Bxb1-*att* Site-Specific Recombination System-Mediated Autoexcision to Prevent Environmental Transgene Escape

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Mona Easterling and Yuan-Yeu Yau

Abstract

Transgenic plants are obtained experimentally with low frequency. Scientists use selectable marker genes (SMGs) and selection agents to select transformed cells from mostly untransformed cells in the production of transgenic plants. The SMG is usually an antibiotic or herbicide-resistance gene. However, the presence of SMGs in GM plants and subsequently in food, feed, and the environment has raised concerns from regulatory agencies and the public. In recent years, several strategies have been deployed to remove SMGs from GM plants. This chapter describes a case study highlighting the Bxb1-*att* site-specific recombination system for SMG removal in tobacco plants. Case study includes development-induced autoexcision cassette designed to delete both the SMG and the *bxb1* gene with a seed promoter derived from common bean *Phaseolus vulgaris* phaseolin (*phas*) gene to drive *bxb1* expression. GUS-positive T_0 lines transferred to soil for setting T_1 seeds. T_1 progeny and their T_2 generation are also obtained for study. Bxb1-mediated autoexcision events are identified in T_1 seeds and T_1 and T_2 plants through junction PCR analysis. Sequencing confirmed successful excision events. Chimeric plants containing both excised and intact T-DNA were observed in both T_1 and T_2 independent lines. However, two homogenous SMG- and *bxb1*-free T_2 lines were also obtained.

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Keywords

Autoexcision · Bxb1-*att* · CRISPR-Cas · Genome editing · Gene stacking · Marker free · Root promoter · Site-specific recombination · Unidirectional SSR · ZFN

35.1 Introduction

Genetic engineering can be used in plants to improve crop traits and yields by gene targeting to modify or control gene expression (Dong and Ronald 2021). Several available essential molecular tools can serve this purpose. Site-specific recombination (SSR) technology is one of them. SSR systems are well suited for use in both heterologous systems and synthetic genetic circuits. This is because that SSR recombination sites are small, easy to use, and do not require host-specific cofactors. They work well on both supercoiled DNA and linear DNA substrates. The most popular plant application for SSR systems is the removal of selectable marker genes from transgenic plants (GMOs) to produce marker-free GM crops. In addition, SSR systems have been used for gene integration and gene stacking.

In planta, the SSR system has been used in five specific tasks. (1) Removal of unwanted DNA fragments, particularly the selectable marker gene (SMG) for identifying successful genetic transformations, is a primary use (reviewed in Gidoni et al. 2008; Yau and Steward Jr 2013; Kleidon et al. 2020). Selectable marker genes serve no useful purpose after the initial selection phase. The constant presence and expression of SMG can be a metabolic burden to the transformed plant. Due to the small number of effective plant SMGs currently available, there is an advantage to marker removal. Transgenic plants with selectable markers removed have the potential for a future round of genetic transformation with the same SMG (reviewed, see Sang et al. 2013). The presence of SMGs in food and feedstock causes concern among consumers and regulatory agencies. SSR systems can be employed for genetic use restriction technologies (GURTs; also known as "terminator technologies") to protect the intellectual property (IP) of genetically modified (GM) crops using SSR system-mediated site-specific deletion (Kaiser 2000; Sang et al. 2013; Lombardo 2014). (2) Integrating genes of interest (GOI) to a characterized locus for predictable gene expression is a secondary use (reviewed in Srivastava and Gidoni 2010). (3) Simultaneous integration of transgenes and excision of SMG in transgenic plants can be accomplished with two SSR systems (Nandy and Srivastava 2012). (4) SSRs are used to stack transgenes at the same locus *in planta* (Li et al. 2010; Ow 2011; Hou et al. 2014; Srivastava 2019; Pathak and Srivastava 2020). (5) SSRs can serve as genetic switches or tunable genetic circuits to regulate gene expression in eukaryotes. Unidirectional SSR systems, like ϕC31-*att* and Bxb1-*att*, are especially suitable for this application. Gomide et al. (2020) employed both ϕC31 and Bxb1 integrases and their *attB*/*attP* sites in *Arabidopsis* plants to serve as genetic switch controllers to turn a gene on or off. Bernabé-Orts et al. (2020) built a memory switch in tobacco plant (*N. benthamiana*)

to control transcriptional states (on or off) of two genes using components of ϕC3-*att* SSR system.

Among these five, removal of transgenic plant SMGs is the most widely used. Application of SMG excision has moved beyond laboratories into commercial farming. Cre-*lox*-mediated SMG-free Monsanto corn LY038 was one of the first successfully produced and approved for marketing (Ow 2007). However, emergence of novel SSR systems has expanded the repertoire of site-specific genomic engineering *in planta*. Researchers recently generated recombinase-expression lines in maize, using Cre, R, FLPe, phiC31 integrase, or phiC31 excisionase gene for a variety of genetic engineering applications (Cody et al. 2020).

In this chapter, SSR systems are described generally, and plant applications are explored. Recombination outcomes for both bidirectional and unidirectional SSR systems are discussed. A case study using the unidirectional SSR system Bxb1-*att* for autoexcision removal of SMG in tobacco is presented. Finally, the combined use of SSR systems and the novel genetic editing tool CRISPR-Cas system is addressed for next-generation genome manipulation.

35.2 The Site-Specific Recombination (SSR) Systems

35.2.1 The Basics of SSR Systems

The majority of SSR systems are found in bacteria and their virus, bacteriophage. Only a few cases of SSR systems are found in eukaryotes, like yeast. These systems carry out various biological functions, including integration of bacteriophage DNA into host genomes. SSR systems are characterized by these three features: (1) recombination exclusively at specific sequences, known as *attachment sites* (or "recognition sites" and "recombination sites" by some authors); (2) no addition or deletion of DNA bases at recombination sites, known as "conservative" recombination; and (3) strand exchange occurring at small regions (or *core regions*) within the recognition sites.

SSR systems typically fall into two major families according to their mechanisms of catalysis—the *tyrosine* and the *serine* recombinase families. The tyrosine family utilizes a C-terminal catalytic tyrosine to mediate recombination, while the serine family utilizes an N-terminal catalytic serine to mediate recombination (Grindley 1997). Members of the tyrosine recombinase family include the well-studied λ integrase, Cre resolvase, Flp invertase, and many others (Grindley et al. 2006). Examples from the serine recombinase family include the mycobacteriophage Bxb1 integrase and the *Streptomyces* phage ϕC31 integrase.

Recombinase from each SSR system can catalyze site-specific recombination between the two corresponding recognition sites. Sequences of both recognition sites for a specific SSR system can be identical or dissimilar. Recombination between the two recognition sites results in "excision," "integration," "inversion," or "translocation" of DNA depending on orientation and location for the two recognition sites (Fig. 35.1).

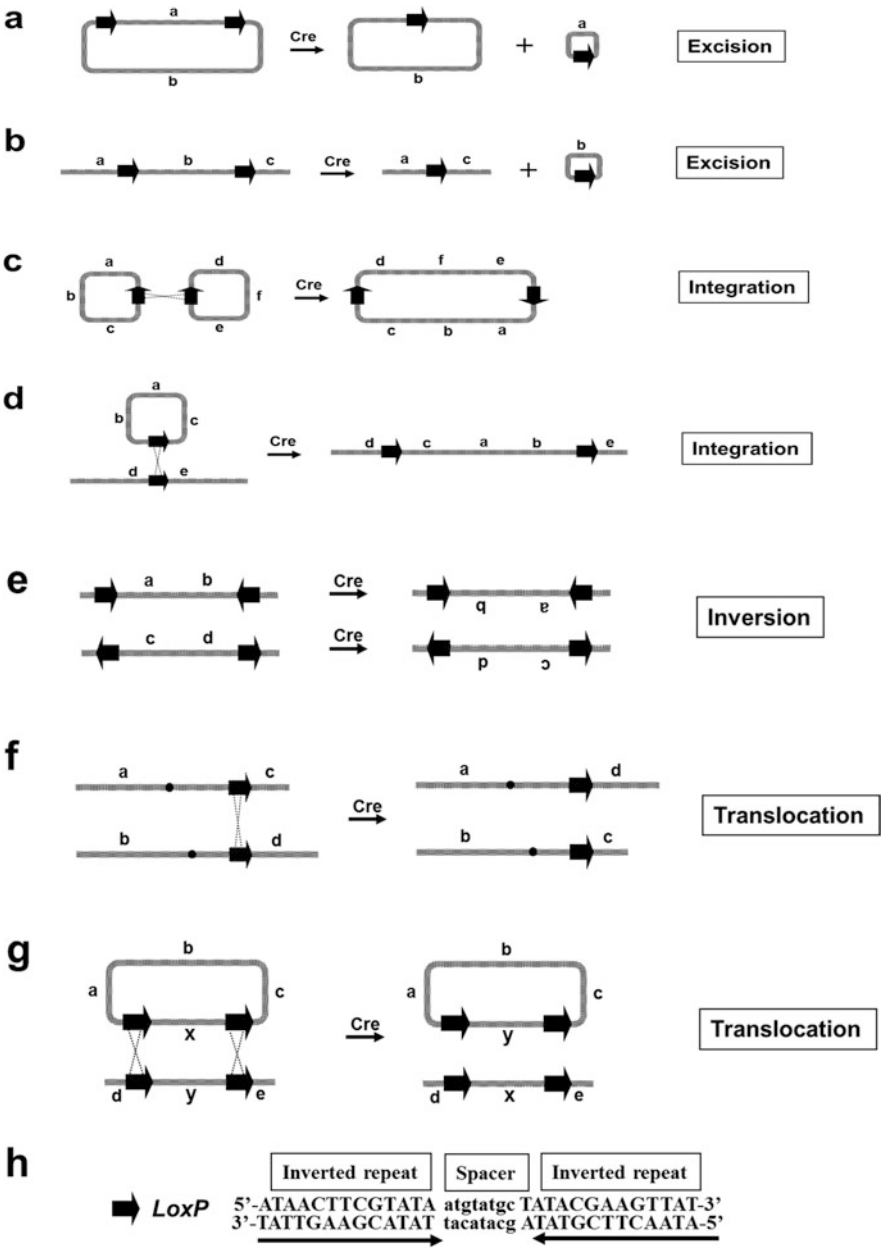

Fig. 35.1 Cre-mediated DNA manipulation. (**a**) DNA fragment *a* flanking two *loxP* sites excised from plasmid. (**b**) DNA fragment *b* flanking two *loxP* sites excised from genomic site. (**c**) Co-integration of two plasmids through *loxP* × *loxP* site-specific recombination. (**d**) Plasmid integrated into a genomic site through *loxP* × *loxP* site-specific recombination. (**e**) Inversion of DNA fragment between two opposite-oriented *loxP* sites. (**f**) Cre-mediated translocation between two chromosomes. (**g**) Exchange of DNA fragments *x* and *y* through recombinase-mediated cassette exchange (RMCE). (**h**) Structure of *loxP* site from Cre-*lox* SSR system

Traditionally, SSR technology manipulated eukaryotic genomes by embedding SSR recognition sites into host genomes before recombination. The common use of *Agrobacterium*-mediating transformation resulted in random insertion of recognition sites. The number and orientation of recognition sites are arranged on the vector to either excise or add a DNA fragment later through site-specific deletion or integration. After sites are transformed into the host genome, successful recombination results in transient or stable expression of a recombinase DNA cassette. Since the recognition cassette is randomly embedded in the host genome, position effect plays a vital role in expression. Experimental results demonstrate that the genomic position of recognition sites determines the efficacy of site-specific recombination (Thomson et al. 2009).

There are numerous SSR systems in nature (Grindley et al. 2006). Recently, approximately 35 new phage integrases active in bacteria have been described (Yang et al. 2014). However, only a few SSR systems have been researched, and even fewer are currently being used for genome manipulation in mammals and plants.

35.2.2 Uni- Vs. Bidirectional SSR Systems

The resulting sequence from two identical recognition sites (e.g., *loxP* × *loxP*) yields the same sequence as *loxP*. This newly formed *loxP* site can again be used as a substrate for Cre-mediated site-specific recombination (Fig. 35.2a). Because of a tendency to catalyze both *integration* and *excision* in the presence of Cre recombinase (Fig. 35.2a), these systems are called *bidirectional* SSR systems. Examples of such SSR systems are Cre-*lox*, FLP-*FRT*, ParA-*MAR* (Gerlitz et al. 1990), and CinH-*R2* (Kholodii 2001).

On the other hand, SSR systems such as serine recombinases ϕC31 or Bxb1 promote unidirectional or irreversible reactions between *attP* and *attB* sites. These differ in sequence and produce two "hybrid sites," *attL* and *attR* (Fig. 35.2b, c). Such SSR systems are termed *unidirectional*. DNA sequences of *attL* and *attR* differ from *attP* and *attB* sequences and do not provide a substrate for their integrases. Once *attR* and *attL* sites are generated, free reversal will not occur in the presence of corresponding recombinase, until a phage-encoded accessory protein or recombination directionality factor (RDF) is provided. For example, the Bxb1-*att* SSR system RDF is gp47 protein (Ghosh et al. 2006, 2008), and the ϕC31-*att* RDF is 27.5 kDa gp3 protein (Khaleel et al. 2011). In the absence of RDF, integrases promote *attP* × *attB* recombination, while in the presence of RDF, the *attP* × *attB* recombination is inhibited, and *attL* × *attR* is stimulated (Ghosh et al. 2006; Khaleel et al. 2011). To date, only members of the tyrosine-recombinase family have shown bidirectional activity. Both the tyrosine and serine recombinase families demonstrate unidirectional function. Some members of the serine recombinase family are dedicated to a deletion reaction not capable of catalyzing inversion or integration reactions (Thomson and Ow 2006).

Bidirectional SSR systems catalyze bidirectional recombination between two identical sites. Figure 35.2a depicts catalysis of Cre protein, shown in the two

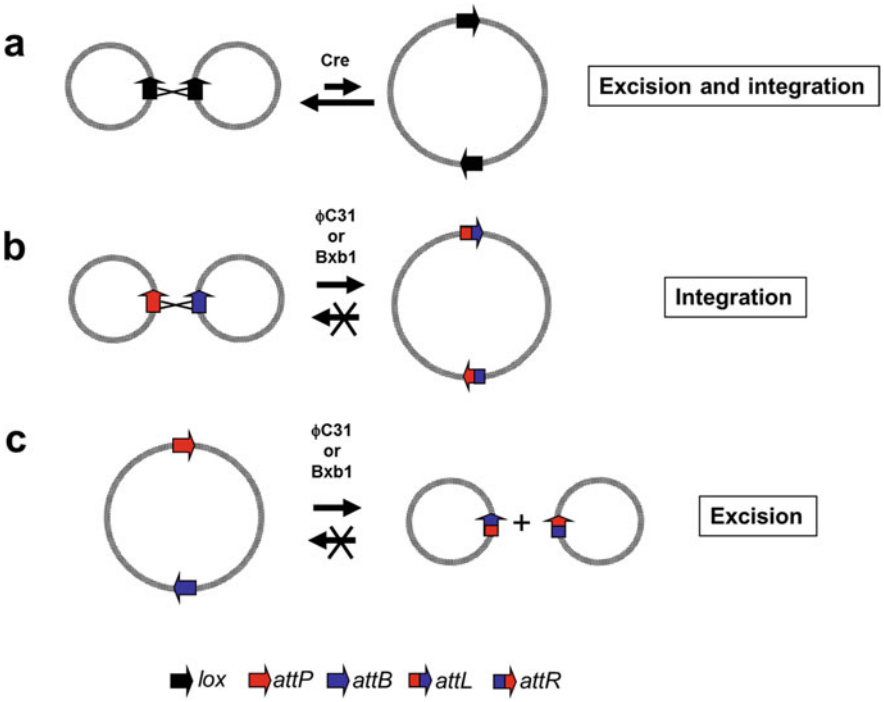

Fig. 35.2 SSR system-mediated DNA integration and excision. (**a**) Bidirectional SSR system (Cre-*lox*)-mediated DNA integration and excision. (**b**) Unidirectional SSR system (ϕC31-*att* or Bxb1-*att*)-mediated two circular DNA co-integration. (**c**) Unidirectional SSR system (ϕC31-*att* or Bxb1-*att*)-mediated DNA excision. Black arrow: *loxP* site. Black arrow: *loxP* site; red arrow: *attP* site; blue arrow: *attB* site; *attL* and *attR*: hybrid sites

small circular DNA molecules each carrying a *loxP* site that co-integrate to form a larger, circular DNA molecule with two *loxP* sites similarly oriented. Continuous presence of Cre protein can perform intramolecular excision to convert the circular molecule back into two smaller DNA molecules through site-specific recombination of the two *loxP* sites. This occurs because the deletion reaction is kinetically favorable to the integration reaction. This system is most useful for carrying out site-specific deletions efficiently (Nagy 2000). In contrast, unidirectional recombinase reactions are irreversible following integration. The transgene is trapped, and the reaction cannot be reversed, as hybrid sites are no longer recognized by the recombinase. This renders these recombinase options especially useful for catalyzing integration reactions. Bidirectional SSR systems are also available for conducting transgene integration but require extra measures to stabilize integrated genes. These measures increase integration efficiency of bidirectional SSR systems in comparison to unidirectional systems. Some measures include the following:

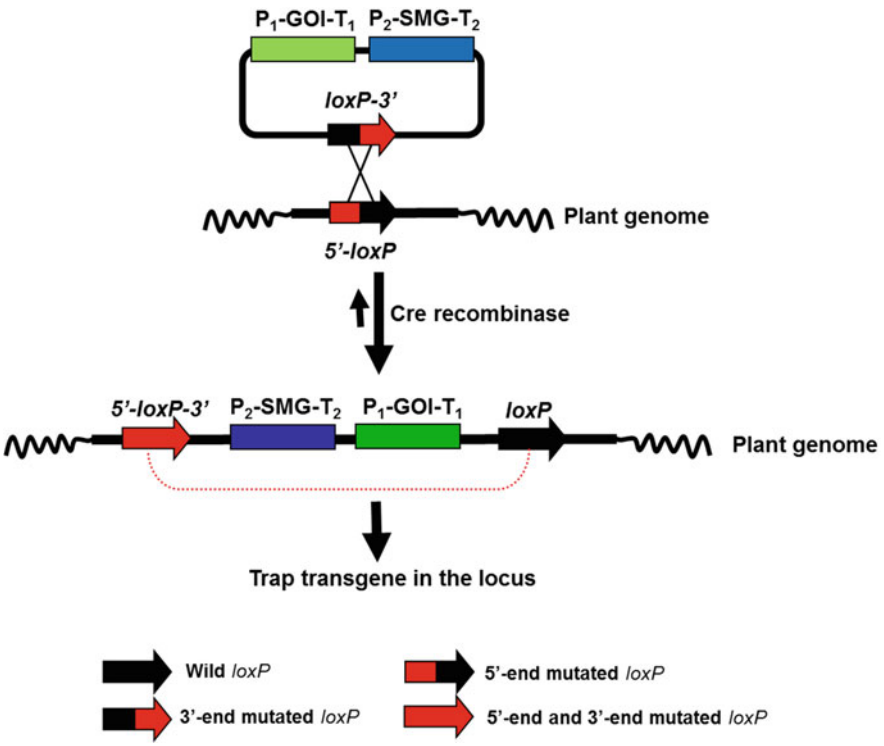

Fig. 35.3 Cre-mediated site-specific integration with mutant *lox* sites. Integrated gene is stably trapped through recombination of two mutated *lox* sites (***loxP-3′*** and ***5′-loxP***), resulting in doubly mutated 5′-*loxP*-3′ and wild-type *loxP* sites, which reduces the reversible recombination reaction significantly. *GOI* gene of interest, *SMG* selectable marker gene

1. The use of partial mutation on the two identical recognition sites to prevent reversible recombination (Fig. 35.3). The brief history of using this strategy *in planta* is described in other reviews (Srivastava and Gidoni 2010; Nandy et al. 2015; Srivastava and Thomson 2016).
2. The use of limited or transient recombinase expression for temporary usefulness. The recombinases are provided temporarily. This is an option when the recombinase gene does not integrate the plant genome or when the *cre*-expression construct present in the target genome is displaced to abolish post-integration Cre activity (Albert et al. 1995).

Although the strategies mentioned above can increase efficacy of bidirectional SSR systems, no known strategy can reach the efficiency of unidirectional SSR systems for trapping an integrated gene. For example, the strategy (1) described above has been studied using reversible recombination (Fig. 35.3). The product derived through Cre-mediated DNA fragment excision between a wild-type *loxP* and a doubly mutated *lox* site (*lox*75/76) has been observed in rice (Srivastava et al.

2004). However, a small number of reversible recombination reactions between wild-type *loxP* and the doubly mutated *lox* site were observed. Reversibility of the inserted transgene reduces integration frequency.

Bidirectional SSR systems (e.g., Cre-*lox*) are also not suitable for iterative integration of multiple genes, due to recombination reversibility (Nandy et al. 2015). Specific strategies are needed to ensure integrated gene trapping when bidirectional SSR systems are used for iterative gene integration, known as transgene stacking. For example, Srivastava's group demonstrated the combined use of three systems (Cre-*lox*, ZFN, and I-SceI) for Cre-mediated transgene stacking at the same locus in rice (Nandy et al. 2015).

35.2.3 Unidirectional Bxb1-*att* SSR Systems

Mycobacteriophage Bxb1 is a temperate phage of *Mycobacterium smegmatis* isolated at Albert Einstein College of Medicine in 1990. The Mycobacteriophage Database contains additional details concerning this phage (Fig. 35.4a) (http://phagesdb.org/phages/Bxb1/). The genome size of Bxb1 mycobacteriophage is about 5 kb in length (GenBank AF271693). Bxb1 integrase catalyzes integration of Bxb1 phage genome into the *Mycobacterium smegmatis* chromosome through recombination of phage *attP* site and bacterial chromosomal *attB* site. Both *attP* and *attB* sites share an 8-bp common core (5′-GCGGTCTC-3′) (Fig. 35.4b), allowing DNA strand exchange during recombination. Recombination between *attP* and *attB* sites generates two hybrid sites, *attL* and *attR*. Hybrid sites flank inserted prophage genomic DNA. Bxb1 integrase belongs to the "large" serine recombinase group, named for their large size (300–500 amino acids). Previously discovered serine recombinases contain only 200 amino acids (Stark 2017).

Mycobacteriophage Bxb1 is a unidirectional SSR system and a powerful eukaryotic genome manipulation tool in several species, including some plants (Xu et al. 2013). Study results show Bxb1-*att* system is efficient *in planta* for DNA excision and integration. The system works well in plants with small genomes, including model plant *Arabidopsis* and more complex genomes like wheat. Bxb1 was first tested for functionality *in planta* using plant protoplasts (Yau et al. 2011). In this study, Bxb1 integrase facilitates site-specific integration. A plasmid (or *integration* plasmid) carrying Bxb1 *attB* sites integrates a transformed tobacco genome pre-embedded with the Bxb1 *attP* site. Integrants were obtained lacking Bxb1 recombinase gene detection, indicating transient Bxb1 recombinase activity. One of these integrants was used in a gene stacking study for further exploration. Later, Bxb1 integrase was used to successfully stack three GOIs (*gus*, *gfp*, *luc*) back-to-back at a tobacco genomic locus by the same research group (Hou et al. 2014). Functionality of Bxb1-*att*-mediated site-specific excision also confirmed in *Arabidopsis* and wheat (Thomson et al. 2012; Blechl et al. 2012). Final excised cassette was transmitted to the next generation of *Arabidopsis* plants. In wheat, Bxb1 gene demonstrated heritability to progeny. In 2014, Shao et al. demonstrated Bxb1-*att* capable of site-specific excision in the tobacco plastid genome (Shao et al. 2014).

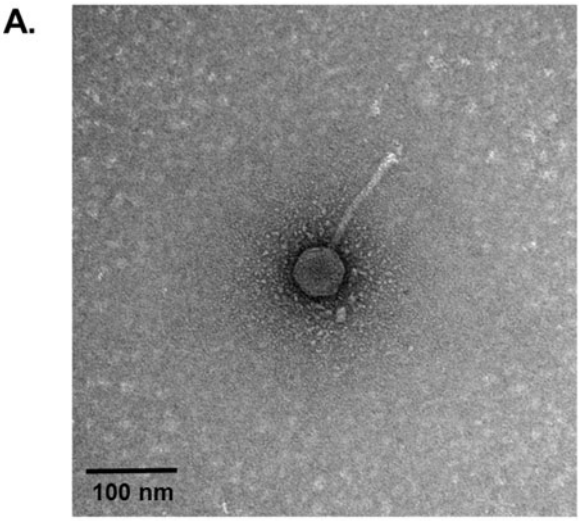

Fig. 35.4 *Mycobacterium* phage Bxb1-*att* system. (**a**) *Mycobacterium* phage Bxb1. Direct Mag: 140,000×. Image obtained from (https://phagesdb.org/phages/Bxb1/) with permission. (**b**) Sequence of the 51 bp *attP* and 42 bp *attB* Bxb1 recognition sites, where the minimal required sequence is underlined and the two-nucleotide **gt** core region of crossover is in bold. Both *attB* and *attP* sites contain an 8-bp common core sequence (-GCGGTCTC-), within which strand exchange occurs (Kim et al. 2003). (Image **b** reproduced from Thomson, J.G., Chan, R., Smith, J. et al. (2012) *The Bxb1 recombination system demonstrates heritable transmission of site-specific excision in Arabidopsis*. BMC Biotechnol 12: 9(2012). The article is an open access article distributed under the terms of the Creative Commons CC BY license, which permits unrestricted use, distribution, and reproduction in any medium)

35.2.4 SSR Systems Used for Plant Research

Since SSR systems carry out recombination of two DNA fragments (recognition sites), researchers can manipulate eukaryotic genomes by incorporating SSR systems into genetic transformation events. Sauer and Henderson were first to demonstrate Cre protein promoting DNA recombination at two *loxP* sites stably residing in a mammalian chromosome (Sauer and Henderson 1989). Since that time, different SSR systems have been discovered and found functional in both

mammalian and plant cells. *In planta*, Ow's group first provided evidence of Cre-*lox* SSR system functionality in tobacco cells (Dale and Ow 1991). Afterward, several SSR systems applications have been described for DNA deletion (such as SMG removal) or integration, either in nuclear genomes or plastid genomes in plants. These systems are Cre-*lox* (Dale and Ow 1991), R-*RS* (Onouchi et al. 1991), Gin-*gix* (Maeser and Kahmann 1991), FLP-*FRT* (Lyznik et al. 1993), λ-*att* (Suttie et al. 2008), HK022-*att* (Gottfried et al. 2005), ϕC31-*att* ((plastid genome, integration) Lutz et al. 2004; (nuclear genome, excision) Thomson et al. 2010), β-*six* (Grønlund et al. 2007), ParA-*MAR* ((nuclear genome, excision) Thomson et al. 2009; (nuclear genome, excision) Zhou et al. 2012; (plastid genome, excision) Shao et al. 2017), Bxb1-*att* ((nuclear genome, integration) Yau et al. 2011; (nuclear genome, excision) Thomson et al. 2012; (nuclear genome, excision) Blechl et al. 2012; (plastid genome, excision) Shao et al. 2014; (nuclear genome, integration) Hou et al. 2014; (nuclear genome, integration) Li et al. 2016), and CinH-*R2* ((nuclear genome, excision) Moon et al. 2011; (nuclear genome, excision) Zhou et al. 2012; (plastid genome, excision) Shao et al. 2017). A history of SSR system development for plant genome manipulation from the early days of SMG removal to more recent transgene stacking has been described in a review paper entitled "The Long Road to Recombinase-Mediated Plant Transformation" (Ow 2016).

In planta, a majority of publications demonstrate the use of SSR for SMG removal, but some studies reported use for integrating transgenes into a predetermined genomic location. SSR systems successfully used for transgene integration include Cre-*lox* (Albert et al. 1995; Vergunst et al. 1998; Pathak and Srivastava 2020), ϕC31-*att* (Lutz et al. 2004), R-*RS* (Nanto et al. 2005), FLP-*FRT* (Li et al. 2009, 2010; Nandy and Srivastava 2011, 2012), and Bxb1-*att* (Yau et al. 2011; Hou et al. 2014; Li et al. 2016). Integrated genes targeting the same chromosomal locus in different lines have demonstrated similar expression levels (Day et al. 2000; Nandy and Srivastava 2012; Hou et al. 2014).

35.2.5 Other Applications of SSR Technology

Beyond genome manipulation, SSR systems are also used for other applications. Three examples are (1) cloning tool, (2) DNA-assembly tool, and (3) a rewriteable digital data-recording device. SSR technology has served as a valuable *cloning tool* enabling DNA circuits to assemble on a vector efficiently, a technique called "recombinational cloning (RC)" (Hartley et al. 2000). A well-known case is the GATEWAY® technology using λ-*att* system to efficiently shuffle genes to expression systems for gene function studies (Liang et al. 2013). As a *DNA-assembly tool*, researchers have used ϕC31-*att* plus Cre-*lox* and *Streptomyces* phage ϕBT1 SSR systems to construct large transgenes, 150 kb and 400 kb, in vivo and in human minichromosome, respectively (Dafhnis-Calas et al. 2005; Xu et al. 2007). Xu et al. assembled a 400-kb transgene from 80-kb mouse DNA through five cycles of iterative SSR-mediated integration (Xu et al. 2007). ϕC31-*att* system was used in the serine integrase recombinational assembly (SIRA) method for rapid and effective

assembly of a metabolic gene set involved in the bacterial carotenoid biosynthesis pathway. This paved the way for others to facilitate reconstitution of many genes involved in transgenic plant biosynthetic pathways. Random assembly of gene orders along these pathways allowed for evaluation of gene product outputs. This method allows researchers to conduct rapid pathway optimization (Colloms et al. 2014). The *rewritable digital data-recording device* harnesses the recombinase's unidirectional ability to "invert or flip" sequences. Bxb1 is being used by bioengineers to generate rewritable digital data stored in cells as part of research involving cancer and aging (Bonnet et al. 2012). Researchers reapply the recombinase to flip specific DNA sequences back and forth at will and visualize switch events through the use of GFP (green color) or RFP (red color) fluorescence. This technology may 1 day give scientists the ability to turn off cell division by stopping cell division cycles. The goal of switching off after reaching a certain threshold is prevention of multiple cancerous mutations (Bonnet et al. 2012).

SSR systems can also be used with novel genome-editing tools such as ZFN, TALEN, and CRISPR-Cas9 for precise gene integration/stacking at engineered sites or SMG deletion. Scientists in animal studies have combined the use of SSR and CRISPR-Cas technologies for genome manipulation, but few studies have emerged in the plant field (Yang et al. 2017). Srivastava (2019) recently described a gene stacking scheme using Cre-*lox* SSR system gene integration into a precise genomic sites and zinc finger nuclease (ZFN) to delete SMG in plants. However, the same group reported potential ZFN toxicity in rice. Inducible expression of CCR5-ZFN did not result in detectable excisions, although it transmitted stably to progeny (Pathak et al. 2019). Therefore, researchers should be careful in choosing an appropriate nuclease for use in any specific plant species.

35.3 Autoexcision of SMG from Potential Energy Crop

35.3.1 Autoexcision Mechanism

Autoexcision is an attractive method for simultaneous removal of several genes in a molecular cassette embedded in a genome. For example, it can be used to delete both the SMG and recombinase gene after their expression is no longer required. This accelerates the process of generating SMG-free transgenic plants, by removing the need for generating another generation of plants to segregate away the SMG and recombinase gene. The primary feature of autoexcision cassette design is placement of the recombinase gene under the control of an "inducible" promoter, while the selectable marker gene is under the control of a separate promoter placed between the two recombination sites (e.g., *loxP* site of Cre-*lox* system) in direct orientation. In this scenario, the gene of interest is inserted outside the recombination sites (Fig. 35.5a). Under controlled activation of the recombinase gene, both the recombinase gene and SMG are eliminated (Fig. 35.5a). The recombinase gene promoter can be inducible or tissue-specific. PCR can be used to evaluate an autoexcision event, by using primers designed outside of the two recombination

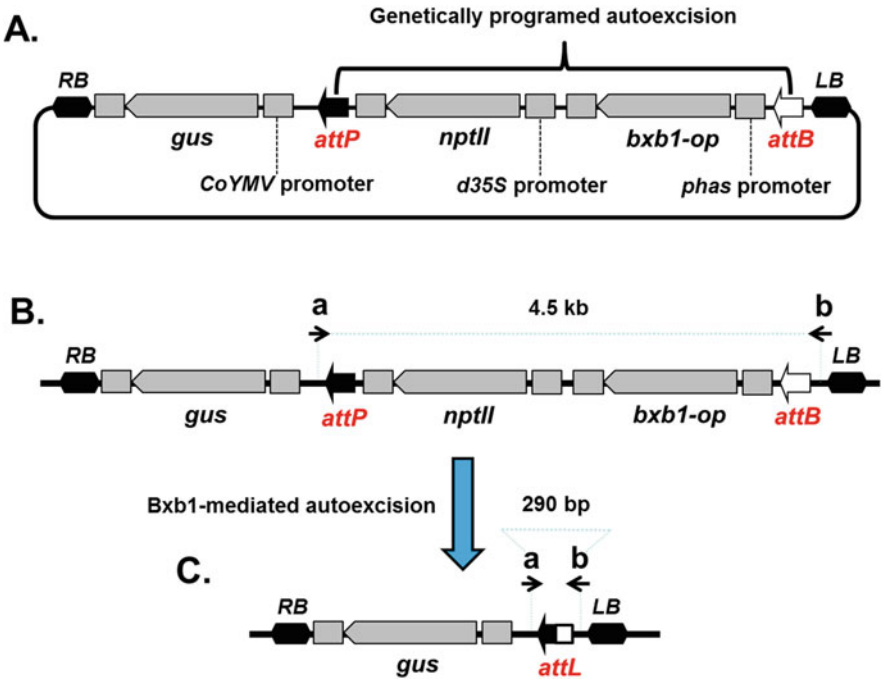

Fig. 35.5 Plant binary vector used in this case study. (**a**) T-DNA structure of plant binary vector pRB140-Bxb1-op. T-DNA consists of β-glucuronidase (*gus*) gene driven by the CoYMV promoter and an autoexcision cassette designed to delete a selectable marker gene (*nptII*) and Bxb1 site-specific recombinase gene (*bxb1-op*, "op": gene codon optimized). *nptII* gene is under control of the double CaMV 35S promoter (d35S). Seed promoter *phas* is used to drive Bxb1 recombinase gene. (**b**) PCR is used to evaluate autoexcision events. Primers used for genotyping outside Bxb1 *attP* and *attB* sites are marked as **a** and **b**. (**c**) The T-DNA configuration generated after autoexcision of *attP* and *attB* flanked DNA fragment. PCR product of a 290-bp band is expected if autoexcision event occurs. *LB* T-DNA left border, *RB* T-DNA right border, *bxb1-op* codon-optimized *bxb1* recombinase gene, *nptII* neomycin phosphotransferase II gene, *gus* β-glucuronidase gene, *attL* footprint of a hybrid site after *attP* × *attB* recombination. Diagram not to scale

sites (Fig. 35.5b). Two examples of tissue-specific promoter are pollen promoter and seed promoter. With pollen promoter, autoexcision of unwanted DNA occurs in the pollen of transformed plants, preventing SMG escape through pollen. Inducible promoters can support regulation by presence of heat (Wang et al. 2005; Du et al. 2019), cold, or chemicals (Hare and Chua 2002).

35.3.2 Case Study: Bxb1-*att*-Mediated Autoexcision in Tobacco Plants

To assess the feasibility of using Bxb1-*att* system and a seed promoter for autoexcision in tobacco plant, a codon-optimized seed promoter is used to drive

Bxb1 integrase gene (Yau et al. 2012; Easterling 2014). A study construct is built and placed in *Agrobacterium* for tobacco plant genetic transformation. GUS-positive putative T_0 transgenic lines are grown in greenhouse soil to harvest T_1 seeds. T_1 seeds of each putative transgenic line are used for kanamycin selection to evaluate transgene copy numbers. Both T_1 seeds and T_1 plantlets are also used for autoexcision evaluation through junction PCR. Subsequent sequencing verification of PCR products is conducted to check for the presence of site-specific recombination footprint via the hybrid site *attL*.

35.3.2.1 Materials and Methods

Construction of Binary Vectors pRB140-Bxb1-op and *Agrobacterium* Strain

Principles and methods of molecular manipulation, such as plasmid isolation, restriction enzyme digestion, DNA fragment ligations, and transformation of *E. Coli*, are conducted as described by Green and Sambrook (2012) and manufacturer instructions. Binary vector pRB140-Bxb1-op (Fig. 35.6) was built for this study. Three plasmids were obtained before the construction of pRB140-Bxb1-op: (1) **pCambia2300** (CAMBIA, Canberra, Australia; https://cambia.org/),

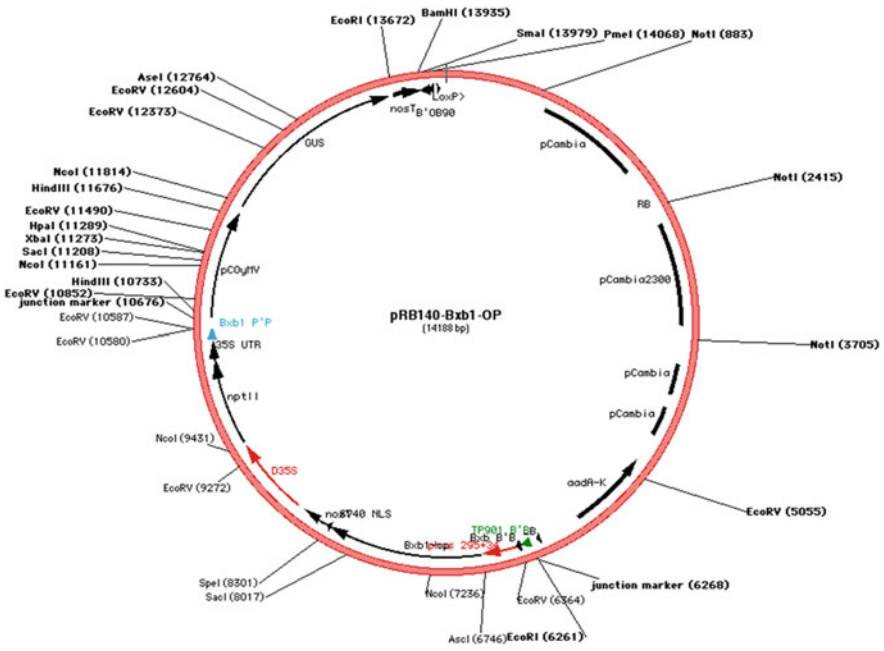

Fig. 35.6 Final construct of pRB140-Bxb1-op vector for use in *Agrobacterium*-mediated tobacco genetic transformation in this study. Plant binary vector pCambia 2300 was used as backbone. Unique restriction sites are indicated. *d35S* double 35S promoter. *Bxb1-op* codon-optimized Bxb1 recombinase gene. *Bxb1 P′P* Bxb1 *attP* site. *Bxb1 B′B* Bxb1 *attB* site. *npII* plant selection marker for kanamycin resistance

(2) **pRB140** (a gift of Dr. Robert Blanvillain; https://www.researchgate.net/profile/Robert-Blanvillain/publications), and (3) **pUC57-phas-Bxb1-op** (customer-ordered and synthesized by GenScript USA Inc. (Piscataway, NJ, USA)). **pUC57-phas-Bxb1-op** contains a synthesized DNA fragment <TP901$_{attP}$-Bxb1$_{attP}$-Phas-Bxb1-op-T$_{nos}$- Bxb1$_{attB}$- TP901$_{attB}$ > as an insert, which was cloned into pUC57 backbone. Recombinase bxb1 gene is codon-optimized (Fig. 35.7). TP901$_{attP}$, TP901$_{attB}$, Bxb1$_{attP}$, and Bxb1$_{attB}$ were recognition sites for TP901-*att* and Bxb1-*att* site-specific recombination systems, respectively.

A seed-specific promoter has been chosen for the Bxb1 recombinase gene in this case study project. The phaseolin (*phas*) gene is native to the common bean plant, *Phaseolus vulgaris* (Bustos et al. 1991). In nature, it encodes the major seed storage protein, β-phaseolin. As a promoter, phas has proven sufficient for yielding high levels of seed-specific expression (Bustos et al. 1991; Odell et al. 1994; Van Der Geest et al. 1995). The 295-bp region incorporates into the vector proximal to the transcription start site (*−295phas*). The wild-type *bxb1* coding region was codon-optimized for adaptive expression in tobacco plants. A nuclear localization signal (NLS) added at Bxb1 recombinase gene terminus to facilitate nucleus entry for site-specific recombination to occur.

Fig. 35.7 Side-by-side comparison of codon-optimized *bxb1* (Bxb1-op in Fig. 35.6) gene sequence with wild-type *bxb1* ORF. Codon-optimized *bxb1* is listed on each top line with Bxb1 ORF listed on each bottom line

Construction Steps

1. Construction of **pYW-Bxb1-op-10:** Restriction fragment d35S-*npt*II-T_{nos} is obtained from **pCambia2300** vector by *Ase*I digestion. After *Ase*I cohesive ends blunted with large Klenow fragment (Cat. No. M0210S, New England Biolabs Inc., USA), fragment is sub-cloned into *Pme*I site between NOS terminus (T_{nos}) and $Bxb1_{attP}$ of **pUC57-phas-Bxb1-op** through blunt-end ligation resulting in plasmid **pYW-Bxb1-op-10**.
2. Constructing **pRB140-GUS**: *Spe*I-*Pst*I fragment from pRB140 containing *nptII* expression cassette was removed. *Spe*I and *Pst*I ends of pRB140 were blunted with Klenow and ligated together with T4-DNA ligase, resulting in plasmid pRB140-GUS.
   To avoid repeat use of d35S promoter in a vector, a *Commelina yellow mottle virus* (CoYMV) promoter was employed to drive d35S promoter. Multiple copies of d35S promoters with identical sequences in a T-DNA might induce promoter silencing in the plants. From a study, while CaMV 35S promoter is active in most cell types, the CoYMV promoter is primarily active at tobacco tissues such as the phloem, the phloem-associated cells, and the axial parenchyma of the roots, stems, leaves, and flowers (Medberry et al. 1992).
3. Constructing **pRB140-Bxb1-op**: *Hpa*I blunt-end fragment ($TP901_{attP}$-$Bxb1_{attP}$-Phas-Bxb1-op-T_{nos}-d35S-*npt*II-T_{nos} $Bxb1_{attB}$- $TP901_{attB}$) was cut from plasmid pYW-Bxb1-op-10 and ligated to *Pml*I site of **pRB140-GUS** (backbone-LB-(*Pml*I)-CoYMV-GUS-T_{nos}-RB-backbone) to form the final plasmid: pRB140-Bxb1-op. Final pRB140-Bxb1-op vector was rechecked with different restriction enzyme digestions.

Agrobacterium tumefaciens LBA4404 is used to deliver binary vectors pRB140-Bxb1-op for transformation in this study. The vector electroporated into ElectroMax™ *Agrobacterium tumefaciens* LBA4404 competent cells (Cat. No. 18313–015, Invitrogen, USA) using an electroporator (Multiporator®, Eppendorf). Forty microliter LBA4404 competent cells and 3 μL vectors were mixed and transferred into a 1-mm-gap electroporation cuvette (Cat. No. 94000100–5, Eppendorf, USA). Electroporation was carried out using a manufacturer preloaded program designated for bacterial electroporation (2000 V, time constant: 5.0 ms). One mL LB liquid medium was added to electroporation cuvette and mixed with electroporated competent cells. Th mixture was then transferred to Falcon® 14-mL polypropylene round-bottom tube (Becton Dickinson Labware, USA) and incubated at 30 °C for 3 h with 225-rpm shaking. After 3 h, bacterial culture of 20 μL, 50 μL, or 100 μL spread onto LB + streptomycin (100 μg/mL) + kanamycin (50 μg/mL) plates. Streptomycin used to select *A. tumefaciens* LBA4404 cells disarmed Ti pAL4404, and kanamycin was used to select pC35. BNK.2. Plates were placed in a 30 °C incubator for 2–3 days to produce colonies. All antibiotics used in this research case study were sterilized with a sterile syringe filter

containing a 0.2 μm cellulose acetate membrane (Cat. No. 28145–477, VWR, Batavia, IL, USA) (Fig. 35.7).

35.3.2.2 Plant Materials and Tissue Culture Conditions

Wild-type tobacco (*Nicotiana tabacum* L.) cultivar "Petit Havana" mutant SR1 used to conduct genetic transformation through leaf disk transformation method. Wild-type tobacco seeds were sterilized with 70% ethanol for 2 min, followed by bleach (sodium hypochlorite) (30% (v/v), added drops of Triton-X 100) for 20 min, and washed thoroughly with sterile distilled water. Sterilized seeds were germinated on MS medium, containing MS mineral salts (Murashige and Skoog 1962; Cat. No. M524, *Phyto*Technology Lab), 3% (w/v) sucrose, 1× Gamborg's vitamin solution (Cat. No. G1019, Sigma-Aldrich), and 0.8% agar. In the present experiment, MS medium was supplemented with B5 vitamins (Gamborg et al. 1976), containing 100-fold thiamine concentration compared to original MS concentration. B5 vitamin-based medium has been reported to enhance growth and solasodine production in hairy root cultures of *Solanum khasianum* Clarke (Jacob and Malpathak 2005) and carrot (Yau and Wang 2012). Plates were sealed with a medical gas-permeable tape (Micropore™ surgical tape; 3 M Health Care, USA) and placed in a 25 °C growth chamber with 16-h photoperiod and 8-h dark.

35.3.2.3 *Agrobacterium*-Mediated Genetic Transformation of Tobacco

Single colonies from plates were selected using a 15-cm sterile cotton-tipped applicator (Puritan Medical Products Company, Guilford, Maine, USA) and streaked on LB plates containing both streptomycin and kanamycin antibiotics. Plates were stored at 30 °C for 1 day to allow for colony growth. For tobacco genetic transformation, bacteria scraped from plates with a sterile inoculation loop and suspended in 35 mL transformation medium (MS mineral salts, 3% (w/v) sucrose, 1× Gamborg's vitamin solution, 3 μg/mL 6-benzylaminopurine hydrochloride (Cat. No. B5920, Sigma-Aldrich), and 100 μM Acetosyringone (AS) (Cat. No. D134406, Sigma-Aldrich)). Transformation medium adjusted to pH 5.8 with 0.1 N KOH or HCl and autoclaved at 121 °C for 20 min. AS was dissolved in 70% ethanol and added to cooled autoclaved medium.

Wild-type leaves were cut and placed in a sterile Petri dish. Each 1-cm-square leaf piece was cut in an ESCO Horizontal Airstream® Laminar flow hood (ESCO, USA).

After inoculation, cotyledons (on leftover stem) were placed abaxial on cocultivation medium (transformation medium solidified with 0.8% agar (Cat. No. A7921, Sigma)) for 3 days in dark, and subsequently transferred to selection medium ("transformation medium" + cefotaxime/carbenicillin (500 μg/mL) + kanamycin (100 μg/mL) and solidified with 0.8% agar). The mixture of 50% cefotaxime (Cat. No. C380, *Phyto*technology Lab., USA) and 50% carbenicillin (Cat. No. C346, *Phyto*technology Lab., USA) was used to remove *Agrobacterium*. Plates were sealed with a 3 M Micropore™ tape and placed in growth chamber with 16-h light/8-h dark photoperiod. Subculturing was carried out every 2 weeks.

Putative transgenic shoots 1 cm in length were cut at base from each explant and transferred to rooting medium (MS mineral salts, 3% (w/v) sucrose, 1× Gamborg's

vitamin solution, 0.8% agar) supplemented with 100 μg/mL kanamycin and 400 μg/mL of mixture of cefotaxime and carbenicillin. Rooted plants were allowed to grow to 5 cm in Magenta® plant tissue boxes and transferred to soil.

35.3.2.4 Kanamycin Selection of T_1 and T_2 Seedlings

T_1 seeds derived from kanamycin-resistant T_0 putative transgenic lines or T_2 seeds from T_1 transgenic lines were sterilized with ethanol and bleach and placed on germination medium (MS mineral salts, 3% (w/v) sucrose, 1× Gamborg's vitamin solution) supplemented with 100 μg/mL kanamycin and 200 μg/mL of mixture of cefotaxime and carbenicillin. Plates were placed in growth chamber with 16-h light/8-h dark photoperiod. Seedlings were displaying stunted growth, pale green to yellowish leaves, and inhibition of the main root extension considered susceptible to kanamycin, while seedlings with healthy green leaves and roots were considered kanamycin resistant.

35.3.2.5 Histochemical GUS Assay

Putative transgenic lines and controls were tested for β-glucuronidase (GUS) expression as described by Jefferson et al. (1987). GUS was assayed in seeds by briefly crushing to allow GUS solution to pass through seed coat. Prepared seeds or leaf discs were placed in wells of 96-well plate containing GUS-staining solution (1 mM 5-bromo-4-chloro-3-indoxyl-β-D-glucuronide (X-gluc)) (Gold BioTechnology, Inc., St. Louis, MO, USA), 100 mM sodium phosphate buffer pH 7.0, 0.5 mM potassium ferricyanide, 0.5 mM potassium ferrocyanide, and 0.1% Triton X-100). After a 10-min vacuum filtration, the plate was incubated overnight at 37 °C. To check GUS staining, the chlorophyll of the leaf tissue was removed by repeated washing in 70% ethanol. Chlorophyll interferes with observation of blue-colored stain. Stained leaf tissues were examined under a dissecting microscope and scored for blue coloration.

35.3.2.6 Genomic DNA Isolation

Forty seeds or a portion (a quarter of the size of a 1.5-mL microcentrifuge) of each leaf were excised from putative transgenic plants or controls in Megenta® boxes harvested into 1.5-mL microcentrifuge tubes. Four hundred μL of grinding buffer (200 mM Tris-HCl, pH 5.7, 250 mM NaCl, 25 mM EDTA, and 0.5% SDS) was added to each tube, and the product was ground with a Kontes Pellet Pestle® (VWR, Batavia, IL, USA) driven by an overhead stirrer (Cat. No. 2572101 *IKA Works*, *Inc.*, USA). Ground samples were centrifuged for 5 min at maximum speed (16,800 × *g*) with an Eppendorf benchtop centrifuge (centrifuge model 5418). 300 μL of supernatant was transferred to a new microcentrifuge tube, and 300 μg/mL of isopropanol was added to precipitate genomic DNA. After inverting several times, the mixture was centrifuged for an additional 15 min. Supernatant was discarded, and 70% ethanol was added to wash DNA pellet. Ethanol was discarded after washing, and microcentrifuge tubes containing DNA samples were allowed to air-dry 20 min before being resuspended in 50 μL of H_2O. For seeds, further purification was performed with a QIAquick® PCR purification kit (Cat. No. 28104, Qiagen,

Valencia, CA, USA). Concentrations of eluted DNA samples were measured using a NanoDrop2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

35.3.2.7 PCR Analysis

Isolated genomic DNA from T_1 seeds and T_1 or T_2 leaf tissues of putative transformants and wild-types was used for junction PCR analysis to identify site-specific excision. Primers were designed and purchased from Invitrogen (USA). A list of primers can be seen in Fig. 35.8. To detect excision event, primer **a** ("deletion 5"; forward primer): 5′- CTT TTT GCT TTT TTT GCC AAA GCT TTC TTC CG-3′; primer **b** ("CoYMV.rev-1"; reverse primer): 5′-TGG GGC TGA AGC TTG ATT TTT GTA CA-3′; or primer **c** ("CoYMV.rev-3"; reverse primer): 5′- AGG TGG TAA CTG AAG ATC AGG GAG ATT-3′ was used. Promega Go*Taq*® Flexi DNA polymerase kit was used for amplification. Each PCR reaction contains 3 µl (approximately 300 ng) of genomic DNA, 2 µL 2.5 mM dNTPs, 2 µL 25 mM $MgCl_2$, 5 µL 5× PCR buffer, 1 µL of each primer (10 µM), 0.12 µL polymerase, and autoclaved water for a total volume of 25 µL. Thermocycle program was used as an initial denaturation at 94 °C for 4 min, followed by 35 cycles of 94 °C (30 s), 65 °C (30 s), and 72 °C (1 min 20 s) and a final extension step at 72 °C for 2 min. A 290-bp PCR product is expected if primers **a** ("deletion 5"; forward primer) and **b** ("CoYMV.rev-1'; reverse primer) were used. A 500-bp PCR product is expected if primers **a** and **c** are used. For PCR amplification between promoter CoYMV and nptII gene, primer **a** ("deletion 5"; forward primer) and primer GUS-4 (reverse primer: 5-′-CGTAATGAGTGACCGCATCGAAACG-3′) were used, to produce a PCR band approximately 1.6 kb in size (see Fig. 35.8). To amplify *nptII* gene, specific primers nptII-F: 5′-ATGATTGAACAAGATGGATTG-3′ (forward primer) and

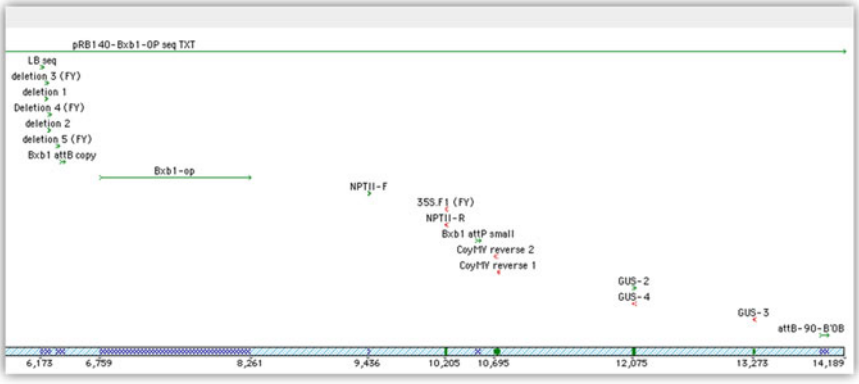

Fig. 35.8 Map of primer loci in T-DNA of binary vector pRB140-Bxb1-op. Primers used to evaluate autoexcision events in transgenic tobacco plants. Forward primers (green arrow heads) or reverse primers (red arrow heads) are indicated with orientation of arrow heads. The map was constructed with a Sequencher® DNA sequence analysis software

nptII-R: 5′-GAAGAACTC GTCAAGAAGGCGAT-3′ (reverse primer) were designed and purchased from Invitrogen (USA).

All PCR performed on Eppendorf's MasterCycler Gradient® PCR machine (Eppendorf, USA). PCR products were separated on 1% TAE agarose gel (Cat. No. 820723, MP Biomedicals, USA) stained with ethidium bromide (Cat. No. E3050, Technova, USA). Gel was photographed with a GelDoc-It™ imaging system (Ultra-Violet Products LLC., USA).

35.3.2.8 Gel Extraction and Sequencing

Bands of junction PCR product (290 bp) were excised from agarose gel and purified with QIAquick® Gel Extraction Kit (Qiagen) following manufacturer instructions. DNA purity and concentration were measured using a NanoDrop2000 spectrophotometer and directly followed by sequencing steps. Primers **a** ("deletion 5") and **b** ("CoYMV.rev-1") were used for sequencing. A mixture of 10 ng purified DNA fragment and 10 ng primer was placed in eight-strip PCR tubes and sent to Genewiz Inc. (South Plainfield, NJ, USA) for sequencing. These sequencing results was analyzed with Sequencher® software (Gene Codes Corporation, Ann Arbor, MI, USA; http://www.genecodes.com/) and Basic Local Alignment Search Tool (BLAST) from the National Center for Biotechnology Information (NCBI).

35.3.3 Results

35.3.3.1 T_0 Putative Transgenic Lines and GUS Staining

One hundred twelve T_0 putative transgenic plants were derived from genetic transformation (with binary vector pRB140-Bxb1-OP) of tobacco leaf disks obtained (Fig. 35.9a–e). Those plant lines were rooted in a rooting medium containing kanamycin (100 μg/mL) (Fig. 35.9f-h). Twenty-three (23) rooted plants were observed. Rooted plants measuring at least 5 cm were transferred to the soil in a greenhouse for acclimation and further growth (Fig. 35.9i). Greenhouse plants were then used for GUS staining. Plants negative for blue stain were discarded. Plants demonstrating no blue stain were considered either non-transgenic ("escapees" from kanamycin selection) or transgenic plants with *gus* gene silencing. Gene silencing caused by position effect is frequently reported in plant genetic transformation (Betts et al. 2019). While in soil, GUS activity were tested multiple times as part of the screening process using different leaves of each transgenic plant at different plant development states. This ensures that blue stains are due to bona fide gene insertion not contamination of residual *Agrobacterium* cells. Leaves of 12 lines of 23 rooted T_0 putative transgenic lines (≈52.2%) were stained blue (Fig. 35.9j). Of the 12 lines, 1 sterile line grew many buds but yielded no seeds. One line did not survive transplant to soil. Therefore, ten seed-producing lines moved forward for future analysis.

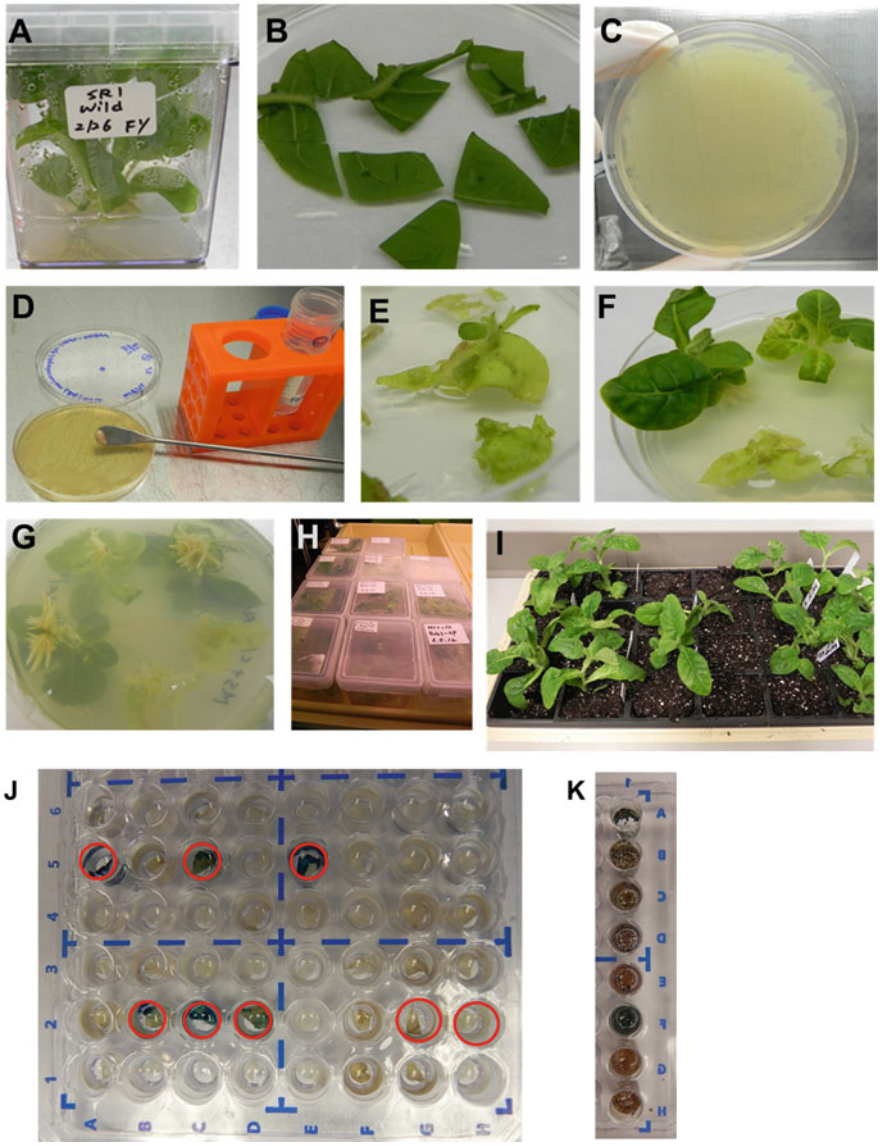

Fig. 35.9 Putative T_0 tobacco transgenic lines. (**a**) Tobacco SR1 used for *Agrobacterium*-mediated transformation. (**b**) Leaf cut into 1 cm × 1 cm disks for disk-dip transformation. (**c**, **d**) *Agrobacterium tumefaciens* LBA4404. (**e**) Putative transgenic shoots on selection medium. (**f**–**h**) Putative T_0 transgenic lines in rooting medium containing 100 μg/mL kanamycin (in plates and in Magenta™ boxes). (**i**) Putative T_0 transgenic lines transferred to soil for ex vitro acclimatization. (**j**) Leaves of putative T_0 transgenic used for GUS staining in a 96-well titration plate. Leaves have been de-chlorophylled with ethanol. (**k**) GUS staining of T_1 seed derived from T_0 putative transgenic lines

35.3.3.2 GUS Staining on T_1 Seeds

Ten fertile, GUS-positive plants grew to produce T_1 seeds that were immediately screened for GUS activity. For comparison, T_1 seeds from randomly selected *GUS-negative* T_0 greenhouse plants were also screened for GUS activity. The *gus* gene region, found outside *attB* and *attP* sites, contains its own CoYMV promoter undisturbed by the excision event. Blue GUS staining results were shown in figure (Fig. 35.9k).

35.3.3.3 PCR Analysis for Autoexcision Events in T_1 Seeds

T_1 seeds from ten GUS-positive T_0 transgenic plants were harvested for PCR analysis to evaluate autoexcision events. Primers **a** ("deletion 5") and primer **b** ("CoYMV.rev-1") were used (Fig. 35.8). PCR product of 290 bp was observed (Fig. 35.10).

35.3.3.4 Autoexcision Evaluation for T_1 Seedlings

To test for excision of recombinase and antibiotic-resistance coding regions embedded in transgenic tobacco genome, T_1 seeds were plated on selection medium containing kanamycin. Four of the ten GUS-positive lines showed ~3:1 ratio of resistant vs. susceptible to kanamycin. The remaining six lines also produced resistant seedlings, indicating *nptII* gene presence in T_1 generation (Fig. 35.11; Table 35.1).

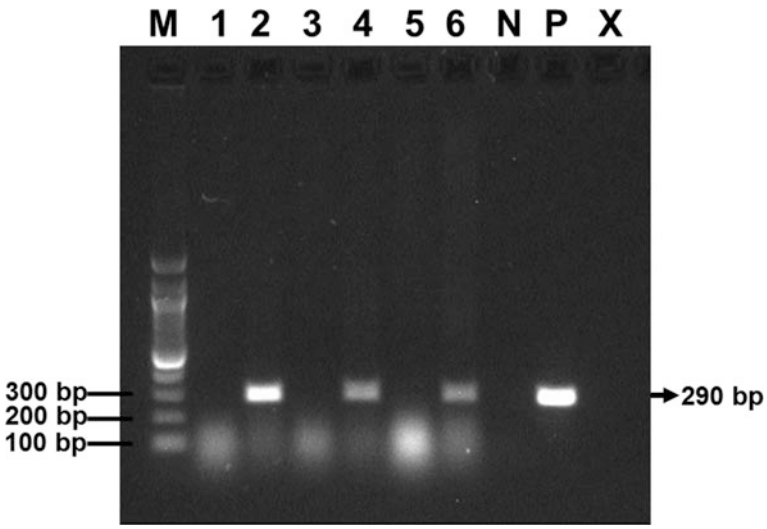

Fig. 35.10 PCR results of gDNA isolated from T_1 seed derived from different T_0 putative transgenic plants. Lane M: 100-bp DNA markers. Lane 1: negative control (wild-type seed gDNA). Lanes 2–6: seed gDNA from different T_0 putative transgenic tobacco plants. Lane N: water. Lane P: positive control. Lane X: no PCR sample loaded. A 290-bp band is expected to be amplified. PCR products run on a 1.3% TAE gel containing ethidium bromide

Fig. 35.11 T_1 seedlings derived from seeds of T_0 transgenic plant Bxb1-op #28 on selection medium (MS + kan (120 μg/mL)). Seedlings transformed with *nptII* gene survived kanamycin selection

Table 35.1 T_1 seed of T_0 putative transgenic tobacco plants (GUS positive) screened on MS + kanamycin (120 μg/mL) (indicated in the table with "MS + K120") medium for progeny test. A 3:1 (resistant vs. susceptible) ratio was observed in some T_0 putative transgenic lines

Line # BXB1-OP	[MS+K120] Resistant: susceptible	[MS only]	T_1 seed junction PCR	
25	25:11	2:0	Yes	T_2
28	93:31	51:0	No	
40	93:18	38:0	Yes	T_2
51	82:28	60:0	Yes Good	
66	95:32	42:0	Yes	
68	42:08	30:0	Yes Faint	
70	55:18	37:0	Yes Good	T_2
82	39:12	28:0	No	
96	18:06	16:0	Yes Strong	T_2
98	47:13	30:0	Yes Faint	
99	Sterile, many buds, no seeds			

Next, T_1 seedlings of GUS-positive lines were used for autoexcision event evaluation. Two hundred ninety bp PCR product was observed when forward primer **a** ("deletion 5") and reverse primer **b** ("CoYMV.rev-1") were used for amplification (Fig. 35.13a). T_1 seedlings with 290-bp band were further checked with other primer pairs to see if DNA (transgenes in T-DNA) rearrangement occurs. PCR product of 500-bp was observed when forward primer **a** ("deletion 5") and reverse primer **c** ("CoYMV.rev-3") were used for amplification (Figs. 35.12 and 35.13b). PCR product of 1.6 kb was also observed when forward primer "CoYMV.rev-1" and reverse primer "GUS-4" were used for amplification, as expected. In the meantime,

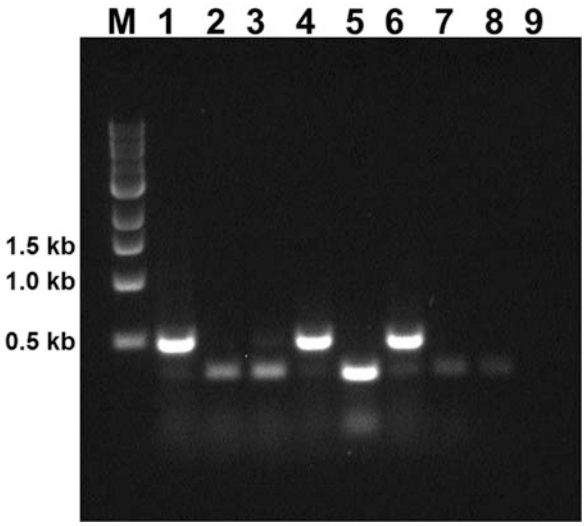

Fig. 35.12 PCR evaluation of six T_1 seedlings derived from T_0 putative transgenic plant **Bxb1-op #96**. Lane M: 1-kb DNA size marker. Lanes 1–6: six T_1 individual plants (T1 Bxb1-op #96–1, #96–2, #96–3, #96–4, #96–7, #96–9). Lane 7: wild-type gDNA. Lane 8: negative control (water, instead of gDNA, was added to the PCR tube)

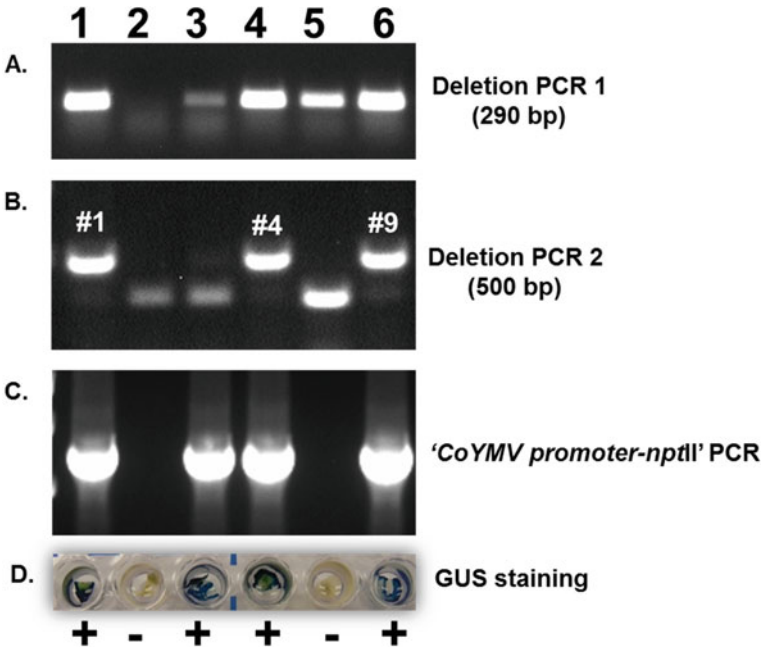

Fig. 35.13 PCR evaluation of autoexcision event in six T_1 seedlings derived from T_0 putative transgenic plant Bxb1-op #96 (T1 Bxb1-op #96–1, #96–2, #96–3, #96–4, #96–7, #96–9). (**a**–**c**) Three different pairs of primers used for genotyping. (**d**) Leaf GUS staining of these six T_1 seedlings

primer pair nptII-F and CoYMV.rev-3 was used to test for *nptII* presence in some cells (Fig. 35.13c). Amplified bands indicate T_1 seedlings are chimeric for excision.

Two GUS-positive fertile lines in the studied T_0 group (ten lines) did not yield PCR results positive for excision in T_1 tissue. Therefore, only 8 of the original 23 lines rooted in the soil (34.7%) and tested positive for GUS demonstrated excision via junction PCR of T_1 tissue.

35.3.3.5 Autoexcision Assay for T_2 Seedlings

An excision event examination of progeny lacking Bxb1 gene followed to determine if the genomic excision event occurred in germline tissue, indicating heritable transmission. From the T_1 plants testing positive for excision, four parental lines (#25, #40, #70, and #96) were chosen to propagate T_2 lines. Six descendants of these lines (#25–3, #25–5, #70–5, #96–1, #96–4, and #96–9) produced seeds in a timely manner, and produced seeds were plated on selection media. Four Bxb1-op T_2 lines (#25–5, #96–1, #96–4, and #96–9) appeared heterogeneous for excision phenotypically as many of their descendants remained resistant to the antibiotic kanamycin, indicating some continued presence of selectable marker. Three lines (#25–5, #96–1, and #96–9) maintained the 3:1 threshold by yielding resistant:susceptible ratios of 13:4, 27:9, and 21:8, respectively. Line #96–4 yielded a ratio of 30:0 resistant seedlings germinating on media containing kanamycin. Two Bxb1-op T_2 lines (#70–5 and #25–3) appeared homogenous for excision phenotypically and tested in triplicate yielding resistant to susceptible rates of 0:86 and 0:47, respectively (Fig. 35.14).

T_2 lines 70–5#3, 96–4#1, and 25–3#13 were used for excision event evaluation with junction PCR. Primer sets **a** and **b** were used to detect 290-bp band. Primers **a** and **c** were also used to detect 500-bp band. Both 290-bp and 500-bp bands were amplified and observed (Fig. 35.15a). These 290-bp and 500-bp bands were excised

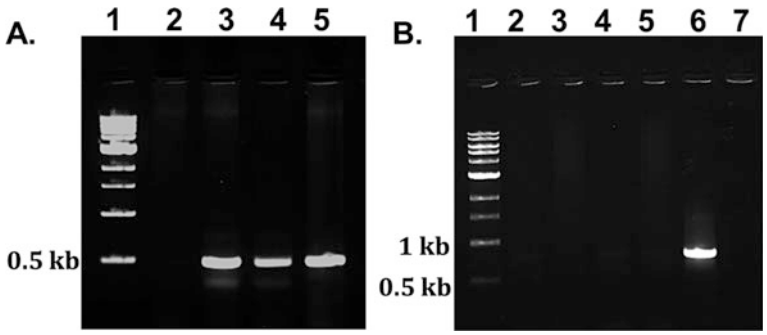

Fig. 35.14 PCR amplification of T_2 lines 70–5#3, 96–4#1, and 25–3#13. (**a**) Gels showing junction PCR products from three T_2 lines. Lane 1: 1 kb DNA ladder. Lane 2: wild type. Lane 3: 70–5 #3. Lane 4: 96–4 #1. Lane 5: 25–3 #13. Bands (excision product) of 500-bp size amplified with primer **a** and **b**. (**b**) PCR amplification of *nptII* gene in T_2 lines mentioned in (**a**). An 890-bp PCR product indicates the presence of the gene. Wild type serves as negative control. Bxb1-op vector serves as positive control. All three putative transgenic lines tested show no band of 890 bp

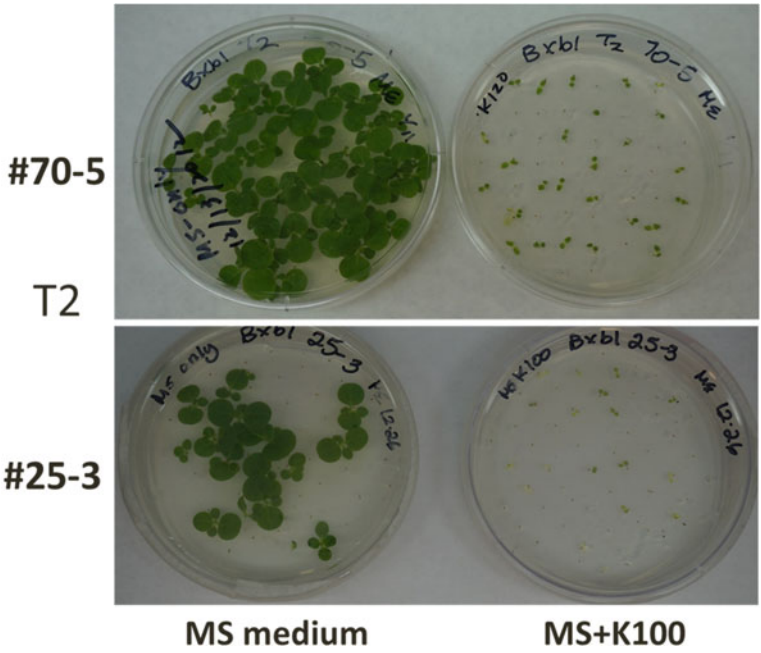

Fig. 35.15 Autoexcision evaluation for T_2 seedlings. Seeds were plated on selection medium containing 120 μg/mL kanamycin. Two Bxb1-op T_2 lines (#70–5 and #25–3) appeared homogenous for excision phenotypically. All seedlings showed susceptibility to kanamycin

from gel, purified, sent for sequencing, and produced results consistent with excision. The three lines further underwent PCR screening using *nptII* primers nptII-F and nptII-R to detect the presence of antibiotic resistance gene. None of the lines tested showed bands for the resistance gene (Fig. 35.15b). These three T_2 lines also tested with nptII-F and CoYMV.rev-1 primers for successful gene excision. 1259 bp indicates the presence of DNA fragment. All five putative transgenic lines tested show no band at 1259 bp.

Seedling descendants of T_2 lines were screened with GUS staining in leaf tissue, yielding positive results for staining in sample batches. For example, #25–3 was tested in 15 different tissue samples with 12 positive results, which scored as 12/15. #25–5 yielded 15/20 positives, and #70–5 yielded 17/20. While three lines gave 100% positive results, #96–1 yielded 12/12, #96–4 yielded 15/15, and #96–9 yielded 12/12. The variation was consistent with the expectation of variety among high and low expression levels in leaf tissue.

35.3.3.6 Sequencing Analysis for Autoexcision Events

290-bp bands were excised from electrophoresis TAE gels and gel-purified for sequencing. Sequence result revealed *attP* × *attB* recombination footprint, via hybrid site *attL* (Fig. 35.16). Hybrid site was observed, and DNA fragment between *attP* and *attB* sites was looped out and deleted. These results confirmed that *phas*

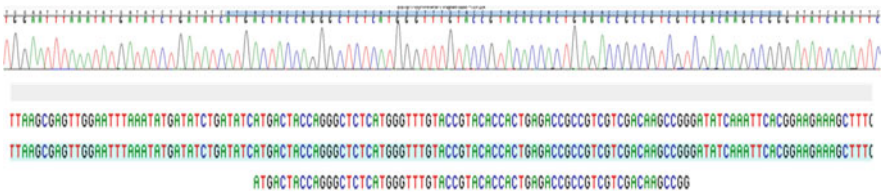

Fig. 35.16 Sequence of a 290-bp PCR product representing the hybrid site *attL* footprint, after Bxb1 *attP* and *attB* site-specific recombination

seed promoter is capable of driving *bxb1* recombinase gene for autoexcision in tobacco plants.

35.3.4 Discussion

This research project case study investigates the Bxb1 recombination system as a potential tool for precise removal of transgenes such as SMG using a tissue-specific promoter. Following successful recombination, cassette containing both *bxb1* recombinase and *nptII* region was completely excised. Absence of these two genes would address concerns surrounding human consumption, and the potential transfer of these transgenes is capable of conferring antibiotic resistance to other organisms environmentally (Yau and Steward Jr 2013). Successful recombinase activity would result in a generation of transformed plants that do not contain the antibiotic resistance (*npt II*) gene or the Bxb1 gene.

This case study collected 112 T_0 putative transgenic tobacco lines. Twenty-three lines grew roots in the rooting medium with kanamycin and was transferred to the soil. Among these 23 lines, 12 lines tested GUS positive. However, one line was sterile and another did not survive soil transplant. Southern analysis has not been performed on these ten T_0 lines, leaving transgene copy numbers unknown in these lines. In theory, T_0 line harboring a single-copy transgene is more likely to produce progeny with a homogenous excision event.

Among the remaining ten lines, two lines did not demonstrate excision events in T_1 seedlings measured by junction PCR tests. This could be due to position effect, indicating T-DNA cassette inserted into a locus preventing effective expression of *bxb1* gene. Position effects impacting gene expression have been reported (Pérez-González and Caro 2019). Of the lines in soil, 8 of the original 23 lines, representing 34.7%, tested positive for GUS and excision by junction PCR in T_1 tissue. However, *nptII* gene was also detected in T_1 tissue, indicating T_1 plants were chimeric. Four of the remaining eight lines, #25, #40, #70, and #96, were used for further study on T_2 progeny. Six descendants of these lines (#25–3, #25–5, #70–5, #96–1, #96–4, and #96–9) produced seeds for kanamycin test. Among them, only two Bxb1-op T_2 lines (#70–5 and #25–3) appeared homogenous for excision phenotypically. In summary, results suggest seed promoter *phas* is capable of driving *bxb1* recombinase gene

autoexcision in tobacco. These excision events were transmitted to the subsequent generation. Two independent lines with homogeneous excision events were obtained in T_2 generation.

Seed promoter have previously been used for SSR-mediated autoexcision. Moravcíková et al. (2008) used a strong, seed-specific *Arabidopsis thaliana* cruciferin C (CRUC) promoter for Cre-*lox*-mediated autoexcision. This promoter is active in seeds and silique but not in any other tissue type, including callus. However, the authors found promoter was leakage in early experimentation. Excision events were observed in callus cells experimentally. T_0 transgenic plants regenerated from calli were chimeric with excision events. T_1 plants derived from seeds of T_0 transgenic lines indicated10.2% of plants with complete removal of *nptII* gene with 86.4% chimera plants survived on kanamycin-containing medium. Autoexcision is effective in low numbers for these early generations of transgenic plants. However, repeated Cre activation in T_2 seeds produced more SMG-free T_2 plants in further study (Moravcíková et al. 2008).

Tissue-specific promoters provide a possibility for stronger *bxb1* recombinase expression. Another SSR-mediated autoexcision study using Bxb1-*att* SSR system is conducted in the monocot energy crop switchgrass (*Panicum virgatum* L.). This study incorporated a rice PS3 pollen promoter (Somleva et al. 2014). Authors report successful deletion of cassette containing recombinase and marker genes in switchgrass pollen through autoexcision, with efficacy range of 22–42%. In another study, pollen-specific LAT52 promoter from tomato was employed to control expression of CinH recombinase for autoexcision in tobacco (Moon et al. 2011). Autoexcision cassette flanked by two *Rs* recognition sites containing a green fluorescent protein (GFP) gene. A successful autoexcision event would delete the gene and remove GFP expression. The authors observed in CinH tobacco T_1 harboring single-copy transgene events, three independent lines exhibited less than 1% GFP-positive pollen, based on flow cytometry (FCM)-based pollen screening of 30,000 pollen grains. In the control event (CinH-Drec), GFP expression was observed in 70% of pollen (Moon et al. 2011). Root promoter approach offers an alternative for SSR-mediated autoexcision in plants.

Overexpression of recombinase causes host cytotoxicity or genotoxicity. Recombinase toxicity were reported in mammalian and plant cells (Loonstra et al. 2001; Coppoolse et al. 2003; Liu et al. 2009; Janbandhu et al. 2014). Loonstra et al. observed Cre expression causing numerous chromosomal aberrations and increasing sister chromatid exchange numbers in mammalian cells. They also found toxicity dependent on Cre activity level. Although Bxb1 toxicity has been reported in yeast (*Saccharomyces cerevisiae*) (Xu and Brown 2016), no abnormal plant phenotypes were observed in either model plants or wheat studies actively expressing Bxb1 integrase. In contrast, ϕC31-transformed *Arabidopsis* developed crinkle leaves (unpublished data), and Cre transgenic plants displayed abnormality (Coppoolse et al. 2003). Native docking sites in eukaryotic genomes can integrate transgene at unintended loci, leading to generation of unwanted GMOs. For example, Bi et al. (2013) reported theinsertion of a plasmid with ϕC31 *attB* site into pseudo *attP* sites in pig genome. In this study, four pseudo *attP* sites were identified, and two gave rise

to increased integration rate of 33%. These unintended insertions might also lead to cell toxicity. It is prudent to remove the recombinase gene with the selectable marker gene once their purposed has been served. In this study, plants actively expressing Bxb1 transgene present phenotypically normal appearance and set seeds in a manner comparable to wild type, thereby demonstrating a lack of detrimental impacts from recombinase constitutive expression.

35.3.5 Conclusion and Future Perspective

Recombinase technology allows for precise levels of genomic integration through more sophisticated applications (Wang et al. 2011). This case study research project demonstrates Bxb1 recombinase successfully performing site-specific genome modification in *Nicotiana tabacum* L. Bxb1 produced conservative site-specific deletions of DNA in regions flanked by *attP* and *attB* within transformed plant genome. Homogenous genotype of excision obtained in T_2 generation, indicating that excised DNA heritably transmitted to next generation.

Tissue-specific promoters (e.g., seed or pollen promoter) drive recombinase for autoexcision, as well as other types of promoters that have been successfully studied. These include inducible promoters, heat shock response promoters, and chemically induced promoters. Du et al. (2019) employed heat shock response promoter (hsp70) for SSR-medicated autoexcision in maize to remove a DNA cassette containing selectable marker gene. Kleidon et al. (2020) used chemically induced recombination for autoexcision of a genetic cassette. This cassette contained both a negative selection gene and R-*RS* recombinase gene to produce selectable marker gene-free Cavendish banana (*Musa* spp.). Steroid-inducer molecule, dexamethasone (DEX), used to activate recombination activity. Tissue-specific promoters have an advantage over inducible promoters, because inducible promoters require an extra step for recombinase activation. Optimal conditions for applying inducers should be determined in advance, as inducers (i.e., chemicals) might have plant side effects or be impractical for large-scale production.

One disadvantage of using SSR technology for transgene integration is the requirement of embedding a landing pad in the target genome in advance. These docking sites, except for native pseudo-docking sites, must be genetically transformed into a genome to begin the process. For example, *attB* site of Bxb1 must first be embedded in the plant genome, and then a plasmid containing gene of interest (GOI) and *attP* site can integrate the *attB* docking site through site-specific recombination to bring in GOI. The genome embedding of *attB* site is random. They can be embedded in undesired sites. Emergence of revolutionary genome editing tool CRISPR-Cas system can change this. CRISPR-Cas system can first be used to deliver *attB* site to a desirable genomic locus X. Following characterization, this *attB* site will serve as a docking site for subsequent GOI integration. Of course, CRISPR-Cas alone can also insert the GOI at locus X through homologous recombination (HR) without SSR-system involvement with low efficiency. Differing optimization factors required to increase CRISPR-mediated HR efficiency.

One important concern for breeders with these recombination systems is freedom to operate (FTO) issue. Dupont's Cre-*lox* patent has expired, and patent application on Bxb1-*att*, previously filed by USDA, has been abandoned (Chen and Ow 2017). With this technology being free from patent licensing fees, breeders and researchers have more affordable tools at their disposal.

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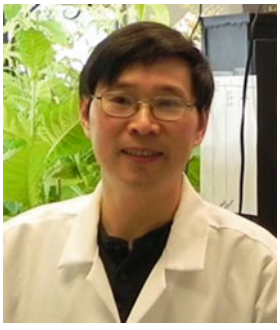

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