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Note

Novel intron-containing luciferase genes for quantitative analysis of mRNA levels in transient gene expression assays

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Abstract Firefly luciferase (Fluc) is commonly used as a sensitive reporter for transient gene expression assays in plants. Although the protein level of Fluc can easily be quantified, it is difficult to quantify the mature mRNA level of the reporter gene in these assays. The mRNA level can be measured using quantitative reverse-transcription PCR (qRT-PCR), but the reaction often amplifies both the target mature mRNA and contaminating DNA or unspliced pre-mRNA, thus providing inaccurate results. To address this problem, we constructed a series of novel *Fluc* genes containing intronic sequences measuring 315 bp or longer. Here, we show that the contaminating DNA and unspliced mRNA were not PCR-amplified due to presence of these introns. Moreover, the intron-containing *Fluc* gene conferred superior Fluc activity to the original intron-less *Fluc* gene. We propose that the novel *Fluc* genes reported here can be used to quantitate both protein and functional mRNA levels in transient gene expression assays in plants.

Key words: Dual-luciferase assay, firefly luciferase, intron-containing reporter gene, qRT-PCR, transient assay.

To characterize a gene or interactions among multiple genes, transient gene expression in plants has advantages over the generation of stably transformed transgenic plants. Transient expression systems are fast and flexible and are not influenced by positional effects of the integrated genes, which can bias gene expression levels. They can be used to express foreign genes in cells at advanced developmental stages and are applicable to plant species resistant to regeneration from a single cell. Such systems can readily introduce plural gene constructs into the same cell, and thus can be used to analyze protein–protein, protein–DNA, and protein–RNA interactions (Fisher et al. 1999). Widely used transient expression systems include electroporation into protoplasts (Fromm et al. 1987), and biolistic bombardment (Altpeter et al. 2005) or *Agrobacterium* infection (Kapila et al. 1997) into intact cells/tissues. The former two systems introduce naked DNA directly into plant nuclei after permeabilization or physical breakage of cell membranes, respectively, while the third method uses a natural soil bacterium, *Agrobacterium tumefaciens*, which has evolved to introduce a fragment of its plasmid DNA into plant nuclei. When using transient reporter gene expression systems combined with the precise quantitation of expressed reporter protein levels, it is important to measure the reporter mRNA levels. mRNA quantitation is especially important when attempting to differentiate between transcriptional and translational expression in response to external factors. Reporter mRNA levels can be measured by quantitative reverse-transcription polymerase chain reaction (qRT-PCR), which can quantify mRNA from a very limited number of cells (Bustin et al. 2005).

One problem with quantitating mature, functional mRNAs using PCR-based methods is that the reaction may amplify non-target nucleic acids with homologous sequences, resulting in an inaccurate estimation of mRNA levels. Non-target nucleic acids include introduced plasmid DNA and transcribed unspliced mRNA when using direct gene transfer methods, and, for *Agrobacterium* infection, binary vector DNA, bacterially-transcribed mRNA (by the leaky transcription of plant promoters in bacteria), transferred DNA within plant nuclei, and transcribed unspliced mRNA. Common methods for addressing these problems including DNA degradation by DNase or mRNA isolation

Abbreviation: CaMV, *Cauliflower mosaic* virus.
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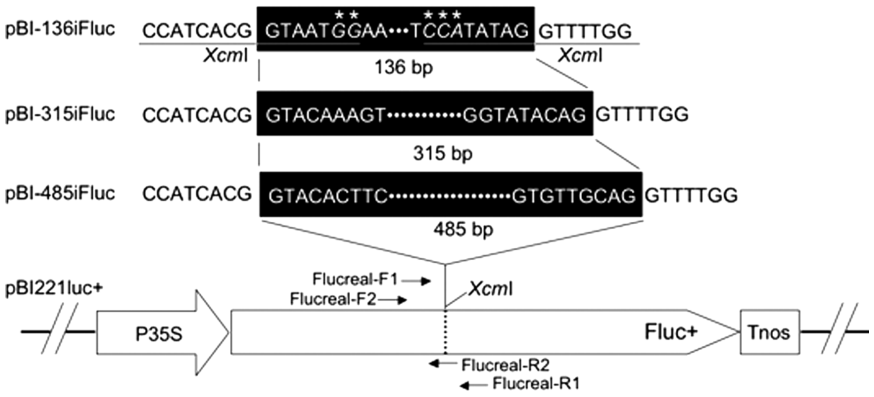

Figure 1. Organization and partial sequences of the *Fluc* gene expression cassettes used in this study. P35S: CaMV 35S promoter. Fluc+: Modified firefly luciferase gene. Tnos: Terminator of the *Agrobacterium* nopaline synthase gene. Filled boxes represent intron sequences from *Arabidopsis thaliana*. The sequences of the exon-intron junctions are shown. Italicized nucleotides with an asterisk are altered nucleotides; the underlined sequences are the created *Xcm*I sites. Filled arrows indicate the annealing sites of the primers used for qRT-PCR. Note that the Flucreal-R2 primer was designed to anneal to the exon-exon junction.

using the poly(A) tail; however, trace amounts of the contaminating non-targets can be problematic, and unspliced nuclear mRNA cannot be removed using these methods. In previous transient expression studies, RT-PCR of intron-containing reporters produced two PCR products: one from unspliced and the other from spliced mRNA after particle bombardment (Mitsui et al. 2003) or *Agrobacterium* infiltration (Eskelin et al. 2010). Therefore, the development of more reliable qRT-PCR protocols that can be used even in the presence of contaminating non-target nucleic acids is important.

The luciferase gene from the North American firefly, *Photinus pyralis*, has been widely used for transient gene expression analysis in plants since its product, firefly luciferase (Fluc), is highly sensitive, has a wide range of amount-activity linearity, and has a short half-life enabling quick responses to changes in mRNA levels (Luehrsen et al. 1992). Thus, we constructed a series of novel *Fluc* gene constructs to prevent the amplification of contaminating DNA and unspliced mRNA and to allow the qRT-PCR quantitation of mature mRNA. The constructed *Fluc* genes contained intronic sequences 315 bp in length or longer. Here, we report optimized protocols for selectively amplifying only spliced, functional reporter mRNAs using a novel set of intron-containing reporter genes, primers, and PCR conditions.

The expression vector pBI-221luc+, harboring a modified-*Fluc* (*Fluc*+) gene (GenBank No. CVU47122) driven by the CaMV 35S promoter, was constructed previously (Matsuo et al. 2001). The intron-containing *Fluc* expression vectors pBI-136iFluc, pBI-315iFluc, and pBI-485iFluc (Figure 1) were constructed by inserting efficiently-spliced introns of the *Arabidopsis thaliana* functional genes into pBI221luc+ as follows. To construct pBI-136iFluc, the second intron (136 bp) of the ribulose 1,5-bisphosphate carboxylase/oxygenase small subunit gene (locus AT1G67090) was amplified using the primers 136iFluc-F (5′-CACCA TCACG GTAAT GGAAC AAAAT TTAAA CATCT-3′) and 136iFluc-R (5′-CACCA AAACC TATAT GGACC ACATG TTACA AACCA-3′) with altered intron sequences (italicized in the primer sequences above and in Figure 1) to create *Xcm*I restriction sites (underlined in Figure 1). The product was digested with *Xcm*I and cloned into the *Xcm*I site in *Fluc*+ in pBI221luc+. To construct pBI-315iFluc and pBI-485iFluc, the first intron (315 bp) of the chaperonin-60 alpha gene (locus AT2G28000) and the first intron (485 bp) of the Rubisco activase gene (locus AT2G39730), respectively, were amplified using the primers 315iFluc-F (5′-GTTCC ATTCC ATCAC GGTAC AAAGT TTGTT CCTTT TTACC TTCC-3′)/315iFluc-R (5′-TAAAC ATTCC AAAAC CTGTA TACCC ACAAC ATCAA TAAAG C-3′) and 485iFluc-F (5′-GTTCC ATTCC ATCAC GGTAC AAAG TTTGT TCCTT TTTAC CTTCC-3′)/485iFluc-R (5′-TAAAC ATTCC AAAAC CTGTA TACCC ACAAC ATCAA TAAAG C-3′), respectively, and cloned into *Xcm*I-digested pBI221luc+ using the In-Fusion PCR Cloning System (Clontech). The expression vector pBI-Rluc (Ono et al. 2004) expressing sea pansy *Renilla reniformis* luciferase (Rluc) was used as a normalization control to minimize experimental variables such as cell viability, transfection efficiency, protein extractability, and pipetting errors.

Cultured tobacco (*Nicotiana tabacum*) bright yellow-2 (BY-2) cells were maintained and their protoplasts were prepared, as described previously (Watanabe et al. 1987). To introduce plasmid DNA into the plant cells, a 700-μl aliquot of 1×10^6 protoplasts per ml of ice-cold electroporation buffer (0.3 M mannitol, 5 mM MES, and 70 mM KCl, pH 5.8) was mixed with 5 μg of one of four Fluc reporter plasmids, 0.5 μg of pBI-Rluc, and 50 μg of sonicated salmon sperm DNA as the carrier. Electroporation was performed in a 0.4-cm gap

cuvette. A single electrical impulse was given to the protoplasts by discharging the electricity stored in a 500-μF condenser at 200 V (Bio-Rad). After electroporation, the protoplasts were kept on ice for 30 min, mixed with 10 ml of protoplast culture medium (4.3 g/l Murashige-Skoog plant salt mixture, 0.4 M mannitol, 1 mg/l thiamine HCl, 0.2 mg/l 2,4-D, and 1% sucrose, pH 5.8), cleared by 3 min of centrifugation at 40×*g*, and cultured for 16 h at 28°C in 10 ml of culture medium in a 50-ml polypropylene tube lying on its side. Two independent electroporation samples were pooled; 25% was used for the luciferase assay and the remainder for qRT-PCR. The activity of the transiently expressed Fluc and Rluc was determined using a Dual-Luciferase Reporter Assay Kit (Promega) with slight modifications. Briefly, protoplasts were harvested by centrifugation, suspended in 100 μl of 1× Passive Lysis Buffer (Promega) and vortexed for 30 s at room temperature for cell lysis. A 10-μl aliquot was mixed with 50 μl of Luciferase Assay Reagent II (Promega) and Fluc-derived luminescence was measured for 5 s using a luminometer (model LB960; Berthold). Next, 50 μl of Stop and Glo Reagent (Promega) were added to quench Fluc activity and provide the Rluc substrate. After 5 s for equilibration, Rluc-derived luminescence was measured for 5 s. Fluc activity is presented as the normalized value after being divided by Rluc activity (Fluc/Rluc). This normalized value did not significantly alter when protoplast culture period was extended up to 24 h (data not shown).

Figure 2 shows the normalized activity of Fluc expressed from the *Fluc* constructs with and without introns. When compared to the Fluc activity from the intron-less construct pBI221luc+ (Fluc), those from pBI-136iFluc (136iFluc), pBI-315iFluc (315iFluc), and pBI-485iFluc (485iFluc) exhibited approximately 27, 96, and 116% activity, respectively.

We next estimated the functional mature *Fluc* mRNA concentrations by qRT-PCR and assessed their correlation with Fluc activity. Total RNA was extracted from harvested cells using an RNeasy Plant Mini Kit (Qiagen). The introduced plasmid DNA remaining in the total RNA preparations was digested with 0.2 U/μg of TURBO DNase (Ambion) for 30 min at 37°C followed by inactivation, as per the manufacturer's protocol. cDNA was synthesized from 500 ng of total RNA by reverse transcription (RT) using a PrimeScript cDNA Synthesis Kit (Takara). Samples that did not undergo RT (non-RT) were used as negative controls. For qRT-PCR, 100 ng of cDNA were mixed with 10 μl of 2× Thunderbird SYBR qPCR Mix (Toyobo), 300 nM PCR primers, and pure water in a total volume of 20 μl. One primer pair (primer pair 1), Flucreal-F1 (5′-CGCAT GCCAG AGATC CTATT TTTGG-3′) and Flucreal-R1 (5′-CTGAA GGCTC CTCAG AAACA GC-3′), was complementary to the *Fluc* cDNA sequences located on each side of the intron insertion site (Figure 1) and amplified different-sized products depending on the template. This primer pair produced a 176-bp product from spliced *Fluc* mRNA template, and 176-bp (pBI221luc+), 312-bp (pBI-136iFluc), 491-bp (pBI-315iFluc), and 661-bp (pBI485iFluc) products from unspliced *Fluc* mRNA or contaminating plasmid DNA. The other primer pair (primer pair 2), Flucreal-F2 (5′-CCAGG GATTT CAGTC GATGT AC-3′) and Flucreal-R2 (5′-GTAGT AAACA TTCCA AAACC GTG-3′), was designed to amplify only spliced *Fluc* mRNA since Flucreal-R2 was complementary to the exon junction of the spliced mRNA (Figure 1) and produced a 156-bp product. qRT-PCR was performed using a Thermal Cycler Dice Real Time System Single (Takara) under the following conditions. After an initial denaturation for 1 min at 95°C, 50 cycles of denaturation (95°C for 1 min) and annealing/extension (60°C for 20 s for primer pair 1 and 1 min for primer pair 2) were performed and the fluorescence was monitored during the 60°C annealing/extension step. To verify successful amplification of the target *Fluc* mRNA, the qRT-PCR products were analyzed by agarose gel electrophoresis (Figure 3A). The relative amount of *Fluc* mRNA was estimated from the threshold cycle (Ct) values of its amplification profile and expressed as a relative value to those of constitutive plant mRNA encoding L25 ribosomal protein amplified with the primer pair L25-F (5′-CCCCT CACCA CAGAG TCTGC-3′) and L25-R (5′-GTCAA CCTCA CATAT GCTTT CTTCG-3′) using the standard curve method (Figure 3B) (Schmidt and Delaney 2010).

In Figure 3A, unexpected products are seen in the negative control samples without RT (non-RT PCR, lanes 5 and 6). This suggested that the total RNA preparation used was contaminated with plasmid DNA even after DNase treatment. When primer pair 1 was used (Figure 3A, top panel), non-RT PCR of RNA from pBI221luc+ (lane 5) yielded a single product with the predicted size (176 bp) for contaminating plasmid DNA. This suggested that the RT-PCR product from the same RNA sample (lane 1) was a mixture of the product derived from *Fluc* mRNA and contaminating DNA. Thus, the *Fluc* mRNA level in this sample as quantified by qRT-PCR (Figure 3B, left panel, lane Fluc) was not reliable. Non-RT PCR of RNA from pBI-136iFluc (Figure 3A, lane 6) yielded a single product at the size predicted for contaminating DNA (312 bp). RT-PCR of the same RNA (lane 2) yielded two products; one was predicted for contaminating DNA (312 bp) or unspliced mRNA (312 bp) and the other for spliced mRNA (176 bp). This suggested that the mRNA quantification of the sample (Figure 3B, left panel, lane 136iFluc) was not accurate. In contrast, non-RT PCR of RNA samples from pBI-315iFluc and pBI-485iFluc (Figure 3A, lanes 7 and 8) yielded no product. Considering that all of the RNA

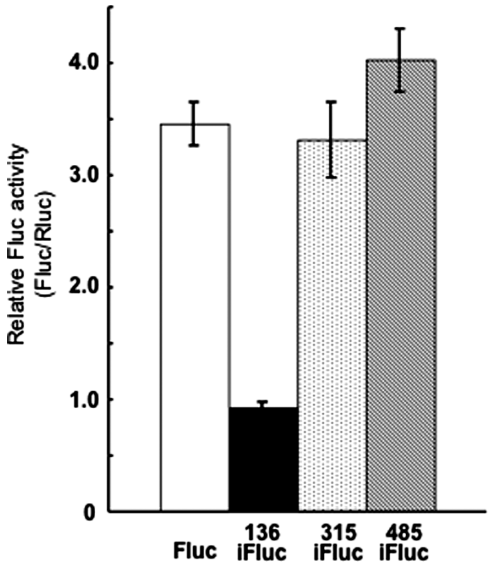

Figure 2. Normalized activities of the Fluc reporter transiently expressed in tobacco BY-2 cells. Tobacco BY-2 cells were electroporated with pBI221Fluc+ (Fluc), pBI-136iFluc (136iFluc), pBI-315iFluc (315iFluc), or pBI-485iFluc (485iFluc) together with pBI-Rluc (a normalization control construct). All values are expressed as the mean±SE of three independent experiments.

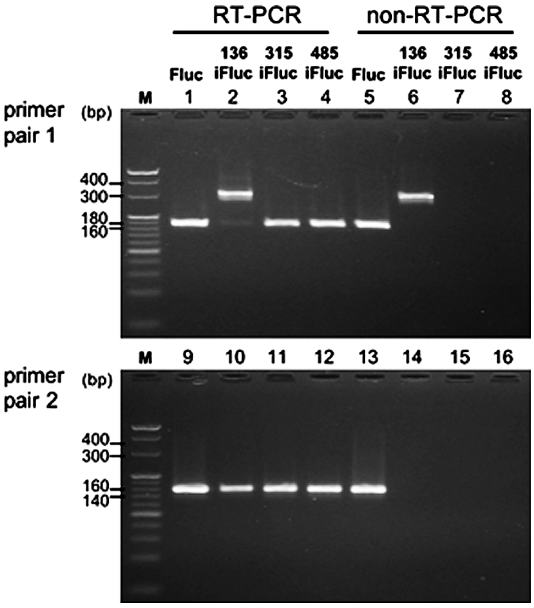

Figure 3A. Analysis of the qRT-PCR products for *Fluc* mRNA by gel electrophoresis. The upper and bottom panels show the products produced using primer pairs 1 and 2, respectively. The 176-bp PCR product produced using primer pair 1 and the 156-bp product produced using primer pair 2 were derived from mature *Fluc* mRNA. Lanes 1–4 and 9–12 are standard qRT-PCR products; lanes 5–8 and 13–16 are those without the RT step. Lane M contains the 20-bp DNA marker.

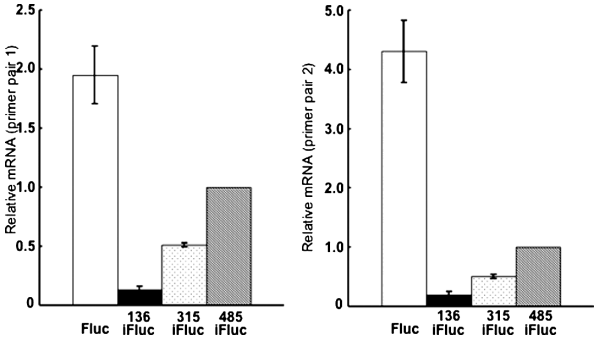

Figure 3B. Relative amount of *Fluc* mRNA as estimated by qRT-PCR using primer pairs 1 (left panel) and 2 (right panel). The amount of *Fluc* mRNA from each construct was normalized to the amount of mRNA encoding the L25 ribosomal protein and expressed as a relative value to the amount of *Fluc* mRNA from pBI-485iFluc construct. All values are expressed as the mean±SE of three independent experiments.

samples were treated with DNase, it is possible that these samples were also contaminated with plasmid DNA. The failed DNA amplification was due to the size of the PCR products; namely, 491 and 661 bp for pBI-315iFluc and -485iFluc, respectively, which were too long to be amplified under the reaction conditions with annealing/extension time of 20 s. This result coincides well with our previous result showing that DNA fragments shorter than 365 bp were amplified, but those longer than 415 bp were not amplified, under the reaction conditions used (data not shown). RT-PCR of the same RNA samples (lanes 3 and 4) both yielded a single product at the size expected for spliced mRNA (176 bp). This indicates that the mRNA quantification of these samples was reliable. Indeed, the mRNA level (Figure 3B, left panel) of pBI-485iFluc was higher than that of pBI-315iFluc, which corroborated the Fluc activity in these samples (Figure 2) where the Fluc activity of pBI-485iFluc was higher than that of pBI-315iFluc.

When primer pair 2 was used (Figure 3A, bottom panel), non-RT PCR of RNA from pBI221luc+ (lane 13) yielded a single product at the size predicted for contaminating DNA (156 bp). This suggests that the *Fluc* mRNA level in this sample, as quantified by qRT-PCR (Figure 3B, right panel, lane Fluc), was not reliable. Non-RT PCR of RNA samples from pBI-136iFluc (Figure 3A, lane 14), pBI-315iFluc (lane 15), and pBI-485iFluc (lane 16) yielded no product. This is because one of the primer pairs (Flucreal-R2 annealing to the exon junction; Figure 1) amplified only cDNA lacking introns and did not amplify cDNA containing introns (from unspliced mRNA) or contaminating plasmid DNA. RT-PCR of RNA samples from pBI-136iFluc (Figure 3A, lane 10), pBI-315iFluc (lane 11), and pBI-485iFluc (lane 12) yielded a single product at the size expected for spliced mRNA (176 bp). This indicated that the mRNA quantitation of these three samples (Figure 3B, right panel, lanes 136iFluc, 315iFluc, and 485iFluc) was reliable. The ratio of the mRNA quantity in these three samples (Figure 3B, right panel) was 1 : 2.6 : 5.1, which was roughly correlated with the ratio of Fluc activity in the three samples (Figure 2) (1 : 3.6 : 4.3).

In this study, we showed that in the presence of contaminating plasmid DNA, functional mRNA levels could be quantified by qRT-PCR using two methods. In the first method, an intron (of any size) was incorporated into the reporter gene and an exon-junction primer (e.g., Flucreal-R2) was used for amplification. In the second method, a long intron was incorporated so that the amplification product was longer than 415 bp, and short annealing/extension times (20 s) were used for amplification. The latter method is especially versatile since designing an exon-junction primer, which is essential for the former method, is not always possible (Bustin et al. 2005). These methods are also applicable to cDNA derived from unspliced bacterial or plant mRNA. The introduction of eukaryotic introns into reporter genes can be used to enhance reporter gene expression (Le Hir et al. 2003), to shut off reporter translation within *Agrobacterium* cells (of mRNA leakily transcribed from plant promoters within bacterial cells) (Vancanneyt et al. 1990), and to isolate spliced cDNA from transiently expressed genomic clones (Wu et al. 2005). Here, we introduced introns to repress the PCR amplification from contaminating DNA and unspliced mRNA.

By the introduction of three *Arabidopsis* introns into the *Fluc*+ gene, we observed a 73% decrease (pBI-136iFluc), and 16% increases (pBI-485iFluc) in Fluc expression compared to pBI221luc+. As mentioned above, the introduction of an intron into a reporter gene often enhances the expression of the reporter in plants and animals via "intron-mediated enhancement" (Le Hir et al. 2003), although the molecular mechanisms are not yet fully understood. Depending on the introns used, the insertion sites within the reporter gene, and the organisms used, the enhancement level varied greatly, ranging from a hundred-fold to a ten-fold decrease (Le Hir et al. 2003). Thus, this effect could have altered Fluc expression from the *iFluc* constructs in this study. Alternatively, the marked decrease in Fluc activity of pBI-136iFluc could result from incomplete splicing since the intron sequence was modified during construction (Figure 1). However, the purpose of this study was not to achieve maximal reporter activity by intron insertion; thus, we did not explore other enhancing introns.

Several *Fluc* genes with an intron inserted into their coding region have already been reported, but the sizes of the introns used were 189 bp (Mankin et al. 1997), 103, 117, and 234 bp (Bourdon et al. 2001), and 234 and 304 bp (Bartlett et al. 2009). Two of the novel intron-containing *Fluc* genes reported here, 315iFluc and 485iFluc, have larger introns than in previous studies and showed similar or superior activity towards the intron-less Fluc+ gene. Therefore, they are applicable to *Fluc* mRNA level determination by qRT-PCR.

References

Altpeter F, Baisakh N, Beachy R, Bock R, Capell T, Christou P, Daniell H, Datta K, Datta S, Dix PJ, Fauquet C, Huang N, Kohli A, Mooibroek H, Nicholson L, Nguyen TT, Nugent G, Raemakers K, Romano A, Somers DA, Stoger E, Taylor N, Visser R (2005) Particle bombardment and the genetic enhancement of crops: Myths and realities. *Mol Breed* 15: 305–327

Bartlett JG, Snape JW, Harwood WA (2009) Intron-mediated enhancement as a method for increasing transgene expression levels in barley. *Plant Biotechnol J* 7: 856–866

Bourdon V, Harvey A, Lonsdale DM (2001) Introns and their positions affect the translational activity of mRNA in plant cells. *EMBO Rep* 2: 394–398

Bustin SA, Benes V, Nolan T, Pfaffl MW (2005) Quantitative real-time RT-PCR—A perspective. *J Mol Endocrinol* 34: 597–601

Eskelin K, Suntio T, Hyvärinen S, Hafren A, Mäkinen K (2010) *Renilla* luciferase-based quantitation of *Potato virus A* infection initiated with *Agrobacterium* infiltration of *N. benthamiana* leaves. *J Virol Methods* 164: 101–110

Fischer R, Vaquero-Martin C, Sack M, Drossard J, Emans N, Commandeur U (1999) Towards molecular farming in the future: transient protein expression in plants. *Biotechnol Appl Biochem* 30: 113–116

Fromm M, Callis J, Taylor LP, Walbot V (1987) Electroporation of DNA and RNA into plant protoplasts. *Methods Enzymol* 153: 351–366

Le Hir H, Nott A, Moore MJ (2003) How introns influence and enhance eukaryotic gene expression. *Trends Biochem Sci* 28: 215–220

Luehrsen KR, de Wet JR, Walbot V (1992) Transient expression analysis in plants using firefly luciferase reporter gene. *Methods Enzymol* 216: 397–414

Ono S, Tanaka T, Watakabe Y, Hiratsuka K (2004) Transient assay system for the analysis of *PR-1a* gene promoter in tobacco BY-2 cells. *Biosci Biotechnol Biochem* 68: 803–807

Kapila J, De Rycke R, Van Montagu M, Angenon G (1997) An *Agrobacterium*-mediated transient gene expression system for intact leaves. *Plant Sci* 122: 101–108

Mankin SL, Allen GC, Thompson WF (1997) Introduction of a plant intron into the luciferase gene of *Photinus pyralis*. *Plant Mol Biol Rep* 15: 186–196

Matsuo N, Minami M, Maeda T, Hiratsuka K (2001) Dual luciferase assay for monitoring transient gene expression in higher plants. *Plant Biotechnol* 18: 71–75

Mitsui M, Murohashi Y, Asano Y, Masada M, Kodama H (2003) Transient assay for *in vivo* splicing in tobacco leaf cells by particle bombardment. *Plant Mol Biol Rep* 21: 21–30

Schmidt GW, Delaney SK (2010) Stable internal reference genes for normalization of real-time RT-PCR in tobacco (*Nicotiana tabacum*) during development and abiotic stress. *Mol Genet Genomics* 283: 233–241

Vancanneyt G, Schmidt R, O'Connor-Sanchez A, Willmitzer L, Rocha-Sosa M (1990) Construction of an intron-containing marker gene: splicing of the intron in transgenic plants and its use in monitoring early events in *Agrobacterium*-mediated plant transformation. *Mol Gen Genet* 220: 245–250

Watanabe Y, Meshi T, Okada Y (1987) Infection of tobacco protoplasts with *in vitro* transcribed tobacco mosaic virus RNA using an improved electroporation method. *FEBS Lett* 219: 65–69

Wu S, Schoenbeck MA, Greenhagen BT, Takahashi S, Lee S, Coates RM, Chappell J (2005) Surrogate splicing for functional analysis of sesquiterpene synthase genes. *Plant Physiol* 138: 1322–1333