Combining higher accumulation of amylopectin, lysine and tryptophan in maize hybrids through genomics-assisted stacking of waxy1 and opaque2 genes

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Waxy maize rich in amylopectin has emerged as a preferred food. However, waxy maize is poor in lysine and tryptophan, deficiency of which cause severe health problems. So far, no waxy hybrid with high lysine and tryptophan has been developed and commercialized. Here, we combined recessive waxy1 (wx1) and opaque2 (o2) genes in the parental lines of four popular hybrids (HQPM1, HQPM4, HQPM5, and HQPM7) using genomics-assisted breeding. The gene-based markers, wx-2507F/RG and phi057 specific for wx1 and o2, respectively were successfully used to genotype BC1F1, BC2F1 and BC2F2 populations. Background selection with > 100 SSRs resulted in recovering > 94% of the recurrent parent genome. The reconstituted hybrids showed 1.4-fold increase in amylopectin (mean: 98.84%) compared to the original hybrids (mean: 72.45%). The reconstituted hybrids also showed 14.3% and 14.6% increase in lysine (mean: 0.384%) and tryptophan (mean: 0.102%), respectively over the original hybrids (lysine: 0.336%, tryptophan: 0.089%). Reconstituted hybrids also possessed similar grain yield (mean: 6248 kg/ha) with their original versions (mean: 6111 kg/ha). The waxy hybrids with high lysine and tryptophan assume great significance in alleviating malnutrition through sustainable and cost-effective means. This is the first report of development of lysine and tryptophan rich waxy hybrids using genomics-assisted selection.

Maize grains are used as food, feed and industrial products worldwide1. Waxy maize, popularly known as ‘sticky’ maize or ‘glutinous’ maize, possesses 95–100% amylopectin compared to 70–75% in traditional maize2,3. Immature waxy cobs and dried grains are an essential part of the human diet in East and South-East Asian countries4,5. It is also used as vegetable, and various breakfast and snack items; and consumed as staple food by various ethnic groups6. Due to its excellent qualities of fresh harvest, waxy maize is extensively used in the frozen food processing industries7. Amylopectin boosts energy levels and restores muscle glycogen quickly in professional athletes8.

Amylopectin is a highly branched polymer with α-1,4 and α-1,6 glucosidic bonds connecting the glucose units in starch molecules9. The Waxy1 (Wx1) gene present on long arm of chromosome-9 encodes granule-bound starch synthase-I (GBSS-I) which controls amylose synthesis in maize endosperm10,11. The dominant/wild type Wx1 gene easily converts ADP-glucose to amylose, but the recessive/mutant wx1 gene greatly impairs the conversion, resulting in increased amylopectin accumulation12. The recessive wx1 gene is also linked to tasty and savory flavor in the kernels13.

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The nutritional value of traditional maize including waxy type is relatively poor due to low level of essential amino acids viz., lysine (0.150–0.250%) and tryptophan (0.030–0.040%) \(^1\). However, specific maize genotypes having \(w_2\) (waxy) mutant gene possess much higher lysine (> 0.300%) and tryptophan (> 0.070%) \(^2\). Symptoms of lysine- and tryptophan deficiency in children include fatigue, delayed growth, loss of appetite, depression, and anxiety \(^3\). Being the building block for protein synthesis, lack of lysine and tryptophan affects normal growth and development in humans, and reduces work efficiency leading to the severe socio-economic implications \(^4\). Furthermore, low levels of lysine and tryptophan aggravate protein energy malnutrition (PEM) that affects more than a billion people worldwide \(^5\). Recessive \(o_2\) gene present on the short arm of chromosome-7 enhances lysine and tryptophan levels by nearly 2-fold \(^6\). The dominant \(o_2\) gene encodes a leucine zipper (bZIP) transcription factor that activates the transcription of α-zein genes \(^7\). Ingression of recessive \(o_2\) coupled with the modifier loci has resulted in the development of large array of quality protein maize (QPM) cultivars that has shown great promise in addressing the PEM \(^8\).

Waxy maize hybrids and landraces rich in amylopectin have been reported in Thailand, Vietnam, Laos, Myanmar, China, Taiwan, Philippines and Korea \(^9\). In India, ‘Mimban’ a waxy landrace is cultivated in the North Eastern Himalayan region, and used as a part of important component of diet \(^10\). However, these waxy cultivars are poor in nutritional quality due to inadequate amount of essential amino acids like lysine and tryptophan \(^11\). So far, no waxy maize hybrid with high lysine and tryptophan has yet been developed and commercialized elsewhere. Here, we report the development of lysine and tryptophan rich waxy hybrids by combining \(wx\) and \(o_2\) genes through genomics-assisted breeding \(^12\). Molecular marker is a preferred option to stack multiple genes into a genetic background without the need for progeny testing thereby accelerates the breeding cycle \(^13\). The present investigation was therefore undertaken to (1) introgress of \(wx\) gene into elite \(o_2\)-based (QPM) inbreds using marker-assisted backcross breeding (MABB), (2) evaluate the MABB-derived inbreds and reconstituted hybrids for amylopectin, lysine and tryptophan, and (3) assess the performance of MABB-derived inbreds and reconstituted hybrids for grain yield and agronomic traits.

Materials and methods

Plant materials. The parental inbreds viz., HKI161, HKI163, HKI193-1, and HKI193-2 were used as recurrent parents. These parents possessed wild type \(wx\) allele and were low in amylopectin. These inbreds are the parents of four popular single cross QPM hybrids [HQP1 (HKI193-1×HKI163), HQP2 (HKI193-2×HKI161), HQP4 (HKI163×HKI193-1) and HQP9 (HKI193-1×HKI163)] in India. These commercial QPM hybrids have been adapted to diverse agro-ecologies of India (Table S1). A waxy inbred, MGU-102-\(wx\) possessed high amylopectin (97.82%) and was used as the recessive \(wx\) gene. MGU-102-\(wx\) had low levels of lysine (0.245%) and tryptophan (0.043%). All the recurrent parents possessed high lysine and tryptophan due to presence of recessive \(o_2\) gene. The donor waxy inbred had white kernels, but all of the recurrent parents were yellow in colour. Recurrent parents were crossed with donor parent, and four backcross populations viz., cross-I (HKI161×MGU-102-\(wx\)), cross-II (HKI163×MGU-102-\(wx\)), cross-III (HKI193-1×MGU-102-\(wx\)) and cross-IV (HKI193-2×MGU-102-\(wx\)), were used to stack \(wx\) and \(o_2\) alleles. The detailed information of the recurrent and donor parents is given in (Table S2).

Backcross- and self-progenies. The recurrent inbreds (as female) and donor inbred (as male) showing polymorphism for gene-based markers specific to both \(wx\) and \(o_2\) genes were crossed during the rainy season (July–November, 2016) at IARI, Delhi (28° 09' N, 77° 13' E, 229 MSL). F₁s were grown during the winter season (December, 2016–April, 2017) at IIMR-Winter Nursery Centre (WNC), Hyderabad (17° 19' N, 78° 25' E, 542.6 MSL). BC₁F₁ progenies were grown at Delhi during the rainy season (2017), and foreground selection was carried out using the \(wx\) and \(o_2\) specific markers. The foreground positive plants along with high recovery of the recurrent parent genome (RPG), maximum phenotypic similarity to recurrent parents and endosperm opaqueness of 25–50% were backcrossed to the respective recurrent parents \(^11\). The BC₁F₁ populations raised at Hyderabad during winter season (2017–2018), and were subjected to foreground-, background- and phenotypic selection were carried out. The foreground positive plants with a maximum RPG, morphological similarity and similar kernel opaqueness (25–50%) to their recurrent parents were selfed. The BC₁F₁ progenies were grown during the rainy season (2018) at Delhi. Foreground positive plants homozygous for \(wx\) and \(o_2\) gene were subjected to the background- and phenotypic- selection including the kernel modification. The selected plants were self-pollinated to generate BC₂F₁ progenies during rainy season (2019) at Delhi (Table S3). White kernel progenies with 25–50% opaqueness in endosperm were chosen in each of the three genetic backgrounds. In all the BC₂F₁, BC₁F₁ and BC₂F₁ generations, kernels with 75–100% opaqueness were not considered \(^11\). The details of backcross- and self- generations grown at different locations and seasons are described in Table S3, while marker-assisted backcross breeding (MABB) scheme \(^12\) followed in the present study is represented in Fig. 1.

DNA isolation and polymerase chain reaction amplification and electrophoresis. The CTAB method was used to isolate genomic DNA from young seedlings (3–4 leaf stage) \(^14\). Polymerase chain reaction (PCR) amplification and electrophoresis of the PCR products for the \(wx\) and \(o_2\) genes were performed using protocol standardized at Maize Genetics Unit, IARI \(^15\). PCR was performed in 20 μl volume on Veriti 96-well thermal cycler (M/s. Applied Biosystems) using GeneDirex OnePCR reaction mixture. Amplification of PCR products was performed with a ‘touch-down 60’ procedure as per Duo et al. \(^1\) Electrophoretic separation of the PCR products was performed using 4% agarose (Lonza, Rockland, ME USA) at 100–120 V for 3–4 h with a 50 bp DNA ladder (MBA-Fermentas). Photographs of the amplified products was captured using gel documentation system (Alphalnnotech, California, USA).
Marker-assisted foreground selection for \( wx_1 \) and \( o_2 \) gene

Hybridity testing was undertaken in \( F_1 \)s using markers specific to \( wx_1 \) and \( o_2 \) genes. Foreground selection was performed in \( BC_1F_1 \), \( BC_2F_1 \), and \( BC_2F_2 \) generations. Gene based \( \text{InDel} \) marker, \( wx-2507F/RG \) was used for selection of \( wx_1 \) gene. Heterozygous plants (\( Wx_1/wx_1 \)) were selected in the \( BC_1F_1 \) and \( BC_2F_1 \), while homozygotes (\( wx_1/wx_1 \)) were selected in \( BC_2F_2 \). Gene based \( \text{SSR} \), \( phi057 \) was used to genotype the populations and homozygotes (\( o_2o_2 \)) were selected in \( BC_1F_1 \). The details information of markers used in foreground selection are presented in Table S4.

Marker-assisted background selection for recurrent parent genome

A set of >320 genome-wide \( \text{SSRs} \) covering all the 10 chromosomes of the maize genome were used for identifying polymorphic markers between the respective recurrent and donor parents (Table 1). The sequence of \( \text{SSR} \) primers was retrieved from the maize genome database (www.maizegdb.org) and was custom synthesized (Sigma Tech., USA). PCR amplification and scoring of amplicons of \( \text{SSRs} \) employed in background selection were carried out as per Hossain et al. Polymorphic \( \text{SSRs} \) between the recurrent and donor parents were used to recover the RPG in individuals from the \( BC_1F_1 \), \( BC_2F_1 \) and \( BC_2F_2 \) populations.

Agronomic evaluation of MABB-derived inbreds.

MABB-derived inbreds (three from each of the four genetic background) and their recurrent parents were evaluated in randomized complete block design (RCBD) with two replications at the IARI, Delhi during the rainy season (2020). Each inbred was grown in a 3 m row, with a 75 cm row-to-row and 20 cm plant-to-plant distance. Inbreds were characterized for five important agronomic traits [days to 50% anthesis (MF), days to 50% silking (FF), plant height (PH), ear height (EH) and grain yield (GY)] and 31 morphological characters pertaining to distinctness, uniformity and stability (DUS). Standard agronomic practices were followed to raise the good crop. Two to three plants per entry were self-pollinated to avoid any xenia effects caused by foreign pollens, and the selfed grains were analyzed for amyllopectin, lysine and tryptophan. Characters namely MF, FF, PH, EH and GY were recorded from open pollinated plants.

Agronomic evaluation of reconstituted hybrids.

Selected three \( BC_2F_3 \) progenies from each of the four inbreds were used to reconstitute 12 \( F_1 \) hybrids during the winter season (2019–20) at Hyderabad. Three versions of the reconstituted hybrids (-A, -B, and -C) and their corresponding original hybrid in each of the four hybrid

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**Figure 1.** Marker-assisted backcross breeding (MABB) scheme followed for development of amyllopectin rich waxy version of HQPM7. RP: recurrent parent; DP: donor parent.

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Absolute amylose content was estimated as per Gibbon et al.\textsuperscript{28} with minor modifications. Around 8–10 dried maize seeds were ground into seed powder with a diameter of < 0.2 mm using seed grinder (Cyclotec Sample Mill-1093, Sweden). Weighted 100 mg of seed powder was treated with 500 µl of 80% ethanol and vortexed for a short time. The sample tubes were centrifuged for 5 min at 10,000 rpm and supernatant was separated. The process was repeated until the supernatant was clear of white layer. The supernatant was discarded, and the residue was fully dried in an incubator at 80 °C for 3–4 h. The resulting residue represented starch with a <5% impurity level. 25 mg of the starch residue was placed into a 50 ml falconer tube. It was solubilized with 2.5 ml 1 M NaOH and mixed properly, and heated for 20 min in a hot water bath at 80 °C. The volume was adjusted to 25 ml with double distilled water after the samples were cooled to room temperature. 1.25 ml samples were transferred from the above sample into a new 50 ml falconer tube and treated with 125 µl of 1 N acetic acid, 100 µl of 1 M NaOH, and 500 µl of I\textsubscript{2}-KI solution. The samples were incubated at room temperature for 20 min to generate colour, then measured at 620 nm for absorbance (G-Biosciences Spectrophotometer, BT-UVS-SBA-E, BenchTop). The percent of amylose was calculated using the average of three technical replicates. The percent amylopectin was obtained by subtracting amylose from 100.

### Analysis of amylopectin
Self-pollinated grains were used to estimate amylopectin from maize kernels. Absolute amylose content was estimated as per Gibbon et al.\textsuperscript{28} with minor modifications. Around 8–10 dried maize seeds were ground into seed powder with a diameter of < 0.2 mm using seed grinder (Cyclotec Sample Mill-1093, Sweden). Weighted 100 mg of seed powder was treated with 500 µl of 80% ethanol and vortexed for a short time. The sample tubes were centrifuged for 5 min at 10,000 rpm and supernatant was separated. The residues of the samples were again treated with 10% toluene and centrifuged for 5 min at 10,000 rpm and supernatant was separated. The process was repeated until the supernatant was clear of white layer. The supernatant was discarded, and the residue was fully dried in an incubator at 80 °C for 3–4 h. The resulting residue represented starch with a <5% impurity level. 25 mg of the starch residue was placed into a 50 ml falconer tube. It was solubilized with 2.5 ml 1 M NaOH and mixed properly, and heated for 20 min in a hot water bath at 80 °C. The volume was adjusted to 25 ml with double distilled water after the samples were cooled to room temperature. 1.25 ml samples were transferred from the above sample into a new 50 ml falconer tube and treated with 125 µl of 1 N acetic acid, 100 µl of 1 M NaOH, and 500 µl of I\textsubscript{2}-KI solution. The samples were incubated at room temperature for 20 min to generate colour, then measured at 620 nm for absorbance (G-Biosciences Spectrophotometer, BT-UVS-SBA-E, BenchTop). The percent of amylose was calculated using the average of three technical replicates. The percent amylopectin was obtained by subtracting amylose from 100.

### Analysis of lysine, and tryptophan
The lysine and tryptophan of maize kernels was estimated using UHPLC (Dionex Ultimate 3000 System, Thermo Scientific, Massachusetts, USA). The selfed seeds were dried and ground into powder, and further used for estimation of lysine and tryptophan\textsuperscript{29}. The flour of the grains was acid hydrolyzed using 800 µl of 6 N HCl, 100 µl of 0.1 N HCl, 100 µl of nor-leucine and 10 µl of phenol for 16 h at 110 °C. Two mobile phases, A and B consisted of buffer and organic phase in the ratio of 9:1 (v/v) and 1:9 (v/v), respectively were used for estimation of lysine. Buffer phase for lysine contained tetra-methyl ammonium chloride and sodium acetate trihydrate (pH 3.5), while organic phase had acetonitrile and methanol (49:1, v/v). In case of tryptophan, alkaline hydrolysis (2 ml of 4 M NaOH and 200 µl of 0.1% ascorbic acid for 16 h at 110 °C) was performed. The mobile phase for tryptophan consisted of water and acetonitrile in the ratio of 95:5. The samples were injected separately in UHPLC through Acclaim 120 C18 column (5 μm, 120 Å, 4.6×150 mm) with a flow rate of 1.0 and 0.7 ml/min, and detected using RS 3000 photodiode array (PDA) detector at 265 and 280 nm, respectively. The concentration of lysine and tryptophan was estimated in three technical replicates by standard regression curve derived using dilutions of external standards (AAS 18-5ML, Sigma Aldrich).

### Statistical analysis
Chi-square analysis was used to test the goodness of fit of the observed segregation pattern of wx1 across segregating populations (BC\textsubscript{1}F\textsubscript{1}, BC\textsubscript{2}F\textsubscript{1} and BC\textsubscript{2}F\textsubscript{2}), as well as o2 in the BC\textsubscript{1}F\textsubscript{1} generation\textsuperscript{22}. The amplicons of SSRs used in background selection were scored as “A” for the recurrent parent, “B” for the donor parent, and “H” for the heterozygous genotype. Recovery of RPG was estimated using formula\textsuperscript{30}, RPG (%) = \[\frac{A + (0.5H)}{A + B + H}\] × 100. Graphical Geno Types (GGT) version 3.0 was also used to determine the

| LG | No. of SSRs screened | HIK161×MGU-102-wx1 | HIK163×MGU-102-wx1 | HIK93-1×MGU-102-wx1 | HIK93-2×MGU-102-wx1 |
|----|----------------------|--------------------|--------------------|--------------------|--------------------|
|    | NP Pol (%)           | NP Pol (%)         | NP Pol (%)         | NP Pol (%)         | NP Pol (%)         |
| 1  | 26                   | 11 42.31           | 10 38.46           | 10 38.46           | 11 42.31           |
| 2  | 29                   | 12 41.38           | 11 37.93           | 10 34.48           | 10 34.48           |
| 3  | 35                   | 11 31.43           | 10 28.57           | 12 34.29           | 13 37.14           |
| 4  | 36                   | 16 44.44           | 14 38.89           | 12 33.33           | 11 30.56           |
| 5  | 38                   | 11 28.95           | 11 28.95           | 11 28.95           | 8 21.05            |
| 6  | 32                   | 11 34.38           | 12 37.50           | 10 31.25           | 9 28.13            |
| 7  | 34                   | 10 29.41           | 9 26.47            | 11 32.35           | 12 35.29           |
| 8  | 36                   | 10 27.78           | 11 38.56           | 9 25.00            | 11 30.56           |
| 9  | 28                   | 10 35.71           | 10 35.71           | 8 28.57            | 9 32.14            |
| 10 | 26                   | 10 38.46           | 9 34.62            | 9 34.62            | 11 42.31           |
| Total | 320                  | 112 35.00          | 107 33.44          | 102 31.86          | 105 32.81          |

Table 1. Percent polymorphism and distribution of SSRs used in background selection. LG Linkage group, NP No. of observed polymorphic markers, Pol. (%) Polyorphism percentage, SSR Simple Sequence Repeats

combinations were evaluated in RCBD with two replications at three diverse maize growing zones of the country namely (1) IARI, Delhi, (2) CSK-HPKVD, Bajaura (31° 85’ N, 77° 16’ E, 1090 m MSL) and (3) IGERI, Jhansi (25° 26’ N, 78° 30’ E, 216 m MSL) during rainy season (2020). Standard agronomic practices were adopted for raising the hybrids. The hybrids were evaluated using 3 m row with a plant-to-plant and row-to-row distance 20 cm and 75 cm, respectively. Two to three plants in each hybrid were self-pollinated to avoid xenia effects. Selfed-seeds were used for the estimation of amylopectin, lysine and tryptophan. Morphological characteristics such as MF, FF, PH, EH, and GY were recorded from open-pollinated plants. The hybrids were also characterized for 31 DUS characters\textsuperscript{27}.

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No. of observed polymorphic markers, Pol. (%) = \[\frac{A + (0.5H)}{A + B + H}\] × 100. Graphical Geno Types (GGT) version 3.0 was also used to determine the
recovery of RPG in selected backcross-derived progenies. Graphical representations on amylopectin, lysine and tryptophan in each genotype were made using Microsoft Excel (2013). Winodstat v10 software was used to analyze the agronomic and biochemical data.

Research involving plants. No approvals were required for the study, which complied with all relevant regulations.

Results

Marker polymorphism among parents. Gene-based InDel marker, wx-2507F/RG was polymorphic between recurrent (HKI161, HKI163, HKI193-1 and HKI193-2) and donor (MGU-102-wx1) parents. wx-2507F/RG amplified 280 bp fragment in all the four recurrent inbreds, while it amplified 260 bp fragment in waxy donor line (Fig. 2A). Gene-based SSR, phi057 produced 165 bp allele in all four recurrent parents, while the donor generated 153 bp allele (Fig. 2B).

Table 2. Segregation pattern of wx1 and o2 in different backcrosses and self-generations. *Significant at $P=0.05$. ns non-significant, N: No. of plants genotyped, df degrees of freedom, Wx1, dominant allele; wx1, recessive allele; O2, dominant allele; o2, recessive allele.

| S. no. | Cross                  | Generation | N   | Wx1/Wx1 | Wx1/wx1 | wx1/wx1 | $\chi^2$ | P-value | N   | O2/o2 | o2/o2 | $\chi^2$ | P-value |
|-------|------------------------|------------|-----|---------|---------|---------|----------|---------|-----|------|-------|----------|---------|
| 1     | HKI161 × MGU-102-wx1   | BC1F1      | 115 | 64      | 51      | -       | 1.47     | 0.23 NS | 51  | 18   | 33    | 4.52     | 0.03*   |
| 2     | HKI161 × MGU-102-wx1   | BC1F1      | 108 | 49      | 59      | -       | 0.93     | 0.34 NS | 59  | -    | 72    | -       | -       |
| 3     | HKI161 × MGU-102-wx1   | BC1F1      | 198 | 43      | 105     | 50      | 1.22     | 0.54 NS | 50  | -    | 50    | -       | -       |
| 4     | HKI161 × MGU-102-wx1   | BC1F1      | 105 | 59      | 46      | -       | 1.61     | 0.21 NS | 46  | 20   | 26    | 1.04     | 0.31 NS |
| 5     | HKI161 × MGU-102-wx1   | BC1F1      | 110 | 62      | 48      | -       | 1.78     | 0.18 NS | 48  | -    | 48    | -       | -       |
| 6     | HKI161 × MGU-102-wx1   | BC1F1      | 273 | 74      | 139     | 60      | 1.53     | 0.47 NS | 60  | -    | 60    | -       | -       |
| 7     | HKI161 × MGU-102-wx1   | BC1F1      | 104 | 57      | 47      | -       | 0.96     | 0.32 NS | 47  | 27   | 20    | 1.16     | 0.28 NS |
| 8     | HKI161 × MGU-102-wx1   | BC1F1      | 114 | 60      | 54      | -       | 0.32     | 0.57 NS | 54  | -    | 54    | -       | -       |
| 9     | HK1193-1 × MGU-102-wx1 | BC1F1      | 207 | 50      | 99      | 58      | 1.01     | 0.60 NS | 58  | -    | 58    | -       | -       |
| 10    | HK1193-1 × MGU-102-wx1 | BC1F1      | 110 | 48      | 62      | -       | 1.78     | 0.18 NS | 62  | 25   | 37    | 5.76     | 0.02*   |
| 11    | HK1193-2 × MGU-102-wx1 | BC1F1      | 102 | 56      | 46      | -       | 0.98     | 0.32 NS | 46  | -    | 46    | -       | -       |
| 12    | HK1193-2 × MGU-102-wx1 | BC1F1      | 195 | 45      | 99      | 51      | 0.42     | 0.83 NS | 51  | -    | 51    | -       | -       |

Genomics-assisted selection. F1 generation. The corresponding polymorphic markers for wx1 and o2 showed hybridity among all the F1s, as all plants revealed 280/260 bp (Wx1wx1) and 165/153 bp (O2o2) ampli-
BC1F1 generation. Foreground selection using wx1 gene in BC1F1 identified 51, 46, 47 and 62 heterozygous plants (Wx1wx1) in HKII61 × MGU-102-wx1, HKII63 × MGU-102-wx1, HKII193-1 × MGU-102-wx1 and HKII193-2 × MGU-102-wx1 populations, respectively (Table 2). The chi-square test revealed Mendelian segregation ratio of 1:1 for the wx1 gene in all four crosses (Table 2). These identified heterozygous plants (Wx1wx1) were further subjected to foreground selection using o2 gene. The PCR assay identified 33 homozygous plants (o2o2) in HKII61 × MGU-102-wx1, while it was 26, 20 and 37 plants in HKI163 × MGU-102-wx1, HKI193-1 × MGU-102-wx1 and HKI193-2 × MGU-102-wx1 populations, respectively. Significant segregation distortion of o2 gene was observed in two crosses (HKII61 × MGU-102-wx1 and HKI193-2 × MGU-102-wx1), while rest two crosses (HKI163 × MGU-102-wx1 and HKI193-1 × MGU-102-wx1) showed 1:1 ratio (Table 2).

Consequently, foreground positive plants with 25–50% opaqueness were studied for background selection using polymorphic markers. Foreground positive plants with 75–100% opaqueness were rejected. Two plants each in HKI161- (82.1% and 83.5% RPG), HKI163- (81.3% and 80.4% RPG), and HKI193-2- (82.4% and 83.3% RPG), while three plants in HKI193-1- (82.4%, 82.8% and 81.4% RPG) based populations were selected for further advancement (Table 3). In BC1F1 generation, the recovery of RPG among the selected individuals varied from 81.3 to 83.5% with an average of 82.2%.

BC2F1 generation. A total of 59 heterozygous plants (Wx1wx1) was identified in HKII61 × MGU-102-wx1, while the same was 48 in HKII63 × MGU-102-wx1, 54 in HKII193-1 × MGU-102-wx1 and 46 in HKII193-2 × MGU-102-wx1 (Table 2). In all four crosses, Mendelian inheritance ratio of 1:1 was observed for the wx1 gene. Background selection among Wx1wx1/o2o2 plants (with 25–50% opaqueness) using polymorphic SSRs led to the recovery of 89.3–93.3% RPG in HKII61 × MGU-102-wx1, 87.9–91.6% in HKII63 × MGU-102-wx1, 88.7–92.2% in HKII193-1 × MGU-102-wx1 and 89.5–92.4% in HKII193-2 × MGU-102-wx1. Two plants in each of HKI161- (92.9% and 93.3% RPG), HKI193-1- (92.2% and 91.7% RPG), and HKI193-2- (91.9% and 92.4% RPG), while three plants in HKI163- (91.6%, 90.7% and 91.1% RPG) based populations were advanced (Table 3). Across BC2F1 generations, the average recovery of RPG was 92.0% with a range from 90.7 to 93.3%.

| S. no. | Cross | Generation | Genotypes | RPG (%) in selected progeny | Range of RPG (%) among all progenies |
|-------|-------|------------|-----------|----------------------------|-------------------------------------|
| 1     | HKI161 × MGU-102-wx1 | BC1F1 | HKI161-99 | 82.1 | 75.9–83.5 |
| 2     | HKI161 × MGU-102-wx1 | BC1F1 | HKI161-107 | 83.5 | |
| 3     | HKI163 × MGU-102-wx1 | BC1F1 | HKI163-99-30 | 92.9 | 89.3–93.3 |
| 4     | HKI163 × MGU-102-wx1 | BC1F1 | HKI163-107-42 | 93.3 | |
| 5     | HKI193-1 × MGU-102-wx1 | BC1F1 | HKI193-1-3 | 82.4 | |
| 6     | HKI193-1 × MGU-102-wx1 | BC1F1 | HKI193-1-6 | 82.8 | 76.5–82.8 |
| 7     | HKI193-1 × MGU-102-wx1 | BC1F1 | HKI193-1-14 | 81.4 | |
| 8     | HKI193-1 × MGU-102-wx1 | BC1F1 | HKI193-1-6-55 | 92.2 | 88.7–92.2 |
| 9     | HKI193-1 × MGU-102-wx1 | BC1F1 | HKI193-1-14-1 | 91.7 | |
| 10    | HKI193-1 × MGU-102-wx1 | BC2F1 | HKI193-1-6-55-9 | 94.1 | |
| 11    | HKI193-1 × MGU-102-wx1 | BC2F1 | HKI193-1-6-55-116 | 95.1 | 91.7–95.1 |
| 12    | HKI193-1 × MGU-102-wx1 | BC2F1 | HKI193-1-14-1-57 | 94.6 | |
| 13    | HKI193-2 × MGU-102-wx1 | BC1F1 | HKI193-2-4 | 82.4 | 75.1–83.3 |
| 14    | HKI193-2 × MGU-102-wx1 | BC1F1 | HKI193-2-6 | 83.3 | |
| 15    | HKI193-2 × MGU-102-wx1 | BC1F1 | HKI193-2-4-39 | 91.9 | 89.5–92.4 |
| 16    | HKI193-2 × MGU-102-wx1 | BC1F1 | HKI193-2-4-20 | 92.4 | |
| 17    | HKI193-2 × MGU-102-wx1 | BC1F1 | HKI193-2-4-39-45 | 94.3 | |
| 18    | HKI193-2 × MGU-102-wx1 | BC2F1 | HKI193-2-4-20-56 | 94.8 | 91.9–95.2 |
| 19    | HKI193-2 × MGU-102-wx1 | BC2F1 | HKI193-2-4-20-111 | 95.2 | |

Table 3. Recovery of recurrent parent genome (RPG) among introgressed progenies.
**BC$_2$F$_2$ generation.** Foreground selection identified 50 homozygous plants ($wx_1wx_1$) in HKI161 × MGU-102-$wx_1$, while it was 60, 58 and 51 in HKI163 × MGU-102-wx, HKI193-1 × MGU-102-wx1 and HKI193-2 × MGU-102-wx1, respectively (Table 2). With regard to the $wx_1$ gene, all four crosses followed the Mendelian segregation pattern of 1:2:1. (Table 2). All the homozygous plants ($wx_1wx_1$) also revealed the presence of $o_2$ gene in homozygous condition. Screening of double-homozygous plants ($wx_1wx_1/o_2o_2$) having 25–50% opaqueness with background markers led to high recovery of RPG in HKI161 × MGU-102-$wx_1$ (91.6–96.4%), HKI163 × MGU-102-$wx_1$ (90.7–94.4%), HKI193-1 × MGU-102-wx1 (91.7–95.1%) and HKI193-2 × MGU-102-wx1 (91.9–95.2%) (Table 3). Three plants each in HKI161- (95.1%, 95.5% and 96.4%), HKI163- (94.4%, 93.5% and 93.9% RPG), HKI193-1- (94.1%, 95.1% and 94.6% RPG) and HKI193-2- (94.3%, 94.8% and 95.2% RPG) based populations were selected for further advancement (Table 3, Fig. 3). Recovery among the selected progenies ranged from 93.5 to 96.4%, with an average of 95.2%.

**Selection of BC$_2$F$_3$ progenies for kernel colour.** BC$_2$F$_3$ seeds borne on BC$_2$F$_2$ plants with $wx_1wx_1/o_2o_2$ genotype were selected for white colour in kernels. Seeds with yellow kernel colour were not considered for the present study. The white seeds homozygous for both $wx_1$ and $o_2$ genes were planted in order to generate BC$_2$F$_3$ progenies. Three progenies each in HKI161-, HKI163-, HKI193-1- and HKI193-2- based populations were finally selected for evaluation and reconstitution of hybrids (Table 4).

**Evaluation of introgressed inbreds for amylopectin.** Amylopectin among MABB-derived progenies of HKI161, HKI163, HKI193-1 and HKI193-2 showed substantial increase (mean: 98.70%, range: 97.68–99.31%) over their respective recurrent parents (mean: 73.76%, range: 72.00–75.17%) (Table 4, Fig. 4). All the introgressed inbreds were statistically superior to their respective recurrent parents for amylopectin content. HKI161 had 73.76% amylopectin, while its waxy versions possessed 98.94% (HKI161-99-30-43-290), 99.00% (HKI161-107-42-1-291) and 98.57% (HKI161-107-42-1-291) amylopectin. HKI163 possessed 72.00% amylopectin, and its MABB versions had 98.24% (HKI163-9-2-13-302), 97.68% (HKI163-9-35-88-303) and 98.46% (HKI163-19-3-107-304) amylopectin. Waxy versions of HKI193-1 had 99.31% [HKI193-1-6-55-9-317], 98.70% [HKI193-1-6-55-116-319], and 99.06% [HKI193-1-14-1-57-320] amylopectin, compared to 74.10% in HKI193-1 (Table 4, Fig. 3S). HKI193-2 had 75.17% amylopectin, and its MABB versions possessed 99.23% [HKI193-2-4-39-45-321], 98.72% [HKI193-2-4-20-56-322] and 98.52% [HKI193-2-4-20-111-325] amylopectin. Overall, an aver-

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**Figure 3.** Graphical genotype of introgressed progenies across the three crosses. RP: recurrent parent; DP: donor parent; IP: introgressed progeny; CHR: Chromosome.
age of ~1.4-fold increase in amylopectin was recorded among introgressed progenies. However, starch content among introgressed- (mean: 69.42%) and original- inbreds (mean: 68.05%) were statistically at par (Table S5).

**Evaluation of introgressed inbreds for lysine and tryptophan.** MABB-derived progenies of HKI161, HKI163, HKI193-1 and HKI193-2 showed higher lysine (mean: 0.367%) and tryptophan (mean: 0.091%) over their respective recurrent parents (lysine: 0.318%, tryptophan: 0.079%) (Table 4, Figs. 4S, 5S). Each of the introgressed progenies had significantly higher lysine and tryptophan over their respective recurrent parents except HKI163-9-2-13-302 and HKI163-19-3-107-304 which had statistically similar lysine with HKI163. HKI161 had 0.315% lysine and 0.079% tryptophan, while its waxy versions viz., HKI161-99-30-43-290 (lysine: 0.364%, tryptophan: 0.093%), HKI161-107-42-1-291 (lysine: 0.367%, tryptophan: 0.089%) and HKI161-107-42-10-293 (lysine: 0.351%, tryptophan: 0.091%) had higher accumulation. Waxy versions viz., HKI163-9-35-88-303 (lysine: 0.378%, tryptophan: 0.094%), HKI163-9-35-88-303 (lysine: 0.365%, tryptophan: 0.093%) and HKI163-19-3-107-304 (lysine: 0.370%, tryptophan: 0.091%) possessed better nutritional quality compared to HKI163 (lysine: 0.338%, tryptophan: 0.084%). In case of HKI193-1, lysine and tryptophan was 0.320% and 0.078%, respectively, while waxy versions viz., HKI193-1-6-55-9-317 (lysine: 0.391%, and tryptophan: 0.092%), HKI193-1-6-55-116-319 (lysine: 0.372%, tryptophan: 0.092%), and HKI193-1-14-1-57-320 (lysine: 0.381%, tryptophan: 0.095%) possessed higher accumulation. MABB-versions viz., HKI193-2-4-20-56-322 (lysine: 0.345%, tryptophan: 0.090%) and HKI193-2-4-20-111-325 (lysine: 0.381%, tryptophan: 0.095%) also possessed superior nutritional quality over original inbred, HKI193-2 (lysine: 0.298%, tryptophan: 0.074%) (Table 4). Overall, introgressed progenies possessed 1.2-fold more lysine and tryptophan over the original inbreds.

**Evaluation of introgressed inbreds for yield and morphological characters.** In general, introgressed progenies and their respective recurrent parents showed statistically similar levels of grain yield, days

| S. no. | Genotypes     | Amylopectin (%) | Lysine (%) | Tryptophan (%) |
|-------|---------------|-----------------|------------|----------------|
| 1     | HKI161        | 73.76           | 0.315      | 0.079          |
| 2     | HKI161-99-30-43-290 | 98.94           | 0.364      | 0.093          |
| 3     | HKI161-107-42-1-291 | 99.00           | 0.367      | 0.089          |
| 4     | HKI161-107-42-10-293 | 98.57           | 0.351      | 0.091          |
| 5     | HKI163        | 72.00           | 0.338      | 0.084          |
| 6     | HKI163-9-2-13-302 | 98.24           | 0.378      | 0.094          |
| 7     | HKI163-9-35-88-303 | 97.68           | 0.365      | 0.093          |
| 8     | HKI163-19-3-107-304 | 98.46           | 0.370      | 0.091          |
| 9     | HKI193-1      | 74.10           | 0.320      | 0.078          |
| 10    | HKI193-1-6-55-9-317 | 99.31           | 0.391      | 0.092          |
| 11    | HKI193-1-6-55-116-319 | 98.70           | 0.372      | 0.092          |
| 12    | HKI193-1-14-1-57-320 | 99.06           | 0.381      | 0.095          |
| 13    | HKI193-2      | 75.17           | 0.298      | 0.074          |
| 14    | HKI193-2-4-39-45-321 | 99.23           | 0.351      | 0.087          |
| 15    | HKI193-2-4-20-56-322 | 98.72           | 0.345      | 0.090          |
| 16    | HKI193-2-4-20-111-325 | 98.52           | 0.364      | 0.088          |
| CD (5%) |                | 8.67            | 0.040      | 0.005          |

Table 4. Nutritional quality attributes among introgressed progenies and their respective recurrent.
to anthesis, days to silking, plant height and ear height (Table 5). The grain yield of HKI161 was 1876 kg/ha, while the same in waxy versions ranged from 1742 to 2142 kg/ha (mean: 1991 kg/ha). HKI163 had grain yield of 2080 kg/ha, while its wx1 introgressed lines produced 1951–2107 kg/ha (mean: 2016 kg/ha) of grain yield. HKI193-1 and HKI193-2 produced grain yield of 1698 kg/ha and 1876 kg/ha, while their introgressed lines had grain yield of 1653–1920 kg/ha (mean: 1754 kg/ha) and 1653–2053 kg/ha (mean: 1949 kg/ha), respectively.

**Table 5.** Morphological characterization of introgressed progenies and their respective recurrent. GY Grain yield, MF days to anthesis, FF days to silking, PH plant height, EH ear height, CD Critical difference

| S. no. | Inbreds       | GY (kg/ha) | MF (days) | FF (days) | PH (cm) | EH (cm) |
|--------|---------------|------------|-----------|-----------|---------|---------|
| 1      | HKI161        | 1876       | 50.5      | 53.5      | 114.5   | 55.2    |
| 2      | HKI161-99-30-43-290 | 1742    | 48.5      | 53.5      | 108.5   | 56.0    |
| 3      | HKI161-107-42-1-291 | 2142    | 51.5      | 54.5      | 113.3   | 51.3    |
| 4      | HKI161-107-42-10-293 | 2089    | 50.0      | 53.0      | 116.7   | 60.0    |
| 5      | HKI163        | 2080       | 54.0      | 57.0      | 125.2   | 75.2    |
| 6      | HKI163-9-2-13-302 | 2107    | 51.5      | 54.5      | 123.8   | 71.7    |
| 7      | HKI163-9-35-88-303 | 1951    | 54.5      | 57.5      | 127.8   | 71.7    |
| 8      | HKI163-19-3-107-304 | 1991    | 54.5      | 57.5      | 126.8   | 77.2    |
| 9      | HKI193-1-6-55-9-317 | 1920    | 50.0      | 53.0      | 104.3   | 48.5    |
| 10     | HKI193-1-6-55-116-319 | 1653   | 50.5      | 53.5      | 116.5   | 51.8    |
| 11     | HKI193-1-14-1-57-320 | 1689    | 50.0      | 53.0      | 109.3   | 57.0    |
| 12     | HKI193-2-1-29-1-1-4 | 1876    | 52.0      | 55.0      | 117.2   | 61.7    |
| 13     | HKI193-2-4-39-45-321 | 1653   | 54.0      | 56.5      | 115.3   | 60.7    |
| 14     | HKI193-2-4-20-56-322 | 2053   | 54.0      | 57.0      | 122.8   | 59.2    |
| 15     | HKI193-2-4-20-111-325 | 2142   | 52.5      | 55.5      | 119.0   | 60.2    |
| 16     | HKI193-2-4-20-111-325 | 2142   | 52.5      | 55.5      | 119.0   | 60.2    |
| CD (5%)|               | 251.60    | 2.91      | 2.88      | 14.25   | 9.25    |

**Figure 5.** Amylopectin content in original- and reconstituted waxy-hybrids.
However, significant difference was observed in few cases viz., (1) grain yield (HKI161 and HKI161-107-42-1-291, HKI193-1 and HKI193-2-4-20-111-325, and (2) days to anthesis and silking (HKI193-1 and HKI193-1-6-55-9-317, HKI193-1-6-55-116-319 and HKI193-1-14-1-57-320) (Table 5). The waxy inbreds also showed a high degree of phenotypic similarity for DUS characters with their recurrent parents (Table S7). However, the marker-assisted selection (MAS)-derived inbreds differed from their original inbreds for few DUS characteristics as well. For example, anthocyanin colouration of brace root was present in HKI161, while it was absent in HKI161-99-30-43-290, HKI161-107-42-1-291 and HKI161-107-42-10-293 (Table S7a). Similarly, anthocyanin colouration of brace root was absent in HKI193-1, while it was found present in all the three versions (Table S7c).

Evaluation of MAS-derived hybrids for amylopectin. The amylopectin of the reconstituted hybrids increased significantly from 72.45% in the original hybrids to 98.84% in the MABB-derived hybrids across three locations (Table S6, Fig. 6S). The newly derived waxy hybrids possessed amylopectin ranging from 98.07 to 99.37% compared to 70.43–74.36% among the original hybrids (Fig. 5). All the reconstituted hybrids showed statistically higher amount of amylopectin from their original hybrids. The original HQPM1 possessed 71.60% amylopectin, whereas its reconstituted hybrids had 98.07% (HQPM1-A), 98.48% (HQPM1-B), and 98.78% (HQPM1-C) amylopectin. HQPM4 had 70.43% amylopectin, while waxy versions of the hybrids possessed 99.04% (HQPM4-A), 98.42% (HQPM4-A) and 98.81 (HQPM4-A) amylopectin. On the other hand, reconstituted hybrids had 99.14% (HQPM5-A), 98.85% (HQPM5-B), and 99.00% (HQPM5-C) amylopectin, compared to 73.44% in HQPM5. Similarly, amylopectin of HQPM7 was 74.36%, and its waxy versions had 98.95% (HQPM7-A), 99.37% (HQPM7-B), and 99.11% (HQPM7-C) amylopectin. Amylopectin levels in the reconstituted waxy hybrids was increased by 1.4-fold over original versions across locations. However, starch content of the original (mean: 70.20%) and reconstituted (mean: 71.66%) versions of the hybrids were statistically at par (Table S5).

Evaluation of MAS-derived hybrids for lysine and tryptophan. The newly derived waxy hybrids had significantly higher lysine (mean: 0.384%, range: 0.347–0.417%) and tryptophan (mean: 0.102%, range: 0.096–0.107%) compared to lysine (mean: 0.336%, range: 0.314–0.361%) and tryptophan (mean: 0.089%, range: 0.083–0.093%) in the original hybrids (Figs. 6, 7). All the reconstituted hybrids possessed statistically higher amount of lysine and tryptophan over the original versions (Table S6). The lysine and tryptophan in HQPM1 were 0.314% and 0.091%, while waxy HQPM1 version of the reconstituted hybrids viz., HQPM1-A (lysine: 0.353%, tryptophan: 0.103%), HQPM1-B (lysine: 0.354%, tryptophan: 0.102%) and HQPM1-C (lysine: 0.347%, tryptophan: 0.105%) were superior in nutritional quality. HQPM4 had 0.334% lysine and 0.083% tryptophan, while the reconstituted hybrids viz., HQPM4-A (lysine: 0.388%, tryptophan: 0.097%), HQPM4-B (lysine: 0.400%, tryptophan: 0.098%) and HQPM4-C (lysine: 0.389%, tryptophan: 0.096%) possessed higher concentration of amino acids. The reconstituted hybrids of HQPM5 viz., HQPM5-A (lysine: 0.382%, tryptophan: 0.101%), HQPM5-B (lysine: 0.372%, tryptophan: 0.103%) and HQPM5-C (lysine: 0.383%, tryptophan: 0.104%) possessed higher nutritional value over the original hybrid, HQPM5 (lysine: 0.337%, tryptophan: 0.090%). Similarly, lysine and tryptophan concentration of HQPM7 was 0.361% and 0.093%, respectively while waxy version of the hybrids viz., HQPM7-A (lysine: 0.417%, tryptophan: 0.107%), HQPM7-B (lysine: 0.416%, tryptophan: 0.107%) and HQPM7-C (lysine: 0.412%, tryptophan: 0.107%) had higher accumulation (Figs. 6, 7). Across loca-
tions, reconstituted hybrids had 1.1-fold and 1.2-fold more lysine and tryptophan, respectively over the original hybrids.

**Evaluation of MAS-derived hybrids for yield and morphological characters.** In general, reconstituted hybrids showed statistically similar grain yield, days to anthesis, days to silking, plant height and ear height with their original version (Table 6). The grain yield of reconstituted waxy QPM hybrids was 6248 kg/ha (range: 5724–7067 kg/ha), whereas in original QPM hybrids it was 6111 kg/ha (range: 5906–6648 kg/ha) across locations.
locations (Table 6). HQPM1 had grain yield of 5953 kg/ha, while its waxy hybrids had 6174 kg/ha (HQPM1-A), 6185 kg/ha (HQPM1-B), and 6056 kg/ha (HQPM1-C). Grain yield of the reconstituted hybrids was 5724 kg/ha (HQPM4-A), 5750 kg/ha (HQPM4-B), and 5971 kg/ha (HQPM4-C), compared to 5935 kg/ha in HQPM4. In case of HQPM5, grain yield was 5906 kg/ha, and its waxy version of hybrid produced grain yield of 5987 kg/ha (HQPM5-A), 6102 kg/ha (HQPM5-B), and 5865 kg/ha (HQPM5-C). Similarly, HQPM7 had grain yield of 6648 kg/ha, whereas the waxy versions had grain yield of 7044 kg/ha (HQPM7-A), 7065 kg/ha (HQPM7-B), and 7067 kg/ha (HQPM7-C) (Table 6, Table S8). The flowering behaviour and plant characteristics of the reconstituted waxy hybrids were quite similar to the original hybrids as well (Table S8). However, in case of plant height and ear height, significant difference was observed between (1) HQPM1 and their reconstitute hybrids viz., HQPM1-C, and (2) HQPM4 and their reconstitute hybrids viz., HQPM1-B and HQPM1-C (Table 6). The improved waxy hybrids were very similar to their respective original hybrids for DUS characters except few traits (Table S9). For examples: anthocyanin colouration of bract root was present in HQPM1, while it was absent in HQPM1-A, HQPM1-B and HQPM1-C (Table S9a). Similarly, anthocyanin colouration of bract root was present in HQPM7, while it was found absent in HQPM7-A and HQPM7-C (Table S9d).

Discussion

Waxy maize rich in amylopectin is highly popular in East and Southeast Asia43. Though large number of waxy maize cultivars are available for commercial cultivation worldwide4, waxy maize protein is poor in nutritional quality due to sub-optimal levels of essential amino acids like lysine and tryptophan44,45. Lack of waxy hybrids rich in lysine and tryptophan limits its great potential as a nutritious food to the resource poor especially in the developing countries4. Here, we used genomics-assisted breeding to combine high amylopectin, lysine and tryptophan in the genetic background of four popular sub-tropically adapted hybrids through marker-aided selection of recessive wz1 and o2 genes.

The gene-based markers viz., wz1-2507F/RG and phi057 helped in precisely selecting individual plants with favourable allele of both wz1 and o2 genes, respectively. Both the markers behaved co-dominantly and distinguished the homozygotes from heterozygotes46. Hussain et al.47 reported polymorphism among Wz1 and wz1 alleles using wz1-2507F/RG. Zhang et al.48 observed polymorphism in wz1 gene among recurrent and donor parents using gene-based SSRs viz., phi027, phi061, and phi022. While, Yang et al.49 reported phi022 and phi027 as polymorphic among the recurrent and donor parents. Several authors have also successfully used gene-based SSRs, phi057 and umc1066 to select o2 allele in the MABB programme22,26. Identification of heterozygotes (BC,F, and BC,F,) and homozygotes (BC,F,) at seedling stage helped in the exclusion of non-target progenies, resulting in significant savings of labour and material cost required for raising crops and pollination activities29,34. In the present study, wz1 gene segregated as per Mendelian ratio of 1:1 in backcross generations and 1:2:1 in selfed generations. Yang et al.13 also reported 1:1 segregation in BC,F1 and BC,F1, while reported 1:2:1 ratio in F1 populations segregating for wz1 gene. However, segregation distortion (SD) was observed for o2 gene in some crosses. Similar observation was also observed by Jompuk et al.35 and Hussain et al.36 while analyzing the segregation of o2 in various backcross populations. This SD could be caused by gametophytic factors, mutants such as faulty kernels, male sterility, and embryo-specific mutations15. SD warrants raising of large population size in order to achieve sufficient foreground positive genotypes in the MABB programme.

Since, o2 and wz1 genes are recessive, traditional backcross approach would have taken 12–14 seasons as each backcross generation would require progeny testing by selfing25. Two generation-based MABB, on the other hand, was efficient enough to generate comparable results in nearly half of the time (5–6 seasons). MABB strategy thus saved significant time and resources besides speeding up the breeding cycle37. Genomics-assisted background transfer parent alleles of SSRs linked to various loci relevant to yield attributing- and agronomic-characteristics29. The high recovery of F1 plants was further validated by great degree of similarity for the large number of DUS and SSRs, respectively. Both the markers behaved co-dominantly and distinguished the homozygotes from heterozygotes46. Hussain et al.47 reported polymorphism among Wz1 and wz1 alleles using wz1-2507F/RG. Zhang et al.48 observed polymorphism in wz1 gene among recurrent and donor parents using gene-based SSRs viz., phi027, phi061, and phi022. While, Yang et al.49 reported phi022 and phi027 as polymorphic among the recurrent and donor parents. Several authors have also successfully used gene-based SSRs, phi057 and umc1066 to select o2 allele in the MABB programme22,26. Identification of heterozygotes (BC,F, and BC,F,) and homozygotes (BC,F,) at seedling stage helped in the exclusion of non-target progenies, resulting in significant savings of labour and material cost required for raising crops and pollination activities29,34. In the present study, wz1 gene segregated as per Mendelian ratio of 1:1 in backcross generations and 1:2:1 in selfed generations. Yang et al.13 also reported 1:1 segregation in BC,F1 and BC,F1, while reported 1:2:1 ratio in F1 populations segregating for wz1 gene. However, segregation distortion (SD) was observed for o2 gene in some crosses. Similar observation was also observed by Jompuk et al.35 and Hussain et al.36 while analyzing the segregation of o2 in various backcross populations. This SD could be caused by gametophytic factors, mutants such as faulty kernels, male sterility, and embryo-specific mutations15. SD warrants raising of large population size in order to achieve sufficient foreground positive genotypes in the MABB programme.

Amylose is a linear homopolymer of glucopyranose units linked by α-(1,4) linkage, whereas amylopectin is a branched homopolymer of glucopyranose with both α-(1,4) and α-(1,6) linkages4. Introgreded inbreds and reconstituted hybrids recorded ~40% increase in amylopectin over original genotypes. Qi et al.49 also reported ~23% increase in amylopectin among waxy lines and hybrids (94.9%) compared to wild type genotypes (76.9%). Accumulation of higher amylopectin in waxy landraces and hybrids have also been reported by Stamp et al.5. Maize starch is composed of amylose and amylopectin fractions5. In maize, wild type Wx1 codes functionally active GBSS-I that catalyzes the formation of amylose from ADP-glucose. However, recessive wx1 leads to impaired activity of GBSS-I which shifts the flux towards synthesis of amylopectin. Mutant wx1 results from various types of mutations including transposon/retrotransposon insertion and nucleotide deletion. These mutations cause formation of premature stop codon or a change in amino acids in a critical region of the transcript, as well as splicing and translational mistakes. Though, MABB-derived wx1-based inbreds and reconstituted hybrids recorded enhanced amylopectin, they also exhibited moderate variation in amylopectin (95–99%) despite the presence of the identical wz1 gene. This difference could be attributed to modifier loci or QTL that influence the accumulation of amylopectin in maize5. However, total starch content remained nearly same among the MABB-derived genotypes over their original versions. This suggested that increase in amylopectin among the
wxl-based genotypes did not pose any negative effect on total starch content, which further justified the similar  

MABB-derived lines and reconstituted hybrids having o2 gene possessed higher lysine and tryptophan than  

Recessive o2 leads to reduction of zein proteins (deficient in lysine and tryptophan), with a concurrent increase in non-zein proteins rich in lysine and tryptophan23. o2 also down regulates the  

sythesis of lysine ketoglutarate reductase (LKR) resulting in increased levels of free lysine24. Besides, it is also  

olved in regulation of various lysine-rich proteins and enzymes45. However, wxlwx1o2o2-based MABB  

derived inbreds and reconstituted hybrids possessed ~11–17% more lysine and tryptophan over the o2o2-based  

original genotypes. Zhou et al.7 introgressed o2 gene into a waxy inbred (Zhao-OP-6/O2O2), and discovered that  

introgressed lines had 51.6% higher lysine than the original waxy line. Yang et al.13 also introgressed recessive  

opaque16 (o16) gene from QCL3024 into two Chinese waxy lines, QCL5019 and QCL5008, and found that lysine  

content of the pyramid lines was 20% higher than the waxy parent. Zhang et al.11 further pyramided o2 and o16  

in a waxy genetic background and found that pyramided lines (wx1wx1/o2o2/o16o16) accumulated 11% more  

lysine than o2o2 genotypes. Thus, stacking of wx1 and o2 provided synergistic effects on accumulation lysine  

and tryptophan, which would provide better nutritional quality to alleviate malnutrition. Wang et al.19 analyzed  

RNA-sequencing of kernels (18th day after pollination) of wx1wx1 and o2o2/wx1wx1 inbreds, and revealed 49  

differentially expressed genes (DEGs) related to mainly catalytic activity and metabolic processes. The o2 gene  

regulated multiple metabolic pathways related to biological processes and molecular function during waxy  

maize endosperm development. In o2o2/wx1wx1 line, the two genes that encode the EF-1a and LHT1 were up-  

regulated, and the gene that encodes sulfur-rich proteins was down-regulated, leading to the elevated levels of  

Yellow1 (Y1) gene on chromosome-6 codes for phytosyn synthase (psy1), which condenses two geranyl-geranyl  

phosphosphate molecules into one molecule of phytoene in the carotenoid biosynthesis pathway53. The dominant  

Y1 allele converts the step thereby leading to the synthesis of carotenoids and eventually yellow colour in the  

endosperm. However, the recessive y1 allele is unable to catalyse the reaction and makes the kernel devoid of any  

carotenoids and eventually kernels look white51. In BC1F1 seeds borne on BC2F2 ears, Y1 gene segregated in four  

forms viz., (i) dark yellow (Y1Y1Y1), medium yellow (Y1Y1y1), light yellow (Y1y1y1) and white (y1y1y1) in the  

endosperm54. We selected only the white kernels to raise the BC2F3 progenies, and eventually develop white  

grained waxy hybrids.  

These newly derived white waxy hybrids possess diverse usage as food and various industrial products. Glob-  

ally immature waxy maize ears are gaining popularity as a breakfast item. It is also widely used to improve the  

viscosity, freeze–thaw stability, uniformity, and appearance of the food products55. Due to high amylopectin  

content, food made from waxy maize is easily digested in the human gut16,19. Amylopectin powder is a preferred  

food after workout in gym and body building industry5. Further, pure amylopectin powder possesses special pasting  

properties, thus used as a popular ingredient in textile, adhesive and paper industries25. Since waxy maize starch  

has a higher hydrolysis rate, it has higher starch-to-ethanol conversion efficiency when used to make ethanol57.  

Further, these white waxy hybrids are also rich in lysine and tryptophan, thus possess superior protein quality.  

So far, large number of QPM hybrids rich in lysine and tryptophan have been developed and commercialized  

worldwide58. But these QPM hybrids do not possess high amount of amylpectin11. On the other hand, several  

waxy landrace and hybrids have been in cultivation especially in East- and South-East Asian countries5. These  

waxy cultivars are poor in nutritional quality as they lack required amount of lysine and tryptophan23. Though  

few studies have improved wxl inbreds for nutritional qualities, the present study possesses novelty on three  

aspects, viz. (1) studies by Yang et al. (2013), Zhou et al. (2016) and Zhang et al. (2013) have mentioned the enhancement  
of only lysine, but we analyzed the effects on both lysine and tryptophan among the waxy genotypes. These two  

are the essential amino acids not synthesized in our body, thus possess paramount importance for growth and  
development in humans, (2) earlier studies have analyzed the levels of amylopectin and lysine only in inbreds,  

but here we combined wxl and o2 genes in elite inbreds, and further developed and evaluated the performance  

of hybrids for amylopectin, lysine, tryptophan, grain yield and agronomic performance, and (3) previous studies  
have combined wxl and o2 genes in temperate background, while lines in the present study are sub-tropically  
adapted. These newly derived waxy hybrids with superior protein quality would help in providing the balanced  
diet and alleviate the malnutrition in a sustainable and cost-efficient manner59. These nutritious waxy hybrids  

are also high yielding and would help the farmers to earn livelihood. The present investigation is the first report  
development of waxy hybrids rich in lysine and tryptophan using accelerated-breeding strategy.  

Conclusions  

Waxy maize rich in amylopectin is becoming increasingly important as a source of human nutrition, livelihood,  

and income generation. However, their usage as a preferred food and industrial product is limited due to lack  
of suitable waxy hybrids. Here, we have developed four high yielding waxy hybrids rich in amylopectin. These
waxy hybrids also possess quality protein, besides high grain yield. The improved waxy QPM hybrids developed in this study can be directly commercialized and used for human consumption. Further, the improved waxy QPM maize inbreds will serve as potential donors for the development of the lysine and tryptophan rich waxy hybrids in the breeding programmes. This is the first report of development of maize hybrids rich in amylopectin, lysine and tryptophan.

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