SUGAR BEET (*Beta vulgaris* L.) PROMOTERS FOR DIRECTED TISSUE-SPECIFIC ROOT TRANSCRIPTION

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Abstract

Several sugar beet (*Beta vulgaris* L.) insect pests and fungal pathogens specifically target the tissues of the taproot. The use of root tissue-specific promoters to confer expression of pest and disease resistance genes in a targeted manner has the potential for genetic improvement of commercial sugar beet varieties. Using suppressive subtractive hybridization (SSH), our laboratory has identified several sugar beet root genes responding to infestations by the sugar beet root maggot (*Tetanops myopaeformis*). This sugar beet EST library has been subjected to macroarray analyses using RNA isolated from various sugar beet tissues. Several genes were identified that exhibit high levels of expression in the root peel, crown and cortex tissues as compared to young roots and vegetative tissues. Based on the macroarray and RT-PCR analysis, we are in the process of cloning several of the most interesting promoters by genome walking. Properties of promoters will be determined by expression of promoter reporter (Pro:: GUS-GFP) constructs in sugar beet hairy roots. Identification of root tissue-specific promoters will facilitate expression of resistance genes in root tissues as a first line of defense targeting root pests and diseases.

Introduction

Sugar beet is an important crop for sugar production, providing approximately a third of all sugar consumed in the world and almost 50% of U.S. sugar production. In addition, the pulp and molasses of sugar beet are widely used as feed supplements for livestock. This economically important plant is subject to attack by more than 150 species of insects and mites (Harry Lange, 1987). Although conventional pesticide application has been effective for the majority of the pests, many of these toxic chemicals are expensive and may be persistent in the environment. The molecular biotechnological approaches are being integrated into conventional approaches by the development of novel transgenic sugar beet lines. Many genes conferring pest resistance are being identified from a diverse range of species and deployed for the generation of transgenic

sugar beet lines. This will lead to the development of transgenic pest resistant sugar beet varieties more quickly, efficiently and with high accuracy. Insect-resistant crops have been one of the major successes of applying plant genetic engineering technology to agriculture (Toenniessen et al., 2003).

Transgenic sugar beet is likely to play an increasingly important role in worldwide sugar production by conferring useful agronomic traits. Efficient genetic modification of sugar beet for agronomic traits will require the use of regulatory sequences, known as promoters, to drive the expression of transgenes in specific plant tissues or at certain developmental stages. The primary regulatory sequences generally are located within 1000 base pairs upstream (5') of the transcription start site in plant genes, although there are cases where regulatory sequences are found further upstream or are downstream (3') of the coding sequences (Dietrich et al., 1992) .

Our ongoing study is focused on cloning and characterizing tissue specific sugar beet promoters. We are using promoter-GUS fusion constructs to transform sugar beet hairy roots. The promoter-GUS fusion analysis will help to identify the promoter sequences which regulate gene expression in tissue specific manner (Jefferson, 1987). Possible applications of promoters for the improvement of sugar beet are discussed.

Materials and Methods

Sugar beet germplasm

Sugar beet germplasm with moderate resistance to the sugar beet root maggot, F1016 (PI608437), and a susceptible, F1010 (PI535818) (Campbell, 1990; Campbell et al., 2000) lines were utilized in this study. Thiram 42S (Gustafson, LLC, Plano, TX) coated seeds were imbibed in water, planted in soil in 7-inch pots, and maintained in a growth chamber at 27°C with a 16 h photoperiod (270 ìml/m2s).

RNA isolation from sugar beet roots and leaves

Sugar beet root tissues from crown, peel and cortex (25% of tissue below the peel) were excised from three month old plants. Also young leaf and root tissue from 17 day old seedlings were harvested and stored at -80°C. Total RNA was extracted from 100 mg of tissue using the RNeasy plant kit (Qiagen, Valencia, CA).

First strand cDNA synthesis and PCR:

A total of .5 µg RNA was used to synthesize single-strand cDNA by reverse transcription according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). The final product was treated with RNase H then stored at -20° C until further use.

Semi quantitative PCR was carried out in a 0.2-ml PCR tube. Each PCR reaction included 1 ìl of 1:5 diluted first strand cDNA, 1x PCR buffer, dNTP, gene specific forward and reverse primers and 2 units *Ex Taq* polymerase enzyme (Takara Bio, Madison, WI). PCR was started at 95°C for 2 min followed by 30 cycles of 95°C (20 s), 55°C (30 s), 72°C (60 s) and final extension at 72°C for 7 min.

Rapid Amplification of cDNA Ends (RACE):

Rapid Amplification of cDNA Ends is a procedure for amplification of nucleic acid sequences from a messenger RNA template between a defined internal site and unknown sequences at either the 3' or the 5' -end of the mRNA (Frohman et al., 1988). The SMARTer RACE cDNA Amplification Kit (Clontech, Mountain View, CA) was used in this study to analyze the 3' and 5' RACE fragment from sugar beet. The 3' and 5' RACE ready cDNA was made using isolated total RNA and SMARTScribe Reverse Transcriptase. For preparation of 5'-RACE-Ready cDNA, 5'-CDS Primer A (5'–(T) 25V N–3') and SMARTer IIA oligo (5'–AAGCAGTGGTATCAACGCAGAGTACXXXXX–3') were used. For the 3'-RACE-Ready cDNA, 3'-CDS Primer A (5'–AAGCAGTGGTATCAACGCAGAGTAC(T)30 V N–3') was used. Amplification of RACE fragment was done using Advantage2 PCR Kit (Clontech). PCR conditions were 5 cycles: 94°C 30 sec, 72°C 3 min; 5 cycles: 94°C 30 sec, 70°C 30 sec, 72°C 3 min; and 25 cycles: 94°C 30 sec, 68°C 30 sec, 72°C 3 min.

Promoter cloning by genome walking

GenomeWalker™ Universal Kit (Clontech, Mountain View, CA) was used to clone the promoters. The sugar beet genomic DNA was separately digested with different restriction enzymes (*Dra*I, *Eco*RV, *Pvu*II, *Stu*I). Each batch of digested genomic DNA was then ligated to the GenomeWalker Adaptor. Two PCR reactions were carried out using the genomewalker library as template. Primer used was adaptor primers and gene specific primers. PCR conditions were 5 cycles: 94°C (25s), 72°C (3 min); 20 cycles: 94°C (25s), 67°C (3 min); and 1 cycle: 67°C (7 min). PCR samples were used to clone the fragment of interest.

Results and discussion

Gene expression analysis

The first step for cloning tissue specific promoter is to identify interesting candidate genes by their tissue specific expression pattern. Our laboratory has identified more than 150 sugar beet root ESTs responding to sugar beet root maggot feeding in moderately resistant, F1016 and susceptible, F1010 genotypes. Many of the identified ESTs corresponding to resistance genes have been reported to be regulated by other pests and pathogens in many different plants (Puthoff and Smigocki, 2007). We screened sugar beet EST clones for root expression by macroarray analyses using RNA isolated from various sugar beet root tissues (Fig.1). Several ESTs were identified that exhibit high levels of tissue specific expression in the root peel, crown and cortex.

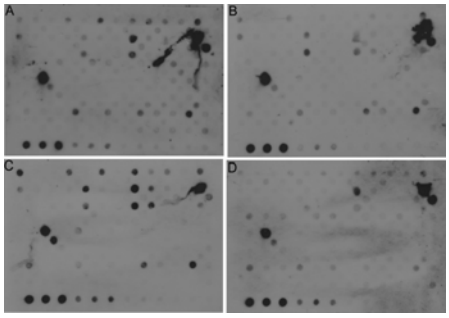

Fig. 1. Macroarray hybridization to identify EST clones differentially expressed in sugar beet root tissues. (A) Young root (B) Root peel (C) Root cortex and (D) Crown

Expression of the identified ESTs was verified by RT-PCR analysis using tissues from sugar beet line F1016 and F1010. Clones BvCL1 through BvCL13 were analyzed (Fig. 2). Expression of BvCL3 and BvCL7 was higher and constitutive in all the root tissues. One clone in particular (BvCL9) was shown to be expressed preferentially in the root but not leaf tissues in sugar beet breeding lines F1010 and F1016. Interestingly, BvCL9 was expressed in both the roots and leaves of line FC607 (data not shown). This implies that cis-acting elements within the BvCL9 promoter in F1010 and F1016 may regulate the expression of the BvCL9 gene in a tissue specific manner.

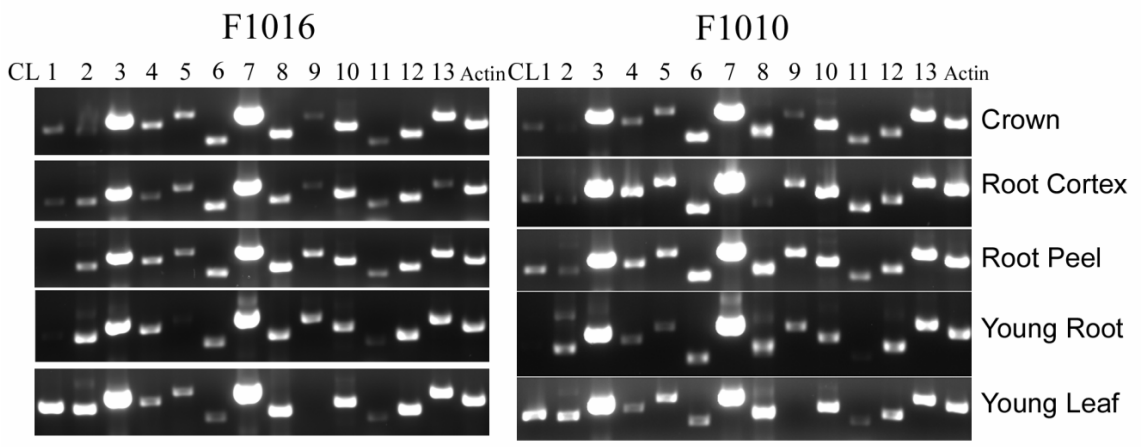

Fig. 2. RT-PCR analysis to analyze differential expression of BvCL1-BvCL13 gene clones in three month old crown, root cortex, root peel and 17 day old root and leaf tissue.

Cloning of root specific promoter

The transcription start site of the selected EST clones was determined using 5' RACE. A gene-specific primer designed from the coding sequence of the EST was used to screen GenomeWalker *B. vulgaris* libraries for upstream sequences (promoters). After two rounds of PCR amplification, each library yielded a specific fragment (Fig. 3). These DNA fragments were cloned in to pCR2.1 TOPO vector and sequenced. The DNA sequence upstream to the cDNA was mapped for promoter specific elements. The

promoter fragments were cloned into pDONR and destination Gateway vectors (Invitrogen) for further characterization in sugar beet roots.

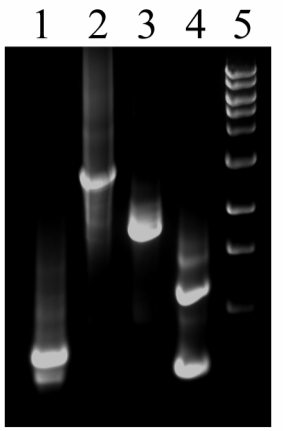

Fig. 3. *Dra*I (lane 1), *Eco*RV (lane 2), *Pvu*II (lane 3), and *Stu*I (lane 4) digested sugar beet genomic DNA ligated to Genomewalker adaptor then PCR amplified using adaptor primer and gene specific primers. Lane 5, 1 kb DNA marker.

Conclusion

Several cDNA clones were identified that display root tissue specific expression in the F1016, F1010 and FC607 sugar beet breeding lines. Among these clones, one is specific to root peel (BvCL9), some are constitutive but more highly expressed in all the root tissue (BvCL3, BvCL7), and some are preferentially expressed in root cortex tissue (BvCL4, BvCL5). Based on the macroarray and RT-PCR analysis we are in the process of cloning several of these most interesting promoters by genome walking.

Root-specific promoters are valuable tools for targeting transgene expression in sugar beet. At present, the most widely used promoter for expression of transgenes in dicot plants is the cauliflower mosaic virus (CaMV) 35S promoter (Odell et al., 1985). The CaMV 35S promoter provides strong constitutive expression in most dicot plants, including sugar beet. However, to develop transgenic sugar beet with specialized agronomic traits such as pest control and more sugar storage, a larger arsenal of constitutive and tissue-specific promoters will be required. The characteristic expression patterns provided by these promoters must be analyzed to determine whether they can be used to express beneficial genes in specific target tissues or developmental stages at maximum levels. An essential tool in sugar beet biotechnology is, therefore, the development of root-specific promoters, which help to overcome undesirable phenotypes generated by constitutive over-expression of a transgene throughout the plant's development. Transgenes driven by tissue specific promoters are preferentially or solely expressed in those tissues where the transgene product is most desired, leaving the rest of the tissues in the plant less likely to be modified by transgene expression. For example, expression of an insect specific toxin gene in the root epithelial layer should serve as a first line of attack against that pest *in planta*, preventing the accumulation of the recombinant protein in other root tissues such as the sucrose storage parenchyma cells.

Acknowledgements

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