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Molecular evidence of gene integration in *Larix decidua* somatic embryos after *Agrobacterium*-mediated transformation

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Abstract

Agrobacterium *was considered to be the method of choice to introduce foreign genes into plant cells. ESM (embryonal suspensor masses) which is composed of actively dividing immature embryos, could probably be very competent for transformation. An average of 1 - 3 clump tissues resistant to hygromycin arose from at least 300 mg ESM of* Larix decidua *after* Agrobacterium *inoculation. These clump tissues were able to proliferate further in the selection medium. PCR analysis was carried out using primers corresponding to HPT and GUS-genes. Both primers amplified an internal fragment in size of 650 and 700 base pair lengths respectively.*

Keywords: *conifer* – Larix decidua – Agrobacterium *-mediated transformation – embryonal suspensor masses*

Introduction

Transformation of plants by introducing novel traits is becoming a routine procedure in commercial use of commercial crops, and basic research (Walden & Schell, 1991). There were three most commonly used transformation systems (Songstad *et al.*, 1995), but *Agrobacterium*-mediated transformation was the method of choice (Stomp *et al.*, 1990). In *Agrobacterium*-mediated transformation, a high percentage of single copy gene is inserted into the chromosome plants (Wenck *et al.*, 1999), which might avoid a silencing phenomena (Meyer, 1995; Mullins *et al.*, 1997; Finnegan *et al.*, 1998).

Wound response to *Agrobacterium* infection leading to a crown gall production has been reported in many conifer species (Ellis *et al.*, 1995; Tzfira *et al.*, 1996). Huang *et al.* (1991) have successfully regenerated transgenic *Larix decidua* using *Agrobacterium rhizogenes* through organogenesis. Somatic embryogenesis is a potential system for producing stable transgenic plants of conifer. Product of somatic embryogenesis in conifer, namely embryonal suspensor masses (ESM), which is composed of actively dividing immature embryos, would provide a relatively high multiplication rate and plantlet recovery (Häggman *et al.*, 1996) and could be competent for transformation

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(Bergmann & Stomp, 1992). The competency of ESM for *Agrobacterium*-mediated transformation has been transiently investigated using GUS (ß-glucuronidase)-gene in *Picea sitchensis* (Drake *et al.*, 1997) and transgenic plantlets have been successfully recovered using kanamycin as a selection agent in *Larix kaempferi x L. decidua* (Levee *et al.*, 1997), *Picea abies* and *Pinus taeda* (Wenck *et al.*, 1999), *Pinus strobus* (Levee *et al.*, 1999), but kanamycin might be sometimes ineffective to select transformed cells (Gutierrez *et al.*, 1997).

Hygromycin showed a broad spectrum of activity against different types of living cells by interfering their protein synthesis (Cabanas *et al.*, 1978). The use of hygromycin has been recommended as a selection agent in conifer transformation using ESM as a target tissue (Levee *et al.*, 1997; Wenck *et al.*, 1999). Since until now there has no report of using hygromycin resistant gene as a selectable marker for the transformation of *Larix decidua* ESM using *Agrobacterium tumefaciens*, therefore construction with such selectable marker gene will be used to conduct the transformation. The success of the transformation will be evaluated by using PCR method.

Materials and Methods

Plant material

The ESM of 2/91, 17/92, 4/93, 14/93, 26/93 and SA90 provided by Institute of Biology, Humboldt University-Berlin were used as materials for the experiments. They were induced from an immature zygotic embryos and propagated by subculture each month on MSG proliferation medium (Klimaszewska, 1989) supplemented with 9,04 µM 2,4-D (2,4-dichlorophenoxyacetic acid) and 2,22 µM BAP (6-benzylaminopurine) solidified with 0,3% gelrite.

Bacterium and culture medium

Agrobacterium tumefaciens strain GV3101 carrying plasmid pPCV812 contains HPT (hygromycin phosphotransferase)-gene expressing hygromycin resistantce under the control of NOS (nopaline synthase)-promoter and GUS-gene as a reporter marker under the control of MAS-promoter was used for the transformation. It was freshly cultured on semi solid YEB medium supplemented with an appropriate antibiotic. Three days before inoculation of the ESM, one colony was suspended in 10 ml liquid YEB medium. After 2 days of culture, 1 ml of *Agrobacterium* suspension was transferred to 24 ml new similar liquid medium and grown overnight at 30°C.

Transformation and selection

Agrobacterium suspension was firstly diluted 1 : 1 with liquid proliferation medium. The ESM were then mixed 3 : 1 (w/v) with diluted *Agrobacterium* suspension and incubated for 15 minutes. One-ml aliquotes were plated on Whatman filter paper discs, chopped with scalpel, co-cultivated for 2 days on solid proliferation medium and incubated in the dark at 25°C. The ESM were afterwards transferred to similar medium supplemented with 500 mg/l cefotaxime to decontaminate the bacterium.

To determine the concentration of hygromycin that would be used for selection, ESM were cultured on proliferation medium containing different concentrations of hygromycin. The minimum concentration of hygromycin that inhibited the growth of ESM was chosen to select the transformed cells. After 10 days of decontamination, transient expression of reporter gene was

evaluated from the surviving ESM and the rest of the transformed ESM were cultured on medium containing this minimal concentration of hygromycin and 500 mg/l cefotaxime. Clumps of surviving ESM were transferred to fresh plates with a few higher concentration of hygromycin to inhibit the growth of unwanted escaped clump tissues. The resistant ESM was further proliferated and used for molecular analysis.

Molecular analysis of resistant ESM

Genomic DNA was isolated from 1 gram ESM based on the modification of published method (Wilke, 1997). Standard PCR (Polymerase chain reaction) was carried out in 30 µl reactions containing at least 100 ng DNA, 3 µl 10 x PCR-buffer; 0,66 mM $MgCl_2$ for HPT-primer and 0,51 mM $MgCl_2$ for GUS-primer, 250 µM each dNTP, 20 pmol of each primer (Table 1) and 1,25 unit Taq DNA-Polymerase (Gibco BRL, Paisly, Schottland). After 1 minute DNA denaturation, the reaction was followed by 30 cycles of 94°C for 45 seconds, 30 seconds in 56°C for HPT-primer and 58°C for GUS-primer and 72°C for 2 minutes. Cycling was closed by 72°C for 6 minutes and kept in 2°C using a MJ MiniCycler (MJ Research, Watertown). PCR products were separated on 1.2% agarose gels and visualized by ethidium bromide staining.

Table 1. Primer sequences for amplification of HPT and GUS-gene.

Primer	Sequence
HPT_1	5'-AAT AgC TgC gCC gAT ggT TTC TAC A-3'
HPT_2	5'-AAC ATC gCC TCg CTC CAg TCA ATg-3'
GUS_1	5'-CTg CgA CgC TCA CAC CgA TAC CAT C-3'
GUS_2	5'-TTC ACC gAA gTT CAT gCC AgT CCA gCg-3'

Results

Decontamination and selection of transformed ESM

Preliminary experiment showed that hygromycin in the concentration of 10 mg/l inhibited the growth of the non-transformed ESM, therefore the levels of concentration were then decreased to 5 and 7,5 mg/l. Five mg/l hygromycin has influenced the growth of the ESM (Table 2). On free hygromycin maturation medium, for instance, the ESM of 4/93 demonstrated fast growth with a white mucilaginous color. However, the concentration of hygromycin at 5 mg/l stopped the growth of ESM and turned it to brownish color. The response of *Larix decidua* ESM to 5 mg/l hygromycin treatment depended on genotypes (Table 2). For 4/93, at this concentration, the ESM have already died, whereas for the other genotypes, they still demonstrated growth despite at a very low rate compared to growth on free hygromycin maturation medium. As ESM were grown on 7,5 mg/l hygromycin-containing maturation medium, all genotypes showed similar response. They have all already died after 20 days of culture. Transferring these ESM on free hygromycin proliferation medium did not revert the growth ability. As hygromycin in the concentration of 5 mg/l has already hampered the growth of the ESM, therefore it was used as a minimal concentration to select cells after transformation. To avoid the growth of unwanted colony after selection, induced clumps of ESM on proliferation medium containing 5 mg/l hygromycin were then transferred to medium containing 7,5 mg/l hygromycin. For further proliferation and selection, resistant ESM was cultured on medium containing similar concentration of hygromycin.

Table 2. Mean and standard error of ESM growth rate (%) of *Larix decidua* grown on proliferation mediu containing different concentrations of hygromycin (0 - 7,5 mg/l) after 30 days of culture (2-10 replications)

Genotypes	Hygromycin (mg/l)		
	0	5	7.5
4/93	2820,250±212,772	-27,469±06,029	-35,421±2,171
14/93	1388,750±135,484	+23,252±01,978	-29,193±5,412
26/93	1489,310±382,697	338,734±96,689	-16,388±3,103
SA90	3778,900±109,554	+19,250±04,593	-11,027±1,655

Growth rate was measured from the different fresh weight at 30 and 0 days of culture to initial fresh weight of ESM.

During cocultivation, the growth of the *Agrobacterium* could be observed at the edge surrounding ESM on the filter paper. The existence of *Agrobacterium* ring on the filter paper was observable after 2 days of culture. As the ESM were transferred to proliferation medium containing 500 mg/l cefotaxime, *Agrobacterium* stopped growing and ESM was sometimes still able to grow. The number of plates with clean growing of ESM after 10 days of culture depended on the genotypes (Table 3). The percentage of clean growing ESM of 2/91 seemed to be the lowest one, whereas genotype 14/93 was found to be the best.

Table 3. Influence of *Agrobacterium* inoculation in the surviving of ESM on decontamination and selection medium

Genotypes	Number of plates	Number of decontamination plates containing clean growing	Number of selection plates inducing new clumps ESM (%)
2/91	15	6,670	6,670
17/92	20	10	10
4/93	120	95,833	45,000
14/93	15	100	66,667
26/93	15	66,667	0
SA90	85	73,750	0

All ESM cultured on decontamination medium were transferred to selection medium. The inhibited ESM on the decontamination medium were not able to grow further on the selection medium. Similar phenomena was also found in the clean normal growing ESM but only during the first 3 weeks of culture. Subsequently, 2 - 3 small white growing clump tissues eventually arose from the inhibited ESM. The number of plates with small white growing clump tissues was dependent on the genotypes (Table 3). Despite the fact that the percentage number of plates genotype 2/91 with clean growing ESM was the lowest, all of these plates were able to produce new clump tissues. For 14/93, new clump tissues could not be induced from all plates but was only inducible from 66,667 % of them. Resistant new clump tissues could not be recovered in genotypes 26/93 and SA90.

As the clump tissues were cultured on proliferation medium containing 7.5 mg/l hygromycin, they were able to proliferate further. The new proliferated ESM grew normally on similar medium. After 6 months on the selection medium supplemented with cefotaxime, ESM were transferred on the selection medium free of cefotaxime, subcultured every month on similar medium and used for DNA isolation.

Molecular analysis

Resistant ESM from which DNA was isolated, were subcultured on free cefotaxime selection medium for at least 6 months and there was no signal of growing bacteria colonies, therefore the probability of the existence of residual *Agrobacterium* in these ESM was not evaluated through PCR. PCR analysis was carried out using primers corresponding to HPT and GUS-genes. Both primers amplified an internal fragment in size of 650 and 700 bp lengths, respectively. Water, pure plasmid of pPCV812, transformed kalanchoe and tobacco calli using similar construct were included in the analysis as control.

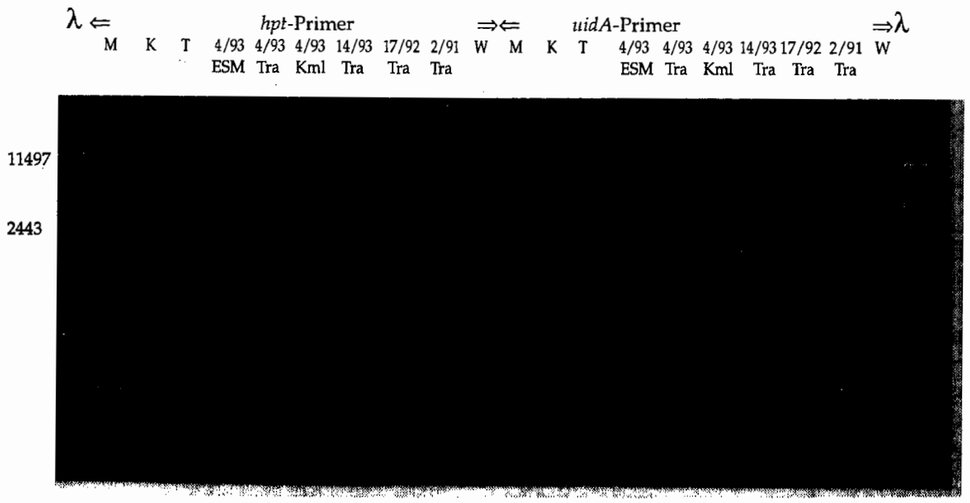

Figure 1. PCR analysis using HPT-primers and GUS-primers. Lane 1. Lambda Pst I Marker; 2 - 11. Bands amplified with HPT-primers (2. Plasmid pPCV812, 3. Resistant kalanchoe callus, 4. Resistant tobacco callus, 5. Non-transformed ESM of 4/93, 6. Resistant ESM of 4/93, 7. Non-transformed plantlet of 4/93, 8. Resistant ESM of 14/93, 9. Resistant ESM of 17/92, 10. Resistant ESM of 2/91, 11. Water); 12-21. Bands amplified with GUS-primers in similar order as HPT; 22. Lambda Pst I Marker

The presence of HPT and GUS-genes were confirmed in transformed tissues and pPCV812, whereas corresponding bands were not detected in non-transformed tissues and water (Figure 1). Further analysis to all resistant ESM showed that similar size of bands could be amplified from almost all of resistant calli but not from the non-transformed one (Table 4).

Table 4. Band produced by PCR amplification using HPT and GUS-primers from non-transformed and resistant genotypes of *Larix decidua*.

Source of DNA	Geno-types	Category	Number of DNA samples	HPT-primer (%)	GUS-primer (%)
ESM	2/91	resistant	1	100,00	100,00
		non-transformed	1	0,00	0,00
	17/92	resistant	3	100,00	100,00
		non-transformed	1	0,00	0,00
	4/93	resistant	49	93,88	93,88
		non-transformed	1	0,00	0,00
	14/93	resistant	7	100,00	100,00
		non-transformed	1	0,00	0,00
Plantlet	4/93	non-transformed	1	0,00	0,00

Percentage of band was the ratio of number of amplified and expected bands from 3 independent amplifications

Discussion

It is well known that in stable transformation only a minor fraction of the treated cells become transgenic and the common way to differentiate the transformed cells was by introducing a selectable marker along with the gene encoding the desired trait (Joerbo & Okkels, 1996). The selection, therefore, appeared to be very important step (Birch, 1997; Gutierrez *et al.*, 1997; Lindsey, 1996; Pena *et al.*, 1997; Songstad *et al.*, 1995; Walden & Schell, 1991) and it was necessary to choose the right selective agents and to define precisely its concentration to produce stable transformation. Hygromycin seems very effective to be used as a selection agent in *Larix decidua* transformation using ESM as a target tissue. At the concentration of 5 mg/l, it inhibited the growth of the ESM (Table 2) and increasing the concentration up to 7,5 mg/l caused the death of ESM. The effectiveness of hygromycin for transformation has been

recognized for a long time. Eady & Lister (1998) reported that low levels of hygromycin inhibited the callus growth of *Allium cepa*. Hygromycin is an aminoglycoside antibiotic, which interfered with the translation step of polypeptide synthesis by affecting the 80 S of ribosomal protein. Hygromycin kills different types of organisms, therefore it is considered as a very toxic agent. The use of hygromycin might decrease the proportion of escaped cell during selection (Kuvshinov *et al.*, 1999). High frequency of escape has been considered as common phenomena in conifer transformation (Ellis *et al.*, 1993; Charest *et al.*, 1996). The use of hygromycin has, therefore, been recommended (Levee *et al.*, 1997; Wenck *et al.*, 1999). At least in this experiment, hygromycin seemed very effective to eliminate unwanted ESM. The growth of ESM was affected at a very low concentration.

Active growth of target tissue at the time of inoculation determines the transformation frequency (Bergmann & Stomp, 1992). Because ESM is composed of actively dividing immature embryos (Wenck *et al.*, 1999), it may be used to produce stable transformed conifer (Häggman *et al.*, 1996). As the ESM of *Larix decidua* were inoculated with *Agrobacterium* and cocultivated on the proliferation medium, the existence of *Agrobacterium* ring on the filter paper was already observable after 2 days of culture. On the decontamination medium, sometimes *Agrobacterium* was still able to grow. It seemed that 500 mg/l cefotaxime in the decontamination medium was not able to decontaminate the bacteria. This result was contradictory to preliminary unpublished experiment, which showed that *Agrobacterium* plated on this medium was not able to grow anymore.

Chopping the ESM could not be homogeneously carried out, as in some cases the ESM might be damaged and died. The death of the ESM might detoxify cefotaxime and caused the over growth of *Agrobacterium*. Rinsing the ESM after cocultivation using liquid proliferation medium containing cefotaxime in similar concentration did not overcome this problem. It seemed that the sensitivity of the ESM to wound was genotype-dependent. Another method of making injury that could wound homogeneously must be developed. Wounding through particle bombardment prior *Agrobacterium* inoculation could be evaluated, although this system did not improve the transformation frequency in *Citrus aurantium* (Gutierrez *et al.*, 1997).

On the selection medium, the growth of the ESM is first inhibited and turned brown, but then 2 - 3 small white new clump tissues arose from inhibited ESM originated from clean normal growing one. The production of small white clumps was also observed in hybrid larch using kanamycin as selective agent (Levee *et al.*, 1997). The number of plates with small white growing clump tissues was genotype-dependent (Table 3). The clump tissues were able to survive on proliferation medium containing 7,5 mg/l hygromycin.

The stable expression of transgene were influenced by the number and structure of integrated copies, chromosomal integration region and its chromatin structure, methylation state, strength and specificity of promoter element. Among of them, promoter in *Agrobacterium*-mediated gene transfer might play an important role (Häggmann & Aronen, 1998). *Agrobacterium* frequently transfers only a limited copy number of DNA without large internal deletion into chromosomal loci that are potentially transcribed (Meyer, 1995), therefore gene silencing might be avoided.

The presence of transgene in the genome, in the long run selected ESM, was demons-

trated by PCR. The size of the gene was also very similar to the same gene from the used plasmid. The GUS has been integrated in the genome. The PCR analysis was performed to all resistant ESM from different genotypes and included kalanchoe, tobacco, plasmid as control. The result was summarized from 3 repeated independent experiments. Even though the percentage of amplified bands in 4/93 was only 93,88%, it could be concluded that the genes were integrated in all resistant ESM. The lanes by which bands was not amplifiable were not constant, it might be only a little technical problem during the preparation of PCR. In another word, there was no escaped colonies. No escaped colonies were also observed using kanamycin as a selectable marker in *Agrobacterium*-mediated gene transfer in conifers (Levee *et al.*, 1997; 1999; Wenck *et al.*, 1999), but this antibiotic was reported to produce a few non transformed resistant colonies after particle bombardment (Charest *et al.*, 1996; Ellis *et al.*, 1993) and sometimes persist *Agrobacterium* contamination in transformed cells (Dandekar *et al.*, 1988).

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