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Efficient seed-specifically regulated autoexcision of marker gene (*nptII*) with inducible expression of interest gene in transgenic *Nicotiana tabacum*

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Abstract: In this study, we developed the seed-specifically regulated autoexcision system based on Cre/*loxP* site-specific recombination for coelimination of *nptII* and *cre* genes from transgenic plants. To accomplish this, a seed-specific gene promoter, BcNA1, from *Brassica napus* was used to drive conditional expression of recombinase. NptII and recombinase cassettes were placed between two directly repeated *loxP* sites. The *loxP*-flanked DNA was located between the SP-DDEE synthetic pathogen inducible promoter and promoterless β-glucuronidase (*Gus*) gene, as a model gene of interest. Upon seed-specific expression of *cre* recombinase, the excision event would eliminate *loxP*-flanked DNA and bring the *gus* reporter gene under the control of the inducible promoter. This Cre/*loxP* system was transformed into *Nicotiana tabacum* via *Agrobacterium*-mediated transformation. The regenerated plants were obtained by selection on kanamycin medium and PCR screening. Based on seed germination assay on kanamycin-containing medium, the transgenic lines were subdivided into homogeneous and heterogeneous categories. Phenotypic and molecular analysis of T1 progeny plants indicated that completely NptII-free plants were obtained in homogeneous transgenic lines. Coelimination of NptII and recombinase cassettes were verified with PCR, sequencing, and histochemical Gus staining assay. Full excision efficiency (100%) demonstrates the effectiveness of this autoexcision system for the production of marker gene-free transgenic plants.

Key words: Cre/*loxP*, site-specific recombination system, seed-specifically regulated autoexcision system, marker-free transgenic plants, BcNA1 promoter

1. Introduction

Plant transformation technology has immense potential for the improvement of crops. Low transformation efficiency necessitates the use of selectable marker genes, especially antibiotic and herbicide resistance genes, for transgenic plant identification (Sundar and Sakthivel, 2008). Once transgenics are identified, these genes are nonessential. The remains of marker genes in transgenic crops have caused several environmental and consumer concerns about the safety of the transgenics harboring these genes (Kuiper et al., 2001; Tuteja et al., 2012). The possibility of horizontal and vertical transfer of marker genes to other microorganisms or crops has been noted as a major biosafety concern in genetically modified plants (Dale et al., 2002). In addition, selectable marker genes remaining in genetically modified crops are a great limitation for gene stacking (François et al., 2002; Halpin, 2005; Manimaran et al., 2011). Therefore, plant improvement for multigene traits, such as disease resistance, is difficult. Elimination of selectable marker genes from transgenic crops both increases public acceptance of genetically modified crops and prepares a gene stacking possibility for the improvement of complex traits.

Several approaches have been developed to produce marker-free transgenic plants. In cotransformation-based strategy, marker and trait genes are introduced into the plant genome by two separate T-DNA and are integrated on unlinked genomic loci. The marker-free transgenic plants are produced with subsequent segregation of the trait gene from the marker gene (Breitler et al., 2004; Wakasa et al., 2012). In transposon-based (Goldsbrough et al., 1993; Cotsaftis et al., 2002), homologous recombination (Zubko, 2000), and site-specific recombination-based (Dale and Ow, 1991; Gleave et al., 1999; Puchta, 2000) strategies, marker and trait genes are integrated into the same genomic locus, although molecular tools are used for marker gene removal.

Various types of site-specific recombination systems, such as Cre/*loxP* (Dale and Ow, 1991; Zhang et al., 2003; Sreekalla et al., 2005), R/*RS* (Endo, 2002), Flp/*frt* (Luo et al., 2000), and PhiC31/*aatB*/*aatP* (Kapusi et al., 2012), have been investigated in plant biotechnology for

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transgene excision. The Cre/*loxP* recombination system is characterized by its ability to function independently of DNA topology through the interaction of Cre recombinase with a pair of identical recognition target sites. Each 34-bp *loxP* site is palindromic, comprising 13-bp inverted repeats surrounding an 8-bp asymmetric spacer region that confers directionality to the site and hence to the recombination reaction (Hoess et al., 1986; Guo et al., 1997). The *cre* function can be introduced in *lox* transgenic plants with several strategies: *loxP*-marker-*loxP* transgenic plants are retransformed with a construct expressing *cre* (Russell et al., 1992) or crossed with *cre* gene-containing plants (Hoa et al., 2002; Zhang et al., 2003; Arumugam et al., 2007). With these strategies, Cre recombinase is driven by constitutive promoters. This provides high recombination efficiencies, but is labor-intensive and time-consuming. Moreover, it was shown that constitutive expression of the *cre* gene can cause unwanted phenotypic and genetic alteration in plants (Coppoolse et al., 2003).

Alternatively, transient expression vectors (*Agrobacterium tumefaciens* and plant Cre-virus vectors) are used for the expression of the *cre* gene in *loxP*-marker-*loxP* transgenic plants (Vergunst et al., 2000; Jia et al., 2006). In this strategy, additional steps are required to develop an efficient transient expression method and regeneration protocol.

Finally, in the autoexcision strategy, the Cre expression cassette and *loxP*-marker-*loxP* DNA can be placed in the same T-DNA and be introduced into the plant genome. In this strategy, *cre* is driven by inducible promoters such as heat-shock inducible promoters (Wang et al., 2005; Cuellar et al., 2006), chemical inducible promoters (Sreekala et al., 2005; Zhang et al., 2006), or tissue-specific promoters (Li et al., 2007; Luo et al., 2007). Due to the leakiness of inducible promoters or the probable side effects of chemical agents on plant proliferation in the inducible autoexcision system, the use of tissue-specific and genetically regulated promoters is recommended for conditional expression of recombinase gene. A number of reports are available on seed-specific promoters of napin genes such as *BcNA1* (Broun et al., 1997; Eccleston and Ohlrogge, 1998; Katavic et al., 2001). Based on these reports, it is considered that the upstream regulatory region of the *BcNA1* gene can be successfully used in tight regulation of recombinase expression.

In this study, we developed a seed-specifically regulated autoexcision system, based on Cre/*loxP* recombination, by using the BcNA1 seed-specific promoter from *Brassica napus* for conditional expression of the recombinase and SP-DDEE pathogen inducible promoter for inducible expression of the gene of interest. We analyzed the efficiency of this system for marker elimination in tobacco. In this strategy, the recombinase gene in seeds is expressed by a seed-specific promoter (BcNA1) without any additional treatment, and the gene of interest is expressed by a pathogen inducible promoter that can be induced by fungal elicitors. Our further goal is to use this system in genetic engineering of target crops for the generation of disease-resistant marker-free transgenic plants.

2. Materials and methods

2.1. Construction of pG-synDDEE-cre^{int}-gus^{int}

All cloning and cell transformation experiments were carried out according to Sambrook et al. (1989). Construction of pG-synDDEE-cre^{int}-gus^{int} was achieved in multiple cloning and subcloning steps. The Gus reporter gene sequence was amplified with KSgusF/Sac. gusR primers (Table 1) with additional *Kpn*I and *Sma*I

Table 1. Oligonucleotide primers used for the amplification of transgenes.

Name	Sequence (5'-3')	Restriction enzyme sites
KSgusF	CTCGAGGGTACCCGGGACCATGGTAGATCTGAGGGTAA	*Xho*I, *Kpn*I, *Sma*I
SacgusR	ATTCGAGCTCGGTAGCAATTC	*Sac*I
creF	CTCGAGATGTCCAATTTACTGACCGTA	*Xho*I
creR	CTCGAGATAACTTCGTATAATGTATGCTATACGAAGTTATCCCCCCTATTTCTCAGGCACA	*Xho*I
napF	AAGCTTTCTTCATCGGTGATTG	*Hind*III
napR	CTCGAGTCTAGATCGCCCATGGTGTATG	*Xho*I
nptIIF	CATACCGTTCCCGAGTAAGGATG	-
nptIIR	TCGAGTGGTGATTTTGTGC	-
E1	GACTAGTTGTCAATGGTCAACATTCAA	-
gusR	CCGGCATAGTTAAAGAAATCATG	-

recognition sites in the 5'-end of forward primer. PCR product was digested with *Xho*I/*Sac*I and subcloned in the pGPTV-gus^{int} vector (Sprenger-Haussels and Weisshaar, 2000); this construct was named pG-KSgus^{int} (Figure 1a). In the second step, the synthetic fragment containing the SP-DDEE inducible promoter sequence (Shokouhifar et al., 2011), the *loxP* sequence, and the nos terminator was digested with *Dra*III and subcloned in pG-KSgus^{int}. The new construct was named pG-synDDEE-KSgus^{int} (Figure 1b). The BcNA1 promoter region was synthesized based on the reported sequence available in the National Center for Biotechnology Information (NCBI) database (GenBank accession no. AF302261), cloned into the *Hind*III/*Xho*I sites of pG-synDDEE-KSgus^{int} and designated as pG-synDDEE-nap-KSgus^{int} (Figure 1c). Then a fragment containing Cre and the nos terminator sequence was amplified using creF and creR primers (Table 1), with the *loxP* sequence at the reverse primer from pLH-cre-gus plasmid (Kopertekh et al., 2009). It was then cloned into the *Xho*I site of pG-synDDEE-nap-KSgus^{int} and designated as pG-synDDEE-cre^{int}-gus^{int} (Figure 1d). *A. tumefaciens* strain LBA4404 was transformed by this construct using the freeze–thaw method. Bacteria were grown overnight at 28 °C in Luria–Bertani liquid medium containing 50 mg/L kanamycin in a water bath shaker (250 rpm) and used for plant transformation.

2.2. Plant transformation and confirmation of primary transgenic plants

Wild type *N. tabacum* 'Samsun' was grown in 1/2 MS medium under sterile conditions. The pG-synDDEE-cre^{int}-gus^{int} construct was introduced to a leaf disk of tobacco via *Agrobacterium*-mediated transformation according to the procedure of Horsch et al. (1985). Transformants were selected on hormone-MS medium supplemented with 100 mg/L kanamycin and 200 mg/L cefotaxime and subcultured every 3 weeks. After 2 months, the regenerated shoots were transferred to a kanamycin-free MS medium for elongation and rooting, and were then transferred to soil for self-pollination.

Genomic DNA from leaf material of primary transgenic plants was prepared using a peqGOLD plant DNA mini kit (PeQlab). The presence of T-DNA in the primary transgenic plants genome was confirmed by PCR. The presence of the *gus* and BcNA1 promoter was detected by PCR amplification using two sets of primers, gusF and gusR (Table 1; Figure 2) for the amplification of the 521-bp fragment, and napF and napR (Table 1; Figure 2) for the amplification of the 1179-bp fragment, respectively. The reaction mixture (25 µL) contained 40 ng of template, 2.5 µL of 10X PCR buffer, 10 pmol of each primer, 200 µL of dNTPs, and 0.5 U of Taq polymerase (Fermentas).

2.3. Seed germination assay

To determine the rate of kanamycin susceptibility in T_1 progeny, a number of seeds from each transgenic line were surface-sterilized with hypochlorite sodium (10%) for 10 min, washed several times with sterile distilled water, and sown on MS medium supplemented with 100 mg of kanamycin. The number of kanamycin-resistant plants (normal seedlings with developed shoot and root systems) and susceptible plants (small seedlings with yellow or pale green cotyledons and undeveloped roots) was counted after 21 days.

2.4. Chemical induction and histochemical β-glucuronidase (*gus*) assay

Three- or 4-week-old T1 progeny plants, which exhibited a kanamycin-susceptible phenotype, were analyzed for *gus* expression. Leaf disks of T1 plants were incubated in 0.2 mM salicylic acid (as a pathogen elicitor) in vacuum for

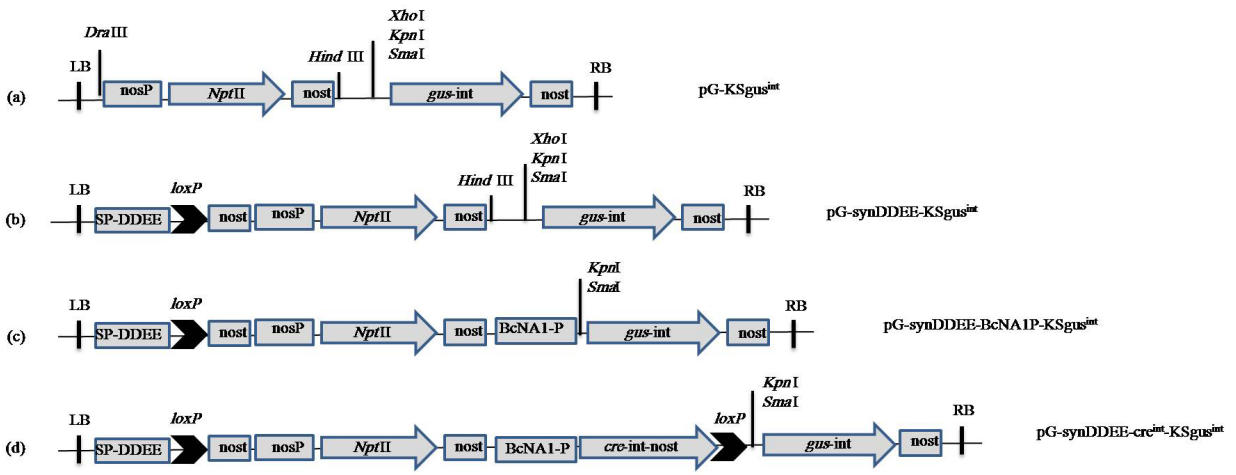

Figure 1. Development of pG-synDDEE-cre^{int}-gus^{int} autoexcision vector: **(a)**, **(b)**, **(c)** T-DNA regions of intermediate vectors, and **(d)** complete T-DNA region of pG-synDDEE-cre^{int}-gus^{int}.

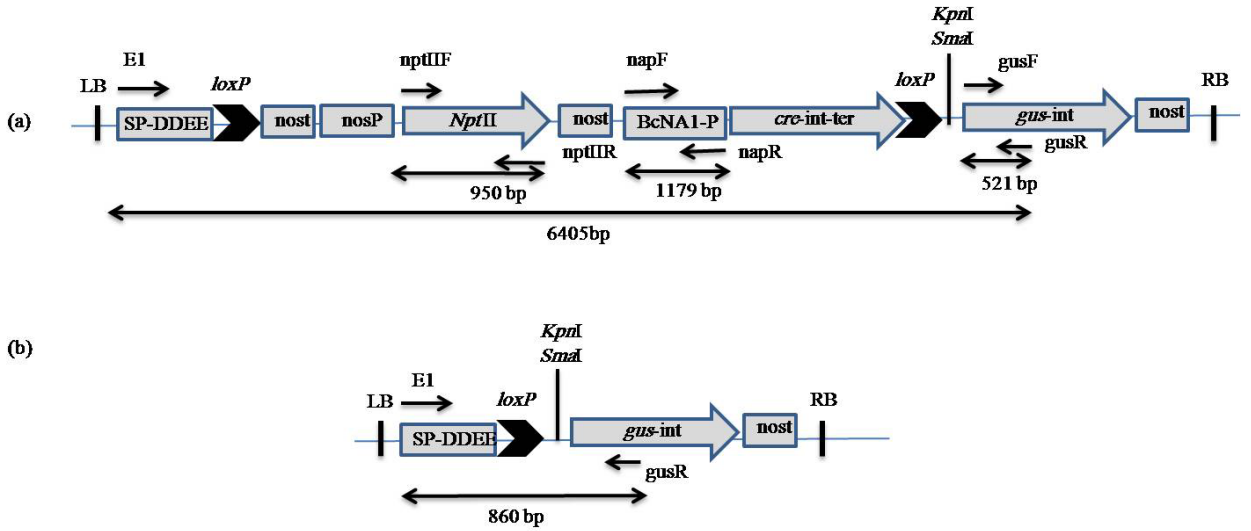

Figure 2. pG-synDDEE-cre^{int}-gus^{int} construct: **(a)** complete T-DNA, **(b)** T-DNA after recombination.

20 min for induction of promoter activity and were then incubated in the dark for 72 h. Induced leaf disks were used for histochemical *gus* staining assay according to the procedure of Jefferson et al. (1987). A noninduced leaf disk was used as the control.

2.5. Analysis of recombination product by PCR and sequencing

A number of T1 progeny seeds from L-7, L-8, L-9, and L-10 transgenic lines were germinated, and genomic DNA for PCR assay was prepared as described earlier. The Cre-mediated recombination was investigated by PCR amplification using E1/gusR and nptIIF/nptIIR primers (Table 1; Figure 2). Thermal cycling was carried out at 95 °C for 1 min, 58 °C for 1 min, and 72 °C for 130 s.

An 860-bp PCR product, derived using E1/gusR primers, was cloned into pTZ 57R/T vector (Fermentas) and sequenced with M13 primers.

3. Results

3.1. Generation and molecular confirmation of transgenic plants

A genetically regulated autoexcision vector for removing the selectable marker gene from transgenic plants genome was designed based on the Cre/*loxP* recombination system. pG-synDDEE-Cre^{int}-Gus^{int} contained the pathogen inducible promoter (SP-DDEE), nos terminator, NptII expression cassette as a selectable marker gene, Cre expression cassette, and promoterless Gus reporter as a model gene of interest (Figure 2a). The seed-specific promoter from the *BcNA1* gene was used to drive conditional *cre* expression in the seeds. The Cre and NptII cassettes of T-DNA were located between two *loxP* sites in the same direction.

Leaf disks of *N. tabacum* were transformed by the pG-synDDEE-Cre^{int}-Gus^{int} vector via *Agrobacterium*-mediated transformation with NptII selection. The *gus* gene was expressed by inducible promoter (SP-DDEE) after all components between the two *loxP* sites were excised. The putative transgenic kanamycin-resistant lines were analyzed by PCR amplification using two sets of specific primers, napF/R and gusF/R (Table 1; Figure 2a).

Eight putative transgenic plants DNA samples were found to be positive by generating the expected amplified bands: 1179 bp for napF/R and 521 bp for gusF/R primers (Figure 3). Nontransgenic plant DNA was used as a negative control.

By excisional recombination event, all the components between the two *loxP* sites would be removed following the seed-specific promoter activity, leading to Cre expression (Figure 2b). Transgenic lines were self-pollinated to obtain T1 seeds. All transgenic lines exhibited healthy phenotype and no abnormality in shape or fertility was observed. Mature seed material from these lines was used for selectable marker gene elimination via phenotypic and molecular analysis.

3.2. Seed germination assay

Seeds from self-pollinated transgenic plants were used to investigate the marker-free plants. Seeds were sown in the presence of 100 mg/L kanamycin to screen the marker-free phenotype. The excision efficiency of the *nptII* gene was figured out based on growth of T1 seeds on the antibiotic-containing medium. Susceptible and resistant germinated seeds were clearly distinguishable 3 weeks after germination (Figure 4). Small seedlings with yellow or pale green cotyledons and undeveloped roots were recognized as susceptible, and normal seedlings

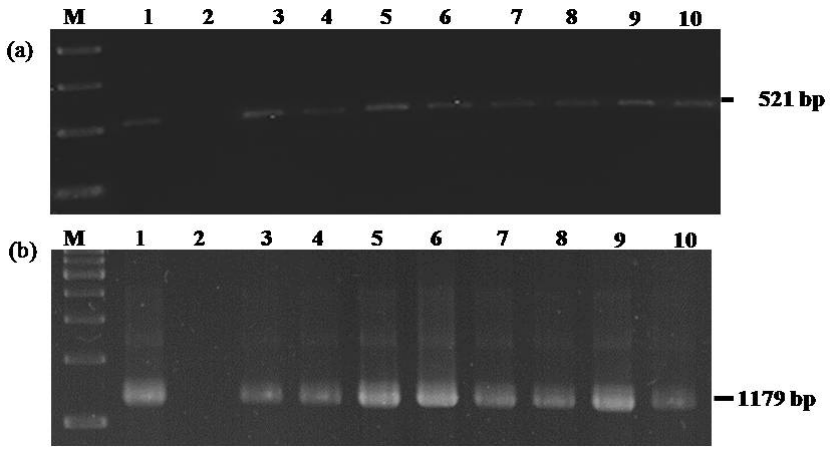

Figure 3. Molecular confirmation of putative transgenic plants: **(a)** PCR results using gusF/gusR primers. M- molecular marker, 1 kb; 1- plasmid pG-DDEE-cre^{int}-gus^{int} as positive control; 2- wild-type tobacco genomic DNA; 3–10- genomic DNA of transgenic lines. **(b)** PCR results using napF/napR primers. M- molecular marker, 1 kb; 1- plasmid pG-DDEE-cre^{int}-gus^{int} as positive control; 2- wild-type tobacco genomic DNA; 3–10- genomic DNA of transgenic lines.

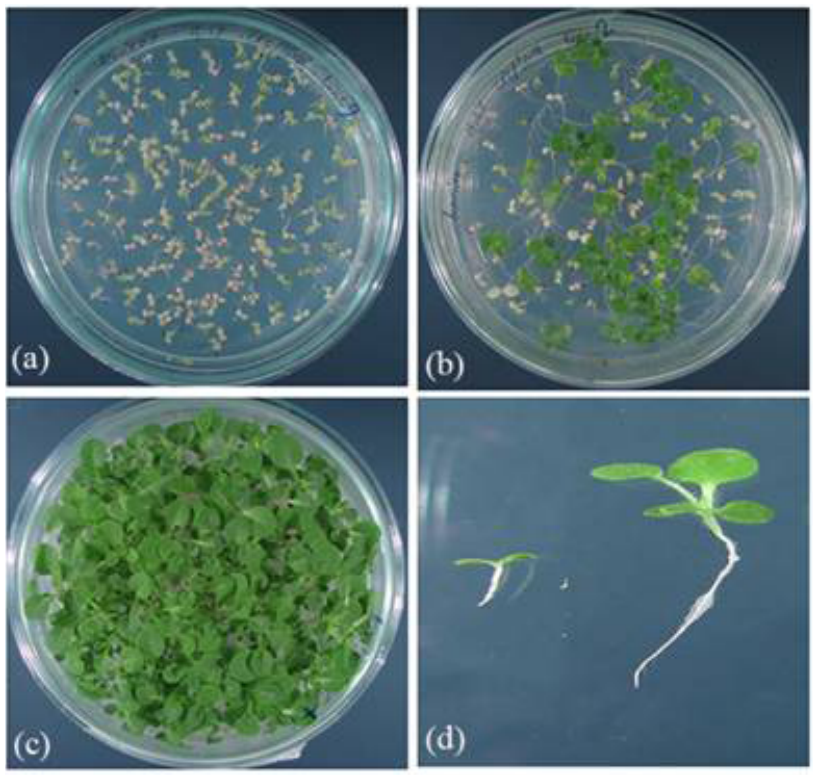

Figure 4. Phenotypic analysis of *nptII* excision on selection condition (100 mg/L kanamycin): **(a)** T_1 progeny plants of L-9 as an indicator for homogeneous lines, **(b)** T_1 progeny plants of L-2 as an indicator for heterogeneous lines, **(c)** pBI-nptII-gus transgenic T_1 progeny plants as a control, **(d)** kanamycin-sensitive versus kanamycin-resistant phenotype.

with developed shoot and root systems were recognized as resistant seedlings. Germination assay data are summarized in Table 2. In this assay, seeds from pBI-NptII-Gus transgenic plants are used as the control; all of them germinated normally and showed kanamycin-resistant phenotype. Four of 8 lines (L-7, L-8, L-9, L-10) demonstrated a kanamycin-sensitive phenotype and were categorized in homogeneous lines; the other lines (L-2, L-3, L-4, L-5) were differentiated according to kanamycin-susceptible and -resistant phenotypes, which were located in the heterogeneous category. However, the frequency of kanamycin-susceptible plants was varied among them: 69 of 96 for L-2, 65 of 109 for L-3, 44 of 147 for L-4, and 31 of 122 for L-5. Segregation analysis for the antibiotic-sensitive and -resistant phenotypes demonstrated that the *nptII* gene had been completely eliminated from the L-7, L-8, L-9, and L-10 transgenic lines.

3.3. Histochemical Gus staining assay

To confirm the results of marker gene elimination, histochemical Gus staining assay was carried out for T1 leaf tissues. The Gus reporter gene was expressed with the inducible promoter SP-DDEE after excision of all components between the two *loxP* sites in pG-synDDEE-cre^{int}-gus^{int} T-DNA (Figure 2b). Analysis of the Gus expression pattern in four kanamycin-susceptible lines (L-7, L-8, L-9, and L-10) exhibited positive blue tissue after the leaf disks of each line were induced by salicylic acid (as a pathogen elicitor) (Figure 5). These observations showed that a successful recombination between the two *loxP* sites had occurred in all four homogeneous lines. The Gus assay indicated that the recombination event in transgenic lines did not interfere with the reporter gene expression.

3.4. Molecular analysis

Phenotypic data verified the removal of the NptII/Cre cassettes, and also confirmed the potential of SP-DDEE inducible promoter for Gus expression after excision. For further confirmation of the phenotypic results, PCR analysis was carried out using T1 progeny genomic DNA, which lost the resistance to kanamycin and also expressed the *gus* reporter gene. The E1/gusR Primers (Table 1; Figure 2a) should amplify a 6405-bp fragment from the complete pG-synDDEE-Cre^{int}-Gus^{int} T-DNA or a 860-bp fragment specifically from the cassette after complete excision of all components between two *loxP* sites (Figure 2b). All four Gus-positive lines (L-7, L-8, L-9, and L-10) were positive for the 860-bp band (Figure 6a), which indicates that recombination occurred in these lines. Since E1/gusR were not able to amplify the 6405-bp fragment from the complete T-DNA and investigated T1 plants (due to the large size of fragment), the nptIIF/R primers (Table 1; Figure 2a) were used for identification of incomplete recombination events. These primers should not amplify any DNA from the excised DNA fragment, since the *nptII* gene should have been lost; instead, they should amplify 950-bp fragments from complete T-DNA (Figure 2a). Eleven T1 progeny plants for each homogeneous line were analyzed to identify an incomplete excision event. Amplification of the 950-bp fragment indicated an incomplete excision event. T1 progeny plants demonstrated that only an 860-bp recombined band would be completely marker-free. Plants that indicated both 860-bp and 950-bp amplification products signified incomplete excision events. The results of the PCR data are summarized in Table 3. According to PCR analysis, putative marker-free

Table 2. Summarized data for seed germination assay.

Line number	Total germinated plants	*npt*II-sensitive plants	*npt*II-resistant plants	Rate of susceptibility
2	96	69	27	71.8%
3	147	44	103	29.9%
4	109	65	44	59.6%
5	122	31	91	25.4%
7	84	84	-	100%
8	104	104	-	100%
9	167	167	-	100%
10	147	147	-	100%

Transgenic T1 seeds were germinated on 100 mg/L kanamycin medium and the numbers of susceptible and resistant plants were counted.

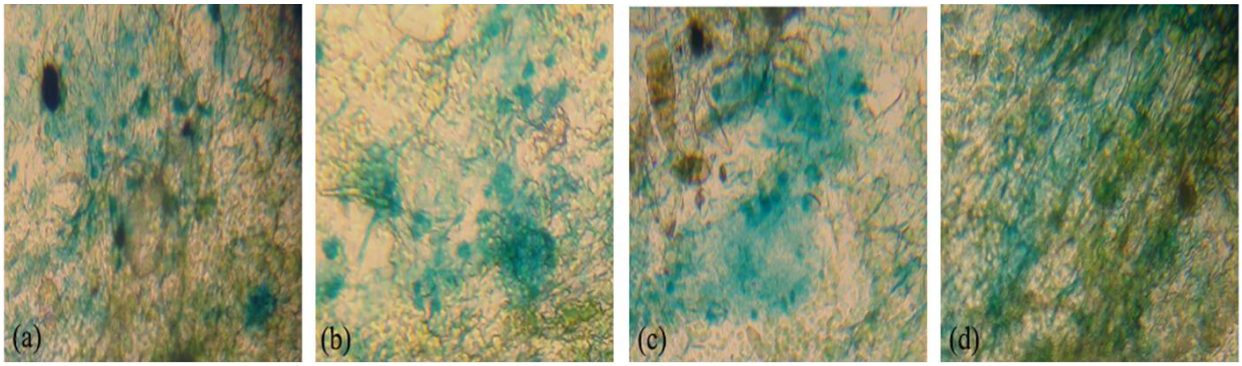

Figure 5. Microscopic appearance of Gus expression in leaf disks of T1 progeny of homogeneous lines. Induced leaf disk of **(a)** L-7, **(b)** L-8, **(c)** L-9, **(d)** L-10.

Table 3. PCR analysis data for marker gene removal.

Line number	Total number of samples	Excision PCR-positive	*npt*II PCR-negative	Complete excision efficiency
7	11	9	9	100%
8	11	8	7	87.5%
9	11	10	10	100%
10	11	10	2	20%

Eleven T1 progeny plants for each homogeneous line were analyzed with PCR using E1/gusR and nptIIF/R primers. E1/gusR amplifies 860-bp postrecombination fragments that are presented in the table as excision PCR-positive (elimination of *npt*II). Positive samples for excision events were analyzed with nptIIF/R primers. *npt*II PCR-negative were scored as plants with complete *npt*II excision. Complete excision efficiency was expressed as a percentage of the number of putative complete marker-free plants relative to the excision-positive plants.

plants were identified in all four homogeneous lines. Two of four homogeneous lines (L-7 and L-9) were completely marker-free. Complete excision efficiency was expressed as a ratio of the putative complete marker-free plants to the excision positive plants. The complete excision efficiency varied from 20% for L-10 to 100% for L-7 and L-9.

Evidence for the precise site-specific recombination event between the two *loxP* sites was obtained by sequencing of the junction regions of four kanamycin-susceptible lines (homogeneous lines). The fragment (860 bp) produced from PCR amplification by E1 and gusR primers containing the junction sequence (SP-DDEE inducible promoter, *loxP*, and Gus) was cloned into the pTZ 57R/T cloning vector and sequenced. As illustrated in Figure 6b, *loxP*-flanked DNA was removed precisely. Only one *loxP* site remained between the SP-DDEE inducible promoter and Gus open reading frame. This fragment also contained the intact recognition sites of *Kpn*I, *Sma*I, and *Xho*I restriction enzymes (Figure 6b).

4. Discussion

Considering the major public biosafety concerns about the remnants of selectable marker genes in transgenic plants (Tuteja et al., 2012), various approaches have been introduced for marker gene elimination (Komari et al., 1996; Park et al., 2004; Parakash et al., 2009; RamanaRao et al., 2010). Site-specific recombination-based strategies have become a promising approach for marker elimination due to their simplicity and accuracy. Different types of site-specific recombination systems have been investigated in various plants (Hoa et al., 2002; Sreekala et al., 2005; Ma et al., 2009; Li et al., 2010).

In this study, we present the feasibility of a designed seed-specifically regulated autoexcision system for NptII cassette excision, based on a Cre/*loxP* site-specific recombination system in *N. tabacum*. In recent studies on marker gene elimination using the Cre/*loxP* recombination system, Cre recombinase was expressed through various strategies including constitutive (Arumugam et al., 2007), transient (Jia et al., 2006), inducible (Wang et al., 2005; Ma et al., 2009), and genetically regulated (Mlynárová et al., 2006; Li et al., 2007; Moon et al., 2010) systems.

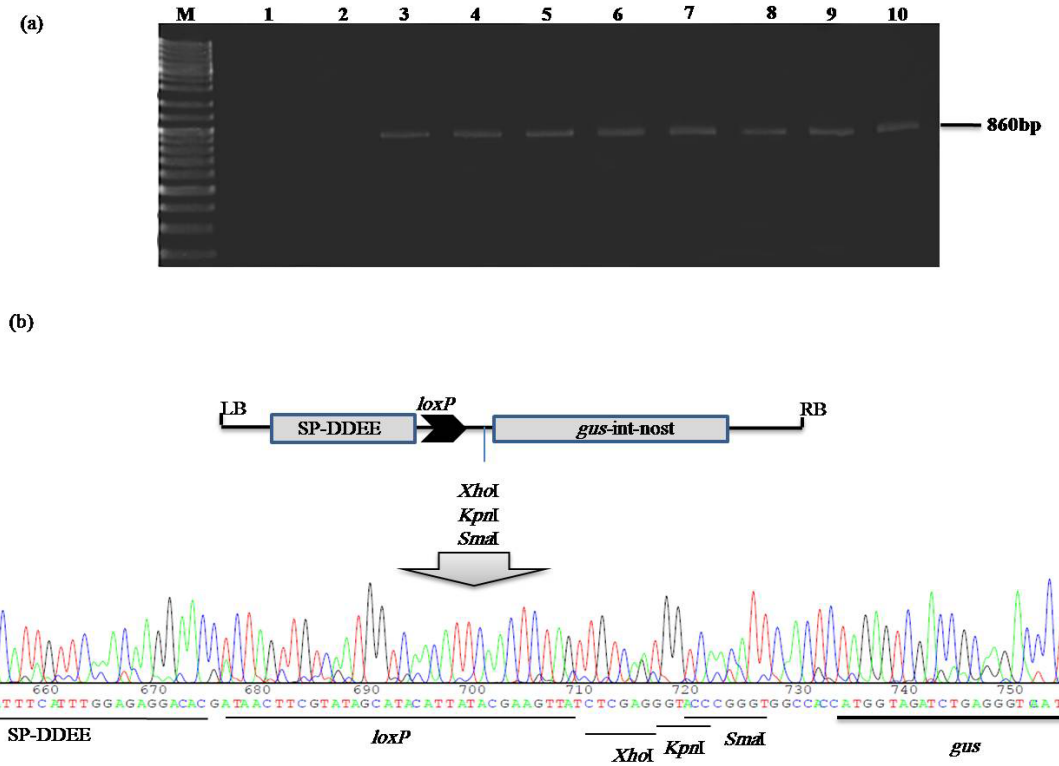

Figure 6. Molecular confirmation of *nptII* excision in homogeneous lines: **(a)** PCR results of T1 progeny plants with E1/gusR primers. M- molecular marker mix; 1- wild-type tobacco; 2- unrecombined pG-synDDEE-cre^{int}-gus^{int} plasmid; 3–10- homogeneous lines (L-7, L-8, L-9, L-10). **(b)** Verification of precise recombination between directed *loxP* sites by sequencing analysis for 860-bp postrecombination fragments amplified using E1/gusR primers.

In our system, seed-specific promoter from the *BcNA1* gene derives from *cre* expression. Some reports have indicated that this tissue-specific promoter is functional in embryos and endosperm without any activity in vegetative tissues (Höglund et al., 1992; Stålberg et al., 1993). The kanamycin-resistant transgenic lines obtained in this study confirmed the tight regulation of this autoexcision system for *cre* expression.

Seed germination assay for T1 progeny showed kanamycin-sensitive phenotypes in all transgenic lines. The investigated lines could be subdivided into two categories based on kanamycin sensitivity: homogeneous (lines L7, L8, L9, and L10), unable to grow on kanamycin-containing medium, and heterogeneous (lines L2, L3, L4, and L5), showing both susceptible and resistant phenotypes (Figure 4; Table 2). *Agrobacterium*-mediated transformation frequently inserts multiple copies of the transgene into the plant genome, and the integration of multiple copies of transgene in heterogeneous lines was confirmed according to Southern blot analysis (data not shown). For marker gene elimination, it is important that all copies of the transgene be completely excised. We hypothesized that the presence of heterogeneous lines could be attributed to the incomplete excision of multiple copies of the transgene in these lines. The same findings have also been reported by Li et al. (2007) about the excision in the self-activating Cre system driven by Arabidopsis embryo-specific promoter. Furthermore, Kopetekh et al. (2009) reported heterogeneous phenotypes for multiple copy transgenic lines in developmentally regulated self-excision systems. Complex integration patterns and genomic positions of T-DNA have been reported as factors influencing the expression of a transgene (Jorgensen et al., 1996; De Buck et al., 2000).

PCR is a sensitive method for the detection of recombination events. PCR amplification (Figure 6a; Table 3) and direct sequencing of the recombined fragment (Figure 6b) were subsequently used to verify excision of the *nptII* marker gene in homogeneous lines. An 860-bp postexcision amplified fragment from DNA of all homogeneous lines confirmed recombination events and marker gene elimination in these lines. PCR amplification with nptIIF/nptIIR primers positioned in *loxP*-flanked DNA was additionally used to identify complete marker gene elimination and, accordingly, complete excision efficiency was 100% for two lines (L-7 and L-9). It can be

speculated that variation in the complete excision efficiency for homogeneous lines might be due to unrecombined transgenes in some transgenic cells, because of various expression patterns of recombinase in embryo cells during seed development. Höglund et al. (1992) demonstrated that the BcNA1 promoter is activated 20 days after pollination and continues until 35 days after pollination. They also reported that the product of this gene (napin protein) was initially detected at the outermost tip of the axis and continued through the axis to the base of the outer cotyledon and further toward its tip. Phenotypic and molecular data in this study indicated that this genetically regulated system was efficient for complete excision of the marker gene. The 20%–100% total gene excision frequency in this study is comparable to results based on other tissue-specific regulated recombinase systems, such as 100% in tobacco using Cre/*loxP* and pollen-specific promoter (Luo et al., 2007), 83%–100% in Arabidopsis using germline-specific promoters (Verweire et al., 2007), 100% in tobacco using microspore-specific promoter (Mlynarova et al., 2006), and 37.5% in rice using floral-specific promoter (Bai et al., 2008).

An efficient system for marker gene removal should not interfere with interest gene expression. To further characterize marker-free plants containing β-glucuronidase (Gus), homogenous lines were analyzed by histochemical gus staining assay. We found that the β-glucuronidase gene was expressed in leaf tissues of all investigated lines (Figure 5). This is similar to the results reported for soybean (Li et al., 2007).

In our autoexcision system, a synthetic inducible promoter (SP-DDEE) drove the *gus* gene after the excision event. This promoter is a pathogen-inducible promoter and its inducibility has previously been confirmed (Shokouhifar et al., 2011).

One of the most significant advantages of this study is the ability to tightly regulate the *cre* gene using a seed-specific promoter, such that the *cre* is not expressed during the early tissue culture period when selective pressure is needed for efficient selection. In this autoexcision system, the *nptII* gene was placed near the left T-DNA border. Thus, we can ensure that primary kanamycin-resistant plants contain complete T-DNA. This autoexcision system might be an efficient tool in transgenic manipulation for producing marker-free transgenic plants.

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