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Contribution of granule bound starch synthase in kernel modification of quality protein maize

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Abstract

The role of *gbssI* and *gbssII* genes, encoding granule bound starch synthase enzyme I and II, respectively, in quality protein maize (QPM) were studied at different days after pollination (DAP). Total RNA was used for first strand cDNA synthesis using the ImpromIISript™ reverse transcriptase. No detectable levels of *gbssI* were observed. The results showed a gradual increase in expression of *gbssII* from 6 to 30 DAPS and *gbss* expression was higher in non-QPM than QPM material. Modification level 1 and 2 had lower expression levels than level 4. In general, grain endosperm opaqueness modification is rated on a scale of 1(completely hard / vitreous) to 5 (soft opaque). Overall, this study showed that *gbss* activity, based on its isoform *gbssII* was high at 30 DAP when maximum accumulation of amylose occurs. It was generally compromised in QPM than in non-QPM materials and appeared to be influenced by opaque donor parent. Protein analysis revealed a mar ked increase of GBSS in both QPM and non QPM. There was a higher proportion of GBSSII than I extracted from developing endosperm in both QPM and non QPM. At 18 DAP, the amounts of extractable GBSSII and I were lower in modified maize line 4 comparable to amounts in level 1 modified germplasm. In contrast, at 30 DAP; the GBSSII and I protein quantities at level 2 were lower than level 4, but comparable to level 3. The major finding was that transcript use was unreliable because the genes under study (*gbssI* and *II*) were influenced by other non-allelic interactions. The stability of both GBSS and zein proteins coupled with the use of SDS-PAGE implied that only one was more reliable and required further validation. It was clear from this study, that kernel modification was regulated by complex genetic interactions. Fairly distinct systems such as the starch synthases, zein proteins and perhaps other non-allelic genetic systems all appeared to act in a complementary manner to regulate kernel texture.

Key words: Amylose, protein maize, zein proteins

Introduction

Quality protein maize (QPM) is derived from natural mutations that increase the level of lysine and tryptophan in maize grain. The rate of QPM development has been slowed down due to frequently poor kernel texture associated with opaque-2. Identification of genes (opaque-2 modifiers) with ability to overcome negative effects of the mutation, while maintaining high nutritional value, paved way for development of commercially attractive high lysine maize genotypes (Paez *et al*., 1969). These have been used to develop modified opaque-2 genotypes. Screening for opaque-2 modifiers by conventional means has been difficult and poses a big challenge to large-scale adoption of the technology (Bjarnson and

Vasal, 1992). The biochemical basis of modified kernel texture in quality protein maize (QPM) is poorly understood. Recent proteomic analysis of several QPM lines indicate increased levels of granule-bound starch synthase I (GBSSI) in the soluble non-zein protein fraction of opaque-2 modified genotypes compared to wild type (Hunter *et al.*, 2002; Gibbon *et al.*, 2003). The report by Gibbon *et al.* (2003) suggests that GBSSI conditions similar counteractive effects on the opaque-2 mutation in a similar manner to the 27 kDa γ–zein class gene. The zein gene counteracts the effects of opaque-2 which interferes with normal transcriptional process by restoring processes that create hard kernels such as filling of empty spaces by gamma-zein-rich protein bodies creating a vitreous kernel phenotype (Dannenhoffer *et al.*, 1995). In the case of GBSSI, normal kernel type is restored through altered enzyme activity which results in cohesion of maize starch granules and hard normal kernel (Hunter *et al.*, 2002). Although conventional breeding procedures have been used successfully to convert commercial lines to QPM, the procedure is highly inefficient and is not directly oriented toward improvement of grain quality but yield. Genetic markers could be used to identify unknown modifiers that contribute to QPM phenotype, both the efficiency and potential for grain quality improvement would be significantly increased. *Granule bound starch synthase* was a candidate gene in this study because of the role it plays in starch synthesis. Therefore this study aimed at understanding the role of *gbssI* in modification of kernel phenotype and its potential use in MAS to facilitate QPM breeding.

Materials and methods

Plant materials

The materials used were the CIMMIT maize lines CML 395, CML 176, CML 173 and the inbred line 136R , and their F3 and F4 progeny with varying levels of kernel modification 1, 2, 3, and 4 (Table 1). Quality protein maize lines (QPM), non opaque-2, and locally adapted maize lines were used. The wild type, non-QPM maize lines included those with and without good modifier gene effects. The QPM genotypes included F3 populations derived from crosses between the QPM inbred

Table 1. Parental materials, their progeny and levels of modification as used in the study

Material	QPM status	Level modification	Generation
CML 395	Non QPM	-	parent[a]
136R	Non QPM	-	parent[a]
CML173	QPM	(2)	parent[a]
CML 176	QPM	(2)	parent[a]
CML395xCML176	QPM	(1)	F_3 progeny
CML395xCML176	QPM	(2)	F_4progeny
CML395xCML173	QPM	(4)	F_4 progeny
CML176x136R	QPM	(2)	F_4 progeny

[a] = these are advanced inbred lines. Genotypes with CML refer to CIMMYT maize lines and 136R is an inbred line derived from an open pollinated maize line, by NaCRRI

line CML 176 crossed with a non QPM recurrent parent 136R and CML395; and F4 populations derived from crosses between CML176/136R and CML395/CML173 (Okello *et al*., 2006). CML 176 is a quality protein maize line donor from CIMMYT that was characterised to be a suitable opaque-2 donor (Okello *et al*., 2006). The maize line 136R is a recycled inbred from the Uganda National Cereals Programme at NaCRRI, improved for resistance to maize streak virus disease, gray leaf spot (*Cercospora zeae-maydis*), Turcicum leaf blight (*Exserohilum turcicum*) and drought resistance (Okello *et al*., 2006).

Experimentation

The experiment was conducted at the National Crops Resources Research Institute. The experimental plots were planted with inbred lines and their progeny. These materials were earlier sorted on a light table to obtain the various levels of modification (1, 2, 3, 4, and 5) based on translucency (Pixley and Bjarnason, 2002). The experiment was established following a randomised complete block design with four plots of size 12 x 6 m with a spacing of 75 x 30 cm. Each maize line was randomised in each plot and replicated twice. The parental lines were established on separate plots. The trial was conducted during the first rain season between April and May 2006. Standard agronomic practices were carried out including regular weeding. At anthesis, the tassels were bagged with pollination bags to collect pollen and the emerging cobs were bagged with polythene sleeves to control extraneous sources of pollen. At silking, the plants were hand pollinated. Six days after pollination (DAP); ears were harvested from each maize line and immediately frozen in liquid nitrogen. Sampling continued at 4-day intervals until 30 DAP. Sampling was also specifically done at 18 DAP, the stage of development when most endosperm cells are differentiated and have high levels of gene expression, as reflected by large amounts of starch and storage protein synthesis (Demason, 1997). This sampling procedure was used to capture the spectrum of enzymatic activities especially starch synthases.

RNA extraction

Kernels from the middle of the cobs were selected and ground into a fine powder using a mortar and pestle. Total RNA was extracted from the seeds using a modified sarkosyl method (Baguma *et al.,* 2003). RNA concentrations were determined using the NanoDrop® ND-1000 spectrophotometer (NanoDrop Technologies Inc. Wilmington, De, U.S.A.) and quality determined by reading absorbance at 260 and 280 nm (i.e. 260:280 ratio).

cDNA synthesis

2 µl of total RNA were used for first-strand cDNA synthesis using the ImpromIIScriptTM reverse transcriptase (Promega, Madison, WI, USA) as recommended by the manufacturer and stored at -20°C until use. The cDNA was quantified using the NanoDrop® ND-1000 Spectrophotometer (NanoDrop Technologies Inc. Wilmington, De, U.S.A.) and aliquots of the first-strand cDNA diluted to a uniform concentration of 5 ng/µl. 2 µl of each sample (10 ng) cDNA were served as templates for semi-quantitative PCR analysis.

Gene specific probes for maize *gbssI* and *gbssII*

Primers to amplify gene specific probes for *gbssI* and *gbssII* were designed based on existing database sequences of respective full length cDNAs to maximise variant detection and binding specificity. Homologous cDNAs probes were reverse transcribed from RNA. The sense and anti-sense primers were 5'-CCACAACCACCAGCGGAACC-3' and 5'-GTAGGAGTGGAAGAAGCGGA-3' for gbss*I* (523 bp) and, 5'-GGGCTGTCGCACTTGTGAGA-3' and 5'-GACCCATCACCACTAACGCC-3' for *gbssII* (225 bp).

cDNA quality assessment

The *actin* gene was used as an internal control for monitoring the quality of cDNA. House keeping genes, *actin* inclusive, are constitutively expressed in the plant cells (Stürzenbaum and Kille, 2001) and can be used in assessing cDNA quality and in normalisation of expression data. The forward primer for the *actin* gene used in this study was 5'-CCCAAGGCAAACCCGAGAGAAG-3' and 5'-GTGGCTCACACCATCACCAG-3'.

Semi-quantitative PCR

Polymerase chain reaction was as follows: initial denaturation 94°C, 2 m; 35 cycles 94°C,1 min, 58°C ,30 s, and 72°C 1 min and a final extension 72°C, 7 min. The PCR was performed in a GeneAmp® PCR System 9700 (Applied Biosystems, Foster City,CA, USA). The amplification products were separated on 3% agarose gels and visualised by ultra violet light (UV) irradiation. The radio graphed gel pictures were scanned using the UMAXTM GS 800 Scanner/ Densitometer (Bio-Rad, Hercules, Ca, U.S.A) and the scanned pictures fed into the ImageQuant TLTM 2005 version (Amersham Biosciences, U.K.) for band intensity analysis and quantification.

Total protein extraction and analysis

Total proteins were extracted with a 3X SDS- PAGE sample buffer as described (Schmidt, 1993). The zein fraction of seed proteins were recovered by extracting with 70% ethanol and 10 mM DTT (1ml per 200 mg sample) (Schmidt, 1993). Protein quantification was done using the spectrophotometer. Electrophoresis was run at 200 V constant voltage in a Criterion TM Cell with 250 kDa Precision Plus Dual Class Standards TM (Bio-Rad, Hercules, Ca, U.S.A.) in 1X MOPS buffer (0.2 M MOPS, 0.05 M sodium acetate, 0.01 M EDTA) until the dye front reached the bottom. The gels were stained overnight on a shaker in 10 gel volumes of 1X Coommasie blue stain solution (Bio-Rad, Hercules, and Ca, U.S.A.). De-staining was done using a 1X R-250 coommasie de-stain until the desired band intensity was attained (Bio-Rad, Hercules, and Ca, U.S.A.).

Results and discussion

Analysis of cDNA quality using actin gene as control

The *actin* gene was used as an internal control for assessing cDNA quality and for normalising expression data. The expression of the *actin* gene was fairly constant in all the test lines during endosperm development (Table 2, Fig. 1). Because of the near uniform expression levels of actin, a second internal control was unnecessary.

Table 2. *Actin* expression in variously modified maize backgrounds during endosperm development

Test lines	DAP	Average band volume
P1 (CML395)	6	7012
1 (CML395/CML176)	6	6988
2 (CML395/CML176))	6	7089
P2 (136R)	18	7083
P1 (CML395)	18	7102
P3 (CML176)	18	7024
1 (CML395/CML176)	18	6996
2 (CML395/CML176)	18	7129
4 (CML395/CML173)	18	7105
P1 (CML395)	28	7103
P2 (136R)	30	7112
2 (CML395/CML176	30	7023

1-4 = Level of kernel modification; P1-P3 = Are parents.CML176 and CML173 are QPM inbred lines. CML 395 is a normal maize inbred line known to have some good traits. It has a semi-dent, big conical white grain that is popular with local farmers. 136R is an inbred line derived from an open pollinated maize line. It is developed by the National Cereals' Programme

Transcript profiling of *gbssII*

Transcript analysis of *gbssI* and *gbssII* were done from 6 to 30 DAP. *gbss I* was not detectable in all the samples.The results showed that there was a gradual increase in expression of *gbssII* from 6 DAP to 30 DAP, *gbssII* expression was higher in non-QPM than QPM material and modification level 1 and 2 had lower expression levels than 4 (Fig. 2). Overall, this study showed that *gbss* activity as investigated, based on its isoform *gbssII* was high at 30 DAP when maximum accumulation of amylose occured but is in general compromised in QPM than in non-QPM materials and appeared to be influenced by opaque donor parent. The data confirmed earlier reports of increased expression of *gbss* in developing endosperm. Those studies show that in storage organs, amylose is typically 26-30 % with most of it synthesised from 14-30 DAP (Tsai *et al*., 1970; Denyer *et al*., 2001). The activities of GBSS however appeared compromised by other proteins as alluded by the proportionately low expression levels. Other studies have

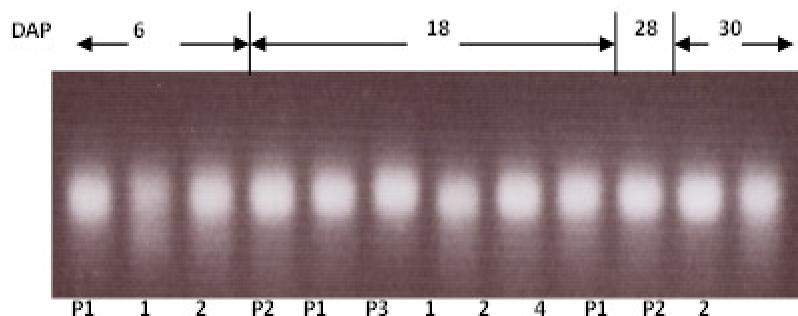

Figure 1. Expression patterns of *actin* gene in the different maize genotypes at different days after pollination.

shown that GBSS has capacity to synthesise amylose and take part in amylopectin metabolism (Denyer *et al.*, 2001). The dual capacity of GBSS, coupled with the negative pleiotropic effects of opaque mutations and the fact that starch synthases interact in complexes to elongate and shape starch molecules account for observations in this study.

Amounts of extractable GBSS in developing maize endosperm

The results showed that GBSS, GBSSI and II proteins were significantly expressed across all the parental lines and progeny used. It was evident that the fluctuations in transcript accumulation did translate to a corresponding oscillation in protein levels. Protein analysis revealed that there was a marked increase of GBSS in both QPM and non QPM (Fig. 3). There was a higher proportion of GBSSII than I extracted from developing endosperm in both QPM and non QPM. The amounts of extractable GBSSII and I were low in modified maize line 4 and were comparable to level 1 modified germplasm. At 30 DAP; level 2 was lower than level 4, but comparable to level 3 (Fig. 3). GBSSI and GBSSII proteins matched *gbss* transcript. The difference, however, is that GBSSII, was detected at transcript and protein level whereas GBSSI *was* only detectable at the protein level. In contrast, Gibbon *et al.* (2005) reported no difference in *gbssI* expression in QPM and non QPM material. They did not study the second isoform of *gbssII* as in this study This study however shows that *gbssII* was expressed at relatively higher levels than *gbssI*. The role of *gbssII* in starch biosynthesis is not known although it is believed to complement *gbssI* activities. Recent evidence suggests that amylose synthesis is responsive to follow besides *gbssI* (Denyer *et al.*, 2001); with

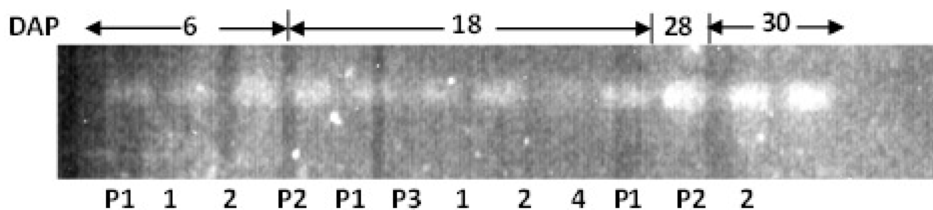

Figure 2. *gbssII* expression patterns in modified maize.

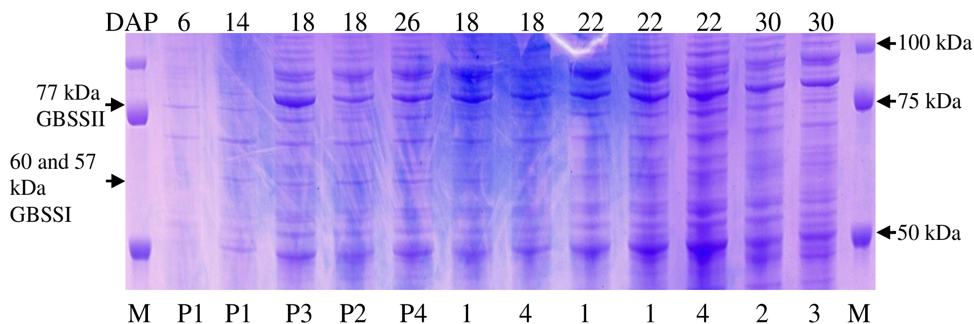

Figure 3. SDS-PAGE analysis of GBSSI and GBSSII proteins at various days after pollination.

isoform *gbssI* as well as *gbssII* playing a role (James *et al*., 2003). Taken together this study provides evidence of stronger role of *gbssII* in starch biosynthesis in maize in general and in QPM in particular. Further studies involving mutants are however still necessary to further elucidate the exact interactions and role of *gbssII*.

Comparison of GBSS with other storage proteins

Temporal analysis of total seed protein partitioning into zein were highly variable although data suggests even at an early stage, the seed begins to partition specific zein classes into different fractions (Fig. 4). In all seeds, the electrophoretic spectra

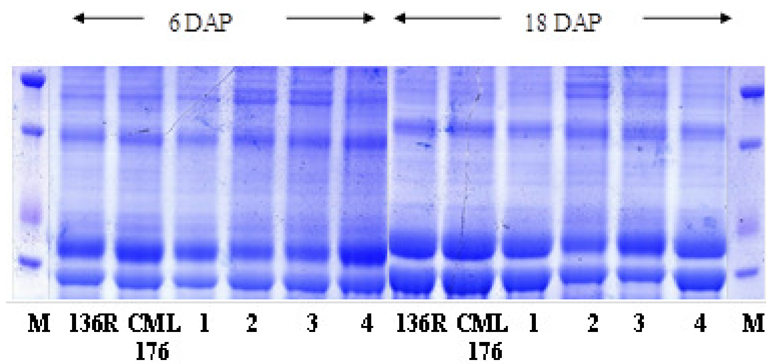

Figure 4. SDS-PAGE analysis of zein proteins at 6 and 18 days after pollination.The numbers 1-4 indicates the level of modification; M-protein standards; 136R and CML176 are the inbreds non-QPM and QPM respectively.

Table 3. Quantified expression levels of *gbssII* based on time course experiments in QPM and non QPM maize lines

Plant materials	Level of modification	DAP	Average band volume	Normalised value	Transcript ratio
CML 395	Non QPM	6	18641.39	2.66	1
CML 395/CML176	1	6	16965.39	2.43	0.91
CML395/CML176	2	6	17605.76	2.48	0.93
136R	Non QPM	18	12432.54	1.76	0.66
CML 395	Non QPM	18	9683.98	1.36	0.51
CML176	QPM	18	4790.98	0.68	0.26
CML 395/CML176	1	18	7632.49	1.09	0.41
CML395/CML176	2	18	5270.2	0.74	0.28
CML395/CML173	4	18	11420.93	1.61	0.6
CML395	QPM	28	3659.61	0.52	0.19
136R	Non QPM	30	17898.02	2.52	0.94
CML 395/CML176	2	30	8568.79	1.22	0.46

1-4 = Level of kernel modification as determined by light table analysis (Pixley and Bjarnason, 2002); P1-P3 = Are parents

for the zeins had a large proportion of low molecular weight proteins. More prominent bands occurred below 25 kDa than at 37 kDa. At 6 DAP; there was a gradual increase in zein protein expression from modification level 1 to 4. At 18 DAP, zein protein expression in modification level 1&4 were indistinguishable from modification level 2&3. Zein protein expression was very similar in modification level 1&4. Similar trend was also observed for modification level 2&3. No differences were noted between the QPM line CML 176 and non QPM line 136 R at both 6 and 18 DAP. In general there was more expression of proteins below 25kDa. The minute amounts of protein detected could have been due to posttranscriptional modifications and probably rapid protein decay rendering the original forms undetectable. The abundance of zein proteins especially below 27 kDa function alludes to their potential role in kernel texture of QPM. Zein proteins particularly the 27 kDa has been shown to increase 2-3 folds on well modified QPM (Dannenhoffer *et al.,* 1995) albeit, the roles of other zeins especially smaller fractions still remains to be elucidated. Thus, the actual relationship between modified starches as conditioned by starch synthases and role of zeins in compacting starch grains in the endosperm remains largely unknown. This is an area that needs further investigation.

Conclusion

The major finding of the study was that transcript use was unreliable because the genes (*gbss I* and *II*) are influenced by other non-allelic interactions. This coupled with the fact that the extraction and handling of RNA can be challenging, may compound the use of gbss transcript in marker assisted selection for quality protein maize. In this study the earlier proposed use of GBSS and zein proteins as potential molecular markers for QPM marker assisted selection has been demonstrated. The stability of both GBSS and zein proteins coupled with the use of SDS-PAGE imply that only one is more reliable and requires further validation. It was clear from this study and others, that kernel modification is regulated by complex genetic interactions. There is therefore a need for further studies to elucidate the interactive effects of three systems, affecting amylose, amylopectin and zein protein synthesis.

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