA for pYLTAC7_C4_NAD-ME, respectively. Isolated plasmid DNA was used as a positive control (K). Generated PCR products were separated on a 1.0 % agarose gel (**a**). Lambda DNA digested with *Pst*I served as DNA size marker (λ). The expected *bands* produced by PCR are shown in the table below (**b**)

an insertion mutation in *recA* to improve recombinant plasmid stability (Lazo et al. 1991). As a stability assay, the destination vector pYLTAC7_PPT_ StPEPC_Oac1, which was obtained after the third round of LR recombination, was transformed into the two *Agrobacterium* strains. Single colonies were streaked out on new agar plates and were tested for presence of the three genes PPT, StPEPC and Oac1 by PCR. As positive control isolated pYLTAC7_PPT_StPEPC_Oac1 plasmid DNA was used, as negative control *Agrobacterium* cells which carried an empty destination vector were used. With GV2260 only 20–30 % of the clones were tested positive for all three genes. In contrast, with AGL1 90–100 % of the cells carried all three genes (data not shown). Hence, AGL1 was used in further experiments. Because of the hypervirulent Ti plasmid of AGL1 also higher transformation rates could be expected with this strain (Nadolska-Orczyk et al. 2000). After transformation of the multigene constructs pYLTAC7_C4_NADP-ME, pYLTAC7_C4_NAD-ME and pYLTAC7_C4_PCK in AGL1 single colonies were tested by PCR for the presence of all genes. Figure 4a shows the results for pYLTAC7_C4_NADP-ME and pYLTAC7_C4_NAD-ME. All PCR products showed the expected size (Fig. 4b). As a positive control, isolated plasmid DNA was used, as a negative control, *Agrobacterium* cells which carried an empty destination vector were used. Hence, although loss of a single gene was observed in some cases, AGL1 is suitable for propagation of the multigene constructs.

Generation of transgenic plants with stable transgene expression up to T2

A total of 66 putative T0 tobacco transformants for pYLTAC7_C4_NADP-ME and pYLTAC7_C4_NAD-ME, 38 for pYLTAC7_C4_PCK and 115 for pYLTAC7_PPT_StPEPC_Oac1 were subjected to expression analysis by real-time PCR (Table 2). About 30 % of the plants transformed with the multigene constructs pYLTAC7_C4_NADP-ME and pYLTAC7_C4_NAD-ME expressed all seven genes located on the T-DNA (Fig. 7 of supplementary material). The rather high number of plants which expressed none of the genes (18 %) can be explained by the strong growth inhibition caused by some genes. HvME and especially EcME lead to serious stunting of plants. Because aim of the multigene insertions was to enhance plant growth by the introduction of a single cell C_4-like CO_2 concentration cycle (Table 4 of supplementary material), non-expressors were privileged in the selection process. For the construct pYLTAC7_C4_PCK with five different genes approximately 40 % of the plants expressed all genes (Fig. 7 of supplementary material). Even higher was the rate of plants which express all genes with the construct pYLTAC7_PPT_StPEPC_Oac1. More than 80 % of

Table 2 Expression analysis of T0, T1 and T2 plants by real-time PCR

T0			T1			T2		
Expressed genes	Number of plants	%	Expressed genes	Number of plants	%	Expressed genes	Number of plants	%
(a) pYLTAC7_C4_NADP-ME + NAD-ME								
7	20	30	7	45	73	7	20	83
6	10	15	6	4	6	6	4	17
5	7	11						
4	6	9						
3	5	8						
2	4	6						
1	2	3						
0	12	18	0 (az)	13	21	0 (az)	0	0
Total	66	100	Total	62	100	Total	24	100
(b) pYLTAC7_C4_PCK								
5	15	39	5	11	69	5	12	75
4	5	13						
3	6	16						
2	4	11						
1	6	16						
0	2	5	0 (az)	5	31	0 (az)	4	25
Total	38	100	Total	16	100	Total	16	100
(c) pYLTAC7_PPT_StPEPC_Oac1								
3	92	80	3	17	77	3	6	50
2	17	15						
1	3	3						
0	3	3	0 (az)	5	23	0 (az)	6	50
Total	115	100	Total	22	100	Total	12	100

A total of 66 T0 plants for pYLTAC7_C4_NADP-ME + NAD-ME (a), 38 T0 plants for pYLTAC7_C4_PCK (b) and 115 T0 plants for pYLTAC7_PPT_StPEPC_Oac1 (c) were tested for expression of the entire T-DNA molecule-encoded genes relative to the endogenous gene Actin2. pYLTAC7_C4_NADP-ME was checked for expression of PPT, StPEPC, Oac1, PPDK, SbMDH, HvME, FbCA, pYLTAC7_C4_NAD-ME for PPT, StPEPC, Oac1, PEPS, EcMDH, EcME, FbCA, pYLTAC7_C4_PCK for PPT, StPEPC, Oac1, PCK, FbCA and pYLTAC7_PPT_StPEPC_Oac1 for PPT, StPEPC, Oac1. 62 (a), 16 (b) and 22 plants of the T1 generation (c) were tested for stable heredity of the transgenes. Furthermore the expression of the transgenes was controlled in 24 (b), 16 (b) and 12 plants of the T2 generation (c). *az* azygous

plants tested by real-time PCR showed expression of all three genes. The results indicated that they are likely to harbor at least one full T-DNA copy. An increase in the activity of PEPC, MDH, ME, PPDK, PEPS and PCK in these plants was also verified by enzyme assays (data not shown). The presence of some genes was also analyzed by PCR (Fig. 8 of supplementary material). In most cases the results were consistent with those from real-time PCR. Only few genes were detected by PCR but showed no expression (<5 %).

We allowed two transgenic lines of each construct that expressed all transgenes to mature and set seed and determined the inheritance and stability of the T-DNA in the next generations. Again the plants were subjected to expression analysis by real-time PCR (Table 2, Fig. 9 of supplementary material). T1 plants of lines L4 and L13 of pYLTAC7_C4_NADP-ME, L25 and L29 of pYLTAC7_C4_PCK and L4 and L11 of pYLTAC7_PPT_StPEPC_Oac1 exhibited a plausible single-insert segregation pattern when grown on a hygromycin-containing medium. In progenies of line

L9 of pYLTAC7_C4_NAD-ME there were plants which expressed 7 genes as well as plants which expressed only 6 genes. They were likely to carry two T-DNAs, an entire and a truncated version. Nearly all progenies of line L22 of pYLTAC7_C4_NAD-ME grew on hygromycin-containing medium and showed expression of all transgenes. Presumably they carried two or even more T-DNAs. Again an increase in enzymatic activities in these plants was verified by enzyme assays (Fig. 10 of supplementary material).

We further analyzed one transgenic line of each construct in the T2 generation to demonstrate inheritance and stability of T-DNA inserts in subsequent generations. Line L13–12 of pYLTAC7_C4_NADP-ME was likely to be homozygous. All progenies expressed all genes located on the T-DNA. T1 plant of line 9–24 of pYLTAC7_C4_NAD-ME exhibited a segregation pattern similar to L9. T1 plants of line L25–39 of pYLTAC7_C4_PCK and line 11–15 of pYLTAC7_PPT_StPEPC_Oac1 showed a plausible single-inherit segregation pattern like the respective parental plants. These results supported the notion that our plants carried full T-DNA inserts and passed them down to next generations. Hence, our data clearly show that multigene expressing plants can be produced using our MultiRound Gateway system.

Discussion

In this work, we present our improved version of MultiRound Gateway technology and demonstrate that it is suitable for the generation of multigene expressing plants. Using our constructed low copy destination vector pYLTAC7_R12_CmR_ccdB we assembled multigene constructs with up to eight transgenes including a selection marker by seven rounds of LR recombination. Each transgene possessed its own promoter and termination region corresponding to more than 26 kb. In contrast, with the high copy destination vector pTRA_R12_CmR_ccdb, similar to that used by Chen et al. (2006), only two rounds of LR recombination were successful. Our proof of principle experiments don't match the limit of the system, because the cloning capacity of the TAC-based destination vector is larger than 100 kb (Liu et al. 1999; Lin et al. 2003), whereas the number of gene-assembly rounds is not a limiting factor within a reasonable range. Dependent on cloning capacity of the destination vector the procedure maybe repeated nearly an infinite number of times. The orientation of transgenes in the destination vector can be freely designed by appropriate cloning into the multiple cloning sites of the entry vectors. We used four different MultiRound Gateway-constructed binary vectors with three, five and seven transgenes located on the T-DNA for stable genetic transformation of tobacco plants. We showed that numerous plants stably expressed the entire T-DNA molecule-encoded transgenes for at least two generations. Thus, our system represents a powerful tool for the construction of multigene plant transformation vectors overcoming the drawbacks of traditional cloning methods.

Another possibility for the assembly of multigene constructs was presented by Lin et al. (2003). The method was based on the Cre/*loxP* recombination system and the introduction of homing endonucleases. Lin et al. stacked ten foreign DNA fragments into a TAC vector by eight rounds of recombination. A major drawback of their system was that every recombination resulted in the integration of the whole donor vector. The undesired backbone of the plasmid and the redundant loxP site had to be removed by digestion with a homing endonuclease. Because of the asymmetric nature of the homing endonuclease sites the ends were not compatible to each other. The recyclization of the TAC vector required the aid of a compatible linker. In comparison to our method, this procedure was quite time-consuming.

It's particularly time-saving when transfer and homologous recombination are performed in vivo in *E. coli* (Muyrers et al. 2001; Warming et al. 2005; Li and Elledge 2005). However, this strategy is not suitable for constructs with repeated elements, because sequences between these elements are prone to deletion. Yet, Chen et al. (2010) developed a technique called MISSA (multiple round in vivo site specific assembly), which relies on a combination of the Cre/*loxP* and the lambda phage recombination system, and which enable assembly of DNA sequences in vivo. The system is based on conjugational transfer, which is driven by donor strains, and two in vivo site-specific recombination events, which are mediated by Cre recombinase and site-specific lambda phage recombination proteins in recipient strains. After introduction of entry vectors into donor strains the genes of interest can be transferred into recipient vectors by mixing bacterial strains. In their

proof-of-principles experiments Chen et al. (2010) constructed a multigene vector, which was generated by fifteen rounds of in vivo recombination. The cloning efficiency of the MISSA system was 60 up to 80 % depending on the used donor vectors. A main source of false clones was the homologous recombination events. Hence the repeated use of the same elements should be avoided as much as possible. Using the low copy destination vector pYLTAC7_R12_CmR_ccdB the cloning efficiency of our MultiRound Gateway system was nearly 100 %, despite the use of several repeated elements. For example the pYLTAC7_C4_NAD-ME construct contained three direct repeated doubled 35S promoters, three direct repeated pA35S termination regions, three direct and one inverted repeated 3′g7 termination regions, two inverted repeated RbcS promoters from *Chrysanthemum morifolium* and two direct repeated SAR elements. Sporadically occurring false clones could be explained as cotransformation of entry and destination vectors. However, by linearization of entry vectors this could be almost completely eliminated.

A drawback of recombination based systems is that plasmids obtained with these strategies contain additional sequences at the junction sites. The Golden Gate cloning method reported by Engler et al. (2008) overcame this problem. The strategy is based on the use of type IIs restriction enzymes, which cut outside of their recognition sequence such as *Bsa*I. With proper design of the cleavage sites, two fragments cut by such an enzyme can be ligated into a product lacking the original restriction site. Other ligation products containing the original restriction site will be re-cut. Besides the feature to leave no additional sequences in the destination vector the method allows ligation of multiple fragments. However, subsequently, no further fragments can be added. It's also not clear, if the method is still efficient for large fragments. However, the method could be used for the construction of the expression cassettes in the entry vectors, which could easily be adapted for this purpose. A worthwhile alternative to simplify construction of entry vectors is the In-Fusion system from Clonetech (Zhu et al. 2007). By recombining entire expression cassettes instead of single elements the additional sequences are located between the cassettes, where no negative effects are expected.

The irreversible nature of the recombination-based reactions does not enable the modification of existing multigene vectors. A versatile and modular system for the assembly of such vectors was recently described by Zeevi et al. (2012). Their system is composed of a destination vector that has been engineered to carry an array of unique recognition sites for zinc finger nucleases (ZFN) and homing endonucleases. Using this method they produced binary vectors that carried up to nine genes. However, the system is limited by the availability of different ZFNs and homing endonucleases, respectively.

In contrast to *E. coli*, the constructed multigene vectors were to some extent unstable in *Agrobacterium*. Sometimes one or more genes were lost during transformation or propagation in *Agrobacterium*. However, the instability should not be attributed to MultiRound Gateway but to the transgenic DNA sequences themselves. DNA sequences between the several repeated elements seemed to be susceptible to deletion due to homologues recombination, although AGL1 is a *recA* negative strain. Maybe a conjugational transfer of the multigene vector would result in gene loss less frequently than electroporation. For the observed gene loss in some transgenic tobacco plants also other factors should be considered, for example, homologues recombination in plants or during the process of T-DNA integration.

We observed transgenic lines which expressed all the transgenes over at least two generations (Table 2). Yet, we could not identify a clear correlation between the expressions of different transgenes located on the same T-DNA or between different lines. For example, pYLTAC7_C4_ NADP-ME lines that showed high levels of PPT expression did not necessarily exhibit high PPDK or FbCA expressions. Thus, it's not possible to determine the efficiency of a specific promoter in a given multigene array due to differences in the gene expression levels which did not correlate with their promoter types. Similar results reported Fujisawa et al. (2009) and Zeevi et al. (2012). Comparably to Chen et al. (2010) variable expression levels of individual transgenes was not only observed between different transgenic lines but also between individual transgenes driven by the same promoter in a given multigene array. Similar promoters seem to behave differently depending on their arrangement in a multigene cluster. Furthermore gene expression is dependent on the integration locus. If they become inserted into a transcriptionally active region (Koncz et al. 1989; Kertbundit et al. 1991), expression may be

influenced by regulatory sequences of nearby host genes.

In summary, the improved version of the Multi-Round Gateway technology described here represents a powerful, highly efficient tool for multigene plant transformation. But its utility is not restricted to plant applications. Any appropriate DNA components can be assembled in various vectors modified according to this study for different purposes, such as transfer of multiple genes of interest to yeast, insect, or mammalian cells.

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