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Note

Differences in intron-mediated enhancement of gene expression by the first intron of cytosolic superoxide dismutase gene from rice in monocot and dicot plants

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Abstract Introns have an enhancing effect on gene expression known as intron-mediated enhancement in various organisms, including plants. Although the mechanism of the enhancement is largely unknown, most enhancing introns are first introns. In this study, we examined whether the first intron of rice superoxide dismutase *sodCc2* gene has an enhancing effect in rice and other plant species. A transient expression assay revealed that the *sodCc2* intron elevated reporter gene expression in rice, wheat, and maize, but not in *Arabidopsis* and tobacco, indicating that the *sodCc2* intron has an enhancing effect in monocot but not in dicot plants. To find the putative signal sequences responsible for the enhancement, we carried out an *in silico* search and found two motifs conserved among the *sodCc2* intron and 11 enhancing introns previously known in rice. The motifs contain a consensus sequence, GATCTG, which also exists in the conserved motif found in *Arabidopsis* enhancing introns. Our results suggest that common motifs are conserved between rice and *Arabidopsis* enhancing introns.

Key words: Intron, intron-mediated enhancement, motif inference, rice (*Oryza sativa* L.), superoxide dismutase.

Eukaryotic genes consist of several elements in addition to the protein-coding sequence. These elements include promoter, 5′- and 3′-untranslated regions (UTRs) and introns, which are involved in the regulation of gene expression. Like the promoter and UTRs, introns also play an important role in gene regulation by exerting an enhancing effect on gene expression known as intron-mediated enhancement (IME; Mascarenhas et al. 1990). IME has been observed in a wide range of organisms including mammals, invertebrates and plants (Le Hir et al. 2003). In plants, the elevation of the expression level by introns has been documented in a number of genes in various species including *Arabidopsis*, rice, maize, petunia, etc. (Morello and Breviario 2008). Although the mechanism of IME is largely unknown, it is recognized that the enhancing effect of introns is correlated to the intron position. Most introns with an enhancing effect (enhancing introns) are first introns (Rose et al. 2008), and some of them are even located in the 5′-UTR. In addition, the enhancing effect decreases as the distance of the intron from the promoter increases (Rose 2004). Thus, enhancing introns tend to be located proximal to promoters. It is shown that introns also participate in the control of tissue specificity (Fu et al. 1995; Gianì et al. 2009; Jeon et al. 2001; Jeong et al. 2006) as well as the control of the expression level.

Introns are also important in terms of biotechnology, since enhancing introns are utilized to elevate the expression of foreign genes in transgenic plants. Also, the effect of IME is more profound in monocot plants, in which Cauliflower mosaic virus (CaMV) 35S promoter is less effective, compared with dicot plants (Mitsuhara et al. 1996). To date, a maize ubiquitin promoter including the 5′-UTR intron has been mainly used for the efficient overexpression of transgene in rice instead of the CaMV 35S promoter (Christensen and Quail 1996).

We have been studying the gene expression of superoxide dismutase (SOD) in rice, a major enzyme involved in reactive oxygen scavenging (Bowler et al. 1994). The rice genome contains 7 SOD genes, of which

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2 cytosolic SOD genes (*sodCc1*; RAP-DB gene ID: Os03g0351500, and *sodCc2*; Os07g0665200) have 5′-UTR introns. We previously demonstrated that *sodCc2* is induced in response to oxidative stress and abiotic stresses (drought and salinity) more prominently than *sodCc1*, suggesting that *sodCc2* plays a more important role in stress defense than *sodCc1* (Kaminaka et al. 1999; Morita et al. 2011). In this study, we examined whether the 5′-UTR intron of *sodCc2* (SOD intron) has an IME effect by means of a transient expression assay in rice seedlings and other plant species.

We constructed reporter plasmids that contained the SOD intron, which was placed between CaMV 35S promoter and reporter genes. The 5′-UTR fragment that includes the first exon, the first intron, and a part of the second exon of the *sodCc2* gene (base Nos. 1356 to 2138, accession No. L19434) was inserted into *Xba*I and *Bam*HI sites of pBI221 between the CaMV35S promoter and *β*-glucuronidase (GUS) coding sequence (Figure 1A), and the resulting plasmid was named p35SINTGUS. Firefly and *Renila* luciferase (LUC and RLUC) reporter plasmids, p35SINTLUC+ and p35SINTRluc, were also constructed by replacing the GUS coding sequence of p35SINTGUS with the LUC or RLUC coding sequence (originating from pSP-luc+NF and pRluc-null, respectively, Promega, Madison, WI, USA). A control LUC reporter plasmid without the SOD intron, p35SLUC+, was constructed from pBI221 by a similar procedure. Since the inserted fragment of the SOD gene contains only a non-coding sequence, GUS, LUC and RLUC proteins are expressed from their own initiation codons in these plasmids. The structures of the reporter plasmids are summarized in Figure 1A.

We then performed a transient expression assay with these plasmids as described previously (Tsulamoto et al. 2005) with modifications. Rice (*Oryza sativa* L. cv Nipponbare) green and etiolated seedlings were grown at 150 μmol photons $m^{-2} s^{-1}$ (16 h light) or in the dark at 28°C for 14 days. The reporter plasmids were introduced into the seedlings by particle bombardment using the PDS-1000/He particle delivery system (Bio-Rad, Hercules, CA, USA). Seedlings were incubated for 18–24 h after the bombardment and subjected to reporter assay. As shown in Figure 1B, the GUS plasmid with the SOD intron (p35SINTGUS) yielded 16.9- to 19.5-fold higher GUS activity in green and etiolated leaves compared with the control plasmid without the intron (pBI221). A similar increase in LUC activity of 4.9- to 27.7-fold was observed in the case of the LUC reporter plasmids (p35SINTLUC+ and p35SLUC+; Figure 1C). These results indicate that the SOD intron has an enhancing effect on the expression of downstream reporter genes.

We examined whether the enhancing effect of the SOD intron is observed in other plant species including wheat (*Triticum aestivum* L. cv Chinese Spring), maize (*Zea mays* L. cv Kingstar), tobacco (*Nicotiana tobacum* L. cv Petit Havana SR1), and *Arabidopsis* (*A. thaliana*, ecotype Wassilewskija [Ws]). Seedlings were grown at 15 μmol photons $m^{-2} s^{-1}$ (16 h light) at 28°C for maize, 25°C for wheat and tobacco, and 23°C for *Arabidopsis*. The LUC reporter plasmids with or without the SOD intron were introduced into green leaves of wheat (14-day-old seedlings), maize (13-day-old), *Arabidopsis* (4-week-old) and tobacco (2.5-month-old), and the transient expression of LUC was monitored after 18–24 h incubation. As shown in Figure 2, 7.6- and 6.5-fold increases in LUC activity due to the SOD intron were observed in wheat and maize seedlings, respectively. However, LUC activity was drastically decreased by 95–88% by the SOD intron in *Arabidopsis* and tobacco. These results indicate that the first intron of the rice *sodCc2* gene has an enhancing effect in monocot but not in dicot species. Similar results have been observed for the castor bean catalase *Cat1* intron and the rice *β*-tublin *OsTub6* intron, which have enhancing effects in rice but not in *Arabidopsis* or tobacco (Morello et al. 2011; Tanaka et al. 1990). The absence of enhancement by these introns is associated with inefficient splicing of the introns in dicot species, suggesting that efficient splicing is required for IME (Morello et al. 2011; Tanaka et al. 1990). It is probable that the rice *sodCc2* intron is not properly spliced in *Arabidopsis* or tobacco, causing the impairment of reporter gene expression.

Although the enhancing mechanism of the SOD intron is unclear, our present results and those of a previous study (Xu et al. 1994) suggest that the signal sequences responsible for IME (enhancing signals) that reside in the enhancing introns are conserved in monocot plants. In order to find putative enhancing signals, we carried out an *in silico* search for conserved motifs in known enhancing introns in rice (Rose et al. 2008) and in the rice SOD intron. We found two conserved motifs, [TC][AG]GATCTG[TC][GT] and GATCTGG (Figure 3A), in 12 enhancing introns using the motif inference software MEME (Bailey et al. 2006). The positions of these motifs varied in each intron (Figure 3B), a finding that supports the notion that the enhancing signals are dispersed throughout the enhancing introns (Rose et al. 2008). The two motifs contain a consensus sequence, GATCTG, which has also been observed in the conserved motif found in *Arabidopsis* enhancing introns (Rose et al. 2008). This result suggests that common motifs are conserved between rice and *Arabidopsis* introns. However, our results and those of previous studies (Morello et al. 2011; Tanaka et al. 1990) indicate that monocot enhancing introns cannot enhance gene expression in dicot species. It is possible that inefficient splicing of monocot introns might impede the enhancing effects in dicot species as

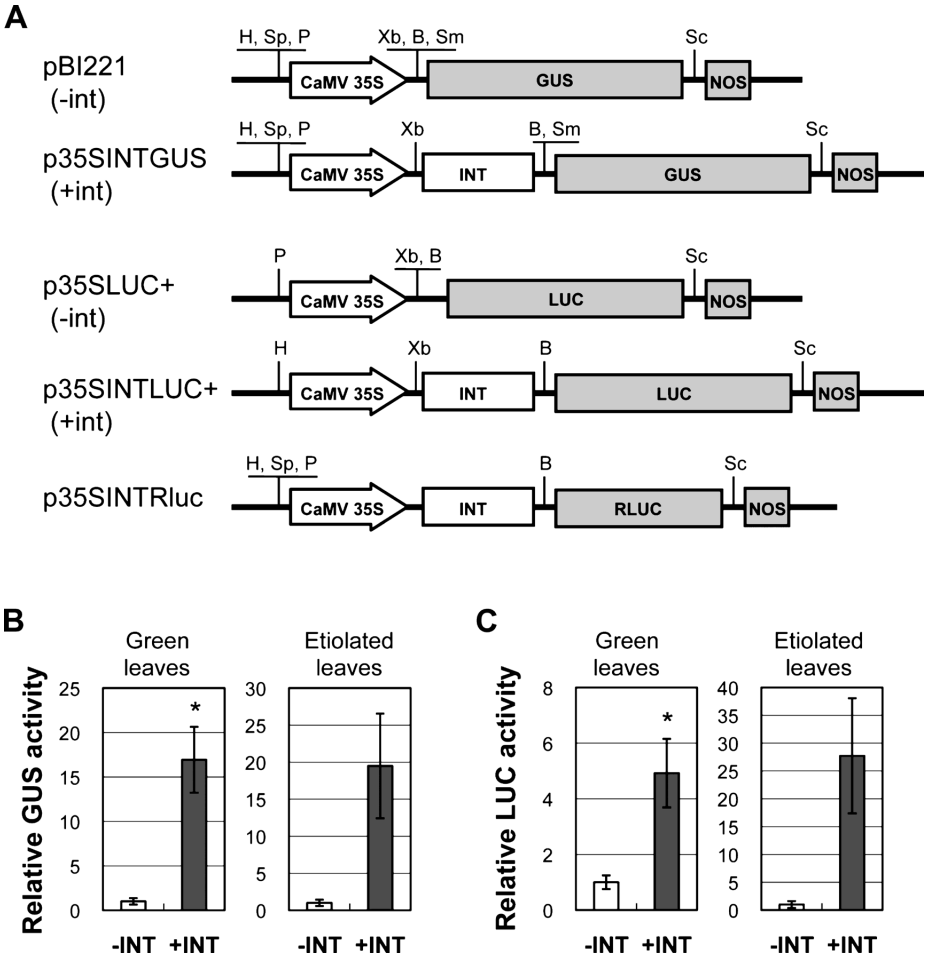

Figure 1. The enhancement of reporter gene expression by the SOD intron in rice. (A) Structure of reporter constructs used in this study. The restriction sites unique in the plasmids are shown. CaMV 35S, CaMV 35S promoter; INT, the first intron of *sodCc2*; NOS, terminator of nopalin synthase gene. H, *Hin*dIII; Sp, *Sph*I; P, *Pst*I; Xb, *Xba*I; B, *Bam*HI; Sm, *Sma*I; Sc, *Sac*I. (B) The effect of the SOD intron on transient GUS expression. The GUS reporter plasmids, pBI221 (−int) and p35SINTGUS (+int), were introduced into green and etiolated leaves of rice seedlings together with p35SINTLUC+ as an internal control. Data are presented as relative GUS activity normalized with LUC activity. (C) The effect of the SOD intron on transient LUC expression. Transient expression assay was performed as described above using the LUC reporter plasmid, p35SLUC+ (−int) and p35SINTLUC+ (+int), and internal control plasmid p35SINTRluc. Data are presented as relative LUC activity normalized with RLUC activity. Error bars indicate SE (n=3–5), and the asterisks indicate statistical significance (*t*-test: $p<0.05$) in (B) and (C).

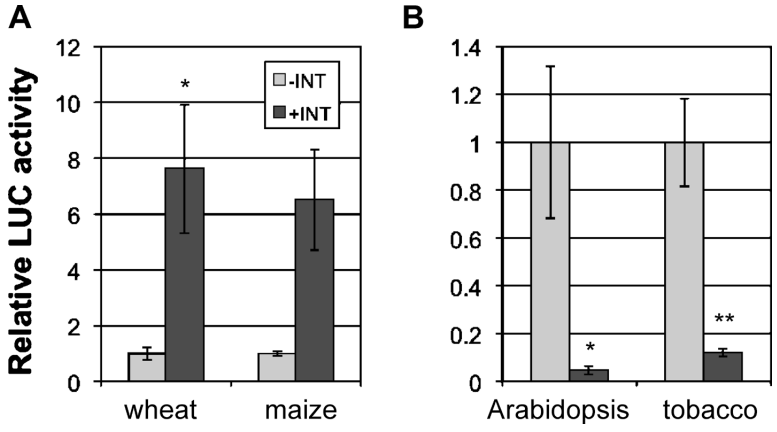

Figure 2. Effect of the SOD intron on LUC reporter expression in monocot and dicot plants. Transient expression assay was performed in monocot (A) and dicot species (B) as described in Figure 1 using p35SLUC+ (−int) and p35SINTLUC+ (+int) and internal control plasmid p35SINTRluc. Data are presented as relative LUC activity normalized with RLUC activity. Error bars indicate SE (n=4–6), and the asterisks indicate statistical significance (*t*-test: $*p<0.05$, $**p<0.01$).

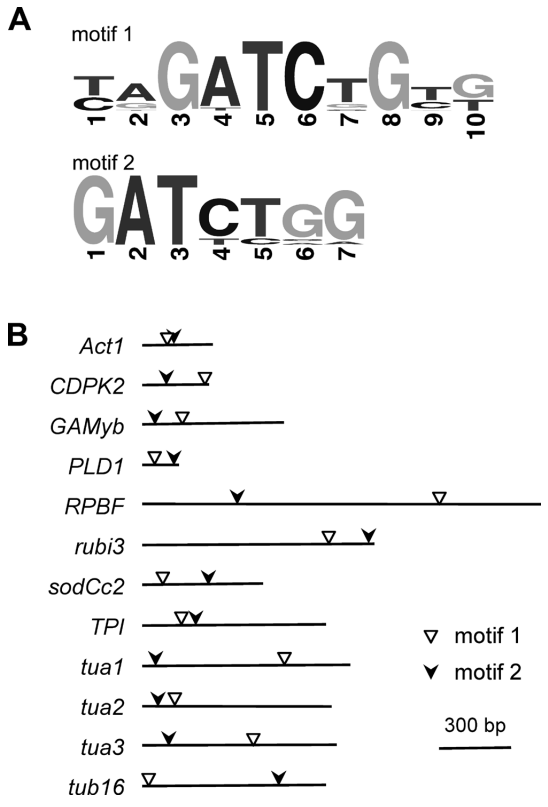

Figure 3. Motifs in rice enhancing introns. (A) Sequence logos of 2 motifs found by MEME in 11 rice enhancing introns listed in Rose et al. 2008 (except for two short introns which are less than 150 bp in length) and the *sodCc2* first intron. The height of the each letter represents the probability of the letter at that position. The total height of the stack represents the information content of that position. (B) The locations of the two motifs in each enhancing intron. The 11 enhancing introns used in this search are as follows: *ACT1* first intron (accession no. S44221), *CDPK2* first intron (Os07g0515100), *GAMyb* first intron (Os01g0812000), *PLD1* first intron (Os01g0172400), *RPBF* first intron (Os02g0252400), *rubi3* first intron (Os04g0628100), *TPI* first intron (Os01g0147900), *tua1* first intron (Os03g0726100), *tua2* first intron (Os11g0247300), *tua3* first intron (Os07g0574800), and *tub16* first intron (Os01g0805900).

observed for the castor bean *Cat1* intron and the rice *OsTub6* intron (Morello et al. 2011; Tanaka et al. 1990).

Our current results indicate that the SOD intron is useful for enhancing transgene expression. We already used the SOD intron in producing stable transgenic rice, resulting in efficient transgene expressions in leaves, roots, and mature seeds (Saito et al. 2009; Sakamoto et al. 1998). The utility of the SOD intron for enhancing transgene expression in other cereal species should be tested as well.

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