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2008

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Publication details

Hill, H, Lee, LS & Henry, RJ 2008, 'Identification of novel starch traits in sorghum', paper presented to 58th Australian Cereal Chemistry Conference, Gold Coast, Qld., 31 August -4 September.

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IDENTIFICATION OF NOVEL STARCH TRAITS IN SORGHUM (*SORGHUM BICOLOR L*): A REVERSE GENETICS APPROACH

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INTRODUCTION

In Australia, sorghum is the 3rd most important cereal grain, after wheat and barley, and is well suited to our hot and dry climate. Grain sorghum plays a key role in providing feed grains to the beef, dairy, pig and poultry industries, and is used in pet foods. It is a good rotation crop that tolerates heat and moisture stress, and performs better than maize on soils with marginal potassium levels. (www.dpi.qld.gov.au)

The use of ionising radiation such as x-rays and gamma radiation to induce variation in plants is an established technique and has been used for over 70 years by plant breeders for crop improvement. (Ahloowalia and Maluszynski, 2001). In this study, sorghum seed has been gamma irradiated to induce random mutation across the genome and DNA sequencing has been used to detect genetic variability in starch genes known to be important in the genetic control of grain quality in cereals.

This work aims to survey starch synthesis genes and discover novel genetic variation, which may subsequently be utilised to enhance the nutritive value of sorghum by breeding through modification of endosperm composition.

MATERIAL AND METHODS

Plant Material

Mature seeds of *Sorghum bicolor* L (cv. MR 43) were bombarded with gamma radiation at a range of dosages between 0 and 950 Gy to induce mutation. Following treatment, over 1000 irradiated seeds were grown in a glasshouse at 25-30°C with 16 hours day length. M2 leaf samples were collected and genomic DNA was extracted utilising a Qiagen 96-well MagAttract kit on the MWG Theonyx® robotic platform.

Primer Design and PCR Amplification

The Genbank sequence accessions used for primer design are listed in Table 1. The PCR reaction volume was 15µl and contained 5ng DNA template, 0.2mM of each dNTP, 1.5mM $MgCl_2$, 0. 2µM of each forward and reverse primer , 0.5 units Platinum® Taq DNA polymerase (Gibco BRL, Invitrogen, Carlsbad, CA) and 1X Gibco® reaction buffer (minus $MgCl_2$). PCR cycling conditions were 94° C for 3 min, 5 cycles of 94 ° C for 30 s, 65 ° C for 45 s (decreasing by 1 ° C per cycle), 72 ° C for 1 min then 30 cycles of 94 ° C for 30 s, 60 ° C for 45 s and 72 ° C for 1min and a final extension at 72 ° C for 7 min. Amplified PCR products were purified before sequencing using 2ul ExoSAP-IT® (USB Corp.,Clevland, Ohio) for 10ul PCR product and incubating at 37 ° C for 15minutes; followed by incubating at 80 ° C for 15 minutes.

Sequencing reactions were carried out using the ABI Big Dye Terminator v3.1 kit (Applied Biosystems, Forster City, CA, USA) in both forward and reverse directions using 2-5 μl of purified product, 3.2 pmol primer, 1X Big Dye sequencing buffer and 0.5 Units BigDye enzyme. Sequencing reactions used an initial denaturation step of 96 °C for 2mins, followed by 30 cycles of 96 °C for 10s, 50 ° C for 5s and 60 °C for 4 mins. Purification of sequenced samples was performed using a standard sodium acetate/ ethanol precipitation protocol.

Sequence Alignment and analyses

Following sequencing all individual data was manually visualised and aligned using SEQUENCHER™ 4.0 Software (Gene Codes Corporation, Ann Arbor, MI, USA to check that there were no errors in the base call. SNP sites and indels were identified in Sequencher from the chromatograms, using both the forward and reverse sequence and by visualising mismatches to the consensus sequence. The consensus sequence was compared to DNA sequences available on the National Centre for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov/blast/) using BlastN and default settings.

RESULTS

Sequence Diversity

We have surveyed over 3.3 kb of the GBSS gene, 2.8 kb region of SBE IIb and 764bp region of SS IIa gene and found 21 single nucleotide polymorphisms (SNP) and 18 insertion/deletions (indels) in intronic regions and 12 SNPs in exonic regions in the genes of interest. The sequence variation revealed among all the putative mutant population is summarised in Table 1.

Table 1. Summary of results for the three genes surveyed.

Gene product	Abbrev.	Genbank Blast Reference/Area surveyed	Gene length (bp)	Region	Length sequence	SNP	INDEL
Granule bound starch synthase	**GBSS**	AF 079258.1 (SB 1410-4561)*	4548	C	1467	3 s / 2 ns	0
				NC	1850	9	8
				Total	3317	14	8
starch branching enzyme	**SBE IIb**	AY 304539.1 (SB 2803-3294)1F/2R	4596	C	885	2s / 3 ns	0
				NC	1920	12	10
		AF 0725725 (ZM 17909-19905)9F/10R	23449	Total	2805	17	10
soluble starch synthase	**SSIIa**	AF 419099 (OS.1990-2634)	2959	C	764	2 ns	0
				NC	0	0	0
				Total	764	2	0

*Concatamer of four primers GBS1F/1R; GBS 15F/17R; GBS 16F/27R and GBS 27F/19R
C-coding
NC- non-coding
s- synonymous,
ns- nonsynomymous
SB- *Sorghum bicolor*, ZM-*Zea mays*, OS-*Oryza sative*

GBSS I
In the GBSS I gene, a total of 22 polymorphisms, including 14 SNPs and 8 indels were found within a total of 3.3 kb of the GBSS I gene scored. Five SNPs occurred within exon regions and 9 SNPs occurred in intron regions, whilst the 8 indels were confined to intron regions. Of the 5 SNPs, three were synonymous with no change in the amino acid codon, whilst 2 were non synonymous with 2 altered amino acids encoded.

For the region amplified by GBS1F/1R there was a C→T SNP which is linked to other SNP and indel events further downstream for a length of 800 bases.

SBE IIb
For the SBE IIb gene, there were a total of 27 polymorphisms, including 17 SNPs and 10 indels within a length of 2.8kb sequenced. All 10 indels occurred in intron regions, and of the 17 SNPs, 5 were in coding region, of which 3 SNPs resulted in 3 nonsynonymous amino acid changes. There was an AA indel directly adjacent to a splice junction and was linked with other SNP and indel events for a length of 500bp.

SSIIa
Primers were designed from rice accession AF419099 SSIIa, exon 8 and were used to amplify a 764bp region (1880-2643), which was known to contain three SNPs related to either low or high gelatinisation temperature in rice. (Waters *et al*, 2006). This 764bp region had two C→T SNP's, and resulted in two non synonymous amino acid substitutions.

DISCUSSION
Whereas mutations, in the form of single nucleotide polymorphisms (SNP) and insertion/deletion (indel) events, predominantly occurred in non-coding introns, in total 12 mutations were also found in coding regions in the starch synthesis genes studied. Of these 12 SNPs, 7 polymorphisms resulted in synonymous amino acid substitutions with no change in the amino acid residues, while 5 polymorphisms resulted in 5 nonsynonymous substitutions in the amino acids in the starch synthesis genes of interest and which we hypothesise may lead to alternative proteins encoded and which may subsequently result in an altered starch phenotype in our mutant sorghum individuals.

In subsequent research, phenotypic analyses of some individuals with the altered genotypes will be evaluated and related back to the sequence variation in the gene of interest. Novel genetic variation may be utilised to enhance the nutritive value of sorghum by breeding through modification of endosperm composition.

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