

*Full Length Research Paper*

# The stability of transgene expression and effect of DNA methylation on post transcriptional gene silencing (PTGS) in birch

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In this paper, we selected transgenic birch (*Betula platyphylla* Suk) plants, which included non-silencing plants, transcriptional silence plants including TP96, TP74, TP73 and the post-transcriptional silence ones (TP67 and TP72). The transcription of the *bgt* gene in different tissues and organs were significantly different. The transcriptional level of *bgt* gene in the different tissues and organs was in the following order: leaf > female flower and male flower > branch bark > phloem > root. The transgenic lines were monitored for foreign gene expression for a long-term period of 8 years during their continuous growth under field conditions. GUS protein expression was not reactivated in the transgene silencing lines TP72 and TP67 when cultured in field conditions for long-term period. Meanwhile, no cases of gene silencing were observed again during the study period in the field conditions. Our results suggest that transgene expression in transgenic birch plants appears to be stable under field conditions. The frequencies of methylated cytosines in the code regions of *gus* gene was studied. Relation of transgene expression and DNA methylation was analysed. The data of restriction enzyme digestion (*Hpa*I and *Msp*I) indicated that DNA methylation resulted in post transcriptional gene silencing (PTGS) in transgenic birch.

**Key words:** Transgenic birch, DNA methylation, gene silencing.

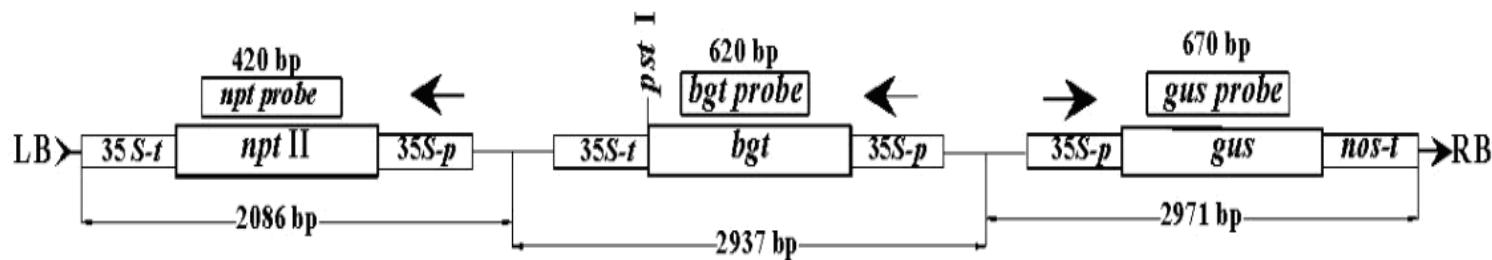
## INTRODUCTION

Genetic transformation of woody plants is a promising tool for their genetic improvement, since their breeding has limitations imposed in general by their high heterozygosity, long juvenile periods and auto incompatibility (Tang et al., 2007). In recent years, inserting foreign DNA into plants expressing insect resistance is a new way to accelerate woody plant breeding. The development of transgenic insect-resistant plants provides a quick and safe approach to pest management. To improve insect resistance of birch and avoid pollution due to insecticides, the fused *bgt* gene consisting of the insecticidal toxin gene from the spider (*Atrax robustus*) and the C terminal of Cry IA (b) gene from *Bacillus thuringiensis* was

transferred into birch by *Agrobacterium*-mediated transformation system (Zhan et al., 2003). Stable expression of foreign gene is important for commercial use of genetic transformation in long-lived tree species as well as for ecological risk-assessment studies. However, analysis of the instable/stable transgene expression in tree is more problematic than in crop plants (Kumer, 2000a). Several studies have been conducted in perennial plants, including poplar trees, over multiple seasons and after vegetative propagation (Gallo-Meagher and Irvine, 1996; Bettany et al., 1998; Cervera et al., 2000; Kumar and Fladung, 2001; Meilan et al., 2002; Hawkins et al., 2003; Leibbrandt and Snyman, 2003) but these studies have given conflicting result (Li, et al., 2008).

This variation highlights the importance of evaluating transgene expression in transgenic plants cultured long term in field conditions. However, some studies did

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**Figure 1.** Schematic representation of the T-DNA, restriction sites and probes. *PstI* restriction endonuclease was used to analyze the copy number of *bgt* and *gus*. 35S-*p*, promoter from the cauliflower mosaic virus; 35S-*t*, terminator from the cauliflower mosaic virus; *nos-t*, terminator from nopaline synthase; *nptII*, bacterial neomycin phosphotransferase II gene; *bgt*, the chimeric gene of a spider insecticidal peptide gene and the C peptide sequence of *Bt* gene; *gus*, β-glucuronidase gene; LB, T-DNA left border; RB, T-DNA right border.

**Table 1.** Amplification primers for multiplex PCR.

Amplified gene	Polarity	Name	primer sequence	Length	Tm	GC%	Product size (bp)
<i>bgt</i>	+	Bgt-F	5'AACGGTAGATTCGCTGGAT3'	19	54.5	47.4	247
	-	Bgt-R	5'CAGAAGTTCCAGAGCCAAG3'	19	52.7	52.6	
<i>nptII</i>	+	NptII-F	5'TCCGGCCGCTGGGTGGAGA3'	20	59.8	58.3	449
	-	NptII-R	5'TGGCGCGAGCCCCTGATGCT3'	20	58.7	58.8	
<i>gus</i>	+	GUS-F	5'GCAACTGGACAAGGCACCA3'	19	53.9	52.6	668
	-	GUS-R	5'AGCGTCGCAGAACATTACA 3'	19	54.7	47.4	

demonstrate that long term culture of transgenic plants in field affected the expression of the transgene (Kumar and Fladung, 2001; Hawkins et al., 2003). This is important in the application of transgenic tree species. Stability of transgene expression levels in transgenic birch lines is therefore an important consideration for transgenic birch cultivar development programs. The purposes of this study were to: (i) reveal the transcriptional level of *bgt* gene in different tissues; (ii) analyze the stability of expression level of transgenes in transgenic birch and determine the effect of long term culture (8 years) on transgene expression; and (iii) explore the relationship between gene silencing and DNA methylation.

## MATERIALS AND METHODS

### Characterisation of the transformed plants

Transgenic plants of *Betula platyphylla* Suk. were obtained via *Agrobacterium* (LBA4404 strain)-mediated transformation. The transgene construct was pCAMBIA-2301, which contained the selectable marker *nptII* gene and reporter gene *gus*, along with a fused *bgt* gene consisting of an insecticidal toxin gene from a spider (*A. robustus*) and the C terminal of the Cry IA(b) gene from *B. thuringiensis* (Figure 1). Transgenic plants were established by *in vitro* propagation and then, together with non-transformed control plants, were cultivated in a greenhouse under natural daylight conditions.

### DNA isolation and multiplex PCR analyses

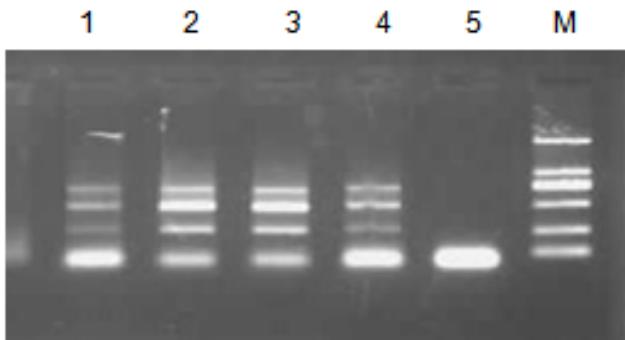
Genomic DNA was isolated from fresh leaf material using the CTAB method. Multiplex PCR was performed in a reaction volume of 30 µl containing 50 ng DNA, 0.5 µM from each primer (Table 1), 200 µM dNTPs and 1 U Taq DNA polymerase (TAKARA). PCR reactions was performed with the following amplification program: 94°C for 3 min; 35 cycles of 30 s at 94°C, 40 s at 58°C, and 1 min at 72°C, followed by 72°C for 10 min. The amplification products were subjected to electrophoresis using a 0.8% (w/v) agarose gel.

### RNA isolation and northern blot analysis

Total RNA was isolated from the fresh leaf material using the CTAB method. For Northern blots, total RNA (10 µg) was denatured with 1 M glyoxal and 50% (v/v) DMSO in 10 mM sodium phosphate (pH 7.0) for 1 h at 50°C, separated in 1% (w/v) agarose-formaldehyde denaturing gels, blotted onto a Hybond-N<sup>+</sup> nylon membrane (Roche) in 20×SSC transfer buffer, and fixed by UV cross-linking. The DIG labelling and detection system (Roche) was used following the manufacturer's instructions. After the hybridisation of the *bgt* probe, membrane was stripped twice for 1 h at 80°C in formamide containing 1% (v/v) SDS to remove the DIG-labelled *bgt* probe, was rinsed for 5 min in 2×SSC, and stored in 2×SSC buffer. Stripped membrane was then prehybridised and hybridised with the *gus* probe using the same procedures as for the *bgt* probe.

### GUS assay

Expression of the *gus* gene was examined histochemically in the



**Figure 2.** Multiplex PCR with total genomic DNA from regenerated transgenic birch plants. Lanes 1 to 4, transgenic plants for 8 years culture; lane 5, negative control; M, DL2000 marker.

leaves or callus from the regenerated plants at different subculture times as previously described (Jefferson et al., 1987). The negative controls were leaves from untransformed birch.

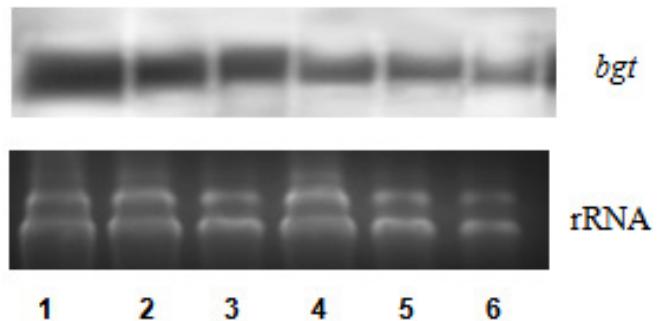
#### DNA isolation and methylation analysis

Genomic DNA was isolated from fresh leaf material with CTAB method. For restriction digests utilizing *Hpa*I and *Msp*I restriction enzymes, 10 µg DNA samples were digested under the conditions recommended by the manufacturer (New England Biolabs Inc) (Figure 1) and were separated in a 0.8% agarose gel. The gel was denatured, neutralized and blotted onto Hybond-N<sup>+</sup> nylon membrane (Roche) in 20×SSC transfer buffer, fixed by UV cross-linking. Southern hybridization was conducted with the DIG labeling and detection system (Roche) following the manufacturer's instructions. Probes were labeled using PCR amplification with DIG-11-dUTP (Roche). A 620 bp *bgt* probe fragment was amplified from plasmid DNA of the transgene construct pCAMBIA-2301 and was labeled using the primers: 5'ACCGGTAGATTGCTGGAT3' and 5'CAGAACCTCCAGAGCCAAG3'. A 670 bp *gus* probe fragment was amplified from the genomic DNA extracted from one transgenic event. The forward and reverse primers used for amplification and labeling were: 5'GCAACTGGACAAGGCCTA3' and 5'AGCGT CGCAGAACATTACA 3'. After the hybridization of the *bgt* probe, membrane was stripped twice for 15 min at 37°C in 0.2 M NaOH containing 0.1% SDS to remove the DIG-labeled *bgt* probe, was rinsed for 5 min in 2×SSC, and stored in 2×SSC buffer. Stripped membrane was then prehybridized and hybridized with the *gus* probe using the same procedure for the *bgt* probe hybridization and detection.

## RESULTS

#### Structural integrity of the transgenes

The multiplex PCR results for the transgenic plants for long-term cultured, using primers specific to *bgt*, *gus* and *npt* II genes, are shown in Figure 2. Gene specific bands appeared in the multiplex PCR products from all the long-term cultured plants. The non-transgenic control lacked the specific band. The transgenes were therefore stably maintained over the long term culture under field condition.



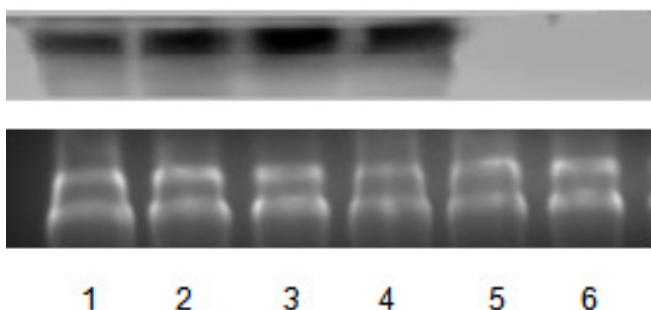
**Figure 3.** Northern blot analysis of transgenic birch plant RNA from different organs. Lane 1, Leaf; lane 2, female flower; lane 3, male flower; lane 4, branch bark; lane 5, phloem; lane 6, root.

#### Transcriptional expression of *bgt* gene in different part

To determine the transcriptional level of the *bgt* gene in different tissues and organs, Northern blot analysis was performed on leaf, female flower, male flower, branch bark, phloem and root isolated from the different transgenic birch. As shown in Figure 3, the transcription of the *bgt* gene in the different tissues and organs were significantly different. The transcriptional level was higher in leaf than other tissues or organs. The expression level was the lowest in the root. The transcriptional level of *bgt* gene in the different tissues and organs was in the following order: leaf > female flower and male flower > branch bark > phloem > root.

#### Transcriptional expression of *bgt* gene in different years

The extent of the transcriptional expression of *bgt* and *gus* genes in transgenic birch plants among the different years was analysed by Northern blot. As reported in Zeng et al. (2009), transcriptional level of *bgt* in TP28, TP46 and TP58 was higher than those of the other lines. Transcriptional gene silencing (TGS) was obtained in transgenic line (TP96) with four copies of *bgt*, and in transgenic line (TP30) with one copy of *bgt*. But TGS has not been found in other transgenic lines with one or four copies of *bgt*. The results demonstrated that there was no absolute correlation between the numbers of transgene copies and the gene expression. As shown in Figure 4, the transcription of the *bgt* genes in TP27 and TP46 plants in 2005 was similar in level to that of the plants in 2009. The results of the comparative analysis of the expression of the transgene in the various plants for the different years showed that transcriptional levels of the transgene in the different years were consistent. The transcriptional expression level of the extraneous genes in the plants did not decrease with increasing culture time. Transcriptional gene silencing (TGS) in transgenic



**Figure 4.** Northern blot analysis of transgenic birch plant RNA with *gus* probe. Lanes 1 and 2, transcriptional level of *bgt* gene of TP27 in 2005 and 2009; lanes 3 and 4, transcriptional level of *bgt* gene of TP46 in 2005 and 2009; lanes 5 and 6, transcriptional level of *bgt* gene of TP96 in 2005 and 2009.

lines (TP96 and TP30) could not be reactivated. TGS was not found in the transgenic lines, which expressed normally, during long time culture in the field.

#### Analysis of GUS activity of transgenic plants during long-term cultured

GUS analysis of 30 randomly selected transformed plants showed that foreign genes were stable through a cycle of dedifferentiation and differentiation and were maintained in long-term. As shown in Figure 5, GUS activity in the plants of the 2008 was similar to that of the original plants. No silenced lines were found in the original non-silenced plants. The silenced lines also could not be reactivated, indicating that expression level of *gus* had stabilised within the eight years culture.

#### Relationship of DNA methylation and PTGS

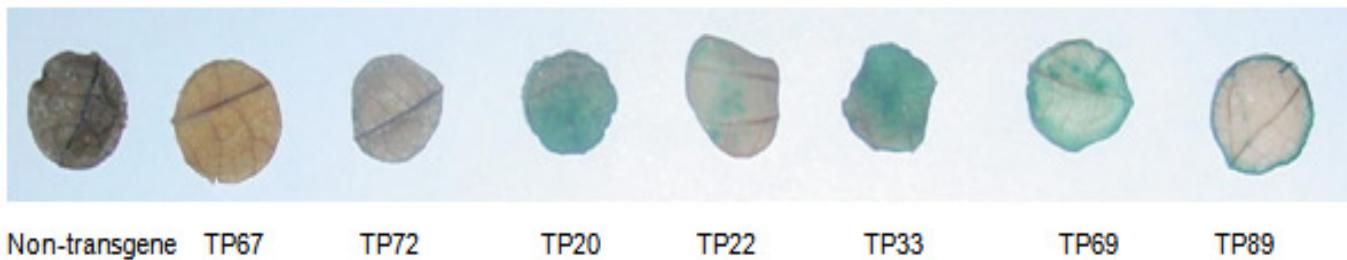
As reported by Zeng et al. (2009), there was no absolute correlation between the numbers of transgene copies and the gene expression. To determine methylation status, Southern blot analysis was performed on leaf DNA isolated from seven transgenic birch. Initially, genome DNA from each plant was cut with either *Hpa*II or *Msp*I restriction enzymes, subjected to gel electrophoresis, transferred to a nylon membrane and hybridised with a DIG labelled *bgt* and *gus* probe (Figure 1). *Hpa*II does not cleave after the addition of a methyl group to the inner C (but not the outer C) of CCGG sequences, whereas *Msp*I is only sensitive to methylation of the outer C. Figure 6 depicts the *Hpa*II/*Msp*I restriction sites in the transgene construct and predicted *Hpa*II/*Msp*I fragment sizes of the unmethylated genomic DNA. TP28, TP46, TP72 and TP73 was found to have similar size fragments for both *Msp*I and *Hpa*II digests, which was consistent with a lack of methylation of *gus* gene for the plant. However, incomplete cleavage with restriction endonuclease *Hpa*II

(different molecular weight fragments observed) showed that these transgenic lines have methylated DNA at CCGG sites contained in the *gus* gene. This restriction pattern revealed methylation of CpG dinucleotides in the transgene and these plants had post transcriptional gene silencing (PTGS) phenotype. Ethidium bromide fluorescence photographs of this gel showed no evidence of incomplete cleavage and the *Msp*I restriction profile was confirmed with repeat digests. So, the results demonstrated that a close correlation existed between the methylation of transgene CDS and PTGS in TP67 and TP72 lines.

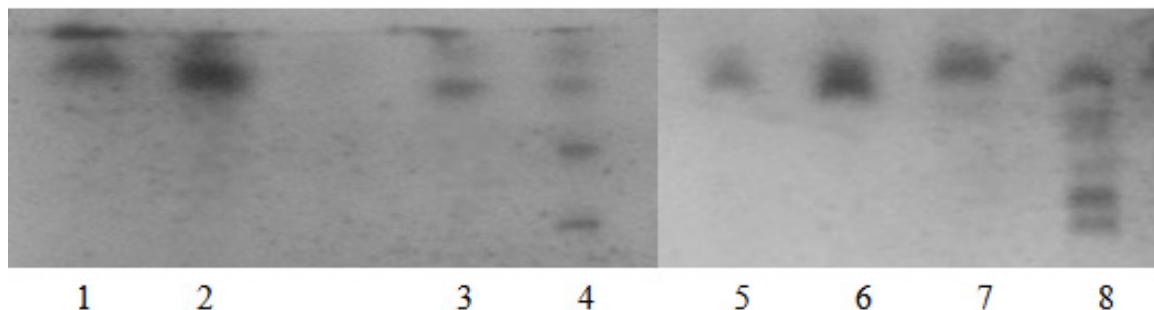
#### DISCUSSION

A major biological concern about transgenic trees is whether transgene expression will be stable over a full harvest rotation, which can span decades. Several studies have been conducted in perennial plants, including poplar trees, over multiple seasons and after vegetative propagation (Kumar and Fladung, 2001; Meilan et al., 2002; Hawkins et al., 2003; Li et al., 2008). These studies have suggested that the expression of transgenes after vegetative propagation can be highly stable over many years in the field. For instance, Meilan et al. (2002) reported high stability of herbicide resistance in 40 poplar transgenic lines over 4 years in the field. Similarly, Li et al. (2008) reported long-term stability of herbicide resistance in poplar over 8 years in the field. Long-term expression stability of the GUS gene was also demonstrated in another study in poplar (Hawkins et al., 2003). However, phenotypic instability of a *rolC* gene was observed in some transgenic aspen lines grown *in vitro*, in greenhouse, or in field conditions (Kumar and Fladung, 2001). Instability of *rolC* expression was most obvious after transfer from *in vitro* to greenhouse conditions. In this report, we utilized the *gus* and *bgt* gene to study the variable transgene expression in transgenic birch plants. The transgenic lines were monitored for foreign gene expression for a period of 7 to 8 years during their continuous growth under field conditions. Our results suggested that transgene expression in transgenic birch plants appeared to be stable under field conditions. Fladung et al. (1999) suggested that gene silencing is relatively rare in woody trees, and indeed our results indicated that transgene expression was stable over a long period under field conditions. Transgene expression remains stable in time and space throughout the lifetime of the plant.

In our study, GUS and BGT protein expression was not reactivated in transgene silencing lines TP72 and TP67 when cultured in field conditions for long-term period. Meanwhile, no cases of gene silencing were observed again during the study period in the field conditions. These results suggested that gene silencing may occur immediately following the integration of a transgene. Since transgene silencing was reported (Finnegan and



**Figure 5.** GUS activity in transgenic birch of eight years.



**Figure 6.** Southern blot analysis of the insect-resistant transgenic birch plant DNA digested with *HpaII* and *MspI* with *gus* gene probe. Lanes 1 and 2, TP27 DNA digested with *HpaII* and *MspI* separately; lanes 3 and 4, TP67 DNA digested with *HpaII* and *MspI* separately; lanes 5 and 6, TP96 DNA digested with *HpaII* and *MspI* separately; lanes 7 and 8, TP72 DNA digested with *HpaII* and *MspI* separately.

McElroy, 1994), the concern for the stability of transgene expression has been increasing. Inactivation of transgene expression has often been observed in annual herbaceous species; relatively little information is available about its occurrence in long-lived perennial species such as trees. Our previous study showed that multiple copies of T-DNA could not trigger transgene silencing and was not correlated with the expression level of *bgt* protein. Some studies indicated that gene silencing was associated with methylation of promoter or coding sequences of the transgene (Meyer et al., 1992; Mette et al., 2000; Lechtenberg et al., 2003). To determine the possible cause of DNA methylation, Southern blot analysis using methylation-sensitive restriction enzymes revealed evidence of DNA methylation in the GUS CDS regions of these silencing transformants. As a result, we described a transgene-silencing phenomenon in birch accompanied by DNA methylation. DNA methylation has been implicated in both gene regulation and transgene silencing in plants (Wassenegger, 2000). In plant genomes, the sequence CpG is also the predominant methylation context, but the symmetric context CNG and asymmetric context CNN can also be methylated (Mathieu and Bender, 2004). RNA-directed DNA methylation (RdDM) has been implicated in TGS that is initiated by dsRNAs containing promoter sequences (Aufsatz et al., 2002; Mette et al., 2000; Kawasaki and Taira, 2004; Cigan et al., 2005). RdDM was observed in protein regions in

many cases of PTGS (Smith et al., 2000; Ebbs et al., 2005). However, the degree to which DNA methylation is relevant to PTGS remains uncertain. In this study, DNA methylation in the coding region was observed in the PTGS silenced events, while other unsilenced events and the TGS silenced events, they were not observed.

## Conclusion

The transcription of the *bgt* gene in different tissues and organs were significantly difference. The transcriptional level of *bgt* gene in different tissues and organs was in the following order: leaf > female flower and male flower > branch bark > phloem > root. Moreover, during the growing season, the trend of the expression level of the extraneous genes was descending. The stability of the transgenic expression was studied in 30 different transgenic plants grown under field conditions over a period of 8 years. Our results suggest that transgene expression in transgenic birch plants appears to be stable under field conditions. The frequencies of the methylated cytosines in the code regions of the *gus* gene was studied. The relation of transgene expression and DNA methylation was analysed. The data of restriction enzyme digestion (*HpaII* and *MspI*) indicated that DNA methylation resulted in post transcriptional gene silencing (PTGS) in transgenic birch.

## ACKNOWLEDGEMENTS

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