Original Paper

Enhancement of Reporter-Gene Expression by Insertions of Two Introns in Maize and Tobacco Protoplasts

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Abstract

The effects of two-intron insertions on reporter-gene expression were examined in transient assays in maize and tobacco. The first introns of a rice gene for phospholipase D (PLD), a maize gene for ubiquitin and a castor bean gene for catalase were tested with a gene for β-glucuronidase (GUS) or a gene for luciferase driven by a cauliflower mosaic virus 35S promoter (35S-GUS or 35S-Luc), the promoter of the maize ubiquitin (Ubi-GUS) or the promoter of a maize pyruvate, orthophosphate dikinase (PPDK-GUS). Although the enhancing effect varied, several intron combinations synergistically enhanced the reporters. When the first and second introns of the PLD gene were inserted in tandem, the reporter was synergistically enhanced. These results imply that expression enhancement by two introns may be observed in wide combinations of promoters, introns, and structural genes and that such enhancement plays certain roles in regulation of gene expression in higher plants.

Key words: Expression enhancement, Gene expression, Intron, Maize (*Zea mays*), Protoplast, Tobacco (*Nicotiana tabacum*).

Abbreviations

CaMV, cauliflower mosaic virus; Cat, catalase; Cat-int1, first intron of a castor bean gene for catalase; GUS, β-glucuronidase; Luc, luciferase; Nos, a gene for nopaline synthase; PLD, phospholipase D; PLD-int1 or 2, first or second intron of a rice gene for phospholipase D; PPDK, pyruvate, orthophosphate dikinase; SI, synergy index; Ubi, ubiquitin; Ubi-int1, first intron of a maize gene for ubiquitin.

Introduction

The enhancement of gene expression by introns has been reported for many plant and animal genes. The effects of introns, mainly the first introns, have been tested by their removal from a native sequence or insertion between a promoter and a reporter gene in transient or stable transformation assays. In plant genes, the first introns of maize genes for alcohol dehydrogenase-1 (*Adh1*) and UDP-glucose 3-O-glucosyl transferase (*bz1*) were first reported to increase gene expression (Callis *et al.*, 1987), followed by the first introns of genes for maize sucrose

synthase (Vasil *et al.*, 1989), rice actin (McElroy *et al.*, 1990) and castor bean catalase (Tanaka *et al.*, 1990). More than twenty introns have been reported to increase gene expression. The enhancing effect of introns is generally higher in monocotyledons than in dicotyledons. The range of enhancement reported is roughly 2–5 fold for dicotyledons and 10–100 fold for monocotyledons (Simpson and Filipowicz, 1996). The use of introns with the adjacent exon segments resulted in even stronger expression in monocotyledons (Mascarenhas *et al.*, 1990; Maas *et al.*, 1991).

Despite the dramatic effect of introns, the mechanism of intron-mediated enhancement of gene expression is not well understood. The degree of enhancement depends on many factors such as the intron used, its location in the transcript, the nature of other sequences in the construct (such as the promoter and exon sequences), the cell type in which expression takes place and the physiological status of those cells (Simpson and Filipowicz, 1996). Furthermore, expression enhancement by an intron was shown to be a process dependent on structural genes (Rethmeier *et al.*, 1997). This phenomenon is associated with an increase in the steady-state level of the mRNA, and several lines

of evidence indicate that introns act post-transcriptionally (Dean *et al.*, 1989; Huang and Gorman, 1990; Rose and Last, 1997). The deletion of internal sequences in introns still retained intron-mediated enhancement (Mascarenhas *et al.*, 1990; Clancy *et al.*, 1994; Luehrsen and Walbot, 1994; Rose and Beliakoff, 2000), which may explain that certain sequences of introns are redundant (Luehrsen and Walbot, 1994) or that most intron sequences are dispensable (Rose and Beliakoff, 2000). The correlation between efficient splicing and intron-mediated enhancement was pointed out (Tanaka *et al.*, 1990; Luehrsen and Walbot, 1994), while an opposite case that splicing is unnecessary for enhancement was reported (Rose and Beliakoff, 2000). Export of mRNA is said to be coupled to pre-mRNA splicing (Reed and Hurt, 2002), so the interaction between pre-mRNA and mRNA export machinery, even if not spliced, might be the key to an efficient export of mRNA from the nucleus to the cytoplasm.

Several genes are known to require an intron for expression at proper levels (Callis *et al.*, 1987; Dean *et al.*, 1989; McElroy *et al.*, 1990; Curie *et al.*, 1993; Norris *et al.*, 1993; Xu *et al.*, 1994; Donath *et al.*, 1995; Rose and Last, 1997). An *Arabidopsis* gene for ribosomal protein kinase (*atpk1*) is expressed in all tissues, but the expression is restricted to cotyledon tips without the intron (Zhang *et al.*, 1994). Removal of the first intron from a potato gene for sucrose synthase (*Sus4*) changed the location of expression in the root tips from the root cap and apical meristem to the procambium (Fu *et al.*, 1995). Tissue-preferential expression of a rice gene for α-tubulin (*OsTubA1*) is mediated by the first intron, where the intron is necessary for the normal expression in actively dividing tissues (Jeon *et al.*, 2000). These studies have shown the evidence that introns play regulatory roles in plant gene expression.

On the practical side, this phenomenon is very useful for obtaining a high expression level of a transgene (Maruta *et al.*, 2001), and the technique for higher expression level with introns is required for the improvement of useful plants. The effect of intron insertions has been studied mainly with single-intron insertions, and the limited trials of inserting two introns in tandem had resulted in less than additive effects (Mascarenhas *et al.*, 1990; Luehrsen and Walbot, 1991; Clancy *et al.*, 1994). Previously, we reported that the insertion of two introns in tandem synergistically enhanced the expression of GUS gene driven by the 35S promoter in rice protoplasts, and that the enhancement was accompanied by an increase in the steady-state

level of the transcript (Ueki *et al.*, 1999). Here, we describe the effect of two-intron insertions on reporter-gene expression using chimeric genes of various combinations of promoters, introns and structural genes in maize and tobacco protoplasts.

Materials and Methods

Construction of chimeric genes

DNA manipulations were carried out by the standard techniques (Sambrook *et al.*, 1989). Unless otherwise mentioned, DNA fragments were blunt-ended with a Klenow fragment when the ligation of DNA fragments with incompatible ends was carried out.

CaMV 35S promoter and GUS

35S-GUS reporters with either one or two of the first introns of a rice gene for phospholipase D (PLD-int1), a maize gene for ubiquitin (Ubi-int1) and a castor bean gene for catalase (Cat-int1) were constructed as described in our previous study (Ueki *et al.*, 1999).

Ubiquitin promoter and GUS

A region that contained the promoter, 5′-exon and first intron (Ubi-int1) of a maize gene for ubiquitin, *Ubi*-1 (Christensen *et al.*, 1992), was excised with *Pst*I and subcloned in pUC18. The promoter region excised with *Hin*dIII and *Bgl*II was inserted between the *Hin*dIII and *Xba*I sites of pBI221 (Jefferson, 1987), pBI[PLD] (Ueki *et al.*, 1999) and pIG221 (Ohta *et al.*, 1990) to yield pUQ, pUQ[PLD] and pUQ[Cat], respectively. The intron fragment excised with *Bgl*II and *Bam*HI was inserted at the *Sma*I sites of pUQ and pUQ[PLD] to yield pUQ[Ubi] and pUQ[PLD/Ubi], respectively. pUQ[PLD] was digested with *Hin*dIII and *Bgl*II, and the resultant fragment was inserted between the *Hin*dIII and *Bgl*II sites of pIG[PLD/Cat] to yield pUQ[PLD/Cat]. The promoter region with the intron was excised with *Hin*dIII and *Bam*HI, and inserted between the *Hin*dIII and *Xba*I sites of pIG221 to yield pUQ[Ubi/Cat]. In plasmids that contain the Cat intron (Cat-int1), splicing is required for the expression of GUS activity (Ohta *et al.*, 1990).

PPDK promoter and GUS

A 1.0 kb DNA fragment that contained a promoter region of a maize gene for PPDK was prepared by PCR with genomic DNA from maize cultivar B73 as template. The amplified fragment was subcloned into PCR1000 vector (Invitrogen), excised with *Sac*I, blunt-ended with T4 DNA

polymerase, and then subcloned at the *Sma*I site of pUC119. The promoter region excised with *Hin*dIII and *Eco*RI was inserted between the *Hin*dIII and *Xba*I sites of pBI221, pBI[PLD], and pIG221to yield pDK, pDK[PLD], and pDK[Cat], respectively. The intron fragment for ubiquitin excised with *Bgl*II and *Bam*HI was inserted at the *Sma*I sites of pDK and pDK[PLD] to yield pDK[Ubi] and pDK[PLD/Ubi], respectively. This intron fragment was also inserted at the *Xba*I site of pDK[Cat] to yield pDK[Ubi/Cat]. A fragment excised with *Hin*dIII and *Bgl*II from pDK[PLD] was inserted between the *Hin*dIII and *Bgl*II sites of pIG[PLD/Cat] to yield pDK[PLD/Cat]. In plasmids that contain Cat intron, splicing is required for the expression of GUS activity (Ohta *et al.*, 1990).

CaMV 35S promoter and luciferase

A fragment containing the coding region of a gene for firefly luciferase was excised with *Bam*HI and *Sac*I from pDO432 (Ow *et al.*, 1986), inserted between the *Bam*HI and *Sac*I sites of pUC19, and excised with *Sma*I and *Sac*I. The fragment was inserted at the *Sma*I sites of pBI221, pBI[PLD] and pBI[Ubi], to yield p35Luc, p35Luc[PLD] and p35Luc[Ubi], respectively. The intron fragment for catalase that had been excised with *Bam*HI and *Sal*I from pIG221was insertedat the*Sma*I sites of p35Luc, p35Luc[PLD] andp35Luc[Ubi] toyieldp35Luc[Cat], p35Luc[PLD/Cat] and p35Luc[Ubi/Cat], respectively. Because the Cat intron was inserted in the 5′ untranslated region, splicing is not required for the expression of GUS activity. The intron fragment for ubiquitin excised with *Bgl*II and *Bam*HI was inserted at the *Sma*I site of p35Luc[PLD] to yield p35Luc[PLD/Ubi].

PLD or CaMV 35S promoter, PLD intron 1 & 2, and GUS

A promoter fragment, the first intron (PLD-int1) and the 2nd intron (PLD-int2) from *PLD1* (Morioka *et al.*, 1997) were prepared by PCR. Primers used were 5′-GTCCAACTTAAGCTTCTTCAGTCA-TTTTCC-3′ and 3′-GGGCGTTAAAATATTC-CTAGGGCCCAGTAT-5′ for the PLD promoter, 5′-AGTCAGGGATCCCCCGGAGAAGACGCC-TCC-3′ and 3′-GTTTAGGTTTAGTCGTCGG -CCTAGGCAGAC-5′ for PLD int1, 5′-AAGTC CCCCGGGCCGCGCCAGCGGAAG-3′ and 3′-GACACCCACAGCCGTCTATAGTTCGTA-5′ for PLD int2. The amplified fragments were subcloned into pCRII (Invitrogen, San Diego, CA, USA). The PLD promoter fragment excised with *Hin*dIII and *Bam*HI were inserted between the *Hin*dIII and *Bam*HI sites of pBI221 (pLDpro). The PLD-int1

fragment excised with *Bam*HI and PLD-int2 fragment excised with *Sma*I and *Eco*RV were inserted into the *Bam*HI site and *Sma*I site of pLDpro, respectively, (pLDpro[1st] and pLDpro[2nd]). The PLD-int1 fragment excised with *Bam*HI was inserted into the *Bam*HI site of pLDpro[2nd] to yield pLDpro[1st /2nd]. Because the length between the 3′ splicing site of the PLD-int1 and the translation initiation site was 37 bases in the native gene for PLD, both intron fragments were flanked by 37 bases each of the exon sequences at both ends. The PLD-int1 fragment flanked by 10 bases each of the exon sequences at both ends is described "PLD" in plasmid names as was the case in the previous study (Ueki *et al.*, 1999).

The PLD-int1 fragment excised with *Bam*HI and the PLD-int2 fragment excised with *Sma*I and *Eco*RV were inserted into the *Bam*HI site and *Sma*I site of pBI221, respectively (pBI[1st] and pBI[2nd]). The PLD-int1 fragment excised with *Bam*HI was inserted into the *Bam*HI site of pBI[2nd] to yield pBI[1st /2nd].

Protoplast isolation, electroporation and analysis of reporter-gene expression

Seed line, growth conditions, and protoplast isolation of maize were as described by Sheen (Sheen, 1991). Protoplasts were isolated from etiolated leaves (yellow protoplasts) and greening leaves under room light for 17 h (greening protoplasts) of maize plants at 10 days after germination, then suspended in electroporation buffer, namely, 0.6 M mannitol and 20 mM KCl in 4 mM Mes-NaOH, pH 5.7 (Sheen, 1991). Electroporation was carried out with 30 μg of plasmid DNA which contained a gene for GUS, 15 μg of pDO432 (Ow *et al.*, 1986) which contained a gene for luciferase, 75 μg of salmon sperm DNA, and 1×10^5 protoplasts in 0.8 ml of electroporation buffer at 125 μF and 400 V cm^{-1} with one pulse, in a Bio-Rad Gene Pulser (Hercules, CA, USA). After electroporation, protoplasts derived from etiolated or greening leaves were incubated for 16 h at 25℃ in darkness or under white light (20 μE m^{-2}), respectively.

Tobacco suspension culture (BY-2) and green leaves (BY-4) were digested for 3 and 16 h, respectively, at 25℃ in darkness with the same solution used for the digestion of maize leaves. Protoplasts isolated from cultured cells (white protoplasts) and green leaves (green protoplasts) were suspended in electroporation buffer, and electroporation was carried out as described above except for the pulse condition at 125 μF and 500 V cm^{-1}. After electroporation, protoplasts derived from the suspension culture and leaves were incubated for 16 h at 25℃

1in darkness or under white light, respectively.

Protoplasts were suspended in 75 μl of 50 mM Tirs-H_3PO_4, pH 7.5. After addition of 75 μl of Lysis Reagent Luc (Toyo Ink MFG., Tokyo, Japan), the mixture was sonicated three times for 10 s with 10 s intervals. Assays of GUS activity were performed as described by Jefferson (Jefferson, 1987). Assays of luciferase activity were performed with PicaGene (Toyo Ink MFG.). GUS activity was normalized by luciferase activity obtained by co-transformation with the plasmid pDO432 (Ow et al., 1986).

Chimeric genes employing the same promoter were introduced to the protoplasts isolated in a single preparation, and the overall response shown as a single experiment here was confirmed with another experiment.

Results

Normalization of measurement of reporter genes activities

Because the expression level of a reporter gene usually varies considerably from experiment to another in transient assays, additional reporter gene was introduced into plant cells together as an internal control (Callis et al., 1987; Leckie et al., 1994; Kovtun et al., 1998). In a preliminary study, the plasmid pIG221, which contained a GUS gene driven by the CaMV 35S promoter (35S-GUS), and the plasmid pDO432, which contained a luciferase gene driven by 35S promoter (35-Luc), were co-introduced into protoplasts isolated from etiolated maize leaves. The standard deviation of GUS activities from six independent experiments was 0.22 relative to the mean value. The ratio of the GUS activity and luciferase activity in each experiment was then taken as a normalized GUS activity. The standard deviation of the normalized GUS activities was 0.04 relative to the mean value. Therefore, the normalized activities were reasonably invariable over experiments. In this study, the luciferase construct was used as the internal control for all assays of GUS constructs and vice versa. Furthermore, because variations in measurements with a single preparation of protoplasts are usually smaller, even after normalization, than those with more than one preparations (Leckie et al., 1994), one set of constructs, which consisted of several constructs with the same promoter and various combinations of introns, were assayed with a single preparation of protoplasts throughout this study.

Effect of introns in maize protoplasts

The effect of introns on reporter-gene expression in maize protoplasts was examined with the chimeric genes that contain one or two introns between a promoter and a reporter gene (Fig. 1A). Values relative to the GUS activity obtained with an intron-less construct (pBI221, pUQ or pDK) are shown in Table 1. A drastic increase in expression of the GUS gene was brought by the insertion of introns, and the highest activity was obtained from one of the two-intron constructs for each promoter in both yellow and greening protoplasts.

For the evaluation of the effects of the two introns inserted in tandem, a synergy index (SI), defined as [enhancement by two introns in tandem]/[enhancement by one intron]+[enhancement by the other intron] was calculated (Table 1). An SI of 1.00 indicates that the effect is additive, and an SI greater than 1.00 indicate that the effect is synergistic.

Combinations of PLD-int1 and Ubi-int1 (abbreviated as PLD-int1/Ubi-int1) and PLD-int1/Cat-int1 synergistically enhanced the expression of the 35S-GUS in both yellow and greening protoplasts (pBI[PLD/Ubi], pIG[PLD/Cat]). The effect of Ubi-int1/Cat-int1 (pBI[Ubi/Cat]) was not much different from the single insertion of Ubi-int1 (pBI[Ubi]) in yellow protoplasts and was merely additive in greening protoplasts. Combinations of PLD-int1/Cat-int1 and Ubi-int1/Cat-int1 synergistically enhanced the expression of the Ubi-GUS in both yellow and greening protoplasts (pUQ[PLD/Cat], pUQ[Ubi/Cat]). The effect of PLD-int1/Ubi-int1 was synergistic only in greening protoplasts (pUQ[PLD/Ubi]). Combination of PLD-int1/Cat-int1 and Ubi-int1/Cat-int1 synergistically enhanced the expression of the PPDK-GUS in both yellow and greening protoplasts (pDK[PLD/Cat], pDK[PLD/Ubi]). Enhancement of the expression of PPDK-GUS by the single insertion of Ubi-int1 was relatively great, and the addition of PLD-int1 to the construct appeared to suppress the expression (pDK[PLD/Ubi]).

Effect of introns in tobacco protoplasts

The effect of introns on reporter-gene expression in tobacco protoplasts is also shown in Table 1. In general, the effect of introns on expression of the GUS gene was much less than that in maize protoplasts. Still, the effect of two-intron insertions was greater than that of single-intron insertions in most cases. Synergistic enhancement was clear in the expression of 35-GUS with PLD-int1/Ubi-int1 in white and green protoplasts (pBI[PLD/Ubi]), and in the expression of PPDK-GUS with PLD-int1/Cat-int1 and PLD-int1/Ubi-int1 in white protoplasts (pDK[PLD/Cat], pDK[PLD/Ubi]).
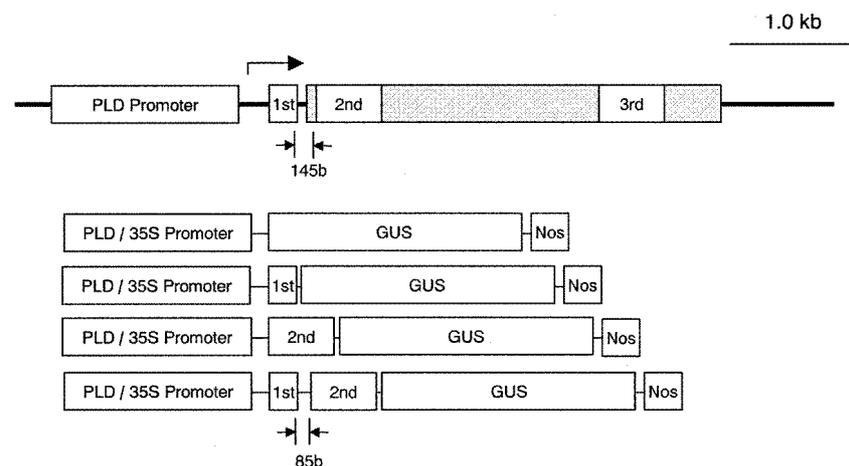
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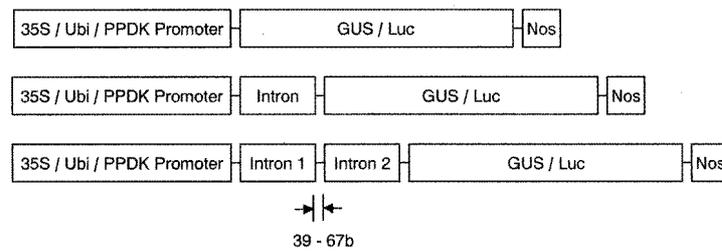
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Fig. 1 Diagrams of genes. (A) Structure of chimeric genes with intron insertions. Promoters of genes for cauliflower mosaic virus, maize ubiquitin or maize pyruvate, orthophosphate dikinase were used to drive reporter genes. GUS and Luc indicate the coding regions of genes for β - glucuronidase and luciferase, respectively. Nos indicates the terminator of a gene for nopaline synthase. Introns were inserted between the promoter core and the coding sequence of GUS or Luc except that the first intron of castor bean catalase gene (Cat - int1) resides within the amino - terminal region of the GUS - coding sequence. (B) Structure of a rice gene for phosphplipase D (*PLD1*) and chimeric genes with PLD introns. Open boxes represent introns and stippled boxes represent the coding sequence of PLD. An arrow denotes the transcription start.

Effect on another reporter gene in maize

Because the intron-mediated enhancement of transgene expression was reported to be a gene-dependent process (Rethmeier *et al.*, 1997), we tested another reporter gene, a gene for luciferase. As shown in **Table 2**, single insertions of PLD-int1, Cat-int1 and Ubi-int1 had enhancing effects, and the effect of Ubi-int1 was the biggest (p35Luc[Ubi]) of the three. SI values greater than 1.0 were observed in the combination of PLD-int1/Cat-int1 (p35Luc[PLD/cat]) in greening proto-plasts in maize.

Effect of PLD introns on the expression of GUS gene driven by PLD or CaMV 35S promoter

PLD1 is one of the PLD genes in rice, and it has three introns. The first (PLD-int1) and second (PLD-int2) are located very close to each other (**Fig. 1B**) (Morioka *et al.*, 1997) in the 5′ end of the transcribed region. It is possible that both of the introns are involved in regulation of the gene. A GUS gene construct, in which the structure of the 5′ end of *PLD1* gene was mimicked, was made. Either the native promoter of *PLD1* or the CaMV 35S promoter was connected to the gene. Under the control of the *PLD1* promoter, single insertions of

Table 1   Relative GUS activities compared to intron-less constructs in protoplasts and synergy. Plasmid pDO432 (35S-Luc) was co-transformed for the normalization of GUS activity. Yellow and greening refer to protoplasts isolated from maize etiolated leaves and greening leaves, respectively. White and green refer to protoplasts isolated from tobacco cultured cells and green leaves, respectively. A synergy index (SI), defined as [enhancement by two introns in tandem]/[enhancement by one intron]+[enhancement by the other intron], is given for each of chimeric genes that contained two introns.

Plasmid	Structure of plasmid					Relative activity							
	Promo-ter	Intron-1	Intron-2	Repor-ter	Termi-nator	Maize				Tobacco			
						Yellow (SI)		Greening (SI)		White (SI)		Green (SI)	
pBI221	35S			GUS	Nos	1.00		1.00		1.00		1.00	
pBI[PLD]	35S	PLD		GUS	Nos	7.36		4.73		3.61		2.85	
pIG221	35S	Cat		GUS	Nos	14.3		14.6		1.38		3.38	
pBI[Ubi]	35S	Ubi		GUS	Nos	47.6		51.7		1.39		1.40	
pIG[PLD/Cat]	35S	PLD	Cat	GUS	Nos	78.4	(3.62)	53.1	(2.75)	2.11	(0.42)	5.79	(0.93)
pBI[PLD/Ubi]	35S	PLD	Ubi	GUS	Nos	97.8	(1.78)	70.5	(1.25)	9.40	(1.88)	5.05	(1.19)
pIG[Ubi/Cat]	35S	Ubi	Cat	GUS	Nos	50.1	(0.81)	70.0	(1.05)	1.38	(0.50)	2.27	(0.47)
pUQ	Ubi			GUS	Nos	1.00		1.00		1.00		1.00	
pUQ[PLD]	Ubi	PLD		GUS	Nos	3.66		7.66		0.64		1.39	
pUQ[Cat]	Ubi	Cat		GUS	Nos	11.5		53.0		1.54		2.72	
pUQ[Ubi]	Ubi	Ubi		GUS	Nos	34.2		35.2		2.26		2.55	
pUQ[PLD/Cat]	Ubi	PLD	Cat	GUS	Nos	18.3	(1.20)	86.5	(1.42)	1.56	(0.71)	2.50	(0.61)
pUQ[PLD/Ubi]	Ubi	PLD	Ubi	GUS	Nos	30.8	(0.81)	100	(2.35)	2.54	(0.88)	2.49	(0.63)
pUQ[Ubi/Cat]	Ubi	Ubi	Cat	GUS	Nos	78.7	(1.72)	229	(2.60)	2.76	(0.73)	2.30	(0.44)
pDK	PPDK			GUS	Nos	1.00		1.00		1.00		1.00	
pDK[PLD]	PPDK	PLD		GUS	Nos	3.42		5.98		0.82		1.22	
pDK[Cat]	PPDK	Cat		GUS	Nos	2.52		8.65		0.93		2.57	
pDK[Ubi]	PPDK	Ubi		GUS	Nos	193		184		2.48		3.78	
pDK[PLD/Cat]	PPDK	PLD	Cat	GUS	Nos	7.29	(1.23)	18.3	(1.25)	3.96	(2.26)	2.43	(0.64)
pDK[PLD/Ubi]	PPDK	PLD	Ubi	GUS	Nos	75.9	(0.39)	124	(0.65)	4.14	(1.25)	3.59	(0.72)
pDK[Ubi/Cat]	PPDK	Ubi	Cat	GUS	Nos	277	(1.42)	223	(1.16)	2.34	(0.69)	4.81	(0.76)

Table 2   Relative Luc activities compared to the intron-less construct (p35Luc) in protoplasts and synergy index. Plasmid pBI221 (35S-GUS) was co-transformed for the normalization of Luc activity. Yellow and green refer to protoplasts isolated from young maize etiolated leaves and greening leaves, respectively. A synergy index (SI), defined in the legend of **Table 1**, was given for the constructs that contained two introns.

Plasmid	Structure of plasmid					Relative activity	
	Promoter	Intron1	Intron2	Reporter	Terminator	Yellow (SI)	Greening (SI)
p35Luc	35S			Luc	Nos	1.00	1.00
p35Luc[PLD]	35S	PLD		Luc	Nos	15.3	7.70
p35Luc[Cat]	35S	Cat		Luc	Nos	23.1	20.6
p35Luc[Ubi]	35S	Ubi		Luc	Nos	110	51.4
pIG[PLD/Cat]	35S	PLD	Cat	Luc	Nos	39.2 (1.02)	32.5 (1.15)
p35Luc[PLD/Ubi]	35S	PLD	Ubi	Luc	Nos	61.1 (0.48)	56.5 (0.96)
p35Luc[Ubi/Cat]	35S	Ubi	Cat	Luc	Nos	59.5 (0.44)	44.5 (0.62)

both of the introns had a small enhancing effect, and the combination of the two introns showed a very high SI value (3.22) (**Table 3**). Under the control of the CaMV 35S promoter, the enhancement by a single insertion of PLD-int2 was very large, and no more enhancement was observed by the addition of the first intron.

Discussion

Our previous study demonstrated that the two-intron insertions of certain combinations of introns synergistically enhanced the expression of the GUS gene driven by the CaMV 35S promoter in rice protoplasts (Ueki *et al.*, 1999). The introns used in the study were PLD-int1, Cat-int1, Ubi-int1 and the first intron (Skn-int1) of a maize *Shrunken-1* gene. The combinations of PLD-int1/Cat-int1, PLD-int1/Ubi-int1, PLD-int1/Skn-int1, Ubi-int1/Cat-int1 and Cat-int1/Skn-int1 had expression-enhancing effect in rice protoplasts isolated from cultured cells or greening leaves. Here, we described that the effects of two-intron insertions were evident also in maize and tobacco protoplasts. For each of the 35S promoter, Ubi promoter, and PPDK promoter, certain combinations of two introns had an enhancing effect on reporter-gene expression in maize protoplasts isolated from both etiolated and greening leaves. When the structural gene was replaced with the luciferase gene, an additive effect was observed with the combination of PLD-int1/Cat-int1 in maize cells. Furthermore, the enhancing effect of two-intron insertions was also observed in tobacco protoplasts, although the effect was much smaller than that in maize proto-

plasts. These results imply that expression enhancement by two introns may be observed in wide combinations of promoters, introns, and structural genes in plant cells.

Expression-enhancing effect of monocotyledonous introns in dicotyledonous protoplasts has not been reported. For example, the presence of Skn-int1 greatly reduced the reporter-gene expression in tobacco protoplasts (Maas *et al.*, 1991). In addition to the enhancement by the single-intron insertions, certain two-intron insertions showed a synergistic effect in protoplasts isolated from tobacco. The fact that the combination of the monocotyledonous introns (PLD-int1/Ubi-int1) showed a synergistic enhancing effect on the expression of the GUS gene driven by the monocotyledonous promoter (PPDK) in tobacco cells suggests that certain regulatory elements derived from monocotyledonons may function effectively in cells of dicotyledonous plants. Expression enhancement by two-intron insertions as well as by single-intron insertions was higher in maize cells than in tobacco cells. The enhancing effect is generally higher in monocotyledons than in dicotyledons, and the reasons for the difference remain to be elucidated. The machinery used for the processing of pre-mRNAs may be different in each plant species (Tanaka *et al.*, 1990), particularly between monocotyledons and dicotyledons.

Until recently, the main focus in the studies of regulatory functions of introns in native plant genes had been in the first introns of genes, because they are located relatively close to the main regulatory elements, such as promoters. Our studies have shown that all of the combinations of introns tested

Table 3 Effect of PLD intron insertions of on the GUS gene expression in maize protoplasts. Relative GUS activities compared to intron-less constructs (pLDpro or pBI221) are shown. Plasmid pDO432 (35S-Luc) was co-transformed for the normalization of GUS activity. Protoplasts isolated from maize etiolated leaves were used. A synergy index (SI), defined in the legend of **Table 1**, was given for the constructs that contained two introns.

Plasmid	Structure of plasmid					Relative activity
	Promoter	Intron1	Intron2	Reporter	Terminator	(SI)
pLDpro	PLD			GUS	Nos	1.00
pLDpro[1st]	PLD	PLD-int1		GUS	Nos	1.31
pLDpro[2nd]	PLD	PLD-int2		GUS	Nos	1.89
pLDpro[1st/2nd]	PLD	PLD-int1	PLD-int2	GUS	Nos	10.3 (3.22)
pBI221	35S			GUS	Nos	1.00
pBI[1st]	35S	PLD-int1		GUS	Nos	3.78
pBI[2nd]	35S	PLD-int2		GUS	Nos	110
pBI[1st/2nd]	35S	PLD-int1	PLD-int2	GUS	Nos	82.5 (0.72)

so far having short spacer sequences (31 – 85 bases) in–between were able to synergistically up–regulate gene expression in at least one combination of promoters, structural genes, and plant species. Therefore, it is possible that combinations of introns, especially introns separated by short sequences, play important regulatory roles in plants. The introduction of an intact genomic fragment that contained maize phosphoenolpyruvate carboxylase (PEPC) gene into rice resulted in a high expression level and that it was likely that the presence of introns contributed to the high expression level (Ku et al., 1999). The maize gene for PEPC contains 9 introns. Three of the exons are very short (around or less than a hundred bases), and the introns adjacent to those exons are closely located to each other. Some of these combinations might contributed to the high–level expression. Many plant genes have introns, which are separated only by short exons like the ones in the PEPC gene. For example, 5 out of 8 exons of a maize gene for alcohol dehydrogenase fall within a size of 47–94 bases (Osterman and Dennis, 1989). In a maize gene for triosephosphate isomerase, 5 out of 7 exons fall within a size of 76–95 bases (Marchionni and Gilbert, 1986). Studies of these introns will certainly provide further insights into regulatory roles of combinations of introns. Our results may suggest that the possible cooperation of introns to achieve higher and/or complicated expression that cannot be conducted with a single intron is present and that the cooperation plays a role in the regulation of gene expression in plant cells.

Several lines of evidence indicate that introns act post–transcriptionally (Callis et al., 1987; Huang and Gorman, 1990; Mascarenhas et al., 1990; Clancy et al., 1994; Donath et al., 1995; Rose and Beliakoff, 2000), however, molecular mechanisms of gene–expression enhancement is not well understood. Since various kinds of factors including unidentified ones appear to be involved in the post transcriptional processes such as capping, transcript elongation, polyadenylation and export of mRNA coupled with pre–mRNA splicing (Simpson and Filipowicz, 1996; Reed and Hurt, 2002; Brown et al., 2003), the difference in enhancement might be the reflection of the difference in type and/or amount of those factors that bind to an intron and adjacent exon sequences. If the transcript including two introns could recruit more factors in type and/or amount than that including a single intron, a synergistic or suppressive effect might occur depending on those factors, possibly through the interaction between those factors. In our study, the same constructs with two introns had different effects de-

pending on the cell state. The enhancement observed with pUQ[PLD/Ubi] was less than that with pUQ[Ubi] in maize yellow cells, but was synergistic in greening cells (**Table 1**). Similar cases were also observed with pDK[PLD/Cat] between white and green cells of tobacco and with pIG[Ubi/Cat] between white and greening cells of rice (Ueki et al., 1999). These results may indicate the presence of factors that reflect cell state and affect the enhancement by two introns.

The present results indicate that insertion of two introns between a promoter and a transgene is a useful tool for obtaining high expression of the transgene in genetic engineering in higher plants, especially in monocotyledonous plants including rice and maize. The production and analysis of stably–transformed plants are underway.

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