

Gene Trapping with Firefly Luciferase in Arabidopsis. Tagging of Stress-Responsive Genes^{1[w]}

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To monitor the expression of T-DNA-tagged plant genes *in vivo*, a collection of 20,261 transgenic lines of *Arabidopsis* (Columbia-0) were generated with the promoter trap vector pTluc, which carries a promoterless firefly *luc* (*luciferase*) reporter gene linked to the right T-DNA border. By detection of bioluminescence in 3-week-old seedlings, 753 lines were identified showing constitutive, organ-specific, and stress-responsive luciferase expression patterns. To facilitate the identification of well-defined luciferase expression patterns, a pooled seed stock was established. Several lines showed sugar, salt, and abscisic acid (ABA)-inducible luciferase activity. Segregation analysis of 215 promoter trap lines indicated that about 50% of plants contained single insertions, whereas 40% carried two and 10% carried three or more T-DNA tags. Sequencing the T-DNA insert junctions isolated from 17 luciferase-expressing lines identified T-DNA tags in 5'- and 3'-transcribed domains and translational gene fusions generated by T-DNA insertions in exons and introns of *Arabidopsis* genes. Tissue specific expression of eight wild-type *Arabidopsis* genes was confirmed to be similar to the luminescence patterns observed in the corresponding luciferase-tagged lines. Here, we describe the characterization of a transcriptional *luc* reporter gene fusion with the WBC-type ABC transporter gene *At1g17840*. Expression of wild-type and luciferase-tagged *At1g17840* alleles revealed similar induction by salt, glucose, and ABA treatments and gibberellin-mediated down-regulation of ABA-induced expression. These results illustrate that luciferase gene traps are well suited for monitoring the expression of stress-responsive *Arabidopsis* genes *in vivo*.

Based on the availability of complete genome sequence (*Arabidopsis* Genome Initiative, 2000), T-DNA- and transposon-induced insertion mutations can now be identified in any *Arabidopsis* gene using PCR-aided mutant screening approaches and large-scale sequencing of amplified insert junctions (Bouché and Bouchez, 2001; Szabados et al., 2002). To screen for insertions in genes, several promoter and enhancer trap technologies are available that use promoterless reporter genes facing the boundaries of T-DNA or transposon insertions. Promoter trap insertions in transcribed chromosomal loci may generate *in situ* fusions between plant genes and reporter genes that can be easily identified by monitoring the spatial and temporal activity of reporter proteins in diverse plant organs. Transcription enhancers located in the vicinity of insert boundaries can be identified similarly by enhancer trap vectors, in which the reporter genes are linked to low-activity minimal promoters (Klimyuk et al., 1995; Campisi et al., 1999).

Although enhancer trapping yields a higher frequency of *in situ* reporter gene activation, promoter trapping is more effective in yielding loss of function gene mutations. This is illustrated by numerous applications in fruitfly (*Drosophila melanogaster*) and mice (*Mus musculus*; O'Kane and Gehring, 1987; Allen et al., 1988; Bellen et al., 1989) and in *Arabidopsis* (Kertbundit et al., 1991), tobacco (*Nicotiana tabacum*; Teeri et al., 1986; Lindsey et al., 1993; Fobert et al., 1994), and potato (*Solanum tuberosum*; Lindsey et al., 1993; Springer, 2000). Because the expression of reporter genes usually faithfully reflects the expression patterns of tagged genes, the promoter and enhancer trap approaches are widely used to generate specific genetic markers for studying cell differentiation and organ development (Topping et al., 1994; Campisi et al., 1999).

The applicability of plant gene fusion technologies was first demonstrated in tobacco and *Arabidopsis* using a promoterless kanamycin phosphotransferase gene [*aph(3')* II; Teeri et al., 1986; Koncz et al., 1989]. The use of β -glucuronidase (GUS; *uidA*; Jefferson et al., 1987) reporter gene provided later a sensitive means for histochemical detection of tissue and cell-type-specific expression of gene fusions (Kertbundit et al., 1991). However, the GUS reporter system is not suitable for nondestructive screening purposes. Therefore, further developments capitalized on the application of light-emitting bacterial (*lux*) and firefly (*luc*) luciferase reporter enzymes, the synthesis of which can be detected *in vivo* by bioluminescence

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imaging with sensitive CCD cameras (Riggs and Chrispeels, 1987; Riggs et al., 1989; Jiang et al., 1992). Firefly luciferase has a rapid turnover with a half-life time of 3 h; thus, it is well suited as a real-time reporter for in planta gene expression studies (Thompson et al., 1991; Millar et al., 1992). Recent identification of *Ds*-transposon enhancer traps in 108 tomato (*Lycopersicon esculentum*) lines illustrates the usefulness of *luc* reporter system in random gene tagging experiments (Meissner et al., 2000).

Here, we describe a T-DNA-based *luc* promoter trapping approach, which was used to generate a collection of 20,261 Arabidopsis insertion mutant lines. This collection was characterized by screening for *luc* gene fusions that show distinct expression patterns in living plants. A representative sample of active *luc* gene fusions was characterized by recovering the boundaries of pTluc T-DNA and identification of tagged genes driving the expression of the *luc* reporter gene. To illustrate the use of *luc* gene fusion system in the identification of specific stress-regulated genes, we have isolated several *luc* gene fusions that show specific induction by stress stimuli. A *luc* reporter-tagged stress-responsive ABC transporter gene, *At1g17840*, is described as a case study. ABC transporters, carrying conserved ATP-binding and trans-membrane domains, transfer molecules across the membrane against a concentration gradient, gaining energy through ATP hydrolysis (Higgins, 1992). ABC transporters play an important role in detoxification and excretion of potentially harmful compounds in eukaryotes (Henikoff et al., 1997; Higgins, 2001). Plant ABC transporters are implicated in ion channel activity, detoxification, chlorophyll biosynthesis, stomatal movement, and various aspects of plant development (Sidler et al., 1998; Gaedeke et al., 2001; Kushnir et al., 2001; see: Martinoia et al., 2002; van den Brule et al., 2002). Transcript levels of ABC transporter genes are often enhanced by chemicals that are substrates for the transporters themselves; therefore, data on expression regulation can indicate their biochemical function (del Sorbo et al., 1997; Higgins, 2001). In Arabidopsis, 129 potential ABC transporters were identified, most of them with unknown function (Sanchez-Fernandez et al., 2001; Kolukisaoglu et al., 2002). Our data show that the tagged *At1g17840* gene encodes a novel stress-regulated ABC transporter of the WBC subfamily, the expression of which is regulated by sugar, salt, abscisic acid (ABA), and GA.

RESULTS

Screening for in Situ Luciferase Gene Fusions and Characterization of pTluc-Tagged Arabidopsis Collection

Using *Agrobacterium tumefaciens*-mediated transformation, 20,261 promoter trap Arabidopsis lines were generated, carrying the T-DNA of the pTluc gene

fusion vector (Fig. 1). All hygromycin-resistant T₁ seedlings were screened for luciferase expression using bioluminescence imaging (Fig. 2). After recording and analysis of organ-specific luciferase expression patterns (Fig. 3), 17,000 seedlings were transferred to Murashige and Skoog Arabidopsis medium containing either 400 mM Glc or 250 mM NaCl and tested again for luciferase activity. This screen identified 38 plants displaying Glc or salt induced and 32 plants showing decreased luciferase expression. Altogether, luciferase expression was detected in 753 seedlings representing 3.7% of the whole collection.

After harvesting T₂ progeny from all individual plants, seed stocks were prepared by pooling equal amount of seed from 100 individual T₂ families. The screening conditions were optimized by germinating 10-mg aliquots of seed stocks at a density of about 500 seedlings per Petri dish and monitoring the frequency of seedlings displaying similar luminescence patterns in each pool (Fig. 2).

To characterize the collection, the segregation of hygromycin resistance marker and luciferase expression pattern was determined in 215 promoter trap lines (see supplemental data, available in the online

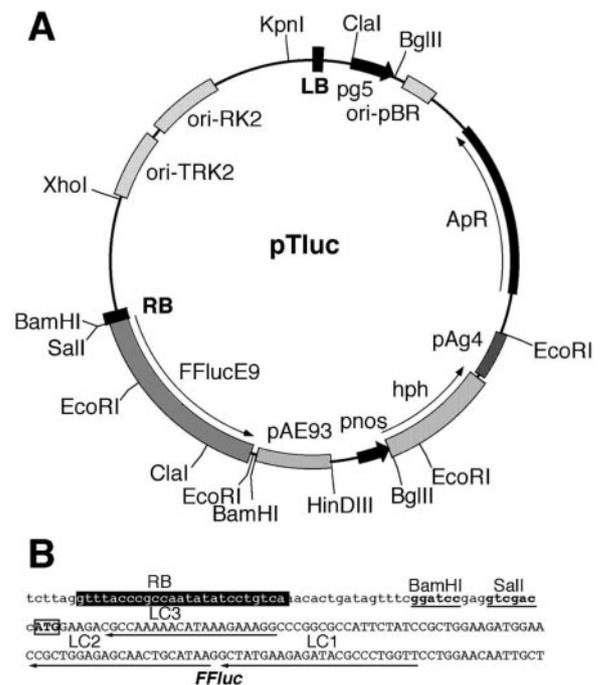
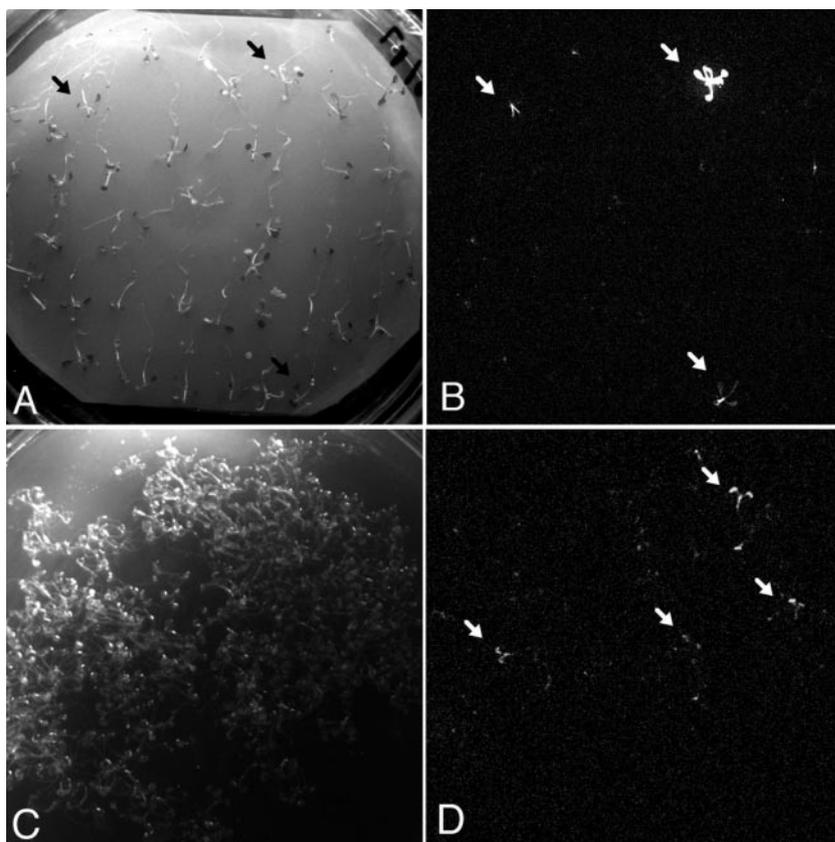


Figure 1. The pTluc promoter trap vector. A, Restriction map of the pTluc vector. pg5, promoter of T-DNA gene 5; ori/pBR, replication origin from plasmid pBR322; ApR/CbR, bacterial ampicillin/carbenicillin resistance marker; pAg4, 3' poly(A⁺) signal sequence of T-DNA gene4; *hph*, hygromycin phosphotransferase gene; *pnos*, nopaline synthase promoter; *FFluc*, firefly luciferase gene; *pAE93*, 3' polyA region of gene E93. B, Sequence of the right border (RB) region. Position of the T-DNA RB is shown by white letters on black background. Sequences of *Bam*HI and *Sall* restriction sites are underlined. ATG of the *FFluc* reporter gene is boxed. The position of oligonucleotides LC1, LC2, and LC3 is indicated by arrows.

Figure 2. Identification of luciferase gene fusions in the pTluc-transformed *Arabidopsis* population. A, Light image of 45 screened T_1 seedlings. B, Luminescence image of the screened plants. Different patterns of luciferase activity are detectable in three plants. C, Identification of a luciferase gene fusion in pooled seed stocks. Image of 500 screened seedlings. D, Detection of luminescence in the pooled seedlings.



version of this article at <http://www.plantphysiol.org>). The hygromycin resistance marker showed 3:1 segregation ratio in 108 lines, whereas 15:1 segregation in 85 lines suggested the presence of two independent T-DNA inserts. The remaining 22 lines displayed a segregation of three or more independent inserts. In lines that carried single inserts, the luciferase expression always cosegregated with the hygromycin resistance marker (data not shown). Hygromycin-resistant T_2 progeny of the selected 215 lines was recurrently subjected to image analysis. Luciferase expression patterns detected in the original T_1 lines were reproducibly observed in the hygromycin-resistant progeny (Table I; for details, see supplemental data). In 22 lines, luminescence was observed in all organs, whereas in 64 lines, a similar pattern was found without detectable expression in the roots. Luciferase activity was predominant in shoot apical region of 73 lines, sometimes accompanied by faint luminescence in hypocotyls or petioles. Luminescence was strongest in stem tissues of 21 lines, in leaves of 32 lines, and in hypocotyls of three lines. Responses to high sugar (400 mM Glc), salt (250 mM NaCl), or cold (4°C) were tested by transferring plants to germination media supplemented with Glc or salt or by incubating plants at 4°C for 6 to 8 h. Stress-responsive luciferase activity could be confirmed in 24 lines, showing enhanced (15 lines) or reduced (nine lines) luminescence in response to one

or several treatments. Examples for such analysis are shown in Figure 3, and features of analyzed lines are depicted in the supplemental data.

Isolation and Characterization of Luciferase Gene Fusions

To analyze the molecular structure of *luc* gene fusions, we have isolated the right T-DNA insert boundaries from 17 T_2 families, which displayed diverse organ-specific luciferase expression patterns, using inverse PCR and Thermally Interlaced (TAIL)-PCR. Sequencing the T-DNA insert junctions localized 5' upstream of the *luc* reporter gene allowed a precise localization of all insertions in the *Arabidopsis* genome by performing Blast homology searches using The *Arabidopsis* Information Resource (TAIR) database. T-DNA inserts were identified in exons and introns of eight *Arabidopsis* genes, providing examples for both translational and transcriptional gene fusions (Koncz et al., 1989, 1994). In five lines, the T-DNA insertions landed in predicted promoter and 5'-UTRs of genes illustrating promoter trap events and formation of transcriptional gene fusions. In three lines, the *luc* reporter gene was localized in 3' transcribed but UTRs of genes. Only in one case was the *luc* gene tag localized in a distance larger than 500 bp upstream of a predicted gene, suggesting that the annotation of corresponding genomic sequence was

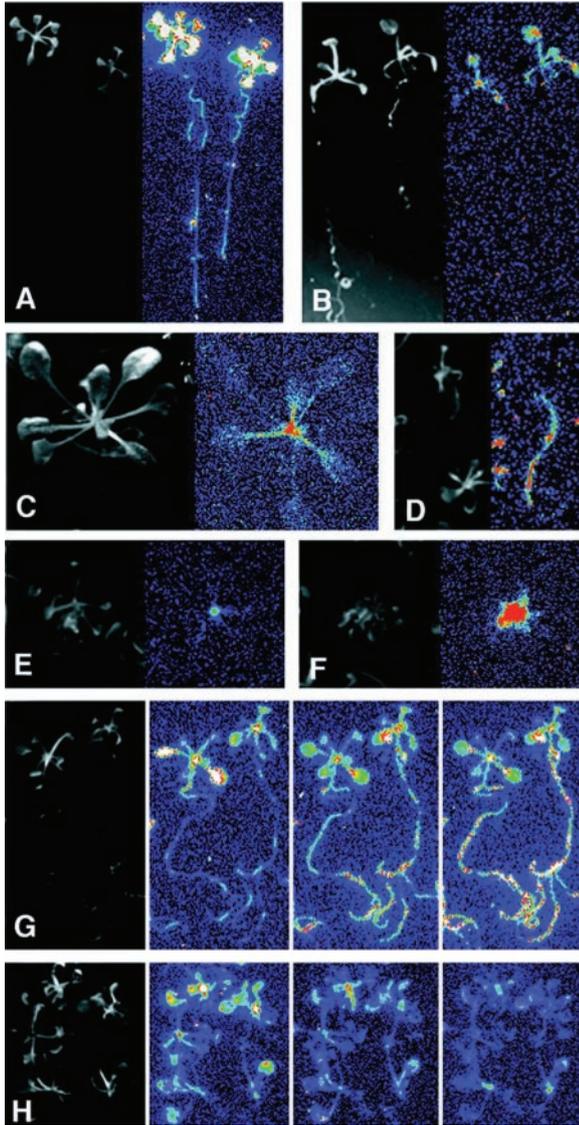


Figure 3. Examples for characteristic luciferase expression patterns in tagged Arabidopsis plants. Images show 3- to 4-week-old plants in light (left) and the corresponding luminescence pattern (right). Colors correspond to increasing luminescence intensity in the following order: blue < green < yellow < red < white. Luminescence patterns: A, constitutive (L3033); B, all green organs (L0213); C, petioles and shoot tip (L6180); D, root (L6318); E, shoot apex (L6331); and F, young leaves (L6365). G and H, Salt-responsive luciferase activity in T_2 plants, which were challenged by high-salt medium. Luminescence was recorded at 0, 2, and 6 h after transfer. G, Salt-induced luminescence in roots (L1518). H, Salt-repressed luminescence (L6177).

either incorrect, or an unknown cryptic promoter led to *luc* gene activation (Table II).

To test whether the expression of luciferase gene fusion precisely reflects the regulation of wild-type alleles of tagged genes, reverse transcription (RT)-PCR analysis of eight such genes was performed (Fig. 4). RNA was prepared from leaf, cotyledon, hypocotyl, root, and stem of in vitro-germinated plants and from cell suspension cultures. Cell cultures are

composed of dividing cells and, therefore, are similar to meristematic regions. cDNA templates obtained by RT were used as templates in RT-PCR reactions. RT-PCR analysis of the 18S rRNA gene *At2g01010* showed constitutive expression and served as a loading marker. T-DNA insertion was localized in the 5' region of the *At2g01010* gene in line L3033, which showed luciferase expression in all tested organs (Fig. 3A). RT-PCR analysis of the other genes revealed tissue-specific alterations in the transcript levels, which were similar to differences observed in luminescence activities of corresponding tagged lines (Fig. 4).

Identification of a Novel Stress-Responsive ABC Transporter

To study a stress-responsive luciferase gene fusion, we performed a detailed analysis of line L0213, which showed salt-inducible luciferase expression in our initial screen. In L0213 plants, luminescence was detected in all tissues except for roots (Fig. 3B). Co-segregation of luciferase activity and hygromycin resistance was tested in 112 T_2 plants. Eighty-five plants displayed luminescence and proved to be hygromycin resistant, whereas 27 plants showing no detectable luminescence were hygromycin sensitive. In addition to salt, the expression of single-copy *luc* gene fusion in line L0213 was also inducible by Glc, mannitol, and ABA treatments (Fig. 5). The tagged gene thus appeared to be controlled by an ABA-dependent regulatory pathway in response to osmotic stress. H_2O_2 repressed luciferase activity, whereas heavy metals, such as $CdCl_2$, and other hormones, including 2,4-D and salicylate, had no apparent effect on the expression of the *luc* reporter gene (Fig. 5).

PCR amplification and sequencing of the right T-DNA insert boundary indicated that the insertion in line L0213 occurred 190 bp upstream of the ATG codon in the 5'-transcribed leader region of a putative gene, *At1g17840*, located in the F2H15 contig of chromosome 1 (Fig. 6). Thus, the sequence analysis indicated that the insertion event resulted in the for-

Table I. Luciferase expression patterns in tagged Arabidopsis lines

Luciferase activity of in vitro germinated 3-week-old T_2 plants was analyzed in 215 selected lines by low-light imaging.

Luminescence Pattern	Lines	%
All organs	22	9.8
Green organs	64	30.2
Leaf	32	14.9
Shoot tip	73	34.0
Stem	21	9.8
Hypocotyl	3	1.4
Total	215	100
Stress induced	15	–
Stress suppressed	9	–

Table II. Sequenced luciferase gene fusions in 17 *Arabidopsis* lines

Positions of T-DNA insertions are shown in relation to the predicted ATG or STOP codons.				
Code	Luminescence	Insertion	Gene	Predicted function
L0202	Green organs	Promoter, -183 bp	At1g01400	Putative UTP-Glc glucosyltransferase
L0211	Green organs	3'-Untranslated region (UTR), +54 bp	At5g20380	Putative inorganic phosphate cotransporter
L0213	Green organs	5'-Untranslated region (UTR), -190 bp	At1g17840	Putative ABC transporter
L0264a	Green organs	3'-Untranslated region (UTR), +157 bp	AT4g25200	Mitochondrion-localized small heat shock protein
L0264b	Green organs	Intron 1, 649 bp	At2g39190	Putative ABC transporter
L0266	Shoot tip	Intron 1,783 bp	At1g08390	YABBY2 protein (2 × transmembrane segment)
L0267	All organs	Intron 3, 995 bp	At3g20000	Putative membrane import protein, TOM40 homolog
L0268	Shoot tip, stem	Promoter, -490 bp	F5E6.8	Hypothetical protein
L0610	Green organs	Intron, 142 bp	At1g56045	60s Ribosomal protein L41
L1518	All organs	Intron, 905 bp	At1g36035	Putative gag-protease polyprotein
L1529	Shoot tip	3' Region, +274 bp	At2g44270	Hypothetical protein
L1538	All organs	Exon, 1,481 bp	T21E2.5	Putative dTDP-D-Glc 4,6-dehydratase
L1551R	Leaf	Exon, 1,713 bp	At4g35260	NAD ⁺ -dependent isocitrate dehydrogenase subunit 1
L1571L	Shoot tip	Promoter, -128 bp	At1g12830	Peroxisomal targeting signal type 2 receptor
L3033	All organs	5' Region, -971 bp	At2g01010	18S ribosomal RNA
L3051	Constitutive	Intron 20, 4,944 bp	At1g17690	Hypothetical protein
L3067	Leaf	Promoter, -219 bp	At3g05760	Hypothetical protein

mation of a transcription gene fusion between the promoterless *luc* gene and the 5'-UTR of gene *At1g17840*. According to gene prediction of TAIR, the *At1g17840* gene encodes a putative ABC transporter protein of 703 amino acids. The predicted ABC transporter carries a conserved nucleotide-binding domain at position 59 to 259 and six trans-membrane domains between amino acid residues 401 and 648. RT-PCR analysis showed that the luciferase activity of line L0213 faithfully reflects the expression of *At1g17840* gene because it is high in most tested organs except in roots, where transcript level proved to be very low (Fig. 4).

To identify homozygous knockout lines, 16 T₂ families were analyzed by PCR amplification using gene- and T-DNA-specific primers (Fig. 6). PCR fragment could not be amplified with the combination of gene- (213R) and T-DNA- (LB21) specific primers in three plants, which showed no luciferase activity. All 13 plants, which carried the amplified T-DNA insert junction of 750 bp, also showed luminescence. From these, four lines proved to be homozygous and nine hemizygous in germination assays on hygromycin-containing medium. The homozygous plants showed no morphological and developmental alterations in comparison with hemizygous and wild-type control lines. In germination assays, differences were not observed in hormone sensitivity (2,4-D, kinetin, ABA, GA₃, and salicylic acid) nor in stress responses (NaCl, mannitol, Glc, H₂O₂, CdCl₂, and paraquat; data not shown).

To compare the regulation of *luc*-tagged and wild-type *At1g17840* alleles, the temporal expression of luciferase reporter and steady-state levels of wild-type *At1g17840* mRNA were tested in 3-week-old

heterozygous T₂ plants. Plants grown on MSAR medium were transferred to fresh medium containing 250 mM NaCl, 400 mM Glc, or 50 μM ABA (Fig. 7). Transferring the plants into new medium resulted in a slight transient increase in luciferase activity within 2 to 3 h, followed by a gradual, steady decline. In response to Glc and salt treatment, luciferase activity was induced three to four times and reached a peak 4 to 5 h after transfer. In ABA-treated plants, maximum luminescence was detected in 2 to 3 h. To compare the luciferase activity with transcriptional regulation of the endogenous *At1g17840* gene, RT-PCR analysis was performed with ubiquitin as internal control. Two to 4 times increase of *At1g17840* transcription was detected upon Glc, salt, or ABA treatments (Fig. 7). Although some differences were observed between the kinetics of stress induction of luciferase-tagged and wild-type *At1g17840* alleles, nonetheless, the RT-PCR assays confirmed that the *At1g17840* gene is activated by the same stimuli, as was indicated by the nondestructive luciferase assays. Therefore, analysis of expression of luciferase gene fusions can provide useful information for further studies. This is illustrated by an additional assay performed with the *luc*-tagged *At1g17840* gene, where sequential treatments were employed to monitor hormonal responses of this gene. T₂ plants were sprayed by 100 μM ABA and subsequently by 100 μM GA₃. Luciferase activity increased 4-fold after ABA treatment, which could be down-regulated by subsequent GA treatment (Fig. 8). Because GA₃ treatment alone had no significant effect on luciferase activity, these results suggested that GA is a negative regulator of ABA-induced *At1g17840* gene expression.

A	gene	template cDNA						PCR cycles	tagged line
		R	H	C	L	S	Cs		
	<i>At5g20380</i>							35	L0211
	<i>At1g17840</i>							29	L0213
	<i>At2g39190</i>							29	L0264
	<i>At1g56045</i>							32	L0610
	<i>At4g35260</i>							35	L1551
	<i>At1g12840</i>							35	L1571
	<i>At1g17690</i>							32	L3051
	<i>At2g01010</i> (18SrRNA)							29	L3033

B	line	luminescence pattern	intensity
	L0211	green organs, mainly leaf, stem	++
	L0213	all green organs	++++
	L0264	green organs: leaf, stem, hypocotyl	++++
	L0610	all green organs	+++
	L1551	all organs, mainly leaf, cotyledon, shoot tip	++
	L1571	mainly shoot tip,	+
	L3051	mainly shoot tip, leaf, hypocotyl, less in root	++
	L3033	all organs, constitutive	++++

Figure 4. Expression analysis of eight Arabidopsis genes. A, Total RNA was isolated from root (R), hypocotyl (H), cotyledon (C), leaf (L), and stem (S) tissues of 3-week-old in vitro-germinated plants and cell suspension cultures (Cs). RT-PCR was performed on equal amount of cDNA templates, using gene-specific primer pairs. PCR fragments are shown after 29 to 35 cycles of amplification. Strong expression levels required lower amplification cycles, whereas more cycles are needed to amplify fragments from low-abundance transcripts. 18SrRNA gene (*At2g01010*) was used as constitutive control. B, Luminescence pattern and intensity in the tested Arabidopsis lines. Approximate light intensities are shown in arbitrary scale from low (+) to high (++++).

DISCUSSION

The Luciferase Gene Fusion System

Using a T-DNA-based *luc* gene fusion vector, we have generated an Arabidopsis insertion mutant collection, which consists of 20,261 transgenic lines. Segregation analysis indicates that about one-half of these lines carry single T-DNA insertions and that the average copy number of T-DNA tags is 1.6 in the collection. These data predict that the collection carries altogether about 32,000 T-DNA inserts. Random sequencing of T-DNA insert junctions in other collections indicates that about 35% of all inserts are located in exons and introns, whereas about 20% of T-DNA tags are found in 5'- and 3'-regulatory regions of genes within 300 bp from the predicted ATG or STOP codons, respectively (Szabados et al., 2002). Gene fusions are only generated when the polarity of promoterless reporter gene within the T-DNA and

the tagged plant genes is identical, whereas for formation of translation gene fusions, the reporter gene should be fused to plant genes in frame. Therefore, the calculated maximum frequency of translation gene fusions in coding regions is: $0.5 \times 0.33 \times 0.35 = 0.058$ (i.e. 5.8% of all inserts), whereas the predicted maximal frequency of inserts in 5'- and 3'-regulatory regions of genes is $0.5 \times 0.2 = 0.1$ (i.e. 10% of all inserts). In our experiments, active *luc* gene fusion was detected in 753 T₁ plants, which represent 3.7% of the population and 15% of the calculated frequency of gene fusions. The frequency of *luc*-expressing lines is lower than the previously reported GUS-expressing enhancer and promoter trap lines (Kertbundit et al., 1991; Campisi et al., 1999; He et al., 2001). Because we screened only in vitro-germinated seedlings, it is likely that our collection contains additional lines that exhibit luminescence in other developmental stages or in different environmental conditions. Furthermore, we used only macroscopic detection, which measures low light intensities in living tissues. Gene fusions that are expressed at very low level or are active only in a few cells could have escaped detection due to limitations in sensitivity and resolution of our luciferase assay

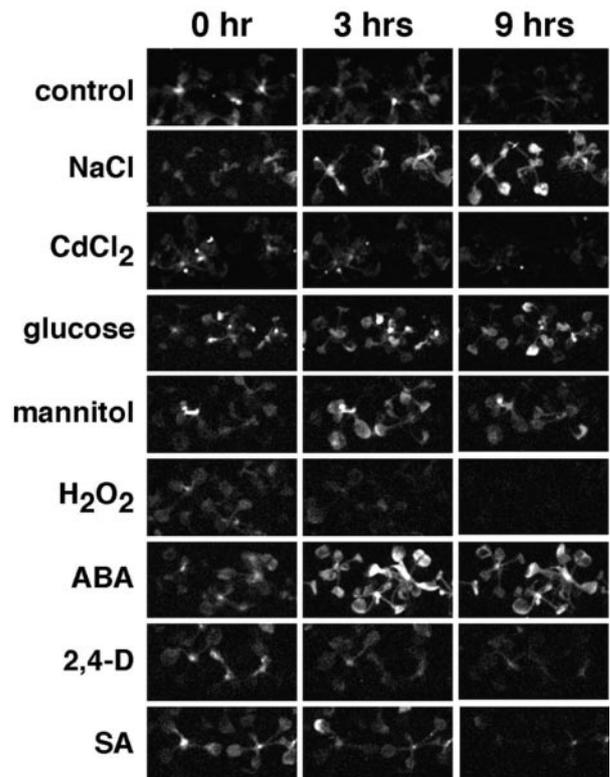
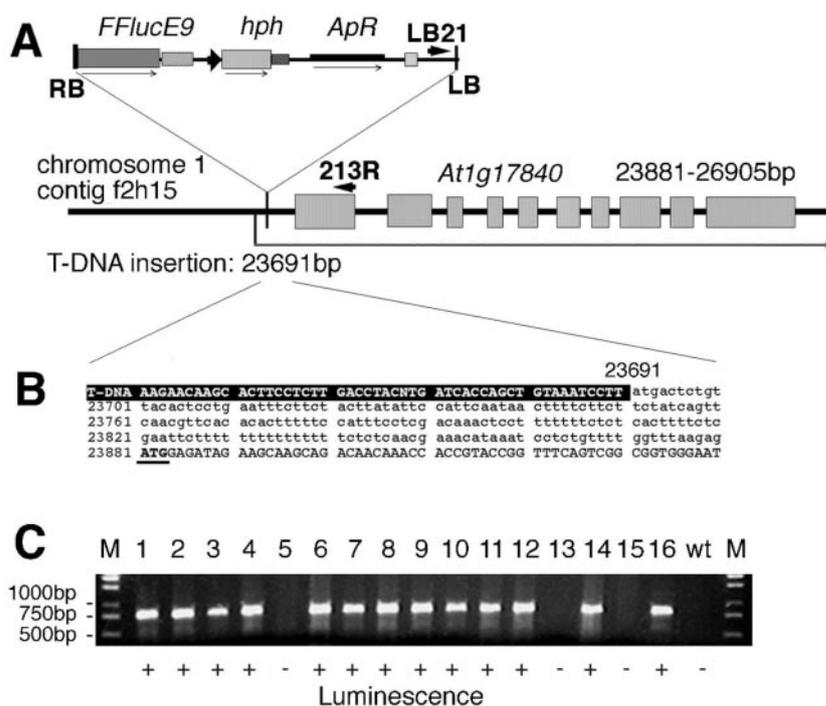


Figure 5. Stress- and hormone-responsive luciferase activity in line L0213. Three-week-old plants were transferred to media supplemented by following additives: 250 mM NaCl, 1 mM CdCl₂, 400 mM Glc, 400 mM mannitol, 8 mM hydrogen peroxide (H₂O₂), 50 μM ABA, 1 μM 2,4-dichlorophenoxyacetic acid (2,4-D), and 1 μM salicylic acid. Luminescence was recorded in multiple time points and is shown here after 0, 3, and 9 h of treatments.

Figure 6. Mapping and segregation of T-DNA insertion in line L0213. A, T-DNA insertion is located in chromosome 1, at 23,691 bp of contig f2H15, in the 5'-UTR of gene *At1g17840*. Exon-intron structure of the *At1g17840* gene, position, and structural elements of the pTluc insert are indicated. Position of LB and gene-specific PCR primers (LB21 and 213R, respectively) are shown by arrowheads. B, Sequence of the T-DNA and plant DNA junction. Left border (LB) sequence of the T-DNA is shown by white letters on black background. Sequence of 5'-UTR is indicated by lowercase, and the first exon is shown by capital letters. The predicted ATG codon is underlined. C, Segregation of the T-DNA insert and luminescence in 16 independent T₂ plants. Test PCR was performed using genomic DNA isolated from individual plants and combination of gene-specific (213R) and T-DNA-specific (LB21) primers. M, DNA size marker; 1 to 16, amplification of 760-bp PCR fragment in independent T₂ plants; wt, wild type Columbia-0 plant. Plants 5, 13, and 15 had no luciferase activity and produced only hygromycin-sensitive progeny. All the other plants showed luminescence.



system. In comparison, microscopic observation of GUS-based histochemical staining is more sensitive, if the screening aims the detection of gene fusions that are active in few cells (Topping et al., 1994; Sundaresan et al., 1995; Campisi et al., 1999; He et al., 2001). The identification of genes responding to nematode infection using a GUS promoter trap illustrates that this system is suited for identification of conditionally expressed cell-type-specific genes as well (Barthels et al., 1997). Because the end product of the β -glucuronidase histochemical reaction is toxic for plant cells, the GUS reporter gene is not, however, optimal for studying regulated gene expression in living organisms. In contrast, the main advantage of the firefly luciferase reporter gene system is that the activity of gene fusions can be detected by bioluminescence imaging in living tissues (Riggs and Chrispeels, 1987; Millar et al., 1992). An important application of luciferase gene trapping is the identification of conditionally regulated plant promoters and creation of molecular markers for the analysis of specific environmental and hormonal responses. Our results demonstrate that the luciferase reporter system can be exploited for identification and tagging of genes, the expression of which is positively or negatively regulated by sugar, salt, ABA, and osmotic stress signaling. To facilitate public screening of pTluc-tagged Arabidopsis collection, we have created seed stocks from each of 100 lines and performed a proof of concept study to screen for *luc*-gene fusions that are regulated by external stimuli.

It is widely accepted that the activity of gene traps faithfully reflects transcriptional regulation of tagged genes (Smith and Fedoroff, 1995; Vielle-Calzada et

al., 2000). However, several examples show that cryptic promoters located in intergenic regions could also activate gene traps (Fobert et al., 1994; Ókrész et al., 1998). In our experiments, sequencing of T-DNA insert junctions indicated that in 16 of 17 cases, the T-DNA integration events resulted in the formation of genuine transcription or translation fusions with the luciferase reporter gene. Only one pTluc T-DNA insertion was localized far from a predicted gene, suggesting that the analysis of gene fusions may identify unknown regulatory sequences in the Arabidopsis genome or point to problems resulting from incomplete annotation of Arabidopsis genes. Comparison of the luciferase expression patterns and transcriptional activity of the corresponding wild-type endogenous genes showed that luminescence could be detected only in those organs where the endogenous gene has abundant transcript levels. Differences in luciferase activity in general corresponded to alterations in tissue-specific expression. Therefore, the firefly luciferase can be considered a suitable reporter gene for gene trapping and in vivo expression studies.

Tagging of Stress-Regulated Genes. A Case Study

Our data indicate that the luciferase reporter gene is well suited to identify in situ gene fusions that are regulated by external stimuli. In our experiments, about 8% of active *luc* gene fusions respond positively or negatively to Glc and salt stress. Thus, further analysis of these *luc*-gene fusions will provide useful tools to gain more insight into interactions between sugar, ABA, and osmotic stress signaling

pathways (Gazzarini and McCourt, 2001; Xiong and Zhu, 2001).

To compare the regulation of *luc*-tagged mutant and wild-type alleles of a stress-regulated gene, we have characterized in detail a mutant line, L0213, that carries a *luc*-tag in a stress-responsive ABC transporter gene. The tagged *At1g17840* gene encodes a WBC-type transporter with one ABC domain and six trans-membrane domains. Thus, according to the classification of Higgins (1992), the protein encoded by *At1g17840* belongs to the "half size" transporter group. After confirmation of cosegregation of luminescence and T-DNA-encoded hygromycin resistance marker, we have examined the regulation of the *luc*-tagged *At1g17840* allele. Analysis of temporal regulation of luciferase expression indicated that the tagged gene is inducible by Glc, salt, and ABA. These

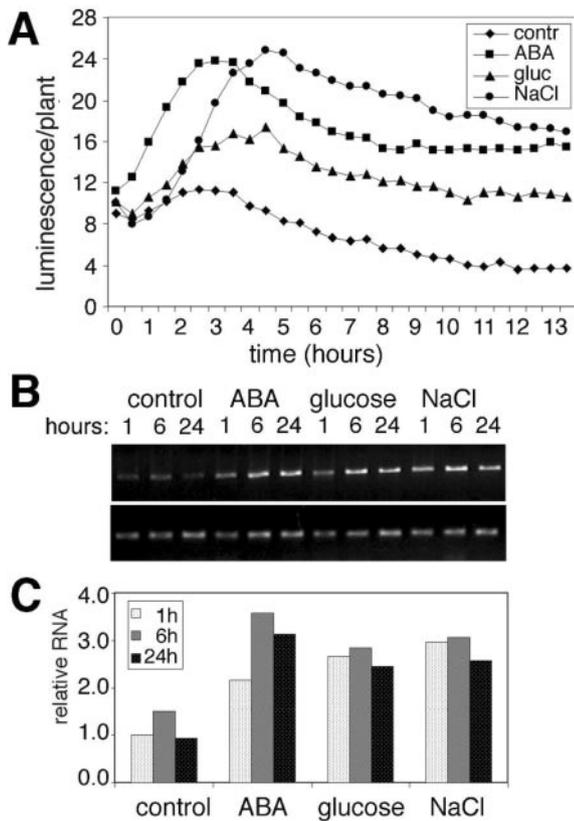


Figure 7. Analysis of *At1g17840* expression. A, Kinetics of salt, sugar, and ABA-responsive luciferase expression in line L0213. Three-week-old plants were transferred to MSAR media containing 250 mM NaCl, 400 mM Glc, or 50 μ M ABA, and luminescence was recorded in 30-min intervals. B, RT-PCR analysis of *At1g17840* expression. Total RNA was isolated from 3-week-old plants treated with 250 mM NaCl, 400 mM Glc, or 50 μ M ABA for 1, 6, or 24 h. Equal amounts of cDNA templates were used for PCR amplification of gene-specific fragments using primers L213A and L213B. Upper row, *At1g17840*-specific PCR fragments are shown after 29 cycles of amplification (ABC). Lower row, PCR fragments corresponding to the ubiquitin 10 gene are shown after 23 cycles (UBI). C, Quantification of the PCR analysis. Average values of three measurements were normalized to control RNA.

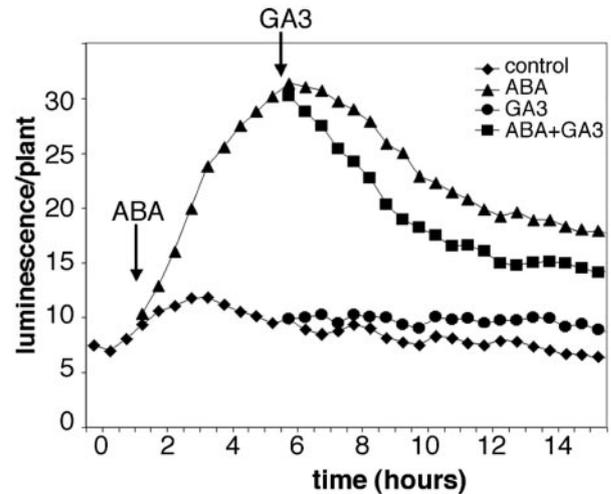


Figure 8. Effect of sequential ABA and GA treatments on luciferase activity of line L0213. Two-week-old plants were sprayed by 100 μ M ABA and 5 h later by 100 μ M GA₃ (see arrows). ABA-responsive activation of *At1g17840::luc* gene fusion could be repressed by GA₃.

properties of the *At1g17840* gene are similar to those of the *SpTUR2* gene, which encodes a PDR-type ABC transporter in the water plant *Spiradella polyrrhiza* (Smart and Fleming, 1996). Remarkably, ABA induction of the *At1g17840* gene was found to be down-regulated by GA, suggesting that stress-regulated expression of *At1g17840* in shoots is also controlled by GA signaling. Comparative analysis of steady-state *At1g17840* mRNA accumulation qualitatively confirmed the observations made by the analysis of expression of the *luc*-tagged allele. These results indicate that the analysis of *luc* gene fusion patterns can be supported by confirmatory studies using the corresponding wild-type alleles. Therefore, the use of *luc* gene fusions can provide a facile means for identification of specifically regulated genes in conjunction with identification of corresponding insertion mutations.

MATERIALS AND METHODS

Vector Construction

To construct the pTluc promoter-trapping vector, an *XhoI-HindIII* fragment from the pSKFFlucE9 plasmid (kind gift of Ferenc Nagy, BRC, Szeged, Hungary) was inserted into the *BamHI-HindIII* sites of the pTgus vector (Koncz et al., 1994). pTluc carries a promoterless firefly luciferase gene, *FFlucE9*, in the vicinity of the right T-DNA border sequence to facilitate nondestructive screening for in planta *luc* gene fusions (Fig. 1). The T-DNA of pTluc carries a hygromycin resistance gene (pnos:hph:pAg4) for selection of transgenic plants, an ampicillin/carbenicillin (Ap/Cb) resistance gene as bacterial selectable marker, and a pBR322 replication origin to enhance the recovery of insertions by plasmid rescue. The pTluc vector was introduced into *Agrobacterium tumefaciens* GV3101 carrying the pMP90RK helper plasmid and used for Arabidopsis transformation as described (Koncz and Schell, 1986).

Plant Transformation and Screening for Activation of Luciferase Reporter

Arabidopsis (Columbia-0) was used for all experiments. *A. tumefaciens*-transformed Arabidopsis lines were generated by in planta transformation

Table III. Oligonucleotides used for PCR amplification and sequencing

Name	Sequence	Purpose
LC1	5'-AACCAGGGCGTATCTCTTCATAGC-3'	TAIL-PCR or iPCR at RB
LC2	5'-TTATGCAGTTGCTCTCCAGCGG-3'	TAIL-PCR or iPCR at RB
LC3	5'-CCTTCTTTATGTTTTGGCG-3'	Sequencing at RB
LCE	5'-TTTGTACCAGATCCTTTGATCGTG-3'	iPCR at RB, used with LC1
LB11	5'-CAGACCAATCTGAAGATGAAATGGGTATCTGGG-3'	TAIL-PCR or iPCR at LB
LB21	5'-GTGAAGTTTCTCATCTAAGCCCCCATTTGG-3'	TAIL-PCR or iPCR at LB
LB41	5'-TTCTCCATATTGACCATCATACTCATTGC-3'	Sequencing at LB
PB	5'-CACATTTCCCGAAAAGTGCCACCTGACG-3'	iPCR at LB, used with LB21
213R	5'-CAGCGAGGCGGCTAGCTA-3'	L213 line segregation test
L0211A	5'-AAGGGTTAAGTGCTCCGCCA-3'	RT-PCR, At5g20380 forward
L0211B	5'-ACCAACCCAGCCACAGATGA-3'	RT-PCR, At5g20380 reverse
L0213A	5'-GAGATGGAGATAGAAGCAAGCAG-3'	RT-PCR At1g17840 forward
L0213B	5'-TTGCGGCCGTTAAGAAGAA-3'	RT-PCR At1g17840 reverse
L0264A	5'-TCGCCGTTGCTGAGTTGAGA-3'	RT-PCR At2g39190 forward
L0264B	5'-AGGAAGGCTGAGGCCAAGAT-3'	RT-PCR At2g39190 reverse
L0610A	5'-CAACAACAAGAACAATGAGAGC-3'	RT-PCR At1g56045 forward
L0610B	5'-AGATAAGAGCACAAGCAAGT-3'	RT-PCR At1g56045 reverse
L1551A	5'-AACGCCGTGGAACAGGTGAT-3'	RT-PCR At4g35260 forward
L1551B	5'-ACCAGGAACAACCTCGTGCT-3'	RT-PCR At4g35260 reverse
L1571A	5'-TAATCGGCGATGTTGGAGCTGA-3'	RT-PCR AT1G12830 forward
L1571B	5'-TCGTTGACATGGCGGTTACTGA-3'	RT-PCR AT1G12830 reverse
L3033A	5'-AGGCGCGCAAATTACCCAAT-3'	RT-PCR At2g01010 forward
L3033B	5'-TCTTCAAAGTAAACAGCCGCGGA-3'	RT-PCR At2g01010 reverse
L3051A	5'-ACCATTGTGGCTCTGACGGAAG-3'	RT-PCR At1g17690 forward
L3051B	5'-ACAACGCGAAAGGCAATGCT-3'	RT-PCR At1g17690 reverse

using vacuum infiltration (Bechtold and Pelletier, 1998). Seeds from infiltrated plants were germinated on selective MSAR seed medium containing 15 mg L⁻¹ hygromycin (Koncz et al., 1994). Three-week-old hygromycin-resistant seedlings were transferred into petri dishes (13-cm diameter, 80–100 seedlings/plate), sprayed with 2 mM D-luciferin solution (Biosynth AG, Staud, Switzerland), and assayed for luciferase activity using low light imaging with a CCD camera system (Visilux Imager, Visitron Systems GmbH, Puchheim, Germany). Bioluminescence images were processed using the Metaview 4.5r6 software (Universal Imaging Corporation, Downingtown, PA). To screen for stress-induced *luc* gene fusions, seedlings were transferred to MSAR medium supplemented with either 400 mM Glc or 250 mM NaCl, and the increase in luminescence was monitored 6 to 8 h later. Plantlets showing light emission were individually registered. After bioluminescence imaging, all seedlings were planted in soil and brought to maturity to obtain T₂ seeds. Pooled seed stocks were prepared by mixing 200 mg of T₂ seeds from 100 transgenic lines. To screen for luciferase-expressing plants, 10 mg of pooled seeds was surface sterilized, germinated, grown for 2 weeks in petri dishes on MSAR medium, and subjected to luminescence imaging as described above.

Measurement and Analysis of Kinetics of Luciferase Activation

Temporal activation of the *luc* reporter was characterized by sequential recording of bioluminescence images. Three-week-old seedlings were sprayed with 2 mM D-luciferin solution and transferred to germination medium supplemented by 50 μM ABA, 400 mM Glc, or 250 mM NaCl. Luminescence of 20 seedlings from each T₂ family was recorded in each experiment every 30 min for at least 12 h. Images were analyzed using the Metaview 4.5r6 software and processed in Microsoft Excel worksheets (Microsoft, Redmond, WA). Each experiment was repeated three times.

Sequencing of T-DNA Insert Junctions

DNA fragments flanking the T-DNA insertions were amplified from purified plant DNA samples using either long-range inverse PCR or TAIL-PCR as described (Mathur et al., 1998; Szabados et al., 2002). Long-range inverse PCR was performed with DNA templates that were previously

digested by *EcoRI* and self-ligated. Nucleotide sequence of the gel-purified PCR fragments was determined using nested sequencing primers (Table I). The positions of T-DNA insertions in the Arabidopsis genome were determined by BLAST homology searches using TAIR's database (<http://www.arabidopsis.org>).

RT-PCR Analysis of Steady-State mRNA Levels

RNA samples were isolated from 3-week-old seedlings as described (Pawlowski et al., 1994). cDNA templates were prepared using 1 μg of RNA and an RT-PCR kit (CLONTECH Laboratories, Palo Alto, CA) according to the supplier's instructions. RT-PCR reactions were performed in 50-μL volume using 2 μL of cDNA template and 0.2 μM each of forward and reverse primers (Table III). The PCR conditions were 94°C for 2 min, 35 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s. Five-microliter aliquots of PCR products were withdrawn after 23, 26, 29, 32, and 35 cycles and separated on 1.2% (w/v) agarose gels. The intensities of ethidium bromide-stained bands were determined using the NIH Image 1.63 software (National Institutes of Health, Bethesda, MD).

Distribution of Materials

All novel materials described in this publication are available for non-commercial research purposes. Obtaining any permissions will be the responsibility of the requestor.

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