Title: Mutation analysis of rice starch synthesis genes.
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Conference Title: Year of Conference: 2007
Conference Location: Melbourne, Australia
Editor: J. F. a. B. Panozzo, C.K.
Publisher: Royal Australian Chemical Institute
Pages: 280-283
URL: http://www.raci.org.au/division/cereal/accc-57/
Keywords: Rice, gene, mutation, starch
Abstract: Starch is the main storage carbohydrate in plants, and it consists of two glucose polymers, amylose and amylopectin. There are several key enzymes involved in starch synthesis and which determine starch structure. Different forms of each of these enzymes, and their encoding genes have been identified in different rice cultivars. The potential for manipulation of the enzymes involved in starch synthesis in order to modify or create desirable starch characteristics and content is a major area of interest. Therefore it may be useful to identify DNA sequence variations in the genes involved in starch synthesis. This project investigated the existence of variation in rice starch synthesis genes in order to identify different gene forms that result in altered starch structure and content.
CSU ID: CSU289140

MUTATION DETECTION IN GENES FOR STARCH ENZYMES OF RICE

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INTRODUCTION

Starch is the main storage carbohydrate in rice. It consists of two glucose polymers, amylose and amylopectin. Amount and structure of these two polymers are determined by a range of starch biosynthetic enzymes, which can have different levels of activities in various rice varieties. These differences in enzyme activities are often due to variations in the corresponding genes (alleles) coding for various starch enzymes. Therefore to understand the genetic basis of starch structure in different rice lines and their cooking properties, it is imperative to look for polymorphisms in the genes coding for starch biosynthetic enzymes.

Genetic variations involve insertions, deletions or single nucleotide polymorphisms (SNP), which can result in altered amino acid sequences of the coded protein/ enzyme and thus affect its activity/ function. In general, insertions and deletions (indels) in a gene can be easily detected by comparing the size of the PCR amplicon in the target region of a gene, however SNP detection requires a more sensitive technique like analysis of single-strand conformation on a sequencing gel (Orita et al 1989) or fluorescent staining detection (Qi et al 2001) followed by confirmation with sequence analysis.

In this study, a Hi-Res Melting ™ technique (Wittwer et al 2003) was tested to detect SNPs in different starch synthetic enzyme genes of rice. This technique is based on the detection of subtle differences in fluorescent signals over a temperature range and permits screening of fragments as a pre-sequencing gene scanning. PCR-amplicons from target genes are subjected to a high-precision denaturation process in the presence of a DNA binding dye LC Green®. Nucleic acid melting is then tracked by monitoring the fluorescence of the mixture across a defined temperature range, generating melting profiles that show the presence of sequence variation within the target sequence (Reed et al 2004). Therefore, the main aim of this project was to validate the application of Hi-Res Melting™ technique in detection of SNPs prior to detailed sequence analysis of rice starch synthesis enzyme genes.

MATERIALS AND METHODS

Plant material and DNA extraction

Two rice cultivars namely; Opus (a *japonica* type) and IR-36 (an *indica* variety) were utilised in this study. Genomic DNA from young leaves was isolated using a standard phenol extraction procedure. DNA samples were first analysed on agarose gels, to check their quality and quantity before proceeding with the PCR amplifications.

PCR amplification and analysis

Three different regions from the soluble starch synthase I (*SSS-I*) were amplified from the two rice lines, using gene specific primer sets. The sequencing of the first two amplicons (Oliver 2000) has already been shown to contain several variations in this gene as follows: a SNP in the sequence of Opus amplified with the first primer set, and an insertion and two SNPs in the sequence of IR-36 amplified with the second primer set. Therefore, these PCR products were included as a positive control for validation of the Hi-Res Melting™ technique.

For a negative control, two identical plasmid clones of Opus corresponding to a third region of the soluble starch synthase I (*SSS-I*) were also analysed with the Hi-Res Melting™ technique.

For PCR, each reaction was performed in a total volume of 20µL containing ~100ng of genomic DNA, 10µL of Bioline BioMix, 0.4µL of each primer (~50ng) and 2µL of dye LC Green® (10X stock solution provided by the John Morris Scientific Ltd.). The LC Green® is a double-stranded DNA binding dye, which has the ability to detect the presence of heteroduplexes formed during PCR, if the sample contains a particular mutation. It is a saturation dye and during DNA melting does not easily jump from a double-stranded DNA of low melting temperature to that of a higher Tm. Thus, the presence of LC Green® allows clear distinction between multiple double-stranded DNA species (in solution) based on their unique melting curve.

RESULTS AND DISCUSSION

Results from the Hi-Res Melting™ analysis of the first two *SSS-I* amplicons from the two rice lines are shown in figure 1. As expected, the melting curves of the first amplicon from Opus (1) and IR-36 (2) show clear differences, indicating that this technique can readily detect variations between the two rice lines. Similarly, differences in the melting curves 3 (Opus) and 4 (IR-36) from analysis of the second amplicon of *SSSI*(Figure 1b) further confirms the capacity of this technique in the detection of variations that have been identified before (Oliver 2000).

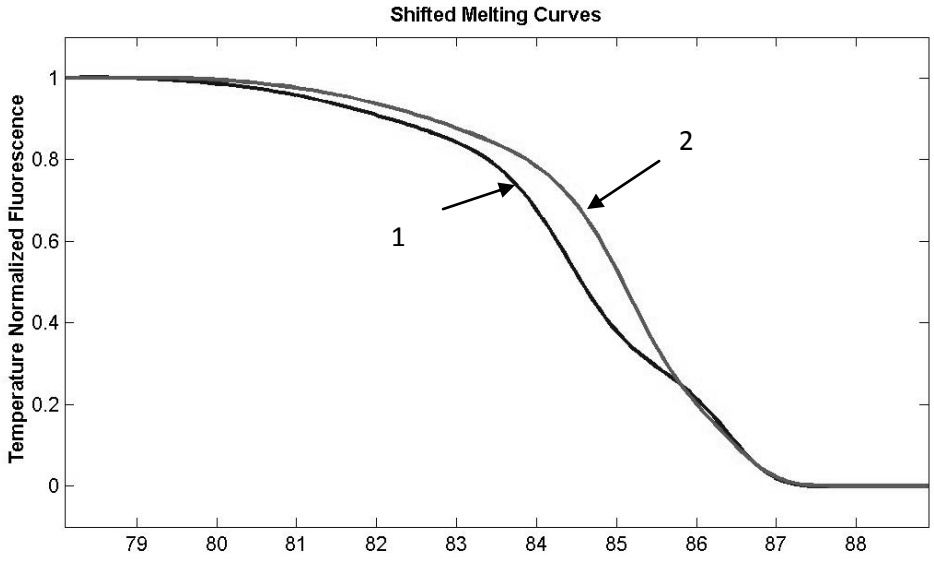

Figure 1a. Melting curves of amplicons from Opus (1) and IR 36 (2), amplified with the first set of *SSS-I* primers.

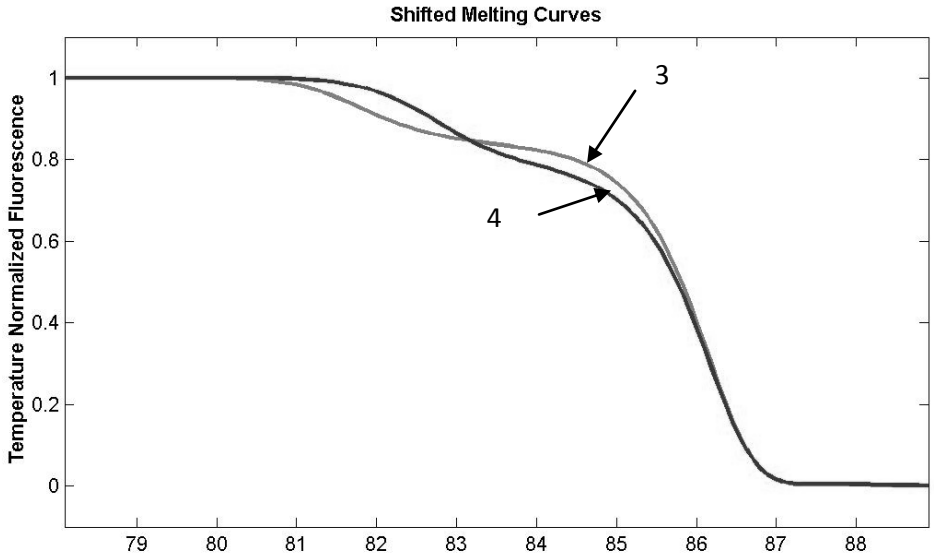

Figure 1b. Melting curves of amplicons from Opus (3) and IR 36 (4), amplified with the second set of *SSS-I* primers.

The melting curves obtained with the Hi-Res Melting™ for the two amplicons of the two identical plasmid clones of Opus rice are perfectly overlapping (Figure 2), indicating that there is no difference in the sequence of the two clones, as expected for the negative control.

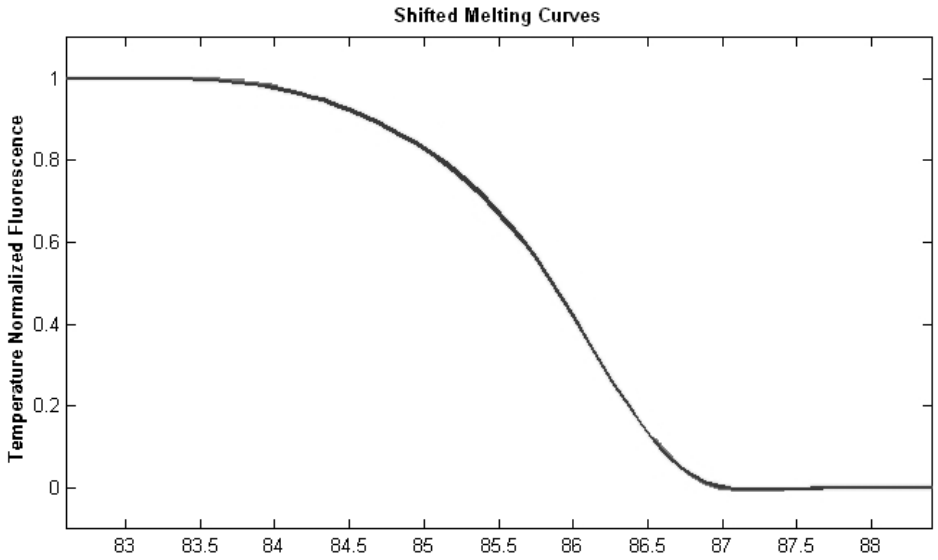

Figure 2. Melting curves of two amplicons from identical clones of Opus, amplified with the third set of *SSS-I* primers.

As the results obtained by previous sequencing of the target regions from the *SSSI* gene of rice (Oliver 2000) and those from the Hi-Res Melting™ technique are corresponding, the latter technique can be successfully employed to detect any subtle changes in the target regions of the genes under investigation.

In order to detect some variations in other starch enzyme genes, analysis of their amplicons with this Hi-Res Melting™ technique is currently in progress (data not shown)

CONCLUSIONS

These results show that analysis of melting profiles of target DNA with the Hi-Res Melting™ technique allows the detection of small sequence variations without the need for sequencing the whole coding region. This technique is likely to improve the efficiency of mutation detection and reduce the cost of unnecessary sequencing of regions that do not harbour any polymorphism.

ACKNOWLEDGEMENTS

The authors wish to thank Sandra Oliver for SSI primers used in this study. Provision of LC Green® dye and analysis of melting curving of PCR amplicons by Mr Jackson Jones from John Morris Scientific is duly acknowledged.

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