Transgenic Res
DOI 10.1007/s11248-007-9148-1

ORIGINAL PAPER

Efficient and stable transgene suppression via RNAi in field-grown poplars

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Received: 12 June 2007 / Accepted: 22 September 2007

Abstract The efficiency and stability of RNA interference (RNAi) in perennial species, particularly in natural environments, is poorly understood. We studied 56 independent poplar RNAi transgenic events in the field over 2 years. A resident *BAR* transgene was targeted with two different types of RNAi constructs: a 475-bp IR of the promoter sequence and a 275-bp IR of the coding sequence, each with and without the presence of flanking matrix attachment regions (MARs). RNAi directed at the coding sequence was a strong inducer of gene silencing; 80% of the transgenic events showed more than 90% suppression. In contrast, RNAi targeting the promoter resulted in only 6% of transgenic events showing more than 90% suppression. The degree of suppression varied widely but was highly stable in each event over 2 years in the field, and had no association with insert copy number or the presence of MARs. RNAi remained stable during a winter to summer seasonal cycle, a time when expression of the targeted transgene driven by an *rbc*S promoter varied widely. When strong gene suppression was induced by an IR directed at the promoter sequence, it was accompanied by methylation of the homologous promoter region. DNA methylation was also observed in the coding region of highly suppressed events containing an IR directed at the coding sequence; however, the methylation degree and pattern varied widely among those suppressed events. Our results suggest that RNAi can be highly effective for functional genomics and biotechnology of perennial plants.

Keywords RNA interference · Stability · Poplar · MAR · Copy number · Methylation · Transgene stability

Electronic supplementary material The online version of this article (doi:10.1007/s11248-007-9148-1) contains supplementary material, which is available to authorized users.

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Introduction

The mechanism of homology-dependent gene silencing induced by double-stranded RNA (dsRNA), widely known as RNA interference (RNAi), was first documented in the nematode *Caenorhabditis elegans* (Fire et al. 1998) and then demonstrated in diverse eukaryotes including plants, fungi, *Drosophila*, and vertebrates (Plasterk and Ketting 2000). RNA silencing is an evolutionarily conserved gene-regulatory mechanism used for cellular defense, genome surveillance, and for guiding development (Vance and Vaucheret 2001; Kusaba 2004; Lippman and Martienssen 2004; Mallory and Vaucheret 2006). The various pathways of RNA silencing involve the cleavage of dsRNA into short interfering RNAs (siRNAs) or microRNAs (miRNAs) (Baulcombe 2004; Matzke and Birchler 2005; Brodersen and Voinnet 2006). These small RNAs act as guides to direct cleavage or translational repression of complementary mRNAs to induce post-transcriptional gene silencing (PTGS), or cause DNA and chromatin modification to induce transcriptional gene silencing (TGS).

Artificial introduction of dsRNA-producing transgenes in plants has been shown to induce sequence-specific gene silencing at high frequency (Waterhouse et al. 1998; Chuang and Meyerowitz 2000; Stoutjesdijk et al. 2002; Kerschen et al. 2004; Wagner et al. 2005). Constructs designed to express hairpin RNA (hpRNA) are highly efficient inducers of silencing (Smith et al. 2000; Wesley et al. 2001; Stoutjesdijk et al. 2002). In an hpRNA-producing vector, partial or full sequences of target genes are placed as an inverted repeat (IR) separated by an unrelated sequence (Kusaba 2004). Use of an intron as the spacer sequence has been shown to increase stability and efficiency of RNAi (Wesley et al. 2001). In addition to coding sequence, dsRNA of promoter sequences can result in TGS accompanied by de novo methylation of homologous sequences (Mette et al. 2000; Cigan et al. 2005). The ability of RNAi to suppress, or silence, expression of specific genes has made it a major new tool for functional genomics and genetic engineering of both plants and animals (Small 2007). However, little is known about the efficiency and stability of RNAi-induced gene suppression in the diversity of organisms where it has been applied.

In *Arabidopsis*, the high degree of silencing of the endogenous Δ12-desaturase gene (*FAD2*) induced by an intron-spliced hairpin transgene was studied in one transgenic event over five sexually propagated generations, and no reversion or reduction of gene silencing was observed (Stoutjesdijk et al. 2002). Temperature-dependent gene silencing has been reported both in plants and animals (Fortier and Belote 2000; Szittya et al. 2003; Kameda et al. 2004; Sós-Hegedűs et al. 2005). In *Nicotiana benthamiana*, both virus- and transgene-triggered RNA silencing (*35S-GFP*/*35S*-ds*GFP*) were inhibited at a low temperature (15°C), and the loss of silencing was accompanied by a reduction in the amount of siRNA (Szittya et al. 2003). This study also reported that temperature affected antisense-mediated endogene inactivation in *Arabidopsis* and potato. However, Sós-Hegedűs et al. (2005) reported both temperature dependence and independence of antisense-mediated gene silencing. Out of 24 potato antisense transgenic lines, nine lines were not influenced by the low temperature (15°C) with respect to antisense-induced gene silencing. These studies suggest that RNAi suppression may be unstable under field conditions, where temperatures can vary dramatically.

There is a dearth of conventional breeding methods in trees that allow the directed mutation of specific genes to produce desired traits. This is a result of their long juvenile period, intolerance of inbreeding, and lack of gene-characterized mutant stocks. By enabling the production of dominant, gene-specific changes in gene expression, RNAi may provide a major advance in the Mendelian genetics and breeding of trees. However, studies of RNAi silencing in woody plants are rare. In the gymnosperm *Pinus radiata*, *cinnamyl alcohol dehydrogenase* (*CAD*), a gene associated with the biosynthesis of lignin, was more efficiently suppressed by an inverted repeat of the *CAD* coding sequence than by either sense or antisense transgenes introduced via biolistic transformation, and suppression level was positively associated with the expression level of the transgene (Wagner et al. 2005). In poplar, RNAi suppression of the endogenous, putative flowering-onset gene *PCENL1* caused early flowering in the field (Mohamed 2006). Brunner et al. (2007) reported the production of RNAi genes directed at single or multiple floral homoetic genes in poplar, with the goal of producing complete sexual sterility.

For RNAi technology to find wide use at imparting new traits in trees, it will be important to demonstrate

stable suppression of target genes over multiple years in the field. Such stability is especially important for altering floral development to achieve transgene containment. Here we report the first study of the stability of RNAi in poplar trees in a field environment. We retransformed transgenic poplars containing the herbicide-resistance gene *BAR*, which encodes phosphinothricin acetyltransferase (PAT), with four kinds of intron-spliced hpRNA (ihpRNA) constructs that contained IR directed at promoter or coding sequence. We also evaluated how flanking matrix attachment regions (MARs)—which have been reported to increase expression levels and stability (Mlynarova et al. 2003; van der Geest et al. 2004; Abranches et al. 2005; Halweg et al. 2005)—influence RNAi efficiency and stability. We report that RNAi directed at the coding sequence, but not promoter sequence, was highly efficient at gene suppression, that RNAi was stable over 3 years of study, and that MAR elements had no discernible influence on suppression efficiency or stability.

Materials and methods

Construction of RNAi stability vectors

Gene- and promoter-specific sequences in sense and antisense orientation were assembled in the suppression vector pHANNIBAL (Wesley et al. 2001; provided by Dr. P. Waterhouse, CSIRO, Australia). For the construction of IR of coding sequence, a 275-bp sequence (Suppl. S1) was amplified using a forward primer 5′ cactcgagggtctgcaccatcgtcaac 3′, and a reverse primer 5′ tgggtacctcagcaggtgggtgtagag 3′, containing *Xho* I and *Kpn* I sites (underlined) introduced in the forward and reverse primers, respectively. The same fragment was again amplified with the primers containing different recognition sites for *Bam* HI and *Cla* I, respectively: 5′ agggatccggtctg caccatcgtcaac 3′ and 5′ catcgattcagcaggtgggtgtgag 3′. Both fragments were cloned into pHANNIBAL to create an IR. Sense and antisense fragments of the *Arabidopsis rbc*S promoter sequence were assembled in the same way. A 475-bp fragment was amplified with the primers: 5′ ggctcgagatatattccacagtttcacc 3′, and 5′ ccggtaccttggtctagtgctttggtca 3′, and cloned into *Xho* I and *Kpn* I sites of the pHANNIBAL to give the sense orientation. The same fragment amplified with the primers 5′ acggatccatatattccacagtttcacc 3′ and 5′ ccatcgatttggtctagtgctttggtca 3′ was cloned into the *Bam* HI and *Cla* I sites to give the antisense orientation.

The binary vector pGreenII (Hellens et al. 2000) was used for assembling the *CP4* selectable marker and RNAi cassettes. Two versions of the vector were used: one with a flanking MAR element derived from the tobacco *RB7* gene (Allen et al. 1996; provided by S. Spiker, North Carolina State University), and one without MARs. For cloning of MARs, a 1,167-bp fragment was cut from the vector pKH10 with the restriction enzymes *Not* I and *Spe* I, filled in with T4 DNA polymerase, and cloned at *Fsp* I and *Sap* I (blunted) sites of the pGreenII to produce pGM.

The *CP4* selectable marker cassette has been previously described (Meilan et al. 2002). It consists of the FMV-*34S* promoter from figwort mosaic virus, the coding region of the *CP4* gene from *Agrobacterium tumefaciens* conferring resistance to the herbicide glyphosate, and the 3′ untranslated region from *rbc*S from pea (Coruzzi et al. 1984). A 3,044-bp cassette was excised from the vector pMON17227 using *Pme* I and *Not* I, blunted with T4 DNA polymerase, and cloned into the *Sma* I site of pGreenII to produce pGC, and the *Xho* I (blunted) and *Eco* RV sites of pGM to produce pGMC.

The IR directed at coding sequence, together with the *35S* promoter and octopine synthase terminator, were cut from pHANNIBAL with *Not* I and inserted into the corresponding site of pGC to produce pGBi (Fig. 1). The same cassette was cut with *Spe* I, and cloned into the *Spe* I site of pGMC to produce pGMBi.

The IR directed at the promoter sequence, together with *35S* promoter, were cut from pHANNIBAL with *Spe* I and *Sac* II and cloned into the same sites of pGC and pGMC to produce pGPi and pGMPi, respectively.

Plant transformation and field trials

Four *BAR*-containing transgenic poplar events (we refer to these four events as parental events) that were previously generated in our laboratory via an *Agrobacterium*-mediated protocol were re-transformed with the RNAi constructs. The four parental events are 353-38, 353-29, 717-A, and 717-C. 353-38 and

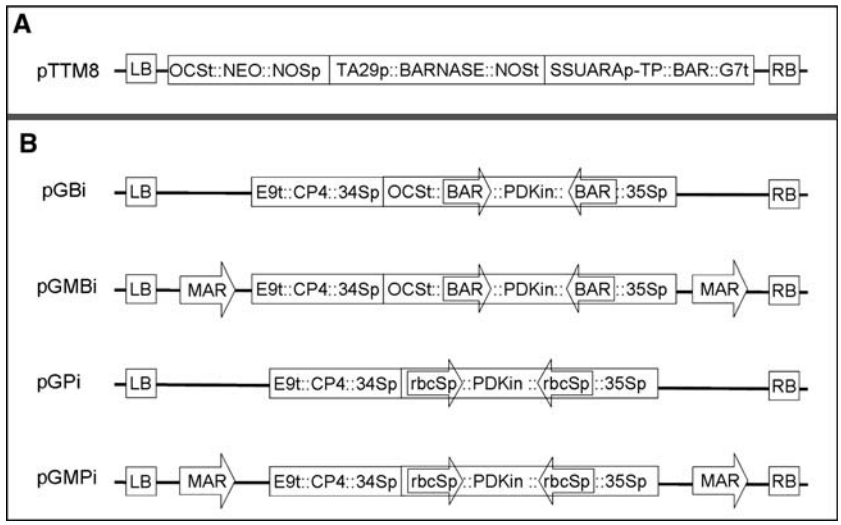

Fig. 1 Schematic diagram of the T-DNA region of the binary vector pTTM8 and RNAi constructs. (**a**) Parental events were previously transformed with pTTM8. OCSt: the 3′ untranslated region from the octopine synthase gene; NEO: neomycin phosphotransferase II; NOSp: the promoter from the nopaline synthase gene; TA29p: the promoter from a tobacco anther-specific gene *TA29*; NOSt: the 3′ untranslated end of the nopaline synthase gene; SSUARAp-TP: the promoter from the *atS1*A ribulose-1,5-biphosphate carboxylase small subunit gene (*rbc*S) from *Arabidopsis thaliana*; G7t: the 3′ untranslated fragment from the TL-DNA gene 7. (**b**) RNAi constructs used to target the *BAR* gene of transgenic parental events. All constructs contain backbone and border sequence from the pGreenII binary vector, and the *CP4* selection gene driven by the FMV-34S promoter. pGBi: RNAi construct with IR of 275 bp of *BAR* coding sequence. pGMBi: RNAi construct with IR of 275 bp of *BAR* coding sequence with flanking MARs. pGPi: RNAi construct with IR of 475 bp of *rbc*S promoter sequence. pGMPi: RNAi construct with IR of 475 bp of *rbc*S promoter sequence with flanking MARs. Abbreviations: E9t: terminator from *rbc*S of pea; *CP4*: EPSPS gene from *Agrobacterium tumefaciens*; 34Sp: promoter from figwort mosaic virus; OCSt: octopine synthase terminator; *BAR*: 275-bp coding sequence of *BAR* from *Streptomyces hygroscopicus*; PDKin: pyruvate orthophosphate dikinase intron; 35Sp: *35S* promoter from cauliflower mosaic virus; *rbc*Sp: 475 bp of promoter sequence from the *rbc*S gene of *Arabidopsis*. RB: right border; LB: left border. T-DNA regions are not drawn to scale

353-29 were made in hybrid poplar clone 353-53 (*P. tremula* × *P. tremuloides*), and the other two in hybrid clone 717-1B4 (*P. tremula* × *P. alba*). The original transgenic poplars had been transformed with the binary plasmid pTTM8 provided by Plant Genetic Systems (Belgium) and contained in the T-DNA two chimeric genes conferring resistance to the antibiotic kanamycin and the herbicide glufosinate-ammonium, respectively, and another chimeric *BARNASE* gene that can impart male sterility (Fig. 1A). The herbicide resistance gene was driven by the promoter of a strongly photosynthesis-associated gene, *rbc*S derived from *Arabidopsis*.

The four events were retransformed with *Agrobacterium tumefaciens* strain C58/pMP90 (GV3101) harboring RNAi constructs as described previously (Filichkin et al. 2006), except that glyphosate selection was at a concentration of 0.4 mM for callus and shoot induction, and 0.012 mM for root induction. The presence of the sense fragments in the transgenic events was confirmed with a forward primer annealing to the *35S* promoter and a reverse primer annealing to the *PDK* intron: 5′ gcacaatcccactatccttcgcaag 3′ and 5′ gatagatcttgcgctttgttatattagc 3′, respectively. The presence of the *BAR* antisense fragment was confirmed with primers homologous to the antisense fragment and the *OCS* terminator: 5′ tagtggttgacgatggtgcagaccg 3′ and 5′ cggccaatactcaacttcaaggaatctc 3′. The presence of the *rbc*S promoter antisense fragment was confirmed with primers to the antisense fragment and the *34S* promoter: 5′ gattaggtgaaactgtggaatatatgg 3′ and 5′ gcgcctaacaattctgcacc 3′, respectively.

Six to eight ramets of each individual transgenic event and non-transformed parental event (NT) were propagated in vitro and transferred to the greenhouse.

Together with the NT controls, four ramets of each transgenic event were coppiced and planted at a field site near Corvallis, Oregon, in June 2004. The plants were distributed in four blocks with a random split-block design because of inherent differences in rate of height growth of the two clones, and transgenic events in the 353-53 background were separated from those in the 717-1B4. Trees were randomly assigned to one of two subplots within each block.

Gene expression measurements

RNA extraction and real-time RT-PCR

Young leaves were sampled from all field plants in 2004 and 2005 and stored at –80°C until assayed. RNA was extracted for two ramets of each individual event using the RNeasy Mini Kit (Qiagen, Valencia, CA; Cat. # 74106) with modifications as described in Brunner et al. (2004). Extracted RNA was treated with DNase I (Ambion, Austin, TX; Cat. # 1906) to remove residual genomic DNA following the manufacturer's instructions. For the synthesis of first-strand cDNA, 1 µg of total RNA was reverse-transcribed using oligo(dT) and random primers with SuperScript First Strand synthesis kit for RT-PCR (Invitrogen, Carlsbad, CA; Cat. # 12371-019) in a volume of 20 µl.

Real-time PCR was performed with SYBR green (Platinum SYBR Green qPCR Super Mix UDG; Invitrogen, Carlsbad, CA; Cat # 11733-046) in a Mx3000 P real-time PCR machine (Stratagene, La Jolla, CA). An endogenous ubiquitin (*UBQ*) gene was used as a reference. We chose the *UBQ* gene as the internal control because poplar *UBQ* is stably and uniformly expressed in a variety of tissues (Brunner et al. 2004). Primers used for amplification of the targeted *BAR* gene were: 5′ tttctggcagctggacttcag 3′ and 5′ atcctagaacgcgtgattcagatc 3′, with a product size of 84 bp. The *UBQ* gene was amplified with primers 5′ gttcaatgtttcgtttcatg 3′ and 5′ taacaggaacggaaacatag 3′, giving a product size of 100 bp.

For each sample, duplicate wells were run for both target and reference genes. Synthesized cDNA of the parent event, 717-C, was serially diluted 5-fold and run in triplicate to create a standard curve. For every reaction, 2 µl of 50 × diluted first-strand cDNA was used in a total volume of 25 µl. The cycle conditions were the same for all PCR reactions: 50°C for 2 min, 95°C for 2 min, and 40 cycles of: 95°C—15 s, 56°C—30 s, and 72°C—30 s. For each transgenic event, the relative expression level of the *BAR* gene was calculated with Mx3000 P system software (version 2) (Stratagene, La Jolla, CA) based on the standard curves and normalized to *UBQ* quantities.

Seasonal development of gene suppression

Tissue samples (bud or leaf) were taken at different seasonal points from two highly suppressed transgenic events and one non-transformed parental event (353-29) to study seasonal changes in gene expression due to RNAi silencing. Samples were immediately frozen in liquid nitrogen, and stored at –80°C until being analyzed.

Molecular characterization of transgenes

Transgene copy number

To estimate transgene copy number in transgenic plants, comparative real-time PCR was performed on all studied events. The single-copy, endogenous *LEAFY* gene (*PTLF*) was used as a reference, which was confirmed by Southern blot (Li 2006). The *35S* promoter driving the IR was chosen for the copy-number analysis so the same primer and probe sets could be used for all four constructs. Real-time PCR was performed using dual-labeled TaqMan® probes (Biosearch Technologies, Novato, CA). All primers and probes were designed using the program Primer 3 (Rozen and Skaletsky 2000). For the *35S* promoter, the primers used for amplification were: 5′ cagtggtg aacatagtgtcg 3′, and 5′ tacaatggacgatttcctct 3′. The hybridization probe was labeled with HEX and BHQ-1: 5′ HEX d(caccttcaccttcgaacttccttc) BHQ-1 3′. For the *PTLF* gene, the primers were: 5′ ggtttctctgaggagccagtacag 3′, and 5′ gcctcccatgtccctcttc 3′. The hybridization probe was labeled with FAM and TAMARA: 5′ FAM d(caaggaggcagcagggagcggt) TAMARA 3′. The primer and probe sets were tested for their amplification efficiency using a standard curve, and were optimized to have a high and comparable efficiency (close to 100%). For the *PTLF* gene, the optimized concentration for both forward and reverse primers was 0.4 µM,

and was 0.2 μM for the probe. For the *35S* promoter, the optimal primer concentration was 0.5 μM for both primers.

All real-time PCR reactions were carried out using QuantiTect Multiplex PCR buffer (Qiagen, Valencia, CA; Cat. # 204545) in a volume of 25 μl. For each sample, 100 ng genomic DNA purified with the DNeasy Plant Mini Kit (Qiagen, Valencia, CA; Cat. # 69106) was used in a duplexed reaction to amplify both *PTLF* and *35S* targets, and run in duplicate wells. The amplification was performed using a Mx3000 P real-time instrument (Stratagene, La Jolla, CA) with the following cycles: 95°C for 15 min, and 45 cycles of: 94°C for 30 s, and 60°C for 1 min. The threshold cycle (Ct) was determined using the Mx3000 P system software (version 2). Copy number of the *35S* promoter was determined by the formula: $2^{(1 - \Delta Ct\ (35S\text{-}PTLF)}$. The use of comparative real-time PCR approach to estimate transgene copy number was verified by Southern blot analysis as described in Li (2006).

Methylation analysis using methylation specific PCR (MSP)

DNA methylation status of promoter and coding regions of the target *BAR* gene were investigated using methylation-specific PCR (MSP). A total of 1 μg of genomic DNA was bisulfite modified with the CpGenome™ DNA Modification Kit (Chemicon, Billerica, MA; Cat. # S7820) following the manufacturer's instructions. In the bisulfite reaction, all unmethylated cytosines are converted to uracils, while methylated cytosines remain unaltered. Specific primers were designed to distinguish methylated from unmethylated DNA. Methylation specific primers for both *rbc*S promoter and *BAR* coding region were designed using the program MethPrimer (Li and Dahiya 2002) based on the sense strand. M primers used for amplification of methylated *rbc*S promoter strand were: 5′ cggaaaaggtataagtaaaatatttaattc 3′ and 5′ taaaccgctaaaataataccacgt 3′. U primers used for amplification of unmethylated *rbc*S promoter sense strand were: 5′ tggaaaaggtataagtaaaatatttaattt 3′ and 5′ cttaaaccactaaaataataccacat 3′. M and U primers amplified the same region of the *rbc*S promoter, and yielded a product size of 726 and 728 bp, respectively (Suppl. S2). M primers used for amplification of the methylated *BAR* coding sequence were: 5′ gaacgacgttcggtcgatattcgtc 3′, 5′ aaaacgtaaaacccaatcc
cgtccg 3′, and a degenerated primer 5′ aaaacgtaaaaccc**agt**cccgtccg 3′ was used in case cytosine in the sequence cag was methylated. U primers used for the amplification of the unmethylated *BAR* sense strand were: 5′ aatgatgtttggttgatatttgttg 3′ and 5′aaacataaaac ccaatcccatccac 3′. Both pairs of the primers amplified the same region of *BAR* coding sequence, and amplified a product 309 bp in size (Suppl. S1).

All PCR amplifications were performed in a volume of 50 μl with the following reagents: 2 μl bisulfite modified DNA, dNTPs (1.25 mM), $MgCl_2$ (6.75 mM), Plantium Taq (2 U; Invitrogen, Carlsband, CA), primers (each 0.4 μM), and 1× PCR buffer. The PCR cycles were: 94°C for 2 min; 39 cycles of: 94°C—20 s, 59°C—20 s, and 68°C—40 s/kb; 72°C for 3 min. The PCR products were purified with the QIAquick PCR Purification Kit (Qiagen, Valencia, CA; Cat. # 28104), cloned into pCR4-TOPO vector (TOPO TA Cloning® Kit for Sequencing with One Shot® TOP10 Chemically Competent *E. coli*; Invitrogen, Carlsbad, CA; Cat. # K4575-01), and sequenced in the ABI 3730 sequence machine at the Center for Genome Research and Biocomputing (CGRB) at OSU.

Statistical analysis

Quantified expression level of the resident *BAR* gene for each RNAi transgenic event was normalized as a fraction of the expression of their corresponding parental event. Therefore, highly suppressed or silenced events had a value close to 0, while those not suppressed had a value near to 1. Except when considering year-to-year stability, mean expression over 2 years for each event was used for RNAi suppression analyses. Stability of RNAi suppression across years was analyzed with regression analysis of $\log_2$ transformed values. Statistical tests of difference in efficiency of RNAi suppression between different IR transgenes were performed using the GLM Type III sums of squares analysis. Differences in variance of RNAi suppression between single- and multi-copy transgenic populations were studied using Levene's test. All statistical tests were carried out using SAS (Cary, NC).

Results

RNA silencing efficiency varies widely between ihpRNA constructs

Four transgenic poplar events (717-A, 717-C, 353-29, and 353-38; referred to below as "parental events") containing the resident *BAR* transgene (Fig. 1A) were generated in another study and showed high, stable resistance to the herbicide glufosinate over several years in the field (Li et al. 2007). Parental events 717-A and 717-C were derived from female clone 717-B4 (*Populus tremula* × *P. alba* genotype), while 353-29 and 353-38 were from male clone 353-53 (*P. tremula* × *P. tremuloides*). These four parental events were retransformed with the four RNAi constructs (Fig. 1B), and a total of 56 transgenic events were produced from the 16 (4 × 4) construct × parental event combinations (Table 1 and Suppl. T1). The number of events produced was smaller than originally planned due to unexpectedly low transformation efficiency (ranging from 0 to 1%), a possible consequence of "leaky" expression during organogenesis of the anther-specific TA29::*BARNASE* transgene (Koltunow et al. 1990) resident in the transgenic parental events. This rate is much lower than the efficiencies (ranging from 5 to 37%) obtained in other transformation projects using similar protocols carried out in our laboratory at about the same time (unpubl. data).

A large difference in RNAi suppression efficiency was observed between promoter (Pi) and coding sequence (Bi) RNAi types (Table 1). RNAi constructs containing IR directed at the coding sequence had a much higher suppression efficiency than IR directed at the promoter sequence. In transgenic events containing Bi constructs, expression of the *BAR* gene was either highly reduced or close to that of the parental events; 80% of the transformed events showed strong suppression. However, transgenic events containing Pi had more variable suppression levels; only a small portion (6%) of transformed events had strong gene suppression. The difference in RNAi efficiency between two types of IR was highly statistically significant ($P < 0.0001$).

The difference in RNAi efficiency between the two types of RNAi constructs did not depend on the parental events used. For example, none of eight transgenic events transformed with pGPi in the 717-A background showed more than 90% suppression of the *BAR* gene, while six of seven (86%) 717-A transgenic events transformed with pGBi showed more than 90% suppression (Suppl. Table T1). A similar difference in suppression efficiency was observed between seven 353-29 transgenic events containing pGPi and pGBi. None of the pGPi group

Table 1 Number and RNAi silencing efficiency for each event and construct combination

Construct	No. of Transgenic Events	% Suppression >90% (% of Total)[a]	% Suppression >50% (% of Total)	% Suppression >0% (% of Total)
pGPi[b]	16	0 (0%)	4 (25%)	10 (63%)
pGMPi[b]	15	2 (13%)	3 (2%)	8 (53%)
pGBi[b]	16	13 (76%)	14 (88%)	14 (88%)
pGMBi[b]	9	7 (78%)	8 (89%)	8 (89%)
Pi[c]	31	2 (6%)	7 (23%)	18 (58%)
Bi[c]	25	20 (80%)	22 (88%)	22 (88%)
M–[d]	32	13 (41%)	18 (56%)	24 (75%)
M+[d]	24	9 (38%)	11 (46%)	16 (67%)

[a] Number of transgenic events with more than 90% expression suppression of the *BAR* gene in each category

[b] Events were pooled by construct type

[c] Events were pooled by IR type

[d] Events were pooled by presence/absence of MAR fragments

Abbreviations: Pi: inverted repeat (IR) of promoter sequence; Bi: IR of coding sequence; M-: without flanking MARs; M+: with flanking MARs

showed more than 90% suppression, while 71% of pGBi transformed events showed a high degree of suppression.

The difference in RNAi efficiency between promoter- and coding region-targeted constructs was also not a reflection of the presence of flanking MAR elements in some of the constructs (P value of IR and MAR interaction term = 0.40). There was also no significant MAR effect on the mean suppression levels ($P = 0.78$). The frequency of events with more than 90% suppression of the *BAR* gene was: 0% for the 16 pGPi and 13% for the 15 pGMPi promoter-directed events, and 76% for the 16 pGBi and 78% for the 9 pGMBi coding sequence-directed events.

Stability of RNAi suppression over time

Based on quantified expression of the resident *BAR* transgene in two different years in the field, the degree of RNAi suppression appeared to be highly stable over time in all constructs ($r = 0.69$, $P < 0.001$; Fig. 2A; $r = 0.75$ for the actual unlogged data). Events with a high level of suppression in the first year stayed highly suppressed in the subsequent year. Among 17 events with more than 90% suppression in the first year, 16 events (94%) retained the same strong degree of suppression; only one event had decreased suppression, going from 92% in year one to 46% in year two. This event contained two copies of the promoter IR without the flanking MAR fragments.

RNAi suppression was also studied at different seasonal points for two highly suppressed events and one non-transformed parental event. Samples were collected from early March (before buds flushed) through mid-summer. Temperature ranged from 14 to 27°C at the time when the samples were taken for RNAi suppression analyses, and mean monthly temperatures ranged from 9.1 to 20.6°C during this period (Fig. 2B). As expected, the temperatures for the specific time when samplings were taken vary considerably more than the weekly and monthly mean temperatures. Plants were actively growing during the last four sample dates. There was substantial seasonal variation in expression of the *BAR* gene in the parental event, a likely consequence

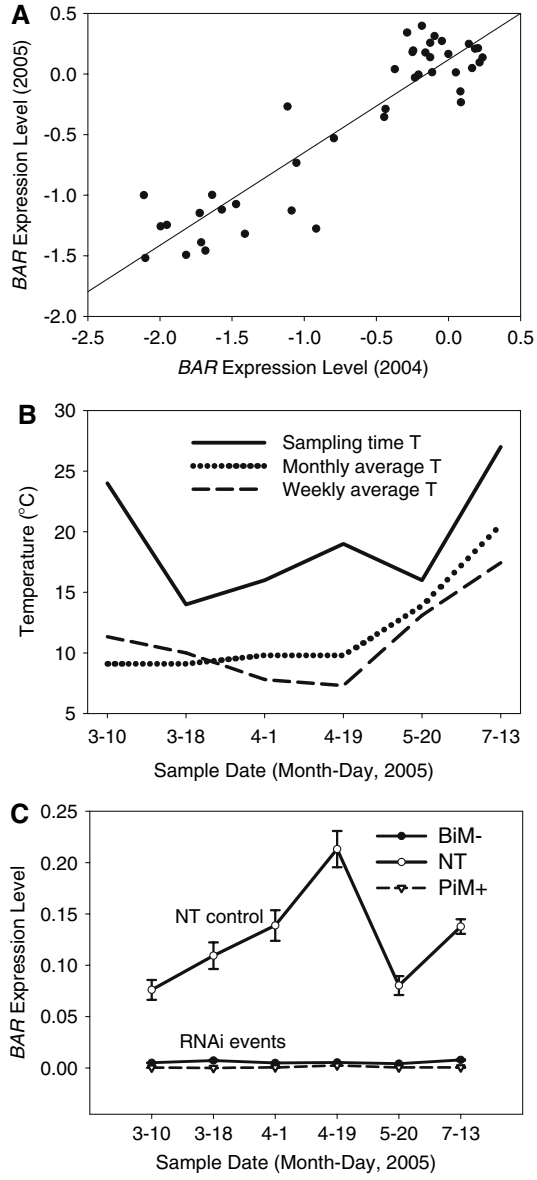

Fig. 2 Stability of RNAi suppression over 2 years in the field and during seasonal development. (**A**) Correlation of *BAR* expression levels between 2 years in the field. Expression levels were determined by qRT-PCR, normalized to expression levels of corresponding parental events used for transformation, and then log transformed. $r^2 = 0.47$ ($P < 0.0001$, n = 42). (**B**) Temperature (T) at the time when samples were taken, mean weekly T (previous 6 days plus the sample day), and mean monthly T. (**C**) Expression of the target *BAR* gene for two strongly suppressed events: one 353-29 transgenic event transformed with pGBi, and one 353-38 event transformed with pGMPi, and parent event 353-29 (NT). Error bars represent one standard error of mean over technical replicates

of its promoter, which was from the *Arabidopsis* small subunit of ribulose bisphosphate carboxylase (*rbc*S) and is most active in mature leaves (Sugita and Gruissem 1987). In contrast, two highly suppressed events (>97%), which included one 353-29 event (pGBi) and one 353-38 event (pGMPi), showed stable suppression over time (Fig. 2C).

Correlation of transgene copy number with RNAi

RNAi transgene copy number was estimated for 53 transgenic events using comparative real-time PCR, which we first validated via Southern blots (Li 2006). Due to the presence of the *BAR* gene in the parental event used for transformation, a sequence within the *35S* promoter that drove the sense and antisense fragments, rather than the *BAR* gene itself, was used to estimate copy number. Among the 53 transgenic events studied, 45 (85%) contained a single copy, five (9%) had two copies, and three (6%) had three copies.

RNAi transgene copy number was not associated with the degree of RNAi suppression of the *BAR* gene (r = 0.015; Fig. 3). The degrees of suppression varied widely among single-copy events. The mean degree of *BAR* suppression was 37% for single-copy integration, 14% for two-copy events, and 58% for three-copy events. The difference in mean suppression level between single- (mean of 0.37, n = 44) and multiple-copy (mean of 0.31, n = 8) events was not statistically significant ($P = 0.82$). The presence of the multiple copies of dsRNA also did not increase the variance of suppression levels among transformants, based on Levene's test (variance of 0.36 for multi-copy events and mean variance of 0.30 for single-copy events, $P = 0.44$).

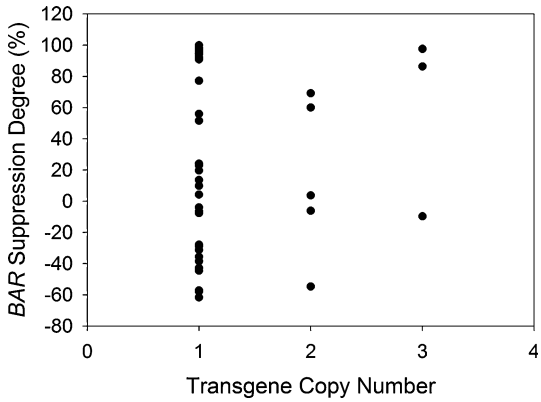

Fig. 3 Correlation of RNAi transgene copy number with RNAi suppression levels (%) of the targeted *BAR* gene (r = 0.015, $P > 0.05$). RNAi transgene copy number of 53 transgenic events was quantified with comparative real-time PCR using poplar *LEAFY* as internal control

DNA methylation analysis

DNA methylation is often associated with TGS and PTGS (Mette et al. 2000). We, therefore, analyzed DNA methylation status of both promoter and coding sequence of the target *BAR* gene in 14 RNAi transgenic events and two parental events. Among 14 studied RNAi transgenic events, four were transformed with the pGMPi, five with the pGBi, and five with the pGMBi. Except for three events in which *BAR* expression was not affected, all other studied events showed high *BAR* suppression, ranging from 86 to 99.8%.

Methylation status was studied with methylation-specific PCR (MSP) (Li and Dahiya 2002) using primers designed to amplify methylated sequence (M), and primers designed for unmethylated sequence (U). The three unsilenced events along with the two parental events served as putative negative controls for M primers, and positive controls for U primers, respectively. The M primers were designed with the expectation that cytosine methylation occurred in all CpG sequence contexts, while the absence of cytosine methylation in any sequence context was assumed for the U primers. As expected, strong, specific amplification of non-RNAi transgenics and unsilenced events occurred with U primers (data not shown). The sequencing of these amplified bands also showed an absence of methylation (data not shown). Because the methylation patterns could vary among different transformants with the same level of gene silencing (Figs. 4 and 5), we sequenced all amplified bands to avoid possible false-negative amplifications.

The three highly suppressed pGMPi events showed DNA methylation in the promoter region of the resident *BAR* gene (Fig. 4), but not in the coding region (data not shown). The methylation occurred only in the sequence sharing identity with the introduced dsRNA, and had not spread to sequences outside this region. The two events that exhibited a 99% reduction of *BAR* expression, pGMPi-1 and

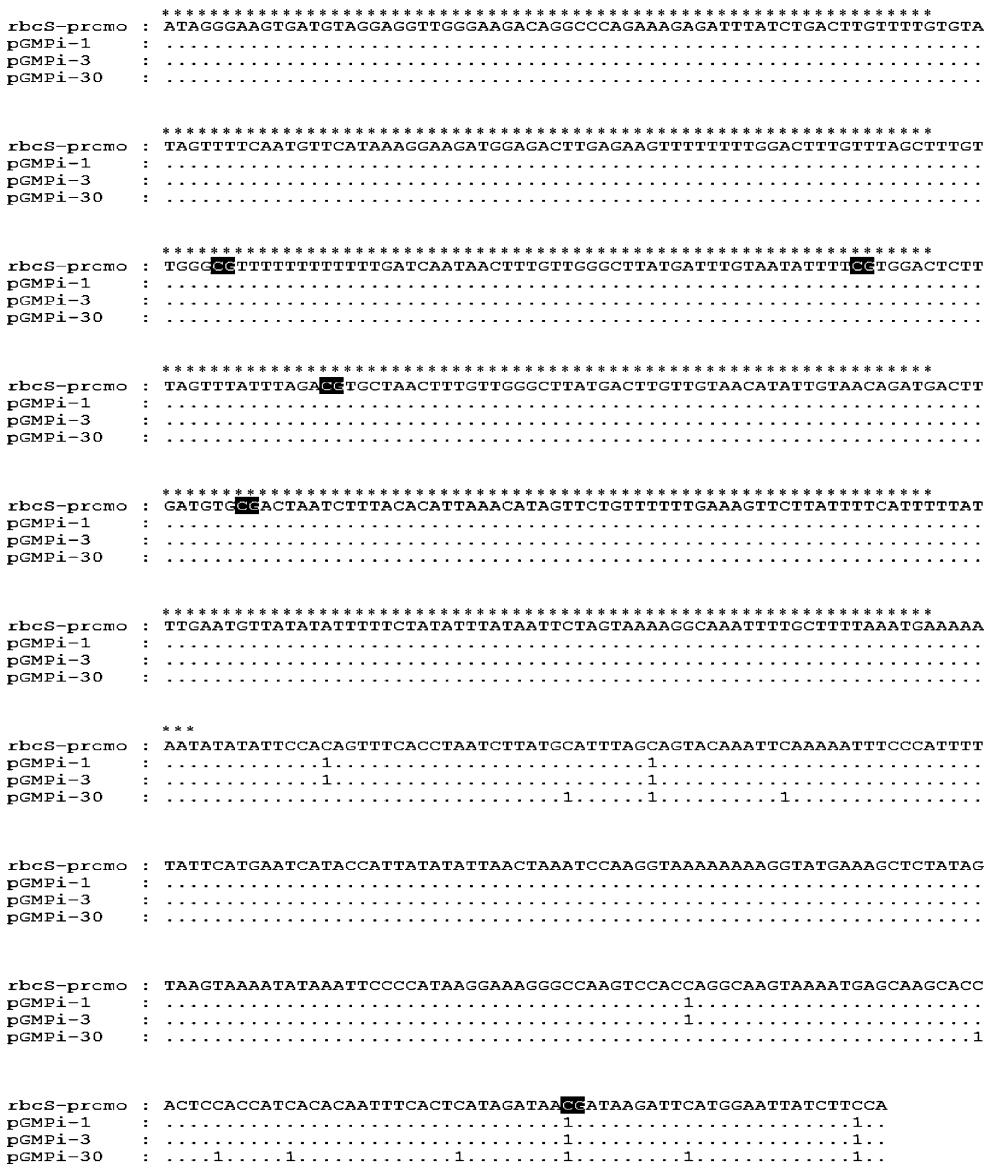

Fig. 4 Promoter methylation of three highly suppressed pGMPi events. CpG sequences are shaded, and methylated cytosines are indicated by the number 1. The sequenced region that was studied for methylation, but not included in the inverted repeat, was indicated by "*". *rbc*S-promo: unmethylated *rbc*S promoter sense strand

pGMPi-3, showed cytosine methylation in CpG and CAG, but rarely in CHH (where H = A, T, or C) sequence contexts (1 out of 50 = 2%). In contrast, event pGMPi-30, which had an 86% reduction in *BAR* expression, showed a different methylation pattern; methylation occurred only in non-symmetric sequences (CHH, 8 out of 50 = 16%).

We also found that DNA methylation occurred in the coding region of the target *BAR* gene in highly suppressed events containing dsRNA directed at the coding sequence (Fig. 5), but it did not spread to its promoter region (data not shown). DNA methylation patterns, however, varied considerably among independent transformants that had a similar level of gene suppression. For example, in two events, pGBi-166 and pGMBi-95, the *BAR* coding sequence was heavily methylated, and the methylation spread to the coding sequence upstream of the dsRNA region. Within the region sharing the sequence identity with the dsRNA, the cytosines in almost all CpG (97%) sequences were

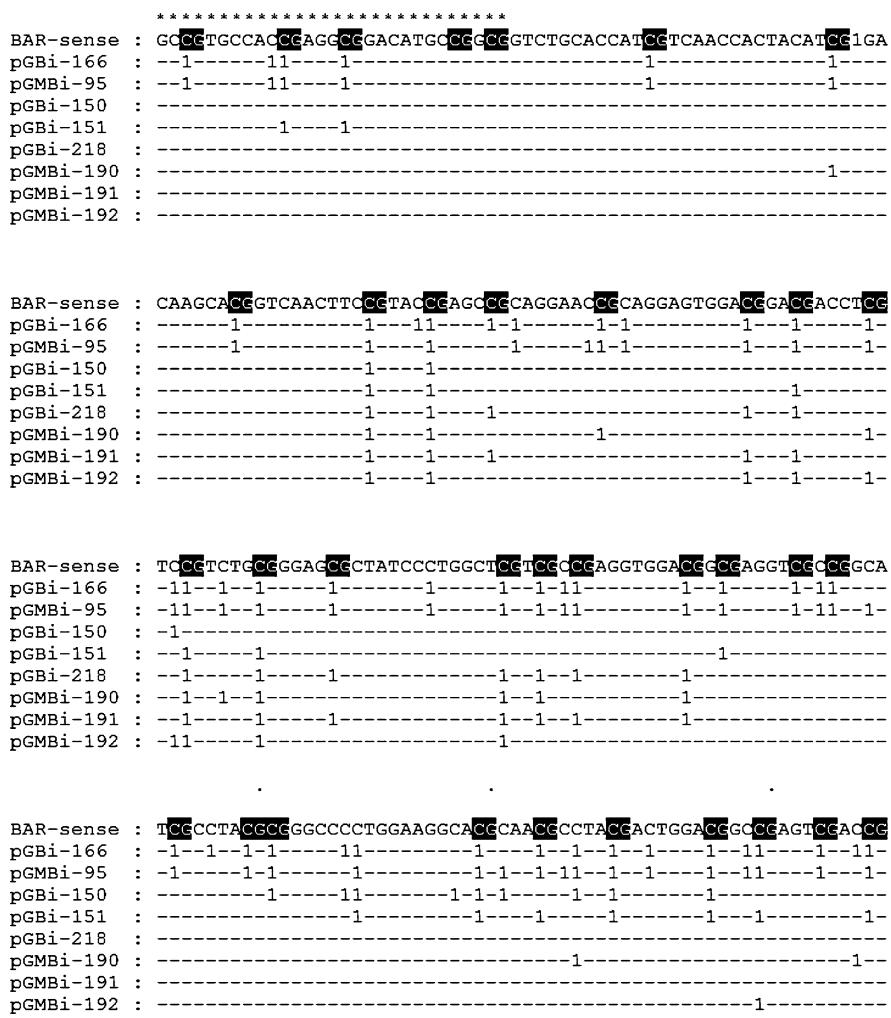

Fig. 5 Methylation patterns of eight highly suppressed pGBi and pGMBi events. CpG sequences were shaded and methylated cytosines were indicated by the number 1. The sequence region that was studied for methylation but was not included in the inverted repeat is indicated by "*". BAR-sense: unmethylated sense strand of the *BAR* coding sequence

methylated. In addition, cytosine methylation also occurred in the symmetric sequence context CNG at a frequency of methylated cytosines of 75% for pGBi-166 and 50% for pGMBi-95. Methylation was infrequent at the non-symmetric sequence CHH (11–14%). In the other six events, cytosine methylation was less frequent, and predominantly occurred in CpG sequences, ranging from 20 to 40%, and rarely in CNG (0–6%) or CHH (0–14%) sequences.

Discussion

Effect of different types of dsRNAs

We have demonstrated that our ihpRNA constructs gave high and stable RNAi suppression. The molecular phenotypes of the Bi transformants generally fell into two categories: highly silenced (more than 90% suppression) and non-silenced, with the former group accounting for 80% of the Bi transgenic populations. Other studies in our lab, however, have reported low frequencies of RNAi gene suppression. Out of 15 transgenic events containing an IR of *PtCENL-1*, the poplar homolog of the *Arabidopsis TERMINAL FLOWER 1* gene, Mohamed (2006) reported that only four events (27%) showed more than 50% suppression of the *PtCENL-1* endogene. Brunner et al. (2007) reported that 18% of 17 transgenic events directed against the poplar *LEAFY* gene, *PTLF*, gave expression below 50% in vegetative tissues. In *Arabidopsis*, the degree of RNAi suppression varied dramatically among 25 targeted endogenes, with some having little or no residual

transcript RNA, and others showing only moderate reductions (Kerschen et al. 2004). Similarly, in maize, RNAi constructs targeting different endogenous genes showed variable silencing efficiency, ranging from non-detectable silencing to nearly complete silencing (McGinnis et al. 2007). As recently reviewed, a number of factors could contribute to the efficacy of siRNA, including the thermodynamic properties of the siRNA and the target RNA structure (Kurreck 2006). Therefore, RNAi efficiency might largely depend on the sequence of dsRNA and the properties and structures of target genes. However, McGinnis et al. (2007) found no correlation of silencing efficiency with expression level of the target gene and sequence features of the dsRNA.

Transcribed dsRNAs directed at promoters have been demonstrated to induce TGS of reporter transgenes and endogenes in plants (Mette et al. 2000; Cigan et al. 2005). Synthetic siRNAs targeted to the promoter of a specific gene can also induce TGS in human cells (Kawasaki and Taira 2004; Morris et al. 2004). Our ihpRNA constructs containing IR directed at the promoter sequence were able to induce suppression of the *BAR* gene, but with a much lower frequency than IR of the coding sequence. Although 58% of produced Pi transgenic events showed some levels of suppression, only 6% of them showed a level higher than 90%. The varying degrees of suppression between events might be caused by varying methylation levels and contexts in the promoter region of the targeted *BAR* gene (discussed below). The frequency of male-sterile phenotypes appeared to vary among different promoter IRs targeted to genes related to pollen development in maize (Cigan et al. 2005). One IR promoter construct was much less efficient in inducing mutant phenotypes than other promoters of maize genes. The size of IR, properties of promoter sequences and regions used to make the IR, including number of cytosine sites that can be methylated, might affect RNAi efficiency. The *Arabidopsis rbc*S promoter is relatively CpG poor, and only contains 22 CpG sites along its 1,731 bp sequence. The sequence from –31 to –506, relative to the translational start site, was used for IR in our transgenic constructs, and includes only 7 CpG sites, a plant TATA box, and the other motifs for binding transcription factors. Further studies using different sizes of IRs, IRs aimed at different regions of this promoter, and IRs to other promoters, might provide a very different picture of promoter-directed RNAi efficiency in poplar. The large difference in RNAi efficiency observed in our study between the coding- and promoter- targeted constructs might be due to the difference in the frequency of CpG sequence, which can be potentially targeted for DNA methylation (discussed below).

Effect of MARs

MARs may elevate RNAi efficiency if they increase transgene expression (reviewed in Allen et al. 2000) and prevent TGS of the RNAi transgene (Levin et al. 2005). However, they may also reduce RNAi efficiency if they impede homology-dependent gene silencing (Allen et al. 1996; Mlynarova et al. 2003). Chuang and Meyerowitz (2000) found that expression levels from both strands of *AG* RNA encoded by an *AG* IR transgene increased in proportion to the severity of floral phenotype. In addition, IRs driven by the *35S* promoter achieved high suppression efficiency, ranging from 87 to 99% of produced transformants, while the weaker *NOS* promoter gave a much lower efficiency (6%). Cigan et al. (2005) reported that only constitutive expression of *MS45* promoter IR under the control of the *UBIQUITIN* promoter resulted in male-sterile phenotypes in maize, while no mutant phenotypes were observed in transformants containing the same IR driven by a maize anther-specific promoter. We found that MARs had little effect on transgene expression (Li 2006), perhaps explaining their lack of effect on RNAi efficiency. In *Arabidopsis*, MARs from the chicken lysozyme gene boosted average transgene expression levels 5- to 12-fold in a transformed PTGS-impaired background, whereas no such boost was observed in a wild-type background, suggesting that MARs do not suppress PTGS, but act as enhancers of expression only when PTGS is suppressed (Butaye et al. 2004).

Effect of transgene copy number

There is very little information on the effect of copy number of integrated IR on RNAi suppression efficiency. Transgene copy number was not related to severity of phenotypes in RNAi T1 *Arabidopsis*

plants (Chuang and Meyerowitz 2000). Also in *Arabidopsis*, Kerschen et al. (2004) reported that single-copy RNAi T4 lines targeting the same endogene generally reduced transcript levels to the same extent, whereas multi-copy RNAi lines differed in the degree of target reduction and never exceeded the effect of single-copy transgenes. We found no obvious association between RNAi transgene copy number and suppression levels of the resident *BAR* gene. Single- and multiple-copy transgenics both showed highly variable degrees of gene suppression. Two of three events that contained three copies showed a high degree of gene suppression, suggesting that multiple copies do not preclude high levels of suppression in poplar.

Stability of RNAi over time

There have been numerous studies on stability of general transgene expression in crop plants (Stam et al. 1997; Kooter et al. 1999) and in trees (reviewed in (Hoenicka and Fladung 2006). However, little information is available on stability of RNAi suppression. Stable suppression of target genes over the long term would be particularly important if RNAi technology is used for transgene confinement (Strauss et al. 1995). Phenotypic changes in a single, highly silenced RNAi transgenic line appeared to be stably inherited over five generations in *Arabidopsis* (Stoutjesdijk et al. 2002).

We observed high stability of RNAi suppression with different constructs over 2 years in the field. RNAi efficiency did not appear to be affected by the changing level of expression of the *rbc*S-driven *BAR* gene over a season, nor was it reduced prior to spring bud flush or during cool spring temperatures. Temperature-dependent RNAi silencing has been reported in plants (Szittya et al. 2003; Sós-Hegedűs et al. 2005), *Drosophila* (Fortier and Belote 2000), and mammalian cells (Kameda et al. 2004). A variety of limiting temperatures were reported in those studies. In *Nicotiana benthamiana*, levels of dsRNA-associated siRNA decreased at low temperature (15°C). In *Drosophila* the RNAi effect on sex differentiation observed at 29°C was strongly inhibited at 22°C. A higher limiting temperature was observed in mammalian cells, where RNAi was observed at 28°C or below. We found that two highly silenced events, one transformed with Bi, and the other with Pi, remained highly suppressed during the period from early March to July, in spite of large temperature differences on the sampling dates. Therefore, it appears that dormancy cycles and environmental variation might not significantly impact RNAi gene suppression in poplar. However, further study of RNAi stability with age and environment, as well as with specific endogene targets of commercial value, such as floral homeotic genes that might be targeted by sterility constructs, are needed. Nonetheless, our results suggest that the RNAi machinery in poplar has evolved to be robust even under the extreme environmental conditions to which temperate-zone trees species must adapt.

Role of DNA methylation

DNA methylation has been implicated in both gene regulation and transgene silencing in plants (reviewed in Wassenegger 2000). In mammalian genomes, methylation occurs almost exclusively at cytosines in the symmetric dinucleotide context CpG. In plant genomes, the sequence CpG is also the predominant methylation context, but the symmetric context CNG and asymmetric context CHH can also be methylated (Mathieu and Bender 2004). RNA-directed DNA methylation (RdDM) has been implicated in TGS that is initiated by dsRNAs containing promoter sequences (Mette et al. 2000; Aufsatz et al. 2002; Kawasaki and Taira 2004; Cigan et al. 2005).

We found that three highly suppressed events containing IR directed at promoter sequence showed methylation in the promoter region. DNA methylation only occurred in the sequence homologous to the IR used in the transgenic constructs, and did not spread to other regions of the same promoter. Aufsatz et al. (2002) also reported that in *Arabidopsis* dsRNA caused do novo methylation within a region of RNA-DNA sequence identity of the *NOS* promoter of a transgene, but in contrast to our study, DNA methylation was much more frequent and affected cytosines in any sequence context. In the present study, DNA methylation affected all symmetric CpG and CNG sequences in two highly suppressed events, whereas cytosines in non-symmetric contexts were rarely affected. However, in the other event studied, DNA methylation affected non-symmetric contexts at

a much higher frequency. Our results are surprising, given previous findings that RdDM affects cytosines in all possible sequence contexts (reviewed in Mathieu and Bender 2004). The maintenance DNA methyltransferase MET1 can maintain CpG methylation in the absense of a DNA methylation trigger, whereas multiple, interacting pathways appear to propagate non-CpG methylation, and endogenous loci appear to require different combinations of pathways to maintain their non-CpG methylation patterns (Chan et al. 2006). Cigan et al. (2005) showed that IRs targeting different promoters varied in their efficiency of silencing and in the degree of suppression among transgenic events, suggesting that inherent properties of the IR fragment or target promoter, such as number and arrangement of potential methylation sites, affect frequency and intensity of suppression.

RdDM was observed in protein-coding regions in many cases of PTGS (Waterhouse et al. 1998; Ebbs et al. 2005). However, the degree to which DNA methylation is relevant to PTGS remains uncertain. Out of eight highly silenced events containing IR directed at coding sequence, two events showed heavy DNA methylation in the coding sequence. DNA methylation occurred at 97% of the CpG sequence contents, more than 50% of the symmetric CNG sequences, and 10 to 14% of the non-symmetric CHH sequences. This is consistent with a previous finding that cytosines at both symmetric and non-symmetric sites can be methylated by RdDM (Pélissier et al. 1999). DNA methylation was not restricted to the sequence used for IR, and spread to the other regions of coding sequence, including the transit peptide. However, DNA methylation did not spread to the untranscribed promoter region. DNA methylation also occurred in the other six highly silenced events but to a lesser extent, with the CpG sequence predominately methylated. Events with the same level of suppression also varied greatly in methylation patterns. Although previous findings and current observation indicated that DNA methylation is involved in RNA silencing, its patterns and degrees of methylation are not clearly associated with RNAi suppression efficiency.

In conclusion, our results suggest that RNAi may be highly stable in perennial plant species whose life cycle requires gene suppression over many dormancy cycles. This indicates that multiple-year functional genomics experiments, such as for wood properties, onset of flowering, and stability of abiotic and biotic stress tolerances, should be reliable. They also suggest that RNAi experiments can be highly efficient, especially when directed at coding regions, without control of insert copy number or use of putative stabilizing elements such as MARs. Further work to test and extend these inferences should consider additional species, a wider variety of target genes and promoters, additional RNAi inducers such as amiRNAs, and comparison of endogene vs. transgene suppression rates.

Acknowledgements We would like to thank Plant Genetic Systems for kindly providing the vector pTTM8, Monsanto for the use of their *CP4* gene, Dr. Steven Spiker for providing MAR fragment, and Dr. Peter Waterhouse for the use of pHANNIBAL. This work was supported by Initiative for Future Agriculture and Food Systems (award # 00-52100-9623) from the USDA Cooperative State Research, Education, and Extension Service; TBGRC/TGERC Research Cooperative; the OSU Foundation Forest Biotechnology Fund; and the Conrad Wessela Scholarship Fund at the OSU College of Forestry.

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