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Development of *opaque-2* introgression line in maize using marker assisted backcross breeding

Swapan K. Tripathy[a,*], Devraj Lenka[b], Dinesh Manohar Ithape[a], Manasmita Maharana[a]

[a] Department of Agricultural Biotechnology, College of Agriculture, OUAT, Bhubaneswar 751003, India
[b] Department of Plant Breeding and Genetics, College of Agriculture, OUAT, Bhubaneswar 751003, India

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ABSTRACT

Two elite normal maize inbreds that produced heterotic experimental hybrid (OML 17-3 × OML 42-9) with 45% yield advantage over the standard check Vivek QPM 9, were used for conversion to QPM status using *opaque-2* donors (CML 176 and CML 186) using marker assisted back cross breeding. Each of the normal maize parental inbreds was used as female parent in separate conversion programme using cross combination OML 17-3 × CML 176 and OML 42-9 × CML 186. Foreground selection (using *opaque-2* specific marker phi 057) combined with phenotypic selection for recipient parent at early back cross generations (BC_1 to BC_3) was carried out for rapid recovery of recurrent parent genotype. The BC_3F_1 plants showing heterozygous status (O_2o_2) were selfed and advanced to BC_3F_2 to enable for identification of homozygous recessive *opaque-2* segregants at seedling stage. The most promising BC_3F_3 introgression lines from either cross combinations were finally selected based on higher tryptophan and lysine content. The newly developed introgression lines (OQL 176-17-3 and OQL 186-42-9) were at par with the QPM donors (CML 176 and CML 186) and nearly double the lysine and tryptophan content as compared to respective normal inbreds (OML 17-3 and OML 42-9). These may serve as valuable breeding materials for development of QPM hybrids.

1. Introduction

Maize is consumed by more than a billion people in many countries (Gupta et al., 2009). Seed proteins in maize serve as the important nutrient source for human and livestock. On an average, maize grains contain 7–9% seed proteins (Sofi et al., 2009) and ~70% of these are classified as seed storage proteins (Flint-Garcia et al., 2009). Major fraction (60%) of seed storage protein in maize is zein (a prolamin group) (Leite et al., 1999) followed by glutelin (34%), while albumin and globulin occur in traces (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. Zeins comprise four subfamilies, α (19 and 22 kD), β (15 kD), γ (50, 27, and 16 kD) and δ (18 and 10 kD) (Esen and Stetler, 1987; Coleman and Larkins, 1999). Among these, α-zeins are the major prolamin subunits in maize due to high expression of α-zein genes in endosperm. A common feature of α-zeins is abundant internal tandem repeats of proline and glutamine which makes it deficient in essential amino acids like lysine and tryptophan leading to over all protein malnutrition. Therefore, maize cannot serve as a balanced dietary protein source for humans and monogastric animals (Mertz et al., 1964). Several spontaneous and induced mutations that affect amino acid composition in maize have been discovered. The *opaque 2* (o_2)-a natural recessive mutation in maize led to nearly double the lysine and tryptophan content in endosperm (Vasal, 1994; Krivanek et al., 2007) due to decrease in the synthesis of zein proteins and increase in the other seed protein bound lysine and tryptophan. The recent studies using RNA interference based down regulation of 22 kD RNAi lines are reported to be more profoundly caused opaque phenotype as compared to 19 kD component(Holding, 2014). However, the *opaque-2* mutation made the endosperm chalky and soft resulting damaged kernel while harvesting,

Abbreviations: OML, Odisha maize line; QPM, quality protein maize; CIMMYT, Centro Internacional de Mejoramiento de Maiz y Trigo (International Maize and Wheat Improvement Center); CML, CIMMYT maize line; F_1, first filial generation; F_1 and F_2, first and second filial generation; BC_1 to BC_3, first to third back cross generation; OQL, Odisha QPM line; MAS, marker assisted selection; kD, kilo Dalton; RNAi, ribonucleic acid interference; O_2 and o_2, *opaque-2* alleles; QTL, quantitative trait loci; DNA, deoxyribonucleic acid; CTAB, Cetyl trimethylammonium bromide; Tris–HCl, tris(hydroxymethyl)aminomethane hydrochloride; pH, hydrogen ion potential; EDTA, ethylenediaminetetraacetic; NaCl, sodium chloride; β-ME, β-Mercaptoethanol; PVP, polyvinyl pyrrolidone; HCl, hydrochloric acid; $MgCl_2$, magnesium chloride; rpm, revolution per minute; dNTPs, deoxynucleotide phosphates; PCR, polymeric chain reaction; RAPD, random amplified polymorphic DNA; ISSR, inter simple sequence repeat; SSR, simple sequence repeat; SNP, single nucleotide polymorphism; bp, base pair; RFLP, restriction fragment length polymorphism; cDNA, copy deoxyribonucleic acid; χ^2 and P-value, chi-square and probability value

* Corresponding author.
E-mail address: swapankumartripathy@gmail.com (S.K. Tripathy).

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poor germination, increased susceptibility to pest 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. Subsequently, the *opaque 2* mutation is reported to be associated with numerous modifiers which togatherly behave as polygenic trait for kernel vitreousness. The identity and mode of action of the o_2 modifiers are still unclear. Two major QTLs associated with endosperm modification have been identified near the centromere and telomere, respectively, on the long arm of chromosome 7(Holding and Larkins, 2008) while the short arm of the same chromosome contains the o_2 mutation (Sofi et al., 2009). Vasal et al. (1980) combined the *opaque-2* allele with QTLs for genetic modifiers and produced elite germplasm with hard kernel and much higher quantity of lysine and tryptophan. This results 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). Since, *opaque-2* is a recessive mutation and endosperm specific, and biochemical analysis of lysine and tryptophan content is expensive; conventional backcross breeding alone is not efficient for nutritional enrichment of maize. However, use of *opaque-2* gene specific markers provided excellent opportunities for conversion of elite inbreds to homozygous o_2/o_2 forms through marker assisted selection (MAS). In India, Vivek QPM-9: a hybrid of two QPM introgression lines was released in 2008 (Gupta et al., 2009) for commercial cultivation. Therefore, a major emphasis was laid on conversion of normal corn genotypes to *opaque-2* versions using marker assisted back cross breeding.

2. Materials & methods

2.1. Plant materials

Two elite normal maize inbreds that produced heterotic experimental hybrid (OML 17-3 × OML 42-9) with 45% yield advantage over the standard check Vivek QPM 9, were used for conversion to QPM status using *opaque-2* donors (CML 176 and CML 186) received from CIMMYT centre, Hyderabad, India. Each of the normal maize parental inbreds was used as female parent in separate conversion programme using cross combination OML 17-3 × CML 176 and OML 42-9 × CML 186. Only the heterozygotes reliably identified by *opaque-2* gene specific primer phi 057 were back crossed to the recurrent parent (normal inbred) till BC_3 to allow rapid recurrent parent genome recovery along with retaining targeted allele (o_2). The BC_3F_1 plants were selfed to advance to BC_3F_2 to identify homozygous recessive *opaque-2* segregants at seedling stage using primer phi 057. These homozygous o_2o_2 plants derived from each of the back cross OML 17-3 × CML 176 and OML 42-9 × CML 186 were further selfed to raise uniform BC_3F_2 families to be used as QPM version of OML 17-3 (designated as OQL 176-17-3) and OML 42-9 (designated as OQL 186-42-9) respectively.

2.2. Isolation of DNA

Genomic DNA of single plant was isolated from tender young leaves at seedling stage on the same day of collection using standard CTAB method. The plant materials were homogenized in liquid nitrogen and extracted with extraction buffer (100 mM Tris-HCl pH 8.0, 20 mM EDTA, 2% CTAB, 1.4 M NaCl, 0.4% β-ME and 2% PVP) at 65 °C for 1 h with occasional shaking and an equivalent volume of phenol-chloroform-isoamyl alcohol (25:24:1) mixture was added and centrifuged at 1000 rpm for 15 min at 4 °C. The supernatant was added with 0.6 volume of ice cold absolute ethanol and kept for 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 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

Each individual genomic DNA sample was primed and amplified using the *opaque-2* gene specific primer phi-057 (F: 5′-CTCATCAGTGCCGTCGTCCAT-3′ and R: 5′CAGTCGCAAGAAACCGTT-GCC3′). 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 the 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 95 °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 and final extension for 10 min at 72 °C followed by storing at 4 °C till loading to the agarose gel. The amplified products were loaded in 2.5% agarose gel containing 0.5 mg/ml of ethidium bromide and electrophoresed at a constant voltage (50 V). PCR conditions were optimized to yield reproducible result. The gels were scanned by 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.

Three random seed samples of each inbred line were considered for biochemical analysis separately in duplicate with respective standard checks to minimize experimental error. Protein and tryptophan content were estimated as per Bailey (1967) and Vivek et al. (2008) respectively, while lysine content in seed was estimated as per Tsai et al. (1972). Amount of these two essential amino acids were expressed as percentage of total crude protein in seed.

3. Results and discussion

Standard backcrossing is required to introgress favorable alleles from a donor plant into a recipient elite genotype (recurrent parent). The targeted *opaque-2* allele under transfer is recessive (Vasal et al., 1993a; Vasal et al., 1993b) and the endosperm modifiers are polygenic with no reliable molecular marker identified for kernel modification. Therefore, molecular marker based screening for QPM status coupled with phenotypic selection for improving endosperm characteristics was adopted as strategy for development of QPM introgression lines.

Opaque-2 allele being recessive, plants in each back cross population would show uniformly similar dominant phenotype, although they genotypically segregate into O_2O_2 and O_2o_2. Therefore, traditional backcross breeding requires raising F_2 population after each back cross generation to trace plants carrying the target allele. In this context, the marker-assisted breeding strategy enables identification of plants carrying donor allele (o_2) in each back cross population itself and thus it eliminates the need to grow F_2 to sort out QPM phenotype (by lysine and tryptophan estimation). In traditional backcross breeding the reconstruction of the recurrent parent genotype requires more than six generations, while this may be reduced to only three generations in marker assisted back crossing (Frisch et al., 1999; Gupta et al., 2013; Tanksley et al., 1989). Thus, it allows rapid introgression of target allele into the recurrent parent. Gupta et al. (2013) used marker assisted selection (MAS) for development of QPM parental lines of Vivek-9 hybrid and could developed QPM hybrid in less than half the time required through conventional breeding.

A number of researchers revealed polymorphic molecular profiles of QPM lines using RAPD (Hemavathy, 2015; Nkongolo et al., 2011), ISSR (Lenka et al., 2015; Nkongolo et al., 2011), SSR (Bantte and Prasanna,