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Full Length Research Paper

Expression of *bgt* gene in transgenic birch (*Betula platyphylla* Suk.)

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Study on the characteristics of integration and expression is the basis of genetic stability of foreign genes in transgenic trees. To obtain insight into the relationship of transgene copy number and expression level, we screened 22 transgenic birch lines. Southern blot analysis of the transgenic birch plants indicated that the copy number of *bgt* varies from one to four, of which 18.2% were single copy. Copy number of *bgt* and *gus* was different in 68% of transgenic birch plants, indicating that rearrangement or partial deletion appeared in the process of T-DNA integration. Transcriptional expression of *bgt* gene in transgenic birch plants was analyzed by Northern blot. TGS has been found in two transgenic lines with one and four copies of *bgt*. The ELISA result showed that the BGT protein expression level in the transgenic birch plants ranged from 0.000 to 0.283% of total soluble protein. In contrary to most studies, this research showed no significant correlation was found between copy number and expression level of *bgt* gene. Effective resistance of transgenic plants against *Lymantria dispar* was verified in feeding bioassays with the insects. Bioassays results were mostly consistent with the expression level of BGT insecticidal protein detected by ELISA and Western blot in transgenic birch. The paper showed transgenic birch had the high lethal effect on gypsy moth larvae (*L. dispar*). The practicality of this work will benefit not only the birch producers, but also the environment worldwide.

Key words: Transgenic birch, transgene, copy number, ELISA, insecticidal activity.

INTRODUCTION

Birch (*Betula platyphylla* Suk.) is the most widely distributed species of Birch (87%) found in Northeastern China, and the most prevalent of the birch hardwoods. Several species of forest insect pests heavily attacked birch. Improving resistance of trees to insect pests by traditional breeding and selection is dif-ficult and takes a long time. Therefore, the transgenic approach would be a valuable strategy for the control of these insect pests because the insecticidal protein is eaten by the pests (Sardana et al., 1996; Cho et al., 2001). In order 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 *Agrobacteriumm*-mediated transformation system (Zhan et al., 2003).

The stable integration of foreign genes in the genome of forest trees and their subsequent stable expression are essential for the further use of transgenic trees in forest tree breeding programs. However, gene silencing is frequently observed in transgenic plants, which impedes the application of transgenic tree (Vaucheret et al., 2001; Kumar and Fladung, 2000; Stam et al., 1997; Holtorf et al., 1995). Several factors that might serve as a trigger for silencing mechanisms include transgene copy number, T-DNA structure and integration sites. The presence of multiple copies of transcripts that are coded by transgenes might cause homology-dependent transgene silencing. Studies on the correlations between transgene

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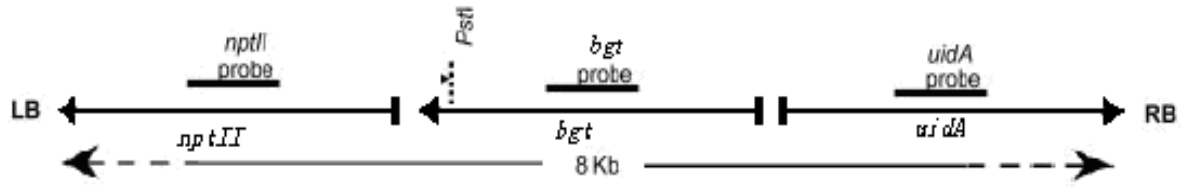

Figure 1. Graphic representation of the T-DNA structure.

silencing and copy number have shown conflicting results. Some studies showed that copy number and transgene expression levels can be positively correlated (Hobbs et al., 1993; Tang et al., 2003; Van der Hoeven and Dietz, 1994; Voelker et al., 1992), negatively correlated (Cervera et al., 2000; Mannerlöf et al., 1997), or uncorrelated (Bauer et al., 2003; McCabe et al., 1999).

Transgene expression level in transgenic birch lines was therefore an important consideration for transgenic cultivar development programs. The purposes of the study were to: (i) determine the copy number in transgenic birch *Agrobacterium*-mediated genetic transformation; (ii) analyse the expression level of *bgt* gene in transgenic birch; (iii) explore the relationship between gene silencing and the copy numbers; and (iv) analyse the lethal effect of transgenic birch plants on gypsy moth larvae and pick out higher resistant clones.

MATERIALS AND METHODS

Transgenic birch

Transgenic plants of *B. platyphylla* had been obtained through mediation with *Agrobacterium* (LBA4404), in which the chimeric gene of spider insecticidal peptide gene and the C peptide sequence of *Bt* gene were used as purpose foreign gene (*bgt)* for the transformation experiment (Zhan et al., 2003).

Nucleic acid isolation and Southern/northern blot analysis

Genomic DNA and RNA (obtained as described) (Zhan et al., 2003) were isolated from fresh leaf material. For Southern blots, 10 µg aliquots of genomic DNA were digested with *Pst*I restriction endonucleases (Figure 1) and separated in 0.8% agarose gels. Gels were denatured, neutralised and blotted onto Hybond-N^+ nylon membrane (Roche) in 20 × SSC transfer buffer, fixed by UV cross-linking. Southern hybridizations were 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 PCAMBLA-2301 and labeled using the primers: 5'aacggtagattcgctggat3' and 5'cagaagttccagagccaag3'. A 670 bp *gus* probe fragment was amplified from genomic DNA extracted from one transgenic event. Forward and reverse primers used for amplification and labeling were: 5'gcaactggacaaggcacta3' and 5'agcgtcgcagaacattaca 3'. After the hybridization of the *bgt* probe, membranes were stripped twice for 15 min at 37 ℃ in 0.2 M NaOH containing 0.1% SDS to remove the DIG-labeled *bgt* probe, rinsed for 5 min in 2×SSC, and stored in 2 x SSC buffer. Stripped membrane was then prehybridized and hybridized with the *gus* probe using the same procedure for the *bgt* probe hybridization and detection.

For northern blots, total RNA (20 µg) from each sample denatured with 1 M glyoxal and 50% DMSO in 10 mM sodium phosphate pH 7.0 for 1 h at 50°C, were separated in 1.0% agarose-formaldehyde denaturing gels and blotted onto Hybond-N^+ nylon membrane (Roche) in 20 × SSC transfer buffer, fixed by UV cross-linking. Southern/northern hybridizations were conducted with the DIG Labeling and Detection System (Roche) following the manufacturer's instructions.

Enzyme-linked immunosorbent assay (ELISA)

BGT protein was quantitative by double-Ab sandwich ELISA using protein G-purified polyclonal rabbit antibodies and antibody linked with Horseradish peroxidase by the sodium periodate method. ELISA was carried out as described previously (Sambrook and Russell, 2001).

Western blot analysis

To extract total protein, 0.5 g young leaves was ground to a fine powder in liquid N_2 and re-suspended in extraction buffer (50 mmol·L^{-1} Tris·HCl, 5% β-mercaptoethanol, 1% PVPP, 0.1% Triton X-100, 6 mmol·L^{-1} ascorbic acid). After extraction at 4℃ for 2 h, protein samples were subjected to SDS-PAGE. Western blot analysis was carried out as described previously (Sambrook and Russell, 2001).

Insect bioassay

Eggs of Lymantria dispar were sterilized with 2% formalin solution for 3~5 min, then rinsed with sterilized water and air dried and placed in Petri dishes. The newly hatched larvae were reared on artificial diets. Thirty newly molted (>24 h) second instar larvae were placed onto the untransformed (the control) and transformed birch, respectively. The larvae fed on the control and transformed seedlings were weighed five days each. The insect bioassay was repeated three times.

RESULTS

Copy number of *bgt* in transgenic birch

In order to determine the copy number, single restriction enzyme *Pst*I was used to digest the genome DNA. Southern blot analysis of the 22 transgenic lines indicated that the *bgt*, *npt*II and *gus* gene had been integrated into the genome of birch. Among the transgenic birch plants,

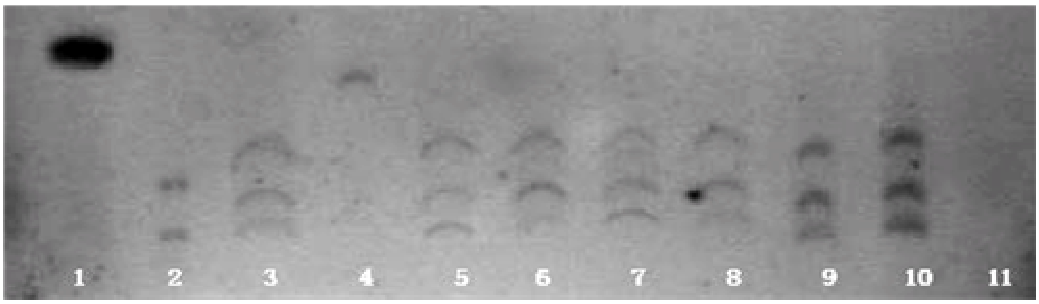

Figure 2. Southern blot analysis of transgenic birch plant DNA digested with *PstI*. Lane 1, positive control (2 kb); lanes 2-10, transgenic lines; lane 11, negative control.

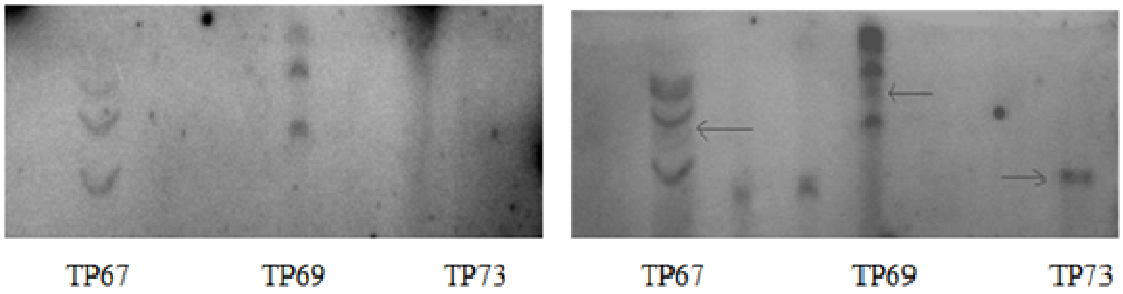

Figure 3. Southern blot analysis of transgenic birch hybrided by *bgt* (left) and *gus* (right) probe. Arrow showed the new strip hybrided by *gus* probe.

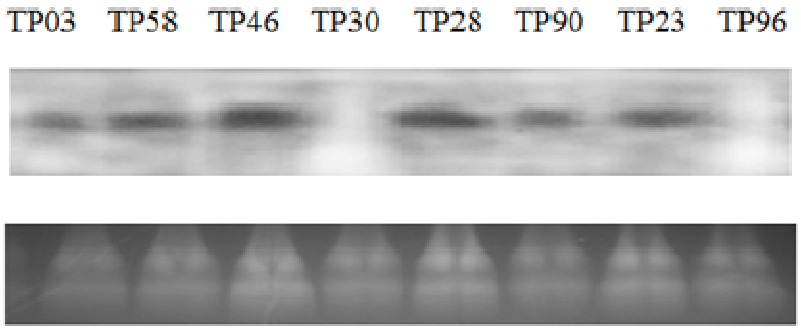

Figure 4. Northern blot analysis of *bgt* in transgenic birch plants.

the copy number of T-DNA varies from one to four (Figure 2).

We found that our *Agrobacterium*-mediated transformation system produced a majority of three copy transformants, with 18.2% of the transformants containing a single copy; 9.1% had two copies; 54.5 had three copies; and the remaining 9.1% contained four copies. None of studied events contained more than four copies of the insert. As shown in Figure 3, the copy number of *bgt* in TP67, TP69 and TP73 was three, three and zero. But the corresponding copy number of *gus* was four, four and one. The copies of *bgt* were inconsistent with the *gus* in 68% transgenic lines, indicating that rearrangement or partial deletion appeared in the process of T-DNA integration.

Transcriptional level of *bgt* gene in transgenic birch

Transcriptional expression of *bgt* gene in transgenic birch plants was analysed by Northern blot. Transcriptional level of *bgt* gene was different in transgenic birch plants (Figure 4). Transcriptional level of *bgt* in TP28, TP46 and TP58 was higher than others. Transcriptional gene silencing (TGS) was obtained in transgenic line (TP96) with four copies of *bgt*, and in transgenic line (TP30) with one

Table 1. Comparison of BGT protein and copy number.

Transgenic birch lines	Copy number	Transcriptional level	Content of BGT protein (%)	Western blot
TP30	1	-	0	-
TP90	3	+	0.145	+
TP46	1	+	0.166	+
TP23	3	+	0.274	+
TP58	4	+	0.239	+
TP03	2	+	0.153	+
TP28	1	+	0.256	+
TP96	4	-	0	-
TP27	3	+	0.197	+
TP71	4	+	0.138	+
TP68	2	+	0.283	+
TP67	3	+	0.116	+
TP63	1	+	0.082	+
TP92	3	+	0.062	+
TP36	4	+	0.068	+
TP52	3	+	0.048	+
TP22	3	+	0.038	+
TP48	2	+	0.054	+
TP69	3	+	0.141	+
TP72	1	+	0.154	+
CK		-	0	-

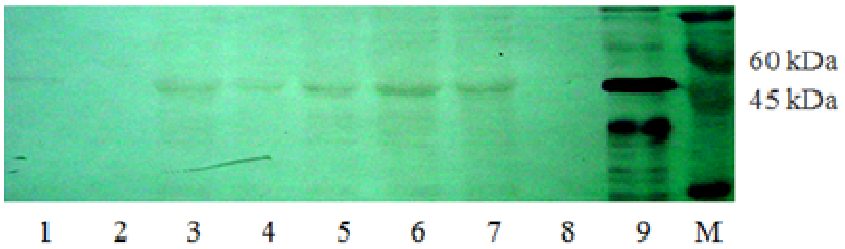

Figure 5. Western blot of BGT protein of transgenic birch. Lanes 1 to 7, transgenic extracts; Lane 8, negative control; Lane 9, positive control; Lane M, molecular mass standards.

copy of *bgt*. But TGS has not been found in other transgenic lines with one or four copies of *bgt*. The results demonstrated no absolute correlation between the numbers of transgene copies and the gene expression.

The level of BGT protein in transgenic birch analysis

To investigate the level of BGT protein in transgenic birchs, all samples extracts were determined by double-Ab sandwich ELISA method that we have established. The expression level of BGT protein was ranged from 0.000 to 0.283% of total soluble protein (Table 1). But three extracts were not detected. That was validated by western blot analysis (Figure 5). The results proved that the *bgt* gene in TP30 and TP96 was silenced. The BGT activity was remarkably higher in TP23 with three copies of *bgt*, TP28 with one copies of *bgt* and TP58 with four copies of *bgt* than others. Correlation between *bgt* copy number and expression level was analyzed. As shown in Figure 6 ($r=-0.125$, $P=0.590$), no significant correlation was found between copy number and expression of *bgt* gene.

Bioassays on *L. dispar* larvae

In order to validate the expression level, bioassays were

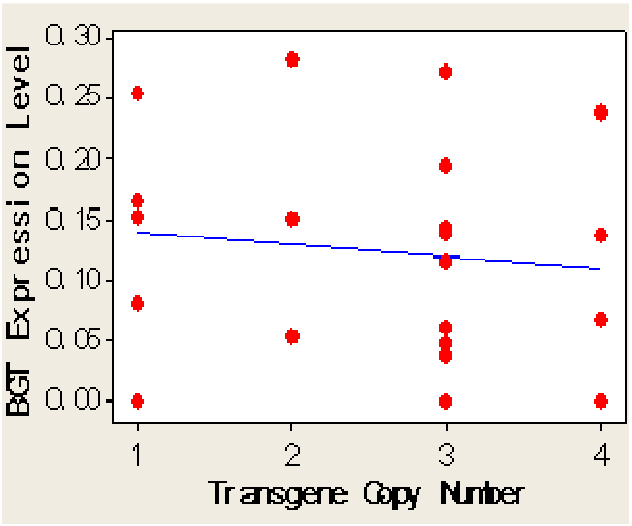

Figure 6. Correlation of transgene copy number and transgene expression level. (r = -0.125, P = 0.590).

Table 2. Result of insect-feeding experiment.

Materials	Mortality (%)	Corrective mortality (%)	Mean body mass (g·head^{-1})
CK	23.33	0	0.0917
TP03	86.67	82.61	0.0411
TP58	96.67	95.65	0.0607
TP46	90.00	86.95	0.0473
TP23	96.67	95.65	0.0496
TP28	93.33	91.30	0.0371
TP90	73.33	65.21	0.0607
TP56	86.67	82.61	0.4030
TP68	96.67	95.65	0.4870
TP67	90.00	86.95	0.0421
TP27	83.30	78.21	0.3890
TP14	96.67	95.65	0.0486
TP69	76.67	69.57	0.6030

carried out. The result is shown in Table 2 and Figure 7. The results indicate that the corrective mortality of the larvae feeding on transgenic birch plants is more than 65% in 30 d. But the toxicity of transgenic birch plants was different. The highest resistant clones with the corrective mortality more than 90% were TP23, TP28 and TP58. The copy number of *bgt* in TP23, TP28 and TP58 was respectively three, one and four. Meanwhile, bioassays results were mostly consistent with the expression level of BGT in transgenic birch, indicating that ELISA and Western blot results could really reflect the expression level of BGT protein in the transgenic birch. Transgenic birch had the highest lethal effect on gypsy moth larvae. No significant correlation was found between copy number of *bgt* and insecticidal activity.

DISCUSSION

Agrobacterium-mediated transformation usually results in fewer copies, while direct DNA transfer methods (e.g., electroporation or particle bombardement) often result in the integration of many copies of transgenes (Kohli et al., 1999). Cheng et al. (1997) transformed wheat using both *Agrobacterium* and particle bombardment. Of 26 *Agrobacterium*-mediated transformants, more than one third contained a single T-DNA insert, half contained 2-3 copies and the remainder contained 4-5 copies. In contrast, from the population of 77 bombarded transformants, only 13 (17%) contained a single copy of the transgene. In our study, the copy number of *bgt* varied from one to four among the 22 transgenic lines. We found that our *Agrobacterium*-mediated transformation system produced a majority of multile copies transformants, with only 18.2% of the transformants containing a single copy. The copies of *bgt* were inconsistent with the *gus* in 68% transgenic lines, indicating that rearrangement or partial deletion appeared in the process of T-DNA integration. These results are generally consistent with previous observations of *Agrobacterium* transformed dicotyledonous and monocotyledonous plants (Tang W et al., 2007; Zhang et al., 2008; Ingham et al., 2001).

Stable transgene expression is a critical parameter for the broad use of transgenic plants in plant biology and agricultural biotechnology. However, the insertion of foreign DNA into a plant genome may lead to alterations in its structure, which may have effect on host and/or transgene expression. Previous studies on the effect of transgene copy number on transgene expression and stability have given conflicting results. Some studies showed multiple copies of T-DNA integrated into the host genome at single or multiple loci could trigger transgene silencing (Fischer et al., 2008; Tang et al., 2007; Mishiba et al., 2005; Cervera et al., 2000; Mannerlöf et al., 1997). Others indicated that copy number and transgene expression levels can be positively correlated (McCabe et al., 1999; Tang et al., 2003; Van der Hoeven and Dietz, 1994; Voelker et al., 1992), or uncorrelated (Lechtenberg et al., 2003; Hawkins et al., 2003; Leibbrandt and Snyman, 2003; Hobbs et al., 1993; Bauer et al., 2003; McCabe et al., 1999). In this paper, transcriptional gene silencing (TGS) was determined in transgenic lines with one and four copies of *bgt*. But TGS has not been found in other transgenic lines with one or multiple copies of *bgt*. Our study showed that copy number was not correlated with transcriptional gene silencing (TGS). There was no significant correlation between copy number and expression level of BGT protein (Figure 6). High levels of insect resistance have been demonstrated in *Bt* toxin gene *Cry3A*-containing transgenic poplars (Meilan et al., 2002) and eucalypts (Harcourt et al., 2000). In this paper, effective resistance of transgenic plants against *L. dispar* larvae was verified in feeding bioassays with insects.

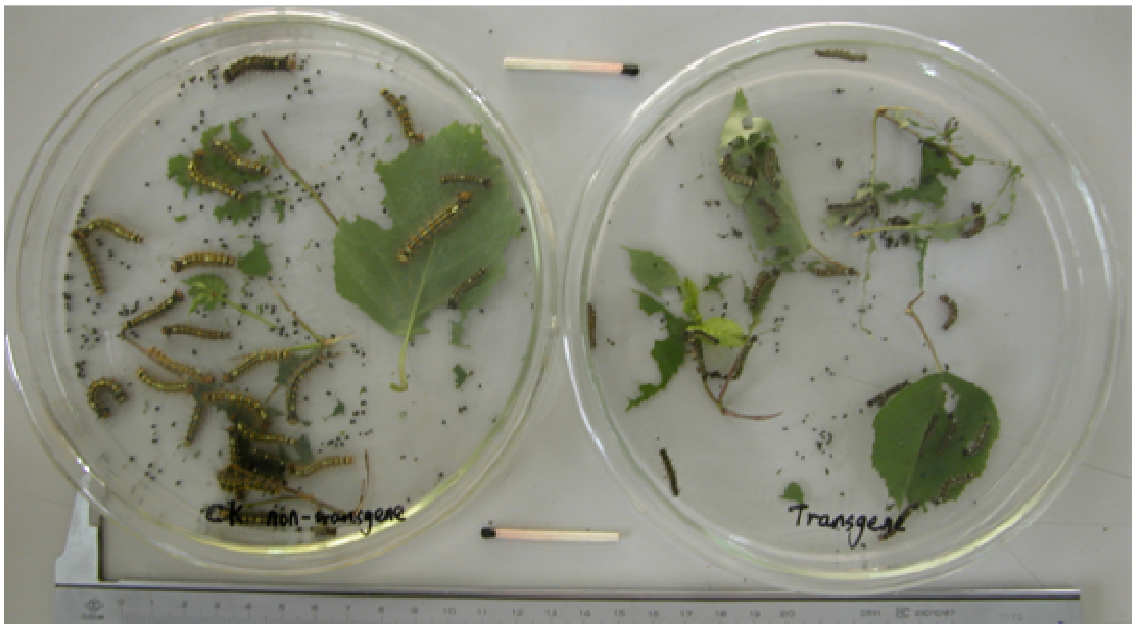

Figure 7. Comparison of insect-feeding with transgenic birch (right) and control (left).

Transgenic birch had high lethal effect on gypsy moth larvae.

Conclusion

The practicality of this work provides the way to new opportunities to improve the birch species. We found that our *Agrobacterium*-mediated transformation system produced a majority of multiple copies transformants, with only 18.2% of the transformants containing a single copy. Re-arrangement or partial deletion appeared in 68% of transgenic birch plants. But no significant correlation was found between copy number and expression level of *bgt* gene. The paper also showed that the corrective mortality of the larvae feeding on transgenic birch plants was more than 65% in 30 d, indicating that transgenic birch had high lethal effect on gypsy moth larvae (*L. dispar*).

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REFERENCES

Bauer M, Libantova J, Moravcikova J, Bekesiova I (2003). Transgenic tobacco plants constitutively expressing acidic chitinase from cucumber. Biologia. 53: 749-758.

Cervera M, Pina JA, Juárez J, Navarro L, Peña L (2000). A broad exploration of a transgenic population of citrus: stability of gene expression and phenotype. Theor. Appl. Genet. 100: 670-677.

Cheng M, Fry JE, Pang S, Zhou H, Hironaka CM, Duncan DR, Conner TW, Wan Y (1997). Genetic Transformation of Wheat Mediated by Agrobacterium tumefaciens. Plant Physiol. 115: 971-980

Cho HS, Cao J, Ren JP, Earle ED (2001). Control of Lepidopteran insect pests in transgenic Chinese cabbage (Brassica rapa ssp. pekinensis) transformed with a synthetic *Bacillus thuringiensis* CRY1C gene. Plant Cell Rep. 20: 1-7.

Fischer U, Kuhlmann M, Pecinka A, Schmidt R, Mette MF (2008). Local DNA features affect RNA-directed transcriptional gene silencing and DNA methylation. Plant J. 53: 1-10

Harcourt RL, Kyozuka1 J, Floyd RB, Bateman KS, Tanaka H, Decroocq V, Llewellyn DJ, Zhu X, Peacock WJ, Dennis ES (2000). Insect- and herbicide resistant transgenic eucalypts. Mol. Breed. 6: 307-315.

Hobbs SLA, Warkentin TD, DeLong CMO (1993). Transgene copy number can be positively or negatively associated with transgene expression. Plant Mol. Biol. 21: 17-26.

Holtorf S, Apel K, Bohlmann H (1995). Comparison of different constitutive and inducible promoters for the overexpression of transgenes in *Arabidopsis thaliana*. Plant Mol. Biol. 29: 637-646.

Ingham DJ, Beer S, Money S, Hansen G (2001). Quantitative real-time PCR assay for determining transgene copy number in transformed plants. BioTechniques, 31: 136-140.

Hawkins S, Leple JC, Cornu D, Jouanin L, Pilate G (2003). Stability of transgene expression in poplar: A model forest tree species. Ann. For. Sci. 60: 427-438.

Kohli A, Gahakwa D, Vain P, Laurie DA, Christou P (1999). Transgene expression in rice engineered through particle bombardment: molecular factors controlling stable expression and transgene silencing. Planta. 208: 88-97.

Kumar S, Fladung M (2000). Transgene repeats in aspen: molecular characterization suggests simultaneous integration of independent T-DNAs into receptive hotspots in the host genome. Mol. Gen. Genet. 264: 20-28.

Lechtenberg BD, Schubert A, Forsbach MG, Schmidt R (2003). Neither inverted repeat T-DNA configurations nor arrangements of tandemly repeated transgenes are sufficient to trigger transgene silencing. Plant J. 34: 507-517.

Leibbrandt NB, Snyman SJ (2003). Stability of gene expression and agronomic performance of a transgenic herbicide-resistant sugarcane line in South Africa. Crop Sci. 43: 671-677.

Mannerlöf M, Tuvesson S, Steen P, Tenning P (1997).Transgenic sugar beet tolerant to glyphosate. Euphytica. 94: 83-91.

McCabe MS, Mohapatra UB, Debnath SC, Power JB, Davey MR (1999). Integration, expression and inheritance of two linked T-DNA marker genes in transgenic lettuce. Mol. Breed. 5: 329-344.
Meilan R, Auerbach DJ, Ma C, DiFazio SP, Strauss SH (2002). Stability of herbicide resistance and *GUS* expression in transgenic hybrid poplars (*Populus* sp.) during several years of field trials and vegetative propagation. Hort. Sci. 37: 277-280.
Mishiba K, Nishihara M, Nakatsuka T, Abe Y, Hirano H, Yokoi T, Kikuchi A, Yamamura S (2005). Consistent transcriptional silencing of 35S-driven transgenes in gentian. Plant J. 44: 541-556.
Sardana R, Dukiandjiev S, Giband M, Cheng XY, Cowan K, Sauder C, Altosaar I (1996). Construction and rapid testing of synthetic and modified toxin gene sequences CRYIA (b&c) by expression in maize endosperm culture. Plant Cell Rep. 15: 677-681.
Sambrook J and Russell DW (2001). Molecular Cloning: A Laboratory Manual, 2nd edition, Cold Spring Harbor Laboratory Press.
Stam M, Joseph NM, Kooter JM (1997). The silencing of genes in transgenic plants. Ann. Bot. 79: 3-12.
Tang W, Newton RJ, Weidner DA (2007). Genetic transformation and gene silencing mediated by multiple copies of a transgene in eastern white pine. J. Exp. Bot. 58: 545-554.
Tang J, Scarth R, Fristensky B (2003). Effects of genomic position and copy number of Acyl-ACP thioesterase transgenes on the level of the target fatty acids in *Brassica napus* L. Mol. Breed. 12: 71-81.
Zhan YG, Wang YC, Wang ZY, Yang CP, Liu ZH, Li CH (2003). Genetic Transformation of Betula platyphylla and insect resistance of the transgenic plants. Zhi Wu, Sheng Li, Yu Fen, Zi Sheng, Wu Xue, Xue Bao, 29: 380-386.
Zhang J, Cai L, Cheng J, Mao H, Fan X, Meng Z, Chan KM, Zhang H, Qi J, Ji L, Hong Y (2008). Transgene integration and organization in Cotton (*Gossypium hirsutum* L.) genome. Transgenic Res. 17: 293-306.
Van der Hoeven C, Dietz A (1994). Landsmann J Variability of organspecific gene expression in transgenic tobacco plants. Transgenic Res. 3:159-165.
Vaucheret H, Béclin C, Fagard M (2001). Post-transcriptional gene silencing in plants. J. Cell. Sci. 114: 3083-3091
Voelker TA, Worrell AC, Anderson L, Bleibaum J, Fan C, Hawkins DJ (1992). Fatty acid biosynthesis redirected to medium chains in transgenic oilseed plants. Science, 257: 72-74.