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Evaluation of the Recombination Efficiencies of FLP Proteins

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EVALUATION OF THE RECOMBINATION EFFICIENCIES OF FLP PROTEINS

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By

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ABSTRACT

Site-specific recombination systems are powerful tools for genetic modification. They have been used to integrate a transgene into a pre-defined locus and to remove marker genes from a transgene locus. Two of the most widely used site-specific recombination systems in plants are the Cre/*lox* system from the bacteriophage P1 and the FLP/*FRT* system from the yeast *Saccharomyces cerevisiae*. The Cre/*lox* system is well-characterized and is the first choice in application of site-specific recombination system. However, some applications such as marker-free site-specific gene integration require the use of two recombination systems. In addition, the availability of alternative recombination systems can offer a flexible choice or the opportunity to develop multiple applications in a single platform. Hence, the FLP/*FRT* system should be evaluated further for its recombination efficiency, particularly in rice, a model crop plant. Some studies using FLP/*FRT* systems, with the wild type FLP called FLPwt recombinase, reported low efficiency for regular application of the system in removal of transgenic locus. However, two improved versions of FLPwt: FLPe (thermostable version of FLPwt) and FLPo (mouse-codon optimized version of FLPe) are available and have not been carefully tested in plants.

To look for the best choice of FLP recombinase variant in the application of the FLP/*FRT* system in crop genetic engineering, the relative recombination efficiencies of FLPwt, FLPe and FLPo for marker gene excision from the transgene locus in rice were evaluated. FLPwt, FLPe, and FLPo transgenic rice lines were generated and FLP activity in these lines was evaluated. These experiments revealed that FLPe and FLPo had much higher activity than FLPwt in removing *FRT*-flanked *npt* segment to fuse *GUS* gene with the promoter. These experiments

also indicated that FLPo is relatively more efficient than FLPe. Thus, based upon results from the present study, I recommend the use of FLPo in plant genetic engineering.

This thesis is approved for recommendation
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INTRODUCTION AND LITERATURE REVIEW

1.1. Genetically Modified (GM) Crops and Agricultural Improvement

World population is projected to reach 9.1 billion by 2050, which is 34 percent higher than today. In order to meet food demand for this increasing number of population, food production is required to increase about 70 percent in 2050 compared to the 2005-2007 period (FAO, 2009). This can be achieved via expansion of cultivated land and improvement of yield. The former target is not achievable since most arable land resources for agriculture have been used, and available land is declining because of the increase in population, urbanization, desertification, and erosion. In addition, climate change with additional abiotic stresses such as drought, cold, and salinity stresses, and emergence of pathogens are contributing to the reduction of food production (reviewed by Mahajan and Tuteja, 2005). Hence, it is urgent to produce crops with higher yield that are able to withstand abiotic and biotic stress.

Traditional breeding has been successful in producing better varieties. However, the process through discovery, selection, and crossing "super" individuals is slow, and is attainable only for those traits available in sexually compatible species. Meanwhile, plant biotechnology allows gene transfer across species boundary, in which, theoretically, a gene from one organism can be isolated and transferred to another organism even if it belongs to a different kingdom in the taxonomy system. The organism that received a gene from a foreign source is called transgenic or a genetically modified organism (GMO).

The first successful efforts to produce transgenic plants trace back to the 1980s, when several groups reported generation of transgenic plants using model species (Wang et al., 2011). Since then, the number of publications in the field increased dramatically, with the

focus shifting to crop plants (Vain, 2007). The traits of agronomic importance include disease and herbicide resistance and tolerance to environmental stresses such as drought, cold, and salinity. Recent research has also focused on increasing crop yield and the nutritional value. These activities hold great promise for food security, and deal with the climate change.

In the year 1994, Tomato was the first GM crop to be grown, but the year 1996 was considered the first year of commercialization as the planted area of GM crops significantly increased and reached 1.7 million hectares (Brookes and Barfoot, 2006). Currently, 160 million hectares of GM crops are planted in 29 countries in all continents of the world. The three countries that have the highest area planted in GM crops are the United States, Brazil, and Argentina. The most widely grown GM crop in the world is herbicide tolerant soybean, followed by stacked traits maize, and Bt cotton (James, 2011). Application of GM crops resistant to insects has resulted in a reduction in pesticide use (Kleter et al., 2007), which is useful to pest management, and beneficial to the environment.

1.2. Current Plant Transformation Technologies

Plant transformation is the process by which foreign DNA is introduced into the excised plant tissues (explants). There are several methods to transfer DNA into plant cells: *Agrobacterium*-mediated T-DNA transfer, particle bombardment of DNA, polyethylene glycol-mediated DNA delivery, microinjection of DNA, and electroporation of DNA. Among these, *Agrobacterium* and particle bombardment are the two common methods (Vain, 2007).

1.3. Transgene Locus Structure

The integration mechanism of transgenes is not fully understood yet. However, there have been a number of research studies that explored these mechanisms. The introduced DNA, normally, will integrate into the host genome randomly and generate multi-copy insertion patterns with linked or unlinked loci (Afolabi et al., 2004; Cluster et al., 1996; Maqbool and Christou, 1999; Pawlowski and Somers, 1998; Svitashev et al., 2002; Zhang et al., 2008). The composition of each locus is also variable and complex, comprising one or more transgene copies with complete, inverted, and/or truncated integration. DNA rearrangement may also occur in the host chromosomal sequences around the transgene locus (Maqbool and Christou, 1999). Within the transgene locus, a vector backbone is often found to be integrated along with genes of interest (Afolabi et al., 2004; Vain, 2007).

Chromosomal positions and the complexity of transgene loci have major effects on the level and stability of transgene expression (Matzke and Matzke, 1998; Stam et al., 1997). A gene will have a greater possibility to be expressed when located in euchromatin as opposed to heterochromatin regions. In fact, transgenes may preferentially integrate into transcriptionally active regions (Kohli et al., 2003), and this may be because of the accessibility of euchromatin to the foreign DNA. Also, multi-copy and complex structure insertion often leads to gene silencing (Stam et al., 1997).

Gene silencing entails suppression or down regulation of gene activity. A number of studies have revealed several circumstances in which gene silencing can take place: a) position effect: the transgenes integrate in unfavorable locations in the genome such as telomeres and centromeres (Matzke and Matzke, 1998); b) gene copy number: presence of multiple copies of

the gene (Stam et al., 1997); and c) transgene over-expression either from multiple gene copies or from a single copy due to strong promoter activity (Que et al., 1997). Hence, in plant transformation, a single copy of the gene of interest (GOI) is expected to generate an optimum level and stable expression; although, a single copy may also undergo silencing if expressed by a strong promoter (Elmayan and Vaucheret, 1996). Further, if multiple copies of a gene are full-length without any rearrangement or truncation, they may be expressed properly to generate higher expression compared to the single copy locus (Akbudak and Srivastava, 2011). Engineering of full-length multi-copy locus, however, requires precise transformation techniques such as recombinase-mediated gene integration.

1.4. Selection Marker Gene (SMG) in Plant Transformation

Plant transformation is a low efficiency process. Therefore, along with the GOI, selectable marker genes are used in plant transformation process to assist the selection of transformed cells. Most commonly used selection marker genes confer antibiotic or herbicide resistance (Table 1). On selection agent-containing media, non-transformed cells cannot survive whereas transformed cells can grow, and therefore, be selected. The presence of these genes in transgenic crops raises serious concern. Horizontal transfer of antibiotic resistance genes from transgenic plants to pathogenic bacteria could potentially occur, which would render the use of these antibiotics in disease treatment impossible. Although, the possibility of DNA transfer from plants to bacteria is debatable, many organizations including the U.S. Food and Drug Administration and European countries recommended removing antibiotic resistant genes from transgenic crops (EFB, 2001; FDA, 1998). Similarly, there is a constant worry of the vertical transfer of herbicide resistance genes from crop to wild and weed species. In addition, once transgenic plants are selected and grown, selectable marker genes serve no

purpose. Meanwhile, the expression of these genes, which is usually driven by strong constitutive promoters such as maize ubiquitin promoter (*ubi*) or cauliflower mosaic virus 35S promoter (CaMV *35S*), could be an unnecessary metabolic burden on plants (Gidoni et al., 2008). Besides, multi-gene transfer to study and engineer a metabolic pathway in plants is increasingly useful approach (Naqvi et al., 2010). However, the number of available of SMG applied routinely in plant transformation is limited, and SMG are needed for selection in each transformation step. Therefore, removal and recycling of SMG will alleviate public and regulatory concerns, and enable gene stacking. Taking all above reasons into account, obtaining marker-free transgenic plants is important for the future GM crops. A number of approaches have been used to produce marker-free transgenic plants, of which two most popular are following:

Table 1: **Commonly used selectable marker genes in plant transformation.**

Marker gene	Enzyme encoded	Selective agent
*npt*II	Neomycin phosphotransferase	Genticin (G418), kanamycin
hpt	Hygromycin phosphotransferase	Hygromycin B
ppt	Phosphinothricin acetyl transferase	Phosphinothricin (Bialophos)
als	Acetolactate synthase	Chlorosulfuron, imidazolinones

1.4.1. Marker-segregation by Co-transformation of GOI and SMGs

In this approach, the SMG and GOI are transferred into explants on separate constructs. If they integrate into separate genomic loci, they can segregate in the progeny and marker-free progeny can be obtained. These constructs can be introduced by *Agrobacterium*-mediated transformation or biolistic transformation. However, the former is preferred as it usually produces distinct T-DNA copies and simpler transgene integrations compared to the latter. Using this approach, several studies have been successful in producing marker-free plants (Daley et al., 1998; Komari et al., 1996; Matthews et al., 2001; Miller et al., 2002).

Using two vectors in one *Agrobacterium* strain, Daley et al. (1998) reported 8 out 40 and 24 out of 41 primary transgenic lines of rapeseed and tobacco, respectively, to contain segregated loci of kanamycin resistance and GUS gene. Thus, marker-free transgene locus could be obtained from 40% and 58% of T0 lines of rapeseed and tobacco, respectively. Miller et al. (2002) generated 87 co-transformed T0 events of maize expressing GUS activity and Liberty^{TM} resistance, using one vector (containing two T-DNA regions) in one strain. Marker-free T1 progeny (GUS positive, Liberty sensitive) were recovered from 55 of these lines, giving an efficiency of ~64%. In rice, Parkhi et al. (2005), using two vectors in two strains, obtained 14 marker-free lines out of a total of 24 co-transformants. In another study carried out to develop sheath blight resistant transgenic rice, Sripriya et al (2008) obtained 4 co-transformed events, of which two were able to generate marker free in T1 progeny. In this study, two vectors in one strain were used.

The advantage of this approach is that no post-transformation modifications are needed to recover marker-free plants. However, this application is limited to sexually propagated species only and in many cases, both T-DNA integrate into the same locus. For example,

Afolabi et al. (2004) selected 50 co-transformed rice lines, representing 98 loci, developed by one strain two vector approach (pGreen and pSoup vectors, where pGreen contained SMG). Transgene inheritance and segregation analysis revealed that 56% of the loci contained linked pGreen and pSoup insertions; while 31% had only the pGreen locus, and 13% had only the pSoup locus in the progeny (Afolabi et al., 2004). Transgene expression analysis showed that overall efficiency of marker-free "active" T-DNA line recovery is ~9%. Previous studies were based on phenotypic analysis, whereas this study carried out a detailed molecular analysis. Thus, efficiency reported by Afolabi et al. (2004) is more reliable. In addition, most transgenic lines produced by the *Agrobacterium* method contain binary vector backbone integrations, which is also an undesirable genetic element.

1.4.2. Recombinase-mediated excision of SMGs

Recombinases are able to delete a DNA segment placed between two directly oriented binding sites. Hence, a site-specific recombination (SSR) system can specifically remove SMGs from a transgene locus. This application was first demonstrated by using the Cre/*lox* system to remove a kanamycin resistance gene from the tobacco genome (Dale and Ow, 1991). Later on, other SSR systems such as FLP/*FRT*, R/*RS* also were also used for SMGs removal (Darbani et al., 2007; Gidoni, Srivastava and Carmi, 2008; Puchta, 2003). Recombinase mediated SMGs excision can be induced under certain conditions or in specific tissue by using inducible or tissue specific promoters (Fladung et al., 2010; Li et al., 2007; Liu et al., 2005; Zuo et al., 2001).

1.5. Approaches for Designing Transgene Locus

High level and stable expression of transgenes is the goal of plant transformation. In order to achieve this, it is important to make sure that transgenes are integrated with a simple locus structure into active regions on chromosomes. Hence, designing a transgene locus, or in other words, gene targeting into a desired locus is a desirable approach in plant transformation. Gene targeting is made possible by homologous recombination (HR) between a transgene and a homologous sequence on the host chromosome (Weinthal et al., 2010). Homologous recombination is the basis of recombination between chromosomes during meiosis. This is also a natural mechanism to repair DNA double strand breaks (DSBs). Homologous recombination involves DNA synthesis directed from a homologous template. However, DSBs can also be repaired by illegitimate recombination (IR) via non-homologous end joining (NHEJ). Thus, HR-mediated gene targeting into mitotically dividing cells is quite inefficient (Cotsaftis and Guiderdoni, 2005). However, gene targeting can be enhanced by introducing DSB into specific genomic sites using specialized nucleases such as Zinc Finger nucleases or TALENs.

1.5.1. Zinc-finger Nucleases (ZFNs)

ZFNs are synthetic restriction enzymes, which combine *Fok*I non-specific cleavage domains with artificially prepared zinc finger domains (Weinthal et al., 2010; Wu et al., 2007). Expression of ZFNs can produce genomic DSBs on the recognition sites (DNA sequences) of the ZFNs, which can be easily designed theoretically for any DNA sequence. ZFN induced DSB become hot spot for gene targeting. ZFN-mediated gene targeting was successfully performed in tobacco (Townsend et al., 2009) and maize (Shukla et al., 2009). In tobacco, based on the number of recombinants recovered, Townsend et al. (2009) achieved ~4% gene

targeting. In the majority of these cases, the distance to mutation (separation of ZFN binding site and the transgene insertion site) was 0.2 kb. In a few cases (0.2%) distance to mutation was 1.5 kb. In another study, based on number of targeted integration events in total transformants, Shukla et al. (2009) reported 18-40% gene targeting in maize using ZFNs. However, the major limitations of ZFN is their toxicity due to off-target activity, and designing of an efficient ZFN (DeFrancesco, 2012). To overcome this problem, TALENs have recently been developed.

1.5.2. TALENs

Transcription activator-like (TAL) effector nucleases (TALENs) are the fusion of the catalytic domain of the *FokI* nuclease with TAL effector targeting domain. The TAL effector target domain is used to direct the *FokI* nuclease catalytic domain to create site-specific DSBs. Since *FokI* functions as a dimer, TALENs are designed in pairs to bind two DNA strands at their target sites separated by a spacer. TAL effectors are found in the genus *Xanthomonas.* Injected into plant cells via type III secretion system, TAL effectors bind and activate expression of genes that facilitate the bacteria colonization (Bogdanove et al., 2010). Most TAL effectors have a 34 amino acid targeting domain involving many repeats. These repeats are polymorphic at a pair of residues, mostly at positions 12 and 13, called the repeat-variable di-residue (RVD). Different RVDs associate with different nucleotides. The number of repeats and the composition of RVDs determine the length and the sequence of target sites (Boch and Bonas, 2010; Bogdanove and Voytas, 2011; Hockemeyer et al., 2011). The repeats in the TAL effectors domain can be customized to target a specific sequence of interest (Cermak et al., 2011). Customized TALENs have been shown to target specifically the sequence of *ADH1* gene from *Arabidopsis* and *gridlock* gene from zebrafish in an *in vivo* assay carried out in yeast

(Christian et al., 2010). While in plant system, TALEN-mediated gene targeting was described recently in rice(Li et al., 2012).

Just as ZFNs, TALENs can also be designed to induce DSBs around marker genes to excise them from the chromosome. The DSBs will be repaired by the cellular DNA repair system. These technologies have not been applied for marker removal to date. Effective design of TALENs for inducing site-specific DSBs, and subsequent repair of these sites will be necessary for the success of these new tools in biotechnology. The effectiveness of these tools in plant genetic engineering remains to be tested.

1.6. Site-specific Recombination (SSR) Systems

SSR systems play a vital role in native biological systems by inserting, excising, and inverting DNA segments. Most commonly used site-specific recombination systems are derived from prokaryotes and lower eukaryotes. They have been utilized widely for experimental research and biotechnology applications in higher eukaryotes. Each SSR system comprises a recombinase and its recognition sites (DNA sequence). In general, SSR systems can be divided into two families: tyrosine or serine recombinase families, according to the presence of tyrosine or serine in the binding site of the catalytic domain of the enzyme (Grindley et al., 2006).

Some representatives of these systems are Cre/*lox* (Control of recombination/locus of x-cross over) and FLP/*FRT* (FLP/FLP Recognition Target) systems in the tyrosine family, and phiC31 in the serine family. The Cre/*lox* system of bacteriophage P1 and FLP/*FRT* system from the 2-µm plasmid of the yeast *Saccharomyces cerevisiae* are well characterized and

commonly used in plant transformation. Each system comprises a single recombinase protein (Cre, FLP) and its target or recognition sequences (*lox, FRT*).

Recombinases carry out the recombination reaction on their target sites and can join or excise a DNA fragment, without adding or losing nucleotides. Hence, they have been used for integrating or removing genes at specific positions. The target sites comprise repeats flanking spacer region. The spacer sequence determines the orientation of recombination sites. Recombinases bind to the inverted repeats and make a cut in the spacer region to initiate recombination. Recombination between two oppositely oriented target sites leads to the inversion of the intervening DNA (Figure 1). In contrast, recombination between two target sites placed in the same orientation on a DNA fragment leads to the excision of the intervening DNA.

The excised DNA has a circular form, which in principle can be re-integrated into the site of origin (Figure 1). However, excision reaction is kinetically favored and re-integration of the excised DNA has not been detected to date. This recombination mechanism/kinetics is appropriate for removing marker genes from transgenic plants. For transgene integration, however, reversibility of recombination must be controlled to prevent excision of the newly integrated DNA. Hence, mutant recombination sites have been developed, which recombine with each other and generate recombination-incompetent products (Albert et al., 1995; Schlake and Bode, 1994). As a result, the forward reaction is much more efficient than the reverse reaction (Figure 1). With the unique ability to cut and join DNA, SSR systems are useful tools for genetic engineering applications such as transgene integration and marker gene deletion.

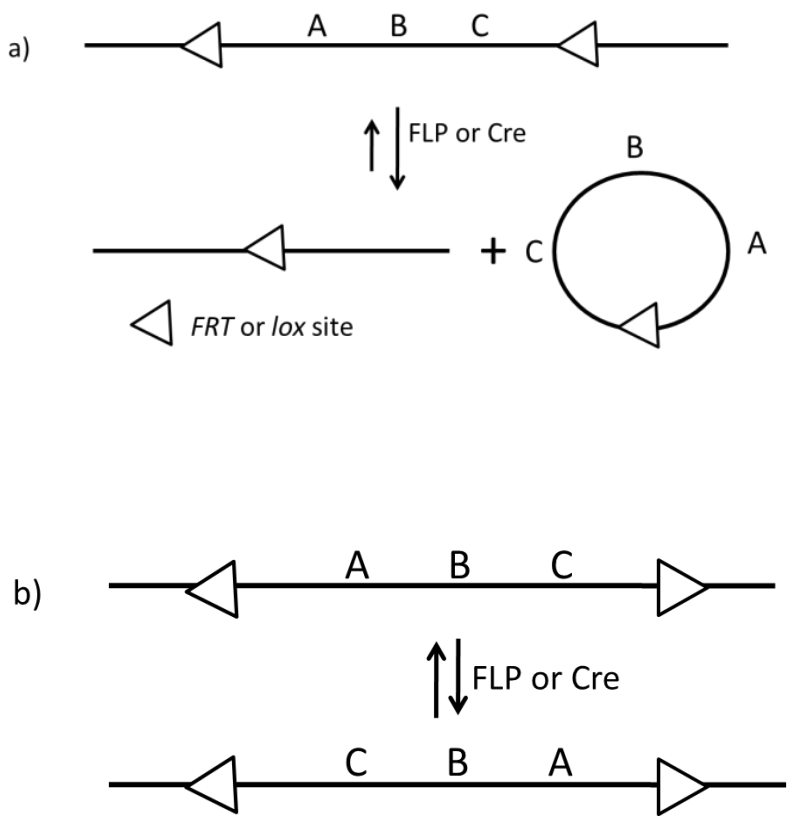


Figure 1: Site-specific recombination. (a) Deletion and integration: Recombination between two directly oriented sites (triangles) on one DNA molecule leads to the excision of the intervening segment as a circular molecule. The reaction is reversible, so the site-specific recombination between a circular DNA molecule and chromosome leads to the insertion of circular DNA into the chromosome. (**b) Inversion:** Recombination between two oppositely oriented sites generates the inversion of the intervening DNA in a reversible reaction.

1.6.1. The Cre/*lox* system

The Cre/*lox* system from *Escherichia coli* phage P1 consists of the Cre recombinase and its 34-bp recognition site, *lox*. Cre is a 343-amino acid protein, with a molecular mass of 38-kDa, belonging to the tyrosine recombinase family. The wild type *lox* site, called *loxP,* consists of two 13-bp binding regions flanking a 8-bp spacer region (Figure 2). The Cre/*lox* system is the first SSR system employed for genomic modification. This system is widely used in both plant and mammalian systems.

A study by Sauer utilizing the Cre/*lox* systems in *Saccharomyces cerevisiae* showed that Cre functions in heterologous systems (Sauer, 1987). Later, the system was shown to function in tobacco (Dale and Ow, 1990). Since then, this system has been used in many species for a variety of applications.

1.6.2. The FLP/*FRT* system

The FLP/*FRT* system comes from the 2-µm plasmid of the yeast *Saccharomyces cerevisiae*. FLP recombinase, belonging to the tyrosine recombinase family, is a 423-amino acid protein, with a molecular mass of 46-kDa. *FRT* is the binding site of FLP recombinase. The minimal *FRT* site consists of 34-bp, with two 13-bp repeats flanking the 8-bp spacer region. The native *FRT* site contains an additional 13-bp repeat, which is non-essential for the recombination (Figure 2).

1.7. New FLP Proteins

There are three different variants of FLP protein: FLPwt, FLPe, and FLPo. FLPwt is a modified version of native FLP found in yeast. In FLPwt, cryptic splice acceptor sites at the 3’

end was eliminated to allow for efficient translation of the FLP protein in mammalian and plant cells (O'Gorman et al., 1991). A study on recombination activity of FLP and Cre at different temperatures revealed that FLP is more thermolabile compared with Cre both *in vitro* and *in vivo* (*E. coli* and mammalian cells) (Buchholz et al., 1998). The optimum temperature for FLP activity was found to be under 30 ^{O}C, and the activity was hardly detectable at temperature above 39 ^{o}C, where as optimum temperature for Cre was 37 ^{o}C and above. Due to the instability of FLP at 37 ^{o}C, FLP/*FRT* recombination in plant and mammalian systems is inefficient. Looking for an improved version of FLP that can stay active at higher temperatures, Buchholz and co-workers (1998) randomly mutated the coding sequence of FLP gene and obtained an thermostable version of FLP, enhanced FLP or FLPe, that had a four-fold higher recombination efficiency than FLPwt at 37^{o}C and 10 fold higher at 40^{o}C in *E. coli* but became fully denatured at 42^{o}C (Buchholz et al., 1998). FLPo, the third variant of FLP recombinase, is the codon-optimized version of FLPe with the codon usage based on the mouse genome (Raymond and Soriano, 2007).

1.8. Site-specific Recombination based Plant Transformation Technologies

Several site-specific recombination systems have been shown be functional in a variety of plant cells. Therefore, they have been used to manipulate transgene locus structure. The most widely used SSR systems are Cre/*lox* and FLP/*FRT*. These systems have been successfully used for removing SMG from plant genomes, and also for integrating foreign DNA into a dedicated genomic site. A review of their applications in plant transformation is provided below:

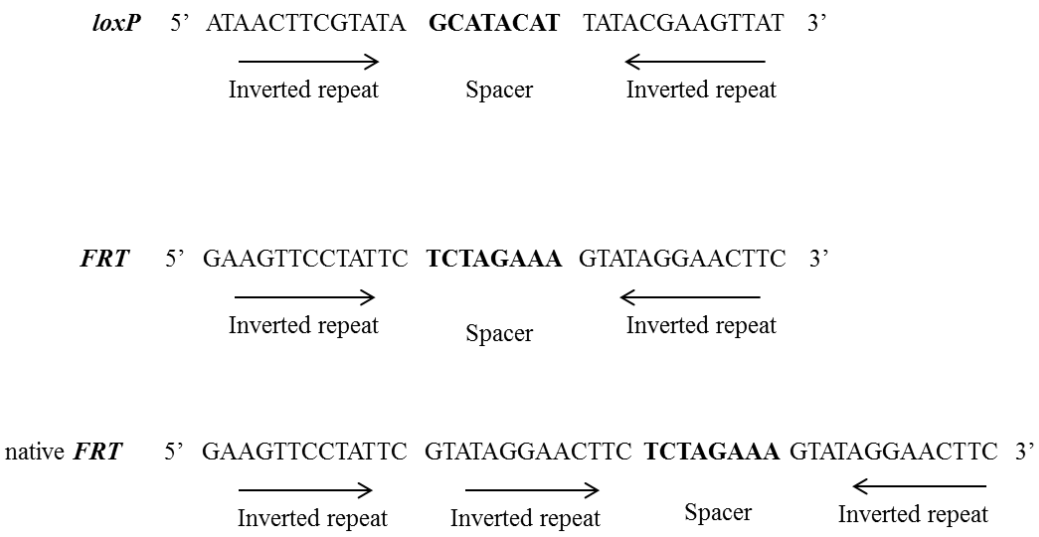


Figure 2: **Nucleotide sequences of *lox* and *FRT* recombination sites.**

1.8.1. Cre/*lox* system based plant biotechnologies

Cre/*lox* system is the first site-specific recombination system used in studies to modify genomes of plants and animals. Based on the function of Cre recombinase, the system has been used widely to insert foreign genes into specific chromosomal locations, delete a transgene from the locus or resolve transgene locus structure.

1.8.1.1. Cre/*lox* system for site-specific integration

In plants, Cre/*lox* have been deployed to generate precise transgene integration in *Arabidopsis* (Louwerse et al., 2007; Vergunst et al., 1998; Vergunst and Hooykaas, 1998), tobacco (Albert et al., 1995; Day et al., 2000), and rice (Akbudak and Srivastava, 2011; Chawla et al., 2006; Srivastava et al., 2004; Srivastava and Ow, 2004; Srivastava and Ow, 2002). Site-specific integration is achieved via two steps: random integration of target *lox* site into the genome followed by Cre-mediated insertion of gene-of-interest in the target *lox* site (Figure 3). Vergunst and Hooykaas (1998) generated target *Arabidopsis* lines, which contained the *nptII* gene without a promoter and ATG start codon upstream. Hence, these lines were kanamycin sensitive. Retransformation of the target lines with two vectors, one containing the promoter-ATG fragment and the other containing the *cre* gene, generated a precise integration locus, in which *nptII* was made functional by the precise placement of the promoter and start codon in promoterless *nptII* gene. In another study, Day et al. (2000) utilized the Cre/*lox* system to achieve precise integration of the *gus* gene in tobacco. In this study, Cre activity was encoded in the target locus; hence, transformation was done with a single vector called a donor vector that contained a promoter-less hygromycin resistance gene (*Hyg*) and a fully functional *gus* gene. Introduction of the donor vector generated hygromycin

resistant cells expressing GUS activity. Cre/*lox* mediated precise transgene integration in rice was first reported by Srivastava and Ow (2002). This approach was further validated in follow up studies by Srivastava et al (2004) and Chawla et al (2006). A similar strategy was adopted in this study involving restoration of *nptII* activity in the site-specific integration locus.

Precise introduction of a transgene to a locus can also be achieved by the exchange of a cassette via recombination between a pair of *lox* sites. In this approach, both target locus and donor vector must have two *lox* sites flanking genes to be exchanged via recombination. Louwerse et al. (2007) utilized this strategy to replace the *bar* gene in a target locus with the *nptII* gene and obtained kanamycin-resistant *Arabidopsis* lines.

As described in section 1.3, single copy integration is highly desired in plant transformation because multi-copy transgene locus integration frequently undergoes gene silencing. The site-specific integration approach generates integration of a single-copy of transgenes into a defined genomic position. Hence, transgenic lines produced by this approach will not only be single copy, but also have predictable level of expression as determined by transgene control elements (e.g. promoters and enhancers) (Chawla et al., 2006; Day et al., 2000; Nanto and Ebinuma, 2008; Srivastava et al., 2004).

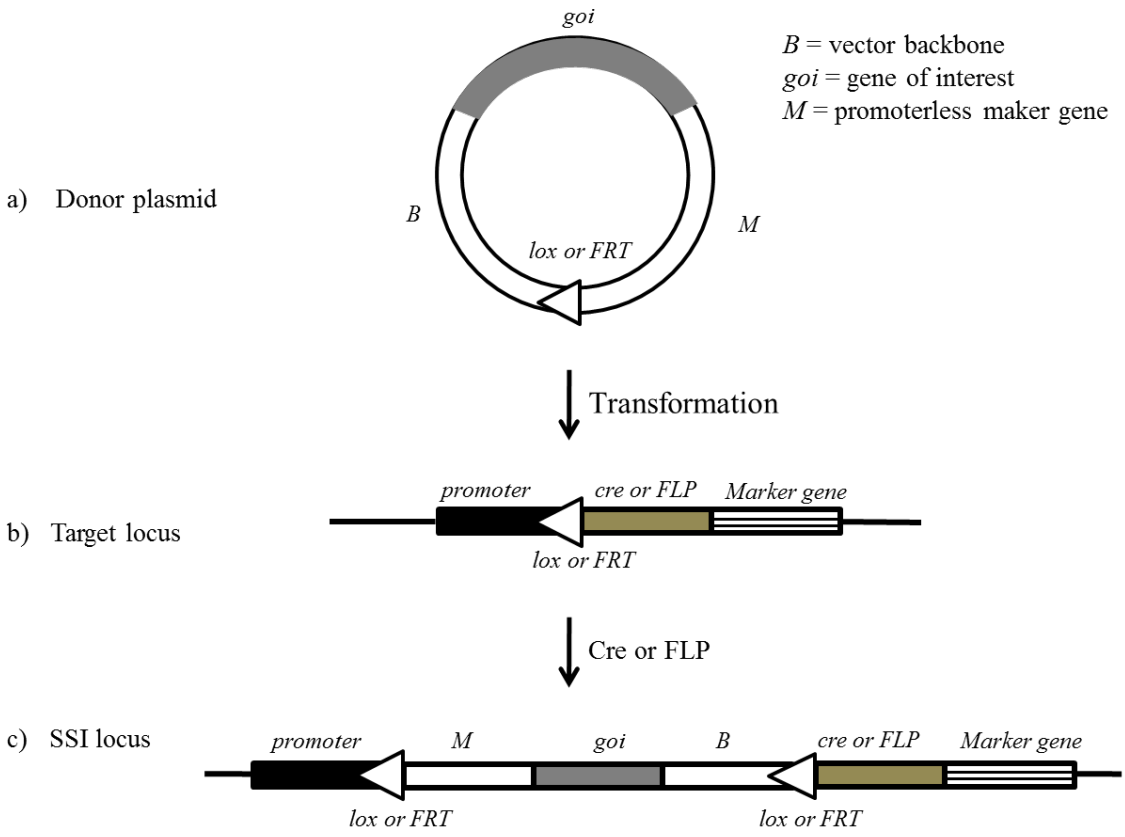


Figure 3: Molecular Strategy of site-specific gene integration. (a) Donor plasmid containing the gene of interest (*goi*) along with a recombination site, *lox* or *FRT*, and promoterless marker gene (*M*). (b) Target locus carrying recombinase gene (*cre* or *FLP*) with a constitutive promoter, a target site (*lox* and *FRT*), and a selectable marker gene. (c) Introduction of the donor plasmid into the target cells results in recombinase (Cre or FLP) mediated site-specific integration of donor circle into target locus generating a site-specific integration (SSI) structure. The SSI structure contains unique fusion of marker gene (M) with the promoter of target locus, making the event selectable on specific drug or herbicide.

1.8.1.2. Cre/*lox* system for marker removal

A number of studies have utilized the Cre/*lox* system to excise DNA fragments or marker genes from transgene loci in various plant species, e.g. tobacco, rice, maize, banana, and wheat (Chong-Pérez et al., 2012; Cuellar et al., 2006; Dale and Ow, 1991; Day et al., 2000; Hoa et al., 2002; Jia et al., 2006; Khattri et al., 2011; Odell et al., 1990; Odell et al., 1994; Sreekala et al., 2005; Zhang et al., 2003). Principle of site-specific recombination based marker removal from transgenic plants was described in Figure 4. The general principle for testing marker removal is that a reporter gene (gene of interest) is separated from its promoter by a *lox*-flanked DNA segment. Upon the introduction of Cre activity, the DNA segment is removed, and a reporter gene is activated through fusion with its promoter. Cre activity can be introduced by re-transformation, crossing with cre expressing lines or conditional expression (tissue-specific or inducible). Utility of Cre/*lox* system for marker removal was demonstrated more than 20 years ago in a set of studies (Dale and Ow, 1991; Odell et al., 1990; Russell et al., 1992). Dale and Ow (1991) generated transgenic lines conferring hygromycin (*hyg*) and kanamycin resistance, in which *hyg* gene was flanked by *loxP* sites. The authors used both re-transformation and cross pollination to introduce Cre activity, and obtained hygromycin sensitive tobacco lines. Using the retransformation approach, 10 out of 11 kanamycin resistant lines were made hygromycin sensitive; whereas only 42 lines out of a total of 78 were made sensitive to hygromycin in the cross pollination approach. Thus, marker-removal efficiency is much higher in the retransformation approach. In rice, Hoa et al. (2002) also applied the cross pollination approach to remove the *hpt* gene from rice and analyzed T1 hybrids. Marker removal in F1 plants in this study was reported to be 26.02% when T0 plants were crossed with Cre plants, and 58.33% when T2 plants were crossed with Cre plants. Zhang et al.

(2003) tested auto-excision approach (removal of *cre* gene and marker gene in a single fragment) in maize by placing the *cre* gene under the control of a heat shock promoter. Both the *nptII* gene and *cre* gene were flanked by *loxP* sites. Heat treatment generated Cre activity, which removed the *loxP*-flanked fragment, and fused the *GFP* gene with its promoter. In this study, the auto-excision was very efficient but not 100% in some plants as they still expressed *nptII* after heat shock treatment. Marker removal using the inducible *cre* gene was also achieved in rice by Sreekala et al. (2005) and Khattri et al. (2011). In the first study, Cre activity was induced by a chemical (β-estradiol), and among 86 independent transgenic T0 plants, 10 plants were found to be marker-free after the induction. In the second study, Cre activity was controlled by the promoter *HSP 17.5 E,*a soybean heat shock promoter. Upon heat shock treatment, *nptII* gene was removed resulting in the activation of *gus* gene. The efficacy of heat-inducible *cre* gene for marker excision was demonstrated in six different cell cultures. Marker-excision and inheritance of marker-free locus in transgenic plants was studied in one line, in which it was found to be 94%. Using Cre/*lox* system, complex transgene locus or multi-copy integrations could be resolved to single-copy locus (De Buck et al., 2007; Moore and Srivastava, 2006; Srivastava et al., 1999; Srivastava and Ow, 2001). Srivastava et al. (1999) flanked a transgene fragment between two oppositely oriented *lox511* sites. The *bar* selection gene in this fragment was flanked by directly oriented *loxP* sites. Four bialaphos resistant wheat lines were generated, which were crossed with a Cre-expressing line. Analysis of F2 progeny from this cross revealed that a multi-copy locus was resolved to a single copy locus, and the *bar* selection marker gene was removed concomitantly. Thus, utilization of two sets of heterospecific *lox* sites (*loxP* and *lox511*) led to marker removal and locus simplification in one step.

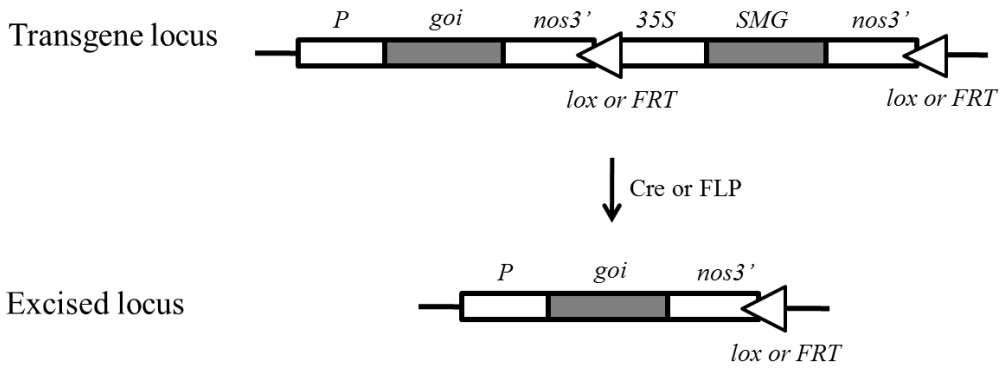


Figure 4: Principle of site-specific recombination based marker removal from transgenic plants. Transgenic lines containing the gene of interest (*goi*) are generated. The transgene construct contains a cassette of a marker gene (*SMG*) flanked by two directly oriented recombination sites (triangles). Then, introduction of recombinase activity into the target lines leads to marker removal and generates transgenic lines containing only the *goi*. *P*, promoter; *nos3'*, nopaline synthase terminator; *35S*, cauliflower mosaic virus 35S promoter; *goi*, gene of interest.

1.8.2. FLP/*FRT* based plant biotechnologies

The FLP/*FRT* system has been shown to function in *Arabidopsis* (Sonti et al., 1995), tobacco (Lloyd and Davis, 1994), rice (Radhakrishnan and Srivastava, 2005), maize (Lyznik et al., 1996), and turfgrass (Hu et al., 2006) among other plant species. Similar to the Cre/*lox* system, FLP/*FRT* system has been employed for: (1) precise transgene integration, and (2) maker removal. However, a smaller number of studies that describe the use of this system have been published compared with the Cre/*lox* system, and most of the reports are on marker excision.

1.8.2.1. FLP/*FRT* system for site-specific integration

The FLP/*FRT* system has been used for site-specific integration in soybean (Li et al., 2009), aspen (Fladung, Schenk, Polak and Becker, 2010), and rice (Nandy and Srivastava, 2011, 2012). For site-specific integration, in general, the target locus containing *FRT* sites are generated by a random transformation approach. Then, a donor construct containing the gene of interest is introduced along with *FLP* gene to produce site-specific integration structure (Figure 3). Li et al. (2009) and Fladung et al. (2010) achieved site-specific integration via the exchange of DNA cassettes between the target locus and the donor DNA placed between a pair of *FRT* sites. With the co-integration approach involving a single *FRT x FRT* recombination, Nandy and Srivastava (2011) generated precise integration of the *gus* gene in rice genome. In this study, FLP activity was introduced via transient expression of FLPe (described in section 1.7). Introduction of FLPe activity was important as the *FLP* gene located in the target locus was inactive. A careful analysis in this study concluded that expression of *FLP* gene is severely down regulated when an *FRT* site is present between the promoter and the *FLP* coding

sequence, a design commonly used in site-specific integration methodology. It should be noted that the presence of the *lox* site between the promoter and the *cre* gene does not significantly affect Cre expression. Since FLPe displays higher recombination efficiency compared to FLPwt (Buchholz et al, 1998), FLPe was chosen to drive site-specific integration of foreign DNA into the rice genome. A recent study by Nandy and Srivastava (2012) developed a marker-free site-specific integration approach in which the marker gene from site-specific integration locus developed by FLPe/*FRT* system was removed by Cre/*lox* recombination. Thus, recombinase-mediated transgene locus manipulation is highly precise and versatile.

1.8.2.2. FLP/*FRT* system for marker removal

For marker removal in plants, the FLP/*FRT* system has been applied in tobacco (Davies et al., 1999; Gidoni et al., 2001; Woo et al., 2009), aspen (Fladung and Becker, 2010), *Arabidopsis* (Kumar and Thompson, 2009), rice (Akbudak and Srivastava, 2011; Hu et al., 2008) and turfgrass (Hu, Nelson and Luo, 2006) among others. The general principle for testing marker removal is the same as described in the Cre*/lox* system. Davies et al. (1999) used this strategy in tobacco to remove *gus* gene and activate the spectinomycin resistance gene (*aadA*). In this study, FLP activity was introduced by crossing the FLP-expressing lines with the target (*FRT*) line. The excision was evident in the somatic cells of F1 hybrids; however, only one of eight F1 plants displayed excision in germinal tissue as stable marker-free (FLP-negative) F2 progeny was obtained only from one F1 parent. Woo et al. (2009) placed a cassette consisting of a hygromycin resistance gene (*hyg*) and *FLP* gene under the control of hydrogen peroxide-induced promoter in the *FRT*-flanking region, and developed transgenic tobacco lines. When hydrogen peroxide was applied, FLP was expressed and *FRT*-flanked region was removed. This recombination resulted in the fusion of the *bar* gene with the *35S* promoter. The authors

reported about 13-41% T1 seeds were marker-free (*hpt*), and grew on media containing phosphinothricin but not hygromycin. A similar strategy was applied by Fladung and Becker (2010) to remove marker genes and activate GUS activity from aspen. They used heat-shock promoter to activate the FLP gene instead of a chemical-induced promoter and obtained GUS expression in 15 lines out of 23 transgenic aspen lines. Hence, marker removal in that study was reported to be about 65%.

Gidoni et al. (2001) applied the FLP/*FRT* system for excision of the functional *rolC* gene in tobacco. *rolC* confers a distinct phenotype consisting of small, male-sterile flowers and some other pleiotropic effects. In this study, *rolC*-expressing line was crossed with FLP-expressing line, and removal of *rolC* was assessed by restoration of male fertility and normal phenotypes in F1 tobacco plants. This study reported restoration of fertility and seed setting to be 52-93% or 20-42% in the progenies displaying normal or sectorial leaf phenotypes, respectively. In *Arabidopsis*, Kumar and Thompson (2009) placed a reporter cassette (*35S-Luciferase-GFP* NosT) between two regions called "to be removed" (TBR). Each TBR was flanked by directly repeated *FRT* sites. This double TBR construct was used to retransform *Arabidopsis* carrying a heat shock-inducible *FLP* gene. With this strategy, the authors obtained TBRs-free *Arabidopsis* plants upon FLP induction, and also showed reduction of transgene copy number in later generations. Marker removal was also successfully done in rice by Hu et al. (2008) and Akbudak and Srivastava (2011). In both studies, the *nptII* gene was removed to activate GUS activity. However, in the first study, FLP activity was introduced by crossing FLP-expressing line with *FRT* recombination-reporter line; while in the second study, marker removal was achieved when marker-containing lines were retransformed with *FLPe* gene. These two studies are further discussed in the next chapter (Part 2).

All of the above studies, except that of Akbudak and Srivastava (2011), utilized the wild-type FLP (FLPwt) for marker excision. Marker excision was observed in all of these studies in somatic cells but transmittance of marker-free locus to the next generation was usually found to be inefficient. Additionally, FLPwt was found to be ineffective in directing site-specific gene integration in the rice genome (Nandy and Srivastava, 2011). These observations indicate that FLP/*FRT* system is not as robust as Cre/*lox* system when FLPwt is used. However, this deficiency has been effectively addressed by the use of FLPe and FLPo proteins.

1.9. Efficiency of FLP Proteins

A study on the fusion of nuclear localization signal into phiC31, Cre, and FLPe in hamster ovary cells revealed FLPe only achieved 10% recombination activity on chromosomal targets compared with Cre (Andreas et al., 2002). In embryonic stem cells, Cre and FLPo have similar recombination efficiency (Raymond and Soriano, 2007). Recently, several researches in mammalian system have successfully utilized FLP (Schaft et al., 2001) and FLPe (Farley et al., 2000; Ponsaerts et al., 2004; Wong et al., 2011) to drive recombination. From protein titration experiments, FLP is required at 10 fold concentration compared to Cre to obtain optimum recombination of the same quantity of substrate (Ringrose et al., 1998). In plant systems, a study on the function of the FLP/*FRT* system in rice showed that FLPwt is as efficient as Cre in removing DNA segment when FLP and *FRT* vectors are co-bombarded into rice cells (Radhakrishnan and Srivastava, 2005). Akbudak and Srivastava (2011) compared FLPwt and FLPe activities on a chromosomal target by bombarding FLP vectors into a *FRT*-transgenic line, and found FLPe to be three to five times more efficient. However, there is no study that directly compared recombination efficiency of different FLP variants, FLPwt, FLPe and FLPo,

expressed from genomic loci on chromosomal *FRT* targets. Evaluation of FLP activity using this design is necessary to assess the efficiency of FLP/*FRT* system for marker removal from plant genomes.

Part 2.

RESEARCH OBJECTIVES

Cre/*lox* has become the first choice for marker-excision and site-specific integration because of its superior efficiency in a wide range of species. However, some applications such as marker-free site-specific gene integration require the use of two recombination systems (Darbani et al., 2007; Fladung and Becker, 2010; Nanto and Ebinuma, 2008; Srivastava and Ow, 2004). Moreover, the availability of alternative recombination systems can offer the opportunity to develop multiple applications in a single platform. Hence, there is a need to develop additional recombination systems that can efficiently carry out both DNA excision and integration reactions. The well-characterized FLP/*FRT* system should be evaluated further for its recombination efficiency in crop plants such as rice. In an earlier study, FLPwt was found to have similar efficiency as Cre in removing marker genes from the extra-chromosomal plasmid molecules in rice cells when expressed from genomic DNA (Radhakrishnan and Srivastava, 2005). In a later study, Hu et al. (2008) crossed seven FLP-expressing transgenic rice lines with 19 *FRT*-target lines and demonstrated that FLP recombinase efficiently catalyzed the excision of *nptII* gene from the target locus, which resulted in the activation of the *GUS* gene. Hu et al. (2008), however, did not analyze the recovery rate of a stable marker-free locus (% of Gus-positive, FLP-negative progeny). The excision efficiency in this study was based on the presence or absence of uniform GUS activity in the progeny. Among 27 hybrids, Hu et al. (2008) found 15 hybrids with complete DNA recombination (uniform GUS activity), and 12 with incomplete DNA recombination (variation in GUS activity). Further, presence of uniform GUS staining in a large number of F2 progeny suggested that the FLP-mediated marker excision took place in the germinal cells of the F1 parent. However, the authors also noted that a number of hybrids that showed uniform GUS staining failed to generate stable marker-free F2 progeny. Hence, the efficiency

of the FLP/*FRT* system in removing marker genes from transgenic line and its progeny was not evaluated by confirming the presence of a recombination footprint in the progeny. In another study, Akbudak and Srivastava (2011) found that transiently expressed, FLPwt was ineffective in recombining *FRT* sites located on the rice genome, whereas FLPe was effective. Thus, a careful evaluation of the FLP activity on chromosomal targets is needed.

There are three different versions of FLP proteins: FLPwt, FLPe (the thermostable derivative of FLPwt), and FLPo (the mouse codon-optimized version of FLPe); however, no previous study compared recombination efficiencies of these variants when they are stably expressed from a genomic locus and act on chromosomally located *FRT* sites. Hence, the overall objective of this research is to stably express the three variants of FLP recombinase (FLPwt, FLPe and FLPo) in rice and evaluate their relative recombination efficiencies for marker gene excision from a well characterized transgene locus, 1.7D (Khattri, 2006).

In order to achieve the above objective, the following steps were executed:

1. Develop transgenic rice lines expressing FLPwt, FLPe, and FLPo.
2. Carry out molecular analysis of the lines.
3. Initiate crosses of the selected FLP lines with the 1.7D line.

However, to the time of this thesis, experiments for only first two steps were completed. In the future, selected FLP lines will be crossed with the 1.7D line, and will be evaluated the efficiency of FLP-mediated transgene excision in F1 hybrids and the progeny.

Part 3.

MATERIALS AND METHODS

3.1. Two Distinct Approaches for Generation of FLP-expressing Lines

Two different approaches were used to generate FLP lines:

(1) Site-specific approach: *FLP* genes will integrate into the defined T5 locus (see 3.2.1) to generate identical transgenic lines. This is the best scenario to compare activity of FLP proteins (see 4.1). FLP lines generated by this approach are referred as FLP-SSI lines.

(2) Standard method for genetic transformation: random integration: *FLP* genes will integrate randomly into different loci and therefore, generate variation in gene expression. FLP lines generated by this approach are referred as Nipponbare FLP lines.

3.2. Material

3.2.1. Rice Cultivars

For the site-specific approach, the T5 line (Taipei-309), a well-characterized Cre/*lox* target line (Srivastava and Ow, 2002) (Figure 5) was used. In the T5 locus, *cre* is controlled by the maize ubiquitin promoter (*Ubi*); one *lox 76* site is placed between the *Ubi* promoter and *cre* gene; next to c*re* gene is the hygromycin resistant gene (*HygR*) (*35S:hpt:nos3'*). The T5 locus provides Cre activity for site-specific integration. On the other hand, the Nipponbare cultivar was used to generate FLP lines in a random integration approach.

3.2.2. Plasmid Constructs

Two different sets of constructs were used in this study. Plasmids used in the site-specific approach were called donor constructs whereas plasmids used in the random integration approach were called random integration constructs. All plasmids were already available in Dr. Srivastava's laboratory.

3.2.2.1. Donor constructs

In the donor vectors, FLP coding sequences are driven by the *Ubi* promoter. The *npt* gene was placed before *FLP* genes for selection on geneticin-containing media. The whole cassettes are flanked by *loxP* and *lox75* sites (Figure 6). The only difference among these constructs is the FLP coding sequence in which:

pAA9 contains the coding sequence for FLPo.

pAA10 contains the coding sequence for FLPe.

pAA11 contains th ecoding sequence for FLPwt.

3.2.2.2. Random integration constructs

The random integration vectors are shown in Figure 7, which contain the coding sequences for the three types of FLP protein in which:

pAA7 contains the coding sequence for FLPo.

pAA8 contains the coding sequence for FLPe.

pUbiFLP contains the coding sequence for FLPwt

Because of the lack of a marker gene for selecting transformed cells, these constructs were used in a co-bombardment with a selection vector containing the hygromycin resistant gene (pHPT).

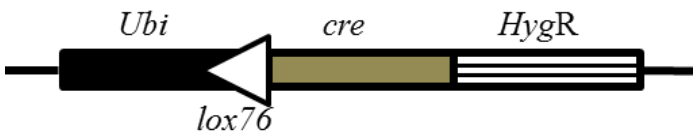


Figure 5: T5 target locus. *cre* is under the control of the maize ubiquitin promoter (*Ubi)*. One *lox76* site is placed between *cre* and the *Ubi* promoter. Hygromycin resistant gene (*Hyg*R = *35S:HPT:nos3'*) is present in the locus for selection.

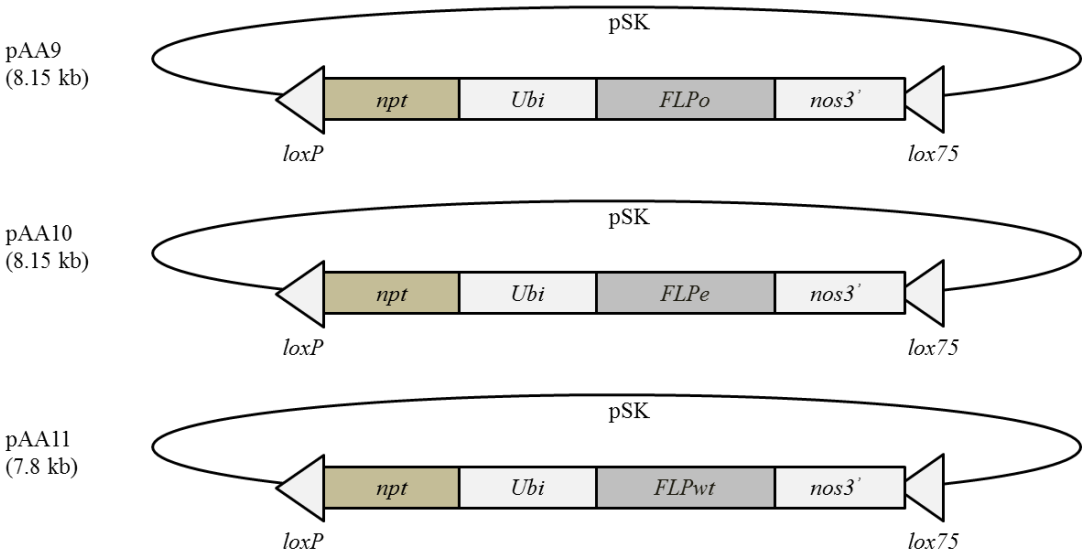


Figure 6: FLP donor constructs. *loxP* and *lox75* sites flank a cassette involving promoterless neomycin resistant gene (*npt*) and *FLP* genes. *FLP* genes are controlled by maize ubiquitin promoter (*Ubi*).

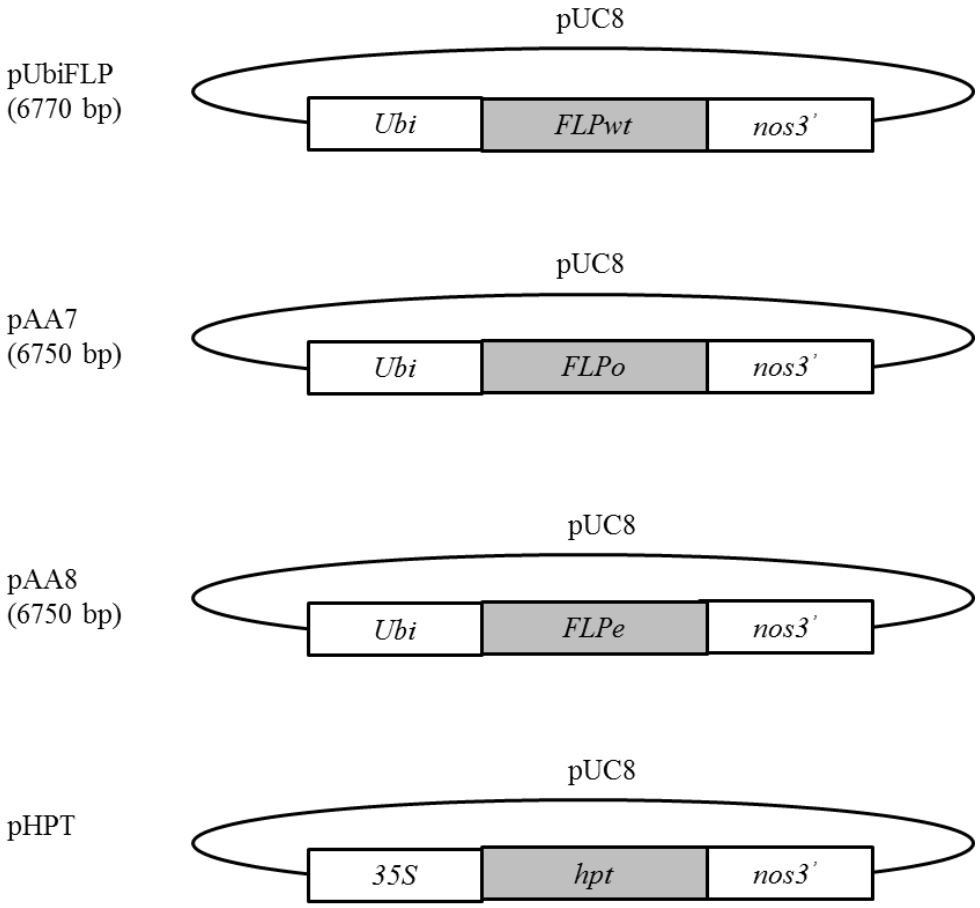


Figure 7: Random integration constructs. *FLP* genes are driven by the maize ubiquitin promoter (*Ubi)*. The *hpt* gene is driven by the *35S* promoter.

3.3. Methods

3.3.1. Rice Transformation, Selection and Regeneration

The rice tissue culture procedure was adopted from Nishimura et al. (2006). Seeds of T5 or Nipponbare cultivar were placed on 2N6D media (Table 2) to induce callus formation. Callus was selected and transferred to bombardment media (Table 2) and kept for two hours before transformation using the Biolistic particle bombardment method with a PDS-1000/He system (Bio-Rad). Each donor construct (10 µg) was separately coated onto 25 µg gold suspension, whereas 5 µg of each random integration construct along with 5 µg p*Hyg* was separately coated onto 50 µg gold suspension. Each DNA-coated gold suspension was included in a mixture with 50 µl 2.5M $CaCl_2$ and 20 µl 0.1M spermidine for each transformation experiment.

After bombardment, calli were kept 24 hours in bombardment media. Then, calli were divided into four equal parts as four possible independent transgene events and transferred to 2N6D media for a week. Subsequently, they were transferred to 2N6D media containing geneticin^{TM} (100 mg/ml) or hygromycin (50 mg/ml) according to the type of plasmid used in transformation, for selection of transformed calli for 4-8 weeks. The growing calli on selection media were selectively transferred to regeneration media (Table 2). Callus induction, selection, and regeration were carried out in Percival^{TM} growth chamber at 25^{o}C with full light. Regenerated shoots were transferred to plant growth media for root induction and shoot elongation. Plants were then moved to the greenhouse.

Table 2: Plant tissue culture media.

Meida	Components (for 1 litter)
2N6D	3.98 g N6 (CHU) basal salt mixture; 0.1 g Myo-inositol; 0.3 g Casaminoacids; 2.878 g Proline; 100 µl 2,4 D (10 mg/mL); 30 g Sucrose; 3 g Phytogel; 10 ml N6 vitamins 100X stock; pH = 5.8
Bombardment	4 g N6 (CHU) basal salt mixture; 0.1 g Myo-inositol; 1 g Casaminoacids; 200 µl 2,4 D (10 mg/mL); 30 g Sucrose; 63 g Sorbitol; 2 g Phytogel; 2 ml N6 vitamins 500X stock; pH = 5.8
Regeneration	4.6 g MS salt mixture; 10 ml of 100X MS vitamin; 0.1 g Myo-inositol; 2 g Casaminoacids; 1 ml of 1,000X NAA; 20 ml of 50X Kinetin; 30 g Sucrose; 30 g Sorbitol; 3 g Phytogel; pH = 5.8
Plant growth	4.6 g MS salt mixture; 10 ml of 100X MS vitamin; 0.1 g Myo-inositol; 30 g Sucrose; 3 g Phytogel; pH = 5.8

3.3.2. DNA Isolation

For molecular analysis, DNA was extracted from callus or leaf tissue of transgenic lines using a CTAB method. Frozen tissues were ground and dissolved in 2 ml CTAB extraction buffer by vortexing and incubating at 55 ^{o}C in 30 minutes. subsequenctly, 1 ml phenol-chloroform was added, mixed and centrifuged at 4000 rpm for 10 minutes. The aqueous phase was collected into 2 ml of chloroform:iso-amyl alcohol (24:1). After centrifugation at 4000 rpm for 5 minutes, the aqueous phase was collected and mixed with double volume of cold 95% ethyl alcohol before DNA was collected by centrifugation at 4000 rpm in 10 minutes. After one wash with cold 70% ethyl alcohol, the DNA was dried and dissolved in autoclaved water.

3.3.3. Polymerase Chain Reaction (PCR)

PCR was carried out on the genomic DNA of geneticin or hygromycin resistant lines with corresponding primers to determine successful integration of the *FLP* genes. Amplified products were separated on 0.8% agarose gel. The components for one 25-µl PCR reaction are 1 µl of 100 ng / µl DNA; 5 µl of Mg-free buffer; 1.5 µl of 25 mM $MgCl_2$; 2.5 µl of 2 mM dNTP; 0.5 µl for each of 25 µM forward and reverse primers; 0.25 µl of 100 µg/µl Taq polymerase; and 13.75 µl of autoclaved water.

Quantitative real time PCR (qRT-PCR) was also carried out for measuring mRNA abundance of FLP genes. Total RNA was extracted from leaf tissue of T1 FLP lines and subjected to qRT-PCR. The components for one 12.5- µl qRT-PCR reaction are: 0.25 µl *Tag* mix; 6.25 µl of 2X SYBR Green reaction mix; 0.05 µl for each of 15 µM forward and reverse primers; 1.5 µl of RNA template (25 ng/ µl); and 4 µl RNase-free water. Combination results

from this experiment and FLP transient assays will provide correct comparison of activity of FLP proteins (see sections 5.4 and 5.5).

3.3.4. Southern Hybridization

To further confirm the presence of the transgene and determine the transgene integration pattern, Southern hybridization was carried out. About 5 µg of genomic DNA (from leaf tissue) was digested with *Eco*RI or *Eco*RV according to random integration or SSI approach, respectively. Digested DNA was separated on 0.8% agarose-ethidium bromide containing gel and then transferred onto nylon membranes (Amersham Hybond^{TM} – N^{+}). These membranes were hybridized with particular ^{32}P labeled DNA probes for *ubi* and *FLP* genes.

3.3.5. FLP Expression Assay

Calli from FLP lines were bombarded with plasmid pRP9 in which the *FRT*-flanked *npt* gene separates the *gus* gene from a *Ubi* promoter (Figure 8). The FLP activity from the FLP lines is expected to remove the *npt* cassette from the beta-glucuronidase (GUS)-negative pRP9 locus and generate the GUS-positive recombination footprint (Figure 8). The GUS activity was detected by incubating bombarded explants in GUS stain solution. The components of GUS stain solution are: 0.1 M sodium phosphate buffer (pH 7); 0.5 mM $K_3Fe(CN)_6$; 0.5 mM $K_4Fe(CN)_6$; 10 mM Na_2EDTA (pH8); and 1 mM X-Gluc (5-Bromo-4-chloro-3-indoxyl-β-D-glucuronide cyclohexylammonium salt).

After bombarding with *pRP9* vector, calli were kept 72 hours at room temperature. Then, the calli were placed in GUS staining solution and kept at 37 ^{o}C. To calculate GUS activity, the total number of blue spots generating on each callus in each staining well was counted.

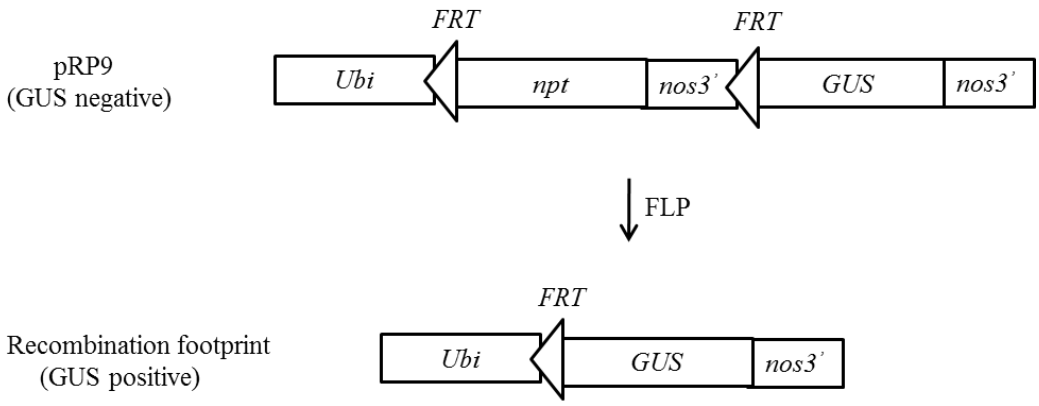


Figure 8: **Molecular strategy for the FLP expressing assay.** FLP-expressing lines were bombarded with the *pRP9* vector. FLP activity is expected to remove the *npt* gene from the vector to put the promoterless *GUS* gene under the control of the *Ubi* promoter. Hence, FLP activity has detected indirectly via GUS expression. *Ubi*, maize ubiquitin promoter; *npt*, neomycin phosphotransferase gene; *nos3'*, transcription terminator from nopaline synthase gene; *GUS*, beta-glucuronidase gene (coding region).

Part 4.

RESULTS AND DISCUSSION

Section 1.

Rice Transformation by the Site-specific Integration Approach

4.1. Rationale for the Site-specific Integration Approach

Different genomic positions and variable copy numbers of transgenes generate variation in gene expression. The genomic 'position effect' should be eliminated to precisely compare recombination efficiency of FLP proteins, and *FLP* genes should be expressed at the same level. *FLP* genes are expected to have same expression level when they are expressed from a locus containing a single copy that was inserted in a specific site within the genome referred to as site-specific integration (SSI). Due to the fixed genomic position and locus structure, SSI is expected to generate equal level of *FLP* expression from different transgenic lines as demonstrated for *GUS* and *GFP* genes in rice SSI lines (Akbudak et al., 2010; Chawla et al., 2006; Day et al., 2000; Nanto and Ebinuma, 2008; Srivastava et al. 2004).

4.2. Molecular Strategy for Site-Specific Integration

In the site-specific integration approach, donor constructs containing *FLPwt, FLPe* or *FLPo* genes were introduced into the T5 line that contains a 'target locus' for Cre/*lox* mediated site-specific integration. Cre activity in T5 cells is expected to split the donor construct via *loxP* x *lox75* recombination, and generate a backbone-free donor circle. This donor circle contains a promoter-less *npt* gene, and a functional *FLP* gene. The donor circle will integrate into the T5 target locus via *lox75* x *lox76* recombination, and form a site-specific integration locus (SSI) (Figure 9). The SSI locus expresses the *npt* gene, and is therefore selectable on geneticin^{TM}. In addition, SSI locus contains a double-mutant *lox* site (*lox75/76* fusion), that is important for stabilizing the SSI locus as the *lox75/76* recombinant is a poor substrate of Cre.

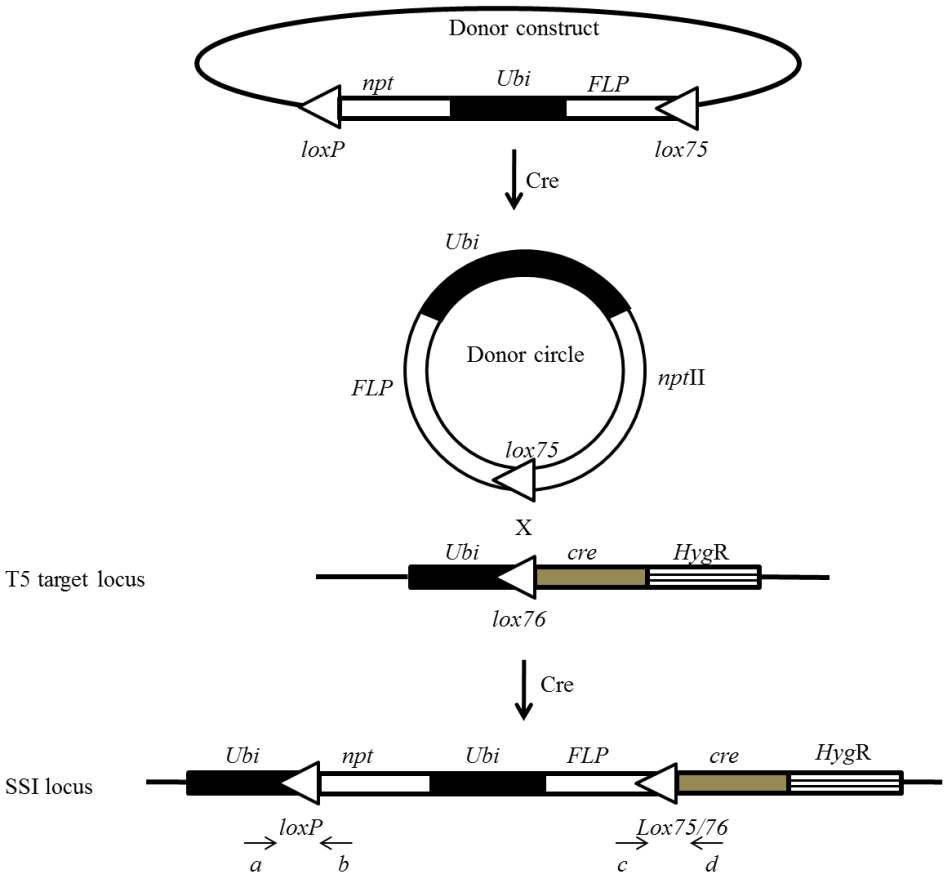


Figure 9: Site-specific integration of the *FLP* gene into the T5 locus via Cre-*lox* recombination. Upon introduction of FLP donor constructs (pAA9, 10, 11) into the T5 callus, Cre activity in T5 cells splits the donor construct via *loxP* X *lox75* recombination, and generates a backbone-free donor circle, which contains the promoter-less *npt* gene and a complete *FLP* gene. Then, the donor circle integrates into the T5 target locus via *lox75* x *lox76* recombination, and forms a site-specific integration locus (SSI). *npt*II, promoterless neomycin phosphotransferase gene; *Ubi*, maize uniquitin promoter; *FLP*, *FLPwt* or *FLPe* or *FLPo* gene; *cre*, Cre recombinase gene; *Hyg*R, hygromycin resistance gene (*35S:HPT:nos3'*); *a, b, c*, and *d* are PCR primers.

4.3. Generation of FLP-SSI Lines in the T5 Background

Seeds from the T5 cultivar were plated on 2N6D media for callus induction. Callus was selected for particle bombardment. FLPwt, FLPe, and FLPo lines were generated by bombarding T5 callus with plasmids pAA11, pAA10, and pAA9, respectively. Bombarded calli were selected on geneticin^{TM} (100 mg/L) -containing 2N6D media. In total, three experiments consisting of 77 plates were conducted to generate FLP-transgenic lines in which 19 plates were for FLPwt, 28 plates for FLPe, and 30 plates for FLPo. These bombardments generated 65 FLPwt, 41 FLPe, and 28 FLPo geneticin-resistant lines (Table 3). Each callus which was able to grow on the selection media was transferred to regeneration media containing Geneticin^{TM} (100 mg/L). At the same time, genomic DNA was extracted from the geneticin-resistant callus lines, and subjected to PCR analysis.

4.4. PCR Analysis of FLP Callus Lines

Geneticin resistant callus lines were subjected to PCR for the detection of two integration junctions: the first junction spanning *loxP*, and the second junction spanning *lox75/76*. Primer pair *a* and *b* was used to detect *junction 1* at *loxP* site, and primer pair *c* and *d* was used to detect *junction 2* at *lox75/76* site (Figure 9 and Table 4). A large number of FLPwt lines was difficult to handle, so for PCR analysis, 20 out of 65 FLPwt lines were randomly selected. Twenty eight FLPe and FLPo were selected for PCR. Eighteen FLPwt, 18 FLPe and 12 FLPo were positive for both *junctions* (Table 3; Figure 32 in Supplementary Work section).

4.5. FLP-activity Assay on Transgenic Callus Lines

FLP expression assay was carried out on all available lines in order to select the lines displaying high FLPwt activity. In this assay, FLP lines were bombarded with pRP9 vector, and stained for GUS activity 72 hours after bombardment. In the pRP9 vector, a cassette of *FRT*-flanked *npt* fragment separates the *gus* gene from the *Ubi* promoter. FLP activity is expected to remove the *npt* fragment and fuse the *gus* gene with the *Ubi* promoter, which leads to GUS expression (Figure 8).

Sixty five FLPwt, 41 FLPe, and 12 FLPo lines were bombarded with pRP9 vector. All 65 FLPwt resistant lines showed a blue background staining without deep blue spots. This result indicated that there was intrinsic or native GUS activity in FLPwt lines, and no activity originating from FLP-mediated recombination of pRP9 was generated (absence of deep blue spots). GUS activity is generally absent in plants. However, a few studies reported intrinsic GUS activity in a number of plant species under specific conditions such as callus cells: sugarbeet, *Arabidopsis*, rice, tobacco, maize, rye, potato, tomato, apple, and almond (reported and reviewed by Sudan et al., 2006; Wozniak et al., 1994).

To further confirm the native GUS activity, GUS staining of FLPwt callus lines was carried out without pRP9 bombardment, which generated dark blue stain in all 65 FLPwt lines. These results are illustrated with a representative line, T20C4, in Figure 10. Twenty one FLPwt SSI lines were selected for regeneration. These lines included 18 PCR-positive lines, and three newly available lines that showed light blue background staining (Table 5). Intrinsic GUS activity was not observed in FLPe and FLPo lines. In the FLP activity assay, none of the FLPwt lines showed the presence of deep blue spots upon pRP9 bombardment, and only background

staining was visible (Figure 10). This indicated absence of detectable FLP activity in FLPwt-SSI lines. Next, 7 out of 41 FLPe and 9 out of 12 FLPo lines displayed GUS activity (presence of deep blue dots) upon pRP9 bombardment (Figure 11 and 12). The FLPo lines generated more blue spots than FLPe lines in this assay, indicating higher FLP activity. Thus, FLPe and FLPo were found to display much higher FLP activity compared to FLPwt, and FLPo appeared superior to FLPe.

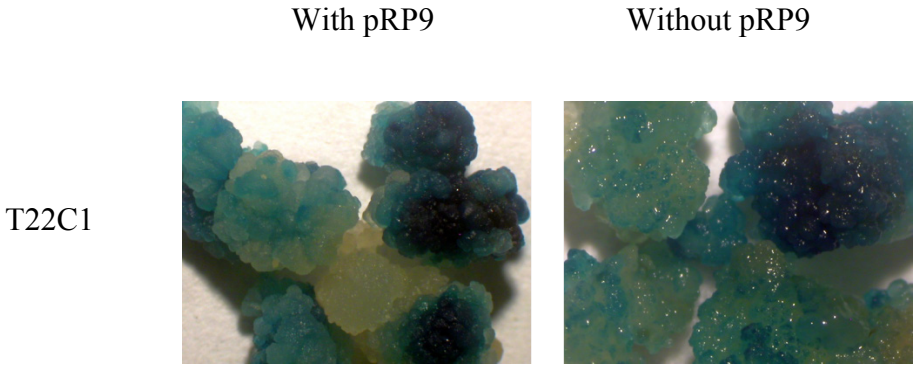


Figure 10: Intrinsic GUS activity in a representative FLPwt-SSI callus line (T22C1). Callus was submerged in GUS stain and incubated at 37^{o}C for a few hours or overnight. GUS activity in callus was observed regardless of pRP9 bombardment.

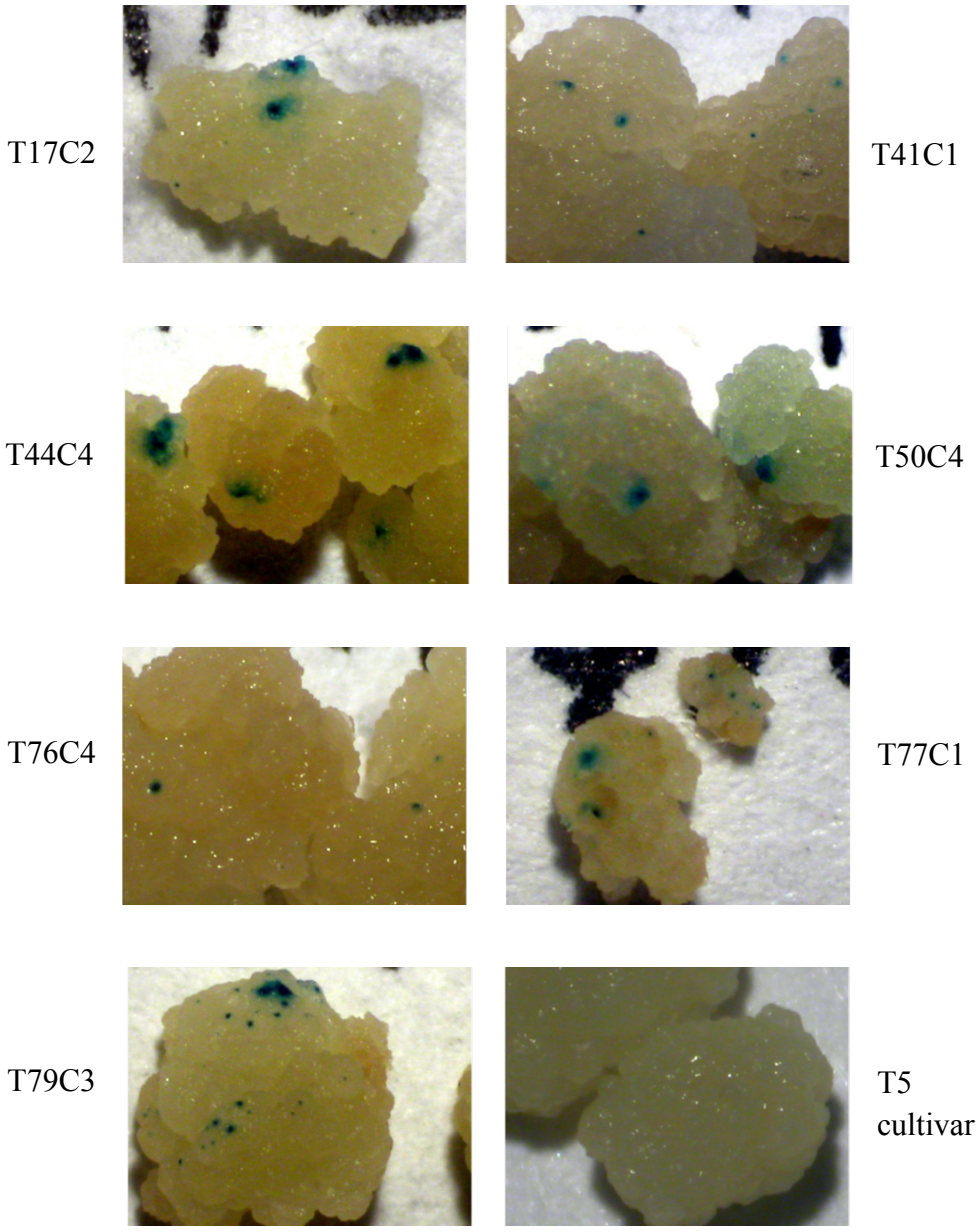


Figure 11: FLP assay in FLPe-SSI lines. Callus bombarded with pRP9 was submerged in GUS stain and incubated at 37^{o}C overnight. Number and size of blue dots indicates FLP activity. Seven FLPe lines that showed GUS activity upon pRP9 bombardment are shown. T5 callus was used as negative control.

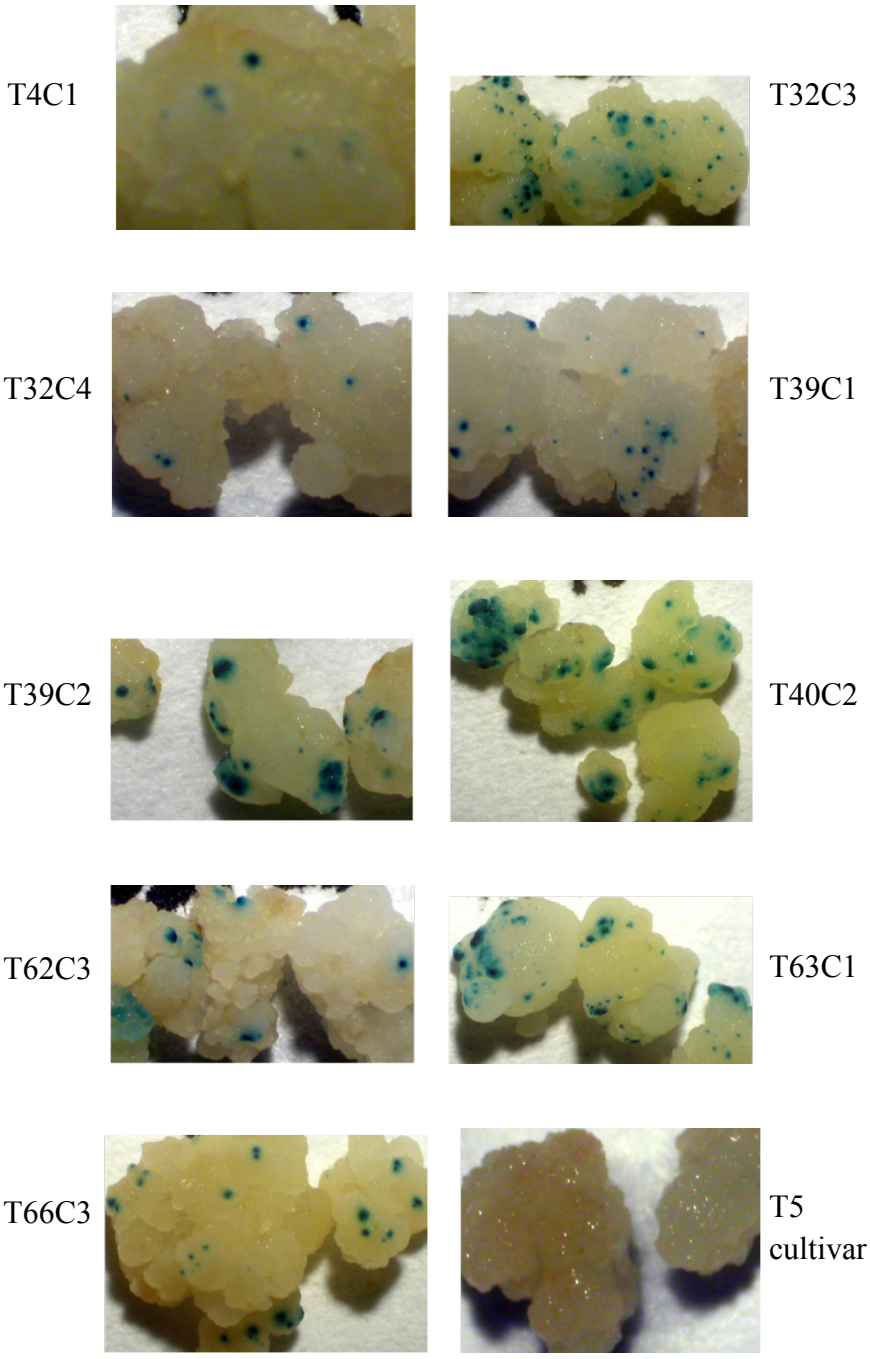


Figure 12: FLP assay in FLPo-SSI callus lines. Nine FLPo lines that showed GUS activity upon pRP9 bombardment are shown. GUS activity indicates FLP activity. T5 callus serves as negative control.

4.6. Generation of FLP Plant Lines

All FLP lines including PCR-positive and newly available lines (whether or not positive for FLP activity based upon the pRP9 bombardment) were considered for plant regeneration. Twenty one FLPwt lines, 33 FLPe lines and 15 FLPo callus lines were transferred to regeneration media. Of which, 17 FLPwt lines, 14 FLPe lines, and 4 FLPo lines were regenerated (Table 5, 6, and 7). Unfortunately, none of FLPe and FLPo lines that displayed positive FLP activity in pRP9 bombardment assay were successfully regenerated (Table 6 and 7). However, FLP-negative lines were considered for further analysis because we assumed that absence of FLP activity in callus may have been due to the reversion of SSI locus. SSI lines are mostly hemizygous for the insertion locus, and express strong Cre activity from the second target allele. This Cre activity may revert SSI locus to generate chimeric transgenic lines. These chimeric lines regenerate into SSI plant lines, which could also be chimeric. However, chimeric SSI lines transmit the SSI locus to progeny at high efficiency (Srivastava et al., 2004). In homozygous progeny, the SSI locus is stably expressed (Srivastava et al., 2004). It was not clear that FLP-negative lines had suffered reversion; therefore, FLP lines were regenerated.

Fifteen FLPwt, 8 FLPe, and 3 FLPo lines were successfully transferred to the greenhouse (Table 3, 5, 6, and 7). Since FLPwt callus showed native GUS activity, all 15 FLPwt plant lines were subjected to GUS staining to check for the presence of GUS activity. Leaf cuttings from 15 FLPwt lines were stained with GUS staining solution at 37^{o}C and observed after 12 or 24 h. Background GUS activity was visible in 9 FLPwt lines, whereas the other 6 FLPwt lines did not show GUS activity (Figure 13). The 9 FLPwt lines with GUS activity were removed from further analysis. Through the whole process from bombardment and tissue culture, 6 FLPwt, 8 FLPe, and 3 FLPo SSI lines were selected for further analysis (Table 5, 6, and 7).

Table 3: Summary of three bombardment experiments on T5 background.

FLP type	Experiment	No. of bombarded plates	No. of geneticin resistant callus lines	No. of callus lines selected for PCR	No. of callus lines positive for PCR	No. of regenerated lines *	No. of plant lines grown in greenhouse
FLPwt	1	10	32	17	16	17	15
	2	9	30	3	2	0	0
	Total	**19**	**65**	**20**	**18**	**17**	**15**
FLPe	1	9	14	12	7	7	5
	2	10	11	8	5	3	1
	3	9	16	8	6	4	2
	Total	**28**	**41**	**28**	**18**	**14**	**8**
FLPo	1	10	5	5	3	2	2
	2	10	12	12	7	1	1
	3	10	11	11	2	1	0
	Total	**30**	**28**	**28**	**12**	**4**	**3**

* : including PCR-positive lines and newly available lines (see Table 5, 6, and 7)

Table 4 : **Primers used for PCR analysis**

Codes	Primers	Sequences
a	Ubi	5’ TCTACTTCTGTTCATGTTTGTG 3’
b	KanR	5’ CTCGATGCGATGTTTCGCTT 3’
c	Gus2	5’ GATTAGAGTCCCGCAATTAT 3’
d	CreUAG	5’ CTAATCGCCATCTTCCAGCA 3’
e	FLP RII	5’ CTCAGTGATCTCCCA GATGC 3’
f	FLPe F	5’ CGCGCCACCATGAGCCAATTT 3’
g	FLPe R	5’ ATGCGGGGTATCGTATGCTTCC 3’
h	FLPo F2	5’ CCCAAGCTTGGATCCATGAGCCAGTTCGACATCCTG 3’
i	FLPo R	5’ GGGGTACCGAGCTCTCAG ATCCGCCTGTTGAT 3’
m	Hygro F	5’ ACCGCGACGTCTGTCGAGAA 3’
n	Hygro B	5’ CCAGTGATACACATGGGGATC 3’

Table 5: Summary of FLPwt-SSI lines

FLPwt	PCR (callus DNA)	Regenerated	Rooted	Green house	Native GUS leaf	Selected for further analysis
T20C3	+	+	+	+	-	*
T20C4	+	+	+	+	+	
T21C1	+	+	+	+	+	
T22C1	+	+	+	+	-	*
T22C2	+	+	+	+	-	*
T23C1	na	+	+	+	+	
T23C3	+	-				
T23C4	+	+	+	+	+	
T24C1	na	+	+	+	-	*
T25C3	+	+	+	+	-	*
T25C4	+	+	+	died		
T27C2	+	+	+	+	+	
T27C3	+	-				
T28C1	+	+	+	+	+	
T28C2	+	+	+	+	-	*
T28C3	+	+	+	+	+	
T28C4	+	+	+	died		
T29C3	na	+	+	+	+	
T29C4	+	+	+	+	+	
T55C1	+	-				
T55C3	+	-				

na: not assayed

Table 6: Summary of FLPe-SSI lines

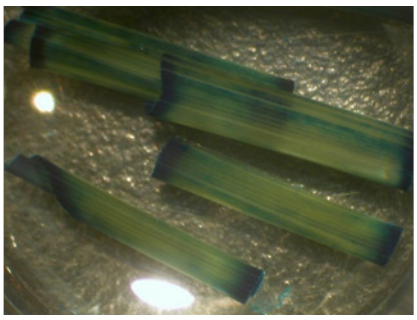
FLPe	PCR (callus DNA)	FLP activity (callus)	Regenerated	Rooted	Greenhouse
T11C3	+	-	-		
T11C4	na	-	-		
T12C2	+	-	+	+	+
T14C1	+	-	+	+	+
T14C3	+	-	+	+	+
T16C1	+	-	+	+	+
T17C2		+	-		
T17C4	+	-	+	+	+
T18C3	+	-	+	lost	
T41C1	-	+	-		
T44C4	na	+	-		
T45C1	+	-	+	-	
T45C4	+	-	+	+	+
T47C2	na	-	-		
T49C4	+	-	-		
T50C2	+	-	-		
T50C3	+	-	+	-	
T50C4	na	+	-		
T71C4	na	-	-		
T72C2	+	-	+	+	+
T72C4	na	-	-		
T73C3	na	-	-		
T74C1	na	-	-		
T75C1	na	-	-		
T75C3	na	-	-		
T76C2	+	-	+	+	+
T76C4	na	+	-		
T77C1	+	+	-		
T77C3	+	-	+	-	
T78C2	+	-	+	-	
T79C2	na	-	-		
T79C3	+	+	-		

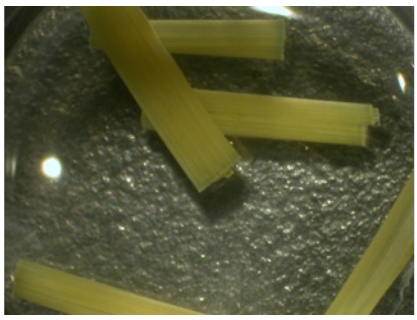
na: not assayed

Table 7: Summary of FLPo-SSI lines

FLPo	PCR (callus DNA)	FLP activity (callus)	Regenerated	Rooted	Greenhouse
T2C3	+	-	-		
T3C2	+	-	+	+	+
T4C1	+	+	+	+	+
T32C3	+	+	-		
T32C4	+	+	-		
T38C1	+	-	+	+	+
T39C1	+	+	-		
T39C2	+	+	-		
T40C1	+	-	-		
T40C2	+	+	-		
T63C1	+	+	+	lost	
T66C3	+	+	-		

T28C1





T28C2

Figure 13: Native GUS activity in representative positive (T28C1) and negative (T28C2) FLPwt lines. Leaf cuttings from 15 FLPwt lines were immersed in GUS staining solution at 37 ^{o}C and observed after 12 h and 24 h.

4.7. Molecular Characterization of FLP- SSI Plant Lines

FLP lines in greenhouse were characterized by molecular analysis. Genomic DNA was extracted from leaves of FLP-SSI lines by the CTAB method, and subjected to PCR and Southern hybridization.

4.7.1. PCR analysis

FLP-SSI lines, which contain integration of FLP donor constructs into the T5 locus, should have two unique junctions at the *loxP* and *lox75/76* sites (Figure 9). To test the presence of these junctions, DNA from FLP-SSI lines were subjected to PCR with primer pair *a* and *b* and primer pair *c* and *d* (Figure 9 and Table 4). Amplified fragments at *junction 1* and *junction 2* are expected to be 1.4 kb and 1.2 kb, respectively. DNA from the parent line, T5 cultivar, was used as negative control. Those lines that showed bands of the two junctions, even of an unexpected size which indicated the intake of the *FLP* genes, are considered as positive, and the lines that did not show any amplification are considered as negative. Based on this criteria, 5 out of 6 FLPwt-SSI lines (T20C3; T22C2; T24C1; T25C3; T28C2) were positive for both junctions, and one (T22C1) negative for both junctions (Figure 14 and Table 8). All eight FLPe-SSI lines (T12C2; T14C1; T14C3; T16C1; T17C4; T45C4; T72C2; T76C2) contained both junctions of the expected sizes (Figure 15 and Table 8). All three FLPo-expressing lines (T3C2; T4C1; T38C1) were positive for the two junctions; although T38C1 had an unexpected size for *junction 2*, which was assumed that there was truncation in adjacent sequences (Figure 16 and Table 8).

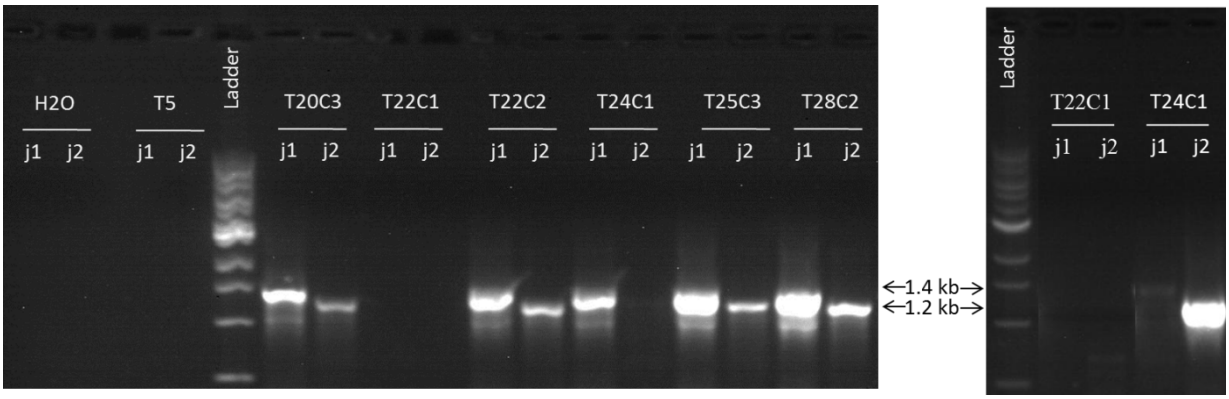


Figure 14 : PCR analysis for two integration junctions in FLPwt-SSI lines. Primers *a* and *b* amplified 1.4 kb fragment at *junction 1*; primers *c* and *d* amplified 1.2 kb fragment at *junction 2*.

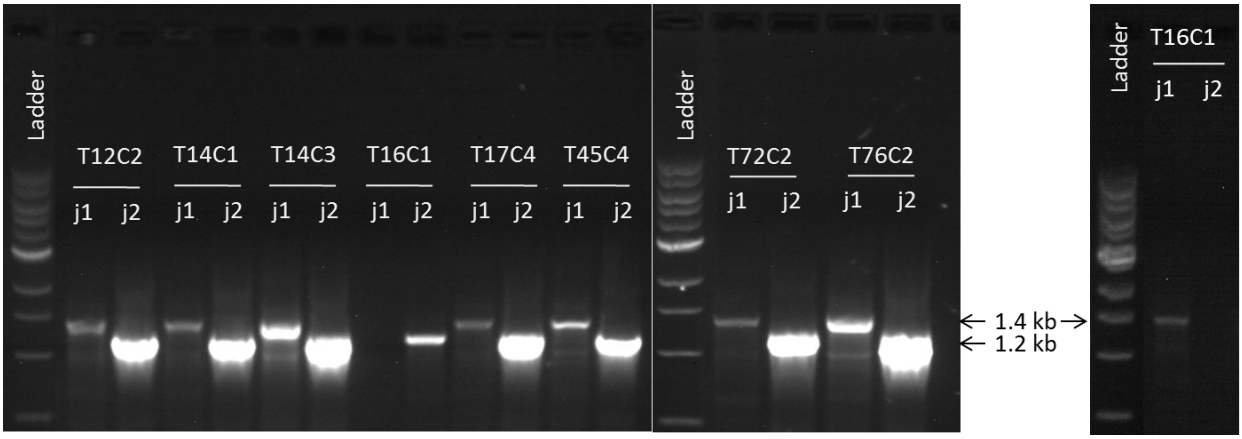


Figure 15 : PCR analysis for two integration junctions in FLPe-SSI lines. Primers *a* and *b* amplified 1.4 kb fragment at *junction 1*; primers *c* and *d* amplified 1.2 kb fragment at *junction 2*.

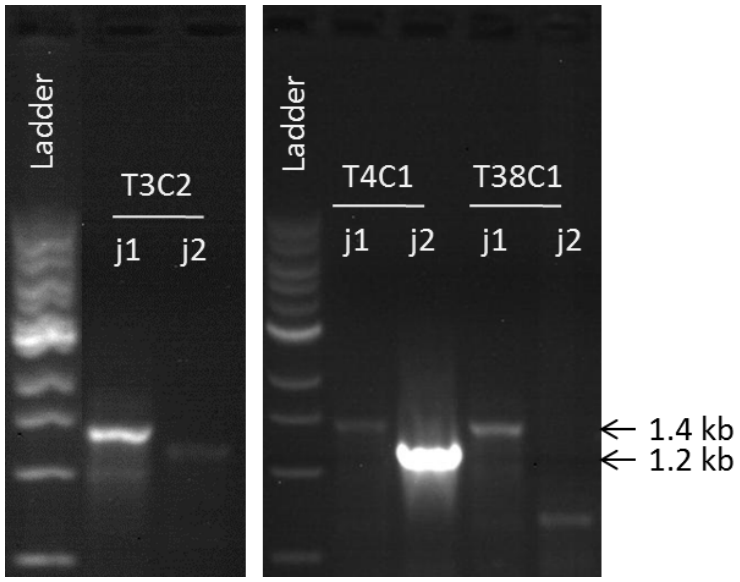


Figure 16: PCR analysis for two integration junctions in FLPo-SSI lines. Primers *a* and *b* amplified 1.4 kb fragment at *junction 1*; primers *c* and *d* amplified 1.2 kb fragment at *junction 2*.

Table 8: Summary of PCR analysis of FLP-SSI lines

FLP type	Positive for 2 junctions	Negative for 2 junctions
FLPwt	T20C3; T22C2; T24C1 *st; T25C3; T28C2;	T22C1*0
FLPe	T12C2; T14C1; T14C3*st; T16C1*st; T17C4; T45C4; T72C2*st; T76C2	
FLPo	T3C2; T4C1; T38C1*u	

*0: none of two junction amplified.
*u: unexpected size of amplified band.
*st: sterile

4.7.2. Southern analysis

After PCR analysis, FLP-SSI plant lines were analyzed by Southern hybridization to further investigate integration of transgenes and determine integration pattern. T24C1 (FLPwt-SSI line), T14C3, T16C1 and T72C2 (FLPe-SSI lines) were sterile, and had been removed; hence, this analysis were carried out on 5 FLPwt-, 5 FLPe-, and 3 FLPo-SSI lines.

Genomic DNA from the FLP-SSI lines and T5 cultivar was digested with *Eco*RV and hybridized with a ^{32}P-labelled probe for *Ubi* promoter. Since the *Ubi* promoter is located out of *Eco*RV fragment (Figure 17, 18 and 19), *Eco*RV-digested DNA from both T5 and FLP-SSI lines should present only one band on the blot, if consisting only of the SSI locus and lacking random integrations (single-copy). However, the hybridized bands for successfully integrated FLP-SSI lines should be of a larger size compared with the band for T5 locus as the SSI locus has an additional donor cassette (*npt-Ubi-FLP*). Furthermore, random integration of donor plasmids is also possible, which would generate a multi-copy integration pattern. Therefore, Southern hybridization results from FLP-SSI lines may fall into following categories:

(1) A 5-kb single band similar to the band of T5 cultivar: this pattern indicates no integration of donor circle into T5 locus, therefore, the line is non-transgenic. T22C1 (FLPwt-SSI line) fell into this category (Figure 17 and Table 9).

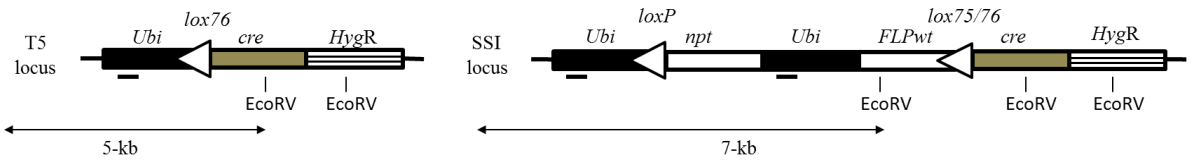
(2) Two bands, one same as that of T5, and the another of a larger size. Based on *Eco*RV maps of SSI locus of FLPwt, FLPe, and FLPo, ~7 kb with FLPwt and ~10 kb with FLPe and FLPo SSI locus is expected (Figure 17a, 18a, and 19a). This pattern indicates integration of donor circle into one of the T5 alleles (hemizygous integration). Southern hybridization revealed that three FLPwt-SSI lines (T20C3; T22C2; T25C3), two FLPe-SSI lines (T17C4; T45C4), and one FLPo-SSI line (T3C2) were hemizygous for

integration locus (Figure 17, Figure 18, Figure 19 and Table 9). However, these integrations were likely to be truncated as the observed bands were of smaller than the expected sizes. Further, the intensity of SSI band in some lines (e.g. T20C3, T25C3, T17C4, T45C4, and T3C2) was less than that of T5 band. This phenomenon is characteristic of "locus reversion" because of the Cre activity from the intact T5 allele in hemizygous SSI lines. This excision in T3C2 (FLPo-SSI line) took place at very high efficiency as integration allele is hardly detected (Figure 19).

(3) There was only one band at a larger size compared to that of T5 cultivar. This band should be at 10-kb for FLPe and FLPo lines or at 7-kb for FLPwt. This pattern indicates the lines contain SSI integration into both T5 alleles (homozygous). One FLPwt-SSI line (T28C2) and two FLPe-SSI lines (T14C1 and T76C2) fell in this category (Figures 17, 18 and Table 9). However, the bands were not at expected size; therefore, these integrations were likely truncated.

(4) More than two bands: this pattern indicates random integration of the donor circle outside the T5 target locus, most likely in addition to SSI. This category is called a multi-copy integration. One FLPe-SSI line (T12C2) and two FLPo-SSI lines (T4C1 and T38C1) fell into this category (Figure 18, 19 and Table 9). T12C2 had three integrations involving one truncated SSI and two random integrations. T38C1 had three random integrations. These random integrations may consist of truncated SSI in both T5 alleles and one random integration of the donor circle (Figure 18, 19). T4C1 had multi-random integration of the donor circle (Figure 19).

a)



b)

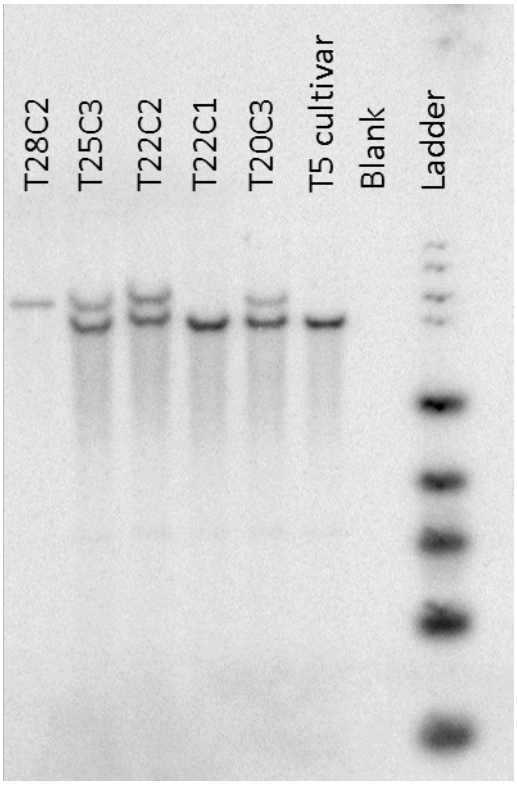
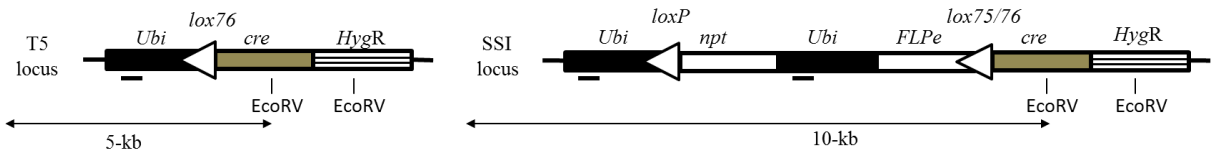


Figure 17: Southern hybridization of *Eco*RV-digested genomic DNA of FLPwt-SSI lines with the Ubi probe. a) *Eco*RV map of T5 and SSI locus. b) Southern hybridization of *Eco*RV digested genomic DNA of FLPwt lines with Ubi probe. T20C3, T22C2, and T25C3 were hemizygous for integration; T28C2 was homozygous for integration; T22C1 was non-transgenic T5.

a)



b)

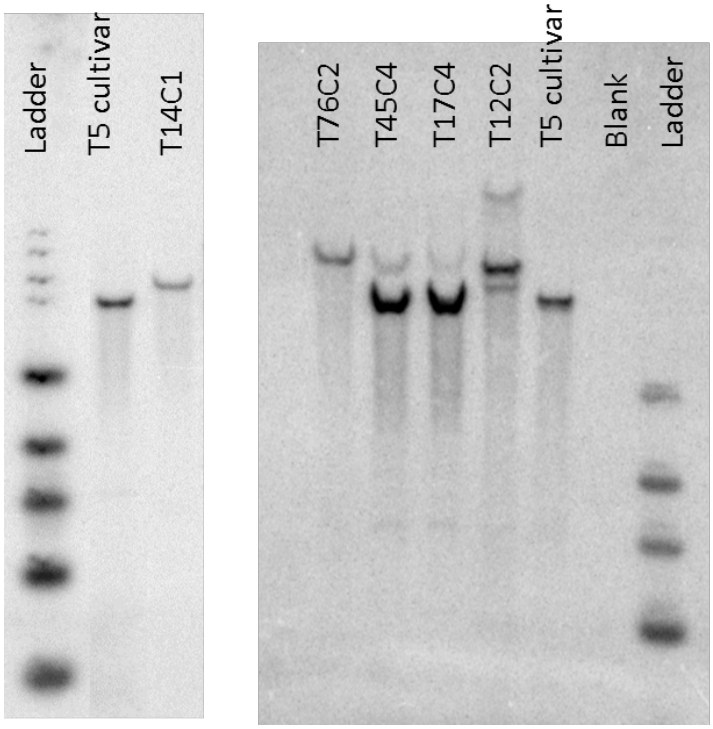
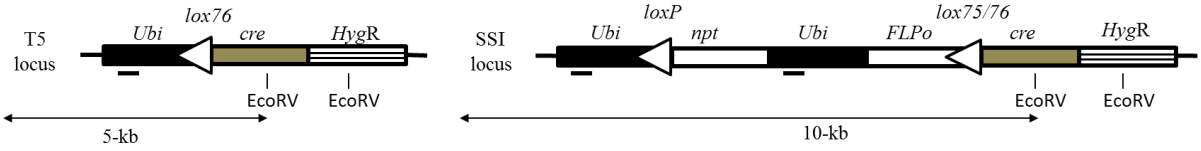


Figure 18: Southern hybridization of *Eco*RV-digested genomic DNA of FLPe-SSI lines with Ubi probe. a) *Eco*RV map of T5 and SSI locus. b) Southern hybridization of *Eco*RV digested genomic DNA of FLPe lines with Ubi probe. T17C4 and T45C4 were hemizygous for integration; T14C1 and T76C2 were homozygous for integration; T12C2 contains random integrations.

a)



b)

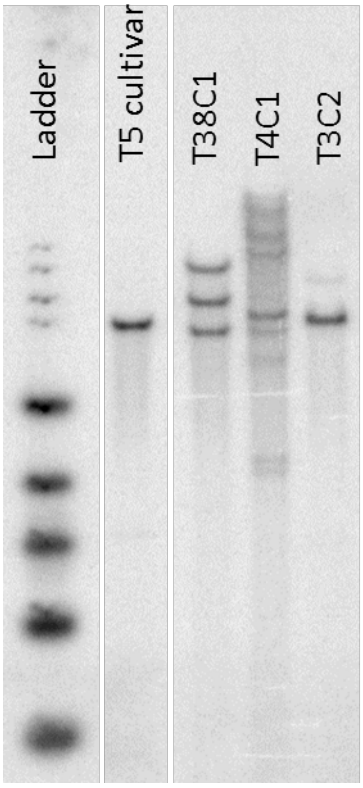


Figure 19: Southern hybridization of *Eco*RV-digested genomic DNA of FLPo-SSI lines with Ubi probe. a) *Eco*RV map of T5 and SSI locus. b) Southern hybridization of *Eco*RV digested genomic DNA of FLPo-SSI lines with Ubi probe. T3C2 was hemizygous for integration; T4C1 and T38C1 had random integrations.

4.8. FLP Expressing Assay on T1 FLP-SSI Lines

In order to detect FLP activity in the progeny of FLP-SSI lines, embryos from T1 seeds or callus derived from T1 seeds were bombarded with pRP9. Ten-twelve embryos from each of T20C3, T22C2, T25C3, T28C2 (FLPwt), and T3C2 (FLPo) lines were isolated. Similarly, callus induced from mature T1 seeds of T28C2 (FLPwt), T12C2, T14C1, T76C2 (FLPe), and T38C1 (FLPo) were used for bombardment with pRP9 and stained 72 hours later with GUS staining solution (Table 9). FLP activity was determined through the number of blue spots produced on explant per bombardment. After staining with GUS staining solution for 12 h, 24 h, and 48 h, no GUS activity (blue spot) was observed on any sample (Figure 20 and 21, and Table 9). This procedure was repeated and the same result was obtained. Therefore, none of FLP-SSI lines was selected for future application.

Table 9: Characterization of FLP-SSI lines

FLP type	Name	Characterized by Southern hybridization	Selected for FLP assay	Result for FLP assay
FLPwt	T20C3	Heterozygous [tc]	*	- [a]
	T22C1	Non-transgenic		
	T22C2	Heterozygous [tc]	*	- [a]
	T25C3	Heterozygous [tc]	*	- [a]
	T28C2	Homozygous [tc]	*	- [a, b]
FLPe	T12C2	Random integration	*	- [b]
	T14C1	Homozygous [tc]	*	- [b]
	T17C4	Heterozygous [tc]	*	- [a]
	T45C4	Heterozygous [tc]	*	- [a]
	T76C2	Homozygous [tc]	*	- [b]
FLPo	T3C2	Heterozygous [tc]		
	T4C1	Contained random integration		
	T38C1	Contained random integration	*	- [b]

tc: truncated integration
- : negative, no blue spot was observed
a : on embryos of T1 seeds
b : on callus derived from T1 seeds

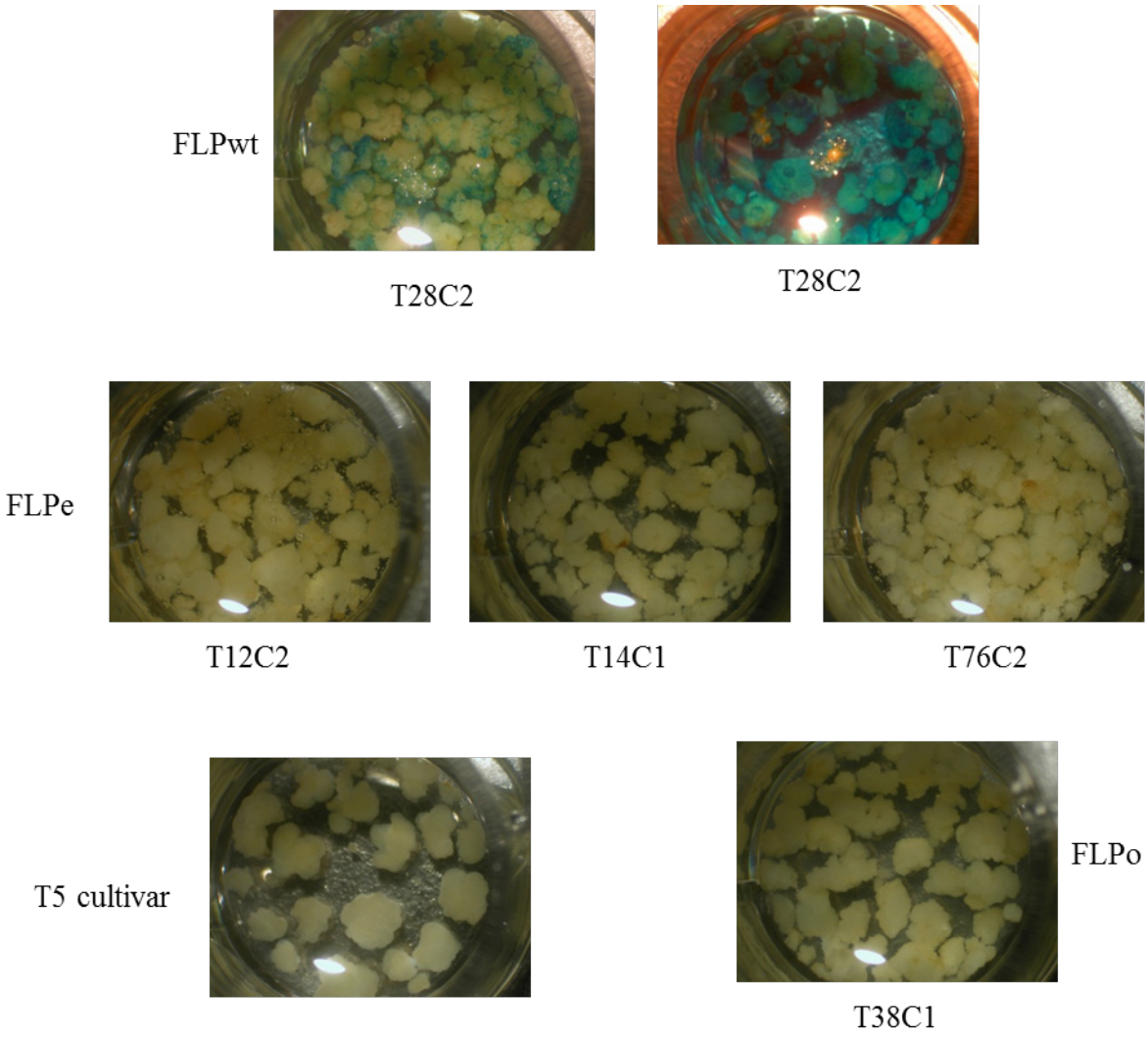


Figure 20: FLP activity assay on T1 callus. Callus was bombarded with pRP9 and stained for GUS activity. FLPwt, FLPe, FLPo lines are shown. GUS activity in FLPwt line T28C2 is intrinsic activity as this activity was observed without pRP9 bombardment.

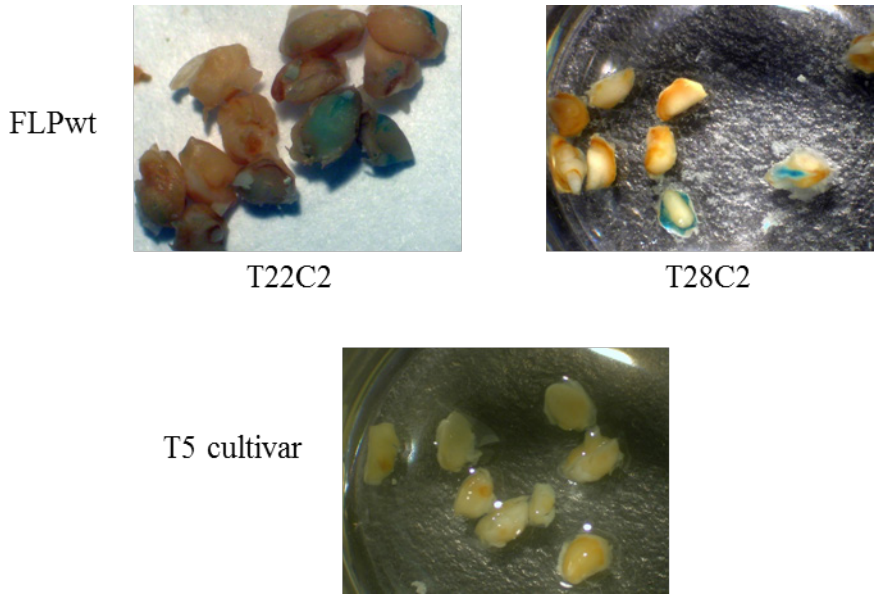


Figure 21: FLP activity assay on T1 embryos of selected FLP-SSI lines. T1 embryos were bombarded with pRP9 and stained with GUS solution. None showed GUS activity. T22C2 and T28C2 showed some background staining (shown above), whereas other lines (T20C2, T25C3, and T3C2) appeared similar to the T5 cultivar control (as shown above).

4.9. Discussion

Three bombardment experiments performed for a total of 28 and 30 callus plates to generate FLPe and FLPo expressing lines, respectively, whereas the number of experiments to generate FLPwt expressing lines was two on a total of 19 callus plates. Sixty five FLPwt, 41 FLPe, and 28 FLPo resistant lines were obtained. However, 21 FLPwt, 33 FLPe, and 15 FLPo lines were transferred to regeneration media after PCR analysis and FLP activity assay. Seventeen FLPwt, 14 FLPe, and 4 FLPo lines were regenerated but only 15 FLPwt, 8 FLPe, and 3 FLPo lines were successfully grown in the greenhouse. However, intrinsic GUS activity was again observed in FLPwt lines that reduced FLPwt lines for further analysis to only 6 lines.

The *GUS* gene is not to be present in plants. However, a number of studies reported background GUS activity in plants: *Arabidopsis*, rice, tobacco, maize, rye, potato, tomato, apple, and almond (reported and reviewed by Sudan et al., 2006; Wozniak et al., 1994). The proposed explanations for this phenomenon were microbial contaminants or endophytes, which may present GUS activity. Background GUS activity has an optimum acidic pH. Hence, native GUS activity is believed to be controlled at neutral or higher pH. In the present study, native GUS activity was still observed on calli with staining solution at both pH 7 and 8.

PCR analysis revealed that all FLP-SSI plant lines except T22C1 (FLPwt) had the integration of donor circles. Later, Southern analysis also confirmed that the *Ubi* promoter was not present in T22C1. Hence, this line was non-transgenic. This means T22C1 (FLPwt) was able to escape the selection with geneticin. Most of the lines had the integration in only one allele (three FLPwt lines, two FLPe lines, and one FLPo line). These lines were heterozygous for the integration loci (Table 9). The intensity of hybridized bands for SSI loci in some lines was less

than that of the T5 locus. The reason for this is that the activity of Cre from T5 locus acted on SSI locus that excised out donor circles after the integration. The excision was at high rate in T3C2 line (FLPo) that made the hybridized band for the SSI locus in this line hardly detectable. The integration in two alleles took place in three lines: T28C2 (FLPwt), T14C1 and T76C2 (FLPe); hence, these lines were homozygous for the integration locus. Unfortunately, all these homozygous or hemizygous integrations appeared to be truncated as the integrated bands were not of the expected size. Beside SSI, random integrations were also present in one FLPe line (T12C2) and two FLPo lines (T4C1 and T38C1).

FLP activity then was assessed in FLP-SSI lines. Calli or embryos from T1 seeds of FLP lines were bombarded with the pRP9 vector and stained with GUS solution 72 hours later. GUS activity was not observed in any samples. Therefore, none of FLP-SSI lines was selected for further application. Looking back at the experiments on FLP activity assays, these lines did not show any GUS activity in the FLP assay (Table 6 and 7). The reason for this could be truncation in the SSI locus as revealed in Southern analysis.

Although comparison for FLP activity from characterized plant lines was not possible, FLP assay on callus lines revealed FLP activity in several transformed lines (Figure 10, 11 and 12). Based on number of blue spots, it can be suggested that FLPe and FLPo removed the *FRT*-flanked *npt* fragment much more efficiently than FLPwt, indicating superior recombination efficiency of FLPe and FLPo.

Section 2.

Standard Genetic Transformation of Rice: Random Integration Approach

5.1. Generation of Nipponbare FLP lines

Calli were induced from seeds of Nipponbare (Nip) cultivar on 2N6D media. Calli were selected for bombardment with FLP constructs. FLPwt-, FLPe-, and FLPo-transgenic lines were generated by co-bombarding four, five, and four Nipponbare callus plates with plasmids pUbiFLP, pAA8, pAA7 (Figure 7), respectively, along with a plasmid containing the selection marker gene, hygromycin phospho-transferase gene (pHPT; Figure 7). Bombarded calli were selected on hygromycin (50 mg/L) containing 2N6D media. In total, 15 FLPwt, 18 FLPe, and 16 FLPo (hygromycin resistant) lines were obtained (Table 10). These lines were taken for a FLP expression assay and allowed to regenerate on hygromycin-containing regeneration media. Thirteen FLPwt, 16 FLPe, and 6 FLPo lines were successfully regenerated, and transferred to growth media to prolong shoot and root growth before growing in the greenhouse. Some lines were infected with fungi or bacteria or had inefficient development of the root system; therefore, only 8 FLPwt-, 13 FLPe-, and 4 FLPo- plant lines were successfully transferred and grown in the greenhouse (Table 10, 11, 12 and 13).

5.2. FLP Expressing Assay on Callus Nipponbare FLP lines

All 15 FLPwt, 18 FLPe, and 16 FLPo lines and Nipponbare cultivar were bombarded with the pRP9 vector and stained with GUS staining solution 72 h later. FLP activity was determined through blue dots produced on the bombarded calli. Two FLPwt, two FLPe and one FLPo lines showed blue dots, indicating the presence of FLP activity (Figure 22 and Table 11, 12, and 13). Other lines showed no GUS activity. Nipponbare (negative control) did not show any GUS activity as expected.

Table 10: Summary of Nipponbare FLP lines

FLP gene	Bombarded plates	No. of hygromycin resistant callus lines	No. of regenerated lines	No. of rooted lines	No. of greenhouse grown lines
FLPwt	4	15	13	12	8
FLPe	5	18	16	16	13
FLPo	4	16	6	6	4

Table 11: List of Nipponbare FLPwt lines

FLPwt (15)	GUS T0 calli	Regenerated	Rooted	Greenhouse
N7C1	-	+	lost	
N7C2	-	+	lost	
N7C3		+	+	+
N7C4	-	+	+	+
N8C1	-	+	+	+
N8C3	-	+	+	died
N8C4	-	-		
N9C1	-	+	+	+
N9C2	-	+	-	
N9C3	-	+	+	+
N9C4	-	+	+	+
N10C1	+	+	+	+
N10C2	-	+	+	+
N10C3	+	-		
N10C4	-	+	+	died

Highlighted lines were analyzed to T1 generation

Table 12: List of Nipponbare FLPe lines

FLPe	GUS T0 calli	Regenerated	Rooted	Greenhouse
N1C1	-	+	+	+
N1C2	-	+	+	died
N1C3	-	+	+	+
N1C4	-	+	+	+
N2C1	-	+	+	+
N2C2	-	+	+	+
N2C3	-	+	+	+
N2C4	-	+	+	+
N3C2	-	-		
N3C3	+	+	+	+
N3C4	+	+	+	+
N4C1	-	-		
N4C2	-	+	+	died
N4C3	-	+	+	died
N4C4	-	+	+	died
N13C1	-	+	+	+
N13C2	-	+	+	+
N13C3	-	+	+	+

Highlighted lines were analyzed to T1 generation

Table 13: List of Nipponbare FLPo lines

FLPo	FLP activity (callus)	Regenerated	Rooted	Green house
N5C1	-	+	+	+
N5C2	-	+	+	+
N5C3	-	+	+	died
N5C4	-	+	+	+
N6C1	-	+	+	died
N6C2	-	-		
N6C3	-	-		
N6C4	-	-		
N11C1	-	-		
N11C2	-	+	+	+
N11C3	-	-		
N11C4	+	-		
N12C1	-	-		
N12C2	-	-		
N12C3	-	-		
N12C4	-	-		

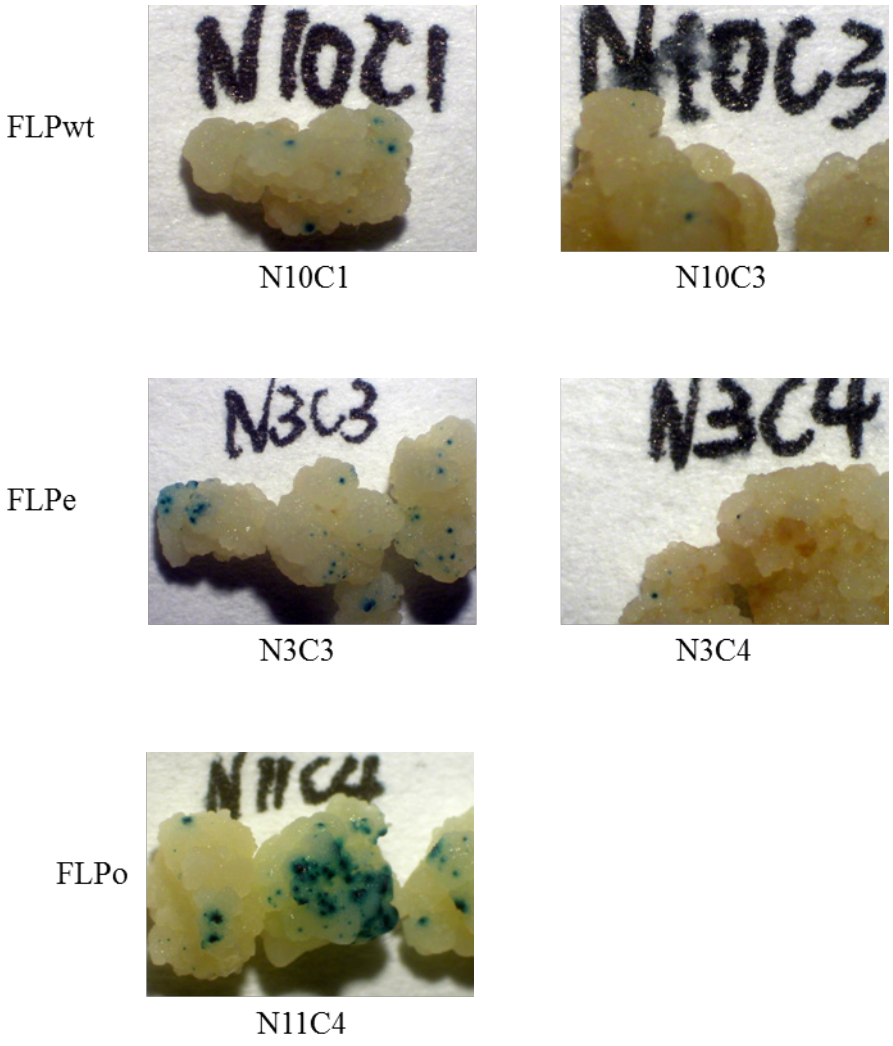


Figure 22: FLP expression assay for Nipponbare FLP callus lines. Only the lines that showed GUS activity are presented.

5.3. Molecular Characterization of Nipponbare FLP Lines

FLP lines in the greenhouse (Table 11, 12, and 13) were analyzed for transgene integration using molecular analysis. Genomic DNA of these lines was isolated from leaves by the CTAB method, and subjected to PCR and Southern hybridization.

5.3.1. PCR analysis

PCR was used to determine the presence of FLP genes in FLP lines. Genomic DNA was used to amplify FLP genes with suitable primers: primer pair *a* and *e* with annealing at 56^{o}C amplified a 1.4-kb fragment of FLPwt; primer pair *f* and *g* with annealing at 60^{o}C amplified a 1.2-kb fragment of FLPe; and primer pair *h* and *i* with annealing at 60^{o}C amplified a 1.3-kb fragment of FLPo (Table 4). Bombarded plasmids (pUbiFLP; pAA8; pAA7) were used as positive controls, whereas the negative control was DNA from the non-transgenic Nipponbare plant. Five out of 8 FLPwt, and 12 out of 13 FLPe lines showed amplification of the FLP genes. All lines showed amplification at the expected size, i.e. same as positive controls, except N3C4 FLPe line that produced smaller bands (Figure 23, 24). None of the four FLPo lines amplified whereas the expected band was present for the positive control, pAA7 (Figure 25). The PCR on FLPo lines was repeated twice. Hence, PCR on *hpt* gene in FLPo lines was carried out with primer pair *m* and *n* (Table 4), and plasmid pHPT was included as the positive control. All 4 FLPo-expressing lines amplified the 0.5-kb HPT fragment (Figure 26). Therefore, FLPo lines were transformed by pHPT but not pAA7.

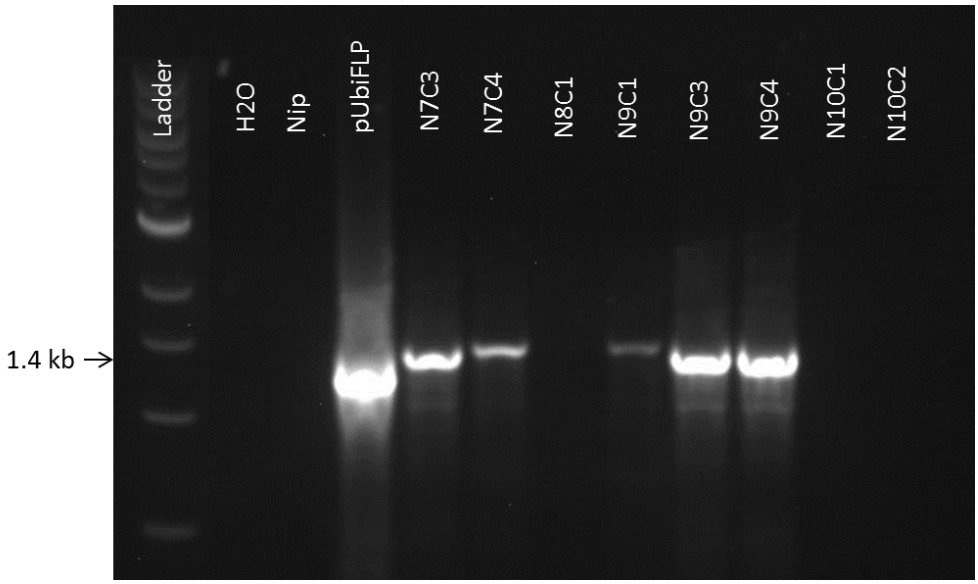


Figure 23: PCR analysis for the presence of *FLPwt* gene in Nipponbare FLPwt lines. DNA from Nipponbare plant (Nip) was used as negative control, and pUbiFLP as positive control. Five (N7C3; N7C4; N9C1; N9C3; N9C4) out of eight FLPwt lines amplified a 1.4-kb FLPwt fragment with primer pair *a* and *e*.

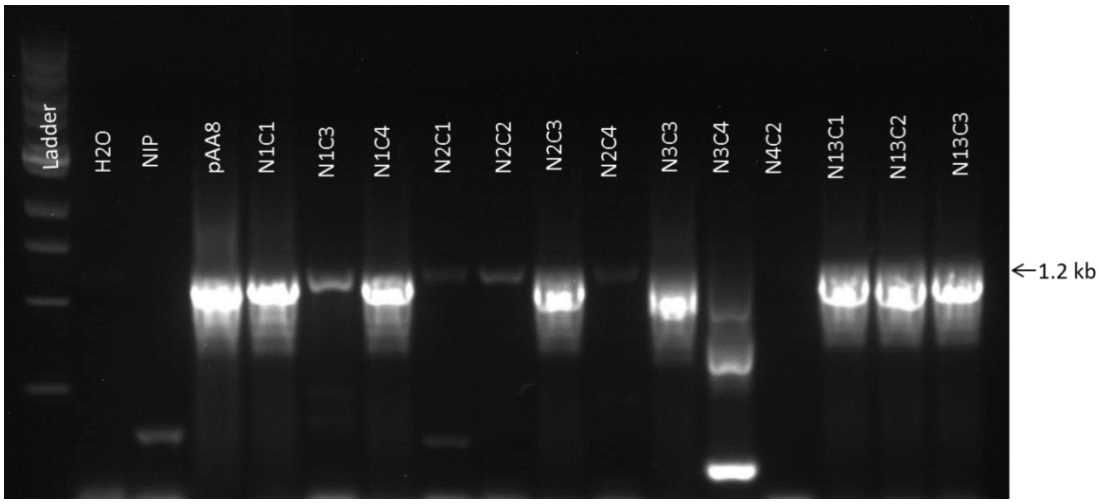


Figure 24: PCR analysis for the presence of *FLPe* gene in Nipponbare FLPe lines. DNA from Nipponbare cultivar (Nip) was used as negative control and pAA8 as positive control. Twelve out of 13 FLPe lines (except N4C2 line) amplified the FLPe fragment, of which 11 amplified the expected 1.2-kb band with primer pair *f* and *g*, whereas line N3C4 amplified smaller bands.

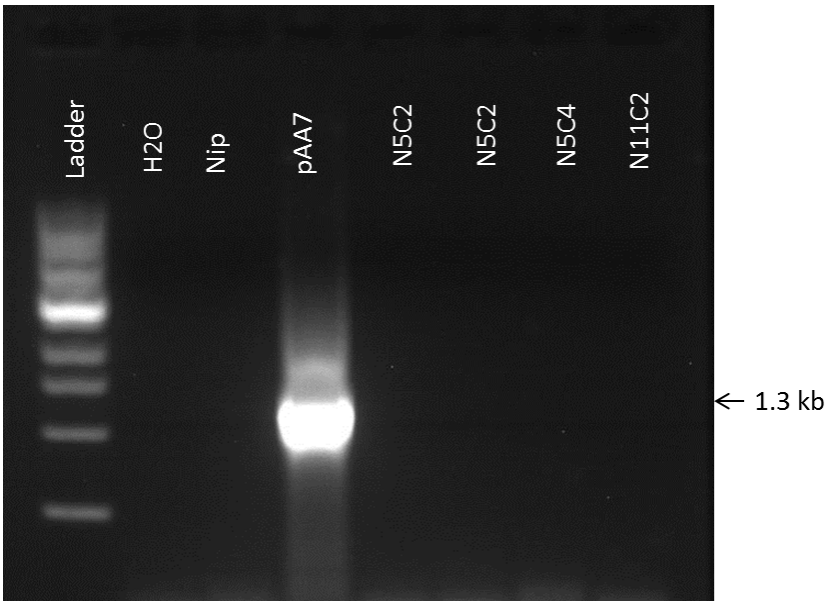


Figure 25: PCR analysis for the presence of *FLPo* gene in Nipponbare FLPo lines. DNA from the Nipponbare cultivar (Nip) was used as negative control and pAA7 as positive control. None of the four FLPo lines (N5C1; N5C2; N5C4; N11C2) amplified any fragment with primer pair *h* and *i*, while pAA7 amplified a 1.3 kb fragment.

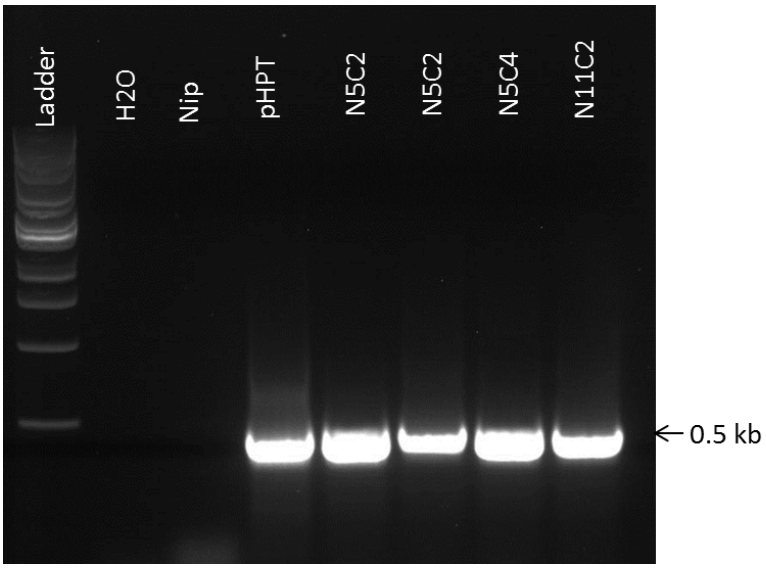


Figure 26: PCR analysis for the presence of *hygromycin phosphotransferase (HPT)* gene in FLPo lines. DNA from Nipponbare cultivar (Nip) was used as negative control, and pHPT as positive control. All four FLPo- lines amplified a 0.5-kb fragment of the HPT gene with primer pair *m* and *n*.

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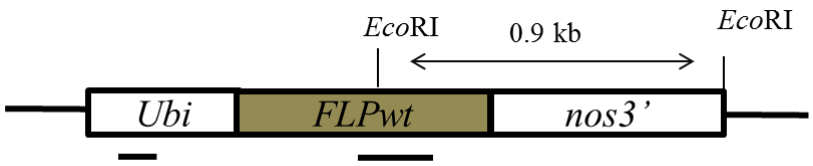
5.3.2. Southern analysis

Nipponbare FLP lines were subjected to Southern analysis. Genomic DNA from these lines was digested with *Eco*RI, and hybridized with probes for FLPwt,FLPe, or the *Ubi* promoter.

Two *Eco*RI sites were present in the FLPwt construct, one in FLPwt sequence and another beside the *nos 3'* terminator (Figure 27). The distance between these two *Eco*RI sites was 0.9-kb. The probe used for FLPwt hybridized with FLPwt region that contains the *Eco*RI sites. Hence, successful integration in FLPwt lines should produce a 0.9-kb hybridized band. In addition, there should be one more band of an unpredictable size in single-copy lines. Hybridized blot revealed that four FLPwt lines (N7C4, N9C1, N9C4, and N10C1) had 0.9-kb, and one to two additional bands, 3 lines (N7C3, N8C1, and N9C3) had hybridized bands but none of these was 0.9-kb. There was one line, N10C2, failed to produce a hybridization (Figure 27). Meanwhile, the *Ubi* promoter is located outside of the *Eco*RI segment (Figure 27); hence, hybridization with the Ubi probe is expected to generate only one band of undetermined size in the single-copy FLPwt lines. Hybridization pattern showed on the blot indicated that these lines had zero to three copies of *Ubi* promoter (Figure 27). Specifically, three lines (N7C3, N7C4, and N9C4) contained three copies; two lines (N9C1 and N9C3) contained two copies; one line N10C1 contained a single copy; however, this line did not contain FLPwt gene according to PCR. Therefore, this line may contain a truncated integration. Finally, two lines (N7C4 and N10C2) did not hybridize with the Ubi probe (Figure 27). The results from FLPwt and Ubi blots showed that there were a total of 6 transgenic lines with copy number ranging from 1–3. However, one of the lines, N10C1 was PCR negative, so it probably contains a truncated FLPwt gene. Thus, a total of 5 FLPwt-Nipponbare lines were available for further analysis (Table 14).

In the FLPe construct, there is only one *Eco*RI site located beside the *nos3'* terminator (Figure 28). Therefore, *Eco*RI-digested DNA from single-copy lines is expected to produce only one band of undetermined size which should be > 4-kb on the blot with FLPe or Ubi probes (see Figure 28). In other words, the number of hybridized bands on the blot would indicate the number of transgene copies. Four Nipponbare FLPe lines (N1C1, N1C4, N2C3, and N13C2) were determined to be two-copy lines, one (N13C3) to be three-copy line in which one copy was truncated (< 4-kb); and three lines (N3C3, N3C4, and N13C1) to be multi-copy lines. Among FLPe lines, N4C2 had no hybridized band with either the FLPe or Ubi probes. With PCR, the presence of *FLPe* gene was not detected in this line. Therefore, this line may only be transformed with pHPT (not determined) or be non-transgenic but able to escape the selection with hygromycin. Two lines, N1C3 and N2C1, that were positive for the *FLPe* gene in PCR, did not hybridize with any of the two probes. PCR analysis may have been flawed due to cross contamination from other samples or plasmid. The remaining lines (N2C2 and N2C4) had one or two integrations, but the presence of < 4 kb bands in these lines suggested the presence of truncated fragments (Figure 28 and Table 15).

a)



b)

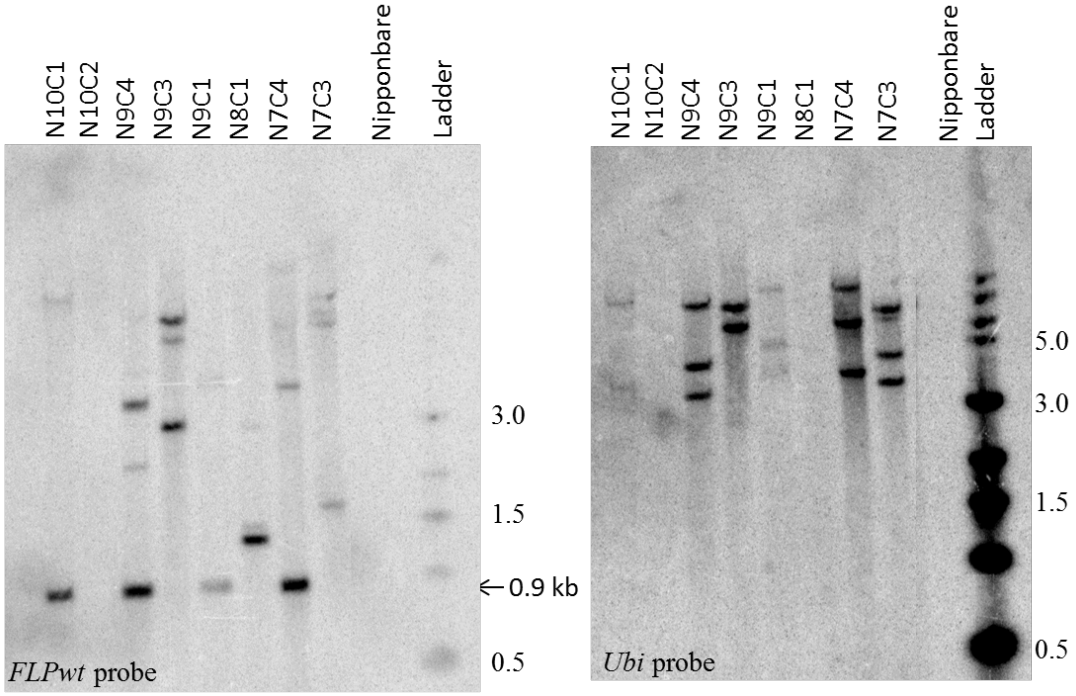


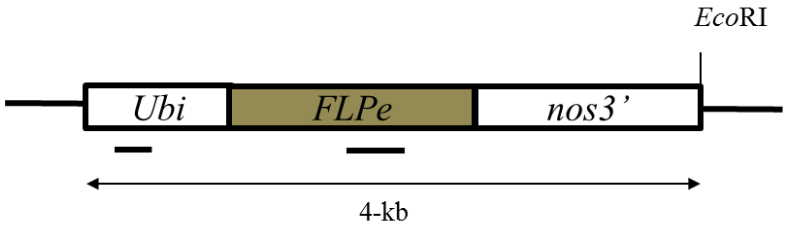
Figure 27: Southern analysis for Nipponbare FLPwt lines. a) *Eco*RI sites in the pUbiFLP plasmid. b) Genomic DNA was digested with *Eco*RI and hybridized with the FLPwt probe and Ubi probe. Nipponbare DNA serves as the negative control; 1-kb ladder was used as the size marker.

Table 14: Summary of Southern analysis for Nipponbare FLPwt lines

FLPwt lines	PCR for *FLPwt* gene	Presence of 0.9-kb band with FLPwt probe	Additional bands with FLPwt probe	No. of bands with Ubi probe	Conclusion
N7C3	+	-	> 2	3	Maybe truncated
N7C4	+	+	> 2	3	3 copies line
N8C1	-	-	-	> 1	Maybe truncated
N9C1	+	+	1	2	2 copies line
N9C3	+	-	3	2	Maybe truncated
N9C4	+	+	2	3	3 copies line
N10C1	-	+	1	1	Single copy (maybe truncated)
N10C2	-	-	-	-	Did not have FLPwt gene

- : no band

a)



b)

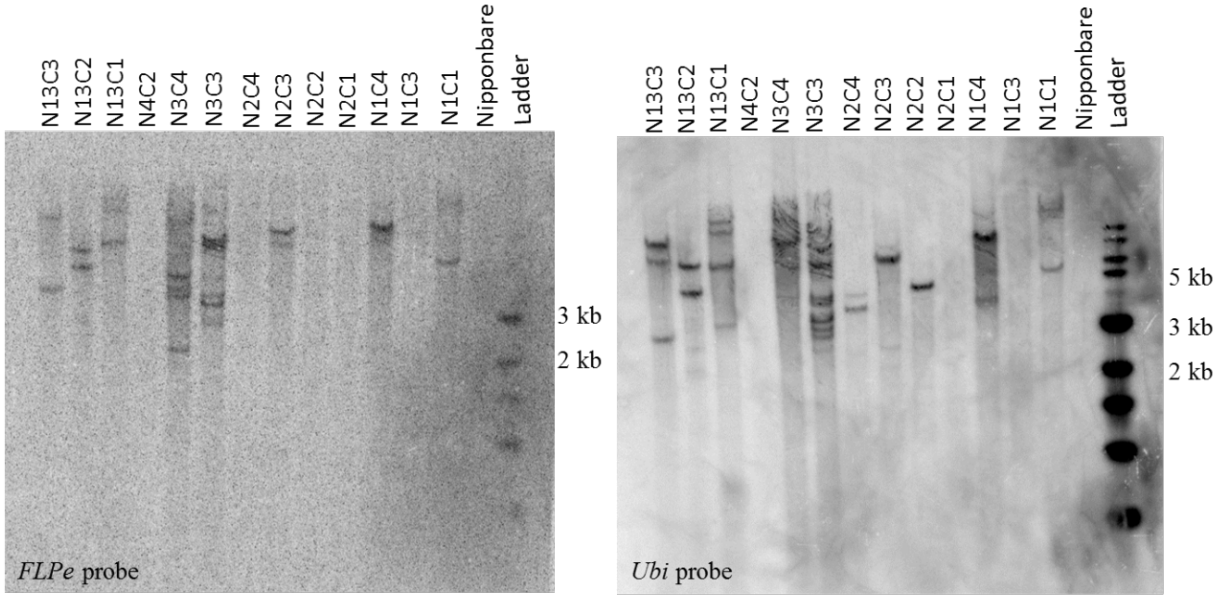


Figure 28: Southern analysis of Nipponbare FLPe lines. a) *Eco*RI site in the pAA8 plasmid. b) Genomic DNA was digested with *Eco*RI and hybridized with FLPe and Ubi probes. Nipponbare cultivar was used as the negative control; 1-kb ladder was used as the size marker.

Table 15: Summary of Southern analysis for Nipponbare FLPe lines.

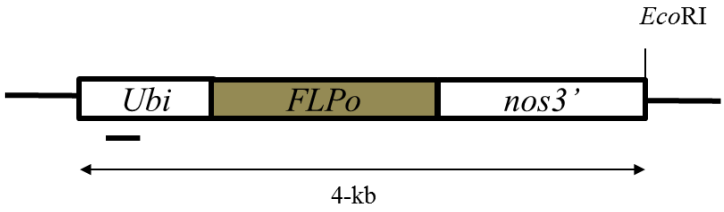
FLPe lines	Hybridized bands with FLPe probe	Hybridized bands with Ubi probe	Conclusion
N1C1	> 1	2	2 copies line
N1C3	-	-	negative for FLPe gene
N1C4	1	2	2 copies line
N2C1	-	-	negative for FLPe gene
N2C2	-	1	Maybe truncated
N2C3	2	1	2 copies line
N2C4	-	2	Maybe truncated
N3C3	Multi	Multi	Multi copies
N3C4	Multi	Multi	Multi copies
N4C2	-	-	negative for FLPe gene
N13C1	> 1	Multi	Multi copies
N13C2	2	2	2 copies line
N13C3	2	3	3 copies line, one maybe truncated

- : no band
* : in combination with PCR analysis

The FLPo construct also has only one *Eco*RI site beside the *nos3'* terminator (Figure 29). Therefore, *Eco*RI-digested DNA from single-copy lines is expected to produce only one band of an undetermined size > 4-kb on the blot with the *Ubi* probe (Figure 29), and the number of hybridized bands on the blot would indicate the number of transgene copies. Of four FLPo lines, N5C1 showed two hybridized bands with *Ubi* probe, whereas N11C2 had one hybridized band. The remaining two lines (N5C2 and N5C4) did not hybridize with the *Ubi* probe. PCR detected the presence of the *hpt* gene but not the *FLPo* gene in these four lines. Therefore, N5C1 and

N11C2 probably contained truncated integration of pAA7, whereas two lines N5C2 and N5C4 were transformed with only pHPT. From these results, hybridization with the FLPo probe was not carried out, and all four FLPo lines were removed from further analysis.

a)



b)

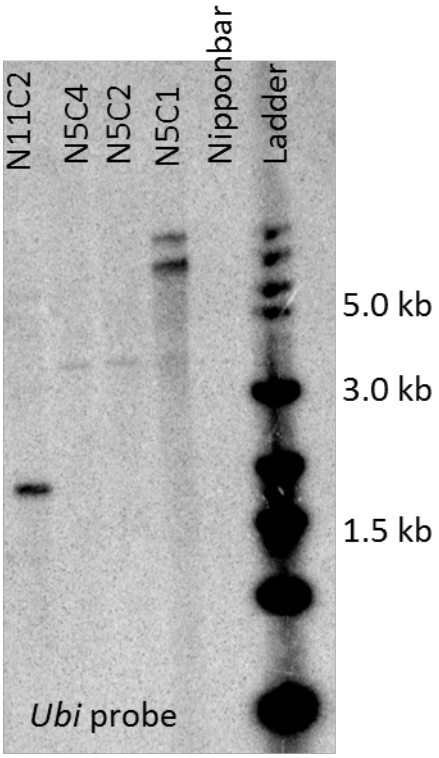


Figure 29: Southern analysis of Nipponbare FLPo lines. a) *Eco*RI site in the pAA7 plasmid. b) *Eco*RI-digested genomic DNA of FLPo lines hybridized with the Ubi probe.

5.4. FLP Expression Assay

FLPwt and FLPe lines were selected to determine FLP activity using the GUS expression assay. Calli induced from T1 seeds of FLP lines were bombarded with the vector, pRP9, and 72 hours later stained with GUS staining solution at 37 ^{o}C. Due to slow growth and partial sterility, N10C1 was not included; therefore, only three FLPwt lines (N7C4, N9C1, and N9C1) were tested. Among the FLPe lines, N1C4 was not included because this line had only four seeds at a very late time point.

Calli from a single T1 seed per line was bombarded and stained separately. With sufficient calli for each seed used in each bombardment that was carried out with a single stock of pRP9-coated gold particles, the highest FLP activity was measured by the number of blue spots produced after staining for GUS activity per bombarded plate. Figure 30 and Table 17 present the results of the experiment. Two out three FLPwt lines produced 15 blue spots per seed whereas three out of four FLPe lines produced 200 - 300 blue spots per callus plate, which is more than 15 fold higher than that of FLPwt line. The higher number of blue spots in FLPe lines may be based on (a) higher *FLPe* gene expression, and/or (b) higher recombinase activity of FLPe. FLP-expression assay was restricted to Nipponbare FLPwt and FLPe lines because there was no FLPo lines based on the negative PCR analysis (see above).

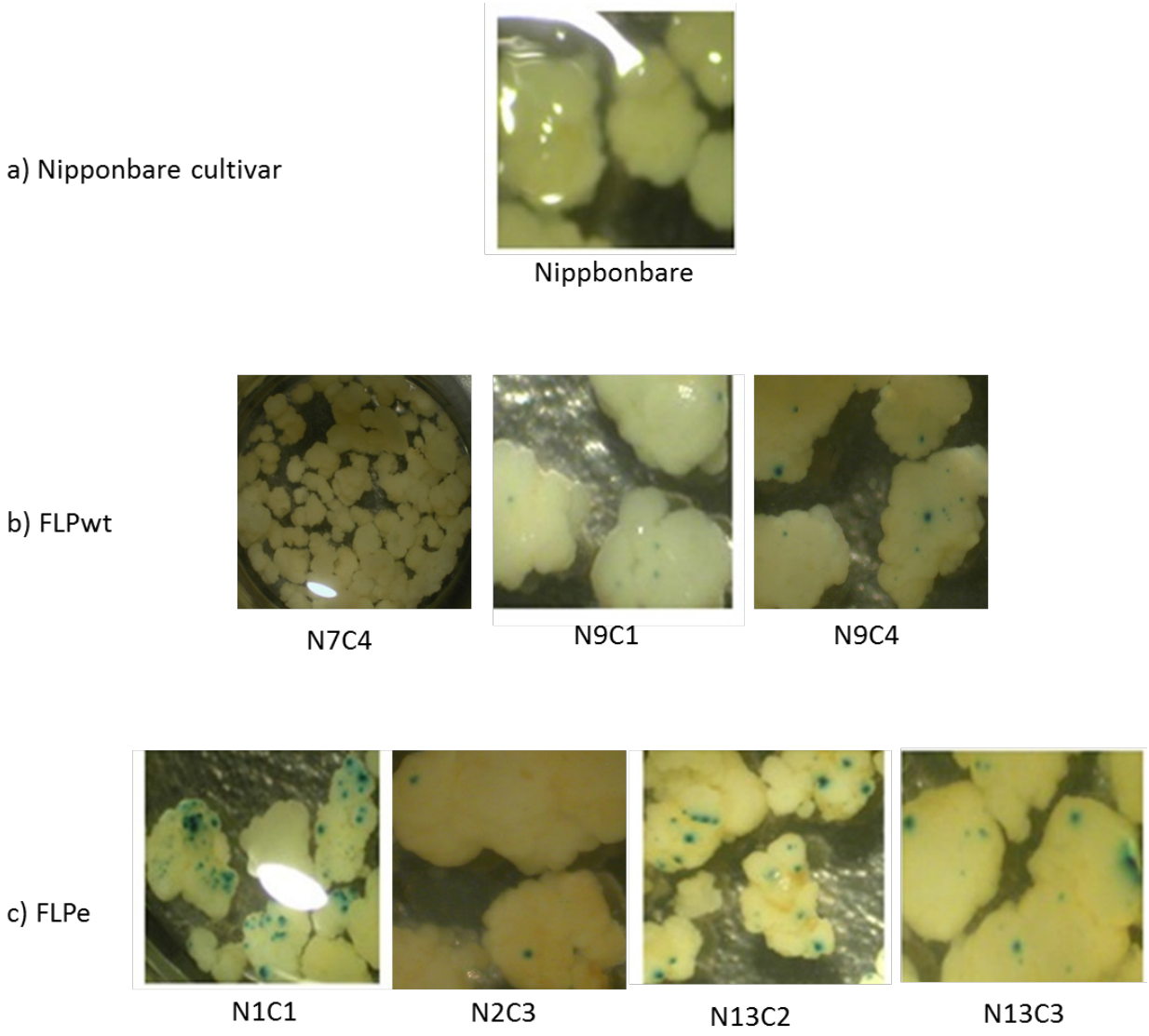


Figure 30: FLP assay in Nipponbare FLP lines. T1 seed derived callus was bombarded with pRP9 and stained 72 h later for GUS activity, which directly correlates with FLP activity. a) Nipponbare callus (negative control); b) FLPwt derived calli; c) FLPe derived calli.

5.5. Quantitative Real Time PCR (qRT-PCR) for FLP Gene Expression Analysis

In order to determine whether superior FLP activity of FLPe or higher *FLPe* gene expression contributed to higher observed FLP activity in FLPe lines, quantitative real time reverse transcriptase PCR (qRT-PCR) was carried out. Total mRNA was extracted from leaves of Nipponbare FLPwt and FLPe lines and the Nipponbare cultivar and subjected to qRT-PCR. For the FLPwt, the primer pair *o* and *p* (Table 16) was used and for FLPe, primer pair *r* and *s* was used. Phytoene Desaturase (PDS) gene (primer pair *t* and *u*) was used to normalize the amount of RNA added to the reactions. The qRT-PCR reactions for all genes were carried out at 60 ^{o}C in duplicates. From the CT value of qRTPCR, fold change in expression of FLPwt and FLPe (Table 17) was calculated using the ΔΔC(t) method (Livak and Schmittgen, 2001):

$$\text{Amount of target} = 2^{-\Delta\Delta C(t)}$$

This analysis showed that FLPwt expression was at least 4 times higher than FLPe expression in their respective lines (Figure 31). Therefore, higher FLP activity in FLPe lines was based on higher FLP recombinase efficiency rather than higher gene expression.

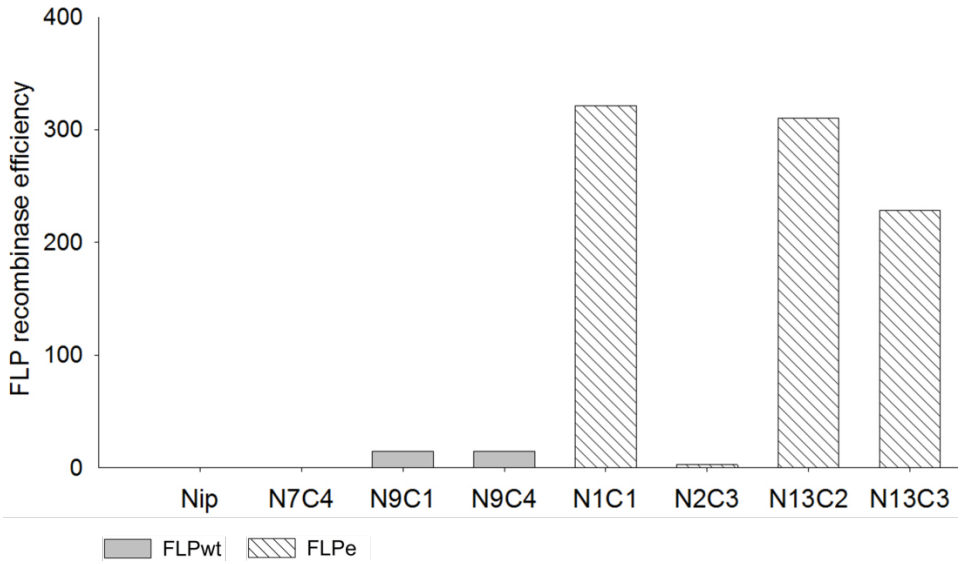
Table 16: **Primers used for qRT-PCR analysis**

Codes	Primers	Sequences
o	FLP FII	5’ GCATCTGGGAGATCACTGAG 3’
p	FLP R641	5’ CTGTCACTAAACACTGGATTA 3’
r	FLPe F796	5’ CCGGCAATTCTTCAAGCAAC 3’
S	FLPe R980	5’ CAACTCCGTTAGGCCCTTCA 3’
t	PDS F1306	5’ GCAGAGGAATGGGTTGGAC 3’
u	PDS R1490	5’ AGAGGTCGGCAAGGTTCAC 3’

Table 17: FLP expression

FLP type	Lines	Number of blue spots per callus plate (single seed)	Fold change in gene expression
FLPwt	N7C4	0	1909
	N9C1	15	2387
	N9C4	15	2439
	Nipponbare	0	1
FLPe	N1C1	321	51
	N2C3	3	1.5
	N13C2	310	36
	N13C3	228	511
	Nipponbare	0	1

a)



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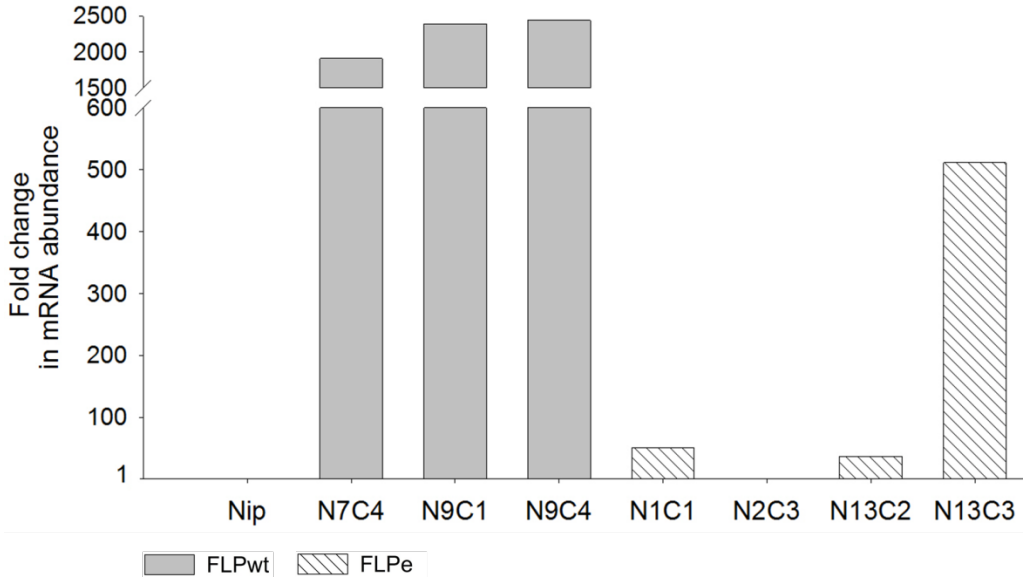


Figure 31: FLP activity and FLP expression in FLPwt and FLPe lines. a) FLP recombinase efficiency as indicated by number of blue dots obtained upon pRP9 bombardment; b) Fold change in FLP mRNA abundance in FLPwt and FLPe lines compared to Nipponbare.

5.6. Discussion

In the random integration approach, a total of 15 FLPwt, 18 FLPe, and 16 FLPo hygromycin resistant callus lines were obtained. thirteen FLPwt lines, 16 FLPe lines, and 6 FLPo lines were regenerated. Although 12 FLPwt lines, 16 FLPe lines, and 6 FLPo lines were rooted, only 8 FLPwt line, 13 FLPe lines, and 4 FLPo lines were successfully grown in the greenhouse because of contamination and poor root system development in several lines.

All Nipponbare FLP lines in the greenhouse were subjected to PCR and Southern analysis to determine integration of *FLP* genes. Southern hybridization pattern indicated that most of the FLPe and FLPo lines were 2-3 copy lines (Table 14 and 15). Only one single-copy line (N10C1) was produced, which was the FLPwt line. Of the four FLPo lines, two appeared to be truncated and two were untransformed; hence, these lines were removed from further analysis with FLP expressing assay.

FLP expression assays were carried out on primary transgenic calli (T0 calli), and later on calli derived from T1 seed of FLPwt and FLPe lines. FLP assays on T0 calli revealed FLP activity in two FLPwt lines (N10C1 and N10C3), two FLPe line (N3C3 and N3C4), and one FLPo line (N11C4). This experiment showed that recombination activity of FLPe and FLPo was higher than that of FLPwt as they generated more blue dots on T0 calli upon pRP9 bombardment (Figuer 22). All FLP lines that showed FLP activity on T0 calli were not available for the FLP assay on T1 calli later. Particularly, N10C3 (FLPwt) and N11C4 (FLPo) were not regenerated, whereas two FLPe plant lines N3C3 and N3C4 contained multi-copy integrations. N10C1, the only single-copy line and that showed FLP activity in T0 calli, was sterile and therefore unavailable for T1 callus induction. Hence, FLP assay on T1 calli was carried out for only three FLPwt lines (N7C4, N9C1, and N9C4) and four FLPe lines (N1C1, N2C3, N13C2, and N13C3),

which did not show FLP activity on T0 calli (Table 13 and 17). However, two of three FLPwt lines (N9C1 and N9C4), and all four FLPe lines (N1C1, N2C3, N13C2, and N13C3) showed FLP activity in T1 calli. This means T0 calli of these lines was chimeric and these plant lines were developed from T0 calli that had FLP activity but this could not be represented in FLP assays on T0 calli. Observation on pRP9-based GUS activity in T1 calli and FLP mRNA abundance indicated higher recombination efficiency for FLPe than FLPwt (Figure 30 and 31).

Part 5.

CONCLUSIONS

The main objective of the present study was to assess efficiency of three types of FLP protein: FLPwt, FLPe, and FLPo for marker removal when they were stably expressed from a rice genomic locus. Hence, FLPwt, FLPe, and FLPo transgenic rice lines had to be generated, confirmed with Southern hybridization, and screened for FLP expression. FLP transgenic lines were generated by two approaches: a site-specific integration (SSI) approach and a standard genetic transformation approach (random integration). This study, through the whole process from transformation to the assessment of FLP activity in the produced FLP lines, is summarized below

(1) FLP assays on both FLP-SSI lines and Nipponbare FLP lines revealed that FLPe and FLPo have higher recombination activity compared with FLPwt in removing the marker from the introduced plasmid. A previous study based on transient FLP expression in rice cells also reported that FLPe and FLPo were more efficient than FLPwt in recombining *FRT* sites located in the rice genome (Akbudak and Srivastava, 2011). This study found FLPe and FLPo to be similar in recombination efficiency. In two other studies, Nandy and Srivastava (2011, 2012) have successfully used FLPe for site-specific integration of foreign genes into *FRT* locus in rice genome. Thus, FLPe and FLPo have been tested in both stable and transient expression assays, and found to have superior recombination activity than FLPwt. The higher stability of FLPe and FLPo at 37^{o}C compared with that of FLPwt presumably contributed to the higher activity in these improved versions of proteins compared with FLPwt. In the present study, FLPo was found to display relatively higher efficiency than FLPe, indicating that mouse-codon optimization also contributed to improvement in recombination efficiency of FLP protein in rice.

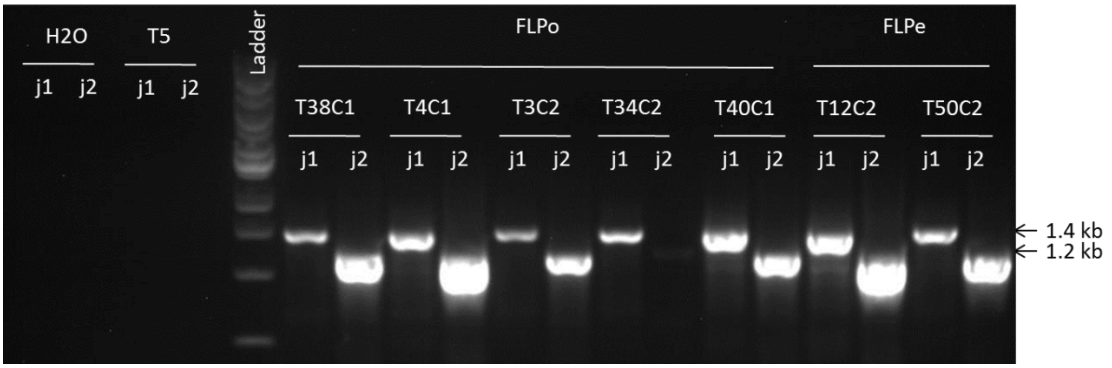
Therefore, if rice-codon optimization may further improve FLP recombination efficiency for rice genetic engineering.

(2) Two Nipponbare FLPwt lines (N9C1 and N9C4) and three Nipponbare FLPe lines (N1C1, N13C2, and N13C3) that showed FLP activity in the T1 generation can be utilized for further research or biotechnology applications (e.g. marker removal from rice genome).

(3) T22C1 generated in the present study was non-transgenic; however, this line was able to escape the selection. T22C1 was likely generated from a chimeric transformed callus. The transformation process may generate chimeric transgenic callus lines. Hence, molecular confirmation of the generated transgenic lines is necessary to select the desired lines.

(4) Site-specific integration (SSI) may also contain random integrations; however, this approach surely generates more single integrations compared with random integration approach which also was observed in this study. Single-copy integrations are known to display stable transgene expression (Chawla et al., 2006; De Buck et al., 2007; Srivastava and Gidoni, 2010). Hence, the site-specific integration approach is more suitable for obtaining uniform protein expression; although in the present study, stable SSI lines were lost in early transformation stages.

SUPPLEMENTARY WORK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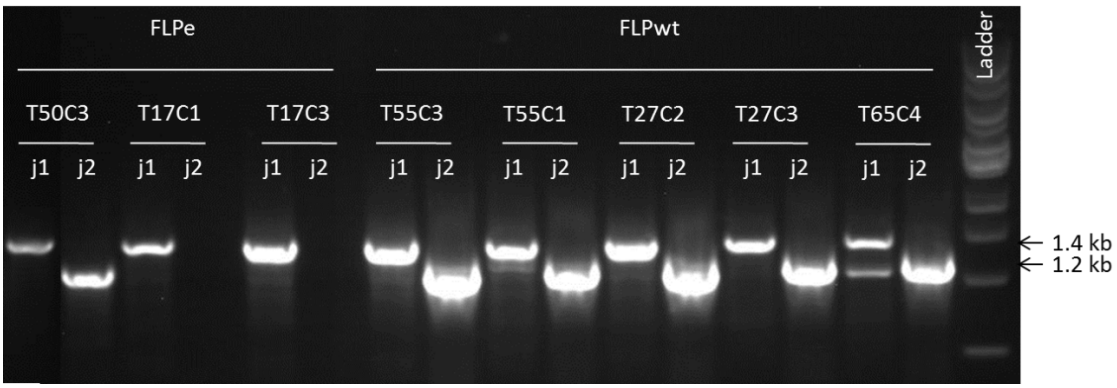
1.2 kb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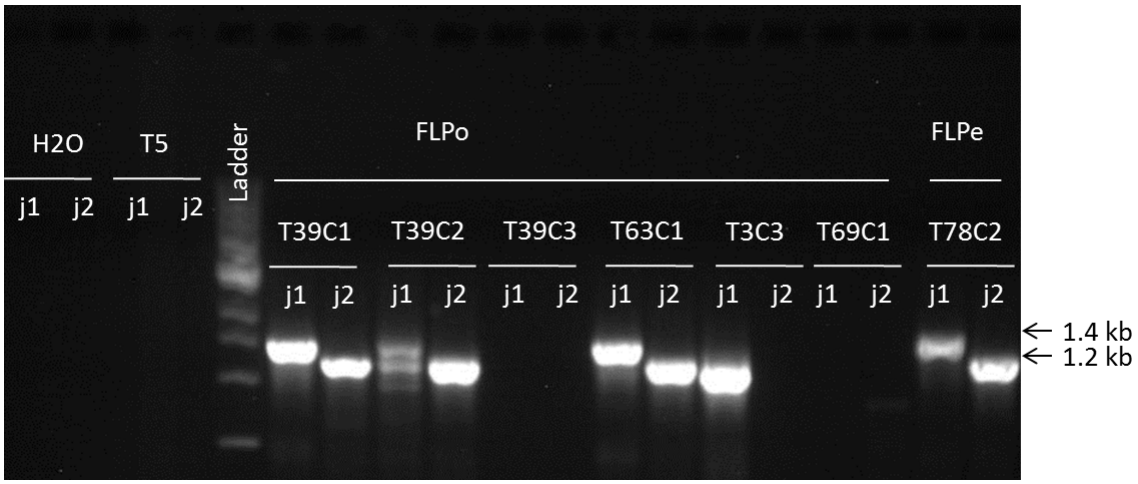
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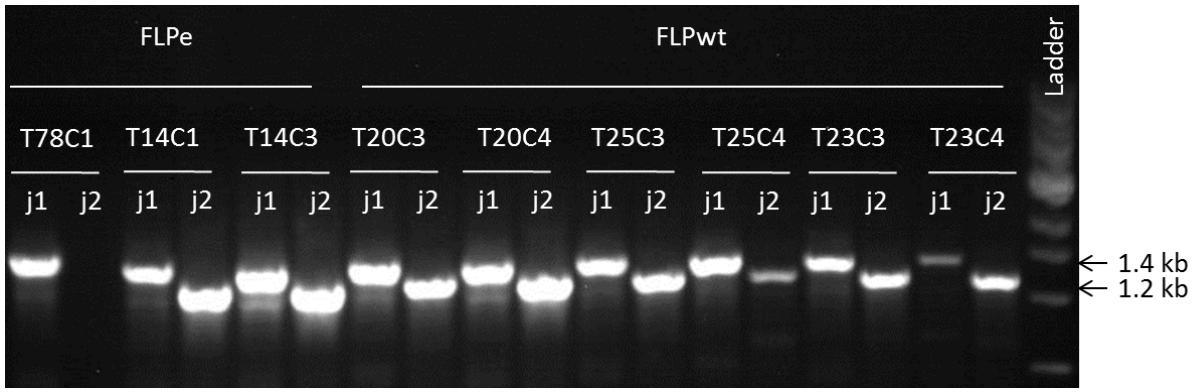
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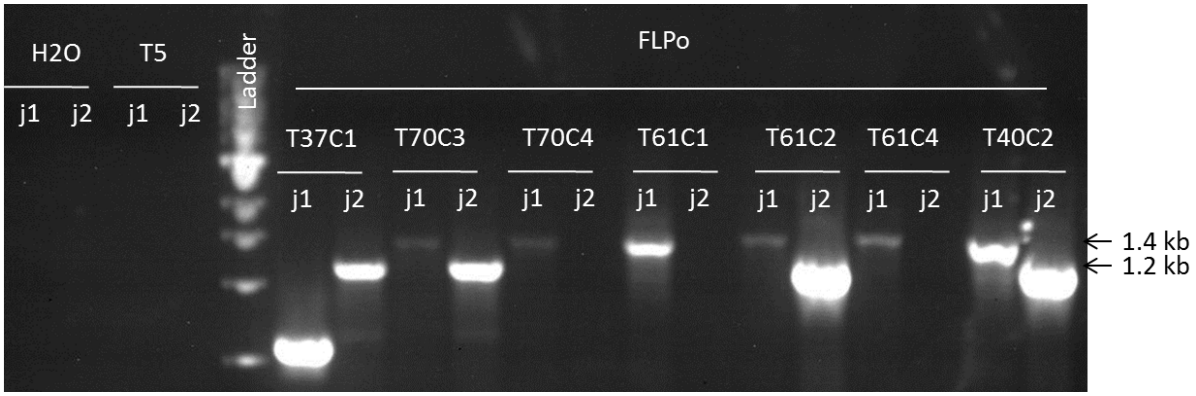
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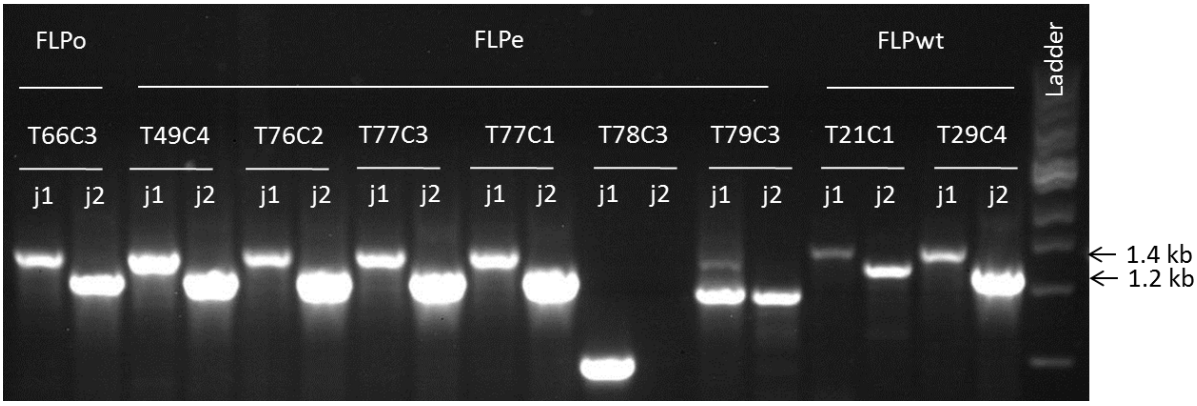
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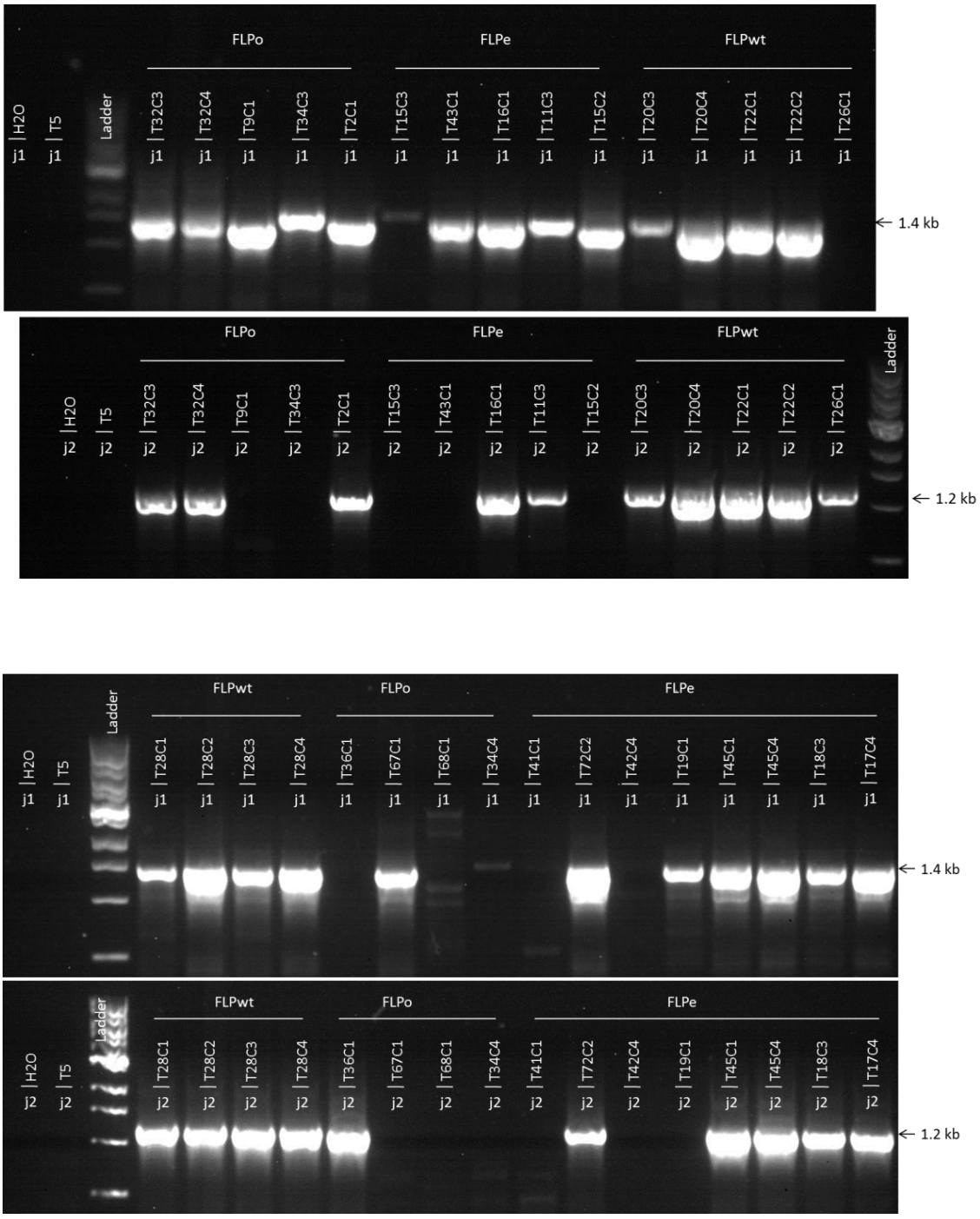

Figure 32: PCR analysis for two integration junctions in FLP-SSI calli. Primers *a* and *b* amplified 1.4 kb fragment at *junction 1*; primers *c* and *d* amplified 1.2 kb fragment at *junction 2*.

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