ISOLATION AND EVALUATION OF A DEVELOPMENTALLY REGULATED SUGARCANE PROMOTER

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Abstract

Young internodal tissue of sugarcane is an ideal target for genetic manipulation, to potentially alter metabolism and control diseases and pests. As no regulatory sequences that specifically drive transgene expression in these young internodes are available, the objective of this study was to isolate and evaluate such potential sequences. The approach followed was to identify an endogenous gene expressed in the desired pattern, and isolate the corresponding promoter. The promoter of a gene encoding UDP-glucose dehydrogenase (UGDH) was selected for isolation. Expression analysis of sugarcane UGDH confirmed that gene expression was correlated with growing and expanding tissues. The promoter region was isolated and subsequently fused to the GUS reporter gene for plant transformation. Preliminary analysis of transgenic sugarcane plants indicates that the promoter is able to drive GUS expression in a tissue-specific manner. This promoter will be a valuable tool for any genetic manipulation of sugarcane.

Keywords: sugarcane, gene promoter, UDP-glucose dehydrogenase, GUS, transgenic plants

Introduction

During harvesting, the top internodes are discarded due to low sucrose content and juice purity. The top of the cane is often also the site of infection for diseases and pests. Although genetic manipulation has the potential to alter metabolism in the tops, a major obstacle limiting progress in this area is the availability of promoters.

The shortage of such regulatory genetic elements has made it necessary to isolate specific promoters from sugarcane. At present, there are no regulatory sequences available that specifically drive transgene expression in developing sugarcane tissues. The aim of this study was to isolate and evaluate a developmentally regulated sugarcane promoter.

The promoter of a gene encoding UDP-glucose dehydrogenase (UGDH) was selected as a potential target for promoter isolation, based on what is known about the function of the enzyme that it encodes.

As UGDH is required for growth and development (Reiter and Vanzin, 2001), the promoter of this gene could possibly be used to drive transgene expression in young developing tissues.

Materials and Methods

Isolation of sugarcane UGDH promoter
A sugarcane UGDH EST that contained the 3' end of the gene was used to screen a sugarcane genomic library constructed from sugarcane cultivar N19. Prehybridization and hybridization were performed in ULTRAhyb^{TM} buffer (Ambion) according to the manufacturer's instructions. A PCR amplified probe was labeled using the Prime-It II random primer labeling kit (Stratagene) and [α-^{32}P] dCTP (Amersham). The membranes were exposed to a Multi Purpose Phosphor Screen (Packard) for 16 hours and visualised using a phosphor imager and analysis system.

Four positive genomic clones were isolated. Lambda DNA of these clones was isolated using the QAIGEN Lambda mini kit. The Deletion Factory™ system Version 2.0 (GibcoBRL) was used, according to the manufacturer's instructions, to generate nested deletions across the isolated genomic DNA inserts. Sequence determination using vector-specific primers was done using the Dye Terminator Cycle Sequencing Ready Reaction with Ampli*Taq* DNA Polymerase FS (Perkin Elmer Applied Biosystems). DNASIS (version 2.1) was used to identify overlaps in the sequences. These sequences were then compared with sequences in international databases using the BLAST program at the National Center for Biological Information (NCBI) to identify the coding region, 5' and 3' ends of the gene.

A larger fragment of the upstream region of sugarcane UGDH was obtained using a gene-specific reverse primer and a forward primer based on a conserved sequence in the 5'untranslated region (UTR) of closely related species (sequences obtained from international databases). This fragment of 1100 bp was sequenced and then used to screen the sugarcane genomic library, as described above. Two further genomic clones were obtained. Restriction enzyme digests of these clones, using known restriction sites close to the 5' end of the gene, followed by blotting and probing with the previously isolated 5' end of the gene, were used to locate the fragment(s) containing the 5' UTR and upstream sequences. A fragment of 3000 bp, located above the translation start site, was cloned.

A combination of gene-specific primers and internal restriction sites were used to amplify and clone the promoter region with and without the intron. These regions were fused to the GUS-reporter gene for further analysis.

Transient expression analysis
Transient assays were performed using maize seedlings that were germinated in the dark at room temperature. The constructs were delivered to five-day old maize seedlings by microprojectile bombardment. Bombarded tissues were kept in the dark on wet filter paper and assayed for GUS activity after 24 hours.

Sugarcane transformation
Standard tissue culture, transformation, and hardening-off protocols were used (Snyman *et al.,* 1996).

Histochemical GUS assays
Bombarded tissue or whole transgenic plants were vacuum infiltrated and subsequently incubated in substrate-containing GUS assay solution (100 mM sodium phosphate buffer (pH 7.0); 5 mM potassium ferrocyanide; 5 mM potassium ferricyanide; 2 mM X-Gluc (5-bromo-4-chloro-3-indolyl-β-glucuronic acid); 0.3 % (v/v) Triton X-100) for 24 hours, followed by dehydration in an ethanol series.

Results

Isolation of the sugarcane UGDH promoter

Four positive genomic clones were isolated, and their sequences revealed that three of these clones contained only a part of the coding region of the gene. One of the clones contained the translation start site of the gene and an additional 720 bp of upstream sequence. The right border of an intron was found 24 bp upstream of the translation start site. This indicated that the genomic clone did not contain the promoter.

A gene-specific primer, used in conjunction with a primer based on a conserved sequence in the 5'UTR region of closely related species, amplified a fragment of approximately 1000 bp from sugarcane genomic DNA. After sequence analysis confirmed that the translation start site and the intron were indeed amplified, this fragment was used to screen the sugarcane genomic library, as described above. Two further genomic clones were obtained. From one of these, a fragment of 3000 bp was cloned, and sequence analysis confirmed that it contained the entire 5'UTR, including an intron of 980 bp and 1500 bp of the promoter region. Based on comparison with known sequences, the transcription initiation site is located approximately 95 bp upstream of the intron.

The 1500 bp promoter region, with (pUGDip) and without (pUGDp) the intron, was successfully fused to the GUS-reporter gene for further analysis.

Transient expression analysis

Very strong transient GUS expression was observed after the germinating maize seeds were bombarded with the intron-containing construct, pUGDip, whereas bombardment with the intronless construct, pUGDp, resulted in very weak transient expression.

Sugarcane transformation

Transformation of embryogenic sugarcane calli with pUGDip and pUGDp resulted in five and nine transgenic lines, respectively. Histochemical GUS assays of whole plants, grown in tissue culture and in the glasshouse, revealed high levels of tissue-specific GUS expression in lines transformed with pUGDip. No GUS activity was detected in lines transformed with pUGDp.

Discussion

Isolation and evaluation of the sugarcane UGDH promoter

The promoter region of UGDH was successfully isolated through screening a genomic library. Although no introns were found in the coding region of the gene, a large intron (980 bp) was located in the 5' untranslated region (UTR), 24 bp above the translation start site. As introns located within the 5'UTR have been reported in many cases to have a large positive effect on gene expression, especially in monocots (Christensen *et al.,* 1992; Maas *et al.,* 1991; McElroy *et al.,* 1991; Rethmeier *et al.,* 1997), two reporter gene constructs were prepared, one including the intron and the 5' UTR, pUGDp, and one excluding the intron, but maintaining the 5' UTR, pUGDip.

The transient expression analysis, after microprojectile bombardment of maize seedlings five days after germination, showed that both constructs were able to drive reporter gene expression. pUGDip showed very strong transient expression, while the intronless promoter construct, pUGDp, showed very weak, but detectable, GUS expression. This shows that the first intron of the sugarcane UGDH gene is able to enhance gene expression in the context of its own promoter, an effect termed intron-mediated enhancement (IME).

Although not investigated in this study, the fist intron of UGDH could possibly be used in conjunction with a promoter that is known to be active in transgenic sugarcane to enhance expression of a transgene, and possibly to selectively enhance expression in young developing tissues.

pUGDp and pUGDip were used also to transform sugarcane. Transgenic plant lines obtained were successfully hardened-off and moved into the glasshouse. Preliminary analysis of transgenic plants, through histochemical GUS assays, showed strong, tissue-specific expression in transgenic lines transformed with pUGDip. In the leafroll, the highest level of GUS expression was visible in the guard cells of the developing stomata. This is to be expected, as thick cell walls are actively being synthesised in these cells. A clear ring of GUS expression was also visible at the bottom of the developing internodes, where the growth ring is located. In leaves, strong GUS expression was observed at the sheath base, where the leaf is attached to the stem, and at the blade joint.

Although the results are preliminary, this is the first demonstrated isolation of a functional, developmentally regulated promoter from sugarcane, and retention of promoter activity and specificity driving a foreign gene when reintroduced into sugarcane. Given that many promoters are silenced when introduced into sugarcane, this is a very encouraging result.

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