THE SITE-SPECIFIC RECOMBINATION SYSTEMS CRE-*LOX* AND FLP-*FRT* ARE FUNCTIONALLY ACTIVE IN POPLAR

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ABSTRACT

We have tested two site specific recombination systems, Cre-*lox* and FLP-*FRT* in a model tree system. A hybrid aspen clone (*Populus tremula* × *P. tremuloides*) was co-transformed with a plasmid containing the 35S promoter controlling FLP, and an excision construct containing the *rolC* gene between two directly oriented FRT sites as a morphological excision marker. Similarly, the Cre-*lox* system was tested using a single plasmid containing two lox sites in direct orientation flanking the heat shock-inducible Cre gene inserted between the 35S promoter and the GUS coding region. In total, 10 independent transgenic lines carrying the FLP-*FRT* plasmid alone, or both constructs, and 36 Cre-*lox* containing transgenic lines were obtained. Out of the 10 transgenic lines carrying the FLP/*FRT* or both constructs, four lines showed phenotypical aberrations whereas in the group of 36 Cre-*lox* transgenic lines no morphological aberration could be detected. In two FLP-*FRT* lines morphological and molecular evidence showed that the fragment between the two FRT sites has recombined and been excised. Molecular investigations of eight heat-shock treated Cre-*lox* transgenic lines also indicate excision of the insert between the two lox sites, however, GUS-staining tests reveal that only six out of the eight lines express the gene. However, sequencing of the 35S-*lox*-GUS region shows no sequence variation, confirming exact excision of the insert. The results presented indicate the usefulness of the two site specific recombination systems in the tree species *Populus* but raise some further concerns with regards to the expression of the "transgene-of-interest" as well as on the occurrence of pleiotropic phenotypic aberrations.

Key words: GUS, marker gene, position effect variation, *rolC*, FLP/*FRT*, Cre/*lox*, targeted gene transfer.

INTRODUCTION

Twenty years after the first publication on the production of transgenic tobacco, genetic transformation of a number of tree species has become a routine method (CAMPBELL *et al.* 2003). Although most of the transgenic trees being produced are used to address basic scientific questions, a significant number is also applied to forest breeding purposes (*e.g.* herbicide and insect resistance, features and/or composition of wood; CAMPBELL *et al.* 2003), and the number is increasing every year.

Using currently available transformation methods, integration of a transgene into the tree genome occurs randomly and in an unpredictable manner (KUMAR & FLADUNG 2002b). The transgene may be integrated as a concatamer leading to transgene silencing abolishing its expression. The integrated transgene may also result in deletions or rearrangements of plant genomic sequences at the site of transgene integration (FLADUNG 1999, KUMAR & FLADUNG 2001a, 2002a, FLADUNG *et al.* 2004). The genomic position where the transgene is integrated also has a profound effect on transgene expression, the so-called "position-effect variations" which has been characterized in transgenic trees (KUMAR & FLADUNG 2001a, FLADUNG & KUMAR 2002a) but also in transgenic plants in general (IGLESIAS *et al.* 1997, MATZKE & MATZKE 1998, VAUCHERET & FAGARD 2001).

Besides this, the presence of unneeded DNA in the transgene locus *e.g.* antibiotic selection marker genes, which are only important during the transformation process itself, has become a severe

problem. This is evident in particular in the context of public acceptance of gene technology. Both problems can be addressed by using site-specific recombination systems (LIU *et al.* 2000, KUMAR & FLADUNG 2001b, MATSUNAGA *et al.* 2002, LYZNIK *et al.* 2003, SRIVASTAVA & OW 2004).

For transformation of annual plants the most utilized site-specific recombination systems are the Cre-*lox* sequence derived from the bacteriophage P1 (HOESS & ABREMSKI 1985, GUO *et al.* 1997), and FLP-*FRT* from yeast (LYZNIK *et al.* 1993). The successful use of recombination systems to either delete the selection marker gene (DALE & OW 1991, RUSSEL *et al.* 1992, SRIVASTAVA *et al.* 1999, EBINUMA *et al.* 2001, PUCHTA 2003, CHEN *et al.* 2004, KOPERTHEK *et al.* 2004) or to target the transgene to predetermined positions in the genome (DAY *et al.* 2000, SRIVASTAVA *et al.* 2004), has been demonstrated.

The significance of site-specific recombination systems for targeted gene transfer or marker gene elimination is evident in particular for tree species. Trees are long-lived plants having long generation times, and the fidelity of transgene expression is required over decades of years. The correct function of the recombination system Cre-*lox* has already been demonstrated in poplar (EBINUMA *et al.* 2001), however, to date there is no report on the use of FLP-*FRT* in trees. Here we describe two recombination systems, Cre-*lox* and FLP-*FRT* applied to the model tree *Populus tremuloides* × *P. tremula*. We provide evidence for successful recombination and excision in both systems in the context of the expression of the "transgene-of-interest".

MATERIAL AND METHODS

Plasmids:

The pJFLO (=FLP) and pFCF (=*FRT*) binary constructs were described in detail in GIDONI *et al.* (2001). In short, the pJFLO construct carried the FLP gene under control of the Cauliflower-35S-promoter (Fig. 1A). Transgenic aspen carrying the pJFLO construct appeared normal in phenotype. The pFCF construct contained the 35S-*rolC* gene flanked by two *FRT* sites (Fig1B). Transgenic aspen transformed with this gene construct revealed the typical *rolC* characteristics as reported previously (FLADUNG *et al.* 1997). Both gene constructs were carrying the *npt*-II selection marker leading to kanamycin resistance. Following co-transformation of both constructs, the 35S-*rolC* was excised in the few independent transgenic lines obtained and, thus, the phenotype reverted to wildtype.

The plasmid pCrox18 included the Cre recombinase under the control of a heat-inducible promoter (HSP) flanked by two *lox* sites (Fig. 2, HOFF *et al.* 2001). The HSP-*Cre* gene was inserted between 35S promoter and GUS coding region rendering the GUS gene inactive in this form. In the presence of the Cre protein the *Cre* gene excised making the GUS gene functional. Outside the *lox* cassette the *npt*-II marker gene as transformation selection marker was located.

All the gene constructs described were transformed into the *Agrobacterium tumefaciens* strain GV3101::pMP90RK (KONCZ & SCHELL 1986, GIDONI *et al.* 2001, HOFF *et al.* 2001).

Plant material, transformation of aspen and molecular analysis:

A leaf disc co-cultivation method was used for *Agrobacterium*-mediated transformation of the hybrid clone (*P. tremula* L. × *P. tremuloides* Michx.; Esch5; (FLADUNG *et al.* 1996, 1997). For selection of transgenic plants, the regeneration media contained Kanamycin (50 mg/L) and Cefotaxime (500 mg/L). The *Agrobacterium* strains containing the constructs pJFLO and pFCF were mixed before transformation. In the following text, independent transgenic lines containing either pJFLO, pFCF, or both, are termed as FLP#X while the ones with pCrox18 are termed as Esch5:pGUSV4#X.

Extraction of genomic DNA from leaves to carry out PCR analysis was performed as described elsewhere (FLADUNG *et al.* 1996, 1997). The sequences of the primer pairs 1/2 and 3/4 used in PCR reactions amplifying fragments of the pJFLO and pFCF plasmids are described in GIDONI *et al.* (2001). In case of the pCrox18, recombination following heat-shock treatment was tested using a 35S-promoter forward primer (5'-TCG GGA AAC CTC CTC GGA TT-3') and a reverse primer from the GUS gene (5'-GTT TAC GCG TTG CTT CCG CCA-3') according to FLADUNG *et al.* (1996, 1997). The transgenic aspen line Esch5:35S-GUS#10 (E51-10) carrying the 35S-GUS gene construct was used as positive control (FLADUNG, unpublished).

Amplification of the *npt*-II gene was performed using the forward primer 5'-ATG GAT TGC ACG CAG GTT CTC-3' and a reverse primer 5'-AAG GCG ATA GAA GGC GAT

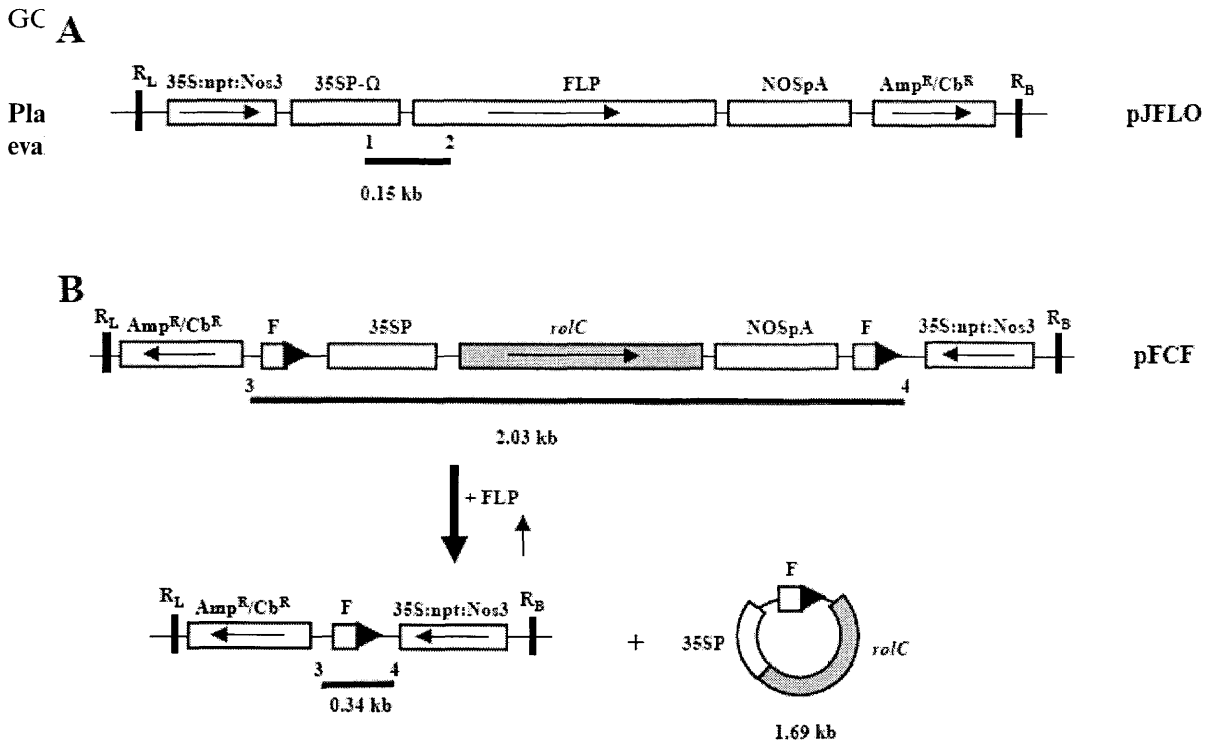

Figure 1. Schematic diagram of the pJFLO (=FLP) and the pFCF (=FRT) constructs (GIDONI *et al.* 2001). A. In the pJFLO construct the FLP gene is under the control of the 35S-promoter and the ΩÙ-translation enhancer sequence, and terminated by NOSpA. Positions of primer 1 and 2 are indicated. In PCR analyses an amplification fragment of 0.15 kb was obtained. B. The pFCF construct carried a 35S-*rolC*-NOSpA cassette flanked by two *FRT* (F) target sites. Positions of primer 3 and 4 are indicated. In PCR analyses a 2.03 kb fragment in the non-recombined or a 0.34 kb fragment in the recombined tissues was amplified. Modified according to GIDONI *et al.* (2001).

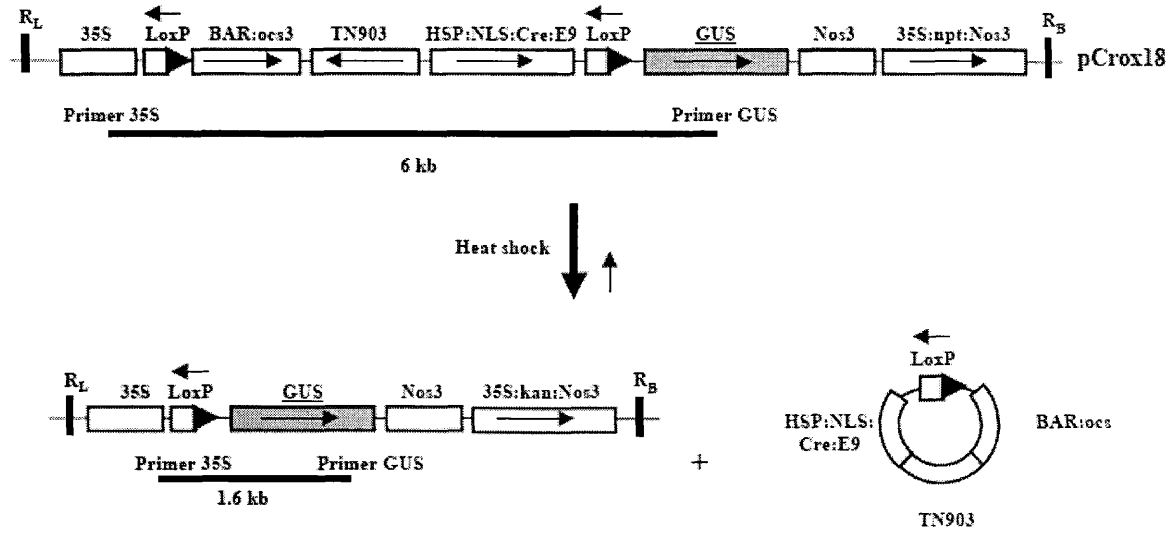

Figure 2. Schematic diagram of the pCrox18 construct (HOFF *et al.* 2001). The Cre recombinase is under the control of a heat-inducible promoter which is located between two *lox* target sites. Upstream of one lox site a promoter-less GUS-Nos3 gene is located. Following heat-shock induction, the 3.4 kb BAR:ocs3-TN903-HSP:NLS:Cre:E9 fragment is excised resulting in the GUS gene coming under the control of the 35S-promoter. A remaining *lox* site is located between 35S and the GUS gene. Positions of primers within the 35S promoter and the GUS gene are indicated. As plant selectable marker the *npt*-II gene leading to kanamycin resistance is used. Modified according to HOFF *et al.* (2001).

Table 1. Summary of the results obtained with FLP-*FRT* transgenic aspen. The phenotype and morphological aberrations were classified following morphological inspection of the transgenic plants. PCR analyses were performed by using the primer pairs as indicated in material and methods.

Aspen line	Phenotype	Phenotypical aberrations	PCR	
			Npt-II	Primer 1/2 / Primer 3/4
FLP1	*RolC*	Chimeric leaves	+	2.03 kb
FLP2	Wildtype	Leaf form	+	–
FLP3	*RolC*	Short reversion	+	2.03 kb
FLP4	*RolC*	–	+	2.03 kb
FLP5	*RolC*	–	+	2.03 kb
FLP6	Wildtype	–	+	–
FLP7	*RolC*	–	+	2.03 kb
FLP8	*RolC*	–	+	2.03 kb
FLP9	Wildtype	–	+	1.8 kb / 0.25 kb / 0.15 kb
FLP11	Wildtype	Chimeric leaves	+	1.8 kb / 0.25 kb / 0.15 kb
Esch 5 (control)	Wildtype	–	–	–

in sterile cultures *in vitro* at 25 °C and continuous light. Regenerated aspen plants derived from tissue culture were used as untransformed controls. Following *in vitro* culture the plants were transferred to soil and cultivated in growth chambers at 25 °C and 16/8 hours day/night cycle. Plants were watered daily. Control and transgenic plants were grown for three to six months side by side in the same growth chamber.

The pJFLO and pFCF transgenic aspen were visually inspected for recombination events in comparison with already available 35S-*rolC* transgenic aspen carrying one integrated copy and which served as *rolC*-transformed control (FLADUNG, 1999, KUMAR & FLADUNG 2000). GUS staining for screening of the heat-shock treated (16 hours at 37 °C) two-months old pCrox18 transgenic aspen plants was done according to the protocol by JEFFERSON *et al.* (1987) with some modifications. Leaves were excised and placed in cold 90 % acetone for 10 minutes. After rinsing with water the leaf tissue was placed in the reaction mix containing 0.5 M $NaPO_4$ (pH 7), 0.1 Na_2EDTA (pH 7), 5mM $K_3Fe(CN)_6$, 5mM $K_4Fe(CN)_6$, and 10mM X-Gluc (dissolved in Dimethylformamide). The leaves were incubated at 37 °C for at least 1 hour to overnight. Chlorophyll was removed by dipping the leaf tissue in 76 % ethanol.

RESULTS

FLP-*FRT* system

In total, ten putative transgenic lines were obtained following co-transformation with the two constructs pJFLO (=FLP) and pFCF (=*FRT*). All transgenic lines were regenerated on Kanamycin-containing medium. Transgenic plants grew well and formed roots on this medium. PCR analyses amplifying part of the *npt-II* gene confirmed the presence of the selection gene, and, thus, one or both constructs in the ten transgenic lines (data not shown). Six lines showed the typical *rolC* phenotype as already described in Fladung *et al.* (1996, 1997) indicating presence of the *FRT* construct. Four lines revealed wildtype plant habitus, which can be due to presence of only the FLP or both constructs.

By testing the 1/2 or the 3/4 primer pair independently in PCR experiment, the integration of either the pJFLO or pFCF was observed (Fig. 1). The primer pairs 1/2 and 3/4 yield fragments of 0.15 kb and 2.03 kb for pJFLO and pFCF, respectively. When both constructs are combined, the theoretical size of the amplification product of 3/4 primer pair should be smaller in plants or tissues (from 2.03 kb down to 0.34 kb) containing recombined product.

Testing the primer pair 3/4 in all six lines with *rolC* phenotype (FLP1, FLP3, FLP4, FLP5, FLP7, and FLP8, Table 1) a 2.03 kb amplification product was observed indicating the presence of only the pFCF construct (Fig. 3 left, arrow). In these transgenic lines no indication for presence of pJFLO was obtained. Out of the four transgenic lines with wildtype plant habitus, two lines (FLP2 and FLP6) revealed only the *npt*-II gene but were negative for both plasmids (Table 1, Fig. 3, 5). Analyzing the two other transgenic lines with wild

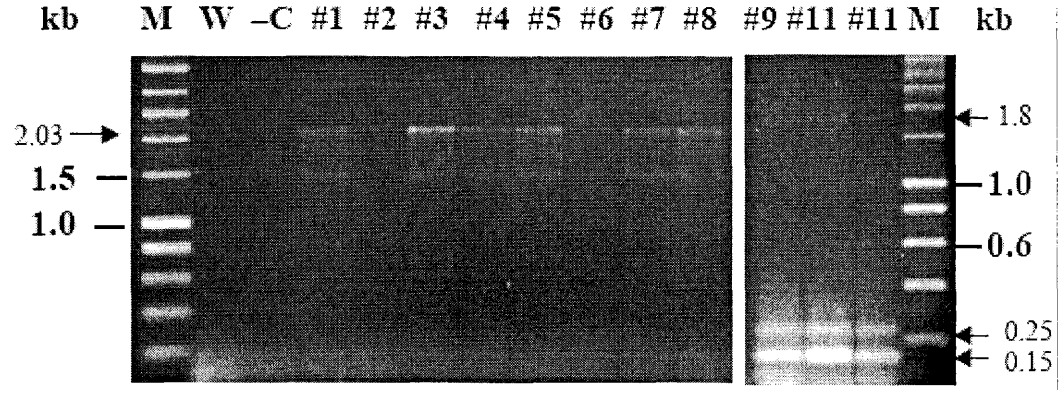

Figure 3. PCR analysis of FLP transgenic lines carrying either the pJFLO or the pFCF constructs, or both, using primer pairs 1/2 or 3/4. Bands of 2.03, 1.8 and 0.25 kb are indicated by arrows. M = molecular weight marker in kilo base pairs, W = water control without any DNA, -C = untransformed (negative) control, #1 to #11 independent FLP transgenic lines.

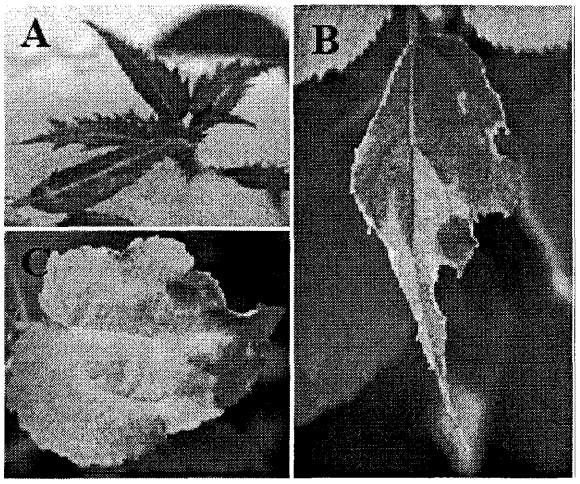

Figure 4. Leaf aberrations observed in the FLP transgenic lines. A. Lanceolate leaves with spiky leaf margins observed in plantthe line FLP2. B. Leaf sectors revealing chlorophylls of deficiency detected in plants of the line FLP1. C. Line FLP11 with light-green, *rolC*-expressing, and "normal" green sectors (wildtype sectors).

type plant habitus (FLP9 and FLP11) in PCR experiments with the two primer pairs, a 0.15 kb product specific for the pJFLO plasmid but also two additional products with unknown origin appeared, one of about 0.25 kb and a second weak one at 1.8 kb (Fig. 3 right, arrows).

During vegetative growth of three lines either phenotypical alterations or sometimes the formation of chimeric leaves was observed. In plants of the transgenic line FLP2 lanceolate leaves with spiky leaf margins were formed (Fig. 4A). This line is phenotypically wildtype but reveals the presence of only the *npt*-II gene. Transgenic plants of the line FLP1 generally revealed *rolC* phenotype but few chimeric leaves with partly chlorophyll deficiency were formed (FLP1, Fig. 4B). Plants of the line FLP11 which showed a wildtype plant habitus but sometimes chimeric leaves with either light-green, *rolC*-expressing and "normal" green wildtype leaf sectors appeared (FLP11, Fig. 4C) Interestingly, following PCR analyses it was revealed that FLP11 belongs to the group where recombination has occurred, thus, appearance of chimeric leaves is possibly caused by recombination in some cells giving wild type sectors whilst *rolC*-sectors are indicative of cells without recombination reaction.

The transgenic line FLP3 originally classified as pFCF transgenic line revealed a shoot reversion (Fig. 5A) as originally described by FLADUNG (1999) for 35S-*rolC* transgenic aspen. Molecular analysis of the *rolC*-expressing tissue revealed the presence of the *rolC* gene. In the wildtype-appearing, reverted plant part, however, both the 35S-*rolC* containing fragment as well as the *npt*-II gene could not be detected (Fig. 5B).

Cre-*lox* system

In total, 36 independent transgenic lines were obtained using the pCrox18 gene construct (HOFF *et al.* 2001) in transformation experiments with the hybrid aspen line Esch5. All transgenic lines regenerated, reveal wildtype phenotype and no morphological variations were observed among this set of transgenic lines. PCR analyses amplifying part of the *npt-II* gene confirmed the presence of the selection gene (data not shown).

From a total of 36 transgenic lines obtained, eight were randomly selected and heat-shock treat-

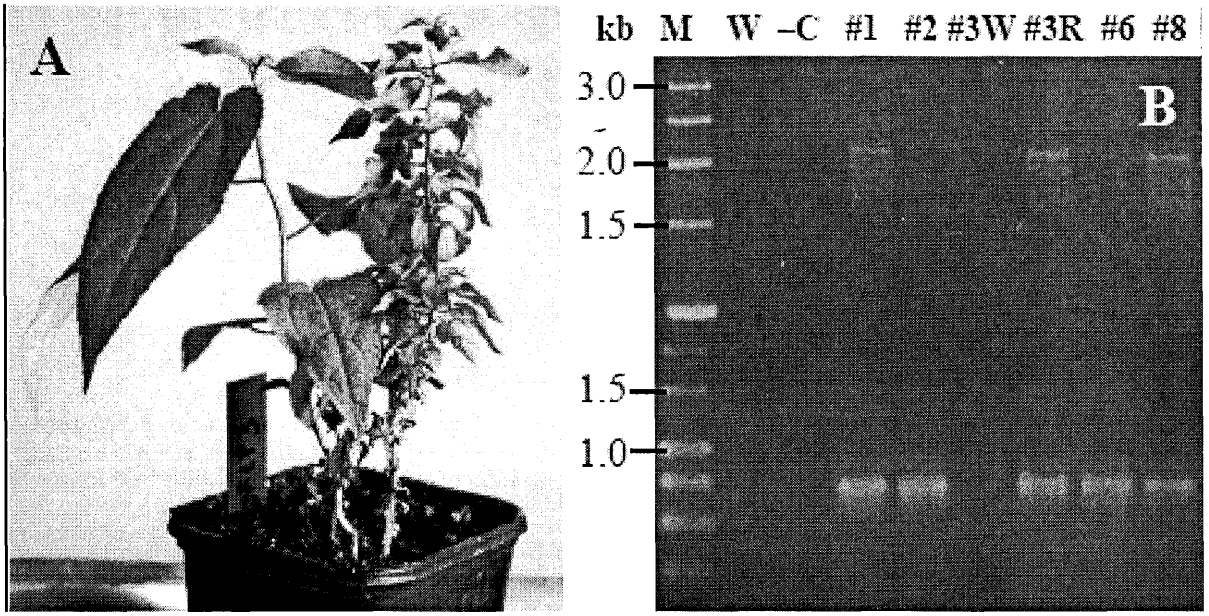

Figure 5. Shoot reversion observed in the transgenic line FLP3. A. The observed reversion occurred directly after transferring the plant into soil (age of plant approximately four months). B. PCR analyses of FLP1, FLP2, FLP3 wildtype (W) and *rolC* expressing (R) shoot, FLP6 and FLP8 using primer pair 3/4 (Fig. 1, top) and a *npt*-II-specific primer pair (bottom).

Table 2. Summary of the results obtained with Cre-*lox* transgenic aspen. The phenotype and morphological aberrations were classified following morphological inspection of the transgenic plants. PCR analyses were performed by using the primer pairs as given in material and methods.

Aspen line	GUS staining	PCR 35S-GUS
Esch5 : pGUSV4#1	+	1.6 kb
Esch5 : pGUSV4#2	+	1.6 kb
Esch5 : pGUSV4#5	–	1.6 kb
Esch5 : pGUSV4#7	mosaic	1.6 kb
Esch5 : pGUSV4#8	+	1.6 kb
Esch5 : pGUSV4#9	+	1.6 kb
Esch5 : pGUSV4#10	–	1.6 kb
Esch5 : pGUSV4#13	+	n.d.
E51-10 (positive control)	+	1.55 kb
Esch5 (untransformed control)	–	–

n.d. – not determined.

ed. DNA was isolated from all the treated lines and the region from the 35S-promoter to the GUS gene was amplified using PCR (Fig. 6). The line Esch5:35S-GUS#10 (=E51-10 in Table 2) was used as a positive control. In all lines analyzed, a single amplification product of about 1.6 kb was obtained indicating that the insert between the two lox sites had recombined and excised. The slightly larger product in the recombined pGUSV4 line as compared to the positive control is due to the presence of one *lox* site between the 35S-promoter and the GUS gene.

To confirm the PCR results indicating recombination, eight heat-shock treated independent pCrox18 transgenic lines as well a positive (Esch5:35S-GUS#10) and a negative control (untransformed Esch5) were stained for GUS expression. Out of the eight pCrox18 transgenic lines analyzed leaf and stem tissues of five lines were blue stained indicating GUS expression (Table 2), and one line (Esch5:pGUSV4#7) showed mosaic staining (not shown, Table 2). Leaf and stem tissues of two further lines revealed no GUS staining at all despite successful recombination indicated

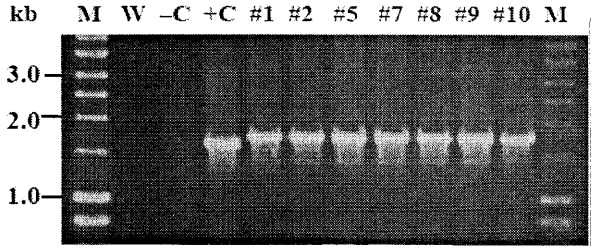

Figure 6. PCR analysis of transgenic lines carrying the pCrox18 gene construct using a GUS-specific primer pair. M = molecular weight marker in kilo base pairs, W = water control without any DNA, -C = untransformed (negative) control, +C = transgenic control carrying 35S-GUS gene, #1 to #10 independent pCrox18 transgenic lines.

by PCR.

To unravel a possible imprecise excision of the insert between the two lox sites the PCR fragment covering the region 35S-lox-GUS of one GUS-positive stained line (Esch5:pGUSV4#1) and the two negative lines (Esch5:pGUSV4#5 and -#10) were sequenced. Surprisingly, the sequences obtained from all three lines were similar (data not shown) indicating that transcriptional control (*e.g.* methylation) may be responsible for GUS inactivation).

DISCUSSION

Besides integration modus of the transgene into the tree genome, the integration locus also plays an important role with regards to the expression characteristics of the transgene (MEYER *et al.* 1992, FLADUNG 1999, MATZKE & MATZKE 1998). In addition, integration in or near another gene may cause an undesired phenotype (KUMAR & FLADUNG 2001a). To address the issue of expression variability and secondary effects, single-copy transgenes need to be placed precisely into pre-defined genomic positions. Expression variability of transgenes placed in these genomic positions should be tested using reporter genes before specific target genes are integrated. Transgenic lines containing transgenes only in the pre-defined genomic positions that are characterized by the absence of expression variability even under extreme stress treatments (light, temperature, etc.) should be used in a commercial forestry environment. This could be important to minimize expression variation among different transformation events, and therefore enhance the economic viability of a tree genetic engineering program.

In annual plants such as tobacco, maize or rice successful site-specific recombination (reviewed in OW 2002) has been used to delete the selection marker gene (DALE AND OW 1991, RUSSEL *et al.* 1992, SRIVASTAVA *et al.* 1999). Also attempts have been undertaken to target the transgene to predetermined positions in the genome already harbouring a *lox* or *FRT* site (DAY *et al.* 2000, SRIVASTAVA *et al.* 2004). For long-lived trees the stability of transgene expression is very important to ensure predictable expression patterns or to avoid undesired silencing phenomena (KUMAR &FLADUNG 2003a). This is in particular important when using transgenes leading to male sterility or inducing resistance to biotic pathogens. However, very little has been done so far to introducing site-specific recombination systems into tree genomes. The only work that has been published so far uses the site-specific recombination systems R-*Rs* or Cre-*lox* to remove the selection marker in transgenic *Populus* (EBINUMA *et al.* 2001, 2003) as it has been confirmed here for Cre-*lox*.

In this paper, two different recombination systems have been successfully tested in transgenic hybrid aspen (*Populus tremula* × *P. tremuloides*). The FLP-*FRT* system was tested for the first time in aspen, the other one Cre-*lox* has been tested before (EBINUMA *et al.* 2001, 2003). Both systems Cre-*lox* and FLP-*FRT* work well in aspen, however, some unexpected observations have been made. In four out of ten transgenic aspen lines transformed with the FLP-*FRT* gene constructs, morphological/phenotypical alterations were detected. The variation observed in FLP2 is clearly independent from expression of the FLP recombinase (because the gene is lacking) but possibly is a T-DNA insert variation (because *npt-II* is present. FLP1 is a putative sectorial mutation (SPENA & SALAMINI 1995). The alteration observed in FLP11 resembles the ones observed either in 35S-*rolC* unstable Lines (KUMAR & FLADUNG 2001a, FLADUNG & KUMAR 2002) or transgenic lines carrying the *Ac* transposon from maize (KUMAR & FLADUNG (2003b). The appearance of the shoot reversion observed in FLP3 as well as the results from molecular analyses are similar to the ones described in FLADUNG (1999).

A second surprising result of the FLP-*FRT* system is that following recombination the PCR of remaining fragment (foot print) revealed a size of about 0.25 kb product instead of the expected 0.34 kb amplification product. Sequencing of the 0.25 kb fragment confirmed the presence of the *FRT* site. Imprecise recombination can be excluded

because the correct excision of sequences using these recombination systems has been reported (GOSH *et al.* 2005). The only explanation is that following T-DNA integration 90 base pairs have been lost which remained undetected in the 2.03 kb fragment. It would be interesting to include other tree species to study the mechanism of the FLP-*FRT* recombination system.

Within the 36 independent Cre transgenic aspen lines, however, no single morphological variation was detected. Using a similar construct no alterations could be detected in transgenic *Arabidopsis* (HOFF *et al.* 2001). In transgenic tobacco and tomato plants carrying the Cre gene under control of the 35S promoter, however, morphological alterations other than these induced by the *rolC* gene were described (COPPOOLSE *et al.* 2003). In the same report no aberrant phenotype was observed when a tissue-specific promoter was used to drive to Cre gene. This leads to the speculation that morphological alterations occurred at higher frequencies generally in recombinase transgenic plants when these genes are driven by the 35S promoter.

The Cre-*lox* system has been tested in a range of plant species including poplar (DALE AND OW 1990, 1991, ODELL *et al.* 1990, HOFF *et al.* 2001, ZHANG *et al.* 2003, Ebinuma *et al.* 2003). Here, we have transformed aspen using a gene construct containing the Cre gene under control of a heat-inducible promoter which has been tested before in *Arabidopsis* (HOFF *et al.* 2001). Eight independent transgenic lines investigated in PCR analyses (out of 36 in total) showed successful excision of a 4.4 kb fragmentcarrying a bar selectable marker gene. However, in GUS staining test, from the eight randomly selected only six lines revealed GUS expression. Partial sequencing of the region covering 35S-lox-GUS obtained from one GUS-expressing and two non-expressing lines revealed precise excision of the 4.4 kb fragment. Thus, it is possible that either there is a deletion in the 3'-region of the GUS gene in the two lines or that probably transcriptional or post-transcriptional silencing of the single copy GUS gene occurs in the two lines.

In this paper, we have successfully tested two site specific recombination systems in hybrid aspen (*Populus tremula* × *P. tremuloides*). The next step is to establish a strategy to initiate a targeted transfer of the "transgene-of-interest" (TOI) into the tree genome as described in KUMAR and FLADUNG (2001b, 2003a). This strategy comprises (a) the transfer of a reporter gene (*e.g.* GFP, GUS) flanked by site-specific recognition site in the genome, (b) characterization of the genomic region for the reporter gene expression and (c) if reporter gene expression characteristics are desirable, the targeted transfer of a TOI to this site. The site-specific recombination reaction requires the action of a site-specific recombinase that can be supplied either by stable or transient transformation. We have shown here that two recombination systems are working in aspen. Once a genomic target is shown to confer stable expression without any secondary effects at certain developmental stages and/or different stress conditions, subsequent deliveries of TOI's can be made to that site.

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