Chemoecology 12:177–183 (2002)
0937–7409/02/040177–07

CHEMOECOLOGY

Agrobacterium-mediated transformation of *Nicotiana attenuata*, a model ecological expression system

Tamara Krügel, Michelle Lim, Klaus Gase, Rayko Halitschke and Ian T. Baldwin

Department of Molecular Ecology, Max-Planck-Institute for Chemical Ecology, Winzerlaer Strasse 10, D-Jena 07745, Germany

Summary. Research into the genetic basis of the ecological sophistication of plants is hampered by the availability of transformable systems with a wealth of well-described ecological interactions. We present an *Agrobacterium*-mediated transformation system for the model ecological expression system, *Nicotiana attenuata*, a native tobacco that occupies the post-fire niche in the Great Basin Desert of North America. We describe a transformation vector and a transformation procedure that differs from the standard cultivated tobacco transformation protocols in its use of selectable markers, explants, media and cultivation conditions. We illustrate its utility in the transformations with genes coding for key enzymes in the oxylipin cascade (lipoxygenase, allene oxide synthase, hydroperoxide lyase) in antisense orientations and present high-throughput screens useful for the detection of altered phenotypes for the oxylipin cascade (green leaf volatiles and jasmonic acid after wounding).

Key words. *Nicotiana attenuata* – *Agrobacterium* transformation – lipoxygenase – allene oxide synthase – hydroperoxide lyase – green leaf volatiles

Introduction

Transformation is rapidly becoming one of the most important tools for the 'post-genomics' era in plant biology. The ability to silence or over-express individual genes remains the most robust tool for determining gene function and has catalyzed dramatic advances in plant biotechnology (Dixon & Arntzen 1997; Pereira 2000). Advances in understanding the traits responsible for the ecological function of plants (in pollinator-, herbivore-, pathogen-interactions, etc.) are similarly dependent on the ability to manipulate the expression of individual genes (Kessler & Baldwin 2002; Roda & Baldwin 2002). However, the model plant systems that are currently readily transformable are frequently not the optimal choices for ecological research. Crop plants have been selected for yield maximization, a process that has likely altered ecological responses and *Arabidopsis thaliana* (L.) Heynh. lacks important ecological interactions, which can be analyzed in near relatives (Mitchell-Olds 2001) but those are currently not readily transformable. Cultivated tobacco, *Nicotiana tabacum* L., was one of the first species to be routinely transformed, but this tetraploid species has never been found outside of human cultivation and has clearly been under strong selection for the particular requirements of smokers. Its diploid relative, *Nicotiana attenuata* Torrey ex Watson, has emerged as a model system for understanding the molecular basis of ecological sophistication in the "agricultural niche" (Baldwin 2001), a niche characterized by synchronized seed germination into nitrogen-rich soils which, in turn, results in intense intra-specific competition, selection for rapid growth and inducible responses to biotic challenges. As a result of *N. attenuata*'s unusual fire-chasing behavior, which, in turn is determined by its unusual seed germination behavior (Preston & Baldwin 2000), this species has evolved in exactly this niche.

Unfortunately, *N. attenuata* belongs to the group of native *Nicotiana* species which rapidly 'habituates' after a short exposure to exogenous hormones either before or after *Agrobacterium*-mediated transformation, and grows vigorously for long periods of time as callus without shoot or root differentiation on media without hormone supplements (Bogani *et al*. 1997). As a consequence of this habituation behavior, the standard procedure for transformation of cultivated tobacco (Gallois & Marinho 1995; Hall 1991; Horsch 1988) does not work for this species. Here we describe a procedure for the efficient transformation and rapid regeneration of this unique model ecological expression system. To illustrate the utility of this transformation procedure for ecological research, we provide examples of transformations that silenced key enzymes in the oxilipin signal cascade (allene oxide synthase: AOS; lipoxygenase: LOX; and hydroperoxide lyase: HPL), a signal transduction cascade that strongly influences *N. attenuata*'s responses to herbivore attack (Baldwin 2001). In a high-throughput (HTP) phenotypic screen, we analyzed wound-induced jasmonate (JA) levels and green leaf volatile (GLV) releases of plants transformed with AOS, HPL, and LOX, in an antisense orientation, to identify lines in which the transformation caused the desired phenotypic effect.

Materials and methods

Materials: Chemicals were from Sigma, oligonucleotides used as primers for PCR and sequencing were synthesized by Sigma-ARK,

Correspondence to: Ian T. Baldwin, e-mail: baldwin@ice.mpg.de

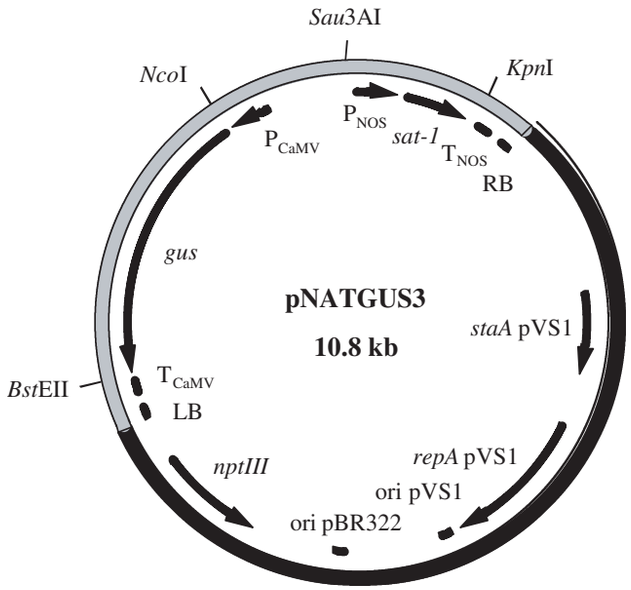

Fig. 1 *Nicotiana attenuata* transformation vector pNATGUS3 with *sat-1* as plant selectable marker gene. DNA to be transcribed can be inserted as *Nco*I-*Bst*EII fragment. Functional elements on T-DNA (grey): LB/RB, left/right border of T-DNA; P_{CaMV}/T_{CaMV}, 35S promoter/terminator of cauliflower mosaic virus; P_{NOS}/T_{NOS}, promoter/terminator of nopaline synthase gene; *sat-1*, nourseothricin resistance gene; *gusA*, *E coli* beta-glucuronidase gene containing intron 1 of *Ricinus communis* catalase gene. Genes outside T-DNA are from pCAMBIA-1301.

DNA modifying enzymes were obtained from New England Biolabs, RNase A was from Machery & Nagel.

Vector Construction: The streptothricin-acetyl-transferase gene (*sat-1*, GeneBank accession number X15995), present on pXGSAT, was PCR amplified with primers SAT3-40 (5'-GGATCTGGATCGTTTCGCATGAAGATTTCGGTGATCCCTG-3') and SAT4-40 (5'-GCGGCGGGTACCGGGCCCGTTAGGCGTCATCCTGTGCTCC-3'). Using primers PBI3-21 (5'-GTTCAATCGGACCAGCGGAGG-3') and SAT2-40 (5'-CAGGGATCACCGAAATCTTCATGCGAAACGATCCAGATCC-3') the nopaline synthase (NOS) promoter sequence of pBI121 (Jefferson *et al*. 1987) was amplified. In a subsequent polymerase reaction, in which both resulting PCR products served as template for each other, a fusion fragment containing *sat-1* downstream from the NOS promoter was synthesized, digested with *Kpn*I and cloned into pUC19, which had been cut with *Sma*I and *Kpn*I, to produce pUCNAT2. The *Nco*I-*Bst*EII *gusA* gene fragment of pCAMBIA-1301 (GeneBank accession number AF234297) was replaced by the adequately digested PCR fragment obtained with primers PMT6-36 (5'-GCGGCGGGTCACCGGTACCAACACAAATGGCTCTAC-3'), PMT7-31 (5'-GCGGCGCCATGGAGCCCTTAAAGACTTGACG-3') and pBI121-ASPMT (Voelckel *et al*. 2001) as template. The 9.0 kb partial *Kpn*I-*Bam*HI fragment of the resulting plasmid pCAMPMT1 served as cloning vector for the 0.9 kb *Kpn*I-*Bcl*I fragment from pUCNAT2. The resulting plant transformation vector pCAMNAT1 contained two selectable plant resistance markers: *hgh* (K01193) *or aph(4)* (V01499) driven by the CaMV 35S promoter and *sat-1* driven by the NOS promoter.

The 8.8 kb partial *Xho*I-*Aat*II-fragment of pCAMNAT1 was ligated with the annealed primers ASV1-23 (5'-GGCCATGGCTGCAGGGTGACCGG-3') and ASV2-31 (5'-TCGACCGGTCACCCTGCAGCCATGGCCACGT-3'). The resulting plasmid pCAMNAT2 was cut with *Xho*I, blunt ended with mung bean nuclease and partially recut with *Bst*EII. The generated 8.8 kb fragment served as vector for cloning the 2.1 kb *gusA* fragment obtained from pCAMBIA-1301 by *Nco*I digestion, blunt ending with T4 DNA polymerase and *Bst*EII digestion. The surplus *Bst*EII site near the 3' end of *sat-1* was removed from the resulting plasmid pNATGUS1 by partial *Bst*EII digestion, T4 DNA polymerase treatment and subsequent recircularization. The obtained cloning vector pNATGUS3 (Fig. 1) carried *sat-1* as plant selectable marker and enabled the transcription of transgenes in *N. attenuata*. For the construction of the antisense gene silencing vectors PCR fragments of *N. attenuata hpl* [primers: HPL1-34 (5'-GCGGCGGGTCACCACACTCATGGCGAAAATGATG-3'), HPL2-33 (5'-GCGGCGCCATGGCACAGGTGGACTAAGTCTAAG-3'); template: *hpl* cDNA cloned on plasmid pSKIIHPL, unpublished result], *N. attenuata lox* [primers: LOX1-34 (5'-GCGGCGGGTCACCGGAACAAGAACAAGGAAGATC-3'), LOX2-32 (5'-GCGGCGCCATGGCTACATGTTACTCCAGGGCC-3'); template: *lox* cDNA cloned on plasmid pNaLoxLSA-II, unpublished result] and *N. attenuata aos* [GeneBank accession number AJ295274; primers: AOS1-35 (5'-GCGGCGGGTCACCGTGTTCTTTCTTATCTTGATCC-3'), AOS2-31 (5'-GCGGCGCCATGGAAGTAGGAAAACCAAGAAC-3'), template: chromosomal *N. attenuata* DNA] were synthesized, digested with *Nco*I and *Bst*EII (*aos* fragment partially) and cloned in pNATGUS3 cut with the same enzymes, yielding pNATHPL1, pNATLOX1 and pNATAOS2, respectively.

Plant DNA Extraction: 200 mg plant material were flash-frozen in liquid nitrogen, ground to powder and suspended in 750 µl of 100 mM Tris/50 mM EDTA (pH 8.0), containing 250 µg/ml RNase A. Eight µl liquid laundry detergent (Ariel, Procter & Gamble, Schwalbach, Germany) were added. After 60 min incubation at 60ºC and subsequent addition of 80 µl of 5 M NaCl, the suspension was centrifuged for 5 min at 16,000 × g. The supernatant was removed carefully and extracted with phenol/chloroform. The DNA was precipitated with 600 µl isopropanol, pelleted 5 min by centrifugation at 16,000 × g, washed with 200 µl 70% ethanol and dissolved in 50 µl of water.

Plant material and Agrobacterium suspension cultures: *N. attenuata* seeds (derived from a 1988 collection from a native population in Washington County, Utah, USA, and subsequently selfed for 6 generations) were used for all transformations and subsequent analysis with wild type plants. Seeds were sterilized for 7 min in 5 ml aqueous solution of 0.1 g dichloroisocyanuric acid (DCCA: Sigma, St. Louis, MO, USA), supplemented with 50 µl of 0.5% (v/v) Tween-20 (Merck, Darmstadt, Germany). Seeds were washed 3 times with sterile water before incubation for 1 h in 5 ml sterile liquid smoke (House of Herbs, Inc.; Passaic, New Jersey, USA) solution, 50 × diluted in water and supplemented with 50 µl of 0.1 M gibberellic acid, GA_3 (Roth, Karlsruhe, Germany). After this treatment, seeds were washed 3 times with sterile water and 25 seeds were transferred individually to a petri dish containing germination medium [Gamborg's B5 medium with minimal organics: (Sigma) and 0.6% (w/v) phytagel (Sigma)]. The plates were maintained in a growth chamber (Percival, Perry Iowa, USA) at 26ºC/16h h 155 µm/s/m²-(measured with a LCA3: ADC, Hoddesdon, England) light, 24ºC/8 h dark. *A. tumefaciens* (strain LBA 4404: Life Technologies-Gibco BRL, Eggenstein, Germany) was maintained on solidified yeast-peptone-NaCl (YEP) medium containing 50 mg/l kanamycin at 4ºC in the dark. Two days before transformation, the desired *A. tumefaciens* strain was grown overnight at 28ºC in YEP medium with 25 mg/l kanamycin. The liquid culture was spread onto solid YEP medium containing 50 mg/l kanamycin and grown overnight at 26ºC. The bacteria were washed with 3 ml liquid MS medium containing 0.02 mg/l indole-3-acetic acid (IAA: Duchefa, Netherlands) and 1 mg/l 6-benzylaminopurine (BAP: Duchefa) and the optical density (OD) was measured at 600 nm. An OD of 0.6 proved to be optimal for transformation. Since hygromycin (Duchefa) is labile, all media containing this antibiotic were freshly prepared just before use, and the media was cooled to 56ºC before the addition of hygromycin. Nourseothricin (NTC: Werner BioAgents, Jena, Germany) is significantly more stable, and media containing NTC can be stored for at least two weeks until use.

Transformation procedure: An extensive analysis of callus derived from different plant material revealed that optimal transformation and regeneration results were obtained when hypocotyls of 8-10 day old seedlings, which have a high potential for cell division, were used for transformation. Each seedling was cut below

Table 1 Steps in *Agrobacterium*-mediated transformation of *Nicotiana attenuata*. Average duration, media composition used, and culture conditions of each stage

Time required (days)	Type of medium	Medium content	Conditions
3	Co-cultivation	[#]Basal medium, 0.02 mg/l IAA, 1 mg/l BAP	Dark, 26°C
14–21	Callus induction	[#]Basal medium, 0.02 mg/l IAA, 1 mg/l BAP, [§]selectable antibiotic, 125 mg/l timentin	16 h day, 30°C; 8 h dark, 26°C
14–21	Regeneration	[#]Basal medium, 0.5 mg/l BAP, selectable antibiotic, 125 mg/l timentin	16 h day, 30°C; 8 h dark, 30°C
14–21	Maturation	[#]Basal medium, selectable antibiotic, 125 mg/l timentin	16 h day, 28°C; 8 h dark, 26°C
21	Rooting	1 × Peter's Hydro-Sol 0.292 g/l, 0.6% (w/v) plant agar, [*]vitamin mixture	16 h day, 26°C; 8 h dark, 24°C

[*]Vitamin mixture:	glycine	2.0 mg/l
	myoinositol	100 mg/l
	nicotinic acid	0.5 mg/l
	pyridoxine HCL	0.5 mg/l
	thiamine	0.1 mg/l
[#]Basal medium:	MS incl Vitamins	4.41 g/l
	sucrose	3% (w/v)
	phytagel	0.3% (w/v)

[§]Selectable antibiotic: hygromycin (20 mg/l) or NTC (50–75 mg/l)

the apical meristem and above the roots. The excised hypocotyl was subsequently cut into 2-3 (ca. 3 mm) pieces and placed on co-cultivation medium (Table 1). Before each cut, the tip of the scalpel was dipped into the *Agrobacterium* suspension. Clean cuts were essential for good infection. After 3 days of co-cultivation with *Agrobacterium*, explants were transferred to callus induction medium (Table 1) containing the antibiotic timentin (*ticarcillin* disodium/potassium clavulanate: Duchefa) to inhibit growth of *Agrobacterium*. After 2-3 weeks, when explants had developed callus with green shoot primodia, the callus was sub-cultured onto regeneration medium (Table 1). When light green shoots started to develop from the shoot primodia (typically within 2 weeks after transfer to the regeneration media), the callus with shoots was transferred to the maturation medium (Table 1), and subsequently sub-cultured every 3 weeks until plantlets were formed. These plantlets were separated into single plants and cultured on rooting medium (Table 1), which contained no antibiotics. At this stage, removal of all traces of callus from the base of the plantlet was essential. Plantlets were sub-cultured on rooting media every 3 weeks until roots appeared, after which plants were carefully removed from the gel and planted into soil (30 l peat moss, 15 l Vermiculite, 125 ml ground limestone, 125 ml bone meal) in Magenta boxes (77 × 77 × 77 mm) maintained in growth chambers at 24ºC/16 h light (200-250 µm/s/m^2), 24ºC/8 h dark. Once established, plants were transferred to 2 l pots in soil [60 l peat moss, 30 l Vermiculite, 30 l Perlite, 250 ml ground limestone, 250 ml bone meal and 400 ml Osmocote 14:14:14 pellets (N:P:K; 2-3 month release rate)] and grown in the glasshouse at 26-28ºC under 16 h supplemental light from Philips Sun-T Agro 400 or 600W Na lights.

Confirmation of transformation: PCRs with chromosomal DNA of potentially transformed plants as template and primer pairs HYG1-18 (5'-CCGGATCGGACGATTGCG-3'), HYG2-18 (5'-CTGACGGACAATGGCCGC-3') for *hgh/aph (4)* and NAT1-18 (5'-CTCTGCTTGCTATGGCGC-3'), NAT2-18 (5'-CGTCATCCTGTGCTCCCG-3') for *sat-1* were used to verify the insertion of the resistance marker and used as an indicator of successfully transformed plants. In addition, leaf disks from T_0 plants were tested for their resistance to the antibiotic under which they were selected. This leaf disc selection procedure provided highly reliable results with a minimum effort and is therefore described in detail. A whole leaf was excised at the petiole from putatively transformed plants and immediately placed into water. The leaf was sterilized for 7 min in DCCA and washed 3 times with sterile water. The leaf was cut into small pieces (ca. 0.5 cm × 0.5 cm) and incubated on MS medium (Murashige & Skoog, 1962) supplemented with 0.1 mg/l IAA, 1 mg/l BAP, selectable antibiotic (250 mg/l NTC or 35 mg/l hygromycin), and 125 mg/l timentin for at least 7 days. Concomitant with each experiment, leaves of wild type plants, which invariably died slowly and developed no callus, were used as a control. The average transformation efficiency, determined from 175 regenerated and evaluated plants was 97%.

Progeny selection: The number of independent loci at which integration occurred can be estimated by the segregation ratio of resistant to sensitive seedlings in the progeny and we used both seed and tissue selection to determine segregation ratios. T_1 seeds from plants transformed with constructs containing *hpt-II* were sterilized and germinated (as above) on Gamborg's B5 medium with 35 mg/l hygromycin. Since roots and shoots appear to have different sensitivities to NTC, we were not able to select seedlings with this antibiotic, but tissue selection worked well for both antibiotics. For tissue selection, half of one cotelydon of 8-10 day old seedlings was removed with a pair of sterile scissors and placed on MS medium supplemented with 0.1 mg/l IAA, 1 mg/l BAP, 250 mg/l NTC or 25 mg/l hygromycin, and 125 mg/l timentin for callus induction at 26ºC under 16 h 125 µm/s/m^2 light. If vigorous callus growth was observed within 7 days, the seedling from which the cotyledon was excised, planted into soil and gradually adapted to the high light levels of the glasshouse. This procedure identified and rescued homo- and heterozygous plants and subsequent crossing and selection was used to identify homozygous plants.

AOS, HPL, and LOX HTP phenotypic screens: Plants silenced in 13-LOX, (because it supplies substrates, 13-hydroperoxides of linoleic and linolenic acid, for AOS and HPL: Fig. 3B) and those silenced in AOS and HPL, were identified by their reduced ability to produce JA and release the GLVs, hexanal and (*Z*)-3-hexenal, after wounding. T_1 plants from independently transformed as-LOX, as-AOS, and as-HPL lines were screened. Wound-induced levels of JA and GLVs of five plants per line for a total of 515 plants from 103 independent lines (one wild type, 35 as-LOX, 30 as-AOS, and 37 as-HPL) were analyzed.

Wound-induced JA analysis: The second fully-developed leaf of rosette-stage plants was wounded with a pattern wheel producing 3 rows of puncture wounds on each leaf half. The wounded

leaves of wild-type *N. attenuata* plants were harvested at the different time points (5; 15; 35; 45; 60; 90; 120; 180; and 300 min) after wounding and immediately frozen in liquid nitrogen. The 0 min time point indicates harvests of leaves from unwounded control plants. The leaves of as-AOS and as-LOX transformed plants were harvested 35 min after the treatment. Samples were analyzed by GC-MS after addition of 172 ng of $^{13}C_{1,2}$-JA as an internal standard and sample preparation as described by Schittko *et al*. (2000) with the following modifications of the extraction procedure. Leaf samples were homogenized in the extraction buffer with the FastPrep extraction system FP120 (Savant Instruments, Holbrook, NY, USA). Tissue was homogenized by reciprocating shaking at 6.0 m/sec for 90 sec in extraction tubes containing 900 mg of lysing matrix (BIO 101, Vista, CA, USA).

Volatile analysis: Volatiles released into the headspace of a leaf during the first minute after wounding were collected and analyzed with a portable gas analyzer. The zNose™ (EST, Newbury Park, CA, USA) is a portable gas chromatograph equipped with an internal sample pump, a pre-concentration trap, a 1 m stainless column (DB-5 stationary phase) and a surface acoustic wave (SAW) detector. The short column length allows rapid chromatographic separation of analytes and is therefore highly suitable for HTP analysis of large sample numbers. The unique sensitivity of the SAW detector obviates requirements for long preconcentration periods and the time-consuming sample preparation of conventional trapping-desorption methods and allows for very short (2 min) sampling intervals.

The treated leaf was placed for 30 sec in an open volatile collection chamber (125 ml) immediately after wounding (see JA analysis) and volatiles were collected by adsorptive trapping on the internal trap of the zNose™ for 30 sec. Sample flow was set to 30 ml/min. Column temperature was maintained at 45°C for 1 sec and raised to 175°C at 5°C/sec. The SAW detector temperature was set to 45°C. The identity of hexanal and (*Z*)-3-hexenal was verified by co-chromatography with synthetic standards.

Results and discussion

The principle difficulty of the transformation and regeneration of native *Nicotiana* species is their taxonomically-determined proclivity to rapidly 'habituate' and grow vigorously for long periods of time without shoot or root differentiation after a short exposure to exogenous hormones (Bogani *et al*. 1997). If the published procedures for *Nicotiana tabacum* callus induction and regeneration (Murashige & Skoog 1962; Zambryski *et al*. 1983; Guerineau *et al*. 1990) are used for *N. attenuata*, no regeneration from callus was observed after more than 4 years of vigorous callus growth. The aggressive addition of auxin-antagonists and transport inhibitors (p-chlorophenoxyisobutyric acid and triiodobenzoic acid) and the complete removal of exogenous auxins did not result in regeneration (data not shown). Two considerations are essential for *N. attenuata* regeneration: the type and length of exposure to auxins and the choice of explant. A minimal exposure (0.02 mg/l, Fig. 2; Table 1) to short-lived auxins (IAA and not 1-naphtylacetic acid) was sufficient to stimulate callus growth and resulted in the highest regeneration rates. This short exposure period produced friable callus of low density that rapidly initiated shoot primordia. High temperatures (30ºC) as well as subculturing to auxin–free regeneration media as soon as shoot primordia were observed, were also required at this stage (Table 1). Since the timing of auxin exposure is critical, it was essential to select an explant tissue that produced consistent regeneration. Only explants from the hypocotyls of germinating seeds (and not leaves, cotyledons or roots) provided

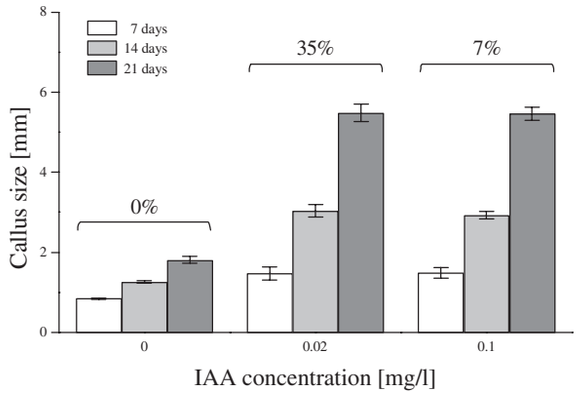

Fig. 2 Size of callus (mean ± SE of 3 independent experiments) regenerated from hypocotyls of seedlings transformed with pCambia1301. Bars indicate callus size at 7, 14, and 21 days after transfer to MS media containing 20 mg/l hygromycin (see Materials and Methods). Numbers in the brackets indicate regeneration rates of the respective experiments, which were determined at day 28 after transformation

reliable material (data not shown), and this reliable response could be due to the particular hormonal balance of this tissue.

Other considerations proved to be important for attaining a high frequency of transformation and regeneration. Using the lowest concentration of selectable antibiotic dramatically increased regeneration rates. The values recommended in Table 1 represent an optimization of maximized regeneration rate and a minimization of the production of non-transformed escapes or chimerical plants. Given hygromycin's lability, the maintenance of consistent selection proved more difficult with this antibiotic than with NTC, but with experience, both antibiotics produced acceptable results (Fig. 3A). Hygromycin selection, on the other hand, has the advantage of being useful in seedling screens that facilitate the analysis of segregation ratios in progeny. Avoidance of the apical meristem in preparing explants proved to be essential in reducing the production of escapes, for this tissue was invariably resistant to *Agrobacterium* transformation. We worked primarily with the LBA4404 strain of *Agrobacterium,* which also works well with other *Nicotiana* species. The choice of antibiotic to control *Agrobacterium* infestation after transformation dramatically influenced regeneration. We found that timentin, at the concentrations listed in Table 1, allowed for regeneration, while others (cefotaxime, gentamicin and vancomycin) dramatically inhibited regeneration. Because of the delicate nature of hypocotyls, we obtained superior results when *Agrobacterium* was introduced on the scalpel during cutting, rather than dipping the explant in the *Agrobacterium* culture. We selected a single regenerating plant from each hypocotyl to guarantee that each plant represented an independent transformation event.

Our analysis of 5 plants from each of 102 independently transformed lines with as-LOX, as-AOS, and as-HPL containing constructs underscored the importance of appropriate HTP screens for ecological research. Only 25 (71%) of the 35 as-LOX lines and 2 (5.4%) of the 37 as-AOS lines exhibited more than an 80% reduction in wound-induced

A

	Antisense expressed gene				
	LOX	AOS		HPL	
Selectable antibiotic	NTC	NTC	HYGRO	NTC	HYGRO
Number of explants	120	120	120	120	120
Number of T_0 plants	62	13	29	37	24
Number of lines	35	11	20	22	9
Lines examined	34	7	8	16	4
Number of transgenic lines*	33	7	8	16	4

*confirmed by antibiotic selection and PCR

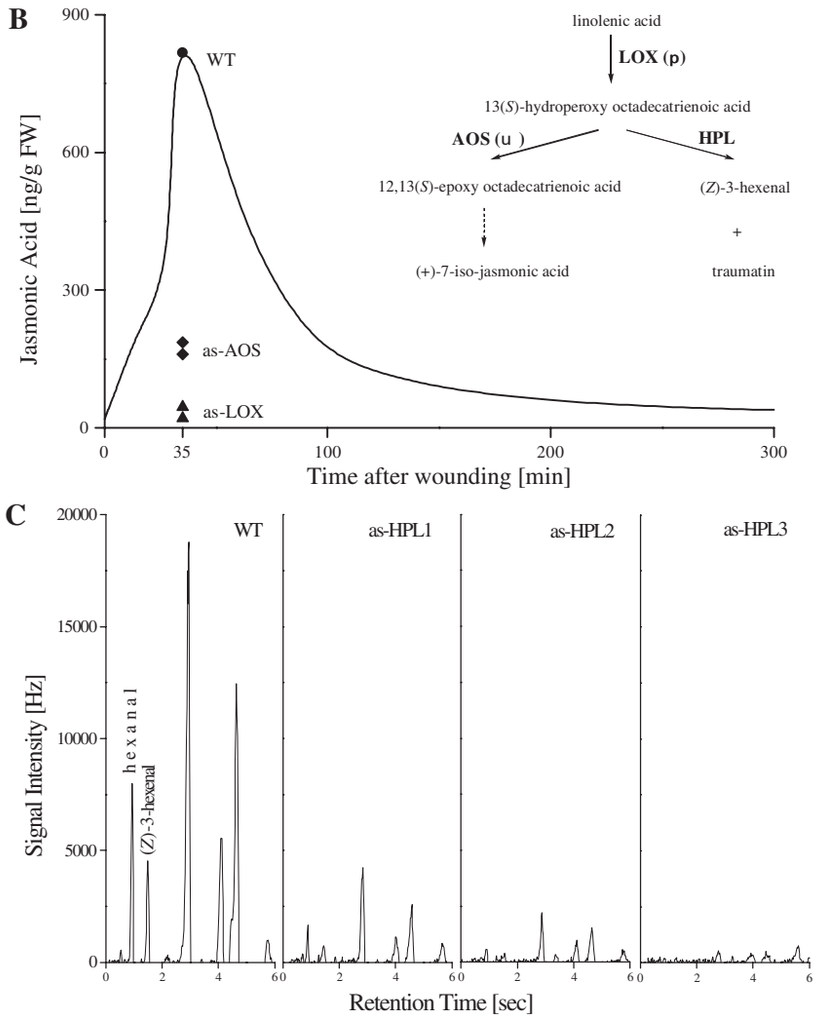

Fig. 3 **A)** Number of T_0 plants regenerating from 120 explants transformed with *N. attenuata* LOX, AOS and HPL constructs in an antisense (as) orientation with either NTC or hygromycin as the selectable antibiotic, and the number of lines examined and found to be transformed (by antibiotic selection and PCR). **B)** Scheme of the biochemical reactions catalyzed by LOX, AOS, and HPL enzymes in the plant oxylipin cascade leading to jasmonic acid and green leaf volatiles. Kinetic of the wound-induced JA accumulation (ng/g fresh mass) in wild-type *Nicotiana attenuata* plants (solid line) and wound-induced JA levels in the treated leaves of the wild type (circle, •), as-AOS (diamonds, ♦), and as-LOX (triangles, Δ) transformed lines. Leaves were harvested 35 min after a standardized mechanical wounding procedure. **C)** Profile of headspace volatiles released by wild type (WT) *Nicotiana attenuata* plants, and three plants representing independently as-HPL transformed lines. Volatiles were sampled and analyzed with the zNose™ during the first minute after leaves had been mechanically damaged by a standardized wounding procedure

JA accumulation observed in wild-type plants and only 12 (40%) of the 30 as-HPL lines exhibited more than a 50% reduction in wound-induced GLVs release (Fig. 3B, C). This phenotypic variation presumably results from positional effects, resulting from the insertion of transgenes into random positions in the genome, which, in turn, differ in transcriptional activity. This phenotypic variation is enormously useful for ecological analyses, for it provides the best controls for the transformation process and allows for quantitative analysis of a trait. Positional variation can produce plants that have been through the entire transformation-regeneration process and contain the transgene, but have wild-type phenotypes, and as such represent the best controls for a phenotypic analysis. Since transposable elements can be activated during callus induction (Grandbastien 1998), control plants used in a phenotypic analysis should have been through the entire transformation-regeneration process. Moreover, since transformation is essentially a mutagenic process, which inserts transgenes at random places in the genome and thereby potentially disrupts other genes in the process, it is important that the phenotypic analysis is conducted with multiple independently transformed lines. If a number of different lines exhibit the same phenotype, it is highly unlikely that the phenotype results from the disruption of an endogenous gene by the insertion of the transgene. If little positional variation is found and all lines show strong phenotypic effects, 'empty-vector' transformants – plants transformed by the same procedure and contain the selectable marker but lack the gene of interest, its promotor and terminator – are the next best controls to use in the phenotypic analysis. Only when the phenotypic analysis includes these controls, one can be certain that the phenotype does not stem from the expression of the selectable marker, disruption caused by the insertion of the plasmids or mutations induced during the transformation-regeneration process.

While we have developed a transformation system for this ecological expression system, it is clear that the system will require additional refinements. In particular, vectors will be required to increase the frequency of phenotypes resulting from reduced expression and we are currently exploring the utility of constructs containing inverted repeats of the gene of interest to this end, as has been pioneered by Waterhouse and associates (Wesley *et al*. 2001). Second, constitute expression strongly limits the choice of genes to those that can be manipulated without deleterious consequences for plant growth or development. The development of an inducible expression system, which could be activated by ecologically-relevant external stimuli would greatly increase the types of genes that could be manipulated. Third, since *Agrobacterium*-mediated transformation can produce multiple-copy insertions and rearrangements of the T-DNA, including deletions from the ends, direct repeats, inverted repeats, and concatamerization, albeit at lower frequencies than other transformation techniques (Barcelo *et al*. 2001), it will be valuable to rapidly analyze the insertion processes in particular transformed lines by rescuing the inserted T-DNA from plant genomic DNA. These three refinements are currently being developed for this transformation system.

Acknowledgements

Supported by Max-Planck-Gesellschaft. PXGSAT was kindly provided by Stephen M. Beverley. Department of Molecular Microbiology, Washington University School of Medicine, St. Louis, Missouri 63110. We are deeply grateful for the helpful discussion with Drs. L. Willmitzer, A. Blau, R. Baran, R. Mendel, R. Hänsch, T. Fenning, V. Reddy, P. Mullineaux into the intricacies of plant transformation during the five years that it took to develop this procedure, and to K. Anand, G. Steinmetzer, C. Nitsche, M. Bader, J. Ehrhardt, K. Kranl, J. Müller for technical assistance.

References

Baldwin IT (2001) An ecologically motivated analysis of plant-herbivore interactions in native tobacco. Plant Physiol 127:1449–1458

Baldwin IT, Zhang Z-P, Diab N, Ohnmeiss TE, McCloud ES, Lynds GY, Schmelz EA (1997) Quantification, correlations and manipulations of wound-induced changes in jasmonic acid and nicotin. *Nicotiana sylvestris.* Planta 201:397–404

Barcelo P, Rasco-Gaunt S, Thorpe CL (2001) Transformation and gene expression. Bot Res Incor Adv Plant Path 34:59–126

Bogani P, Lio P, Intrieri MC, Buiatti M (1997) A physiological and molecular analysis of the genus *Nicotiana* Mol Phylog and Evol 7:62–70

Dixon RA, Arntzen CJ (1997) Transgenic plant technology is entering the era of metabolic engineering. Trends Biotech 15: 441–444.

Gallois P, Marinho P (1995) Leaf disc transformation using *Agrobacterium tumefaciens*-expression of heterologous genes in tobacco. In: Jones H (ed.), Methods in Molecular Biology (pp 39–48) Humana Press, NY

Grandbastien MA (1998) Activation of plant retrotransposons under stress conditions. Trend Plant Sci 3:181–187

Guerineau F, Brooks L, Meadows J, Lucy A, Robinson C, Mullineaux P (1990) Sulfonamide resistance gene for plant transformation. Plant Mol Biol 15:127–136

Hall RD (1991) The initiation and maintenance of callus cultures of carrot and tobacco A2: 1-19. Kluwer Academic Publishers, Dordrecht

Horsch RB (1988) Leaf disc transformation A5: 1–9. Kluwer Academic Publishers, Dordrecht

Jefferson RA, Kavanagh TA, Bevan MW (1987) GUS fusions: ß-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO J 6:3901–3907

Kessler A, Baldwin IT (2002) Plant responses to herbivory: the emerging molecular analysis. Ann Rev Plant Phys Plant Mol Biol 53:299–328

Mitchell-Olds T (2001) *Arabidopsis thaliana* and its wild relatives: A model system for ecology and evolution. Trends Ecol Evol 16:693–700

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plantar 15:473–497

Pereira A (2000) A transgenic perspective on plant functional genomics. Transgenic Res 9:245–260

Preston CA, Baldwin IT (2000) Positive and negative signals regulate germination in the post- fire annual, *Nicotiana attenuata* Ecology 81:293–293

Roda A, Baldwin I (2002) Molecular technology reveals how the induced direct defenses of plants work. Basic Appl Ecol, in press

Schittko U, Preston CA, Baldwin IT (2000) Eating the evidence? *Manduca sexta* larvae can not disrupt specific jasmonate induction in *Nicotiana attenuata* by rapid consumption. Planta 210:343–346

Voelckel C, Krügel T, Gase K, Heidrich N, van Dam NM, Winz R, Baldwin IT (2001) Anti-sense expression of putrescine N-methyltransferase confirms defensive role of nicotine in *Nicotiana sylvesris* against *Manduca sexta.* Chemoecol 11:121–126

Wesley SV, Helliwell CA, Smith NA, Wang MB, Rouse DT, Liu Q, Gooding PS, Singh SP, Abbott D, Stoutjesdijk PA, Robinson SP, Gleave AP, Green AG, Waterhouse PM (2001) Construct design for efficient, effective and high-throughput gene silencing in plants. Plant J 27:581–590

Zambryski P, Joos H, Genetello C, Leemans J, Vanmontagu M, Sell J (1983) Ti-Plasmid vector for the introduction of DNA into plant-cells without alteration of their normal regeneration capacity. EMBO J 2:2143–2150

Received 14 March 2002; accepted 10 May 2002.