Effectiveness of RNA interference in transgenic plants

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Abstract RNA interference (RNAi) can be used to study gene function by effecting degradation of the targeted transcript. However, the effectiveness of transgene-induced RNAi among multiple target genes has not been compared systematically. To this end, we developed a relative quantitative RT-PCR protocol that allows use of a single internal standard over a wide range of target gene expression levels. Using this method in an analysis of transgenic *Arabidopsis thaliana* RNAi lines targeting 25 different endogenes revealed that independent, homozygous, single-copy (sc) T4 lines targeting the same gene generally reduce transcript levels to the same extent, whereas multi-copy RNAi lines differed in the degree of target reduction and never exceeded the effect of sc transgenes. The maximal reduction of target transcript levels varied among targets. These observations suggest that each target sequence possesses an inherent degree of susceptibility to dsRNA-mediated degradation.

Keywords: RNA interference; Functional genomics; RT-PCR internal control; Amplification inhibitor; Target transcript accumulation; Transgene copy number

1. Introduction

A preferred method for delivering dsRNA to plants for inducing RNA interference (RNAi) is to introduce transgenes engineered to express self-complementary transcripts that can 'fold back' to form dsRNA molecules [1]. The principal advantage of integrated transgenes over transiently introduced dsRNA is that the silencing phenotype is sexually transmissible, and so permanent collections of stable RNAi lines can be produced and archived in public repositories.

RNAi induced by dsRNA-producing transgenes targeting a variety of endogenous genes in plants has been shown to result in a high frequency of mutant phenotypes among independent transformants [2–5]. The effectiveness of RNAi was categorized primarily on the basis of the phenotypic effects observed for a given target gene, and the plants analyzed were mostly mixed populations of transformants carrying single or multiple inserts of the dsRNA producing transgene in either hemi- or homozygous condition.

Here, we analyzed the effectiveness of RNAi among multiple target genes in *Arabidopsis thaliana* systematically by identifying RNAi lines that each carry only a single-copy (sc) of the transgene, in the homozygous condition, and by using a single internal control for relative quantitative RT-PCR analysis of target transcript concentration. We found that independent, sc RNAi lines targeting the same gene generally reduced target transcript levels to a similar extent, and the maximal degree of reduction of target transcripts appears to be target-specific. By contrast, multi-copy (mc) RNAi lines analyzed side-by-side with sc lines frequently reduced target RNA levels to a lesser extent and with more variability between lines than did sc lines.

2. Materials and methods

2.1. Generation of RNAi lines

RNAi vectors pFGC1008 and pFGC5941 (GenBank Accession Nos. AY310333 and AY310901, respectively; Arabidopsis Biological Resource Center (ABRC) stock numbers CD3-446 and CD3-447, respectively) were derived from pCAMBIA1200 and pCAMBIA1300, respectively, and used to produce dsRNA-producing transgenes. The constructs are available from the ABRC and maps are displayed at www.chromdb.org. Each RNAi construct contained a cDNA fragment (0.4–0.8 kb) derived from the respective target gene and oriented as an inverted repeat with each repeat separated by either a fragment from the β-glucuronidase gene (pFGC1008) or an intron from the *Petunia hybrida* Chalcone synthase A gene (pFGC5941).

RNAi lines were produced using *Agrobacterium tumefaciens* LBA4404-mediated germ-line transformation of *A. thaliana* accession Wassilewskija (Ws) essentially as described by Clough and Bent [6]. sc transformants were identified by DNA gel blot hybridization using plant genomic DNA digested with *Nco*I (pFGC1008-derived constructs) or *Eco*RI (pFGC5941-derived constructs) and random-primed probes specific for regions adjoining the left and right T-DNA borders to discriminate mc insertions from sc insertions. sc transformants were allowed to self-pollinate and homozygous lines were identified by segregation analysis. A list of all available RNAi lines, including ABRC stock numbers, is available upon request or can be viewed at www.chromdb.org.

2.2. Total RNA extraction

Total RNA was extracted from 10-day-old seedlings grown on MS medium supplemented with 3% sucrose under standard growth conditions (24 °C, 16 h light). Approximately 150 mg of seedlings was transferred to 1.5-ml microfuge tubes and subsequently frozen in liquid nitrogen. The frozen tissue was ground using a disposable small plastic pestle. 0.5 ml of NTES buffer (100 mM NaCl, 10 mM Tris, pH 7.5, 1 mM EDTA, and 1% SDS) was added to each tube, the tissue vortexed, and the contents of the tubes extracted with phenol:chloroform:iso-amyl alcohol (25:24:1) and chloroform:iso-amyl alcohol (24:1). The RNA was precipitated in the presence of 2 M lithium acetate for a minimum time of 3 h on ice to a maximum time of overnight at 4 °C. The RNA was pelleted by centrifugation for 15 min at 4 °C, the supernatant removed, and the pellet resuspended in water. Finally, the RNA was precipated in the presence of 0.3 M sodium acetate and 2.5

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Abbreviations: RNAi, RNA interference; *GAPC*, glyceraldehyde-3-phosphate dehydrogenase C subunit gene

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volumes of 95% ethanol, rinsed with 70% ethanol, dried, and resuspended in 50 μl of water.

2.3. Reverse transcription

5 μg of total RNA was denatured at 70 °C for 10 min in the presence of 3.33 μM oligo(dT)$_{12–18}$ primers. The tube was immediately chilled on ice and reverse-transcribed with 5 U MMLV reverse transcriptase (Promega Co., Madison, WI), 0.5 mM of each dNTP, and 20 U RNasin (Promega, Madison, WI) in a total reaction volume of 30 μl at 42 °C for 50 min. The reaction was heat-inactivated at 70 °C for 15 min. The resultant first-strand cDNA was purified by subsequent extractions with phenol:chloroform:isoamyl alcohol (25:24:1) and chloroform:isoamyl alcohol (24:1), precipitated in the presence of 0.3 M sodium acetate and 2.5 volumes of 95% ethanol, and resuspended in 25 μl of water.

2.4. Polymerase chain reaction

Polymerase chain reaction (PCR) was performed using 0.5 μl of undiluted or diluted cDNA template, 0.5 U *Taq* polymerase (Sigma–Aldrich Co., USA), 1× *Taq* polymerase reaction buffer (Sigma–Aldrich, USA), 2 mM $MgCl_2$, 100 μM of each dNTP, and 500 nM target-specific primers (MWG Biotech AG, Ebersberg, Germany). Thermocycler conditions included an initial denaturation at 94 °C for 2 min, followed by 32 cycles consisting of 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s, and a final synthesis step at 72 °C for 7 min. PCR products were run in 1.2% agarose gels using 0.5× TBE and stained with ethidium bromide. PCR product amounts were estimated visually by comparison with DNA standards or quantified using the UVP LabWorks™ 3.0 image analysis software (UVP Inc., Upland, CA). The efficiency of amplification for a range of PCR cycles within the exponential phase of amplification was calculated according to the formula $E_{n1-n2} = (A_{n2}/A_{n1})^{1/(n2-n1)} - 1$, where $n1$ and $n2$ are the first and last cycle number of a given range, A_{n1} and A_{n2} are the amounts of amplicons produced after $n1$ or $n2$ cycles and E is the efficiency of amplification (a value of 1 would indicate 100% efficiency).

2.5. Relative quantitative RT-PCR

During the exponential phase of PCR-amplification, a target template is expected to amplify according to the equation:

$$A_n = A_0(1+E)^n \quad (1)$$

where A_0 is the amount of initial template DNA, n is the number of cycles of amplification, A_n is the amount of specific product after n cycles, and E is the efficiency of each cycle of amplification [7]. When co-amplified with a biologically invariant reference transcript B, the ratio of amplicons produced from A and B can be expressed as:

$$A_n/B_n = A_0(1+E_A)^n/B_0(1+E_B)^n \quad (2)$$

where E_A and E_B represent the specific efficiencies of amplification of transcripts A or B, respectively.

The factor X by which the initial amount of transcript differs between two samples can be expressed as

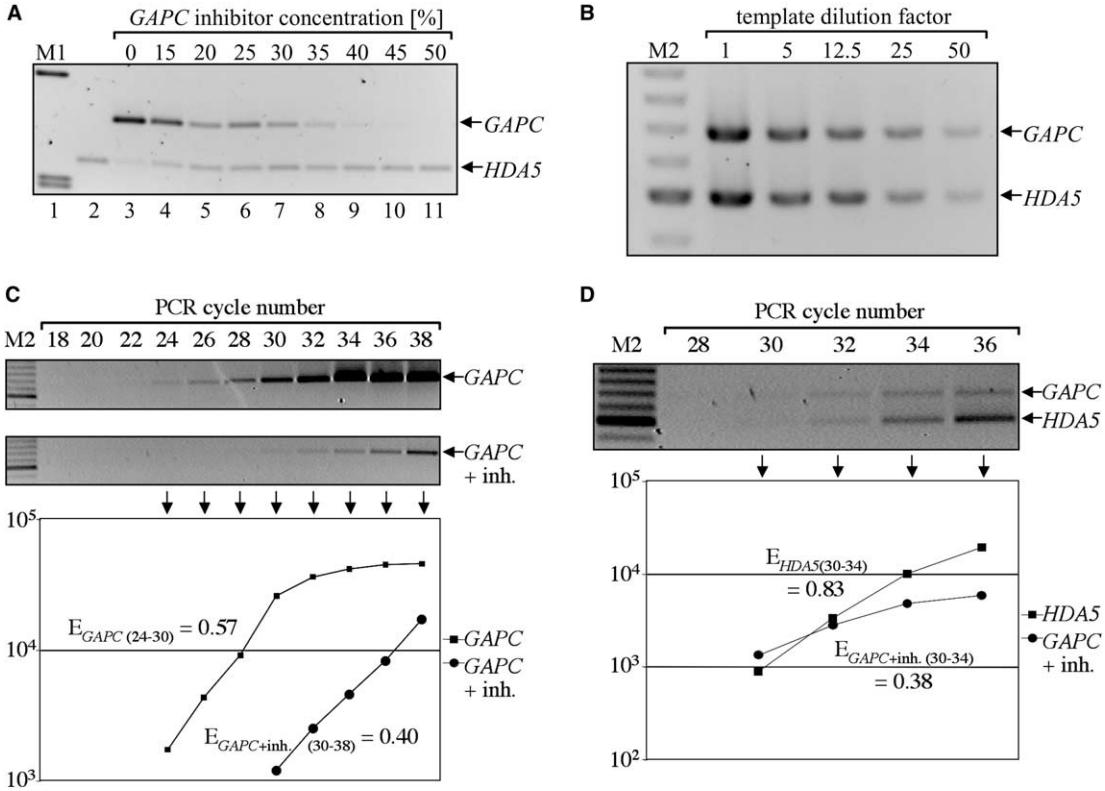

Fig. 1. RT-PCR amplification of *HDA5* and control transcripts using amplification inhibitors. (A) Transcripts derived from the *A. thaliana HDA5* gene were co-amplified with *GAPC* as an endogenous control transcript using increasing concentrations of *GAPC* amplification inhibitors. *A. thaliana* wild-type first-strand cDNA diluted 1/25 was used as template for 32 cycles of PCR amplification, and amplification products were separated by gel electrophoresis. Comparable amounts of the *GAPC* and *HDA5* amplicons were produced when 30–35% *GAPC* amplification inhibitors were included in the reaction. Lanes 2–11, PCR products resulting from amplification with *HDA5* primers alone (2), or co-amplification with *HDA5* primers and *GAPC* primers F1 and R using 0% (3), 15% (4), 20% (5), 25% (6), 30% (7), 35% (8), 40% (9), 45% (10) or 50% (11) of *GAPC* inhibitors F1′ and R′. (B) *GAPC* and *HDA5* were co-amplified in the presence of 32% *GAPC* amplification inhibitors and using decreasing concentrations of the first-strand cDNA template. The amplification product ratio of the two transcripts remained similar over the range of template concentrations tested. (C) *GAPC* amplification kinetics. *GAPC* was amplified without (top) and with 32% of amplification inhibitors (bottom) over a range of PCR cycle numbers (18–38). Signal intensities of the ethidium bromide-stained amplification products were quantified using the UVP LabWorks™ 3.0 image analysis software and plotted against cycle numbers (y-axis = background-corrected total density values). The efficiencies of amplification for a range of PCR cycles within the exponential phase of amplification were calculated according to the formula given in Section 2.4. (D) Co-amplification kinetics of *GAPC* and *HDA5*. *GAPC* and *HDA5* were co-amplified using 32% of *GAPC* amplification inhibitors over a range of PCR cycle numbers (28–36), and signal intensities were quantified and plotted against cycle numbers as described in (C). M1, 1-kb ladder (New England Biolabs, Inc., Beverly, MA); M2, 100-bp ladder (Invitrogen Co., Carlsbad, CA); inh., *GAPC* amplification inhibitors.

$$X = \frac{A1_n/B1_n}{A2_n/B2_n} = \frac{A1_0(1+E_A)^n B2_0(1+E_B)^n}{B1_0(1+E_B)^n A2_0(1+E_A)^n} = \frac{A1_0}{A2_0}\frac{B2_0}{B1_0} \quad (3)$$

where $B2_0/B1_0$ represents the normalization factor that accounts for sample to sample variability. X was determined experimentally by quantification of the PCR products $A1_n$, $B1_n$, $A2_n$ and $B2_n$ as described in Section 2.4.

For the purpose of this study, glyceraldehyde-3-phosphate dehydrogenase C subunit gene (*GAPC*) was chosen as an internal standard for relative quantitative RT-PCR assays. The following generalized procedures describe the relative quantitative RT-PCR protocol used for all RNAi lines assayed in this study.

Primer and inhibitor design. *GAPC* was amplified using either primer pair F1 (5′-CTGTCAACGACCCCTTCATC-3′) and R (5′-CCTGTTGTCGCCAACGAAGTC-3′) or primer pair F2 (5-CACTTGAAGGGTGGTGCCAAG-3′) and R, yielding amplification products of 785 or 543 bp, respectively. The corresponding *GAPC* amplification inhibitors were F1′ = 5′-GCTCGTCGCTGTCAACGACCCCTTCATC-dideoxyC-3′, F2′ = 5′-CTGCAGCTCACTTGAAGGGTGGTGCCAAG-dideoxyC-3′, and R′ = 5′ AATGCTCGACCTGTTGTCGCCAACGAAGTC-dideoxyC-3′. *HDA5* was amplified with forward primer 5′-CATAAATGTTCCATGGGATCAAG-3′ and reverse primer 5′-ATCAGCTCTCCAAGATGTAGATGC-3′ to give a 601-bp amplification product. Primers specific for all endogenous transcripts targeted by RNAi were designed to (a) amplify a fragment of the target transcript that only partially overlaps with the fragment incorporated into the RNAi construct, so that transgene transcripts would not be amplified, (b) distinguish spliced transcripts from unprocessed precursors or genomic DNA contamination, and (c) allow separation of the amplification products from the *GAPC* control by standard agarose gel electrophoresis. A list of all target gene-specific primers is available upon request.

Template dilution test. To standardize the RT-PCR assay for all targeted transcripts, all PCRs were carried out under identical conditions (see Section 2.4). Because the majority of chromatin gene transcripts yielded detectable amplification products after 32 cycles of amplification, this cycle number was chosen for the standardized procedure described here. Relative quantitation of transcript levels by RT-PCR is not accurate once the stationary phase of PCR amplification is reached [8], so the end-points of PCR amplification being assayed need to fall within the exponential phase of amplification. Since the level of expression varied widely between the chromatin genes assayed, the template dilution required for PCR amplification to remain within the exponential phase was determined for each transcript of interest. To this end, PCRs with primers specific for individual genes of interest were performed with template dilution series consisting of undiluted first-strand cDNA from wild-type *A. thaliana* Ws seedlings as well as dilutions of 1/5, 1/25, and 1/125. PCR product amounts were estimated on agarose gels by comparison with DNA standards. The exponential phase of amplification ends at $\sim 10^{12}$ molecules of product under standard PCR conditions [8]. 10^{12} molecules of a 0.5–1.0 kb PCR product (the size range of amplicons being tested) are ~550–1100 ng. To ensure that PCR amplification remained within the exponential phase, a dilution was selected that would produce <100 ng of PCR product and less PCR product than the next more concentrated template of the dilution series.

Inhibitor concentration test. Multiplex PCRs using *GAPC* as the internal control were carried out at the selected dilution and under the same PCR conditions but with the addition of *GAPC*-specific primers (500 nM each) and increasing concentrations of inhibitors (15–50% of total *GAPC*-specific oligonucleotides) (MWG Biotech AG, Ebersberg, Germany). The inhibitor concentration that produced similar amounts of the target and the control amplicon using wild-type first-strand cDNA as template was selected to assay RNAi lines.

3. Results and discussion

RNAi lines targeting genes predicted to encode chromatin-associated proteins were produced by transformation of *A. thaliana* with dsRNA-producing transgenes by the Plant Chromatin Functional Genomics Consortium (www.chromdb.org). To assess the effectiveness of these transgenes systematically, we identified transformants that each carry only a sc of the transgene. These transformants were propagated by self-fertilization, and homozygous progeny plants were assayed in the T4 generation. Moreover, to assay the effect of the transgene in a standardized manner that does not rely on phenotypic effects specific for individual target genes, we determined to what extent the concentration of the target transcript was affected. Relative quantitative RT-PCR can be used to compare target transcript levels in different biological samples (e.g., wild-type vs. mutant plants) relative to another endogenous transcript that serves as an internal standard to control for sample-to-sample variability [8,9]. Rather than attempt to identify and characterize many different control transcripts to cover the full range of target gene expression levels, we devised a method that inhibits amplification of a single, abundant control transcript to whatever extent is necessary to match its amplification to that of any target transcript of lower abundance.

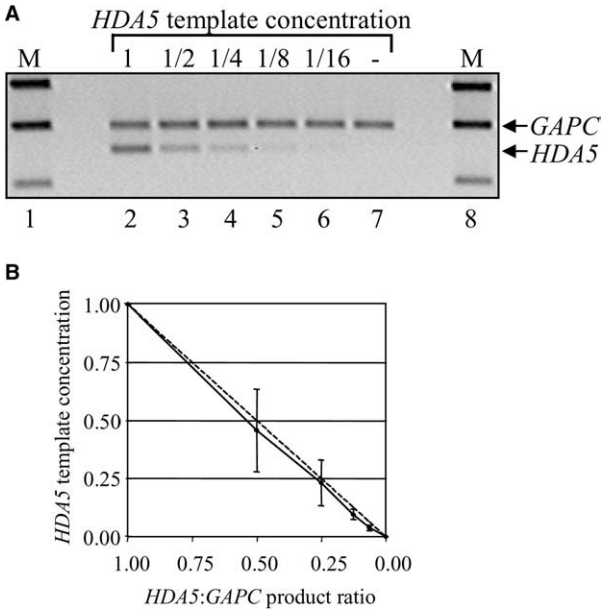

Fig. 2. Co-amplification of *HDA5* and control cDNA using decreasing concentrations of *HDA5* cDNA. (A) *HDA5* and *GAPC* were co-amplified from a mixture of purified *HDA5* and *GAPC* cDNA fragments. *HDA5* and *GAPC* fragments were amplified separately from wild-type first-strand cDNA and gel-purified (QIAquick Gel Extraction Kit, Qiagen, Hilden, Germany). The purified cDNA fragments were diluted to ensure that PCR amplification remained within the exponential phase after 32 cycles (see Section 2.5) and then mixed at a ratio that produced similar amounts of amplicons after 32 cycles of PCR (using a *GAPC* amplification inhibitor concentration of 32%) (lane 2). In parallel reactions, the concentration of *HDA5* cDNA that was added to the *GAPC* cDNA was reduced by factors of 2, 4, 8, and 16 (lanes 3–6), while the concentration of the *GAPC* cDNA was kept constant. In lane 7, no *HDA5* cDNA was added. (B) The ratio of *HDA5* amplicon to *GAPC* amplicon signal intensities (as quantified using the UVP LabWorks™ 3.0 image analysis software and normalized against the ratio of the *HDA5* to *GAPC* signals in the sample with the highest (undiluted) *HDA5* concentration as template) averaged over five replicate experiments was plotted against the *HDA5* template concentration (solid line). The standard deviation is indicated by error bars. The dashed line corresponds to the diagonal that would indicate perfect linear proportionality. M, Low DNA mass ladder (Invitrogen, Carlsbad, CA).

3.1. Relative quantitative RT-PCR using amplification inhibitors of an internal control

The relative quantitative RT-PCR protocol we used achieves inhibition of control amplification with non-extensible oligonucleotides (see Section 2.5) identical in sequence to the control's amplification primers except that the non-extensible oligonucleotides terminate in a 3′-dideoxynucleotide and are extended at the 5′ end in order to compete more effectively for annealing sites on the template cDNA than would inhibitors of the same length as the primers. As control transcript, we utilized *GAPC* (*A. thaliana* locus At3g04120), encoding glyceraldehyde-3-phosphate dehydrogenase C subunit and expressed at moderately high levels in *Arabidopsis* seedlings. Fig. 1A shows the results of RT-PCR amplification with increasing concentrations of 3′-dideoxyoligonucleotides in the presence of primers specific for *HDA5* (*A. thaliana* locus #At5g61060), demonstrating that *GAPC* amplification is reduced in the presence of inhibitors (lanes 4–11). At an inhibitor to primer molar ratio of approximately 0.5:1 (inhibitor concentration = 30–35% of total oligonucleotides), the inhibitors were found to reduce *GAPC* amplification sufficiently to produce similar amounts of *HDA5* and *GAPC* products, whereas *HDA5* amplification was not reduced (compare lanes 2 and 7–8). Under these conditions, co-amplification of *GAPC* and *HDA5* was independent of cDNA template concentration over a 50-fold range (Fig. 1B), showing that the method is robust against sample-to-sample variation in cDNA quantity (as would be expected from Eq. (2)). The kinetics of *GAPC* and *HDA5* amplification in the presence of *GAPC* amplification inhibitors are shown in Fig. 1C and D. The efficiency of *GAPC* amplification was reduced from 0.57 to 0.40 (Fig. 1C). *HDA5* amplification efficiency was approximately twice that of the co-amplified *GAPC* transcripts (Fig. 1D). (For calculation of amplification efficiencies, see Section 2.4.)

In view of the reduced concentration expected for the targeted transcript in RNAi lines, we considered that due to competition for resources during co-amplification of target and control molecules, target abundance might be underestimated in samples in which the target transcript was much less abundant than the control transcript. Thus, the effect of dilution of the target template was investigated, keeping the concentration of the control template constant, as shown in Fig. 2. The target to control product ratio was found to be directly proportional to initial target template concentration over a ~10-fold range, and so provides an accurate measure of initial template concentration.

3.2. Effectiveness of RNAi transgenes in A. thaliana

Using sc, homozygous T4 RNAi lines and relative quantitative RT-PCR with a single internal control transcript as described above, transcript accumulation was analyzed in 2–6 independent RNAi lines for each of 25 target genes (Table 1). Representative results are shown in Fig. 3A. These results demonstrate that the degree of reduction in transcript accumulation is similar for most sc RNAi lines targeting the same

Table 1
Target transcript accumulation in independent, homozygous sc RNAi lines

Target gene	At locus number	RNAi lines	Target accumulation relative to wild-type				IR/cds	In-frame	Spacer fragment
			<20%	20–50%	>50%–<100%	No reduction			
MFP1	At3g16000	6	6				1435–2019/2136	+	CHS A
HDT2	At5g22650	4	4				307–919/921	–	CHS A
CHR6	At2g25170	3	3				3594–4154/4155	+	CHS A
HAG3	At5g50320	3	3				117–887/1698	–	GUS
HDT4	At2g27840	3	3				1–603/612	+	CHS A
NFD3	At1g20696	3	3				28–423/426	+	CHS A
NFD5	At4g35570	3	3				11–375/375	–	CHS A
HDA9	At3g44680	2	2				28–675/1257	+	CHS A
HDT1	At3g44750	2	2				1–732/738	+	GUS
SGA1	At5g38110	2	2				22–657/657	+	GUS
NFC4	At2g19520	5	4	1			262–975/1524	+	CHS A
HAG2	At5g56740	3	3				2–782/1405	–	CHS A
HAG5	At5g09740	3	2			1	559–1056/1335	+	CHS A
NFA2	At2g19480	3	2			1	1–593/1140	+	GUS or CHS A[a]
GTE1	At2g34900	4		4			319–951/1161	–	CHS A
HXA1	At4g16420	4		4			118–762/1464	+	CHS A
HDA2	At5g26040	2		2			442–1020/1023	+	CHS A
CHE1	At3g17590	3		2	1		1–723/723	–	GUS
HXA2	At3g07740	2		1		1[b]	1–639/1647	+	GUS
CHR2	At2g46020	2		1		1	3282–3945/6582	–	GUS
DMT5	At4g19020	3			3		2973–3804/3807	–	CHS A
HAF1	At1g32750	4				4	2581–3345/5757	+	CHS A
CHR4	At5g44800	3				3	2764–3431/6687	–	GUS
NFA3	At5g56950	3				3	58–606/1122	+	CHS A
HAC4	At1g55970	2				2	286–882/4406	+	CHS A
Total number of lines:		77	42	15	4	16			

Target transcript accumulation in RNAi lines relative to wild-type *Arabidopsis* was determined by relative quantitative RT-PCR (see Section 2.5). The position of the cDNA fragments inserted into RNAi constructs relative to the full-length coding sequences of the genes and the origin of the spacer fragment between inverted repeats are indicated (GUS, β-glucuronidase gene fragment; CHS A, intron of the *P. hybrida* chalcone synthase A gene).
[a] Of the two *NFA2* lines with strong reduction in target RNA accumulation, one carries the GUS fragment and one the CHS A intron. The line that did not show any detectable reduction carries the GUS fragment as spacer.
[b] Partial deletion at the right border of the T-DNA insert. [+]In-frame insertion of the target gene cDNA fragment at the ATG of the *NcoI* cloning site.

gene, whereas it varies dramatically among different target genes, with some showing little or no residual transcript RNA (e.g., *HAG3*), some showing little or no reduction (e.g., *CHR4*), and others showing intermediate degrees of reduction (e.g., *HXA1* and *HDA2*).

The observation that target transcript reduction in RNAi lines relative to wild-type varies among targets suggests that each target sequence possesses an inherent degree of susceptibility to RNAi. In *Caenorhabditis elegans*, strong RNAi effects as assessed by a phenotypic analysis were found to correlate with high expression levels of the targeted genes [10]. RNA gel blot analysis of the genes targeted in our study suggests that several of the genes with strongly reduced transcript levels in RNAi lines are expressed at moderate to high levels in wild-type *Arabidopsis* seedlings (e.g., *HDT1*, *HDT2*, and *NFD3*), whereas the four target genes whose transcript levels were not detectably reduced are normally expressed at moderately low levels (*CHR4*, *HAF1*, and *NFA3*) or at a level below the detection threshold (*HAC4*), respectively (see www.chromdb.org). However, we also identified genes with low expression levels in wild-type seedlings that exhibit strongly reduced target transcript levels in RNAi lines (e.g., *HDA9*, *HDT4*, and *SGA1*), suggesting that endogenous transcript accumulation of the targeted gene is not the only target-specific determinant of RNAi effectiveness in *Arabidopsis*. Other factors that may affect RNAi effectiveness in a gene-specific manner include sequence composition, spatial and temporal gene expression patterns, and the normal RNA turnover rate of the targeted gene.

In addition to sc RNAi lines, we analyzed several lines carrying multiple copies of the RNAi construct (Fig. 3B). Although some mc lines showed reduction in transcript accumulation similar to that in sc lines targeting the same transcript, in four out of 12 mc lines analyzed target transcript levels were higher than in most sc lines. Also, mc transgenes did not reduce target levels more than did sc transgenes. Previous studies demonstrated that RNAi lines targeting endogenous genes can produce a series of mutant phenotypes that vary from weak phenotypes to phenotypes resembling known null mutants of the targeted gene [2,3,5]. Our observation that much of the variation among RNAi lines (with respect to level of reduction of target RNA) is due to variation among mc transformants is consistent with the likelihood that mc lines are subject to some degree of transcriptional silencing of the RNAi transgene, thereby reducing its effectiveness. Chuang and Meyerowitz [2] showed that RNAi constructs driven by a

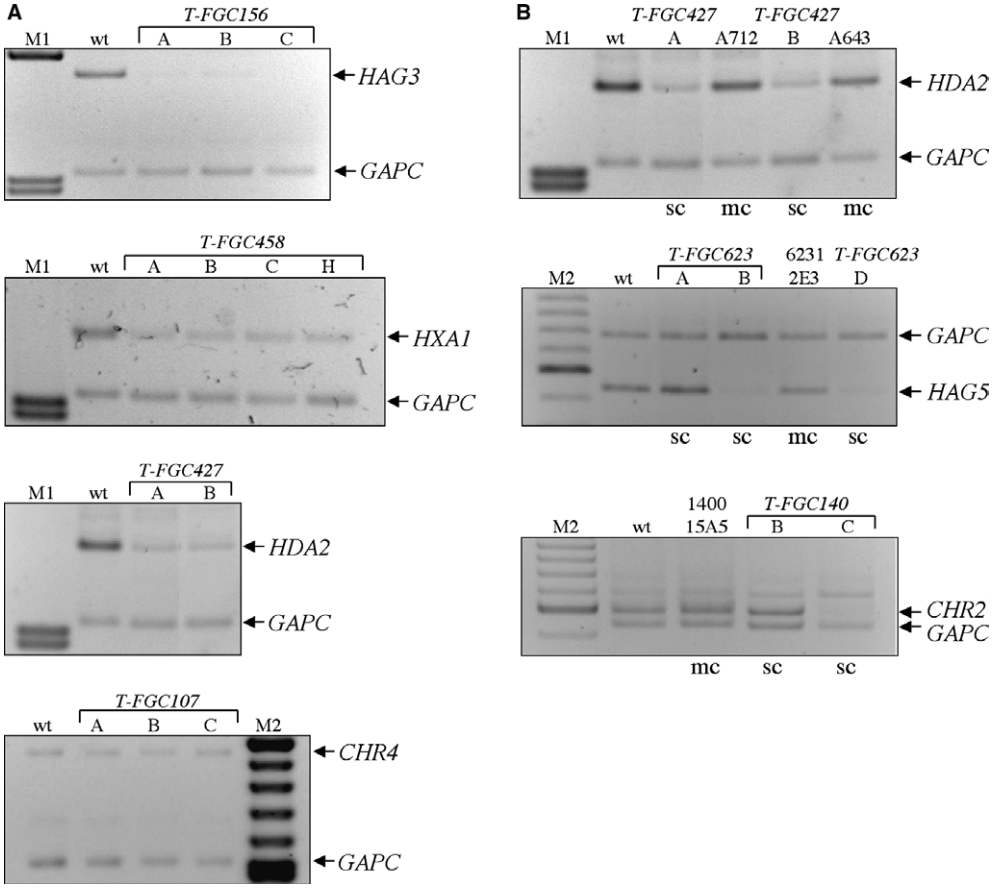

Fig. 3. Relative quantitative RT-PCR of endogenous transcripts targeted by RNAi. (A) sc RNAi lines. The panels show target transcript accumulation in wild-type (wt) *A. thaliana* and independent transgenic lines carrying homozygous, sc inserts of RNAi constructs targeting *HAG3*, *HXA1*, *HDA2*, and *CHR4*. Amplification of *GAPC* as an internal standard was adjusted to be compatible with that of the target transcript by addition of appropriate concentrations (35–45%) of *GAPC* amplification inhibitors. (B) sc and mc RNAi lines. For target genes for which both sc and mc RNAi lines were assayed, the mc lines showed clearly less reduction in target transcript accumulation than at least one corresponding sc line. For sc inserts of the RNAi construct, the T-DNA locus number is given. For mc inserts, the RNAi line number is given. M1, 1-kb ladder (New England Biolabs, Beverly, MA), M2, 100-bp ladder (Invitrogen, Carlsbad, CA).

weaker promoter are less effective than those driven by a stronger promoter. Also, transcriptional transgene silencing can be progressive and increase over generations and may exhibit reversibility and somatic variability [11]. Thus, the usefulness of mc RNAi lines may be compromised. Although mc transgenes might cause greater target reduction than sc transgenes in T1, this advantage may disappear over generations.

Based on our findings, we recommend that functional genomics programs seeking to produce permanent collections of RNAi lines generate sc transgenic lines in order to maximize RNAi effectiveness and stability. mc lines may be useful to achieve intermediate effects on the targeted gene, although with the caveat that these effects may be unreliable due to progressive loss over generations, reversibility, and somatic variability. An alternative approach to vary the degree of effect of RNAi may be to express dsRNA from transgene promoters of different strengths.

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References

[1] Waterhouse, P.M. and Helliwell, C.A. (2003) Nat. Rev. Genet. 4, 29–38.
[2] Chuang, C.-F. and Meyerowitz, E.M. (2000) Proc. Natl. Acad. Sci. USA 97, 4985–4990.
[3] Levin, J.Z., de Framond, A.J., Tuttle, A., Bauer, M.W. and Heifetz, P.B. (2000) Plant Mol. Biol. 44, 759–775.
[4] Wesley, S.V., Helliwell, C.A., Smith, N.A., Wang, M., Rouse, D.T., Liu, Q., Gooding, P.S., Singh, S.P., Abbott, D., Stoutjesdijk, P.A., Robinson, S.P., Gleave, A.P., Green, A.G. and Waterhouse, P.M. (2001) Plant J. 27, 581–590.
[5] Stoutjesdijk, P.A., Singh, S.P., Liu, Q., Hurlstone, C.J., Waterhouse, P.M. and Green, A.G. (2002) Plant Physiol. 129, 1723–1731.
[6] Clough, S.J. and Bent, A.F. (1998) Plant J. 16, 735–743.
[7] Cha, R.S. and Thilly, W.G. (1995) in: PCR Primer: A Laboratory Manual (Dieffenbach, C.W. and Dveksler, G.S., Eds.), pp. 37–51, Cold Spring Harbor Laboratory Press.
[8] Gause, C.G. and Adamovicz, J. (1995) in: PCR Primer: A Laboratory Manual (Dieffenbach, C.W. and Dveksler, G.S., Eds.), pp. 293–311, Cold Spring Harbor Laboratory Press.
[9] Stürzenbaum, S.R. and Kille, P. (2001) Comp. Biochem. Physiol. B 130, 281–289.
[10] Cutter, A.D., Payseur, B.A., Salcedo, T., Estes, A.M., Good, J.M., Wood, E., Hartl, T., Maughan, H., Strempel, J., Wang, B.M., Bryan, A.C. and Dellos, M. (2003) Genome Res. 13, 2651–2657.
[11] Meyer, P. and Saedler, H. (1996) Annu. Rev. Plant Physiol. Plant Mol. Biol. 47, 23–48.