TRANSFORMATION OF HAPLOID AND DIPLOID POPLAR WITH DIFFERENT GENE CONSTRUCTS AND ANALYSIS OF TRANSGENIC PLANTS [1]

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ABSTRACT

In previous experiments we have transferred a series of different gene constructs to various poplar genotypes. Transgenic lines were analysed in respect to T-DNA integration and T-DNA flanking genomic regions. Transgenic aspen carrying the *Ac* transposon from maize were also obtained. In these lines the excision of the *Ac* element as well as its re-integration was studied. The new positions of *Ac* in the genome were identified and flanking genomic regions sequenced. Interestingly, about one third of the sequences obtained show high homology to known genes from databanks. A similar study was undertaken for the genomic sequences flanking the T-DNAs. The results show a lower frequency of T-DNA tagged genes compared to *Ac* tagging.

To apply the technique of transposon tagging to *Populus* the problems of dioecy and long generation cycles are major deterrent. One possible solution is the establishment of haploid *Populus* lines and transfer of transposons in these lines. Initial experiments on the production and establishment of haploid *Populus* lines are reported.

Key words: genomics, *Populus, RolC*, transgenic aspen, transposition

INTRODUCTION

The objectives of our work are the identification, cloning and sequencing of genes and promoters from the model tree species *Populus* spp. The sequences obtained will be compared to those known from databanks, and new sequences with known functions can be subject for patenting. The strategy used is based on our earlier experiments (FLADUNG 1999b) using constructs containing the *Ac* transposon from maize and the phenotypically visible marker genes *rolC* or *iaaL*. The excision of *Ac* during leaf development causes restoration of the marker gene, for example, *rolC* expression was indicated in the 35S-*Ac*-*rolC* transgenic aspen plants by light-green sectors in green leaf background.

Transposon tagging is a powerful tool for the identification of genes in many plant species but its use is limited by the low transformation efficiency and low regeneration capacity during tissue culture. In particular, in trees the practical use of transposons for gene tagging is limited due to the long vegetative cycles and thus the hemizygous status of the element in the genome. Furthermore, the species of the genus *Populus* are dioecious and therefore no selves are possible. In order to overcome these difficulties haploid plants can be used for gene tagging in *Populus*. In annual plant species haploids play an important role in modern plant breeding. The induction of haploids in woody plants has also been reported (CHEN 1987) but, unfortunately, no haploid poplar line was made available upon request.

The activities of our work can be summarised as following: (1) detailed molecular analysis of variant phenotypes from T-DNA tagged transgenic aspen lines, (2) sequencing of genomic regions flanking the new *Ac*-insertion sites, (3) induction and establishment of a haploid *P. nigra* hybrid line, and (4) transformation of haploid poplar lines M22-1c5.

MATERIAL AND METHODS

Plant material

Different *Populus* genotypes were used for the

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transformation experiments with various constructs. Transgenic aspen (*Populus tremula* [pure aspen]; genotypes W52 and Brauna11) and hybrid aspen (*P. tremula* × *P. tremuloides*; genotype Esch5) trees were produced which carry a morphological marker based on the *rolC* gene of *Agrobacterium rhizogenes* (FLADUNG *et al.* 1997). In addition, five other constructs were used for transformation experiments of the hybrid aspen genotype Esch5 (FLADUNG & AHUJA 1996). For transformation of the double hybrid {*Populus tremula* × *P. tremuloides*} × *P. alba*, and haploid *P. nigra* L. ′ hybrid the 35S-*Ac-rolC* and rbcS-*Ac-rolC* constructs were used (SPENA *et al.* 1989, FLADUNG *et al.* 1997).

For induction of a haploid poplar line, in total, six male trees of *Populus* spp. (three individuals of *P. nigra* L. ′ hybrid, two of *P. tremula* L. ′ *tremuloides* Michx, one of *P. balsamifera* L.) were used. The isolation of immature pollen was carried out from January to April in the years 2001 and 2002, respectively. After preliminary culture experiments (data not shown) two black poplar hybrid trees (genotypes 'Aue 1' and 'Aue 2') identified as responsive for callus and embryo formation were used for all subsequent experiments.

Molecular methods and flow cytometry

DNA was extracted from leaves according to the CTAB-based method and PCR analysis was performed as described earlier (FLADUNG *et al.* 1996, 1997). As a positive control in all PCR reactions primers amplifying an aspen-specific genomic region were added (FLADUNG & KUMAR 2002). Electrophoresis and visualisation of bands were done as described elsewhere (FLADUNG *et al.* 1996, 1997).

For Southern blot analyses DNA was digested of with the restriction enzymes *Eco*RI and *Hind*III. Electrophoresis and blotting of DNA on Biodyne A membranes (Pall Europe Limited, Portsmouth, UK) were performed as described elsewhere (FLADUNG *et al.* 1996, 1997). Prehybridization and hybridization were done with the non-radioactive DIG (digoxigenin) system using DIG-dUTP labeled purified *rolC* (FLADUNG & AHUJA 1995, FLADUNG *et al.* 1997).

To demonstrate *Ac* transposition in aspen a large light-green sector available from the transgenic line 35S-*Ac-rolC* #5 was used for the Southern blot analysis (FLADUNG 1999b). Empty donor sites were determined using PCR amplification of DNA from leaf sectors using 5′-35S and 3′-*rolC* primers as described by FLADUNG and AHUJA (1997). T-DNA flanking regions were determined using Inverse-PCR (OCHMAN *et al.* 1988) with some modifications (FLADUNG 1999a). For *Ac* insertion, thermal asymmetric interlaced (TAIL)-PCR (LIU *et al.* 1995) was performed with DNA extracted from the leaf sectors and primers as described in KUMAR and FLADUNG (2003). Each of these primers was used in combination with three arbitrary primers; AD1, AD2, and AD3 described in LIU *et al.* (1995). The Inverse- and TAIL-PCRs were performed using the Expand[a] Long Template PCR system (Roche Molecular Biochemicals, Mannheim, Germany) according to manufacturer's recommendations.

For application of the microsatellite markers genomic DNA was isolated from about 50 mg of callus or leaf tissue (DEUTSCH *et al.* 2004). For the PCR reaction each 25 µl PCR reaction mixture contained 1x PCR reaction buffer, 1.6 mM $MgCl_2$, 0.2 µM of each primer, 0.1 mM of each dNTP, 0.5 U of Taq-DNA-polymerase (Eurogentec, Cologne, Germany) and 20 ng of genomic DNA. The initial denaturation for 4 min at 94 °C was followed by 30 cycles of denaturation (1 min at 92 °C), annealing (1 min at 60 °C) and extension (1 min at 72 °C), and a final extension step of 10 min at 72 °C. PCR was run in the Touch-Down TM Thermal System (Hybaid Limited, Teddington, UK).

To determine the ploidy level of calli and plantlets, small pieces of tissue were chopped with a razor blade into an extraction buffer (Partec, Münster, Germany) in order to release the nuclei. A DNA-binding dye (Partec, Münster, Germany) containing DAPI (4,6-Diamidino-2-phenylindole) was added. The whole suspension was filtered through a 30 µm-nylon mesh before the ploidy level in 5000 nuclei was analysed using a flow cytometer (CAII Cell Analyzer, Partec, Münster, Germany). Tissues not showing the diploid status were measured at least twice. Haploid tissues were measured every month. Young leaves of *in vitro* cultured plantlets from the immature pollen donor tree 'Aue 2' were used as internal (prepared and measured together with the sample) and as external diploid control (measured alone). The G1 DNA peak of the control was set at channel 100. Analysis of ground pollen confirmed that the peak at channel 50 resulted from haploid tissue.

RESULTS

Detailed molecular analysis of putative T-DNA tagged variant phenotypes

Transgene flanking genomic regions of in total 30 transgenic lines including the lines showing variant

phenotypes were analysed using Inverse-PCR strategy. In total, 27 right T-DNA/plant junctions, 20 left T-DNA/plant junctions, and ten target insertions from control plants were obtained. The right end of the T-DNA in 18 transgenic lines was conserved up to the cleavage site and the right border repeat was deleted in nine junctions. Nucleotides from the left border repeat were present in 19 transgenic lines out of 20 cases analysed (KUMAR & FLADUNG 2002).

Comparison of the genomic target sites prior to integration to the T-DNA revealed that T-DNA inserted into the plant genome without any notable deletion of genomic sequence in three transgenic lines. However, deletions of a few nucleotides to more than 500 bp were observed in other transgenic lines. Filler DNA of up to 235 bp were observed on left and/or right junctions of six transgenic lines which in most of the cases originated from the nearby host genomic sequence or from T-DNA. Short sequence similarities between recombining strands near break points without any bias to the left or the right T-DNA end were obtained in most of the lines analysed suggesting that both T-DNA ends may interact during the integration process. Small sequence similarity occurring at the T-DNA 3′ end could act as a primer for DNA fill-in synthesis, whereas at the 5′ end it could adjoin the recessed strand of the partner terminus and be fixed by ligation. The integration model is reminiscent of the model for the repair of genomic DSB in somatic plant cells based on synthesis-dependent strand-annealing (SDSA).

The *Ac* "Flanking Sequence Tags" (FSTs) sequences obtained for the T-DNA genomic regions were compared to public databases for similarity to known proteins and genomic sequences (KUMAR & FLADUNG 2002, Table 1). Only 16 % of the T-DNA insertions landed into or near known or predicted genes.

Sequencing of genomic regions flanking the new *Ac*-insertion sites

Three independent transgenic lines (35S-*Ac-rolC*#2, #3 and #10) containing a single copy of 35S-*Ac-rolC* transgene as shown by Southern analysis (FLADUNG *et al.* 1997) and showing light-green sectors on leaves indicating active excision of *Ac* were selected for the determinations of the FSTs. The light-green sectors were harvested for further molecular analyses (KUMAR & FLADUNG 2003). DNA from several independent light green leaf sectors was used in Inverse-PCR or TAIL-PCR reactions to amplify aspen genomic DNA flanking the *Ac* insertions. The sequences obtained were compared to public databases for similarity to known proteins and genomic sequences. The sequences analyzed in three independent transgenic lines and the BLASTx search results are summarized in Table 2.

The data show that out of 75 sequences analysed 22 sequences gave significant BLASTx hits. This might be a minimal estimate keeping in view the small amount of information available on aspen genome in the public databases. However, we can expect DNA sequences disrupted by these insertions to become available as the poplar genome sequencing project progresses.

Such a catalogue of *Ac* flanking DNA sequences

Table 1. T-DNA tagged aspen genomic sites (out of 32 transgenic lines analysed) with similarity to sequences in public databases.

Transgenic line	Hit blast × similarity search	Organism	Accession number	Positives (% amino acids)	E-Value
Brauna11-*35S-rolC*#2	Probable methyltransferase	*A. thaliana*	E85112	41/46 (88%)	2e-21
Esch5-*35S-rolC*#5	Receptor-like kinase	*Oryza sativa*	AF238472	19/22 (86%) 16/24 (65%)	3e-04
W52-*35S-rolC*#3	Polyprotein	*Sorghum bicolor*	AF061282	44/60 (72%) 9/10 (90%)	3e-11
Esch5-*35S-rolC*#11	Potassium channel 2	*P. tremula* × *P. tremuloides*	PTR2714-47	17/17 (100%)	0.003
Brauna11-*35S-rolC*#5	Hypothetical protein F9E11.7	*A. thaliana*	H96769	128/269	7e-27

Table 2. Frequency of *Ac* insertions into predicted genes (KUMAR & FLADUNG, 2003).

Transgenic line	FSTs analyzed	Significant BLAST×hit
Esch5:35S-*Ac-rolC*#3	41	12 (29%)
Esch5:35S-*Ac-rolC* #10	24	6 (25%)
Esch5:35S-*Ac-rolC* #2	10	4 (40%)
Total	75	22 (29%)

could be used to make a FST database, suitable for aspen reverse genetics. The total frequency of FSTs in predicted genes, summarized in Table 2, was calculated to be 29 %. Details of the *Ac*-tagged hits are given in KUMAR and FLADUNG (2003). The frequency seems to be consistent among individual transgenic lines ranging from 25–33 %. This suggests that about one third of all the *Ac* insertions are in genes that can be predicted on the basis of homology to the public databases. FSTs in predicted genes like DNA methyltransferase can provide information on the unique function of these genes in a long-lived tree system. Poplar is a model plant for the discovery of gene functions in trees. The preferential insertion of the *Ac* element in the coding regions of the aspen genome as indicated by our results suggests that transposon mutagenesis will be a very important tool to discover gene functions by reverse genetics or forward genetics strategies.

This frequency obtained for *Ac* is double as that of the frequency of T-DNA insertion hitting coding sequences in the same aspen system. Hence, the *Ac* system seems to be more effective for gene tagging and promoter trapping studies in aspen.

The T-DNA/*Ac* FSTs obtained from aspen genome were searched for T-DNA tagged *Arabidopsis* lines in GABI-KAT database (http://www.mpiz-koeln.mpg.de/ GABI-Kat/). We obtained five *Arabidopsis* lines where T-DNA appears to be inserted into coding regions that are similar to aspen T-DNA/*Ac* FSTs (Table 3). Three of these five FSTs belong to T-DNA out of which two transgenic lines (Brauna11:35S-rolC#2, Esch5:35S-rolC#5) show variant phenotype. We have ordered seeds of these *Arabidopsis* lines for a comparison and in order to obtain quick results from *Arabidopsis*, which has a very small generation cycle compared to aspen.

Induction and establishment of a haploid *P. nigra* hybrid line

Experiments were carried out to induce haploidy in poplar based on the following activities:

1. Gynogenesis: *In-vitro* culture of non-fertilised ovules
2. Androgenesis: *In-vitro* culture of immature anthers.
3. Pseudogamy: Induction of parthenogenesis.
4. Isolation and culture of microspores.

The experiments (1) to (3) were not successful, and no haploid line was obtained following gynogenesis and androgenesis. The progeny which were obtained after induction of parthenogenesis (3) contained plants with haploid leaves. However, the haploid status was not stable but switched to diploid as detected in flow cytometer measurements made later.

The microspore cultures (4) of the year 2001 produced 113 calli larger than 1 mm, 19 calli per responsive bud (DEUTSCH *et al.* 2004). The cultures of the year 2002 produced 1375 calli larger than 1 mm, 81 calli per responsive bud, four times more than the cultures of 2001. The yields of the microspore cultures are shown in Table 4.

The data of the regeneration from calli and embryos of the years 2001 and 2002 are also summarized in Table 4. In the year 2001, calli with at least 1 mm in size (approximately after four to eight weeks) were transferred onto semi-solid regeneration media "Woody Plant Medium" (WPM) and Gamborg B5 without growth regulators. One third of the number of calli and embryos turned green in light and some embryos formed green cotyledons and hairy roots. Most of them died, but few were rescued using 0.02 $mg \cdot l^{-1}$ NAA and 0.5 $mg \cdot l^{-1}$ BAP. Two embryos and two calli formed adventitious leaves or shoots.

For the year 2001 only one line (M22-1c5 regenerative callus) turned to be haploid in flow cytometer measurements and microsatellite analysis. The apical meristem of M22-1c5 formed regeneration buds with small leaves (Figure 1). Occasionally evolving shoots from these buds could be rooted and transferred into soil one year after culture initiation (Figure 2). The regenerants are dwarfish and slow growing. Unfortunately, these regenerated plantlets

Table 3. GABI-KAT search results of T-DNA/*Ac* flanking genomic sequences obtained from hormone initiated organogenesis

Aspen sequence (GABI-PD code)	Transgenic line	T-DNA/*Ac*	Gene annotation	Gene code	GK line ID
B2_2_RE_B	Brauna11:35S-rolC#2	T-DNA	Putative methyltransferase	At4g10760	353B10
E2_5RE_B	Esch5:35S-rolC#5	T-DNA	Receptor-like kinase	At5g38280	254G07
SK316CUP	Esch5:rbcS-rolC# 11	T-DNA	potassium channel	At4g22200	152C05
SK467	Esch5:35S-*Ac*-rolC#2	Ac	putative phospho-ribosylanthranilate transferase	At4g11610	004G08 147G08 234C05
SK510	Esch5:35S-*Ac*-rolC#3	Ac	Hypothetical protein	At4g03540	301G06

Table 4. Yield of microspore culture 2001 and 2002: Induction of callogenesis.

Donor tree	Buds [a] processed	Responsive buds	Calli (>1 mm)	Calli/Responsive buds
Year 2001				
Aue 1	8	4	104	26
Aue 2	3	2	8	4
Total of 2001	11	6	112	19
Year 2002				
Aue 1	14	8	358	45
Aue 2	19	9	1017	113
Total of 2002	33	17	1375	81

[a] Only buds without infection were considered.

Table 5. Ploidy level and 5 microsatellite loci of 66 regenerative calli obtained in 2002.

	Haploids	Diploids	Tetraploids
Homozygotic loci	5	52	2
Heterozygotic loci as the donor trees		2	1
Homo-and heterozygotic loci		4	
In total	5	58	3

are double-haploid. However, the regenerative callus line M22-1c5 is still haploid in *in vitro* culture for so far 18 months.

In the year 2002, 1375 calli were subcultured on hormone containing media. More than one third (Table 4). No significant increase in frequency of green calli formation was achieved with media supplemented with sucrose or maltose as carbohydrate source. Most of the transparent calli turned brownish but some formed green cores of solid

Figure 1. Regenerants from the embryo M22-1c5 (DEUTSCH *et al.* 2004).

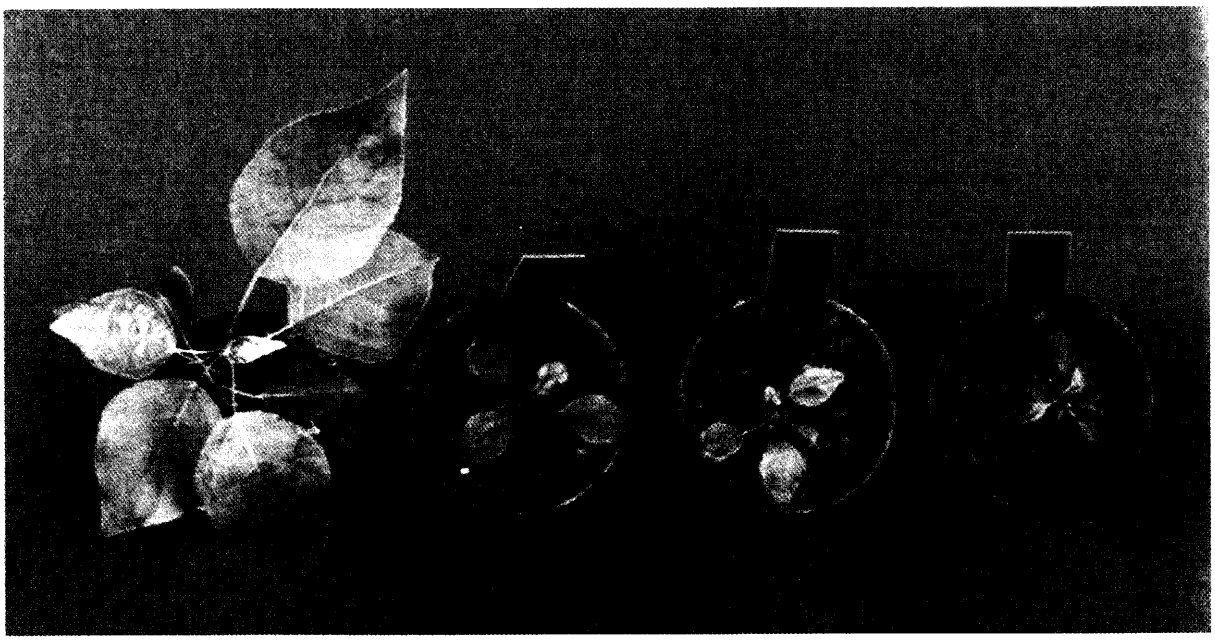

Figure 2. Regenerants from the embryo M22-1c5 in soil. The large plant (left) is a control diploid plant regenerated *in vitro* from the "father" poplar (DEUTSCH *et al.* 2004).

brownish but some formed green cores of solid

regenerative cells and shoots. So far, twelve lines have rooted *in vitro*. Flow cytometer measurements of 66 regenerative calli obtained in 2002 revealed that five calli were haploid, 58 diploid (including two calli showing a second peak at n and two calli at 4n),

two tetraploid, and one callus was aneuploid (Table 5). All regenerative calli analysed in flow cytometer measurements were investigated for their haploid origin using five microsatellites- (SSR-) markers. The microsatellites were heterozygous in the "father" trees 'Aue 1' and 'Aue 2'. All haploid and most diploid and tetraploid calli were homozygous for either alleles, but some diploids and tetraploids revealed heterozygous genomes (Table 5).

One haploid regenerative callus line (FD25) regenerated also haploid plants. The line FD25 is well growing in *in-vitro* culture despite its haploid status. At present (a) measurements are repeated to confirm long-term haploidy in the regenerative callus line, and (b) regeneration experiments from leaves of regenerated haploid plants are underway. The haploid plantlets are currently multiplied for transformation experiments.

Transformation of poplar haploid lines M22-1c5

Transformation experiments using callus and leaf tissue from the haploid clone M22-1c5 with the 35S-*Ac-rolC* construct were performed. Two stable transgenic haploid *Populus* lines carrying *Ac* were obtained that are being tested for *Ac*-excision (Table 6). One *Ac*-transgenic regenerative callus line is haploid, the second is double haploid. Regeneration of *Ac*-transgenic plantlets is in progress. The haploid M22-1c5#23-1 transgenic line was investigated for *Ac* transposition. *Ac* insertion in one case has been found in the *npt-II* gene of the T-DNA, however, Southern analysis are underway to confirm this result. Using the haploid regenerative callus FD25 so far three transgenic calli carrying the 35S-*Ac-rolC* gene construct were obtained (Table 6).

DISCUSSION

T-DNA integration in *Populus*

In order to test previously published hypothesis about T-DNA integration in plants and to obtain insight into the mechanism of T-DNA integration in a long lived tree species we analysed 30 aspen and hybrid aspen transgenic lines. In total, 27 right T-DNA/plant junctions, 20 left T-DNA/plant junctions, and 10 target insertions from control plants were obtained. The right border of the T-DNA was conserved up to the cleavage site in 18 out of 27 transgenic aspen lines. This finding is consistent with previous reports in *Arabidopsis* and tobacco (GHEYSEN *et al.* 1987; GHEYSEN *et al.* 1991; MAYERHOFER *et al.* 1991), and to our earlier results describing T-DNA integration in four transgenic aspen lines (FLADUNG 1999a) as well as T-DNA repeats in aspen (KUMAR & FLADUNG 2000). However, in nine junctions the right border repeat was missing and the number of nucleotides deleted varied from 2-23 bp. We did not observe any major changes in the left border repeat and nucleotides from the left border repeat were present in 19 transgenic line out of the 20 analysed. The deletions on the left border varied from 2-24 bp. In nine transgenic lines the left border repeat was found conserved containing 13 nucleotides from the 24 bp border repeat.

Taking the results of the T-DNA ends in the present study together, there are no major differences for the terminal nucleotide deletions between the left and right T-DNA ends integrated into host plant genome. These findings are not consistent to previous reports in crop plants where analysis of 15 independent integration events in *Arabidopsis* and tobacco showed that right T-DNA border was more frequently conserved compared to the left border postulating T-DNA-specific ligation of the right end (MAYERHOFER *et al.* 1991; reviewed in TINLAND & HOHN, 1995; TINLAND, 1996).

Table 6. Transgenic M22-1c5 and FD25 lines carrying the 35S-*Ac-rolC* gene construct.

Transgenic line	Agro strain	Ploidy level	Regenerated plants
M22-1c5#23-1	35S-*Ac-rolC*	haploid	yes
M22-1c5#23-2	35S-*Ac-rolC*	double haploid	No
FD25-23-2	35S-*Ac-rolC*	haploid	In regeneration process
FD25-23-3	35S-*Ac-rolC*	haploid	Yes
FD25-23-5	35S-*Ac-rolC*	haploid	Yes

Transposition of Ac *in Aspen*

Several independent transgenic aspen lines carrying the 35S-*Ac-rolC* and rbcS-*Ac-rolC* constructs were obtained (FLADUNG *et al.* 1997). Transposition of *Ac* is indicated in about half of the lines obtained by the appearance of light-green leaf sectors indicating *rolC* expression due to *Ac* excision. However, transposition of mobile elements in general comprises two different events: excision of the elements as well as their subsequent reintegration into new genomic positions. An excision event generates an empty donor site frequently with small sequence alteration. DNA extracted from the light-green sectors was used for the PCR amplification of the empty donor site using primer located at 35S promoter and *rolC* gene. A 1.5 kb fragment was obtained giving first molecular evidence of primary *Ac* excision in aspen (FLADUNG & AHUJA, 1997). Sequencing of these fragments revealed the precise *Ac* excision for the majority of the events analysed. However, very small modifications were observed in some of the excision events (FLADUNG 1999b). Further, in northern analyses a *rolC*-specific transcript was detected in light-green leaf sectors, and no *rolC*-specific signal was observed form the dark green leaf tissue (FLADUNG & AHUJA 1997).

In order to confirm reintegration of the *Ac* element in aspen genome and to obtain FSTs, DNA from the light green leaf sectors from three different transgenic lines, carrying a variety of insertions, was used in TAIL-PCR to amplify aspen genomic sequences flanking the *Ac* insertions (KUMAR & FLADUNG, 2003). The analysis of these sequences revealed the exact reconstitution of the *Ac* element including Terminal Inverted Repeats (TIRs) flanked immediately by new genomic sequences for majority of the reintegration events. However, besides these correct excision/reintegration events, a few aberrant transpositions were also observed in all three transgenic lines analysed. The new genomic sequences in these events are flanked by the additional sequences rather than TIR of the *Ac* element. From these results it appears that *Ac* is excised not precisely from the TIR but aberrantly from the sequence flanking the transposon. Aberrant transposition of plant transposable elements has been well documented in the literature (SAEDLER & NEVERS, 1985).

In total, 75 FSTs were obtained and subjected to BLASTx searches (ALTSCHUL *et al.* 1990, 1997) of the GenomeNet Kyoto, Japan. About one third (22/75) of all FSTs identified are significantly similar to sequences represented in public databases (KUMAR & FLADUNG, 2003). FSTs in predicted genes like "probable 24-sterol C-methyltransferase or "Acetyl-CoA:benzylalcohol acet-yltransferase" can provide information on the unique function of these genes in aspen. The frequency of *Ac* insertion in or near predicted genes appears to be lower while compared to similar analyses made in *Arabidopsis* (PARINOV *et al.* 1999), rice (GRECO *et al.*, 2001), tomato (MEISSNER *et al.*, 2000) and maize (Cowperthwaite *et al.*, 2002). About one half of the total insertions were found to disrupt genes encoding proteins in these studies. The per cent of *Ac* insertions hitting known genes in our analysis might be an underestimate keeping in view little information available about *Populus* genome in the public databases. This is obvious from the fact that majority of the BLASTx hits we obtained are from *Arabidopsis*. However, we can expect DNA sequences disrupted by these insertions to become available as the poplar genome sequencing projects progress.

T-DNA versus Ac integration

A similar analysis was also made for FSTs from the T-DNA tagged transgenic aspen lines (KUMAR & FLADUNG, 2003). We analysed 32 transgenic lines, 15 for both left and right borders' flanking genomic sequences while others for either left or right border genomic sequences. The BLASTx searches for the sequences obtained revealed 5 sequences that were similar to known or predicted genes. This suggests that only 16% of the T-DNA insertions landed into or near known or predicted genes. This frequency is about one half of the frequency of *Ac* insertion hitting coding sequences in the same aspen system. Hence, the *Ac* system seems to be more effective for gene tagging and promoter trapping studies in aspen. This is despite the fact that average length of the genomic sequence analysed for T-DNA was 400 nucleotides compared to 200 nucleotides for the *Ac* tags. Further, for half of the T-DNA tagged lines sequences were available from both the ends for the BLASTx searches.

Induction of haploid poplar lines

An *in vitro*-regeneration system from isolated immature pollen of a black poplar hybrid (*P. nigra* L. ' hybrid) was established in our lab. Evidence was given for at least one long-term stable haploid regenerative callus as well as for the haploid origin of most diploid regenerated calli and plantlets using microsatellite markers (DEUTSCH *et al.* 2004). As haploid material has become more interesting for tree genomics because of established transformation techniques and modern molecular diagnostic tools,

the production of haploids will have a great impact for future developments in forest biotechnology and forest tree breeding.

Four different protocols to induce haploidy in poplar have been followed. Although evidence was obtained that haploid tissues was present in plants obtained following pseudogamy, no long-term haploidy was detected. Similar results were found for the other methods. Only regenerants resulting from microspore and immature pollen culture revealed haploid for more than 20 months of development so far (DEUTSCH *et al.* 2004).

To test haploidy in regenerants of anther cultures in trees, isozymes were used first (STOEHR & ZSUFFA 1990 (*Populus*), MÜLLER-STARCK & JÖRGENSEN 1991 (*Quercus*), BALDURSSON *et al.* 1993 (*Populus*). However, microsatellites have become the marker of choice due to a much higher degree of heterozygosity than allozymes have ever exhibited. Thus, they have been developed an elegant tool for individual identification, paternity analysis, genome mapping and many more applications (review in: VENDRAMIN *et al.* 2002).

Maintenance and use of haploid *Populus*

For anther culture of *Populus* BALDURSSON and AHUJA (1996) reported that haploids double their chromosomes spontaneously and very early in plant development. Our results confirm this observation because the majority of regenerated calli was already doubled haploid when analysed by flow cytometry and microsatellite markers. To obviate diploidization during plant development, especially rooting, the regenerative calli have been cultured on hormone containing media. Additionally such calli are in the appropriate stage for transformation as their regeneration potential is already known.

However, six out of eight regenerative calli have been stable haploid for six to 20 months to date. The haploid regenerative callus lines M22-1c5 and F025 were employed for transformation, and transgenic lines were obtained (DEUTSCH *et al.* 2004). Few of the transgenic lines revealed still haploid and will be used for "functional genomics" approaches.

We have shown that *Ac* is actively reinserted, frequently into or near coding regions in *Populus* and, therefore, can be used for gene tagging studies. To overcome the barrier of long vegetative phase in trees we are testing an alternative gene tagging strategy based on a self-stabilizing *Ac* derivative (SCHMITZ & THERES, 1994; SUZUKI *et al.*, 2001) using morphologically detectable and/or antibiotic sensitive excision marker. The somatic-tagged plant population will be regenerated from the somatic leaf sectors indicating *Ac* excision. The tagged plants can be screened for the dominant gain-of-function mutant phenotypes in the same generation. For the loss-of-function mutants we will be using haploid aspen lines which has been described in this paper. The method when found successful could also be extended to other tree species.

Links between Functional Genomics and Population Genetics

Functional Genomics and Population Genetics are strong disciplines each, however, exchange of methods and knowledge are very poor until now. For the future a challenge would be to bring both disciplines together. On the basis of a very few statements and questions links between Functional Genomics and Population Genetics are obviously:

- Search for genomic sequences involved in adaptation processes (genes, promoters, regulatory sequences?)
- Role of Single Nucleotide Polymorphisms (SNPs) for observed variations in adaptation capacity
- Do play transposons, retrotransposons a role for adaptive diversity?
- What are adaptive QTLs?
- Can we use the natural gene pool for tree improvement
- Are risk assessment studies with transgenic trees feasible (gene flow)?

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