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A GUS/Luciferase Fusion Reporter for Plant Gene Trapping and for Assay of Promoter Activity with Luciferin-Dependent Control of the Reporter Protein Stability

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A gene-trapping vector carrying a *GUS/Luciferase* dual reporter gene was developed to establish an efficient and convenient screening system for T-DNA-based gene trapping in plants. A key feature of this gene trap scheme is to place two different types of reporters, luciferase (Luc) and β-glucuronidase (GUS), as a fusion protein within a trapped gene to probe the activity of the gene. Luc is then utilized as a non-invasive, vital and highly sensitive screening reporter to identify trapped lines, including direct screening of the trapped lines from the primary T-DNA mutant pools. GUS is utilized as a histochemical assay reporter to analyze detailed cellular expression patterns. Transgenic expression studies in *Arabidopsis* showed that this fusion reporter protein retains functional enzyme activity for both GUS and Luc. Using this system in *Arabidopsis*, we were able to identify 3,737 trapped lines from 26,900 individual T-DNA insertion lines. Sequence determination of the T-DNA insertion loci in the genome of 78 trapped lines identified *GUS/Luc* fusions with 27 annotated *Arabidopsis* genes which included a subset of transcription factors, protein kinases, regulatory proteins and metabolic enzymes. Of these, particular expression patterns of four tagged genes were further confirmed by analyzing putative promoter regions of the corresponding wild-type genes. Furthermore, the protein stability of the GUS/Luc fusion reporter was controlled by application of luciferase substrate (luciferin), overcoming the excessive stability problem of GUS that causes misrepresentation of the transcriptional activity of a promoter. These results demonstrate the utility of the *GUS/Luc* dual reporter system as a gene trap reporter for studying plant genome function and also as a convenient dual reporter system for study of gene expression.

Keywords: *Arabidopsis* — Dual reporter — Firefly luciferase — Gene trapping — GUS.

Abbreviations: GFP, green fluorescent protein; GUS, β-glucuronidase; HR, hypersensitive response; Luc, firefly luciferase; MU-GlcA, 4-methylumbelliferyl β-d-glucuronide; PPT, phosphinothricin; UTR, untranslated region; Xgluc, 5-bromo-4-chloro-3-indolyl glucuronide.

Introduction

Several types of gene-trapping systems that utilize random integration of reporter gene constructs into the genome have been developed (Springer 2000). Insertion of a promoterless reporter gene within a transcriptional unit of the genome may generate in situ gene fusions, allowing the identification of individual genes and their regulatory elements based on the distinct expression patterns of the tagged reporter gene (Teeri et al. 1986, Koncz et al. 1989, Lindsey et al. 1993, Sundaresan et al. 1995, Mathur et al. 1998, Yamamoto et al. 2003, Alvarado et al. 2004). Alternatively, a gene trap insertion may occur in such a way that the gene function is disrupted. However, loss-of-function mutations in plant genes do not always lead to an easily recognized phenotype due to functional and genetic redundancy of plant genes. Therefore, in certain circumstances, gene trap-aided functional analysis provides considerable advantages because expression patterns of trapped lines may provide information useful for examining the phenotype (Rajani and Sundaresan 2001, Springer et al. 1995). The efficacy of this approach was demonstrated in generating various cell- and tissue-specific markers that are not easily accessed by traditional methods which rely on RNA hybridization or genechip analysis (Springer et al. 1995, Topping and Lindsey 1997, Nagawa et al. 2006).

A number of reporter genes including jellyfish green fluorescent protein (GFP), bacterial β-glucuronidase (GUS) and firefly luciferase (Luc) have been employed in gene traps to visualize the expression patterns of plant genes. Although GFP is a useful reporter for monitoring subcellular localization of proteins (Haseloff and Amos 1995, Moriguchi et al. 2005), it is not useful in performing large-scale expression analysis at the whole-plant level because of the high levels of autofluorescence in various plant organs (Quaedvlieg et al. 1998). GUS is frequently utilized as a reporter because it offers several advantages, such as a high stability in translational fusion with other

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proteins and a fine resolution in histochemical staining that allows detection of signals even in single cells (Jefferson et al. 1987, Lindsey et al. 1993). However, it is not suitable for the observation of conditionally or temporally regulated expression patterns due to its low turnover rate (Jefferson et al. 1987); the destructive nature of the histochemical staining and destaining procedure also prohibits its use in dynamic measurements of transcriptional activity in living tissues. As an alternative, the Luc reporter was recently exploited to monitor the real-time in planta gene expression with high sensitivity, speed and simplicity. Indeed, its utility was well demonstrated in studies profiling dynamic gene expression in response to internal or environmental fluctuations, dissection of signaling pathways and circadian rhythms (Riggs and Chrispeels 1987, Thompson et al. 1991, Millar et al. 1992, Chinnusamy et al. 2002, Yamamoto et al. 2003, Alvarado et al. 2004, Onai et al. 2004). However, detection of Luc activity based on bioluminescence is not appropriate for monitoring expression patterns regulated in tissue- or cell-specific manners. Therefore, the selection of reporters should be made on the basis of the intended study purpose and end-point measurements.

Although Luc and GUS have proved to be excellent reporters for monitoring gene expression, each has advantages and disadvantages as described above. To utilize the advantages of both reporters in *Arabidopsis* gene trapping, we developed a GUS/Luc dual reporter system by creating a translational fusion of GUS and Luc, joined with a flexible linker. Transgenic expression studies on the *GUS/Luc* dual reporter revealed that it exhibits strong GUS and Luc activities and has a short half-life. The strategy that uses Luc as a screening reporter and GUS as an assay reporter over generations demonstrates high efficiency, accuracy and reproducibility in gene traps. Furthermore, establishment of gene trap collections directly from the T_1 generation significantly reduces the time and effort needed for GUS staining and destaining procedures in the T_2 generation. Taken together, our results demonstrate the utility of the GUS/Luc reporter as a novel tool for plant functional genomics as well as other gene expression studies.

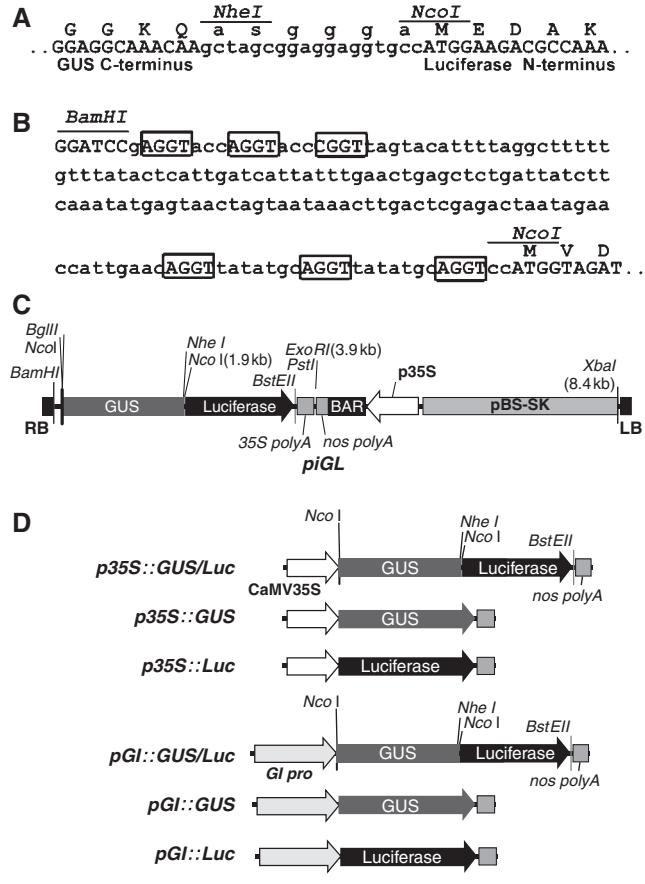

Fig. 1 Structure of the *GUS/Luc* reporter gene and physical map of plant transformation vectors. (A) Nucleotide and amino acid sequence between the GUS and Luc reporter. Lower case letters indicate the ASGGGA flexible linker sequence. (B) Nucleotide sequence at the 5′ end of the *GUS/Luc* reporter. Lower case letters indicate the *Arabidopsis actin2* intron sequence. Boxes indicate multiple splicing donors and acceptors. (C) Physical map of the piGL gene trap vector. RB and LB indicate the right and left border of the T-DNA, respectively. pBS-SK, pBluescript-SK vector; p35S, CaMV 35S promoter. The *Xba*I site used for plasmid rescue is unique in the vector. (D) Physical map of the *GUS/Luc* expression cassettes for transforming *Arabidopsis* plants. Each cassette was cloned into a pCAMBIA1300 vector. pGI indicates the promoter of the circadian-regulated *GI* gene.

Results and Discussion

Design of the GUS/Luc dual reporter and vector constructs

In order to develop a high throughput system for efficient gene trapping, a *GUS/Luc* dual reporter gene was constructed by fusing the full coding sequence of firefly *luciferase* (*Luc*) to bacterial *GUS*. It was noted that mutations or fusions introduced in the N-terminus of Luc may affect the stability and activity of the enzyme (Robinson and Sauer 1998, Sung and Kang 1998, Yamamoto et al. 2003). Thus, an ASGGGA flexible linker was incorporated between the two reporters to minimize the loss of enzymatic activities and conformational interference in the fusion protein (Fig. 1A). Then, a multiple splicing unit (Sundaresan et al. 1995) with the *actin2* intron was linked to the 5′ end of the *GUS/Luc* gene to enable expression via transcriptional and translational fusion after insertion into introns or exons of plant genes (Fig. 1B). This chimeric reporter gene was finally juxtaposed to the right border of a pGA2707 backbone (Jeong et al. 2002) to yield a gene trap vector, piGL (Fig. 1C).

Expression analysis of the GUS/Luc fusion gene in transgenic plants

To evaluate the potential utility of a *GUS/Luc* fusion gene as a dual reporter system in gene trapping, the reporter activities in transgenic *Arabidopsis* plants were examined. A subset of transgenic plants expressing the fusion reporter or the respective single reporter gene under the control of the cauliflower mosaic virus (CaMV) 35S promoter (p35S) was generated using the constructs shown in Fig. 1D. Forty individual *p35S::GUS/Luc* T_1 transgenic plants showed a variable range of GUS and Luc activities (data not shown). This may be attributed to positional effects of T-DNA insertion, as a similar degree of variation was observed in the GUS and Luc activity of transformants carrying *p35S::GUS* and *p35S::Luc* constructs, respectively (data not shown). Based on segregation of the antibiotic resistance trait, 10 homozygous *p35S::GUS/Luc* lines with a single locus T-DNA insertion were selected for further study. The *p35S::GUS* and the *p35S::Luc* transgenic lines showing the highest level of activity among 40 individual lines were selected as controls. GUS activity observed in 10 *p35S::GUS/Luc* lines ranged from levels 0.5- to 1.3-fold higher when compared with that of the control *p35S::GUS* line (Fig. 2A). Similar results were observed for Luc activity in the *p35S::GUS/Luc* lines compared with the control *p35S::Luc* line (Fig. 2C). Moreover, statistical analysis revealed a strong positive correlation between the GUS and Luc activities ($r = 0.779$, $P \leq 0.005$) among the *p35S::GUS/Luc* lines. This was further confirmed by the expression pattern of the *GUS/Luc* gene which was easily visualized by histochemical GUS staining and bioluminescence imaging (Fig. 2B, 2D). To examine further the relationship between enzyme activity and protein expression, GUS/Luc protein accumulation in four individual *p35S::GUS/Luc* transgenic plants was analyzed. Western blot analysis with anti-GUS and anti-Luc antibody demonstrated a single protein band at approximately 135 kDa, corresponding to the molecular weight predicted for a full-length fusion protein between GUS and Luc (Fig. 2E). The profile of GUS/Luc protein accumulation showed a similar trend to those of GUS and Luc enzyme activities, suggesting that the fusion protein is bifunctional without significant loss of enzyme activity.

Luc was shown to have a rapid turnover rate (Thompson et al. 1991, Millar et al. 1992), whereas the turnover rate for GUS was reported to be significantly slower (Jefferson et al. 1987). In order to be useful for monitoring temporally and conditionally regulated gene expression, the turnover rate of the GUS/Luc fusion reporter protein is particularly important. To examine this, *GUS*, *Luc* and *GUS/Luc* chimeras were constructed based on the circadian clock-controlled *GI* promoter (p*GI*) to facilitate dynamic expression of reporter genes (Fig. 1D). The antibiotic-resistant *pGI::GUS/Luc* T_1 transformants exhibited a similar level of GUS and Luc activity when compared with those of the *pGI::GUS* and *pGI::Luc* transformants, respectively. Four homozygous *pGI::GUS/Luc* lines were selected for detailed analysis of their rhythmic expression patterns. Interestingly, the time-lapse Luc imaging of these four *pGI::GUS/Luc* transgenic lines revealed that each line clearly exhibited robust oscillations in Luc activity with a peak time similar to those observed in the *pGI::Luc* plant, although lines 3 and 9 showed a slightly delayed peak after 2 d (Fig. 3A). On the other hand, GUS activity consistently increased during the assay period without any rhythmic patterns in the absence of luciferase substrate, luciferin, in both the *pGI::GUS/Luc* and *pGI::GUS* transgenic plants. However, treatment with luciferin led to rhythmic GUS activity in the *pGI::GUS/Luc* transgenic plant, but not in the *pGI::GUS* transgenic plant (Fig. 3B), suggesting that luciferin-mediated Luc protein turnover controls the overall stability of the GUS/Luc fusion protein. GUS has been extensively used in the study of plant development due to its high sensitivity as a visual marker even in a single cell. However, slow protein turnover may diminish its advantages for studying genes regulated by certain growth conditions or developmental stages. Thus, a novel character of GUS/Luc protein that controls protein stability by luciferin treatment might facilitate application of the GUS reporter for studying dynamically regulated gene expression during plant development. Taken together, our data indicate that the GUS/Luc fusion reporter can be utilized in gene expression analysis as a novel reporter that retains the individual advantages of both GUS and Luc reporters.

Non-invasive gene trapping based on Luc activity in the T_1 generation

A collection of *Arabidopsis* gene trap lines were generated using the piGL trap vector (Fig. 1C) that carries the *GUS/Luc* fusion reporter and the *bar* gene as a selectable marker. Based on the phosphinothricin (PPT)-resistant trait, approximately 26,900 independent T-DNA insertion mutants were initially selected. These lines were subjected to Luc activity screening at two distinct developmental stages, young seedling and reproductive stages. Two-week-old T_1 plants grown on medium containing PPT were screened for Luc activity using a high-performance CCD camera. This assay identified 3,457 Luc-positive lines. Fig. 4A shows examples of representative lines that exhibited distinct *Luc* expression patterns, including those in whole roots, the root tip region, shoot apical meristem region, petiole, leaf tip region and cotyledons, and those with ubiquitous expression (Fig. 4A). The diversity of *Luc* expression patterns in the trapped

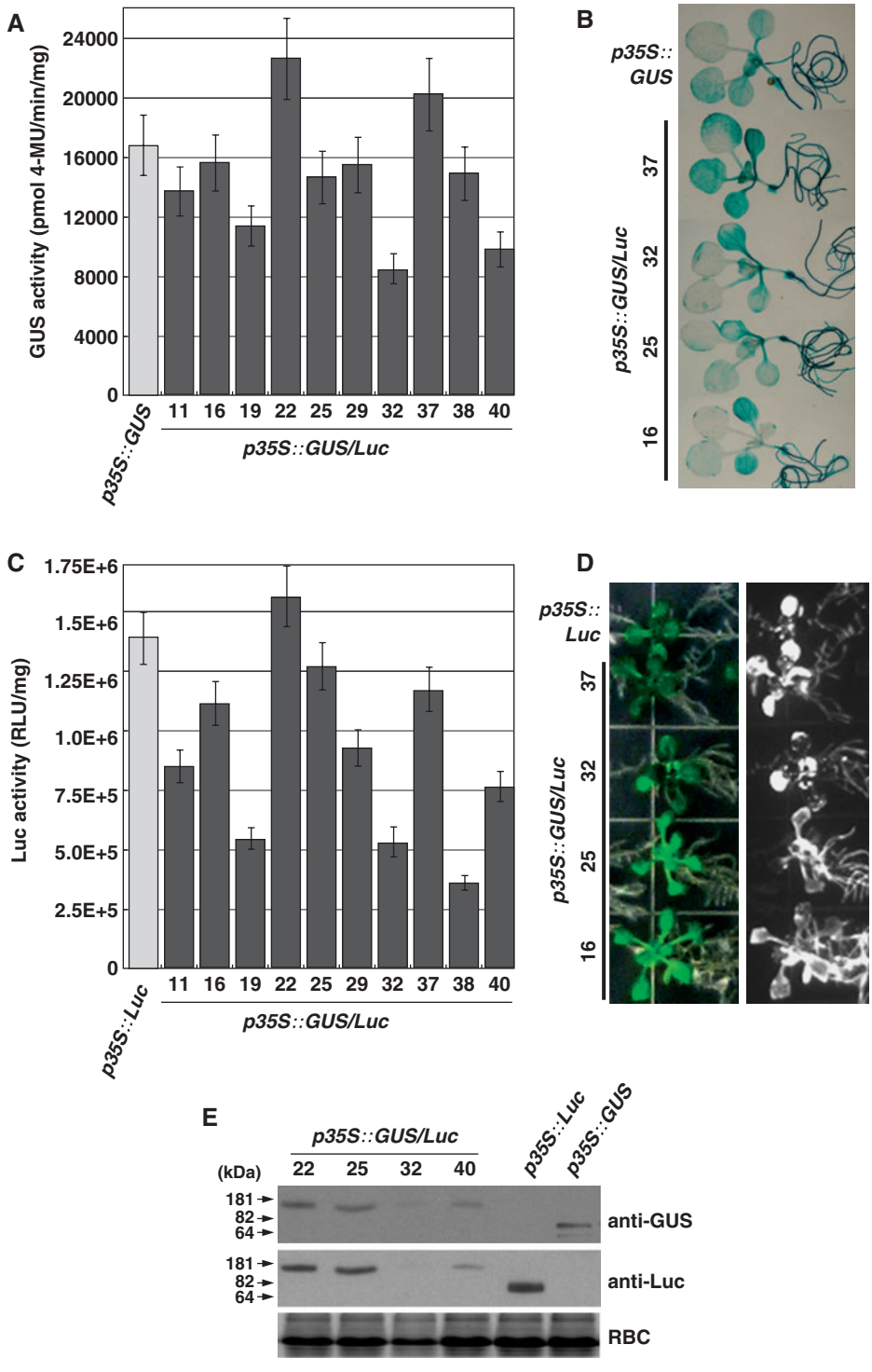

Fig. 2 Expression analysis of transgenic *Arabidopsis* plants expressing *GUS/Luc* under control of the CaMV35 promoter. Two-week old plants grown on half-strength B5 medium were used in GUS and Luc assays. (A) Fluorometric GUS assay of homozygous *p35S::GUS/Luc* transgenic plants. The *p35S::GUS* transgenic line that showed the strongest activity among the 40 individual transformants was included as a control. Total proteins were extracted from the rosette leaves and used for enzyme assays. Numbers indicate individual transgenic lines ($n = 3$). (B) Histochemical analysis of GUS activity in representative *p35S::GUS/Luc* transgenic plants. (C) Luc enzyme assay. Aliquots of total protein from samples used in GUS assays (A) were used to analyze Luc activity. The *p35S::Luc* transgenic plant that showed the strongest activity among the 40 individual transformants was used as a control ($n = 3$). (D) Visualization of Luc activity of representative *p35S::GUS/Luc* transgenic plants by bioluminescence imaging. (E) Western blot analysis of *GUG/Luc, GUS* and *Luc* transgenic transgenic plants using the anti-GUS or anti-Luc antibody. The molecular weights are indicated on the left. Coomassie blue stainining of the Rubisco (RBC) band was used for relative gel loading.

lines suggests that gene trap insertions may be localized in a variety of types of plant genes. These Luc-positive lines were transferred to soil and grown to set seeds. The remaining Luc-negative plants were also transferred to soil and grown to the reproductive stage, at which time detached rosette leaves were assayed for Luc activity (Fig. 4A). Among the 5,800 lines tested, 281 lines showed active *Luc* expression in their senescing leaves. Taken together, a total of 3,737 Luc-positive lines were identified from T_1 screening, which represents 13.8% trapping efficiency. This value is comparable with that of the Luc-based trapping system that showed 12–20% trapping efficiency (Yamamoto et al. 2003). Seeds were harvested from individual Luc-positive plants to establish a collection of gene trap lines.

Confirmation of gene trap lines in the T_2 generation by histochemical GUS staining

In the T_2 generation, the established gene trap lines were further analyzed for their detailed expression patterns using histochemical GUS staining, and in some cases also using the Luc assay. In an experiment with the T_2 plants of 982 Luc-positive lines, GUS activity was detected in 776 lines (79%) (data not shown). In most cases, the GUS staining patterns of T_2 plants were similar to those observed in the T_1 Luc imaging assay (Fig. 4). However, the reason for the absence of detectable GUS signal in 206 lines (21%) is unclear. It is possible that highly stressful conditions during T_1 selection on PPT medium could affect the expression of stress-responsive genes. Alternatively, Luc imaging-based screening might be more sensitive than GUS

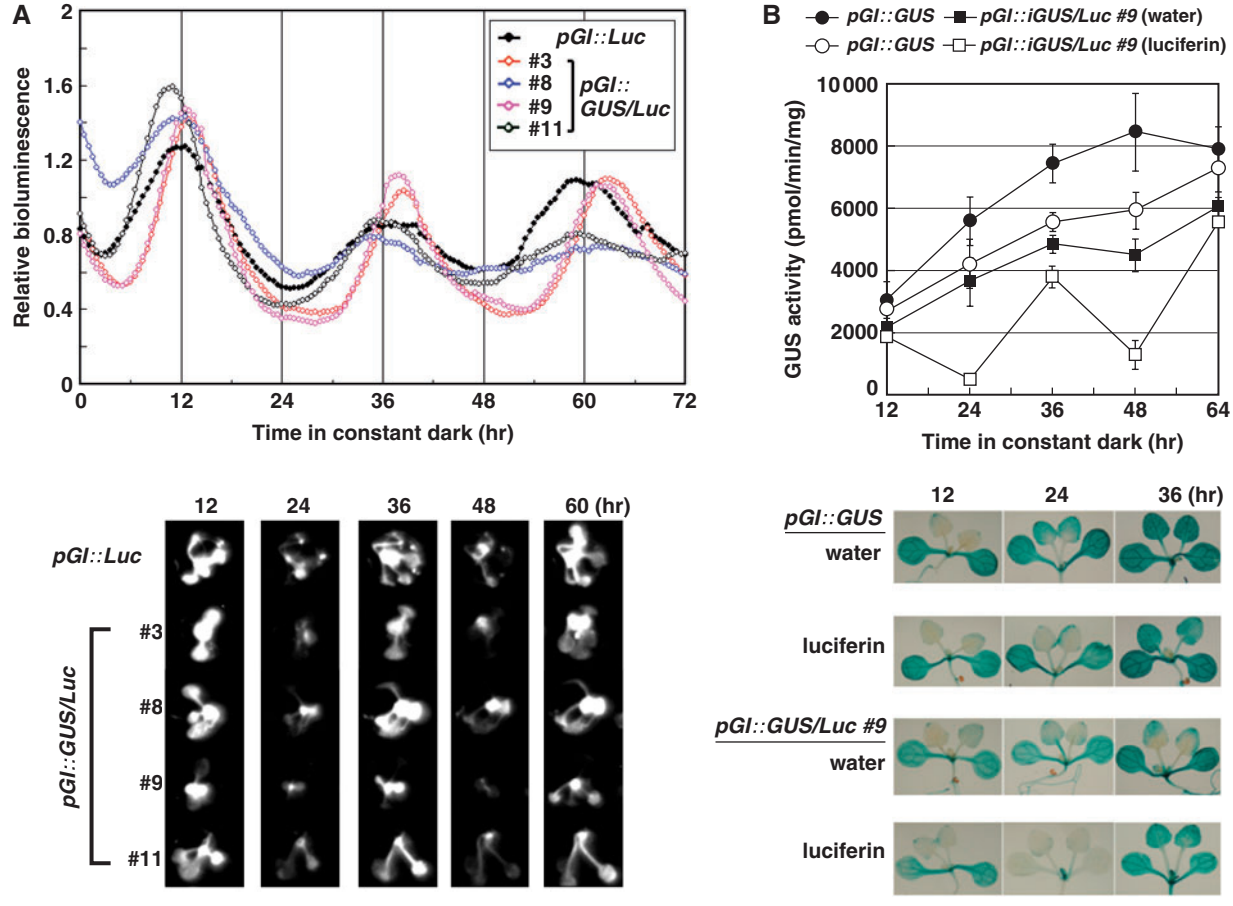

Fig. 3 Rhythmic Luc and GUS activity in *pGI::GUS/Luc* transgenic plants. The homozygous *pGI::Luc*, *pGI::GUS* and *pGI::GUS/Luc* transgenic plants were grown on half-strength B5 agar medium in 12 h light/12 h dark cycles for 10 d. Seedlings were transferred to medium containing luciferin in a 96-well plate at dusk and incubated under constant darkness. Measurement of luminescence was started at ZT 0, corresponding to the start of subjective day. (A) Dynamic luciferase activity in *pGI::GUS/Luc* transgenic plants. Luminescence levels were monitored every 30 min for 3 d. Representative records (upper) and images (lower) of bioluminescence showed a robust oscillation pattern in the *pGI::GUS/Luc* transgenic plants. (B) Luciferin-mediated controls of GUS activity in *pGI::GUS/Luc* transgenic plants. Total proteins were extracted from the aerial parts of seedlings incubated in the medium containing luciferin or water at the indicated time points. GUS activity was measured with a fluorometric assay ($n = 6$). Typical GUS staining images (lower) were taken from three independent experiments.

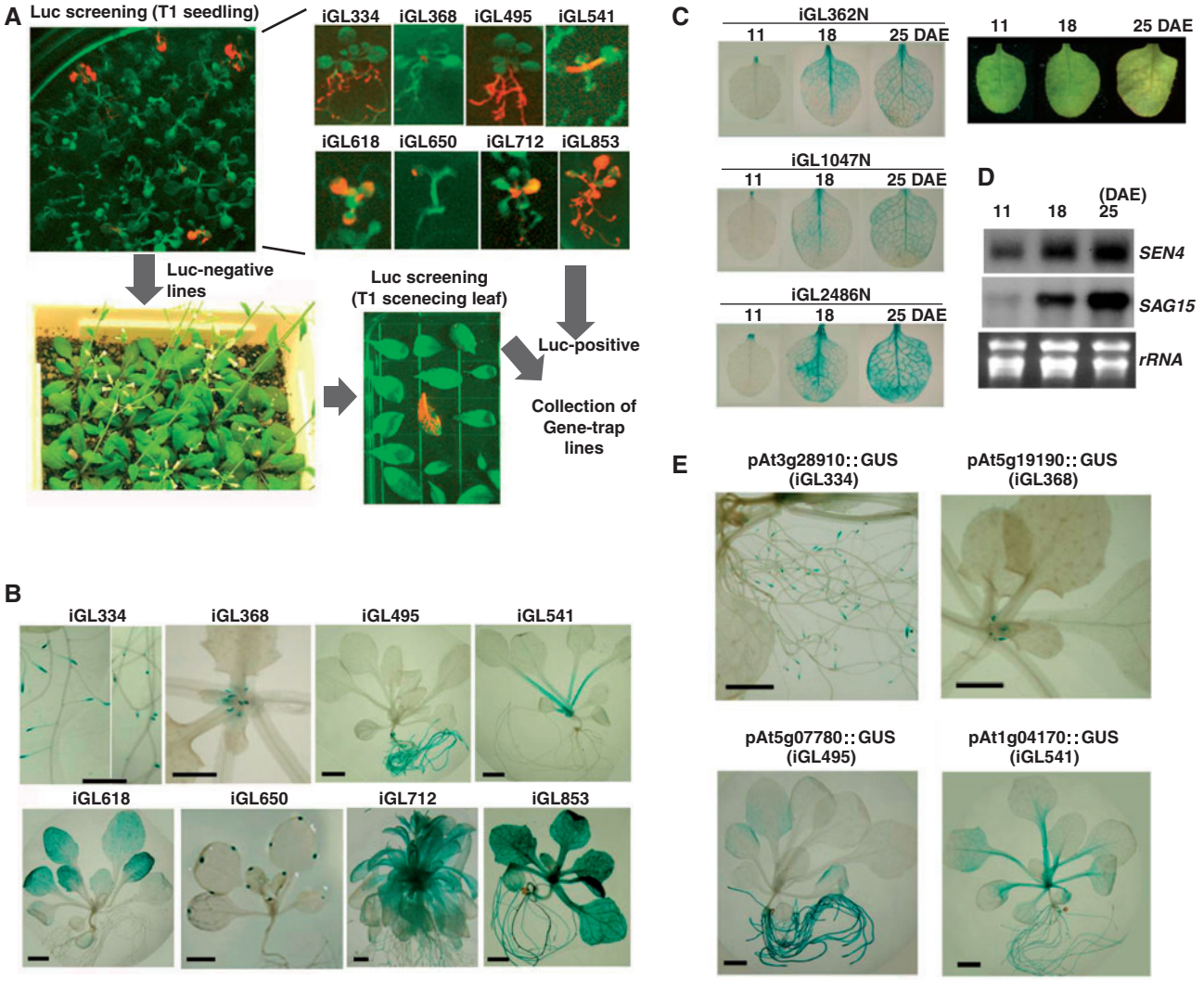

Fig. 4 Establishment of gene trap lines at two developmental stages. (A) T_1 screening using Luc activity. A visible (artificially colored green) image was overlaid with that of CCD-captured bioluminescence (red). Luc-positive lines were identified from the 2-week-old PPT-resistant T-DNA mutant pool. Luc-negative lines were planted in soil and subjected to screening for Luc activity in senescing leaves at the reproductive stage. All Luc-positive lines were maintained individually to establish a collection of gene trap lines. (B) GUS expression analysis of representative Luc-positive lines in the T_2 generation. Some lines such as iGL334 (right panel) and iGL712 showed distinct expression patterns that may reflect mutant phenotypes among the T_2 population. (C) GUS expression analysis in T_2 plants that showed detectable Luc expression in senescing leaves of T_1 plants. Third rosette leaves were detached at 11, 18 and 25 d after leaf emergence (DAE) and used in GUS staining. Representative GUS staining images at each time point are shown. (D) Northern blot analysis of *SEN4* and *SAG12* genes during leaf senescence. Total RNA was isolated from the third and fourth rosette leaves of wild-type Col-0 plants at the indicated time point. (E) Confirmation of tissue-specific promoter activity of the GUS/Luc-tagged alleles. The 1.5–2 kb genomic fragments containing putative promoter regions of the GUS/Luc-tagged alleles were fused to GUS and used to transform *Arabidopsis* plants. Tissue-specific GUS expression patterns were analyzed using 2-week-old transgenic plants harboring *promoter::GUS* constructs. Scale bar = 1 mm.

staining; a few lines that exhibited weak Luc signals in T_1 screening still showed weak yet positive Luc signals in the T_2 generation, but no GUS staining patterns were observed in those plants (data not shown). Nevertheless, the data demonstrate that a dual reporter system is very useful and efficient for analyzing expression patterns of T-DNA-tagged alleles to establish gene trap lines.

The precise expression pattern for some lines was not easily determined by the Luc imaging assay, especially in those where expression was localized in small areas of tissues. As expected, histochemical GUS staining was effective for visualizing the expression in those same lines (Fig. 4). For example, the Luc imaging assay results were inconclusive for cell-type specific expression in lines iGL368

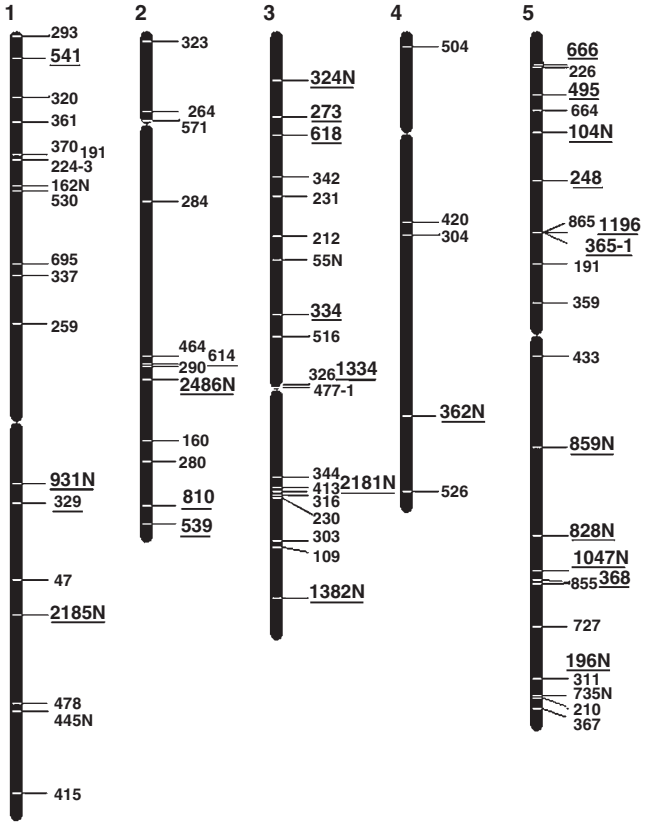

Fig. 5 Distribution of gene trap T-DNA insertions in *Arabidopsis* chromosomes. Analysis of T-DNA insertions revealed a clear bias towards intergenic regions. The locations of individual T-DNA insertions in intragenic (underlined) and intergenic (lower case letters) regions are indicated.

and iGL650, while GUS staining clearly showed their localization in stipules of shoots and in the hydathodes of cotyledons and/or rosette leaves, respectively. In other lines it was possible to identify distinct expression patterns as well as phenotypic mutants. The iGL334 plant exhibited strong GUS activity in root tip regions; closer examination showed that 20% of the T_2 plants (three out of 15 plants) exhibited reduced lateral root elongation (Fig. 4B, right panel). Similarly, the iGL712 plant exhibited an increased number of rosette leaves and preferential *GUS*/*Luc* expression in shoot apex regions, roughly suggesting that the function of the tagged gene(s) might be involved in controlling shoot meristem activity. These examples demonstrate the utility of the dual reporter gene trap system, and its feasibility for studying relationships between gene expression patterns and function.

Determination of gene trap insertion sites

To analyze the molecular nature of the gene fusions made between the endogenous plant genes and the *GUS*/*Luc* reporter, the DNA sequence flanking T-DNA insertion sites was determined in GUS/Luc-positive lines by a plasmid rescue method. For this experiment, 78 GUS/Luc-positive lines with a single-locus T-DNA insertion that showed tissue-specific or senescence-associated expression patterns were selected based on the PPT-resistant trait. T-DNA flanking sequences were obtained by sequencing followed by the plasmid rescue method. The precise positions of T-DNA insertions in the *Arabidopsis* genome were determined by BLAST homology searches and the Chromosome map tool using The *Arabidopsis* Information Resource (TAIR) database (Fig. 5). A survey of these T-DNA insertion sites showed that 27 gene trap insertions (35%) were in intragenic regions of annotated *Arabidopsis* genes, as summarized in Table 1. A literature review indicated that a majority of these (70%) are novel genes that have not yet been reported.

Determination of the T-DNA insertion site showed that 65% of the gene trap insertions were localized in intergenic regions of the genome (Fig. 5 and Supplementary Table S1), an outcome also reported in other gene-trapping studies with *Arabidopsis* (Plesch et al. 2000, Yamamoto et al. 2003, Nagawa et al. 2006). Such reported gene activation may be caused by unknown cryptic promoters in those regions or by promoters of unannotated genes, as observed in several examples where cryptic promoters located in intergenic regions caused guard cell-specific and procambium-specific *GUS* expression in gene trap assays. This would also explain why some gene trap lines (iGL248, 539 and 2185N) carrying T-DNA insertions in the antisense orientation of coding regions (6.1%) exhibited GUS/Luc expression (Table 1).

Several trapped genes, whose expression was previously characterized, strongly validated the accuracy of our gene trap assay. For example, the gene trap insertions of iGL362N and iGL1047N lines, which showed *GUS*/*Luc* expression in senescing leaves (Fig. 4C), were found within the *SEN4* and *SAG15* genes, respectively. These genes are typical senescence markers (Nakashima et al. 1997, Park et al. 1998). This was further confirmed by Northern blot analysis which showed senescence-enhanced expression patterns during age-dependent leaf senescence (Fig. 4D). Another interesting example was found in line iGL334 (Fig. 4B). The gene trap insertion site of iGL334 was found in the *At3g28910* (*AtMYB30*) gene whose expression is highly induced during the hypersensitive response (HR) upon infection with avirulent pathogens (Daniel et al. 1999). Genetic study revealed that it acts as a positive regulator of HR cell death in response to pathogen attack (Vailleau et al. 2002). Under normal growth conditions, iGL334 showed strong expression in root tip regions with mutant phenotypes of reduced lateral root elongation (Fig. 4B). To confirm whether the *GUS*/*Luc* expression pattern in

Table 1 List of *GUS*/*Luc* gene fusions

Line no.	Gene	Predicted function	Insertion site[a]
Young seedling stage			
iGL248[b]	At5g17530	Phosphoglucosamine mutase family protein	Exon, +256
iGL273	At3g10700	Putative galactokinase	Intron, +2,111
iGL329	At1g49180	Similar to MAP/ERK kinase kinase 3	Exon, +2,275
iGL334	At3g28910	Myb family transcription factor (MYB30)	5′-UTR, −7
iGL365-1	At5g23170	Putative serine/threonine protein kinase	Exon, +978
iGL368	At5g51910	TCP family transcription factor	Exon, +54
iGL495	At5g07780	Formin homology 2 domain-containing protein	Intron, +1,219
iGL539[b]	At2g46180	USO1-related protein	Exon, +1,239
iGL541	At1g04170	Eukaryotic translation initiation factor 2 (eIF2) γ	Exon, +98
iGL614	At2g29200	Pumilio/Puf RNA-binding domain-containing protein	Exon, +2.611
iGL618	At3g12600	MutT/nudix family protein	Intron, +127
iGL666	At5g04830	Unknown protein	Exon, +673
iGL1196	At5g23150	Putative transcription factor (HUA2)	Exon, 1,005
iGL1334	At3g33076	Gypsy-like retrotransposon family	5′-UTR, −101
Reproductive stage			
iGL104N	At5g12180	Calcium-dependent protein kinase	Promoter, −264
iGL196N	At5g62070	Calmodulin-binding family protein	Promoter, −504
iGL324N	At3g06380	F-box family protein/tubby family protein	Promoter, −488
iGL362N	At4g30270	Xyloglucan endo-1,4-β-D-glucanase (SEN4)	Exon, +2,146
iGL810N	At2g44190	Unknown protein	Promoter, −512
iGL828N	At5g47910	Respiratory burst oxidase protein D (RbohD)	Exon, +2,763
iGL859N	At5g39960	Mitochondrion, GTP-binding family protein	Exon, +541
iGL931N	At1g47480	Unknown protein	Exon, +32
iGL1047N	At5g51070	ERD1 (early responsive to dehydration)/SAG15	Exon, +1,462
iGL1382N	At3g58990	Aconitase C-terminal domain-containing protein	Exon, +165
iGL2181N	At3g47660	Regulator of chromosome condensation (RCC1) protein	Exon, +1,367
iGL2185N[b]	At1g60990	Aminomethyltransferase	Exon, +576
iGL2486N	At2g31510	ARIADNE-like protein ARI7	Exon, +3,481

[a] Positions of T-DNA insertions are shown in relation to the predicted ATG codon.
[b] Gene trap lines carrying T-DNA insertions in the antisense orientation in coding regions.

the iGL334 line reflects that of the wild-type *At3g28910* (*AtMYB30*) allele, *pAt3g28910::GUS* transgenic plants were generated to analyze promoter activity. Fig. 4E shows that *pAt3g28910::GUS* transgenic plants exhibited nearly identical GUS expression patterns, especially in root tip regions, when compared with the iGL334 line. Taken together, these data suggest that *At3g28910* (*AtMYB30*) may play a role in lateral root development as well as the HR, although further studies are required to elucidate its molecular function. Similar experiments with three additional promoters of tagged genes confirmed that the tissue-specific *GUS*/*Luc* expression patterns of lines iGL368, iGL495 and iGL541 precisely reflect the regulation of their corresponding wild-type alleles *At5g51910*, *At5g07780* and *At1g04170*, respectively (Fig. 4E).

Using advantages of the GUS/Luc reporter system, a significant number of novel genes could be identified even in a pilot experiment with a small set of gene trap lines. Thus, further large-scale study of the lines in this gene trap collection may provide valuable resources for elucidating *Arabidopsis* genome function.

Materials and Methods

Plasmid constructs

Gene trap vector piGL carrying a *GUS*/*Luc* dual reporter gene was constructed using synthetic linkers and PCR-based mutagenesis with appropriate primers. DNA manipulations were performed according to standard procedures described by Sambrook et al. (1989). Complete sequence data are available at GenBank (accession No. EF426459), and further information for the construct is available upon request. Briefly, *Luc* was amplified by PCR using two primers, 5′-AAGCTAGCGGAGGAGG TGCCATGGAAGACGCCAAAAACATAAAGAAAGGCC-3′ and 5′-TAGGTGACCTAGACGGCGATCTTTCCGCCC-3′,

with 5xG-M35S::Luc (Padidam et al. 2003) as a template. The underlined sequences indicate *Nhe*I and *Bst*EII sites, respectively. The resulting PCR product was digested with *Nhe*I and *Bst*EII, and ligated into the same sites in the modified pLAU6-GUS vector (Verdaguer et al. 1996) to yield a GUS/Luc gene fusion (Fig. 1A). A synthetic double-stranded linker containing the *Arabidopsis actin2* intron with a multiple splicing unit (Sundaresan et al. 1995) was inserted at the 5′ end of the *GUS*/*Luc* gene (Fig. 1B). The stability and activity of the GUS/Luc reporter was examined by transgenic expression under the control of the CaMV 35S promoter or the circadian clock-controlled *GIGANTEA* (*GI*) promoter (nucleotides −1,989 to −1 from ATG; Park et al. 1999) using CAMBIA 1300 (CAMBIA, Canberra, Australia) as a vector backbone. The *actin2* intron–*GUS*/*Luc* and the pCaMV35S::BAR/t35S cassette from pCAMBIA3200 were placed in the backbone of binary vector pGA2707 (Jeong et al. 2002) to yield a promoter trap vector, piGL (Fig. 1D).

Plant growth and transformation

Arabidopsis thaliana (Col-0) was grown in an environmentally controlled growth room at 22°C with a 16 h light/8 h dark cycle. The piGL and plant transformation constructs were introduced into *Agrobacterium tumefaciens* GV3101 (Koncz and Schell 1986) by electroporation. Plants were transformed using the *Agrobacterium*-mediated floral dipping method (Clough and Bent 1998), and seeds were harvested when plants were completely dry.

GUS and Luc assays

Frozen leaf tissue was homogenized in 0.1 M K-phosphate buffer (pH 7.8). The homogenate was centrifuged at 12,000×*g* for 15 min, and the supernatant was used for enzyme assays followed by protein quantification. GUS activity was measured using MU-GlcA (4-methylumbelliferyl β-D-glucuronide) as described (Gallagher 1992). The luciferase assay was performed with aliquots of the same protein extracts used in the GUS assay. Luciferase activity was measured using the dual luciferase assay system (Promega Corp., Madison, WI, USA) according to the manufacturer's instructions. For analysis of statistical correlation between GUS and Luc activities in p35S::GUS/Luc transgenic plants, the data were presented as means ±SD. Correlation analysis was used to determine a sample correlation coefficient (r). The t-test and P-value for statistical significance of r were calculated. Statistic analysis revealed a strong correlation between GUS and Luc activities ($r = 0.779$, t-test $= 3.522$ with $P < 0.005$).

Analysis of rhythmic patterns of GUS and Luc activity in pGI::GUS/Luc transgenic plants

Homozygous *pGI::Luc*, *pGI::GUS* and *pGI::GUS*/*Luc* transgenic plants were grown on half-strength B5 agar medium in 12 h light/12 h dark cycles for 10 d. To examine the dynamic reporter activity of the GUS/Luc fusion protein, the seedlings were then transferred to a 96-well plate containing 0.5 mM D-luciferin in half-strength B5 medium. Seedlings were then shifted to constant dark conditions, and measurement of luminescence was initiated at ZT 0 using a high-performance CCD camera (VersArray system, Roper Scientific Ltd, Hemel Hempstead, UK). Images were analyzed by MetaVue version 5.2 software (Universal Imaging Corporation, Downing Town, PA, USA) and processed in Microsoft Excel worksheets (Microsoft, Redmond, WA, USA). Each experiment was repeated three times.

T_1 screening based on Luc imaging

A collection of gene trap lines was established directly from primary T-DNA insertion mutants by detection of Luc activity. Briefly, seeds from *Agrobacterium*-infiltrated plants were germinated on half-strength B5 agar medium containing PPT (Duchefa, Haarlem, The Netherlands) and cefotaxime. After 2 weeks, the PPT-resistant T_1 plants were carefully transferred into new B5 agar medium (100 plants per 18 cm diameter plate) and acclimated for 2 d under the same growth conditions. The plants were sprayed with 100 μM luciferin (Duchefa) to visualize bioluminescence images as described (Chinnusamy et al. 2002). The resulting Luc-positive plants were individually transferred to soil. At the same time, Luc-negative plants were transferred to soil for further screening at the senescence stage. When plants exhibited yellowing signs in rosette leaves during senescence, the leaf was detached and subjected to Luc imaging as described for young plants. All identified Luc-positive lines were self-pollinated, and the resulting T_2 seeds were harvested. The gene trap lines described in this report will be made available upon request for non-commercial research in the future.

Expression analysis by histochemical GUS staining in the T_2 generation

Luc-positive T_1 lines were further confirmed in the T_2 generation by histochemical GUS staining; 15 plants and nine senescing leaves from each T_2 family were used for expression analysis. For GUS staining, 2-week-old plants or senescing leaves were immersed in 1 mg ml^{-1} XGluc (5-bromo-4-chloro-3-indolyl glucuronide) solution in 50 mM sodium phosphate buffer (pH 7.2), 0.1% Triton X-100, 10 mM EDTA, 5 mM $K_4Fe(CN)_6$, 5 mM $K_3Fe(CN)_6$, and incubated overnight at 37°C in the dark as described (Naleway 1992). Tissue was cleared using 70% ethanol, and GUS images were taken using an Olympus SZ-40 dissecting microscope with a digital camera.

Northern analysis

Total RNA was isolated from 2-week-old plants or senescing leaves using Trizol reagent (Gibco-BRL, NY, USA) according to the manufacturer's instructions. Probe DNA fragment was obtained by reverse transcription–PCR using 5′-CCTGGTA ACTCTGCAGGAACAGTCAC-3′ (SEN4-F) and 5′-GCATTC CTTAGGAGCTCCCTGTGG-3′ (SEN4-R) primers for *SEN4*, and 5′-GCATGGAGGTGTTATCT-3′ (SAG15-F) and 5′-GCGC ATATCTTTTCCCAA-3′ (SAG15-R) primers for SAG15. DNA gene fragments were labeled with [^{32}P]dCTP by random priming according to the manufacturer's instructions (Stratagene, La Jolla, CA, USA). Hybridization and other procedures were performed as described previously (Koo et al. 2004).

Western blot analysis

Total protein samples were extracted from the aerial part of 2-week-old plants directly in 2× loading buffer and separated by 8% SDS–PAGE. Proteins were transferred to membranes (Amersham) and blocked in 5% skim milk in TBS (10 mM Tris pH 8.0, 150 mM NaCl). A polyclonal anti-GUS rabbit IgG (Molecular Probes, Eugene, OR, USA) or anti-Luc IgG (Sigma) was used at a 1/5,000 dilution. Proteins were detected using a 1/15,000 dilution of goat anti-rabbit IgG conjugated to horseradish peroxidase using chemiluminescence substrate.

Isolation of T-DNA tagged chromosomal DNA fragments by plasmid rescue

Genomic DNA was extracted from leaves by the cetyltrimethylammonium bromide (CTAB) method (Doyle and Doyle 1990). DNA was digested with *Xba*I, which is unique in the piGL vector, in a final volume of 100 µl overnight. The digested DNA was extracted with phenol and precipitated with ethanol. The linearized DNA was self-ligated in a final volume of 10 µl at 16°C overnight, and the resulting ligation mixture was electroporated into *Escherichia coli* to obtain plant DNA segments. Sequencing was carried out using the GUS primer 5′-CACGGGTTGG GGTTTCTACAGG-3′ by an ABI3730 automatic sequencer (Applied Biosystems, Foster City, CA, USA). The positions of T-DNA insertions were analyzed using the Blastn program in the TAIR database (http://www.arabidopsis.org).

Promoter analysis

To determine whether the diverse *GUS*/*Luc* expression patterns in gene trap lines were driven by the regulatory sequences of the tagged genes, putative promoter regions (approximately 1.5–2 kb of sequence upstream of the ATG codon) of four representative tagged genes, iGL334 (*At3g28910*; AtMYB30), iGL368 (*At5g51910*; TCP family transcription factor), iGL495 (*At5g07780*; protein containing FH domain) and iGL541 (*At1g04170*; eIF2), were isolated from the wild-type genome by PCR and analyzed in transgenic *Arabidopsis* plants. The primers used (F, forward; R, reverse) for PCRs were 334-F (5′-gtatcaactgc atgtgcgcttgcagtgc-3′), 334-R (5′-GGAGGCCTCACCATTATG ATCTTGAACTCCC-3′), 368-F (5′-ccactggtccaggtatgattaacctcg tacctaccg-3′), 368-R (5′-TATCGCGTTGCCTTCGTGATTCG ATTCC-3′), 495-F (5′-CCGAGGTCCTTGCTTCCAAGGCAGC AG-3′), 495-R (5′-AGCGCCAGAGATATCGACAAGCGACAT TGG-3′), 541-F (5′-GAGCATGCGTGCGCTCATGACAG AAG-3′), and 541-R (5′-GAAGCTTCTGCTCCTGCTTAAAA CCG-3′). PCR was performed in a DNA Thermal Cycler 480 (PE Biosystems, Foster City, CA, USA) as follows: one cycle at 95°C for 3 min; 25 cycles at 94°C for 30 s, 58°C for 30 sc and 72°C for 4 min; and 72°C for an additional 10 min after the final cycle. The amplified DNA products were cloned into the *Sma*I site or appropriate restriction enzyme sites of pBI101 vector containing a promoterless GUS (Clontech, Palo Alto, CA, USA). The resulting constructs were introduced into *Arabidopsis* via *Agrobacterium*-mediated transformation.

Supplementary material

Supplementary material mentioned in the article is available to online subscribers at the journal website www.pcp.oxfordjournals.org.

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