Construction of expression vectors based on the rice actin 1 (Act1) 5' region for use in monocot transformation

David McElroy¹, Alan D. Blowers ², Barnabás Jenes ³, and Ray Wu ⁴

¹ Field of Botany, Cornell University, Ithaca, NY 14853, USA
² Plant Science Center, Biotechnology Building, Cornell University, Ithaca, NY 14853, USA
³ Institute for Plant Sciences, Agricultural Biotechnology Center, Gödöllő, 2101 Szent-Györgyi Albert út 4, P.O. Box 170, Hungary
⁴ Section of Biochemistry, Molecular and Cell Biology, Biotechnology Building, Cornell University, Ithaca, NY 14853, USA

Received March 1, 1991

Summary. It has been previously reported that the 5' region of the rice actin 1 gene (Act1) promoted high-level expression of a β-glucuronidase reporter gene (Gus) in transformed rice cells. In this paper we describe the construction of Act1-based expression vectors for use in monocot transformation. As part of the development of these vectors, we have evaluated the influence of the Act1 first intron, the Act1-Gus junction-encoded N-terminal amino acids, and the sequence context surrounding the Act1 and Gus translation initiation site on Act1-Gus gene expression in rice and maize cells. We have found that addition of Act1 intron 1 to the transcription unit of a Gus reporter gene under control of the cauliflower mosaic virus (CaMV) 35S promoter stimulated Gus activity more than 10-fold in transformed rice cells. Optimization of the sequence context around the Gus translation initiation site resulted in a 4-fold stimulation of Gus expression in transformed rice cells. By utilizing both the Act1 intron 1 and optimized Gus translation initiation site, a 40-fold stimulation in Gus expression from the CaMV 35S promoter has been achieved in transformed rice cells; very similar results were obtained in transformed maize cells. Taken together these results suggest that the Act1-based expression vectors described here should promote the expression of foreign genes in most, if not all, transformed monocot cells to levels that have not previously been attainable with alternative expression vectors.

Key words: Actin gene – Monocot transformation – Expression vectors – Intron – Translation initiation

Introduction

One of the major limitations in the application of gene transfer technology to cereals has been the lack of efficient promoters for the expression of foreign genes in transgenic monocots. Although promoters from CaMV 35S RNA (Guilley et al. 1982) and the maize alcohol dehydrogenase 1 gene (Adh1) (Dennis et al. 1984) have been widely used in monocot transformation (Fromm et al. 1990; Gordon-Kamm et al. 1990; Kyozuka et al. 1990; Spencer et al. 1990; Tereda and Shimamoto 1990), both have been reported to show relatively low activity in transformed rice cells (Peterhans et al. 1990; McElroy et al. 1990c). Furthermore, in transgenic rice plants the CaMV 35S promoter is not active in all cell types (Tereda and Shimamoto 1990) and the maize Adh1 promoter is more highly active in root tissue than in leaf tissue (Zhang and Wu 1988; Kyozuka et al. 1990, 1991). With a view towards overcoming the limitations of the CaMV 35S and maize Adh1 promoters in monocot transformation, we have previously reported that the 5' region of the rice Act1 gene directed high levels of Gus reporter gene expression in transformed rice protoplasts (McElroy et al. 1990c). (Although Escherichia coli β-glucuronidase is encoded by the uidA gene, we have denoted this gene as Gus for the purposes of this publication.) In rice plants, the endogenous Act1 transcript is abundant in all tissues and development stages of the plant examined (McElroy et al. 1990b). Likewise, relatively high levels of GUS activity were observed in virtually all tissues of transgenic rice plants expressing an Act1-Gus chimeric gene (Zhang et al. 1991). We have now developed a set of Act1-based expression vectors for foreign gene expression in transformed monocot cells.

Previously, the Act1 5' region was described as a promoter (McElroy et al. 1990c). However, this region contains a number of Act1 transcribed elements, including the first exon (non-coding), an intron contained within the mRNA 5' untranslated leader (intron 1) and a portion of the second, ATG-containing exon of the Act1 gene (McElroy et al. 1990a). From previous studies, the contribution that each of these elements may have made in promoting high levels of Gus expression in transformed rice was not evaluated. As part of the development of the Act1-based expression vectors described in this report, we set out to investigate the influence on
Gus expression of (i) the ActI first intron; (ii) the sequence context surrounding the ActI and Gus translation initiation sites; and (iii) the ActI-Gus junction-encoded N-terminal amino acids. Earlier studies demonstrated that deletion of the ActI first intron from an ActI-Gus chimeric gene resulted in nearly complete loss of Gus activity in transformed rice cells (McElroy et al. 1990c). This suggested that insertion of ActI intron 1 into a transcription unit originating from a heterologous plant promoter might stimulate gene expression in transformed monocot cells, as has been observed for other monocot introns (Callis et al. 1987; Oard et al. 1989; Vasil et al. 1989; Kyozuka et al. 1990; Mascalinas et al. 1990; Tanaka et al. 1990; Luehrsen and Walbot 1991; Maas et al. 1991). Finally, it is well-established that the sequence context surrounding the initiation codon in eukaryotes (Kozak 1984, 1986, 1987) and the identity of the N-terminal amino acids of a protein (Bachmair et al. 1986; Gonda et al. 1989) can have dramatic effects on transcript translatability and protein stability, respectively.

In this report we show that (1) addition of rice ActI intron 1 to the transcriptional unit of a 35S-Gus chimeric gene can stimulate Gus expression more than 10- and 7-fold in transformed rice and maize cells, respectively; (2) optimization of the sequence context around the Gus initiation codon leads to a 4- and 10-fold enhancement in Gus activity in transformed rice and maize cells, respectively; (3) a chimeric 35S-ActI intron 1-Gus gene (with an optimized translation initiation region) produced 40- and 65-fold more Gus activity in transformed rice and maize cells, respectively, than a 35S-Gus chimeric gene; and (4) the intron-mediated stimulation of Gus expression was limited to rice and maize cells, as tobacco cells showed reduced Gus activity after transformation with intron 1-containing test constructs (compared to intronless Gus genes). Based on these results we have constructed a set of ActI 5'-based expression vectors for use in monocot transformation. Both translational, containing either the efficient ActI translation initiation codon region or a modified initiation codon region, and transcriptional expression vectors are described. The vectors contain a wide range of poly linker sites to simplify cloning and use of these vectors results in (relatively) high levels of foreign gene expression in transformed monocots.

Materials and methods

General methods. All enzymatic DNA manipulations, including subcloning strategies, exonuclease III and mung bean nuclease digestions, Klenow DNA polymerase fill-in reactions, and double-stranded sequencing of construct junction regions were performed following standard protocols (Maniatis et al. 1982) using modifications recommended by the enzyme manufacturers.

Construction of ActI-Gus fusion constructs. Construction of the ActI-Gus translational fusion plasmid pActI-F has been described previously (McElroy et al. 1990c). A 317 bp BamHI restriction fragment from pCOR103, containing the modified ActI translation initiation codon region (ACCATGG) replaced the corresponding ActI region of pActI-F, thereby creating the translational fusion plasmid pActI-F2.

Plasmid pActI-F2 was linearized by digestion with NcoI at the modified ActI translation initiation codon (C/CATGG), the ATG codon removed by digestion with exonuclease III, the ends blunted with mung bean nuclease, and ligated to produce plasmid pActI-F3. Plasmid pActI-F3 now contains an out-of-frame translation initiation codon (relative to the Gus ATG) located 29 bp upstream of the Gus coding region, and could potentially encode a polypeptide of 14 amino acids. Plasmid pActI-F2 was digested with NcoI and SmaI to remove the ActI-Gus junction region and associated Gus coding sequence and replaced with an NcoI-SmaI restriction fragment from pRAJ275 (Sleat et al. 1987). This new plasmid, pActI-F4, now contains an ActI-Gus gene that possesses the eukaryotic consensus translation initiation sequence (ACCATGG), rather than the bacterial sequence (CTTATGG), around the Gus initiation ATG codon. Plasmid pActI-F2 was digested with AccI and SmaI, which removes a 32 bp fragment containing the modified ActI translation initiation codon region, five adjacent codons, and a part of the poly linker sequence. The plasmid was ligated after repair synthesis of the AccI site with Klenow enzyme to produce the ActI-Gus translational fusion plasmid pActI-F5.

The ActI-Gus sequence from plasmid pActI-F was excised as an EcoRI-XbaI restriction fragment and sub cloned into the vector pSP72 (Promega) to produce plasmid pActI-G72. Plasmid pActI-G72 was then digested with ClaI and BstEII to remove the ActI 5'-flanking region and first noncoding exon. The remaining plasmid was treated with Klenow enzyme prior to ligation to create the promoterless translational fusion constructs pActI-F6, which contains the ActI 5' intron (and associated splice site junction sequences) fused to the Gus-nos reporter gene.

ActI intron 1 was isolated from plasmid pActI-F2 as a 449 bp BstEII-ActI restriction fragment, treated with Klenow enzyme, and cloned into ClaI- and Kpn I-digested and Klenow enzyme-treated pSP72 vector DNA to reconstitute the pActI exon 1-intron 1-exon 2 junction region, thus creating plasmid ActI-I72. The ActI intron was reisolated from pActI-I72 as a 459 bp EcoRV-SmaI restriction fragment and then inserted in the reverse 3' to 5' orientation into BstEII- and Accl-digested and mung bean nuclease-treated pActI-F2, to produce plasmid pActI-F7. The same operation was carried out to insert the intron in the correct 5' to 3' orientation into the SstI site (Klenow enzyme-treated) of ActI exon 1 in plasmid pActI-R (McElroy et al. 1990c), creating the ActI-Gus translational fusion plasmid pActI-R.

Construction of CaMV35S-Gus fusion constructs. The 3.0 kb HindIII-CoeRI restriction fragment, which contains the CaMV 35S promoter and a portion of the 5' transcribed leader sequence fused to the Gus coding region and the nos polyadenylation sequence, was removed