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High resolution melting (HRM)-based STMS marker analysis for rapid identification of radiation induced mutants of sugarcane

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ABSTRACT

Molecular marker based selection has become the method of choice to find undiscoverable natural variations and induced mutations in plants. Routine gel based molecular marker screening procedures involve complex steps which require considerable investment of time, money and efforts. Most often, genomic configuration of a plant species complicates the ultimate output of such screening programme. Therefore, a reliable and rapid high-throughput method is required to facilitate ease in identification of variants in a large number of samples. Here we demonstrate the application of a high-resolution melting (HRM) method to detect gamma radiation induced mutants of sugarcane (*Saccharum* spp.) which is a complex aneu-polyploidy plant. We have screened nine distinct mutants using sequence-tagged microsatellite site (STMS) markers and the analyses based on gel electrophoresis and HRM curve were compared. All the six STMS microsatellite markers coupled with HRM analysis were found highly informative and generated a unique melting curve for each of the marker tested. Of these, two markers NKSCSSR 22 and NKSCSSR 42 exhibited differential melting curve among the mutants and these results were well correlated with the gel based banding pattern. Taken together these results highlighted potential of HRM based marker screening and demonstrated reliability and robustness in detecting induced mutations in a complex polyploidy plant like sugarcane.

1. Introduction

Sugarcane (*Saccharum* spp.) is cultivated in the tropical and sub-tropical regions, and spreads over 90 countries across the world which accounts for around 70–80% of the world's sugar production (Lakshmanan et al., 2005; Barnabas et al., 2015). Years of rigorous and extensive research and development in intergeneric and interspecific hybridizations resulted in marked increase in overall sugar production. For the present and the future, sugarcane is serving as an important food, industrial and bioenergy (bioethanol) resource which has a significant contribution to the trade and economy of many tropical and subtropical countries. Considering its global significance, sugarcane breeding programmes are targeted to improve biomass production, sugar content and (a)biotic stress tolerance (Ming et al., 2006). Although the conventional methods of improvement have greatly contributed to some extent to increase the biomass and (a)biotic stress tolerance, the evaluation of sugarcane breeding programs indicated that a plateau has been reached for the genetic potential (Grof and Campbell, 2001) and further gains are becoming less pronounced (Burnquist et al., 2010).

Induced mutagenesis has been the core approach to introduce desirable genetic variability and to produce new plant types with unique traits (Suprasanna et al., 2014, 2015). The constraints in conventional breeding that are impending sugarcane crop improvements (such as loss of vigour, narrow gene pool, complex genome, poor fertility and the long breeding/selection cycle) can be addressed through induced mutagenesis. But while performing induced mutagenesis, identification and selection of mutants/variants with desirable traits assume great importance. In case of sugarcane, this task further complicates due to masking effect of polyploidy genomic content. Biotechnological advancements have shown to play a crucial role to unravel some of the limitations of classical breeding (Suprasanna et al., 2011; Dal-Bianco et al., 2012; Devarumath et al., 2013). Current and future research avenues include use of high-throughput research methodologies for marker assisted breeding, gene discovery and genomics, pathway modification through transgenics and/or induced mutagenesis,

Abbreviations: HRM, High-resolution melting; RFLP, Restriction fragment length polymorphism DNA; AFLP, Amplified Fragment Length Polymorphism; RAPD, Random Amplification of Polymorphic DNA; TRAP, Target Region Amplification Polymorphism; STMS, Sequence-tagged microsatellite sites; PCR, Polymerase chain reaction; EMS, Ethyl methane sulfonate; CTAB, Cetyl Trimethyl Ammonium Bromide; PAGE, Polyacrylamide Gel Electrophoresis

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improved agronomic practices and crop physiology modelling, all of which ultimately help together to relieve the bottleneck that is ceasing the yield threshold (Mirajkar et al., 2017).

With the advent of molecular marker techniques, discerning genetic information has become fascinating and reliable with elimination of genotype and environment interactions, and thus allowing precise identification of individuals across environments (Nadeem et al., 2017). Different molecular markers such as restriction fragment length polymorphism (RFLP) (Burnquist et al., 1992), amplified fragment length polymorphism (AFLP) (Rodriguez et al., 2003), random amplification of polymorphic DNA (RAPD) (Kawar et al., 2009), target region amplification polymorphism (TRAP) (Alwala et al., 2006; Hemaprabha and Lavanya, 2015) and microsatellites (Singh et al., 2011) have been utilized most frequently to evaluate genetic variability in sugarcane germplasm and cultivars. Over the years, significant progress has been made in harnessing sugarcane genomic information using molecular markers which led to collection of valuable genomic resource such as SUCEST database (Telles et al., 2001; Nishiyama et al., 2012).

Among the various markers, sequence-tagged microsatellite sites (STMS) markers are considered highly specific, efficient and can distinguish even closely related germplasm lines. STMS markers were used for assessment of genetic diversity among induced mutants and somaclones of sugarcane but shown to detect lesser divergence compared to divergence among cross bred and segregating progeny populations (Hemaprabha et al., 2005, 2006; Lavanya and Hemaprabha, 2010a,b; Lavanya and Hemaprabha, 2012). Although considerably significant morphological differences were found to exist between the donor and the induced mutants, lack of proper marker system left this area as a grey area of research.

DNA melting analysis was introduced for the first time in 1997 as closed-tube and instantaneous method to characterize PCR amplicons. The technique relies on determination of thermal denaturation profile of PCR products rather than assessing amplicon size by physical separation methods (agarose/polyacrylamide gel/capillary electrophoresis) (Croxford et al., 2008). The melting of a DNA occurs at a particular melting temperature (*Tm*) depending on the GC content, length and sequence of the duplex. Subtle changes in the shape of the melting curve indicate heterogeneity in the target DNA sequence. Owing to its simplicity, robustness (requires only few minutes after PCR) and low cost, HRM has becoming a choice for high-throughput scanning and genotyping (McKinney et al., 2010; Simko, 2016). The HRM analysis is particularly applicable in plant species with limited or less genetic information available. HRM based genotyping has been demonstrated successfully in various plant species viz., grapevine (Mackay et al., 2008), almond (Wu et al., 2008, 2009), olive (Muleo et al., 2009; Xanthopoulou et al., 2014), rice (Li et al., 2011), sorghum (Mofokeng et al., 2012), common beans (Ganopoulos et al., 2012), sweet cherry (Ganopoulos et al., 2014), pea (Knopkiewicz et al., 2014), and eggplant (Ganopoulos et al., 2015).

Studies on the use of HRM are relatively scarce for studying mutations in plants. Jeong et al. (2012) demonstrated the application of HRM to detect natural variations and ethyl methane sulfonate (EMS)-induced mutations in *Capsicum*. Single polymorphic mutations carrying natural allelic variation and EMS-induced mutations in the exonic region of gene conferring resistance to potyviruses were identified using HRM analysis. In polyploidy plant species, the effects of mutation in single homoeologues is often masked by inherent genetic redundancy (Fitzgerald et al., 2010), therefore forward genetic screening for mutation detection requires extensive screening based on effective and convenient isoenzymatic or molecular markers. A potential application of HRM based genotyping for mutation detection in polyploidy crop plant was rarely attempted, such as in hexaploid wheat (Dong et al., 2009; Botticella et al., 2011), and tetraploid Alfalfa (Han et al., 2012). Due to higher ploidy genome and repetitive DNA content, sugarcane genomic complexity makes mutation detection difficult. The present study aims to demonstrate the efficiency of HRM based STMS marker analysis, which should be efficient in discerning variability in the induced sugarcane mutants.

2. Material and methods

2.1. Plant material and assessment of morphological attributes

A commercial sugarcane variety CoC-671 (popular for high sucrose content), nine mutants (viz. AKTS-01, 02, 06, 08, 11, 13, 16, 19 and 20) derived from CoC-671 through in vitro mutagenesis using gamma irradiation (Suprasanna et al., 2008) and a low sucrose check genotype MS-6847 were used for the STMS based molecular marker profiling studies. Variations in different qualitative morphological attributes were recorded at full maturity stage (12th month after planting) as per the descriptors suggested by Artschwager and Brandes (1958).

2.2. Genomic DNA extraction

The genomic DNA was extracted from leaf tissue of field grown plants following modified CTAB (Cetyl Trimethyl Ammonium Bromide) extraction method (Doyle and Doyle, 1987). Integrity and intactness of extracted DNA was checked on 0.8% agarose gel and quantity was measured by recording absorbance ratio $A_{260/280}$ using Nanophotometer™ (Implen Inc). Individual samples were then diluted in sterile Milli-Q water to final concentration of 50 ng/μl and stored at 4 °C refrigerator for further use.

2.3. Polymerase chain reaction (PCR) and STMS marker polymorphism

The PCR reaction was set for 20 μl reaction volume comprised of 10 μl 2× ReadyMix™ Taq PCR Reaction Mix with $MgCl_2$ (Sigma Aldrich), 1 μl of each forward and reverse primers, 1 μl of template DNA (50 ng/μl) and 7 μl of sterile Milli-Q water. The primer sequences for STMS markers were designed by NRCPB, New Delhi, India. Altogether six STMS microsatellite markers were used and their details are presented in Table 1. The PCR reaction conditions were, initial denaturation at 94 °C for 5 min, 35 cycles of denaturation at 94 °C for 1 min, annealing at 50–60 °C (depending on primer pair used) for 1 min and extension at 72 °C for 1 min, followed by final extension at 72 °C for 5 min and reaction was hold at 4 °C.

2.4. Polyacrylamide gel electrophoresis (PAGE) and silver staining

The PCR amplified DNA fragments were resolved on 6% polyacrylamide gel prepared in 1× TBE buffer. Macrokin PAGE (Techno Source™) gel assembly with 1.0 mm gel thickness was used. After gel was completely polymerized, 10 μl of PCR product was loaded in respective wells and electrophoresed at a constant voltage of 200 V or 35 mA current for 4–5 h at room temperature. The procedure of silver staining modified after Creste et al. (2001) was used to stain the gel. After staining, the gel was visualized using white-light transilluminator and image was recorded.

2.5. Amplicon sequence confirmation analysis

Two of the promising PCR amplicons generated by NKSCSSR 42 were gel purified (GenElute™ Gel Extraction Kit, Sigma) and sequenced directly using custom sequencing (Eurofins Genomics, India). The sequences were subjected to in silico sequence analysis using the BLAST homology search and multiple alignments were performed using MultAlin version 5.4.1 (Corpet, 1988).

2.6. Scoring of bands and dendrogram construction

Scoring of bands was done on the basis of presence (scored as “1”) and absence (scored as “0”) of bands and alleles amplified per primer