Plant Cell Rep (2016) 35:369–384
DOI 10.1007/s00299-015-1890-x

ORIGINAL ARTICLE

Level of tissue differentiation influences the activation of a heat-inducible flower-specific system for genetic containment in poplar (*Populus tremula* L.)

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Received: 23 July 2015 / Revised: 4 September 2015 / Accepted: 16 October 2015 / Published online: 31 October 2015

Abstract

Key message **Differentiation level but not transgene copy number influenced activation of a gene containment system in poplar. Heat treatments promoted *CRE* gene body methylation. The flower-specific transgene deletion was confirmed.**

Abstract Gene flow between genetic modified trees and their wild relatives is still motive of concern. Therefore, approaches for gene containment are required. In this study, we designed a novel strategy for achieving an inducible and flower-specific transgene removal from poplar trees but still expressing the transgene in the plant body. Hence, pollen carrying transgenes could be used for breeding purposes under controlled conditions in a first phase, and in the second phase genetic modified poplars developing transgene-free pollen grains could be released. This approach is based on the recombination systems *CRE*/loxP and *FLP*/frt. Both gene constructs contained a heat-inducible *CRE*/loxP-based spacer sequence for in vivo assembling of the flower-specific *FLP*/frt system. This allowed inducible activation of gene containment. The *FLP*/frt system was under the regulation of a flower-specific promoter, either CGPDHC or PTD. Our results confirmed complete *CRE*/loxP-based in vivo assembling of the flower-specific transgene excision system after heat treatment in all cells for up to 30 % of regenerants derived from undifferentiated tissue cultures. Degradation of HSP::*CRE*/loxP spacer after recombination but also persistence as extrachromosomal DNA circles were detected in sub-lines obtained after heat treatments. Furthermore, heat treatment promoted methylation of the *CRE* gene body. A lower methylation level was detected at CpG sites in transgenic sub-lines showing complete *CRE*/loxP recombination and persistence of *CRE*/loxP spacer, compared to sub-lines with incomplete recombination. However, our results suggest that low methylation might be necessary but not sufficient for recombination. The flower-specific *FLP*/frt-based transgene deletion was confirmed in 6.3 % of flowers.

Keywords Biosafety · *Populus* · Recombination · Gene flow · Heat shock

Abbreviations

GM	Genetic modified
HSP	Heat shock promoter
exDNA	Extrachromosomal DNA
T-DNA	Transfer DNA
GUS	Beta-glucuronidase
DIG	Digoxigenin

Communicated by D.-B. Zhang.

Electronic supplementary material The online version of this article (doi:10.1007/s00299-015-1890-x) contains supplementary material, which is available to authorized users.

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Introduction

Pollen-mediated gene flow, also called vertical gene transfer, regularly occurs between commercially improved plants and their wild relatives. However, this gene flow was not considered a serious point of concern until the development of plant biotechnology (Hoenicka and Fladung 2006). Since then, many approaches have been proposed for the avoidance of pollen-mediated gene flow from genetic modified plants. Plastid transformation, sterility or cleistogamy (non-opening flowers) induction, and transgene excision in pollen grains belong to the hitherto available methods (rev. in Hüsken et al. 2010). There are only few reports on genetic containment for tree species. The main reason is probably that tree species require many years or even decades to develop first flowers. However, despite of this, some studies have evaluated flower and pollen ablation with different levels of success in poplar (*Populus* spp) (Meilan et al. 2001; Skinner et al. 2003; Hoenicka et al. 2006), pine (*Pinus radiata*) (Zhang et al. 2012) and birch (*Betula pendula*) (Lännenpää et al. 2005).

Transgene excision in reproductive organs is based on site-specific DNA recombination systems. Examples of these systems are bacteriophage P1 *CRE*/loxP (Sternberg and Hamilton 1981) and *Saccharomyces cerevisiae FLP*/frt (McLeod et al. 1986). Traditionally, these systems have been applied either to excise marker genes and short spacer sequences in higher plants (Gilbertson 2003; Srivastava and Ow 2004), or to target transgenes to desired positions in the host genome (Nanto and Ebinuma 2008; Li et al. 2009; Fladung et al. 2010). Recombinase activity can either be initiated by a heat or chemical treatment by using inducible promoters (Moore et al. 2006) or directly by endogenous activation of appropriate gene promoters (Luo et al. 2007; Gidoni et al. 2008). Site-specific recombinases are now becoming increasingly important in the development of gene containment strategies (Mlynárová et al. 2006; Moravcíková et al. 2008; Luo et al. 2008; Herzog et al. 2012). Transgene excision and gene targeting have been demonstrated in many crops, including wheat, tobacco, tomato, soybean and maize (Hüsken et al. 2010), but also in tree species like poplar (Kumar and Fladung 2001; Deng et al. 2009; Fladung and Becker 2010) and apple (Herzog et al. 2012).

One important drawback of the hitherto available approaches for genetic containment derives from their difficult integration into breeding programs. This is due to the early promoted sterility or transgene elimination in reproductive organs. The development of reliable approaches for gene containment allowing a tissue and time-specific T-DNA elimination is convenient for the integration of transgenic lines into breeding programs. A solution could be provided by inducible systems allowing the specific excision of transgenes in pollen grains. There are

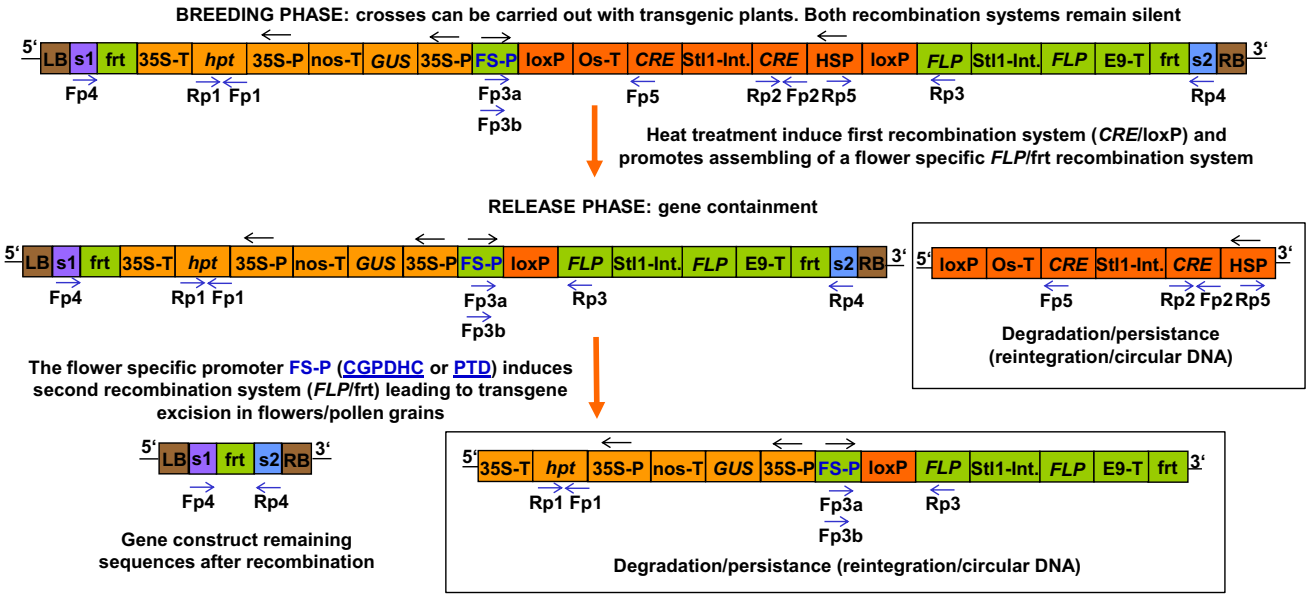

Fig. 1 General structure of gene constructs used for genetic transformation. Recombination genes are silent during the breeding phase. Heat treatment induces the first recombination system (*CRE*/loxP) and activates the *FLP*/frt-based gene containment in flowers. Recombination systems: *FLP*/frt, *CRE*/lox-P. Promoters: FS-P (flower-specific promoters PTD or CGPDHC), 35S-P (35S promoter), HSP (heat-inducible promoter). *Black arrows*: orientation of gene transcription. *Blue arrows*: location of forward (Fp) and reverse (Rp) primers used for PCR analysis. Terminators: 35S-T (35S terminator), nos-T (nos terminator), Os-T (octopine synthase terminator). Marker genes: *hpt* (hygromycin resistance), *GUS* (*uidA*; *beta-glucuronidase*). Others: transgene excision detection sequences (s1, s2), Intron (Stl1-Int.), T-DNA border (LB/RB) (colour figure online)

Table 1 Primers used in this study (P1–P5: see Fig. 1, M1–M4: see Fig. 3)

Primer pairs	F/R		Sequence 5′–3′
Recombination			
p1	Fp1	*HPT*	AAA GCC TGA ACT CAC CGC GA
	Rp1		TCG GTT TCC ACT ATC GGC GA
p2	Fp2	*CRE*	CGA GTG ATG AGG TTC GCA AG
	Rp2		TAA CAT TCT CCC ACC GTC AGT A
p3	Fp3a	CGPDHC	ATG ACT TTC TCC GCC TCC TT
	Fp3b	PTD	AAT ACT TCT CAC GCT ACC CTT CAG
	Rp3	*FLP*	ACT GTG GCT ATT TCC CTT ATC TGC
p4	Fp4	TEDS	CCG GGA AAT CTA CAT GGA TCA
	Rp4		CCC AAG ATC TGG CCC TTA G
p5	Fp5	exDNA	CCG GGA AAT CTA CAT GGA TCA
	Rp5		CCC AAG ATC TGG CCC TTA G
Methylation			
M1	F1	Amplicon 1	GTT AGT TTT TTA AGA GAT AGA ATT T
	R1		TAA AAG TTT CRA AAT TAA
M2	F2	Amplicon 2	GTG GGY GGT ATG GTG TAA GTT GAA TAA
	R2		ATATCTTTAACCCTRATCCTRRCAATTTC
M3	F3	Amplicon 3	AGA AAG AAG TGA AGG TAT YGT GG
	R3		TTC ACC RRC ATC AAC RTT TTC TTT TC
M4	F4	Amplicon 4	TYT AGT TGY GGT TYG AAG AAG T
	R4		CCA CRA TRC CTT CAC TTC TTT CT

F/R forward/reverse, special nucleotides: Y (mixture of C or T) and R (mixture of A or G)

HPT hygromycin resistance gene, *CRE* recombinase gene, *CGPDHC* cytosolic glycerol-3-phosphate dehydrogenase promoter, *PTD* promoter, *FLP* recombinase gene, *TEDS* transgene excision detection sequences, *exDNA* HSP::*Cre*/loxP spacer persistence after recombination as exDNA

several reports of such pollen-specific systems (rev. in Hüsken et al. 2010). The *CRE*/loxP system regulated by a microspore-specific promoter was successfully used to achieve a tissue-specific transgene excision from pollen grains in tobacco (Mlynárová et al. 2006). LoxP-frt fusion sequences, combining wild type loxP and frt, allowed higher transgene deletion efficiency from tobacco pollen and seeds than wild type systems when used as flanking sites for *FLP* or *CRE* recombinase (Luo et al. 2007). An inducible tissue-specific gene containment approach was reported in wheat (Hinze and Becker 2012).

Epigenetic factors must be seriously considered when developing strategies for genetic containment. Epigenetic mechanisms, like methylation of cytosine in DNA, regulate gene expression (Sekhon and Chopra 2009; Becker et al. 2011; rev. in Ahmad et al. 2010; Köhler et al. 2012; Wollmann and Berger 2012) and may affect the expression of the recombinases. Highly dynamic mechanisms of chromatin remodeling occur during cell de-differentiation and differentiation processes on which in vitro adventitious plant regeneration systems are based (rev. in Miguel and Marum 2011). In poplar, substantial changes were reported in DNA methylation during in vitro regeneration (Vining et al. 2013). Non-uniform, mosaic gene expression patterns of genes (Robbins et al. 2009; Sekhon and Chopra 2009) and transgenes (Kaufman et al. 2008; Freeman et al. 2011), triggered by epigenetic modifications, are often linked to transcriptional silencing.

In this study, we studied a new approach for specific transgene removal from poplar flowers. The long period of time usually required for flower development in poplar (>7 years) was overcome by using early flowering HSP::*AtFT* poplar lines (Hoenicka et al. 2014). These lines allowed a fast evaluation of gene containment. Our gene constructs are based on the recombination systems *CRE*/loxP and *FLP*/frt. The *FLP*/frt system was under the regulation of the flower-specific promoter CGPDHC or PTD. Both gene constructs contained a *CRE*/loxP-based spacer sequence controlled by a heat-inducible promoter. Activation of *CRE*/loxP recombination following a heat treatment promoted in vivo assembling of the *FLP*/frt-based flower-specific transgene excision system. This strategy aimed at the incorporation of an additional time-specific compound to gene containment. Hence, transgene containing pollen could be used in a first phase for breeding purposes under controlled conditions. In the second phase, plants producing transgene-free pollen grains could be released. Our results confirmed complete *CRE*/loxP-based

Table 2 Activation of transgene excision systems CGPDHC::*FLP*-FRT or PTD::*FLP*-FRT in sub-lines after recombination of the heat-inducible *CRE*/loxP spacer

		Transgenic line	T-DNA copy number	Complete activation of excision system in sub-lines		Number of transgenic sub-lines
				Yes	No	
Hsp::*CRE*-LoxP	CGPDHC::*FLP*/FRT	N138-1	4	2	0	2
		N138-2	4	1	2	3
		N138-3	1	0	3	3
		N138-4	1	0	4	4
		N138-5	4	1	0	1
		N138-6	2	0	4	4
		N148-1	2	0	2	2
		N148-2	1	1	1	2
		N148-3	3	0	4	4
		N151-1	3	1	3	4
		N151-2	2	4	0	4
		N151-3	1	1	3	4
				11 (29.7 %)	26 (70.3 %)	37
Hsp::*CRE*-LoxP	PTD::*FLP*	N133-1	2	4	0	4
		N140-1	1	1	0	1
		N140-2	2	2	1	3
		N140-4	3	0	4	4
		N140-5	3	3	1	4
		N140-6	3	0	4	4
		N146-1	2	3	1	4
		N180-1	3	0	4	4
		N180-2	3	0	4	4
		N180-3	3	0	4	4
		N180-4	2	0	4	4
		N180-5	1	1	2	3
				13 (30.2 %)	30 (69.8)	43

Complete activation of transgene excision systems was achieved in 11/37 (29.9 %) and 13/43 (30.2 %) transgenic sub-lines with both excision systems, respectively. Other transgenic sub-lines showed incomplete activation, i.e., recombination did not occur in all plant cells. No correlation was found between T-DNA copy number and complete/incomplete activation of the transgene excision systems

in vivo assembling of the flower-specific transgene excision system after heat treatment in all cells of some regenerants derived from undifferentiated tissue cultures but not in differentiated in vitro plants. Complete degradation of HSP::*CRE*/loxP spacer after recombination but also persistence, as an extrachromosomal DNA circle (exDNA) (Srivastava and Ow 2003) or possibly due to reintegrations, were detected in sub-lines obtained after heat treatment. Furthermore, heat treatment promoted methylation of the *CRE* gene body. A lower methylation level was detected at CpG sites in transgenic sub-lines showing complete *CRE*/loxP recombination and persistence of *CRE*/loxP spacer, compared to sub-lines with incomplete recombination. Our results suggest that low methylation might be necessary but not sufficient for recombination. The flower-specific *FLP*/frt-based transgene deletion was also confirmed but the efficiency was low.

Results

Molecular analyses of transgenic lines

Transgenic poplar lines were produced with both gene constructs (Fig. 1). The transgenic plants obtained showed a similar phenotype as control plants. In total, 25 independent transgenic lines were obtained after genetic transformation for each gene excision system. All transgenic lines were regenerated on hygromycin containing media. PCR analyses with primer pairs p1 and p2 (Fig. 1; Table 1), amplifying parts of the *HPT* and *CRE* genes (not

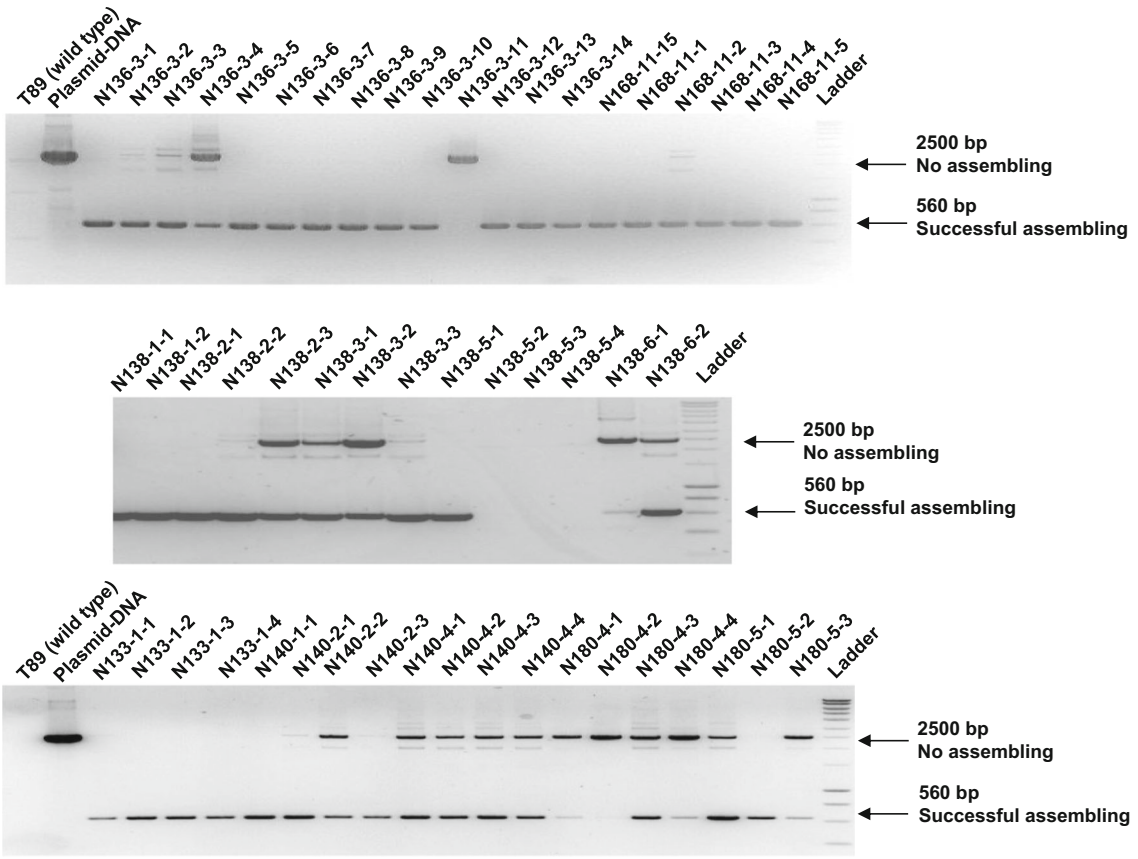

Fig. 2 In vivo assembling of flower-specific transgene excision system. PCR analysis of leaves from sub-lines obtained after heat treatment of undifferentiated poplar tissue cultures containing the gene excision system CGPDHC::*CRE* (*above* and *middle*) or PTD::*FLP* (*below*). *Small amplicons* (~560 bp) indicate the assembling of flower-specific transgene excision system. The presence of a *big amplicon* (~2500 bp) indicates no assembling of transgene excision system. The presence of both amplicons (~560 and ~2500 bp) indicates incomplete activation of the transgene excision systems

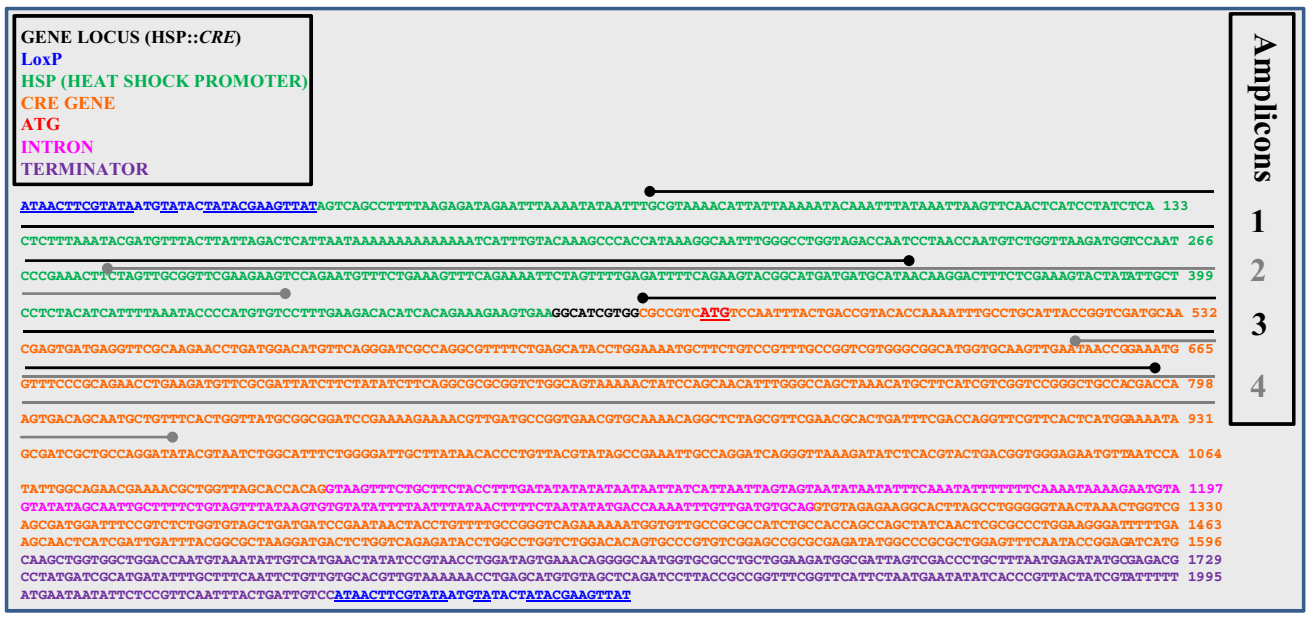

Fig. 3 Amplicon design for methylation analysis of heat-inducible *CRE*/loxP recombination system. Four amplicons were generated: (*1*) and (*2*) HSP promotor, (*3*) *CRE* gene start, (*4*) *CRE* gene body

Table 3 Summary of the DNA methylation studies of the HSP::*CRE*-loxP spacer in selected transgenic lines (before heat treatment: N138-2, N180-5, N138-3, N140-2) and sub-lines (obtained after heat treatment: N138-2-1, N138-2-2, N138-2-3, N180-5-1, N180-5-2)

			Complete *Cre*/loxP recombination	DNA methylation [%]								
				Promoter [1]			Gene start[2]			Gene body[3]		
				CG	CNG	CHH	CG	CNG	CHH	CG	CNG	CHH
Transgenic	lines	(before HT)	N138-2	60	45	63	11	3	0,3	6	6	2
			N138-3	34	15	49	0,4	0,4	0,6	4	0,3	1
			N140-2	75	88	74	8	15	3	8	0,2	0,4
			N180-5	48	50	47	1	1	0	1	2	1
Transgenic	sub-lines	(after HT)	N138-2-1	+	50	36	55	0,6	9	0,1	42	48	42
			N138-2-2	-	69	67	20	11	14	3	99	100	99
			N138-2-3	-	71	68	19	10	2	0,4	31	25	2
			N180-5-1	-	n.a.	n.a.	n.a.	21	2	0,4	99	100	99
			N180-5-2	+	66	70	66	0,5	1	0,3	76	73	67

DNA methylation was assessed in CpG, CHG and CHH sites and averaged between sites and amplicons. No full correlation was found between a complete *CRE*/loxP recombination and the methylation degree of transgenic lines. However, transgenic sub-lines N138-2-1 and N180-5-2, showing complete *CRE*/loxP recombination and spacer persistence (Table 4), have a twenty times lower CpG methylation of the *CRE* gene start region (detail at Fig. 4), compared to sub-lines with incomplete recombination. Heat treatment (HT) promoted a strong methylation of the *CRE* gene body. Amplicons studied (see Fig. 3): [1] Methylation was analyzed at amplicon 1 for all samples except N180-5, where amplicon 2 was used, [2] Methylation was analyzed at amplicon 3, [3] Methylation was analyzed at amplicon 4

shown), and Southern blot analyses (Table 2, Supplementary Fig. 1a, b) with a hygromycin probe, confirmed the presence of the CGPDHC::*FLP* and PTD::*FLP*-based gene constructs in 24 and 21 independent transgenic lines, respectively. Southern blot analyses showed a variable T-DNA copy number in the studied transgenic lines, ranging from one to more than three copies for both constructs (Table 2). Twelve transgenic lines for each gene construct, with one to three and more T-DNA copies, were selected for further studies.

In vivo assembling of *FLP*/frt transgene excision system through a *CRE*/loxP recombination

According to our experimental design, a heat shock treatment should activate the *CRE*/loxP recombination in transgenic lines. This first recombination allowed in vivo assembling of the flower-specific *FLP*/frt transgene excision system (Fig. 1). Plants regenerated after heat treatment of transgenic lines were called "transgenic sub-lines". The obtained transgenic sub-lines were studied with PCR to confirm complete assembling of the flower-specific *FLP*/frt transgene excision system. PCR analyses of heat-treated transgenic sub-lines using specific primers (p3 primers, Table 1; Fig. 1) showed a 2500 bp amplicon if recombination did not occur, whereas a single 560 bp amplicon confirmed *CRE*/loxP recombination. A mix of two amplicons (2500 and 560 bp) indicated an incomplete activation of the *CRE*/loxP recombination system (Fig. 2), i.e., recombination did not take place in all plant cells.

First, heat treatments were applied to differentiated in vitro plants. However, only a partial or even no *CRE*/loxP recombination could be achieved (not shown). In this case, only a single 2500 bp, or two amplicons of 2500 and 560 bp, was obtained. Heat treatments of undifferentiated (regenerative callus) in vitro cultures allowed an improved activation of the *CRE*/loxP recombination system (Fig. 2; Table 2). PCR analyses of resulting 80 transgenic sub-lines revealed both two amplicons of 2500 and 560 bp, and a single 560 bp amplicon in some sub-lines, confirming activation of the *CRE*/loxP recombination system in all plant cells (Table 2). Using this procedure, we confirmed complete activation of the transgene excision system in 11/37 (29.7 %, GPDHC::*FLP*) and 13/43 (30.2 %, PTD::*FLP*) of transgenic sub-lines (Table 2). Thus, about two-thirds of the heat-treated transgenic sub-lines still showed a partial activation or no activation at all (Table 2). No relationship was found between T-DNA copy number of transgenic lines and the successful activation of the transgene excision system (Table 2, Supplementary Fig. 1a, b).

Methylation analysis of the heat-inducible *CRE*/loxP recombination system

Epigenetic inactivation may impede complete *CRE*/lox recombination in transgenic sub-lines. Therefore, the level of DNA methylation at the *CRE* gene was studied in selected transgenic lines and sub-lines. As expected, in plasmid DNA (control) only methylation at CCWGG sites was observed, which originated from the dcm methyltransferase present in *Escherichia coli* (Marinus and Morris 1973). The methylation state of the HSP promoter, the *CRE* gene start as well as the gene body were determined in

Table 4 HSP::*CRE*-loxP spacer persistence or elimination in transgenic sub-lines showing complete recombination after heat treatments

	Before heat shock		After heat shock (*CRE*-loxP spacer persistance or elimination)				
	Transgenic lines	T-DNA copies*	Transgenic sub-lines	*CRE*-loxP activation**	exDNA**	CRE**	CRE*
Hsp::*CRE*-LoxP CGPDHC::*FLP*/FRT	N138-1	4	N138-1-1	+	-	+	+
			N138-1-2	+	-	+	+
	N138-2	4	N138-2-1	+	-	+	+
	N138-5	4	N138-5-1	+	-	-	-
	N148-2	1	N148-2-1	+	-	-	-
	N151-1	3	N151-1-3	+	-	-	-
	N151-2	2	N151-2-1	+	-	-	-
			N151-2-2	+	-	-	-
			N151-2-3	+	-	-	-
			N151-2-4	+	-	-	-
	N151-3	1	N151-3-4	+	-	+	-
Hsp::*CRE*-LoxP PTD::*FLP*/FRT	N133-1	2	N133-1-1	+	-	+	-
			N133-1-2	+	-	-	-
			N133-1-3	+	-	-	-
			N133-1-4	+	-	+	-
	N140-1	1	N140-1-1	+	-	-	-
	N140-2	2	N140-2-1	+	+	+	+
			N140-2-3	+	-	+	+
	N140-5	3	N140-5-1	+	+	+	+
			N140-5-2	+	-	+	+
			N140-5-3	+	+	+	+
	N146-1	1	N146-1-1	+	+	+	+
			N146-1-2	+	+	+	+
			N146-1-4	+	+	+	+
	N180-5	1	N180-5-2	+	-	+	-

Selected transgenic sub-lines, showing complete *CRE*-loxP recombination, were further studied to detect potential *CRE*-loxP persistence. PCR and Southern blot analyses confirmed complete spacer activation and elimination in 7/11 (63.6 %) and 4/14 (28.6 %) of sub-lines for both systems. However, Southern blots with *CRE* gene probes and PCRs with *CRE* primers seem to indicate spacer reintegration after recombination in some transgenic lines. PCR analyses showed presence of HSP::*CRE*-loxP spacer as a exDNA in several transgenic lines. Single asterisk according to Southern-blot analyses/qPCRs (T-DNA copy number verification), double asterisks according to PCR analyses with specific primers (see Table 1). Transgenic sub-lines with complete spacer elimination (blue background) were selected for the induction of early flowering, using a second round of heat treatments. Transgene elimination through *FLP*/frt recombination was studied in selected flowers obtained from these sub-lines (Figs. 5, 6)

transgenic lines (N138-2, N138-3, N140-2, N180-5), and five transgenic sub-lines derived from N138-2 (N138-2-1, N138-2-2, N138-2-3) to N180-5 (N180-5-1, N180-5-2). Methylation was detected in CpG, CHG and CHH sites of four different amplicons (Fig. 3). Our analysis included two transgenic lines (N138-2, N180-5), and their respective sub-lines (N138-2-1, N180-5-2), which showed complete *CRE*/lox recombination after heat treatment and spacer persistence, i.e., incomplete spacer degradation after recombination (Tables 3, 4; Fig. 4).

Results obtained show that the HSP promoter was highly methylated in most transgenic lines and sub-lines (Table 3; Fig. 4). The *CRE* gene (gene start and body) showed a low methylation level in transgenic lines before heat treatments (Table 3). Heat treatments caused strong changes on the methylation level. All transgenic sub-lines showed a high methylation of the *CRE* gene body (Table 3). DNA methylation of the *CRE* gene body changed from 1 to 8 % in transgenic lines (before heat treatments) to 31–99 % in transgenic sub-lines, obtained from transgenic lines after heat treatments (Table 3). The *CRE* gene start region showed less pronounced changes on methylation. In this case, even a twenty times lower methylation level was found in the CpG sites of persistent HSP::*CRE*/loxP spacer in transgenic sub-lines N138-2-1 and N180-5-2, compared to sub-lines with incomplete recombination (N138-2-2, N138-2-3, N180-5-2) (Table 3). Both lines showed complete *CRE*/loxP recombination and possibly spacer reintegration (Table 4). This result shows that sub-lines with complete *CRE*/loxP recombination have lower DNA methylation levels of *CRE* gene start at the CpG sites compared to those sub-lines without complete recombination (Fig. 4b). No complete *CRE*/loxP recombination was detected in sub-lines derived from

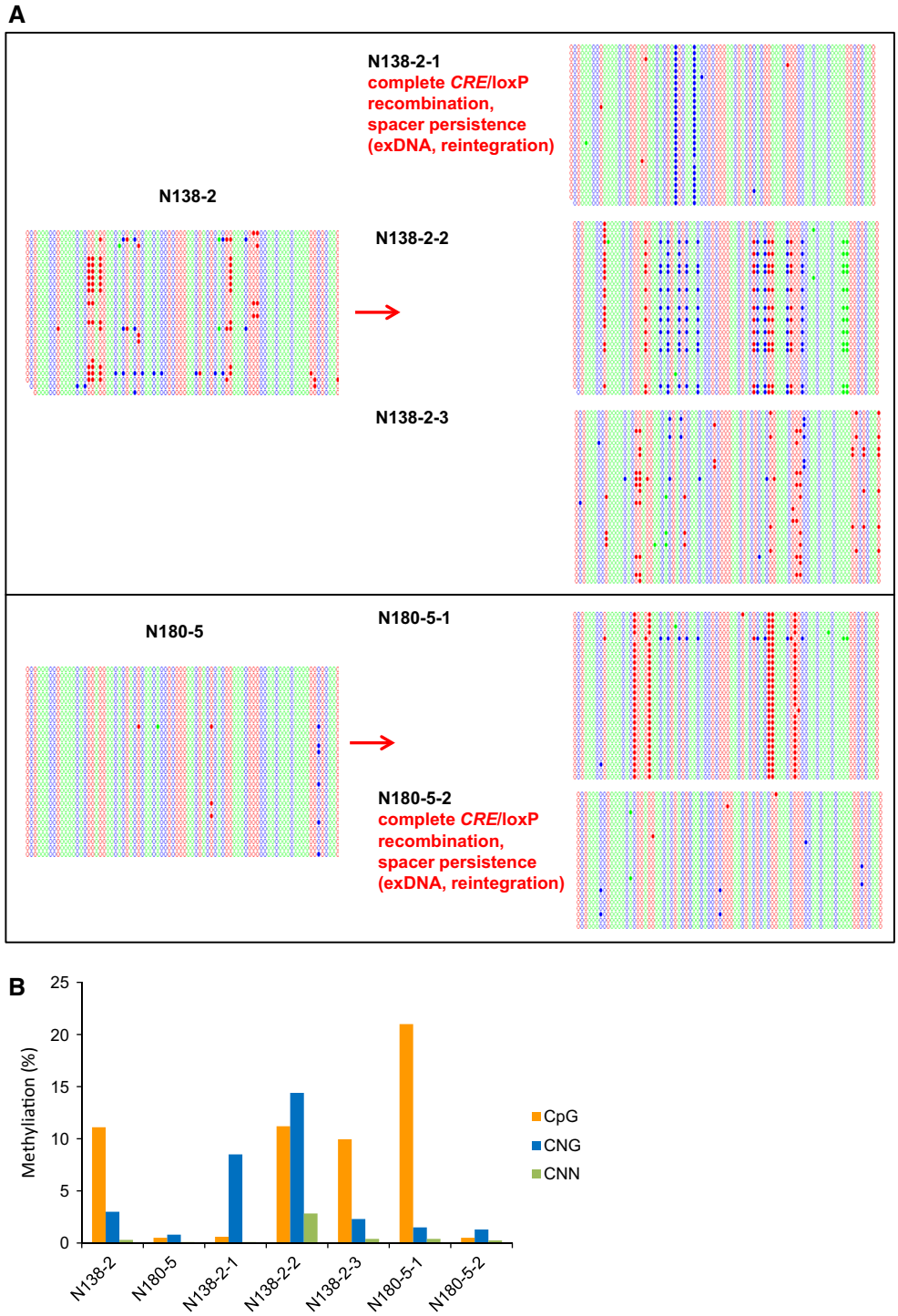

Fig. 4 Methylation level at *CRE* gene start in transgenic lines (N180-5, N138-2) and sub-lines (obtained after heat treatment: N138-2-1, N138-2-2, N138-2-2-3, N180-5-1, N180-5-2). CpG sites are shown in *red*, CNG in *blue* and CNN in *green*. **a** Methylation sites are indicated by *filled circles*, unmethylated sites by *empty circles*. This *figure* was generated with the program KISMETH (Gruntman et al. 2008). **b** The sum of methylation data revealed that transgenic sub-lines with complete *CRE*/loxP recombination have lower DNA methylation levels of *CRE* gene start at the CpG sites compared to those sub-lines without complete recombination (colour figure online)

Table 5 GUS histochemical staining in anthers from transgenic sub-lines

Transgene excision system	GUS+	GUS−	Anthers studied
CGPDHC::*FLP*	98 (94.2 %)	6 (5.8 %)	104
PTD::*FLP*	138 (28.8 %)	342 (71.2 %)	480

Anthers with no or a very weak GUS staining (GUS−) indicated a possible transgene excision

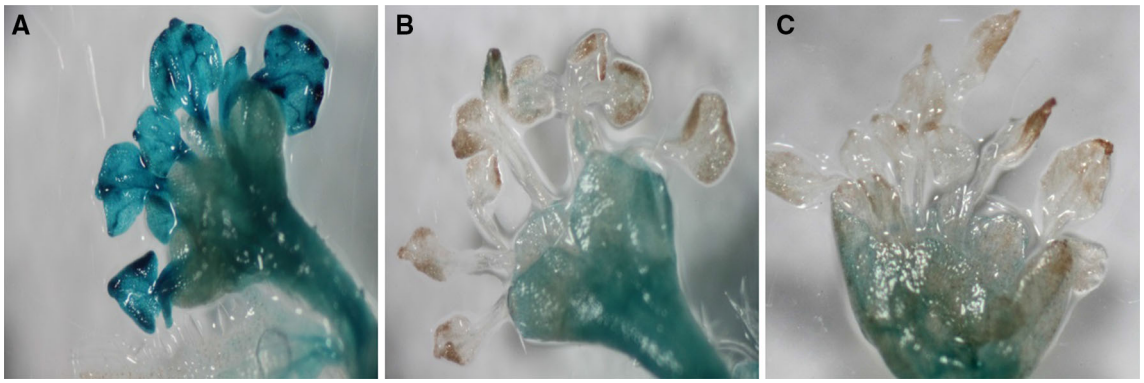

Fig. 5 GUS histochemical staining in anthers from transgenic sub-lines. Different GUS expression levels were obtained in the studied anthers: **a** strong expression, **b** weak expression, **c** no expression detected. In all three cases, anther carrying stalks are *blue stained* (colour figure online)

transgenic line N138-3, even though this transgenic line showed the lowest CpG site methylation level at the *CRE* gene start region (0.4 %) (Tables 2, 3). After heat treatment of transgenic line N138-2 (Tables 2, 3), a complete *CRE*/loxP recombination was detected in the sub-line N138-2-1, despite of the higher CpG site methylation level in N138-2 (11 %).

HSP::*CRE*/loxP spacer after recombination

Further, PCR and Southern blot analyses were carried out to verify elimination of HSP::*CRE*-loxP spacer in transgenic sub-lines showing complete *CRE*-loxP recombination, i.e., N138-2-1, N138-5-1, N148-2-1, N151-1-3, N151-2-1, N151-2-2, N151-2-3, N151-2-4, N133-1-2, N133-1-3, N140-1-1 (Table 4). Specific primers (Table 1) were designed to detect potential spacer persistence. Spacer elimination was confirmed in 7/11 (63.6 %, CGPDHC::*FLP*/frt) and 3/14 (21.4 %, PTD::*FLP*/frt) of transgenic sub-lines (Table 4) showing complete *CRE*-loxP recombination, i.e., 7/37 (18.9 %, CGPDHC::*FLP*/frt) and 3/43 (7.0 %, PTD::*FLP*/frt) of the total number of transgenic sub-lines obtained after heat treatment (Table 2). However, persistence of HSP::*CRE*-loxP spacer was confirmed in the rest of transgenic sub-lines (Tables 2, 4). Amplicons were obtained with PCR reactions using *CRE* gene specific primers (Table 4). Southern blot analyses confirmed the presence of HSP::*CRE*-loxP for most of them. However, HSP::*CRE*-loxP spacer persistence was only detectable with PCRs but not with Southern blot analyses in sub-lines N151-3-4 and N133-1-1 (Table 3). Furthermore, the presence of exDNA was detected in transgenic sub-lines N140-2-1, N140-5-1, N140-5-3, N146-1-2.1, N146-1-2, and N146-1-4 (Table 4).

Detection of transgene excision in poplar flowers

Following *CRE*/loxP recombination, the *FLP* gene comes under the control of a flower-specific promoter (either CGPDHC or PTD; Fig. 1). Selected transgenic sub-lines, showing a complete assembling of the transgene excision system, were subjected to a second heat treatment phase. This heat treatment aimed at the activation of the gene construct HSP::*FT* to induce early flowering. Flowers obtained from the selected transgenic sub-lines were subjected to GUS tests and PCR analyses to confirm *FLP* recombination and successful transgene excision (Figs. 5, 6; Tables 4, 5). GUS tests confirmed GUS staining in anthers indicating no successful *FLP*/frt recombination, and thus the continued presence of the T-DNA-fragment between the two frt-sites. However, anthers without GUS staining were also found (Fig. 5; Table 5). GUS tests showed that no GUS expression was detected in 6/104 (CGPDHC::*FLP*, 5.8 %) and 342/480 (PTD::*FLP*, 71.2 %) of anthers. PCR tests and DNA sequencing of the empty T-DNA cassette confirmed transgene excision in 4/63 (6.3 %) flowers studied in plants containing the gene excision system based on CGPDHC::*FLP* (Fig. 6; Table 6). Despite lacking GUS staining in some flowers from plants transformed with the PTD::*FLP*-based system

Table 6 Transgene excision rates in flowers from heat-treated transgenic sub-lines with both systems were tested

Transgene excision system	Confirmed transgene excision	Catkins studied
CGPDHC::*FLP*	4 (6.3 %)	63
PTD::*FLP*	0	62

Sequence of 457 bp amplicons obtained with PCR analyses was confirmed using DNA sequencing. Transgene excision was confirmed in 4/63 flowers obtained with the CGPDHC::*FLP* system

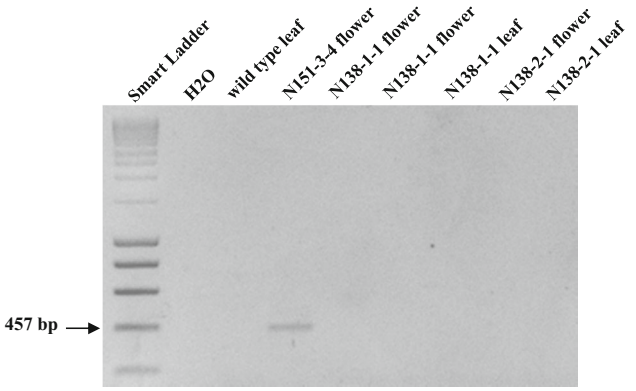

Fig. 6 Transgene excision in poplar flowers. PCR analysis of flowers and leaves was carried out with several heat-treated transgenic sub-lines. *Amplicons* obtained were isolated from agarose gel and sequenced. Transgene excision was confirmed in four flowers but not in leaves from transgenic sub-lines transformed with the CGPDHC::*FLP* gene excision system. In this *photo*, a 457 bp amplicon (*arrow*) and DNA sequencing of this fragment confirmed transgene excision in flowers of the transgenic sub-line N151-3-4

(Table 5), no transgene excision could be confirmed in flowers using PCR analysis (Table 6).

Discussion

The differentiation process strongly influenced in vivo assembling of the flower-specific transgene excision system

The transgene excision system tested in this study contained a heat-inducible *CRE*/loxP-based spacer sequence. The spacer regulated activity of the flower-specific *FLP*/frt transgene excision system. The promoter of the heat shock gene *HSP6871* activated assembling of *FLP*/frt recombination system in vivo. This approach may allow separating the breeding phase, requiring pollen carrying transgene, and the release phase, when transgenic plants are considered for either field testing or commercial application, releasing transgene-free pollen (Fig. 1).

Our results confirmed the activation of the transgene excision system after heat treatment. However, the differentiation level showed a strong influence on in vivo assembling of the transgene excision system. A complete *CRE*/loxP recombination, i.e., in all plant cells, was confirmed in up to 60 % of transgenic sub-lines when heat treatment was applied to regenerants obtained from undifferentiated in vitro cultures (Table 4) but not in differentiated plants. Similar results were reported with a heat-inducible *Ac*/Ds-transposon activation tagging system in poplar (Fladung and Polak 2012). On the other hand, transgenic sub-lines obtained after heat treatment of undifferentiated tissues still showed a high proportion of incomplete, or even no assembling of *FLP*/frt excision system.

Epigenetic factors may be playing an important role in the incomplete recombination obtained. Decreasing gene expression during tissue differentiation has been reported before. For instance, *GUS* expression in barley, under the control of the heat-inducible promoter Hvhsp17, showed decreasing gene expression in older tissues (Freeman et al. 2011). Also in transgenic birch (*B. pendula*), *GUS* activity decreased to 0.3–7 % of the original values after 10 months in vitro culture (Lemmetyinen et al. 1998). Quantitative determination of *GFP* (green fluorescence protein) expression in tobacco showed a mosaic expression pattern (Bastar et al. 2004). The reason for the mosaic expression could not be sufficiently related to well-known factors causing gene silencing, such as copy number, promoter activity or polyploidy. This kind of "epigenetic mosaic", occurring in many eukaryotes, has also been found in non-transgenic plants (Robbins et al. 2009). In general, the need for external induction, low efficiency, and chimerism has hampered widespread field applications of many advanced recombination approaches (Mlynárová et al. 2006). Ideally, auto excision should be driven by an endogenous trigger that is an intrinsic part of plant development (Mlynárová et al. 2006). However, despite the difficulties for the activation of the gene excising system in differentiated poplar cells, recombination was achieved in our study using undifferentiated in vitro cultures.

Influence of methylation on *CRE*/loxP recombination

Epigenetic factors are probably behind the variable efficiency of *CRE*/loxP recombination in poplar. Epigenetic regulation of gene expression is accomplished by multiple factors, like DNA methylation, histone modifications,

histone variants, chromatin remodeling, and may involve small RNAs (Ahmad et al. 2010). We studied the influence of methylation on *CRE*/loxP recombination using selected transgenic lines and sub-lines. The start region of *CRE* was less methylated than the middle in both transgenic lines and sub-lines (Table 3). A similar methylation pattern has been reported in genes from other plant species (Becker et al. 2011). Gene body methylation gradually increases towards the 3′-end, before sharply decreasing at the end of the last exon (Becker et al. 2011; Cokus et al. 2008; Lister et al. 2008; Zilberman et al. 2007; Zhang et al. 2006).

Comparisons with an expression microarray dataset in poplar showed that genes methylated at both promoters and gene bodies had lower expression than genes that were unmethylated or only promoter methylated (Vining et al. 2013). However, DNA methylation patterns of transgenic lines obtained in this study did not correlate with a complete or incomplete *CRE*/loxP recombination. Our results revealed that the HSP promoter was highly methylated but the *CRE* gene was low methylated in the transgenic lines studied (Table 3). Transgenic sub-lines with persistent HSP::*CRE*/loxP spacer showed that heat treatment promoted a high methylation in the middle of the *CRE* gene body but not in the start region (Table 3; Fig. 4). A twenty times lower methylation level was detected in transgenic sub-lines showing complete *CRE*/loxP recombination and persistence of *CRE*/loxP spacer compared to sub-lines with incomplete recombination (Fig. 4; Table 3), suggesting that low methylation might be needed but not sufficient for recombination. There is evidence that stress responses in plants affect epigenetic regulation (Chinnusamy and Zhu 2009). On the other hand, stress factors (UV, cold, and heat stress) can result in the reactivation of silent transgenes and endogenous transposable elements, albeit without the reduction of DNA methylation and repressive histone marks (Pecinka et al. 2010; Tittel-Elmer et al. 2010).

Transgene excision was confirmed but at low frequency

The *FLP*/frt transgene excision system, assembled in vivo after heat treatment, should be only active in poplar flowers. GUS staining indicated that transgene excision had taken place in around 5.8 % of anthers for the gene construct CGPDHC::*FLP*. This result was later confirmed with PCR analyses and sequencing of the obtained amplicons in 6.3 % of the catkins (Table 6; Fig. 6). Both flower-specific promoters were heterologous. The recombination construct containing the CGPDHC from *Cuphea lanceolata* was active and allowed transgene excision in poplar flowers. However, no recombination was confirmed with the PTD promoter from *Populus trichocarpa*. The lacking transgene excision with the PTD promoter was very surprising, as the PTD promoter shows a strong and flower-specific gene expression in *P. trichocarpa* (Sheppard et al. 2000).

Prolonged heat treatments may have played a role in the reduced performance of the transgene excision system. Stress-induced transgene inactivation has already been reported in plants (Walter et al. 1992; Broer 1996). In our study, the complete activation of the transgene excision system and the induction of early flowering required two rounds of heat treatments over several weeks. On the other hand, a promoting effect of heat stress on gene expression has also been reported before (Lang-Mladek et al. 2010; Pecinka et al. 2010; Tittel-Elmer et al. 2010; Ito et al. 2011). Factors related to T-DNA structure can also have an influence on performance of the transgene excision system (Yoo et al. 2005). The 35S promoter, used in our study for the regulation of the *hpt* marker gene, has been found to trans affect and alternate the expression pattern of transgenes (Yoo et al. 2005). It has been suggested that this interference results from the 35S enhancer located within the 35S promoter (Yoo et al. 2005).

Potential of recombination systems for genetic containment in transgenic trees

Most hitherto available gene containment approaches allow either a tissue- or a time-specific transgene excision. There is only one previous report on inducible tissue-specific gene containment in plants (Hinze and Becker 2012). The combination of *CRE*/loxP and *FLP*/frt recombination systems described in our study allowed inducible tissue-specific transgene elimination in flowers, maintaining the GM status of the plant body. However, the recombination efficiency was still low.

Removal of transgene sequences in plants using a recombination strategy should ensure that transgenes are not detectable after recombination. The time-specific component of our containment approach, based on a heat-inducible *CRE*/loxP spacer, allowed a complete activation of transgene excision in only 30 % of transgenic sub-lines (Table 2). Even lower but also higher transgene excision rates have been reported before with the *CRE*/loxP system in transgenic tobacco (Wang et al. 2005), rice (Khattri et al. 2011) and potato (Cuellar et al. 2006). However, all hitherto available reports show that the heat-inducible promoters do not promote a complete activation of the *CRE*/loxP system. We expected to achieve a complete *CRE*/loxP recombination with a prolonged heat treatment. Nonetheless, a longer heat exposure, over fourteen instead of 2 days, did not improve recombination efficiency compared to previous reports.

The elimination of HSP::*CRE*/loxP spacer after recombination was confirmed in 11/25 transgenic sub-lines

showing complete *CRE*/loxP recombination (Tables 1, 4). However, molecular analyses detected spacer persistence in 14/25 transgenic sub-lines (Table 4). The spacer was still present as exDNA in 6/25 sub-lines (Table 4). It is generally assumed that after recombination the elimination product is lost upon cell division. However, the presence of exDNA after *CRE*/loxP recombination has been reported before (Srivastava and Ow 2003). Furthermore, our results seem to indicate that reinsertions of HSP::*CRE*/loxP spacer ocurred in 8/25 of sub-lines (Table 4). In this case, transgenic sub-lines showed complete *CRE*/loxP recombination, no exDNA was detected but PCR analyses still detected presence of spacer. Reintegration events have been reported before for the FLP recombinase (Logie and Stewart 1995). Insertion products seem to be unstable in the presence of high levels of CRE recombinase (Siegal and Hartl 1996). However, absence of CRE protein after heat treatments may promote reinsertion of exDNA. A reintegration may occur through homologous recombination. Although only partially loxP homologous sequences are reported in the *P. trichocarpa* genome (own BLAST results; http://www.phytozome.net), this kind of unspecific event cannot be excluded. The *CRE*/loxP recombination efficiency is greatly reduced when only a few nucleotides in the *lox* spacer region are different (Hoess et al. 1982). Nevertheless, CRE is capable of binding nonspecifically to any DNA, especially at high concentrations (Hoess et al. 1982). The recombination between *lox* sites located at unlinked chromosomes is less efficient (Qin et al. 1994). However, the presence of exDNA may promote recombination events at unlinked chromosomes.

More research is required to improve the transgene excision efficiency by using gene constructs containing, e.g., homologous flower-specific promoters, stress-free inducible promoters for recombination induction or different site-specific recombination systems. LoxP-frt fusion sequences, combining wild type loxP and frt, can provide higher recombination rates than wild type systems, when used as flanking sites for *FLP* or *CRE* recombinase (Luo et al. 2007). Temporal or developmental regulation of *CRE*/loxP recombination decreases/eliminates side-effects of CRE protein that have been reported before (Coppoolse et al. 2003; Kopertekh and Schiemann 2012). PCR-based analysis of gene activity, as performed in this study after *CRE*/loxP recombination, allow a much more reliable strategy for the avoidance of genetic mosaics. GUS staining is prone to over- but also underestimating transgene expression. The influence of the differentiation process, epigenetic and environmental factors on the activity of gene containment constructs should be studied in more detail.

Experimental procedures

Plant material and genetic transformation

Leaf disc co-cultivation method was used for the *Agrobacterium*-mediated transformation (Fladung et al. 1996, 1997) of transgenic early flowering hybrid poplar (*P. tremula* L. × *P. tremuloides* Michx.) clone T89, containing the heat-inducible *Flowering locus T* (HSP::*FT*) gene (Hoenicka et al. 2012). Two gene constructs carrying a transgene excision system based on the recombination systems *CRE*/loxP and *FLP*/frt (for details see next two sections), differing in the flower-specific promoter (FS-P: CGPDHC or PTD) (Fig. 1), were used for genetic transformation of early flowering poplar. Selection of transgenic plants was carried out in McCown woody plant medium (WPM) regeneration media (Lloyd and McCown 1980) (Duchefa, Haarlem, The Netherlands) containing hygromycin (20 mg/l) and Cefotaxime (500 mg/l) (Duchefa).

Transgenic plants carrying the transgene excision system were obtained and propagated in vitro in a growth chamber at 25 °C and continuous light. Regenerated plants from the poplar clone T89 were used as wild type controls. Following in vitro culture, plants were transferred to growth chambers and cultivated at 25 °C/16 °C and 16/8 h day/night cycle (relative humidity of air: 70 %). The plants were transferred to a larger pot (soil culture) and watered daily. At age 3–6 months, plants with 20–40 cm in height were transferred to the greenhouse.

Undifferentiated tissue cultures were established for experiments on heat-inducible *CRE*/loxP recombination. In vitro plants and liquid WPM medium were put into a Waring Micro Blender (Eberbach Corporation, Ann Arbor, MI, USA) and blended for 5 s at low speed. The blender content was spread on a solid WPM medium. The obtained cultures were maintained in a growth chamber.

Plant material was collected from transgenic lines for molecular analyses.

Transformation vectors

We designed two excision approaches for specific T-DNA removal from poplar flowers (Fig. 1). Both approaches are based on recombination systems *CRE*/loxP from Bacteriophage P1 (Sternberg and Hamilton 1981) and *FLP*/frt from yeast (Broach and Hicks 1980). Both genes were fitted with the STLS-1 intron from potato (Vancanneyt et al. 1990) to avoid premature activation in *Agrobacterium*. Gene promoters *PTD* (Sheppard et al. 2000) from the black cottonwood (*P. trichocarpa*) or *CGPDHC* (cytosolic glycerol-3-phosphate

dehydrogenase) from *C. lanceolata* (Hausmann and Töpfer 1999), showing specific activity in poplar flowers (Hoenicka et al. 2006), were used for the activation of the *FLP*/frt system. Both gene constructs, which should remain silent during the breeding process, contained a *CRE*/loxP-based spacer sequence, under the control of the soybean heat-inducible promoter of gene *HSP6871* (Schöffl et al. 1984). Once concluded, the breeding process, a *CRE*/loxP recombination, could be activated by a heat shock treatment. This first recombination promotes in vivo assembling of the flower-specific *FLP*/frt recombination system (Fig. 1). Furthermore, T-DNA contained two selectable marker genes, the *HPT* (*hygromycin phosphotransferase*) (Van den Elzen et al. 1985) and *uidA* (GUS) (Ashwell 1962) under the control of the 35S promoter (Guilley et al. 1982). T-DNAs were synthesized and inserted into the binary vector pB-BA by DNA-Cloning Service (Hamburg, Germany).

Molecular analyses of transgenic lines

Genomic DNA isolation

DNA was extracted (Dumolin et al. 1995) from leaves from transgenic lines, sub-lines and wild type poplar. DNA was quantified using spectrophotometric OD260 measurements with a Nanodrop 1000 (Thermoscientific, Wilmington, USA). RNA quality was assessed by OD260/OD280 and OD260/OD230 ratios (both ratios were maintained between 1.8 and 2.1). PCR analyses were carried out with specific primers (Table 1) using annealing temperatures between 58 and 60 °C, following a procedure described before (Hoenicka et al. 2012).

Southern blot analyses

Twenty µg genomic DNA was digested with the restriction enzymes *SacI* (T-DNA copy number) or *SFi*I (T-DNA integrity) (Fermentas, Waltham, USA) for Southern blot analysis, according to the supplier's instructions. DNA electrophoresis and transfer of DNA to Biodyne A membranes (Pall Europe Limited, Portsmouth, UK) were performed as described elsewhere (Fladung et al. 1996, 1997). Prehybridisation and hybridisation of Southern blots were performed with the non-radioactive DIG (digoxigenin) system using a DIG-dUTP PCR partial-labeled *Hyg*-probe as described before (Fladung and Ahuja 1995; Fladung et al. 1997). The gels were stained with Roti-Safe (Roth, Karlsruhe, Germany) shortly before blotting to confirm similar DNA amounts loaded and uniform restriction patterns. Mainly transgenic lines with one T-DNA copy, but also some with two to three copies, were selected for this study.

qPCR

Transgene copy number was verified using qPCRs (Beltrán et al. 2009). Specific primers for the *FLP* gene, contained in the T-DNA, and the reference genes *PtActII* and *UBQII* were used (supplemental data). Primer design was carried out with QuantPrime (Arvidsson et al. 2008, http://www.quantprime.de/) or Primer3plus (Rozen and Skaletsky 2000, http://primer3plus.com/cgi-bin/dev/primer3plus.cgi) with melting temperatures around 60 °C (Table 2). qPCR reactions were done in a 20 µl volume containing 225 nM of each primer, 5 µl of gDNA (100 ng) and Fast Plus Evagreen Master Mix (Biotium, Hayward, USA). qPCR were performed on the Stratagene Mx3000P (Stratagene, La Jolla, USA) in 96-well reaction plates using the following parameters: 10 min at 95 °C and 40 cycles of 95 °C for 30 s, 60 °C for 1 min and 72 °C for 1 min.

In vivo assembling of *FLP*/frt transgene excision system through a *CRE*/loxP recombination

The *FLP*/frt transgene excision system, for specific transgene elimination in poplar flowers, was assembled in transgenic plants after heat treatment (Fig. 1). This treatment aimed at the induction of *CRE*/loxP recombination in all plant cells. Two-month-old plants and undifferentiated callus from transgenic lines, both growing under in vitro culture conditions, were used for heat treatments. Heat treatments (40 °C for 2 h/1–14 days) were carried out in a growth chamber (Weiss, Leicestershire, UK).

After heat treatments, plant material was collected from transgenic sub-lines, i.e., regenerants obtained from undifferentiated callus and two-month-old plants. PCRs were performed in shoots from 84 in vitro cultures for detection of transgenic sub-lines showing a complete activation of transgene excision system. Specific PCR primers (p3, Table 1) were designed near to the HSP::*CRE* sequence in both gene constructs for this aim (Fig. 1). The successful assembly of the CGPDHC::*FLP* or PTD::*FLP* recombination system was confirmed with PCR after amplification of a small amplicon (~560 bp), instead of a large amplicon (~2500 bp), detectable before activation of the transgene excision recombination system. PCR reactions were carried out with the Expand Long Range dNTPack (Roche, Germany). Transgenic sub-lines showing a complete assembling of the transgene excision system were selected for further studies.

Methylation analysis of the heat-inducible *CRE*/loxP recombination system

Methylation of the HSP promoter, the *CRE* gene, and the gene body was analyzed by bisulfite conversion

(Henderson et al. 2010) in nine selected samples (transgenic lines and sub-lines) using several amplicons (Fig. 3) located in the HSP promoter and *CRE* gene (start and middle region). Primers used after bisulfite conversions are shown in Table 1. The PCR products were subcloned and 20–50 individual colonies were selected for each amplicon and sample, from which the DNA was prepared by alkaline lysis and sequenced using the Sanger protocol as described by Zhang et al. (2009). Data were analyzed using KISMETH (Gruntman et al. 2008) with respect to methylation at CpG, CNG and CNN sites. Plasmid DNA purified from *E. coli* with the same sequence was used as control for conversion.

Detection of *CRE*/loxP spacer persistence in transgenic sub-lines

PCR and Southern Blot analyses were carried out with transgenic sub-lines showing complete *CRE*/loxP recombination to evaluate potential spacer persistence. *CRE* gene primers p2 (Table 1) were used for PCR reactions and DIG-probe labeling. The presence of exDNA was confirmed with primers p5 (Table 1).

Induction of early flowering in poplar

Selected transgenic sub-lines, showing a complete assembling of flower-specific transgene excision system, were subjected to a second heat treatment phase to induce early flowering (40 °C for 2 h/4 weeks; for details see Hoenicka et al. 2012). Heat treatment was carried out with plants of a size >40 cm under growth chamber conditions.

Detection of transgene excision in poplar flowers

GUS assay

Both gene constructs used in this study contain the *uidA* (GUS) gene under control of the 35S promoter. This gene construct should be active in transgenic plants only but not in flowers after T-DNA deletion. Thus, transgene excision can be detected using the GUS histochemical staining. GUS staining was performed following the protocol described by Jefferson (1987) with some modifications. Leaves and flowers were treated with 5-bromo-4-chloro-3-indolyl glucuronide (Roth, Germany) stain solution (0.1 M sodium phosphate buffer pH 7.0, 0.5 mM ferricyanide, 0.5 mM ferrocyanide, 10 mM Na_2EDTA, 0.05 mM X-Gluc) at 37 °C overnight. The stained leaves were fixed with FAA (60 % EtOH, 10 % formalin, 5 % acetic acid) and chlorophyll was removed by passing tissue through EtOH baths (75 % overnight). Microscopic evaluations were carried out using a Zeiss stereo microscope Stemi 2000-C (Jena, Germany).

PCR

Flowers from selected transgenic sub-lines were studied with PCRs using specific primers (p4, Table 1; Fig. 1). The successful activation of the CGPDHC::*FLP* or PTD::*FLP* system should leave a 457 bp T-DNA remaining (s1 and s2 sequences, Fig. 1) located close to the right (RB) and left (LB) borders (Fig. 1). This sequence was consciously left to facilitate detection of successful transgene deletion in flowers. The obtained amplicons were isolated from the agarose gel using the Agarose Gel DNA extraction Kit (Roche, Germany) and sequenced to confirm the empty T-DNA cassette.

Author contribution statement HH: experimental design, data analyses, photography, qPCR, and manuscript preparation, DL: genetic transformation, molecular analysis and tissue cultures, SN: methylation studies (bisulfite conversion, PCRs and cloning), RR: methylation studies (sequencing), AJ: methylation studies (experimental design, data analyses), VB: Southern-Blot-Analyses and qPCR, MF: experimental design and manuscript preparation. All authors have read and approved the manuscript.

Acknowledgments This work was supported by the German Ministry of Education and Research (Biosafety Research; project number 0315210C). We thank S. Strauss (Oregon University, USA) for kindly providing the PTD gene promoter, D. Ebbinghaus and A. Schellhorn, for helpful technical assistance in the lab, and the greenhouse staff (M. Hunger, G. Wiemann, R. Ebbinghaus, M. Spauszus) for plant cultivation.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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