LucTrap Vectors Are Tools to Generate Luciferase Fusions for the Quantification of Transcript and Protein Abundance in Vivo[1]

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Proper plant growth and development strongly rely on the plant's ability to respond dynamically to signals and cues from the intra- and extracellular environment. Whereas many of these responses require specific changes at the level of gene expression, in recent years it has become increasingly clear that many plant responses are at least in part also controlled at the level of protein turnover. It is a challenge for signal transduction research to understand how distinct incoming signals are integrated to generate specific changes at the transcript or protein level. The activity of luciferase (LUC) reporters can be detected in nondestructive qualitative and quantitative assays in vivo. Therefore, LUC reporters are particularly well suited for the detection of changes at the transcript and protein level. To the best of our knowledge, the number of plant transformation vectors for LUC fusions is very limited. In this article, we describe the LucTrap plant transformation vectors that allow generation of targeted and random transcriptional and translational fusions with the modified firefly LUC reporter LUC+. We demonstrate that LucTrap-based fusions can be used to monitor rapid changes in gene expression and protein abundance in vivo.

Plants are sessile organisms that need to respond quickly to signals and cues from their intra- and extracellular environment. Many of these responses require the transcription of specific subsets of downstream genes (Schwechheimer and Bevan, 1998; Schmid et al., 2005). At the level of the individual gene, the spatial and temporal control of gene expression is mediated by transcriptional activators and repressors that regulate promoters and enhancers, and the integration of these activities determines the resulting gene expression changes. To understand how these changes are brought about and how individual signaling pathways modulate gene expression at the level of the individual gene is a big challenge for signal transduction research.

Gene expression can be monitored at the level of the individual gene by northern blotting or reverse transcription (RT)-PCR, or at the genomic level using microarrays (Hennig et al., 2003; Zhu, 2003; Schmid et al., 2005). In recent years, microarray data originating from hundreds of experiments conducted with the plant model species Arabidopsis (*Arabidopsis thaliana*) have been obtained and collected in specific databases so that an overview of a gene's expression pattern can now be gained by simple database analysis (Schmid et al., 2005; Zimmermann et al., 2005). Nevertheless, the comparatively high cost of a microarray experiment adds restrictions to the number of experimental conditions that can be tested in such studies. Therefore, these techniques cannot be used extensively to understand the expression of a single gene of interest, its transcriptional regulation over time, and its responses to complex signaling events. In these cases, transgenic plants expressing transcriptional or translational fusions between the promoter of the gene of interest and the reporter proteins β-glucuronidase (GUS), green fluorescent protein (GFP) and its derivatives, and the luciferases (LUCs) are suitable alternatives (for review, see de Ruijter et al., 2003).

Because each reporter has specific advantages and disadvantages, the goal of the specific experiment generally determines the choice of the reporter. Transcriptional and translational fusions of a promoter or gene of interest to the GUS reporter allow assay of gene expression in a quantitative and qualitative manner (Jefferson, 1987). GUS activity can be quantified in protein extracts in fluorometric assays and tissue-specific and, in some cases, subcellular GUS activity can be assayed using chromogenic assays (Jefferson et al., 1987; von Arnim et al., 1997). Due to its relatively long half-life and its property of forming stable aggregates

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in vivo, the GUS reporter is being considered a stable and reliable reporter protein. Conversely, and for the same reasons, the GUS reporter is not well suited to follow transcriptional repression and protein degradation events because loss of reporter protein activity cannot be detected against the background of aggregated GUS (Gray et al., 2001). GFP and other fluorescent reporter proteins can easily be monitored in vivo in a noninvasive manner by fluorescence microscopy (Haseloff and Amos, 1995; Shaner et al., 2005). These proteins are generally best suited for determination of the subcellular localization of a protein of interest and, in combination with other fluorescent protein-tagged proteins, these reporters are ideal tools for studying protein colocalization as well as in vivo protein-protein interactions (Haseloff and Amos, 1995; Shaner et al., 2005). However, the quantification of fluorescent protein levels, in absolute or relative terms, as is required for some applications, such as fluorescent resonance energy transfer, can only be achieved by the skilled user of sophisticated software (Haseloff, 1999; Shaner et al., 2005). In addition, it has been observed that the folding of GFP is temperature dependent and that many fluorescent proteins photobleach during analysis (Shaner et al., 2005). Therefore, it is difficult to correctly quantify the amount of fluorescent protein that is produced or present within a cell.

In contrast, LUCs can be detected and quantified in vivo in a highly sensitive manner using photomultipliers or highly sensitive cameras. The reaction with the LUC substrates luciferin, ATP, and oxygen causes the release of a photon at 592 nm in 90% of the catalytic cycles (DeLuca and McElroy, 1986). Luciferin can be supplied to plants as a media supplement or a luciferin-containing solution can be sprayed or painted onto the plant material for imaging. One interesting and important feature of LUC is that it is inactivated after the LUC reaction has taken place. For this reason, LUC activity only reveals the amount of de novo synthesized protein rather than the amount of protein that has accumulated over time (Millar et al., 1992; de Ruijter et al., 2003). Therefore, transcriptional or translational LUC fusions are excellent tools to monitor dynamic changes in transcript or protein abundance.

To the best of our knowledge, the number of plant transformation vectors for LUC fusions is very limited. In this article, we report on the LucTrap vectors and describe their use for the analysis of plant response mechanisms that lead to changes in transcript and protein abundance. The LucTrap and LucTrap-3(GW) vectors are designed for the cloning of transcriptional and translational LUC fusions. Using selected examples, we demonstrate that these vectors serve to monitor and quantify positive and negative changes in gene expression as well as changes in protein abundance in planta. We also describe and characterize a collection of 700 Arabidopsis lines that we generated with the gene trap vector LucTrap-2, and we demonstrate that these lines can serve to uncover novel regulatory mechanisms that, in our specific case, are controlled by unstable regulators.

RESULTS

The LucTrap Vector for in Vivo Gene Expression Analyses

To obtain a LUC reporter vector suitable for transcriptional and translational fusions, we constructed the LucTrap vector, which is a derivative of the previously described plant transformation vector pGREEN0029-II (Fig. 1A; Hellens et al., 1999). In LucTrap, a unique *Nco*1 restriction site is positioned at the start codon of the modified firefly *LUC+* gene, and this site can be used to generate transcriptional and translational *LUC+* fusions. To test the performance of LucTrap, we inserted an 800-bp *GH3-2* (At4g37390) promoter fragment into the vector to obtain GH3-2:LucTrap (Fig. 1B). Several members of the *GH3* gene family, including *GH3-2*, have previously been shown to be induced by auxin (Tian et al., 2003). We therefore tested auxin-induced LUC expression in transgenic Arabidopsis seedlings containing GH3-2:LucTrap. Whereas no significant LUC activity was detected in the absence of auxin, 18 of 20 transgenic lines showed LUC expression as early as 45 min following induction with the synthetic auxin 2,4-dichlorophenoxyacetic acid (2,4D; Fig. 1C). To confirm that LUC+ activity driven by *GH3-2* correlates with the expression of the endogenous *GH3-2* gene, we analyzed *GH3-2* mRNA accumulation by semiquantitative RT-PCR (Fig. 1, D and E). In these experiments, auxin-induced *GH3-2* mRNA accumulation was detected as early as 15 min after auxin induction and the overall kinetics of auxin-induced *GH3*-2 expression were found to be comparable between the RT-PCR analysis and the LUC assays. Because the detection of the *GH3-2* transcript by RT-PCR precedes the detection of the active LUC+ protein by approximately 30 min, we suggest that this delay corresponds to the time required for transcript maturation and protein biosynthesis (Fig. 1, C–E). We therefore conclude that the LucTrap vector can serve to faithfully report on the presence and absence of a gene product and its accumulation over time.

The LucTrap-3(GW) Vector for Gateway-Compatible LUC Fusions

To generate a vector that is compatible with the increasingly popular Gateway cloning technology, we inserted the Gateway cassette (rfB) upstream of the *LUC+* open reading frame of LucTrap to obtain LucTrap-3(GW) (Fig. 2). We subsequently tested LucTrap-3(GW) with five different entry clones and achieved full cloning efficiency in all cases, suggesting that LucTrap-3(GW) is a fully functional Gateway vector (data not shown).

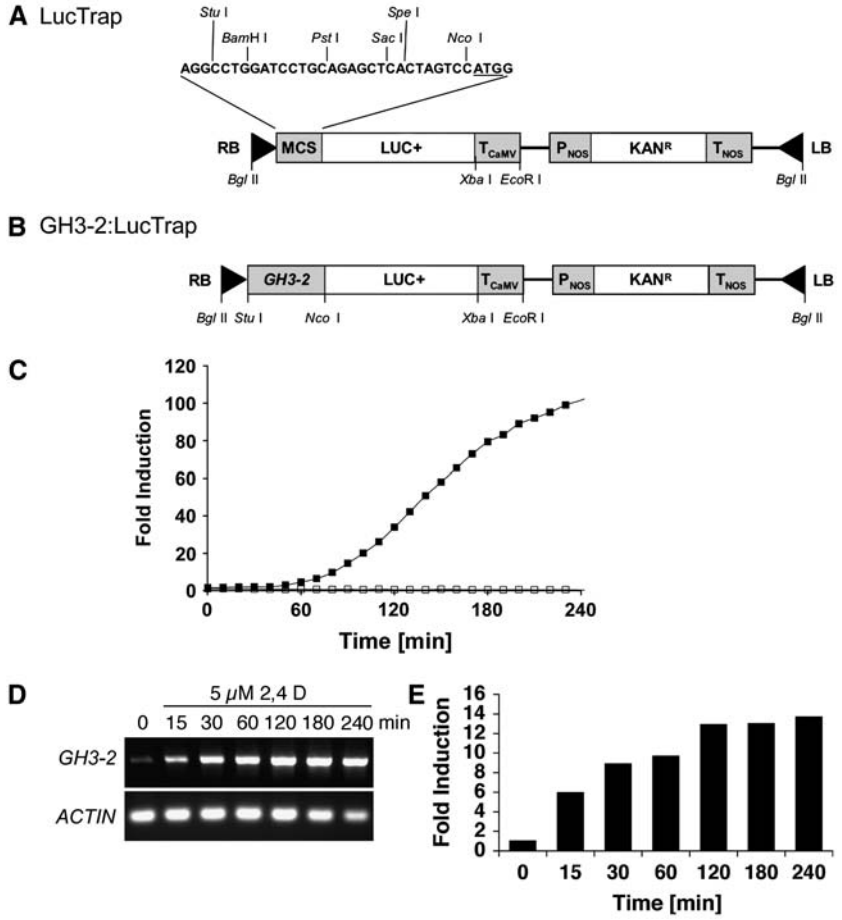

Figure 1. The plant transformation vector LucTrap allows generation of transcriptional and translational LUC reporter fusions. A, Schematic representation of the LucTrap vector T-DNA. Black triangles mark the T-DNA right border (RB) and left border (LB), respectively. The *Nco*1 site of the LucTrap MCS overlaps with the ATG start codon of the modified firefly *LUC* gene (LUC+). CaMV 35S terminator, T_{CaMV}; nopaline synthase (NOS) promoter, P_{NOS}; NOS terminator, T_{NOS}; neomycin phosphotransferase II/Kanamycin resistance gene, KAN^R. The GenBank accession number of LucTrap is DQ073044. B, GH3-2:LucTrap carries a *GH3-2* (At4g37390) gene fragment corresponding to the 800 bp upstream of the predicted *GH3-2* start codon. C, Typical result of an auxin-induction experiment with 5-d-old seedlings of a selected transgenic Arabidopsis GH3-2:LucTrap line. White squares, LUC activity without induction; black squares, LUC activity following induction with 5 μM 2,4D. LUC activity at $t = 0$ min of the untreated sample was set as 1. D, *GH3-2* gene expression following induction with 5 μM 2,4D as monitored by semiquantitative RT-PCR. *ACTIN* was used as an internal standard for cDNA amounts used in the experiment. E, Quantification of the RT-PCR results. *GH3-2* expression at $t = 0$ min was set as 1.

Next, we examined whether translational fusions obtained with LucTrap-3(GW) can be used to determine protein abundance in vivo. To this end, we generated transgenic Arabidopsis lines that carry the construct *REPRESSOR-OF-ga1*-3 (RGA):RGA:LUC. RGA:RGA:LUC lines express a fusion protein of Arabidopsis RGA with LUC+ under the control of a 2-kb *RGA* promoter fragment. RGA is a predominantly nuclear-localized downstream regulator of the gibberellic acid (GA_3) signaling pathway and it is known to be degraded by the 26S proteasome in response to GA_3 (Silverstone et al., 2001; Dill et al., 2004). We therefore tested whether GA_3 and the GA_3 biosynthesis inhibitor paclobutrazol (PAC) have an effect on RGA:LUC abundance in vivo. We found that light-grown RGA:RGA:LUC seedlings show moderate expression of the RGA:LUC fusion protein, whereas RGA:LUC abundance is increased after PAC application, a finding that

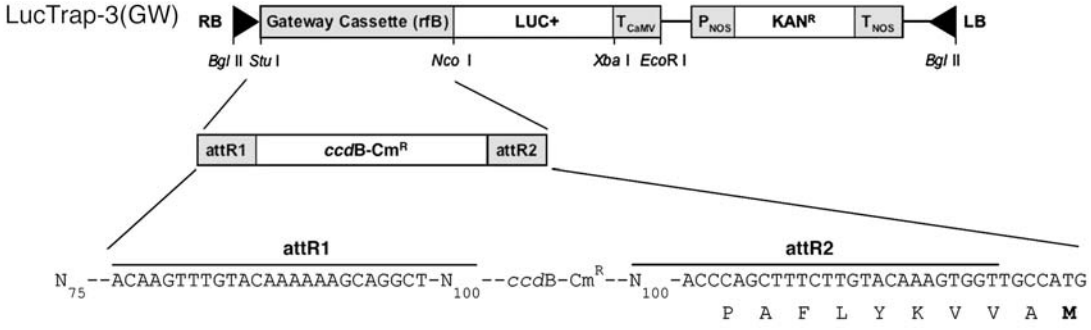

Figure 2. The Gateway destination vector LucTrap-3(GW) for plant transformation. Scheme of the LucTrap-3(GW) T-DNA with the *att*R1 and *att*R2 recombination sites of the Gateway rfB cassette. *ccd*B, *Escherichia coli* DNA gyrase for negative selection; Cm^R, chloramphenicol resistance gene for positive selection. The first amino acid of LUC+ is underlined. For other abbreviations, refer to Figure 1 legend. The GenBank accession number of LucTrap-3(GW) is AY968054.

may be explained by the expected stabilization of RGA:LUC (Fig. 3A). In turn, RGA:LUC stabilization could be reversed by the concomitant application of GA_3, a treatment that counteracts the reduction in endogenous GA_3 resulting from PAC treatment. Finally, and in line with the notion that RGA:LUC requires proteasomal activity for its degradation, we were also able to stabilize RGA:LUC by application of the 26S proteasome inhibitor MG132 (Fig. 3A).

RGA protein abundance has so far almost exclusively been studied using transgenic Arabidopsis lines that contain RGA:GFP:RGA (Silverstone et al., 2001). RGA:GFP:RGA lines express a fusion protein between RGA and the reporter GFP under the control of a *RGA* promoter fragment. As a control experiment, we therefore subjected RGA:GFP:RGA lines to the same treatment we had applied to the RGA:RGA:LUC lines. We found that treatments with the inhibitors PAC and MG132, as well as treatments with GA_3, had the same effect on the previously established GFP:RGA reporter as on RGA:LUC (Fig. 3B). We therefore propose that LucTrap-3(GW) allows generation of LUC+ fusions that can serve to detect changes in protein abundance in vivo.

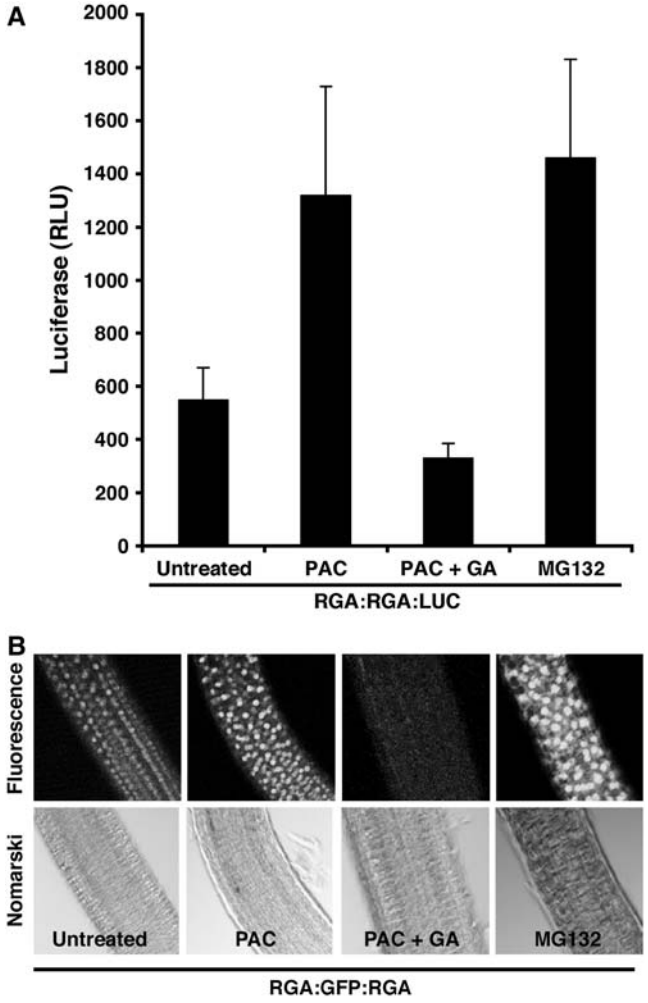

Figure 3. Translational LUC+ fusions with the RGA protein allow detection of protein degradation events. A, Representative result of a transgenic line expressing the LUC gene fused to the *RGA* open reading frame under the control of a 2-kb *RGA* promoter fragment (RGA:RGA:LUC). LUC activity was measured in 5-d-old seedlings (untreated) and, after 12-h treatment with 100 μM of the GA_3 biosynthesis inhibitor PAC, 100 μM PAC, and 100 μM GA_3 (PAC + GA_3), as well as 100 μM of the 26S proteasome inhibitor MG132, as indicated. $n = 4$. B, Fluorescence microscopy and Nomarski images of root cells of 5-d-old Arabidopsis seedlings expressing the RGA:GFP:RGA fusion protein. Treatments were as described in A.

The LucTrap-1 and LucTrap-2 Vectors for Promoter and Gene Trapping

Promoter, enhancer, or gene traps are genomic tools to generate untargeted reporter gene fusions (Evans et al., 1997; Durick et al., 1999; Springer, 2000). Promoter, enhancer, or gene traps are designed in a way that the random insertion of a promoterless reporter gene in a gene (gene trap) or in the proximity of a promoter or enhancer element (promoter or enhancer trap) will lead to the detectable expression of the reporter either as a result of a transcriptional (enhancer or promoter trap) or a translational (gene trap) fusion. In Arabidopsis, such unbiased trapping approaches have been successfully used for the discovery of genes and reporter lines that are expressed in specific tissues, in specific developmental stages, or in response to specific signals, as well as for the discovery of proteins that localize to specific subcellular structures (Kertbundit et al., 1991; Sundaresan et al., 1995; Campisi et al., 1999; Parinov et al., 1999; Cutler et al., 2000; Geisler et al., 2002; Birnbaum et al., 2003; Yamamoto et al., 2003; Alvarado et al., 2004; Tian et al., 2004; Nakayama et al., 2005).

We generated LucTrap-1 as a vector for promoter and gene trapping in plants. LucTrap-1 contains a modified intron of the Arabidopsis G-protein α-subunit gene (Gα; At2g26300) that was inserted between the T-DNA right border and the *LUC+* open reading frame (Fig. 4A). In the context of a similar arrangement, this Gα intron had previously been used successfully for promoter and gene trapping in Arabidopsis with the *GUS* reporter (Sundaresan et al., 1995). LucTrap-1 also contains one splice donor site (D) located directly adjacent to the T-DNA right border as well as three splice acceptor sites (A1, A2, and A3) located upstream of the *LUC+* gene (Fig. 4A). The acceptor sites are spaced in the three different forward reading frames and this spacing should result in the formation of alternatively spliced transcripts between a splice donor site of the trapped gene or the LucTrap-1 D site and the LucTrap-1 acceptors A1, A2, and A3 (Fig. 4C). Hence, LucTrap-1 is designed such that insertion of its T-DNA will result in the expression of *LUC+* or a *LUC+* fusion transcript under the spatial and temporal control of the trapped promoter (Fig. 4C). Furthermore, we generated LucTrap-2, which has all the features of LucTrap-1, but lacks the *LUC+* ATG

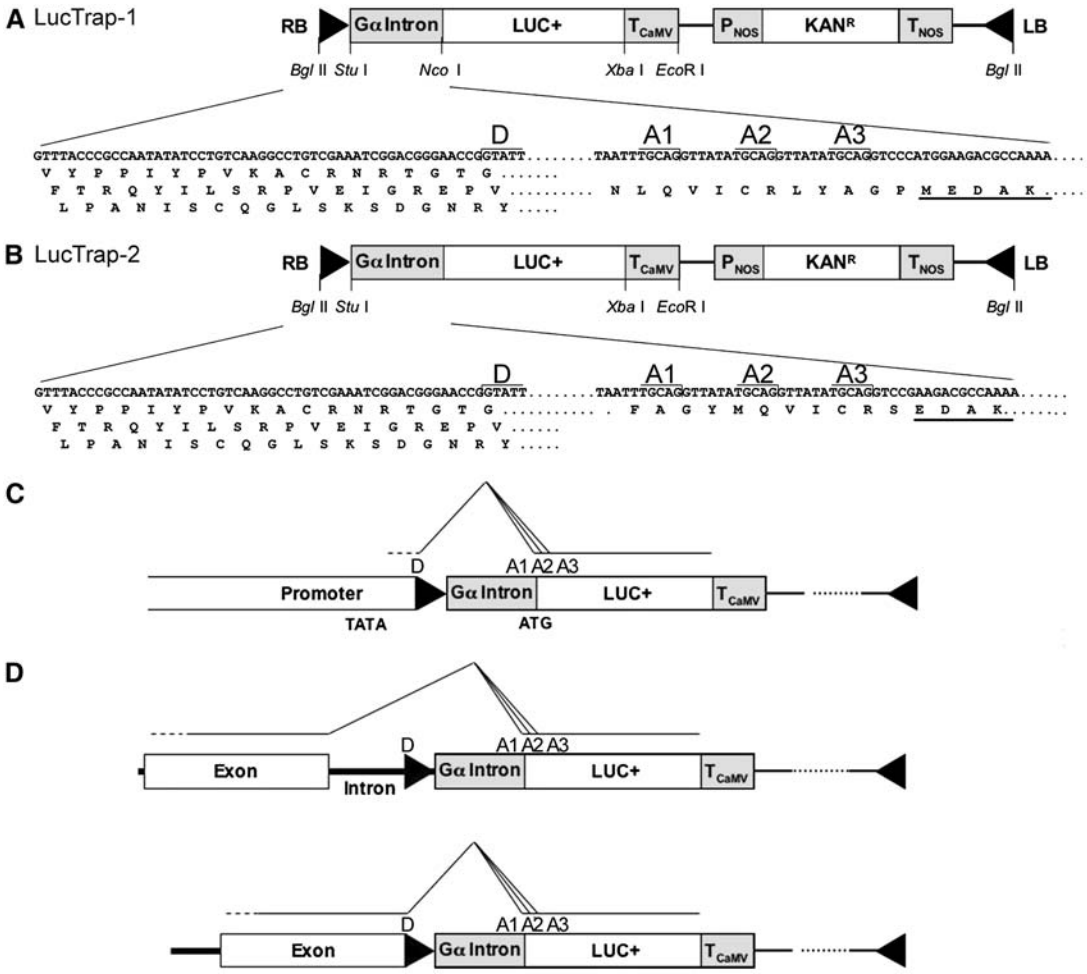

Figure 4. LucTrap-1 and LucTrap-2 plant transformation vectors for promoter and gene trapping. A, Schematic representation of the LucTrap-1 T-DNA. The intron of the Gα subunit gene was placed between the T-DNA right border (RB) and the *LUC+* gene. The artificial splice donor (D) and three splice acceptor sites (A1, A2, and A3) flanking the Gα intron are indicated. The A1, A2, and A3 sites are spaced in a manner that will permit the formation of three alternatively spliced products, one of which will be in frame with the *LUC+* reporter and will therefore generate productive LUC+ fusions. The first amino acids of the LUC+ protein are underlined. The GenBank accession number of LucTrap-1 is AY944581. B, Schematic representation of the LucTrap-2 T-DNA. The vector is identical to LucTrap-1, except that the *LUC+* start codon was deleted. The initial amino acids of the LUC+ protein are underlined. The right border (RB) sequence and the adjacent Gα intron sequence lack stop codons in any of the three reading frames to avoid premature chain termination during translation. The GenBank accession number of LucTrap-2 is AY944582. C, Rationale of the LucTrap-1 promoter trap vector where LucTrap-1 T-DNA insertions in transcriptionally active regions will result in the formation of *LUC+* fusion mRNAs. D, Rationale of the LucTrap-2 gene trap vector where forward LucTrap-2 T-DNA insertions in an exon (top section) or intron (bottom section) will result in the formation of productive LUC+ fusions as indicated by the line drawing.

start codon (Fig. 4B). Because the start codon for the initiation of translation needs to be provided by the trapped gene, we reason that such an arrangement will favor the identification of in-gene T-DNA insertions and therefore LUC+ protein fusions (Fig. 4D).

Characterization of a LucTrap-2 Collection

To test the performance of LucTrap-2, we generated and analyzed a collection of 700 transgenic Arabidopsis lines carrying LucTrap-2. The segregation of the kanamycin resistance trait in the T_2 progeny of these lines indicated that the vast majority of lines have single locus insertions. We then tested 5-d-old light-grown seedlings for LUC+ expression. In this analysis, we found 90 lines (12.8%) to express LUC+ at levels that are at least 2-fold above the levels detected in nontransgenic control plants (Fig. 5A). This group included 46 lines (6.6%) that express LUC+ at levels at least 10 times above that detected in nontransgenic seedlings (Fig. 5, A and B). This shows that the *LUC+* gene of LucTrap-2 is functional in Arabidopsis in the context of genomic insertions.

We then adopted previously established strategies for the amplification and identification of LucTrap-2 flanking sequence tags (FSTs; Table I; Devon et al.,

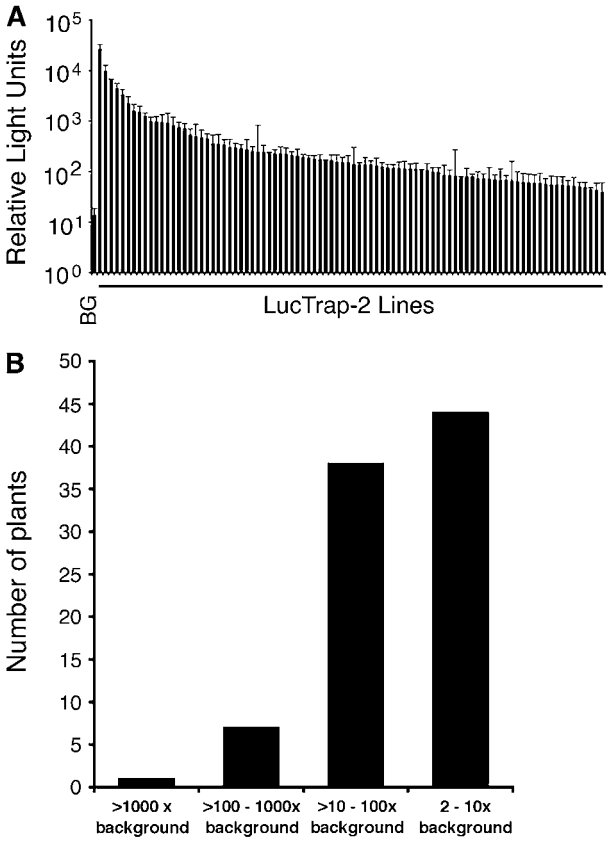

Figure 5. Quantitative analysis of 700 Arabidopsis LucTrap-2 gene trap lines identifies 90 LUC-expressing lines. A, Distribution of LUC activity in the 90 LUC-expressing LucTrap-2 lines. The average and SD of four replicate measurements is shown. Background (BG) activity in this particular experiment was 14 relative light units. Please note the logarithmic scale of the graph. B, Absolute number of LucTrap-2 lines with LUC expression levels above specified BG activities.

1995; Strizhov et al., 2003). Because our main interest lies in the identification of FSTs from LUC+-expressing lines, we preferentially determined FSTs from these lines and, consequently, our sample is not necessarily representative of the entire collection. Furthermore, we would like to point out that, using this strategy, we were unable to identify FSTs for several LucTrap-2 lines, including the three lines LT028, LT032, and LT095 described in more detail below. This may be due to the absence of the appropriate restriction sites in the proximity of the insertion site, and alternative enzyme-primer combinations may have to be used for the successful identification of FSTs from some LucTrap-2 lines (Devon et al., 1995; Strizhov et al., 2003).

We were successful in identifying FSTs from 49 lines and we analyzed these using BLASTN searches (Table II). Based on the position and orientation of the LucTrap-2 T-DNA, we predict that 27 of the 49 lines will give rise to productive fusions between the trapped gene and *LUC+* (Table II). Indeed, the lines predicted to produce LUC+ fusions include 12 lines that we had identified as LUC-expressing lines, suggesting that the trapped genes are expressed during the seedling stage. We also found that the genes that are trapped in 10 of the remaining 14 lines had been reported to be expressed only at low levels during the seedling stage, a finding that may explain the absence of LUC activity in our assays (Zimmermann et al., 2005). Whereas the lack of LUC activity in three remaining lines (LT140, LT200, and LT210) cannot be explained without further analysis, we noticed that line LT005 carries the LucTrap-2 insertion in the 3′-untranslated (UTR) region of At5g40730, and that this gene is composed of a single exon. Because the insertion in LT005 is not in the gene's coding region and because At5g40730 does not contain any introns, this insertion is not expected to result in the formation of productive LUC fusions due to the absence of a splice donor site. In addition, we cannot rule out that the lack of LUC activity in these lines is the result of a *LUC+* fusion transcript or LUC+ fusion protein instability or an impairment of enzymatic activity in the fusion protein context.

Our FST analysis also identified 22 LucTrap-2 lines that we do not predict to give rise to productive LUC+ fusions (Table II). Nevertheless, two lines (LT178 and LT414) display very strong LUC activity, whereas the remaining eight lines have comparatively low LUC levels. This may indicate that the expression of *LUC+* can also be driven from cryptic promoters and cryptic open reading frames, which we would expect to provide the ATG start codon that had been deleted from the *LUC+* gene in LucTrap-2. Alternatively, it may be envisioned that these lines have a duplicated T-DNA insertion in the respective locus so that the right border of the second insertion is oriented such that productive *LUC+* fusions can be formed. Such more complex T-DNA insertion events have frequently been reported for T-DNA insertions (De Neve et al., 1997; Forsbach et al., 2003; Lechtenberg et al., 2003; Windels et al., 2003). In summary, we

Table I. *Primers for the amplification of LucTrap-2 T-DNA flanking sequences*

RB, Right border; LB, left border.

Name	Sequence
Vectorette Primers	
TopL	5′-CGAATCGTAACCGTTCGTACGAGAATTCG-TACGAGAATCGCTGTCCTCTCCAACGAGC-CAAGG-3′
BamHI	5′-GATCCCTTGGCTCGTTTTTTTTTGCAAAAA-3′
VEC1	5′-CGAATCGTAACCGTTCGTACGAGAA-3′
VEC2	5′-TCGTACGAGAATCGCTGTCCTCTCC-3′
LucTrap-2 Right Border Primers	
LucR1	5′-CAATCAATTTTCCTTGTGGACTTGG-3′
LucR2	5′-GTTTTCATGTGTGATTTTACCGAAC-3′
LucR3	5′-GGTTCCCAGTCCGATTTCGACAGG-3′
LucTrap-2 Left Border Primers	
LucL1	5′-CGATAGAAAACAAAATATAGCGCGC-3′
LucL2	5′-CTAGGATAAATTATCGCGCGCGG-3′
LucL3	5′-CTAGATCGACCGGCATGCAAGC-3′

Table II. *Insertion sites identified in LucTrap-2 lines*

LucTrap-2 lines with FSTs identify genomic insertions. The E-values obtained in BLASTN searches using FST reads and primers used for FST identification are indicated. Productive LUC fusions are expected in 27 lines (lines expected to give rise to LUC+ fusions), nonproductive LUC fusions are predicted in 22 lines (lines not expected to give rise to LUC+ fusions). RLUs and SD as detected in 5-d-old seedlings are indicated. $n \geq 4$. LT, LucTrap-2; RLU, relative light units; BG, background activity.

LT No.	Locus	Position	E-Value	Primer	Fusion	RLUs
LucTrap-2 Lines Expected to Give Rise to LUC+ Fusions						
LT001	At4g21750 (ML1-specific homeobox gene)	First intron	3.00E-73	LUCR3	Yes	4,368 ± 1,170
LT005	At5g40730 (arabinogalactan-protein AGP24)	3′-UTR	0	LUCR3	Yes	BG
LT033	At5g45775 (60S ribosomal protein L11)	Third intron	0	LUCR3	Yes	150 ± 42
LT042	At3g08810 (F-box family protein)	3′-UTR	1.00E-105	LUCR3	Yes	BG
LT055	At4g32450 (pentatricopeptide repeat-containing protein)	Unique exon	0	LUCR3	Yes	BG
LT134	At3g23260 (F-box family protein)	Unique exon	1.00E-18	LUCR3	Yes	BG
LT136	At2g44260 (expressed protein)	Second exon	8.00E-26	LUCR3	Yes	BG
LT140	At1g77440 (20S proteasome β-subunit PBC2)	Fifth intron	3.00E-26	LUCR3	Yes	BG
LT155	At5g57399 (UbiE/COq5 methyl transferase)	Third exon	3.00E-15	LUCL3	Yes	105 ± 39
LT171	At4g18570 (Pro-rich family protein)	First intron	2.00E-68	LUCL3	Yes	221 ± 70
LT174	At3g19510 (homeobox protein HAT3.1)	Sixth intron	1.00E-102	LUCR3	Yes	BG
LT179	At3g02820 (zinc knuckle [CCHC-type] family protein)	Fourth exon	1.00E-139	LUCR3	Yes	BG
LT184	At3g11580 (B3 domain transcription factor)	First exon	1.00E-21	LUCR3	Yes	240 ± 0
LT186	At1g05630 (At5PTase 13 inositol 5-P)	Fifth intron	3.00E-126	LUCR3	Yes	BG
LT188	At1g65365 (putative protein kinase, pseudogene)	Unique exon	0	LUCR3	Yes	BG
LT189	At5g10520 (protein kinase)	Seventh intron	2.00E-78	LUCR3	Yes	BG
LT200	At5g67420 (LOB domain protein 37)	Third exon	1.00E-48	LUCR3	Yes	BG
LT206	At1g75840 (Rac-like GTP-binding protein ARAC5)	3′-UTR	4.00E-43	LUCR3	Yes	BG
LT210	At1g73230 (NPAC BTF3 transcription factor)	3′-UTR	1.00E-109	LUCR3	Yes	BG
LT301	At5g65110 (Acyl-CoA oxidase ACX2)	5′-UTR	4.00E-32	LUCL3	Yes	467 ± 195
LT316	At4g33620 (Ulp1 protease family SUMO protease)	Seventeenth exon	1.00E-124	LUCR3	Yes	BG
LT332	At1g48900 (SRP-54C signal recognition particle)	Seventh exon	1.00E-101	LUCR3	Yes	241 ± 58
LT334	At3g02470 (*S*-adenosylmethionine decarboxylase)	First intron	7.00E-57	LUCR3	Yes	26,391 ± 6,355
LT348	At1g21065 (expressed protein)	First intron	6.00E-22	LUCL3	Yes	490 ± 122
LT368	At1g49880 (Erv1/Air family protein)	Fourth exon	8.00E-40	LUCL3	Yes	225 ± 48
LT430	At4g20410 (γ-SNAP)	First intron	3.00-E83	LUCR3	Yes	940 ± 382
LT649	At5g48560 (basic helix-loop-helix transcription factor)	Fifth exon	3.00E-70	LUCR3	Yes	72 ± 26
LucTrap-2 Lines Not Expected to Give Rise to LUC+ Fusions						
LT004	At2g18700 and At2g18690	Intergenic region	1.00E-21	LUCR3	No	BG
LT037	At2g44260 (expressed protein)	Second exon	1.00E-34	LUCR3	No	BG
LT046	At5g10980 (expressed protein)	5′-UTR	1.00E-167	LUCR3	No	BG
LT062	At5g40270 and At5g40260	Intergenic region	0	LUCR3	No	BG
LT104	At3g58500 (Ser/Thr protein phosphatase subunit)	Seventh intron	4.00E-71	LUCR3	No	BG
LT117	At5g38200 and unannotated open reading frame	Intergenic region	6.00E-69	LUCR3	No	BG
LT173	At5g40260 and At5g40270	Intergenic region	6.00E-67	LUCR3	No	BG
LT178	At1g47600 (thioglucosidase)	Thirteenth exon	9.00E-61	LUCL3	No	3,270 ± 951
LT190	At3g23900 (RNA recognition motif-containing protein)	Sixth intron	2.00E-45	LUCR3	No	BG
LT196	At1g04830 and At1g04840	Intergenic region	6.00E-101	LUCR3	No	BG
LT221	At3g53450 (decarboxylase)	Fourth intron	2.00E-06	LUCL3	No	51 ± 25
LT224	At1g13260 (DNA-binding protein RAV1)	5′-UTR	1.00E-93	LUCR3	No	BG
LT263	At4g25620 and At4g25630	Intergenic region	6.00E-56	LUCR3	No	200 ± 75
LT278	At5g15460 (expressed protein with ubiquitin domain)	Second exon	2.00E-14	LUCR3	No	61 ± 30
LT297	At5g34960 and At5g34965	Intergenic region	3.00E-10	LUCL3	No	154 ± 59
LT303	At5g53570 (RabGAP/TBC domain-containing protein)	Fifth exon	2.00E-28	LUCR3	No	BG
LT322	At4g33520 (metal-transporting P-type ATPase)	Fifteenth exon	0.002	LUCR3	No	112 ± 46
LT340	At4g38730 and At4g38740	Intergenic region	4.00E-11	LUCR3	No	70 ± 50
LT414	At4g38710 (glycine-rich protein cylicin II)	First exon	6.00E-05	LUCR3	No	9,730 ± 3,000
LT510	At1g79430 and At1g79440	Intergenic region	6.00E-43	LUCR3	No	122 ± 29
LT516	At3g03700 (expressed protein)	3′-UTR	5.00E-83	LUCR3	No	42 ± 16

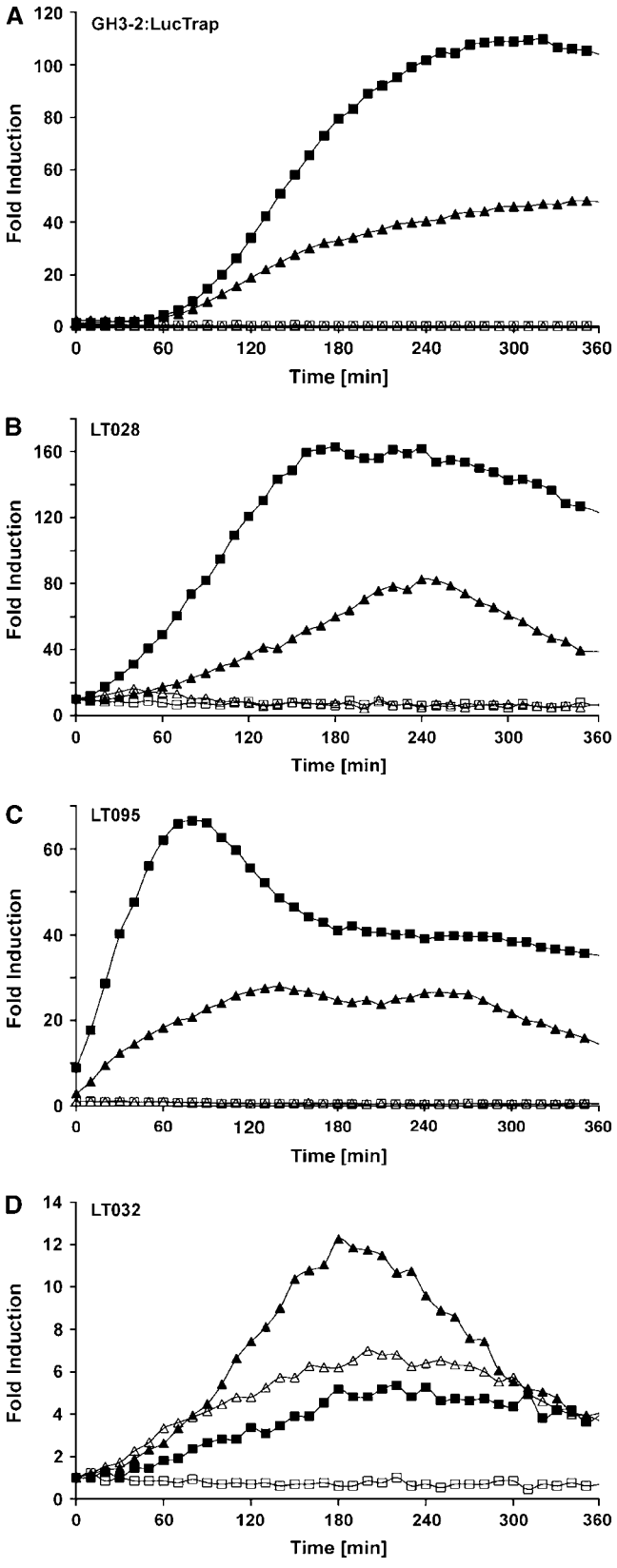

Figure 6. MG132 proteasome inhibitor treatments reveal the role of unstable repressors and activators in controlling auxin-induced gene expression. Relative LUC expression of GH3-2:LucTrap (A), LT028 (B), LT095 (C), and LT032 (D) as detected over time in 5-d-old seedlings (white squares), after auxin induction (5 μM 2,4D; black squares), after proteasomal inhibition (100 μM MG132; white triangles), and after auxin induction (5 μM 2,4D) with concomitant proteasomal inhibition (100 μM MG132; black triangles). The result of a typical induction experiment is shown. The data for GH3-2:LucTrap, uninduced, and auxin treated are identical to those shown in Figure 1C. LUC activity at $t = 0$ min of the untreated sample was set as 1.

suggest that LucTrap-2 can be used as a gene trap vector that will allow generation of random LUC fusion proteins. However, we also have to conclude that LUC expression does not necessarily correlate with the apparent occurrence of such fusion events.

Unstable Negative and Positive Regulators Control Auxin-Induced Gene Expression

In recent years, it has become increasingly clear that many signaling events are controlled by unstable regulators that are degraded by the ubiquitin-proteasome system (Schwechheimer and Calderon-Villalobos, 2004). Signal transduction in response to auxin is currently one of the best characterized cases for proteolysis-dependent signaling in plants. Genetic and biochemical studies have led to the identification of the AUXIN/INDOLE ACETIC ACID (AUX/IAA) proteins as transcriptional regulators that repress gene expression in the absence of auxin (Gray et al., 2001; Tiwari et al., 2001). In response to auxin, AUX/IAA degradation is promoted by the activity of the E3 ubiquitin ligase SCF^{TIR1} whose F-box protein subunit TIR1 also functions as an auxin receptor (Gray et al., 2001; Dharmasiri et al., 2005; Kepinski and Leyser, 2005). Following AUX/IAA degradation, AUXIN RESPONSE FACTOR transcription can activate auxin-induced gene expression (Tiwari et al., 2001). Auxin response is subsequently turned off when de novo synthesized AUX/IAA proteins are available for the repression of AUXIN RESPONSE FACTOR activity (Abel et al., 1994, 1995; Tian and Reed, 1999). The role of protein degradation in auxin-induced gene expression is nicely illustrated in our GH3-2:LucTrap lines, where the application of the 26S proteasome inhibitor MG132 together with auxin results in decreased auxin induction (Fig. 6A). We attribute this effect to a MG132-dependent stabilization of AUX/IAA repressors such as SHORT HYPOCOTYL2 (SHY2)/IAA3, which had previously been shown to control *GH3*-2 expression (Tian et al., 2003).

To examine whether the LucTrap-2 collection contains auxin-induced genes, we examined the effect of auxin on LUC gene expression in all 700 LucTrap-2 lines. In this analysis, we identified three LucTrap-2 lines, namely, LT028, LT032, and LT095, whose LUC expression was activated in response to 2,4D (Fig. 6, B–D). We then went on to study the effect of MG132 application on auxin-induced gene expression in these lines. In agreement with a model where MG132 causes

the stabilization of transcriptional repressors such as the AUX/IAAs, we found that auxin-induced gene expression is impaired in LT028 and LT095 following MG132 application (Fig. 6, B–C). In contrast, our studies indicate that auxin-induced gene expression in LT032 may be governed by a different mechanism. Whereas MG132 alone does not have an effect on the expression of *LUC+* in LT028 or LT095, MG132 is sufficient to induce *LUC+* expression in the absence of auxin in LT032 (Fig. 6D). Furthermore, MG132 superinduces the expression of *LUC+* in LT032 when applied together with auxin (Fig. 6D). Such a result cannot be explained by the activity of unstable transcriptional repressors, but rather points to the activity of an unstable transcriptional activator that is stabilized in response to auxin. Such an unstable activator could be stabilized independently by auxin and by inhibition of proteasomal activity and, in combination, these treatments may then lead to the observed superinduction.

In all four cases examined, auxin-induced *LUC+* expression was followed by negative feedback regulation (Fig. 6, A–D). Such a negative feedback mechanism may be due to the activity of de novo synthesized AUX/IAA repressors whose transcription is known to be promoted by auxin (Abel et al., 1994, 1995; Tian and Reed, 1999). Interestingly, the auxin induction in line LT032, which we hypothesize to be under the control of an unstable activator, is also subject to negative feedback regulation. Therefore, this gene expression mechanism may be negatively controlled by AUX/IAAs or by other as yet unknown repressors. Alternatively, it may be envisioned that the expression of the hypothetical and as yet unidentified activator is down-regulated in response to auxin. As far as we are aware, an auxin-induction mechanism as reported here for LT032 has not been described as yet. LT032 may now be used for the isolation of mutants that show altered *LUC+* expression and that carry defects in genes whose gene products are required for auxin- and proteasome-dependent gene expression in this line.

DISCUSSION

Dynamic Detection of Transcript and Protein Abundance Using the LucTrap Vectors

In this article, we introduce the LucTrap vectors that make use of the modified firefly LUC+ as a reporter for regulated gene expression and protein abundance. Using transgenic Arabidopsis lines that express a promoter fragment of the auxin-inducible *GH3-2* gene, we demonstrate that LucTrap is well suited to follow gene expression patterns in a dynamic and time-resolved manner (Fig. 1). Furthermore, we show that a protein fusion between the unstable GA pathway regulator RGA and LUC+ expressed from LucTrap-3(GW) responds to changes in GA levels and that these changes can be quantified in transgenic lines expressing the fusion protein (Fig. 3; Dill et al., 2001). Furthermore, analysis of a collection of 700 transgenic Arabidopsis lines harboring the vector LucTrap-2 revealed that this vector can be used to generate random LUC+ fusions (Figs. 4 and 5). In 12 of 22 LUC-expressing lines, we were able to provide evidence for LucTrap-2 insertions that are predicted to give rise to productive LUC+ fusions as judged by the T-DNA insertion position and orientation (Table II). Taken together, we provide strong evidence that LucTrap vectors are functional vectors and that LucTrap-based LUC fusions can be used to follow changes in gene expression and protein abundance in vivo.

In comparison to other reporter proteins, such as GFP and GUS, LUC reporters including LUC+ offer the important advantage that they can report on changes in reporter abundance in a time-resolved manner. A number of specific features of LUC contribute to this important advantage. First, LUC reactions are not toxic to the organism under investigation. Furthermore, none of our experiments suggest that the amount of luciferin, its penetration into the plant tissue, and its distribution within the plant are rate-limiting steps in in vivo experiments. For example, in our experiments, we have been able to measure LUC activities as early as 2 min after luciferin application to the plant. Since LUCs including LUC+ are inactivated after the first LUC reaction has taken place, LUC activity measurements report on the current synthesis of LUC rather than on its accumulation over time. Several of our experiments clearly demonstrate that LUC measurements allow the detection of positive and negative changes in LUC synthesis rates at minute intervals when luciferin is continuously supplied. In contrast, the detection of protein degradation events, as exemplified in our case with the RGA:LUC fusion protein, requires measurements of absolute LUC activities and therefore single-point LUC activity measurements (e.g. a comparison of untreated and treated samples). In the same context, we would like to point out that these measurements can be made in a high-throughput manner with seedlings grown in microtiter plates with extremely short measurement times ($<$1 s). Whereas the dynamic nature of LUC expression and the ease of its quantification in a high-throughput manner are certainly great advantages of the LUC reporters, LUCs cannot be used to detect changes in the subcellular localization of a LUC fusion protein. Therefore, whereas LUCs may be optimally suited to detect changes in gene expression rates or protein abundance, they may only allow understanding of some aspects of gene expression or protein behavior.

Protein Degradation as a Regulatory Mechanism

The analysis of the Arabidopsis genome sequence allows the prediction that plant growth and development is regulated to a large extent at the level of protein degradation (Schwechheimer and Calderon-

Villalobos, 2004). The identity of the vast majority of protein degradation-dependent processes, however, remains to be uncovered. The control of transcription in response to auxin is one of the best understood plant-signaling processes, and auxin response has been shown to be dependent on the degradation of the AUX/IAA transcriptional repressors (Gray et al., 2001; Dharmasiri et al., 2005). Through the application of the 26S proteasome inhibitor MG132, we demonstrate that auxin-inducible gene expression in GH3-2:LucTrap as well as in two LucTrap-2 gene trap lines is protein degradation dependent (Fig. 6, A–C). This effect can best be explained through the stabilization of AUX/IAA proteins following proteasomal inhibition with MG132 (Worley et al., 2000; Ramos et al., 2001; Kepinski and Leyser, 2005). In the case of GH3-2:LUC regulation, this hypothesis is also supported by the previously published observations that *GH3-2* expression is negatively regulated in the Arabidopsis *shy2* mutant, which expresses a stabilized form of the AUX/IAA protein IAA3, as well as in mutants of the COP9 signalosome, a protein complex required for proper AUX/IAA degradation (Schwechheimer et al., 2001; Tian et al., 2003; Dohmann et al., 2005).

Interestingly, we also discovered one LucTrap-2 line, LT032, where the inhibition of proteasomal activity by MG132 was sufficient to induce gene expression and where MG132 treatment resulted in a superinduction of auxin-induced gene expression (Fig. 6D). The induction kinetics of the single and the combined treatments strongly suggest that the induction is direct and that both substances act on the same protein. Such induction kinetics cannot be explained through the activity of an unstable repressor, but may best be explained through the activity of an unstable activator that is stabilized by MG132 and stabilized or activated by auxin. As far as we are aware, such a regulatory mechanism for auxin-induced gene expression has not been described as yet and LT032 may now be used for genetic screens that aim at isolation of the factors that control gene expression in LT032.

CONCLUSION

In this article, we introduce the four LucTrap plant transformation vectors. We provide evidence that transcriptional and translational LUC fusions expressed from the LucTrap vectors allow the monitoring of changes in gene expression and protein abundance in vivo. We also demonstrate that LUC measurements can be used to quantify changes in transcript and fusion protein abundance in response to proteasomal inhibition. The Arabidopsis genome encodes for hundreds of proteins with clear homology to known components of the ubiquitin-proteasome pathway (Bachmair et al., 2001; Gagne et al., 2002). The vast majority of processes that require proteasomal activity remain to be identified. The detailed analysis of the already-identified protein degradation-dependent pathways, as well as that of the many as yet unidentified ones, will require novel or complementary tools for the quantification of transcripts and proteins in vivo. We propose that LucTrap vectors will be an essential part of this tool kit.

MATERIALS AND METHODS

Biological Material

Arabidopsis (*Arabidopsis thaliana*) ecotype Columbia was used for all plant transformations described in this study. Arabidopsis transformation was performed using the floral-dip method (Desfeux et al., 2000).

LucTrap Vector Cloning

To generate LucTrap-1, the intron sequence of the Gα (At2g26300) was PCR amplified from the previously published CD126 vector using the primers intron-FW, 5′-AGATCTAGGCCTGTCGAAATCGGACGG-3′ and intron-RV, 5′-CCATGGACCTGCATATAACCTG-3′ (Sundaresan et al., 1995). The intron fragment was cloned into pGEM-T (Promega), sequence verified, and inserted as a *Bgl*II/*Nco*1 fragment upstream of the *LUC+* gene in pSP-LUC+ (Promega). Subsequently, the cauliflower mosaic virus (CaMV) 35S terminator (TER) sequence was obtained by PCR from the vector pCAMBIA-1391Z with the primers CaMV TER-FW, 5′-GAATTCCAGATAAGGGAATTAG-3′ and CaMV TER-RV, 5′-CCATGGCAACCACTTTGTACAAGA-3′, cloned into pGEM-T (Promega), sequence verified, and subcloned as *Xba*1/*Eco*R1 fragment into the Gα intron containing pSP-LUC+. The resulting *LUC+* gene cassette was then inserted as a *Stu*1/*Eco*R1 fragment adjacent to the T-DNA right border of previously published plant transformation vector pGREEN0029-II (Hellens et al., 1999). The resulting vector was designated LucTrap-1 (GenBank accession no. AY944581).

LucTrap-2 (GenBank accession no. AY944582) is derived from LucTrap-1 and was obtained by religation of the *Nco*1-digested and S1 nuclease-treated LucTrap-1 vector. The presence of the desired 4-bp deletion, including the ATG start codon of *LUC+*, was confirmed by DNA sequencing.

LucTrap (GenBank accession no. DQ073044) is derived from LucTrap-1 and was obtained by insertion of the phosphorylated and annealed oligonucleotides LucTrap multiple cloning sites (MCS)-FW, 5′-CCTGGATCCTGCAGAGCTCACTAGTC-3′ and LucTrap MCS-RV, 5′-CATGGACTAGTGAGCTCTGCAGGATCCAGG-3′ into the *Stu*1/*Nco*1-digested LucTrap-1 vector.

LucTrap-3(GW) (GenBank accession no. AY968054) was obtained by insertion of a modified rfB Gateway selection cassette (Invitrogen) into LucTrap-1. To this end, the Gateway rfB cassette was PCR amplified using the primers attR1-*Stu*I, 5′-AGGCCTATCAACAAGTTTGTACAAAAAAG-3′ and attR2-*Nco*I, 5′-CCATGGCAACCACTTTGTACAAGA-3′, cloned into pCR-TOPO (Invitrogen), sequence verified, and subsequently subcloned as a *Stu*1/*Nco*1 fragment into LucTrap-1. LucTrap-3(GW) confers resistance to kanamycin in *Escherichia coli*, and therefore LucTrap-3(GW) works best in combination with the Gentamycin-resistant donor vector pDONR207 (Invitrogen). Because all LucTrap vectors are based on the previously published pGreen0029-II vector, plant transformation requires the presence of the helper plasmid pSOUP (Hellens et al., 1999).

LucTrap-Derived Constructs

To generate GH3-2:LucTrap, an 800-bp *GH3-2* (At4g37390) fragment was PCR amplified from Arabidopsis genomic DNA using the primers GH3-1, 5′-CCATGGTTGTTTTTTTTTCTAAAAGAAAAAGTG-3′ and GH3-2, 5′-AGATCTGTCGACATGCTATAGATTGATATAAGAAAAAAG-3′. The resulting PCR fragment was cloned into pGEM-T (Promega), sequence verified, and subcloned as a *Nco*I/*Stu*I fragment into LucTrap-1. Twenty independent transgenic lines that harbor GH3-2:LucTrap were generated and analyzed.

For RGA:RGA:LUC, a 3,600-bp genomic fragment that comprises the *RGA* (At2g01570) open reading frame and a 2,000-bp promoter fragment were amplified from genomic DNA of Arabidopsis ecotype Columbia with the primers RGA-FW, 5′-AGGCCTTTTATGTTTTCGATGGCTGAGCTTC-3′ and RGA-RV, 5′-CCATGGGCGCCGCCGTCGAGAGTTTCCAAGCGGA-3′. The resulting fragment was inserted into pENTR/D-TOPO (Invitrogen), sequence verified, and subcloned into LucTrap-3(GW). Ten transgenic lines that harbor RGA:RGA:LUC were generated and analyzed.

LUC Activity Measurements

LUC activity was measured using 5-d-old seedlings that had been grown on moist filter paper in 96-well microtiter plates in continuous light (Thermo LabSystems). Seedlings were assayed in a Berthold Mithras LB940 luminometer in the presence of 80 μL Murashige and Skoog medium (Duchefa), supplemented with 5 mM D-luciferin (PJK), 2,4D, or GA_3 (Duchefa) or the inhibitors PAC (Duchefa) and MG132 (Sigma-Aldrich) as indicated. For gene expression experiments, seedlings were incubated with luciferin and LUC activity was measured at regular intervals over the course of the experiments. Changes in LUC+ fusion protein levels were quantified in single-point measurements from samples that had been subjected to the respective treatments for 12 h. The result of one typical experiment is shown in each case.

Fluorescence Microscopy

Transgenic seedlings expressing RGA:GFP:RGA were treated for 12 h with GA_3, PAC (Duchefa), and MG132 (Sigma-Aldrich) as indicated and then imaged using a Leica TCS SP2 confocal microscope. Representative images are shown in each case.

Identification of LucTrap-2 Flanking Sequences

For the determination of flanking sequences from LucTrap-2 transgenic lines, previously established procedures were adapted (Devon et al., 1995; Strizhov et al., 2003). In brief, genomic DNA was digested using the restriction enzymes *Bam*HI, *Bgl*II, or *Bcl*I. Subsequently, an asymmetric adaptor obtained by annealing the TopL and phosphorylated *Bam*HI primers was ligated to the digested genomic DNA (Devon et al., 1995). LucTrap-2-specific fragments were amplified in two or three PCR rounds with the nested vectorette primers VEC1 and VEC2 in combination with LucTrap-2-specific primers. Amplification products were sequenced using LucR3 or LucL3. Sequence reads were analyzed using the BLASTN algorithm at http://www.arabidopsis.org/blast. All primer sequences are provided in Table I.

RT-PCR Analysis

Auxin-induced *GH3-2* (At4g37390) gene expression was examined by semiquantitative RT-PCR. Total RNA was prepared using the RNeasy kit (Qiagen) from 5-d-old seedlings that had been treated with 5 μM 2,4D. One microgram of total RNA was used in combination with the oligo(dT) adaptor primer 5′-GACTCGAGTCGACATCGA(17xT)-3′ for RT as previously described and GH3-2 transcription was examined by PCR (28 cycles) using the *GH3-2* gene-specific primers GH3-2-FW, 5′-GTTTCAGCGACGACTTCTGAGAAAGATGT-3′, and GH3-2-RV, 5′-TCTTCGCTCATAAGAGCATTGCT-3′ (Frohman et al., 1988). RT-PCR results were quantified using ImageJ software available at http://rsb.info.nih.gov.

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers AY944581, AY944582, AY968054, and DQ073044.

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