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Biochemical analysis and validation of molecular markers for identification of quality protein maize

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ABSTRACT

Twenty two inbreds of maize comprising ten OQL(Odisha QPM Lines), ten non-QPM and two standard QPM inbreds (CML 176 and CML 186) were tested for crude protein, tryptophan and lysine content in seed. Seed protein in OQL inbreds was qualitatively rich in tryptophan and lysine content (0.77% and 3.13%), and it was nearly double as compared to non-QPM inbreds (0.40% and 1.61%). OQL 17-3 and OQL 42-9 had total crude seed protein > 8% and harbour high tryptophan (> 0.90%) and lysine content (> 3.60%). These may serve as valuable material for development of QPM hybrids. PCR products amplified by phi 057 primer in non-QPM and QPM inbreds co-segregated with the expected variation in lysine and tryptophan content. Thus, the marker phi 057 could be reliably used for marker-assisted selection to track inheritance of QPM status.

1. Introduction

Among cereals, maize (*Zea mays* L.) ranked high after wheat and rice (Sleper and Poehlman, 2006) in terms of production. It has great worldwide significance as human food, animal feed and as a source of a large number of industrial products. At the global level, maize accounts for 15% of proteins and 20% of calories in the world food diet. Seed storage proteins in maize serve as an important nutrient source for human and livestock. On average, maize grains contain 7–9% protein (Sofi et al., 2009). A Major fraction (60%) of seed protein in maize is zeins (a prolamin group) (Leite et al., 1999; Freitas et al., 2005) followed by glutelin (34%), while albumin and globulin occur in trace amounts (3% each). Zeins are the products of multigene families (Lending and Larkins, 1989) and located within protein bodies on the rough endoplasmic reticulum. Amino acid balance determines the quality of any food and feed. Zein is particularly rich in glutamic acid (21–26%), leucine (20%), proline (10%) and alanine (10%), but deficient in important essential amino acids e.g., lysine and tryptophan, leading to protein malnutrition. α- zeins are the major prolamin subunits in maize (Vasal, 1999), although other minor groups (β, γ and σ -zeins) (Coleman and Larkins, 1999; Leite et al., 1999) are also present in seeds. In normal maize, α- zeins consist of two major sub-classes e.g., 19kD and 22kD zeins. Several mutants (*opaque 2*, *floury-2* and *De B30*) encode a defective signal peptide (transcriptional factor) which is known to change the amino acid profile in endosperm seed protein. In this context, the *opaque 2* (o_2)-a natural recessive mutation encoding a defective basic-domain-leucine-zipper transcription factor in short arm of chromosome 7(Gibbon and Larkins, 2005) led to nearly double the lysine and tryptophan content in maize endosperm (Mertz et al., 1964; Vasal, 1994; Krivanek et al., 2007; Gupta et al., 2009) due to a decrease in the synthesis of zein protein (particularly 22kD α-zein fraction) from 47.2% (in normal maize) to 22.8% (in o_2 mutants). The increased level of lysine in o_2 mutants is due to higher levels of an elongation factor in protein synthesis (eEF1A). The recent studies using RNA interference based down regulation of 22kD RNAi lines are reported to be a more severe opaque phenotype as compared to the 19kD component (Holding, 2014). However, the pleiotropic effects of *opaque 2* mutation made the endosperm chalky and soft (starch loosely packed with lot of air spaces) resulting in reduced kernel weight and more damaged kernel while harvesting, an increased susceptibility to pests and diseases, inferiority for food processing and in general, reduced yield. For many years, this became the major bottleneck in genetic improvement for higher productivity with enriched nutritional quality. However, later remedial strategies entail improving the hardness of the kernel and grain yield by modified backcrosses and recurrent selection. In this context, molecular marker based screening for QPM status coupled with

Abbreviations: QPM, quality protein maize; OQL, Odisha QPM line; PCR, polymeric chain reaction; kD, kilodalton; OML, Odisha maize line; CIMMYT, Centro Internacional de Mejoramiento de Maiz y Trigo(International Maize and Wheat Improvement Center); pH, hydrogen potential; Tris–HCl, tris(hydroxymethyl)aminomethane hydrochloride; EDTA, ethylene diamine tetra-acetic acid; β-ME, β-Mercaptoethanol; SDS, sodium dodecyl sulphate; dNTPs, deoxynucleotide phosphate; rpm, revolution per minute; bp, base pair; RAPD, random amplified polymorphic DNA; ISSR, inter simple sequence repeat; SSR, simple sequence repeat

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phenotypic selection for improving endosperm characteristics was adopted as a strategy for development of improved QPM lines. Vasal et al. (1980) combined the *opaque-2* maize with genetic modifiers and produced qualitatively superior maize germplasm with hard kernels and with a much higher quantity of lysine and tryptophan. This resulted in the birth of Quality protein maize (QPM) (Vivek et al., 2008) which paves the way for availability of a good number of nutritionally enriched food products for children and adults in the market (Atlin et al., 2011). Therefore, an attempt was undertaken to screen a set of maize inbred lines for QPM status using molecular markers to constitute QPM hybrids for a future breeding programme.

2. Materials & methods

2.1. Plant materials

A set of 88 QPM maize populations were initially received from the Directorate of Maize Research, New Delhi and CIMMYT Centre, Hyderabad, India. Fifty eight inbreds were developed by an ear to row method and subsequently further purified and maintained by selfing at Economic Botany-II section, Department of Plant Breeding and Genetics, College of Agriculture, Orissa University of Agriculture & Technology, Bhubaneswar. Out of these, ten inbreds with protein content > 8%, high lysine (> 2.6%) and tryptophan (> 0.6%) content were selected. These pure breeding quality protein maize (QPM) lines (designated as OQL: Odisha QPM lines) along with ten normal (Non-QPM) inbreds (designated as OML: Odisha maize lines) and two standard CIMMYT inbreds (CML 176 and CML186) with known QPM status were laid out in randomized block design (RBD) with three replications following a standard row to row (60 cm) and plant to plant (25 cm) spacing.

Three random seed samples of these lines per replication were considered for biochemical analysis separately in duplicate with respective standard checks to minimize experimental error in a Colorimeter (Spectron 20). The endosperm separated from embryo in each seed was ground to fine powder and defatted for 48 h with n-hexane in Soxhlet apparatus. The dry defatted samples were subjected to estimation of protein, tryptophan and lysine content.

Protein content was estimated using Folin-Ciocalteu reagent (Bailey, 1967). The color yield of protein was estimated at 500 nm using a colorimetric method. Protein concentration was determined by referring the O.D. obtained for the sample versus a standard curve using bovine serum albumin (BSA).

Tryptophan content was determined using the colorimetric method (Nurit et al., 2009; Vivek et al., 2008). The color was developed in a reaction of flour hydrolysate (obtained by overnight digestion with papain solution at 65 °C) with 2 ml of reagent containing 56 mg of Fe3+ dissolved in 1 l of glacial acetic acid and 2 ml of 15 M H2SO4. After incubation at 65 °C for 15 min, absorbance was read at 560 nm. Tryptophan content was calculated using a standard calibration curve, developed with the known amounts of tryptophan, ranging from 0 to 30 μg/ml. Finally, tryptophan content of each sample was expressed as a percentage of total crude protein in seed.

Lysine content in seed was estimated by nitropyridine method as per Tsai et al. (1972). The method utilizes 2-chloro 3, 5- dinitropyridine as a reagent for the Є-amino group of lysine after enzymatic hydrolysis of the proteins and blocking the α-amino groups of free amino acids with copper. After acidification, the absorbance of the resultant Є-dinitropyridyl-lysine was measured at 400 nm. A standard calibration curve, developed with known varying amounts of LysineHCl (0 to 250 μg/ml) added to a sample. The added lysine raised the absorbance in a manner linearly proportional to the amount of lysine added. Finally, the lysine content of each sample was calculated using the standard calibration curve and expressed as percentage of total crude protein in seed.

2.2. Isolation of DNA

Genomic DNA of each inbred was isolated from tender young leaves at seedling stage on the same day of collection using a standard sodium dodecyl sulphate method (Dellaporta et al., 1983). The plant materials were homogenized in liquid nitrogen and extracted with extraction buffer(100 mM Tris-HCl pH 8.0, 20 mM EDTA, 0.5 M NaCl, 7 M Urea, 0.1% β-ME and 2% SDS) at 65 °C for one hour with occasional shaking and an equivalent volume of phenol-chloroform-isoamyl alcohol (25:24:1) mixture was added and centrifuged at 10000 rpm for 10 min. at 4 °C. The supernatant was mixed with an equal amount of ice cold absolute ethanol and kept at −20 °C overnight to precipitate DNA. The intact genomic DNA was hooked out and washed with 70% ethanol 2–3 times and finally re-dissolved in TE buffer (10 mM Tris-HCl, pH-8.0 and 1 mM EDTA). The DNA was purified by DNase free RNase-A (GeNei) @ 20 μg per ml. of DNA extract to remove contaminating RNAs. Finally the DNA was quantified through a UV–vis Nanodrop-2000 spectrophotometer (Thermo Electron Scientific Instruments LLC, USA) at 260 nm and the quality of DNA was checked using the ratio of absorbance at 260 nm and 280 nm. Each DNA sample was diluted to a working concentration of 10 ng/μl for PCR analysis.

2.3. PCR analysis

Genomic DNA samples were primed and amplified using *opaque 2* gene specific primers e.g., phi 057(F: 5′-CTCATCAGTGCCGTCGTC CAT-3′ and R: 5′CAGTCGCAAGAAACCGTTGCC3′), umc1066 (F: 5′ ATGGAGCACGTCATCTCAATGG-3′ and R: 5′-AGCAGCAGCAACGTCTA TGACACT-3′) and phi112 (F: 5′TGCCCTGCAGGTTCACATTGAGT3′ and R: 5′-AGGAGTACGCTTGGATGCT CTTC3′)(www.idtdna.com). PCR amplification was performed in a reaction volume 25 μl containing 1 × reaction buffer (10 mM Tris HCl, pH 9.0, 1.5 mM $MgCl_2$, 50 mM KCl, 0.01% gelatin), 2.5 mM each of dNTPs, 10 ng of random primer pair, 20 ng of genomic DNA and 1 unit of Taq polymerase (Genei, Bangalore). DNA amplification was carried out in the Gene Pro Thermocycler (Bioer Tech. Co., Ltd., Japan), programmed for 5 min at 94 °C for initial denaturation, 40 cycles of 1 min at 94 °C for denaturation, 1 min at 60 °C for annealing and 2 min at 72 °C for synthesis; a final extension for 4 min at 72 °C followed by storing at 4 °C till loading to the agarose gel. The amplified products were loaded in a 2.5% agarose gel containing 0.5 mg/ml of ethidium bromide and electrophoresed at a constant voltage (80 V). PCR conditions were optimized to yield a reproducible result. The gels were scanned by a gel doc system (Fire Reader-Uvtec, Cambridge, UK) for detection of *opaque 2* gene specific alleles. The size of the amplicons was determined by comparing with the lambda DNA ladder (50 bp) with known size (bp) fragments.

3. Results and discussion

Seed protein content and status of two important essential amino acids e.g., tryptophan and lysine (in seed) in twenty two inbreds are presented in Table 1. Total seed storage protein content showed a wide range of variation (7.25 to 9.09%). It ranged from 7.25% to 8.80% in normal maize lines and 8.05 to 9.09% in new QPM test entries versus 8.2 to 8.5% in standard QPM inbreds (CML 176 and CML 186). On average, the newly developed OQL quality protein maize inbreds showed a 5% increase in protein content (8.34%) over the normal maize lines (7.94%) and it was almost equivalent to standard QPM inbreds (8.35%). In the present investigation, OML 68–7 (normal maize) and OQL 176-17-3(QPM) had the maximum seed storage protein content (8.80% and 9.09% respectively). Vivek et al. (2008) reported protein content was at least 8% in a set of CIMMYT QPM inbreds.

As a crop, maize is deficient in tryptophan and lysine. In the present investigation, normal (non-QPM) maize had 0.4% tryptophan and