Chapter 6
Novel Potential Candidate Promoters and Advanced Strategies for Sugarcane Transformation

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Abstract Plant transformation technology offers unique prospects to transfer a wide spectrum of functionally relevant genes in plants. Expression of genes is regulated by a number of factors among which promoter strength, specificity, and *cis*- and *trans*-acting elements play a critical role. The choice of promoter is a key determinant for the levels and specificities of gene expression. In sugarcane, the maize ubiquitin promoter has been the workhorse promoter for decades. The availability of limited promoters for sugarcane transformation is critical in sugarcane crop improvement through genetic engineering. However, recent advancements in biotechnology have provided greater insights into promoter validation from wild and commercially cultivated sugarcane, which is evident from an array of different promoters reported. This review describes the various promoters isolated from sugarcane and its wild relatives that would benefit future genetic engineering studies in sugarcane. In addition, the challenges ahead and improved strategies for sugarcane transformation are discussed.

Keywords Enhanced expression • Promoter • Silencing • Sugarcane • Transformation • Transgene

6.1 Introduction

Sugarcane (*Saccharum* spp. hybrid) belonging to Poaceae family is an economically important food and energy crop grown worldwide. Large genome size, polyploidy, low fertility, complex environmental interactions, slow breeding advances, and nobilization hinder the breeding for this crop. In addition, several issues like

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low cane and sugar yields; susceptibility to abiotic stresses such as drought, cold, and salinity; and biotic stresses such as pest insects and fungal diseases are the major constraints in sugarcane cultivation (Tiwari et al. 2010). Transgenic technology provides an effective tool for sugarcane crop improvement. Both biolistic and *Agrobacterium*-mediated transformation methods have been well established and are widely used to develop transgenic sugarcane. Several factors have to be considered in the development of transgenic sugarcane among which the choice of promoter plays a crucial role.

Promoters are the regulatory sequences present upstream of the genes and are involved in the regulation of the gene expression. In recent years, an array of promoters from plant and viral origin have been characterized and extensively used in transgene expression in plants. They can be broadly grouped into constitutive, spatiotemporal, and inducible promoters based on their activity. The CaMV 35S promoter has been well described and widely used for constitutive transgene expression (Potenza et al. 2004). However, in monocots, the CaMV 35S promoter confers lower levels of transgene expression. The maize ubiquitin promoter (M-ubi1) is the promoter of choice for sugarcane transformation and has been widely used for over two decades. Sugarcane ubiquitin promoters (Ubi4 and Ubi9) when expressed in sugarcane have led to posttranscriptional gene silencing (PTGS) (Wei et al. 2003). The promoters used currently for the development of transgenic sugarcane are limited in number and very few provide tissue-specific expression. Limited tissue-specific promoters have been characterized so far for sugarcane transformation (Damaj et al. 2010). Hence, there is a need for identification of more promoters for specific applications, and from unrelated sources, which would be of great value for future genetic engineering studies in sugarcane. This review discusses the various advancements that took place in the past decade pertaining to sugarcane transformation and promoter validation which will benefit researchers aiming to develop transgenic sugarcane with desirable traits.

6.2 Plant Promoters: Structure and Function

Promoters are defined as regions upstream of a gene's coding region and are involved in the regulation of the frequency of transcription. They usually contain specific DNA sequences and regulatory elements and are the key regulators of transcription, also called as molecular switches. Promoters can be broadly classified into constitutive, tissue specific, and inducible based on their activity. Another type of promoters which are currently of importance is the synthetic promoters that combine the available core promoters with different motifs and are designed for specific expression. A typical plant promoter is composed of a transcription start site (TSS), the core promoter region, the proximal region (or upstream regulatory elements), and the distal regulatory region (or long-range regulatory elements). Plant promoters and their *cis*-acting regulatory elements have been reviewed

extensively (Hernandez-Garcia and Finer 2014; Porto et al. 2014; Grunennvaldt et al. 2015; Shah et al. 2015; Naqvi et al. 2016; Biłas et al. 2016) and hence are not focused in this review.

6.3 Promoters for Enhanced Transgene Expression in Sugarcane

In sugarcane, the maize ubiquitin (Zmubi1) promoter is being used worldwide for more than two decades for genetic engineering. However, it has failed to drive sustained transgene expression throughout the sugarcane growth cycle (Wang et al. 2005). Promoters of viral origin such as sugarcane bacilliform virus promoter (Braithwaite et al. 2004), banana streak virus promoter (Schenk et al. 1999), and CaMV35S:Zmubi1 tandem promoter (Groenewald and Botha 2008) conferred enhanced expression in mature canes. Some of the other promoters used were the enhanced maize ubiquitin promoter and maize carboxylase promoter (Kinkema et al. 2014a, b) which conferred enhanced transgene expression than the Zmubi1 promoter (five and fourfold). The advent of novel tools for promoter discovery, next-generation sequencing boom, and advanced bioinformatics techniques have led to isolation of new promoters from sugarcane and its wild relatives that could drive enhanced transgene expression than the routine promoters. Table 6.1 lists the different promoters characterized from sugarcane and its wild relatives that would enable researchers to develop GM sugarcane with enhanced transgene expression. Yet, the number of promoters is very limited when compared to other plant species. Mudge et al. (2013) characterized three promoters from sugarcane which conferred preferential transgene expression in mature stems and thus have practical application in sucrose-targeted metabolic engineering.

6.4 Challenges Ahead in Sugarcane Biotechnology

Sugarcane is affected by several biotic and biotic stresses which lead to losses in productivity. Using conventional breeding practices, the release of a new variety typically takes 12–15 years after rigorous testing of performance, sugar content, agronomic traits, and genetic stability (Gazaffi et al. 2010). With the advent of transgenic technology, considerable progress has been made in the recent years. Biolistic and *Agrobacterium* methods of transformation, though routinely used, have several disadvantages. Biolistic bombardment method usually generates multiple transgene integration sites. *Agrobacterium*-mediated transformation is rather a time-consuming laborious process, has low transformation efficiency (Joyce et al. 2010), has high variability between experiments, and is genotype dependent (Anderson and Birch 2012). In fact, the time taken from DNA delivery till whole-plant regeneration is longer than any other crop plants.

Table 6.1 Potential candidate promoters from sugarcane and its wild relatives with enhanced transgene expression

Type	Promoter	Source	Gene and function	Strategy for promoter isolation	Inference	References
Constitutive	ScMybR1	Sugarcane	Myb transcription factor gene	BAC library screening and PCR	Identified and validated 8 alleles; 3 found expressive	Mudge et al. (2009)
	EaUbiD7	*Erianthus arundinaceus*	Ubiquitin	RAGE PCR	Deletion ubiD7 drove higher *GUS* expression in sugarcane	Chakravarthi et al. (2015)
Inducible	EaPR10	*E. arundinaceus*	PR-10 gene	RAGE PCR	Induced upon wounding; also constitutive	Chakravarthi et al. (2016)
	ScMybAS1	Sugarcane	Myb transcription factor gene	cDNA library and PCR	Induced upon exposure to abiotic stress and hormones	Prabu and Prasad (2012)
	SoCIN1	Sugarcane	Cell wall invertase	RACE PCR	Induced expression	Niu et al. (2015)
	Sc-ERS	Sugarcane	Ethylene receptor gene	TAIL PCR	Induced upon circadian rhythm, hormone, drought, and light	Li et al. (2013)
	EaMybAS1	*E. arundinaceus*	Myb transcription factor gene	PCR	Revealed enhanced stress tolerance in transgenic tobacco	Kharte et al. (2016)
	alcA	Sugarcane	Ethanol-inducible gene switch	NA	Identified an effective ethanol-inducible promoter	Kinkema et al. (2014b)

Type	Promoter	Source	Gene and function	Strategy for promoter isolation	Inference	References
Stem specific	Sc-c22a	Sugarcane	Dirigent-like protein	Inverse PCR	Conferred mature culm-specific expression	Abraha (2005)
	ScLSG	Sugarcane	Loading stem gene	BAC library screening and PCR	9 alleles of LSG identified and validated	Moyle and Birch (2013)
	ShDIR16	Sugarcane	Dirigent protein	BAC library screening and PCR	Both conferred stem-regulated expression; induced expression in leaf and root upon hormone exposure	Damaj et al. (2010)
	ShOMT		O-methyl transferase			

Another major challenge in sugarcane transformation is the transgene inactivation/silencing problem. Several promoters failed to drive transgene expression in mature canes despite showing activity in callus (Wei et al. 2003). Moreover both transcriptional and posttranscriptional gene silencing has been reported in sugarcane (Ingelbrecht et al. 1999). Mudge et al. (2009) characterized eight distinct promoters of MYB family of which three were expressive. Interestingly, their results illustrated that multiple copies of promoter do not trigger silencing and polyploids may have intrinsic silencing mechanisms that are yet to be deciphered. Birch et al. (2010) reported that silencing in sugarcane is 5′-sequence specific, independent of copy number, developmentally regulated, and posttranscriptional in T_0 transgenic lines. Transgenes fused with strong tissue-specific promoters may alleviate the silencing problem.

Sugarcane genome is about 10 Gb size with homologous genes ranging from 8 to 12 copies (Souza et al. 2011) and the monoploid genome size being 750–930 Mb (D'Hont and Glaszmann 2001). Currently there is a lack of whole genomic data in sugarcane. The major factors that make the whole-genome sequencing of sugarcane difficult are (1) polyploidy—80% of sugarcane genome is inherited from *S. officinarum* and 10% from *S. spontaneum*; (2) high level of recombination—more than 10% of sugarcane genome is mosaic and unknown; (3) heterozygosity—leads to variations that deter genome assembly; and (4) repeats—high number of repetitive sequences present throughout the genome. Moreover, sugarcane lacks diploid progenitors that aid in a faster and easier genome assembly (Garcia et al. 2013) unlike banana (D'hont et al. 2012). In addition, it is difficult to employ shot-gun sequencing such as Illumina which generates shorter reads. Thus, the large and complex genome, high ploidy levels, and high content of repetitive DNA make sugarcane an unusually recalcitrant crop species for both forward and reverse genetic studies.

6.5 Improved Strategies for Sugarcane Transformation

Several researchers are striving hard to overcome the challenges in sugarcane transformation which has led to a breakthrough with an array of advanced techniques, modified protocols, and strategies for efficient sugarcane transformation. This section of the review discusses some of the significant studies which will have a greater impact on sugarcane transgenic research in the near future. Joyce et al. (2010) optimized different parameters for *Agrobacterium* transformation in sugarcane and observed that selection and cocultivation systems were critical factors that affected sugarcane transformation. Jackson et al. (2013) compared both the methods using whole plasmids and minimal cassettes and observed that both the procedures were high expressing and yielded single-gene insertions at a reasonable transformation efficiency (TE).

Taparia et al. (2012a, b) used minimal expression cassettes for biolistic gene transfer and with reduced plasmid concentration and achieved simple transgene integration and stable transgene expression. They also described a rapid transformation procedure that only needs 3 months from culture initiation to potting of transgenic sugarcane. Use of minimal cassettes has shown to be effective since

they are devoid of prokaryotic backbone sequences that may contribute to recombination or induce methylation, thereby leading to transgene silencing.

Anderson and Birch (2012) studied several parameters that are critical for transformation of sugarcane variety Q117. They reported that the key factors influencing transformation efficiency in *Agrobacterium* method were minimal handling of callus during cocultivation and the use of a super-binary vector in AGL *Agrobacterium* strain which led to the highest transformation efficiency reported so far for *Agrobacterium*-mediated transformation in sugarcane. Recently, Mayavan et al. (2015) have developed a rapid, efficient, and genotype-independent *in planta* transformation protocol using sugarcane setts as explants. They have claimed a maximum of 32.6% TE which is so far the highest TE in sugarcane. Their group had earlier developed a seed-based transformation protocol which also proved to be efficient to develop transgenic sugarcane in a shorter duration (Mayavan et al. 2013).

Dong et al. (2014) developed a robust protocol that could be applied on a larger industrial scale for sugarcane improvement through genetic engineering. This protocol employs desiccation during cocultivation that leads to higher TE and has also been tested in several varieties and in several laboratories proving its versatility. In addition, the transgenes were stable across multiple generations and growing seasons that further proves the great utility of the protocol. Sandhu et al. (2016) have recently reported single-step direct transgenic plant regeneration from agro-infected spindle leaf roll segments of sugarcane with a very short period of 8 weeks since it avoids the callusing phase. Stable integration was observed in the transgenics making the protocol reliable for sugarcane transformation.

Jackson et al. (2014) presented a set of rules to achieve sustained transgene expression and validated them in sugarcane. They used the following methods independently or in combination—removal of rare codons, removal of RNA instability sequences, blocking of putative endogenous sRNA-binding sites, and randomization of non-rare codons. This technique can be applied in sugarcane effectively to alleviate transgene silencing. Recently, Lowe et al. (2016) reported an efficient monocot transformation strategy wherein they over-expressed the maize morphogenic regulators *Baby boom* (*Bbm*) and *Wuschel2* (*Wus2*) genes in previously non-transformable maize inbred lines and achieved high transformation frequencies. They also successfully employed this approach to enhance transformation frequency in sorghum, sugarcane, and rice.

Other notable advances worth mentioning are (1) development of synthetic reporter genes in order to alleviate silencing effects to validate promoter expression in sugarcane (Chou and Moyle 2014); (2) use of alternate monocot models such as *Setaria viridis* that yields higher transformation efficiency, has shorter duration, and contains a similar cell wall composition to that of sugarcane. Hence it can be used as an alternate model plant for sugarcane-applied research for stress resistance, improved biomass, and bioethanol production (Martins et al. 2015); (3) use of novel promoters that drive higher levels of transgene expression than the routine promoters and exploiting codon-optimized target genes specific for sugarcane to enhance transgene expression (Kinkema et al. 2014a); (4) application of RNAi technology to

develop improved sugarcane for desired traits (Gan et al. 2010; Gao et al. 2013; Jung et al. 2012); (5) use of a combinatorial approach wherein multiple promoters/enhancers/terminators/5′UTRs are employed to achieve higher transgene expression (unpublished data); and (6) use of systems biology and metabolic modeling approach to unravel gene regulatory networks underlying key mechanisms such as sucrose synthesis and accumulation.

6.6 Conclusion and Future Perspectives

Sugarcane biotechnology has advanced rapidly over the years and transgenic lines for various biotic and abiotic stresses have been developed and are being tested in laboratories worldwide. Commercial testing of transgenic sugarcane has already been approved in Indonesia and is in pipeline in several other countries. Several recombinant proteins have already been produced using sugarcane as a bio-factory. With the advances in transgenic technology, genome sequencing tools, and systems biology coupled with bioinformatics, it is now feasible to manipulate the metabolic pathways in sugarcane, thereby enhancing the crop productivity and increased sugar content. Although obstacles including transgene inactivation, lack of whole genome, and long duration for transformation are certainly a hindrance, genetic engineering combined with the novel advanced strategies would undoubtedly be instrumental in helping the sugarcane industries develop into a stronger bio-economy.

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