Development of low-phytate maize inbred lines through marker-assisted introgression of *lpa1*

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Handling Editor:
Rajeev Varshney

ABSTRACT

Context. Phytic acid is the major storage form of phosphorus in cereals and is considered an anti-nutritional factor because it chelates major mineral micronutrient cations, resulting in micronutrient malnutrition in humans. For monogastric animals fed maize (*Zea mays* L.) grains, the stored phosphorus does not release into the digestive tract, leading to phosphorus deficiency and environmental pollution. **Aims.** The aim of the study was to develop maize lines with a lower level of phytic acid that might substantially enhance the nutritional value of maize. **Methods.** The *lpa1* mutant allele conferring low phytic acid was transferred into the parental lines of popular maize hybrid DMH 121 (i.e. BML 6 and BML 45) through marker-assisted backcross breeding. Foreground selection was performed using a co-dominant single nucleotide polymorphism marker through a high-resolution melting approach, and background selection was undertaken using 50–55 polymorphic sequence-tagged microsatellite site markers. **Key results.** Near-isogeneic lines were produced with >90% recurrent parental genome and reduction of phytic acid content by up to 44–56% compared with the original lines. **Conclusions.** The near-isogeneic lines carrying *lpa1* can be used to reconstitute DHM 121 with low phytate content. **Implications.** The low-phytate maize hybrids produced can be useful in reducing micronutrient malnutrition in humans, as well as environmental pollution.

Keywords: biofortification, high-resolution melting, inorganic phosphorus, maize, malnutrition, marker-assisted backcross breeding, near isogenic lines, phytic acid.

Introduction

Maize (*Zea mays* L.) is the third-most important cereal crop for food and nutritional security, after wheat and rice. It is widely used for feed and starch-making, and is grown in at least 171 countries across the globe on an area of 201.98 Mha. Among major crops, it has the highest global production of 1162 Mt, with average productivity of 5755 kg/ha (FAOSTAT 2020). It is the primary energy source and a major staple food crop for humans, and feed and fodder crop for livestock, in many countries across Africa, Latin America and Asia, including India. Apart from food, feed and fodder-based industries, it also has multi-faceted uses as raw material in industries such as biofuel and starch. Thus, it occupies a prominent position in global agriculture and trade. Being a highly cross-pollinated crop, maize possesses considerable genetic diversity in terms of geographical adaptability and morphological variability (Dhillon 1998). However, the bioavailability of major vital micronutrients including iron (Fe) and zinc (Zn) is very low owing to various genetic and anti-nutritional factors in maize grains (Gupta *et al.* 2015; Mallikarjuna *et al.* 2015, 2020).

Phytic acid (PA) is the major storage form of phosphorus (P) in plant seeds, especially in cereals (Guttieri *et al.* 2004; Coulibaly *et al.* 2010; Cerino Badone *et al.* 2012; Borlini *et al.* 2019). In maize, it varies to greatly from 1.7 to >4.5 mg/g (Lorenz *et al.* 2008; Yathish *et al.* 2021). Mature seeds are the main storage organs of PA, which is synthesised in the endoplasmic reticulum and deposited as a mixture of phytate salts, mainly with

significant cations such as iron (Fe^{2+}), potassium (K^+), magnesium (Mg^{2+}) and zinc (Zn^{2+}), in specific organised structures known as globoids (Raboy 2002; Sparvoli and Cominelli 2015; Borlini *et al.* 2019). During germination, seed phytate is hydrolysed by the enzyme phytase through sequential hydrolysis, leading to the release of minerals, free phosphate and myoinositol. Germination is an energy-demanding metabolic process that requires sufficient quantities of adenosine triphosphate (ATP). The free phosphates released through phytate hydrolysis are utilised to synthesise ATP (Cerino Badone *et al.* 2012; Landoni *et al.* 2013). The other hydrolysed compounds, myoinositol and minerals, are also essential for the growth and development of seedlings, and are utilised in different metabolic processes. However, in the absence of phytases, the phytate salts and PA are excreted without hydrolysis. Thus, PA is considered a significant anti-nutritional factor in many crops, including maize (Zhou and Erdman 1995; Kishor *et al.* 2019). For example, monogastric animals such as swine, poultry birds and humans cannot process PA owing to low levels or absence of phytase activity. This affects the bioavailability of essential mineral cations such as Zn^{2+}, Mg^{2+}, Fe^{2+}, calcium (Ca^{2+}) and K^+ as well as phosphate in monogastric animals. The undigested PA, which is excreted along with bound P enters water bodies, leading to major environmental problems including eutrophication (Sharpley *et al.* 1994). Therefore, the development low-PA (LPA) maize cultivars not only plays as significant role in enhancing the nutritional value of maize grain but also reduces environmental pollution.

Donors for the LPA trait in the active germplasm of most crop plants are low in frequency. However, applications of different approaches, for example mutagenesis and transposon tagging, have led to the identification of several LPA mutants in different crops including maize (Raboy and Gerbasi 1996; Larson *et al.* 1998, 2000; Rasmussen and Hatzack 1998; Raboy *et al.* 2000; Wilcox *et al.* 2000; Pilu *et al.* 2003; Shi *et al.* 2005; Liu *et al.* 2007; Yuan *et al.* 2007). In maize, three *lpa* mutants (*lpa1*, *lpa2* and *lpa3*) have been isolated (Raboy 2007). The mutants *lpa1* and *lpa2* were generated through chemical mutagenesis (Raboy and Gerbasi 1996; Pilu *et al.* 2003) and *lpa3* by transposon tagging (Shi *et al.* 2005). Detailed molecular characterisation of these LPA mutants in maize has led to identification of mutations in different genes encoding different proteins. The first mutant, *lpa1*, is the result of a mutation in the *ZmMRP4* gene, which encodes a multidrug resistance-associated protein (MRP) belonging to the subfamily of ATP binding cassette (ABC) *trans*-membrane transporters (Shi *et al.* 2007; Cerino Badone *et al.* 2012). On the other hand, *lpa2* is the result of a mutation in the maize inositol phosphate kinase (*ZmIpk4*) gene (Shi *et al.* 2003). The first allele of *lpa2*, *lpa2-1*, was caused by genomic sequence rearrangement in *ZmIpk4*, whereas the second, *lpa2-2*, was due to nucleotide transition (C to T) at position 158 in *ZmIpk4*, which generates a stop codon

(Shi *et al.* 2003). The third LPA mutant, *lpa3*, is a knockout mutation in the *myo*-inositol kinase (MIK) producing gene, with a Mu transposon insertion in the coding region of exon 1 (Shi *et al.* 2005).

Biochemical analysis for PA content in the three mutants has revealed a reduction in PA content by 66% (*lpa1*), 50% (*lpa2*) and 45–50% (*lpa3*) (Raboy *et al.* 2000; Shi *et al.* 2005). This suggests that greater reduction in PA content can be achieved through targeting *lpa1* rather than the other two mutants (Cerino Badone *et al.* 2012). The modern maize hybrid DMH 121 is of *kharif* adaptation and was released in India in 2014 for Bihar, West Bengal, Odisha and Jharkhand though to dry central-western zones comprising Chhattisgarh, Madhya Pradesh, Rajasthan and Gujarat. Although quite popular among farmers, it has a PA concentration >3.5 mg/g, making it nutritionally poor. Hence, the present study was undertaken to introgress *lpa1* from an LPA mutant line into the parental lines of DHM 121 (BML 6 and BML 45) to reconstitute a new LPA version of DHM 121.

Materials and methods

Plant materials

The recurrent parents, BML 45 and BML 6, were chosen to transfer the low-phytate trait from the mutant donor line LPA 1 carrying the *lpa1* gene. The donor line was obtained from ICAR-Vivekananda Parvatiya Krishi Anusandhan Sansthan (VPKAS), Almora, Uttarakhand, India. Table 1 contains additional information on the genetic material used in the present study.

Genotyping and marker-assisted selection

DNA extraction

DNA was isolated from 12–15-day-old, field-grown, healthy seedlings by following a modified CTAB protocol (Dellaporta *et al.* 1983). The RNase-treated extracted DNA dissolved in 1× TE buffer was checked for quality by running 1% agarose gel and recording OD at 260 and 280 nm. High-quality DNA was normalised to 50 ng/μL and stored at −20°C.

Foreground selection

The PCR-based sequence-tagged microsatellite site (STMS) marker linked to *lpa1* revealed no detectable polymorphism between recurrent and donor parents at the locus, owing to low resolution to differentiate the target single nucleotide polymorphism (SNP). Thus, 5′-ATAACTGGAGCGTGGGACA G-3′ and 5′-CTGCGGATGATCTTTTGGAT-3′ sequences were employed as forward and reverse primers, respectively, for amplification of *lpa1* in qPCR. The PCR amplifications were performed in 50 μL reaction volumes consisting of ~2 ng genomic DNA template, qPCR MasterMix E1 (GeneON, Ludwigshafen, Germany), and 200 nM forward and reverse

Table 1. Description of donor and recurrent maize inbred lines used in the study.

S. N	Line	Pedigree	Source	Remarks
1	BML 45 (female parent)	Derived from cross NH 6240 × BH 1620	PJTSAU, Hyderabad	Flint, yellow
2	BML 6 (male parent)	SRRL 65-B96-1-1-2-#- 2-2-1-×-1-1-×b-×b	PJTSAU, Hyderabad	Flint, yellow
3	LPA 1 (donor line)	Low-phytate mutant line	ICAR-VPKAS, Almora	*lpa1* mutant

primers. The PCR amplification was initiated with a 10 min hold at 95°C as an initial denaturation step, followed by 40 cycles of 95°C for 10 s, 55°C for 15 s, and 72°C for 20 s. High-resolution melt (HRM) analysis was employed to detect polymorphism between the donor and recurrent parents for the selection of *lpa1* in F_1s and different backcross generations (BC_1F_1, BC_2F_1, and BC_2F_2) (Naidoo *et al.* 2012). HRM analysis was performed automatically after the PCR and programmed to ramp temperature from 72°C to 95°C, raised by 0.2°C/step after the final extension step. PCR amplification and HRM analysis were performed by using AriaMx 96 Real-Time PCR equipment (Agilent Technologies, Santa Clara, CA, USA). The melting curves created were retrieved with AriaMx 96 Real-Time PCR system software to discriminate genotypes carrying wild and mutant forms of *lpa1* based on melt-curve temperature.

Background selection

An initial polymorphism survey was conducted using a set of 450 STMS markers selected from the maize genome database (www.maizegdb.org), ensuring their distribution throughout the maize genome covering all 10 chromosomes at regular intervals. Based on the polymorphism survey, 50 and 55 STMS markers were chosen and used for background selection in different backcross generations (BC_1F_1, BC_2F_1, and BC_2F_2) for BML 45 and BML 6, respectively.

Marker-assisted backcross breeding (MABB) program

Development of F_1s

Recurrent parents, BML 45 and BML 6, as well as donor parent LPA 1 were raised during *rabi* 2015–16 at the Winter Nursery Centre of the ICAR-IIMR (Indian Institute of Maize Research), Hyderabad, Telangana, India. Three staggered plantings of parental lines were used to attempt successful F_1 crosses between recurrent and donor parents.

Development of backcross population

The F_1s were grown along with parents during *kharif* 2016 at the experimental site of ICAR-IIMR in the IARI farm, New Delhi, India. The F_1s were confirmed for the presence of the SNP marker, that is the presence of mutant gene *lpa1* in the heterozygous condition, through HRM analysis. The confirmed F_1 plants were selectively crossed to recurrent parents, BML 45 and BML 6, to generate BC_1F_1 seeds. The backcrossed F_1 plants were grown to maturity, BC_1F_1 seeds were harvested at

physiological maturity, and the BC_1F_1 populations were grown along with the parents during *rabi* 2016–17 at the Winter Nursery Centre of ICAR-IIMR, Hyderabad. HRM analysis was performed to detect the SNP marker linked to the *lpa1* locus in order to identify and select BC_1F_1 plants heterozygous at the locus (foreground selection). The BC_1F_1 plants harbouring the desired gene in the heterozygous condition were screened using polymorphic STMS markers to identify and select BC_1F_1 plants with a higher proportion of the recurrent parent genome (RPG; background selection).

For development of the BC_2F_1 generation, the selected BC_1F_1 plants based on the foreground and background selection were backcrossed with the respective recurrent parent. The BC_2F_1 populations were raised during *kharif* 2017 at the experimental site of ICAR-IIMR in the IARI farm, New Delhi. The heterozygous form of the *lpa1* locus was identified using HRM analysis. BC_2F_1 plants heterozygous at *lpa1* were selected through foreground selection, and background selection was performed in the selected BC_2F_1 plants to identify those with the highest proportion of RPG. BC_2F_1 plants with the highest proportion of RPG were selfed to produce the BC_2F_2 generation. The BC_2F_2 seeds were harvested from BC_2F_1 plants after attaining physiological maturity. Finally, the BC_2F_2 plants were raised during *rabi* 2017–18 at the Winter Nursery Centre, ICAR-IIMR, Hyderabad. BC_2F_2 plants homozygous for *lpa1* were selected based on foreground selection through HRM analysis. The selected BC_2F_2 plants homozygous for *lpa1* were screened using polymorphic STMS markers to identify plants with the highest proportion of RPG recovery. BC_2F_2 plants that were homozygous for *lpa1* with highest proportion of RPG were advanced to the BC_2F_3 generation and maintained by self-pollination.

Phenotyping for PA and inorganic phosphate (P_i)

A modified colourimetric assay (Lorenz *et al.* 2007) was used to estimate maize grain PA and P_i contents. The detailed procedure for biochemical estimation of PA and P_i was followed as reported by Yathish *et al.* (2021). For extract preparations, seed samples (10 g) were ground to a fine powder, and subsamples (10 mg) of the maize flour were taken and put into 2-mL centrifuge tubes, then 0.65 M HCl (200 μL) was added to the tubes. The mixture was incubated for ~12 h at room temperature on a shaker/rocker, and the tubes were centrifuged at 553*g* for 20 min after incubation. For PA estimation, 30 μL extract was placed after centrifugation into a 96-well microplate, then 200 μL diluted

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(1:4) Wade reagent was added to each well. For P_i estimation, another 30 μL extract was placed in a separate 96-well microplate, with 130 μL deionised water and 100 μL Chen's reagent added to each well. Sodium phytate (HiMedia, Mumbai, Maharashtra, India) and potassium dihydrogen phosphate (Supelco; Merck, Darmstadt, Germany) were used as PA and P_i control standards, respectively. The 96-well plates containing control standards and samples of different genotypes were allowed to stand for 15–20 min, after which OD490 nm and OD820 nm were recorded for estimation of PA and P_i, respectively, using a BioTek Epoch 2 Microplate Spectrophotometer (BioSPX, Abcoude, Netherlands).

Data analyses

Data were subjected to analysis using SAS ver. 9.2 software (SAS Institute, Cary, NC, USA) for calculation of the coefficient of variation (CV), honest significant difference (HSD) and standard error (s.e.), and for analysis of variance (ANOVA). GGT ver. 2.0 was used to represent background recovery of recurrent parents (van Berloo 2008).

Results

Validation of foreground marker for *lpa1* and parental polymorphism survey

The co-dominant SNP-based molecular marker located within the gene determining the low-phytate trait was used to differentiate the wild-type (*LPA1*) from the mutant (*lpa1*) allele. The PCR amplification pattern of the SNP marker could not differentiate the donor (LPA 1) and recurrent parents (BML 45 and BML 6). At the same time, qPCR-based HRM analysis of the SNP marker differentiated the donor and recurrent parents with different melt curve peaks with specific temperatures (Fig. 1). The recurrent parents, BML 45 and BML 6, showed melt temperature peaks at 84.6°C and 84.4°C, respectively. The donor parent (LPA 1) had a melt temperature peak of 84.2°C (Fig. 1). The co-dominant nature of the SNP marker means that it can identify the homozygotes and the heterozygotes for the gene.

The parental polymorphism survey was conducted with 450 STMS markers, to identify and use polymorphic markers for background selection to accelerate the recovery of RPG during backcrossing. The number of STMS markers showing polymorphism between donor and recipient parents was 62 (Supplementary Materials, Table S1). Graphical representation of selected polymorphic markers used for background analysis is provided in Fig. S1. These polymorphic STMS were employed for background selection in BC_1F_1, BC_2F_1 and BC_2F_2 generations in order to identify the progenies with the highest proportion of RPG.

Molecular analysis of the F_1 and BC_1F_1 generations

The F_1 hybrids generated between recurrent and donor parents (BML 6 × LPA 1 and BML 45 × LPA 1) were tested for hybridity by using a few random polymorphic STMS
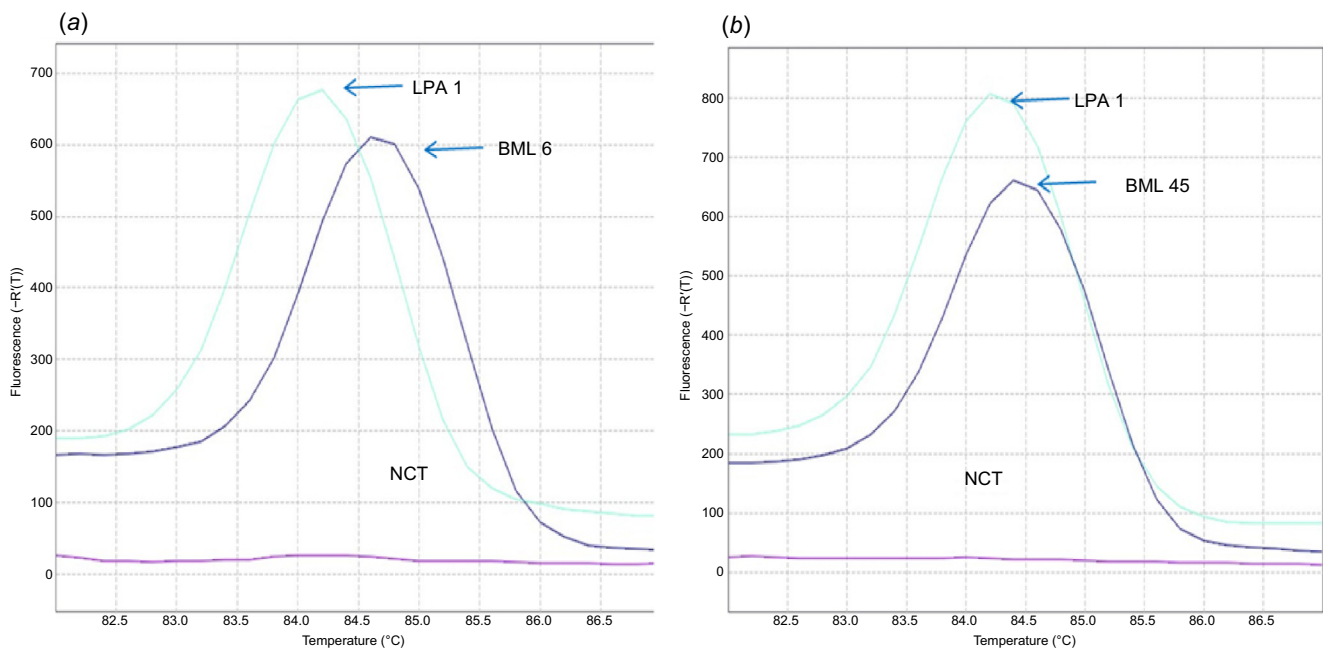

Fig. 1. High-resolution melting (HRM) analysis of the SNP marker differentiating the low-phytate (*lpa1*) from the wild-type (*LPA1*) allele between the donor mutant line LPA 1 and recurrent parental inbred lines: (*a*) BML 6 and (*b*) BML 45. NCT = no-template control.

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markers over the rest of the genome, and for heterozygosity at the *LPA1* locus by using the co-dominant, gene-based SNP marker through HRM analysis (Fig. 2). Based on HRM analysis, hybrid plants #1 (1, BML 6/LPA 1) and #4 (4, BML 45/LPA 1) were selected and backcrossed with their respective recipient parents, BML 6 and BML 45, to generate BC_1F_1 plants. The individual plants in the BC_1F_1 generation were regularly observed after germination for overall growth and development with regard to expression of various morphological traits. Based on the close resemblance of BC_1F_1 plants to their respective recurrent parents in overall morphological and phenotypic characters, the 30 best BC_1F_1 plants were selected for foreground selection through HRM analysis, using the co-dominant, gene-based SNP marker. BC_1F_1 plants showing melt curve peaks towards *lpa1* (donor parent melt curve peak) were considered heterozygous; BC_1F_1 plants showing melt curve peaks of 84.4°C (for recurrent parent BML 6) and 84.2°C (for recurrent parent BML 45) were selected for subsequent generation of BC_2F_1 plants (Fig. 3). With recurrent parents BML 6 and BML 45, respectively, 11 and 12 BC_1F_1 plants were found to be heterozygous for *lpa1*.

Following foreground selection in BC_1F_1, 10 of the plants heterozygous for *lpa1* and with relative morphological resemblance to their recurrent parent in the genetic backgrounds of BML 6 and BML 45 were used to conduct background selection. Fifty polymorphic STMS markers were used for background selection in the BC_1F_1 generation of BML 6, and 55 in BML 45. The percentage of RPG recovered in the BC_1F_1 generation ranged from 74.00% to 79.00% in the BML 6 genetic background, and from 74.55% to 79.09% in BML 45. Fig. 4 is a pictorial depiction of RPG recovery on chromosome 1 from the BML 6 and BML 45 crosses. BC_1F_1 plant #13 (13, BML 6*/(1, BML 6/LPA 1)) and plant #220 (220, BML 45*/(4, BML 45/LPA 1)) with highest genome recovery from BML 6 and BML 45, respectively, were selected and backcrossed with their respective recurrent parents to derive BC_2F_1 populations.

Molecular analysis of BC_2F_1 generation

A similar procedure to that followed in the BC_1F_1 generation was adopted in BC_2F_1. The BC_2F_1 plants with greater resemblance to their respective recurrent parents in as many morphological traits as possible and resemblance in overall growth, development and phenology were selected in each backcross population. Forty BC_2F_1 plants were selected in each of the BML 6 and BML 45 genetic backgrounds for foreground selection using the gene-based SNP marker. Foreground screening identified 27 and 32 plants with the *lpa1* allele in BML 6 and BML 45 backgrounds, respectively. Among the plants heterozygous for *lpa1*, ten with greater resemblance to their respective recurrent parents were chosen for background selection using the same set of polymorphic STMS markers as in the BC_1F_1 generation. The percentage of RPG in the BC_2F_1 population, derived from the genetic background of BML 6 and BML 45, was 85.00–89.00% and 86.36–89.09%, respectively. Fig. 5 is a graphical representation
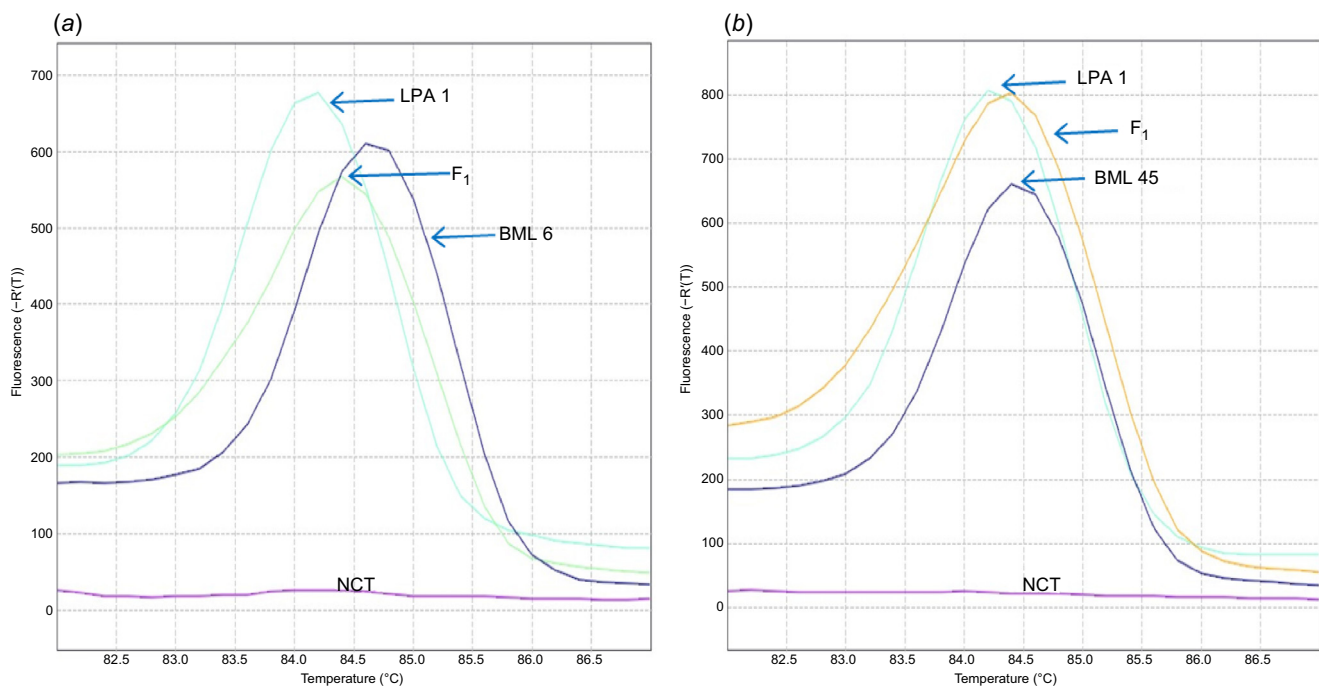

Fig. 2. Identification of F_1 in a hybridity test employing the SNP marker through HRM analysis for *lpa1*. LPA 1 crossed with: (*a*) BML 6, and (*b*) BML 45. NCT = no-template control.
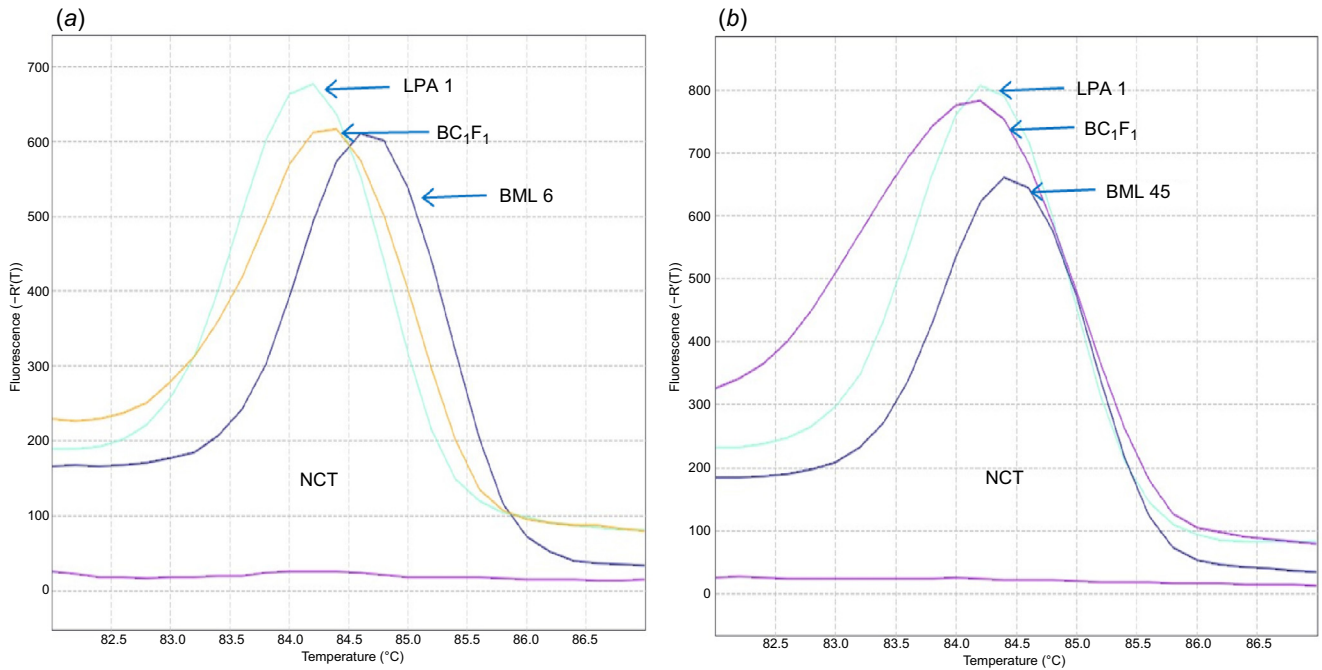
Fig. 3. Foreground selection for *lpa1* using the SNP marker through HRM analysis in the BC$_1$F$_1$ generation for recurrent parents: (*a*) BML 6, and (*b*) BML 45. NCT = no-template control.
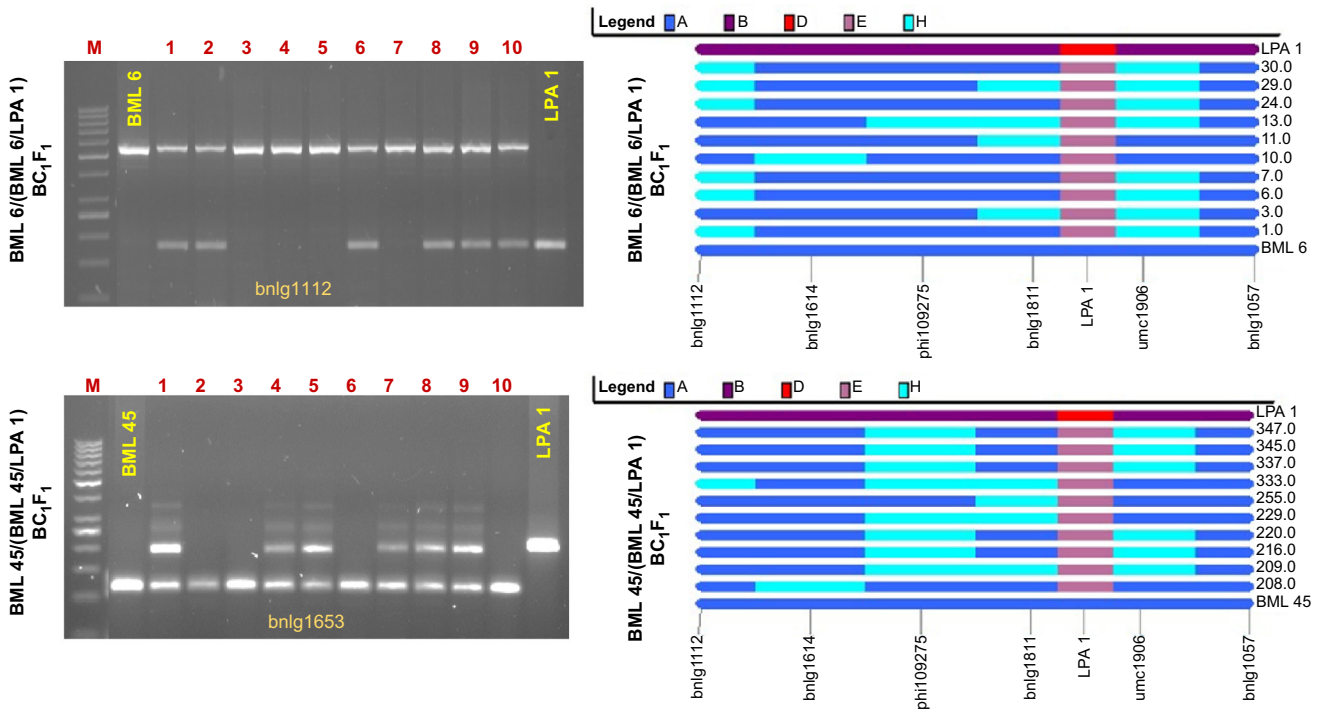

Fig. 4. Recurrent parent genome recovery on chromosome 1 in the BC$_1$F$_1$ generation for recurrent parents BML 6 and BML 45. Donor line LPA 1 and recurrent parental lines are also shown. A, Recurrent parent specific allele; B, *LPA1* allele; D, *lpa1* in homozygous state; E, *LPA1/lpa1*, heterozygous state; H, heterozygote.

of RPG recovery on chromosome 1 of BML 6 and BML 45 crosses. Two BC$_2$F$_1$ plants of BML 6, namely #2875 (2875, (13, BML 6*/(1, BML 6/LPA 1))) and #2878 (2878, (13,

BML 6*/(1, BML 6/LPA 1))) with 89.00% BML 6 genome, and two BC$_2$F$_1$ plants of BML 45, namely #2222 (2222, (220, BML 45*/(4, BML 45/LPA 1))) and #2241 (2841,
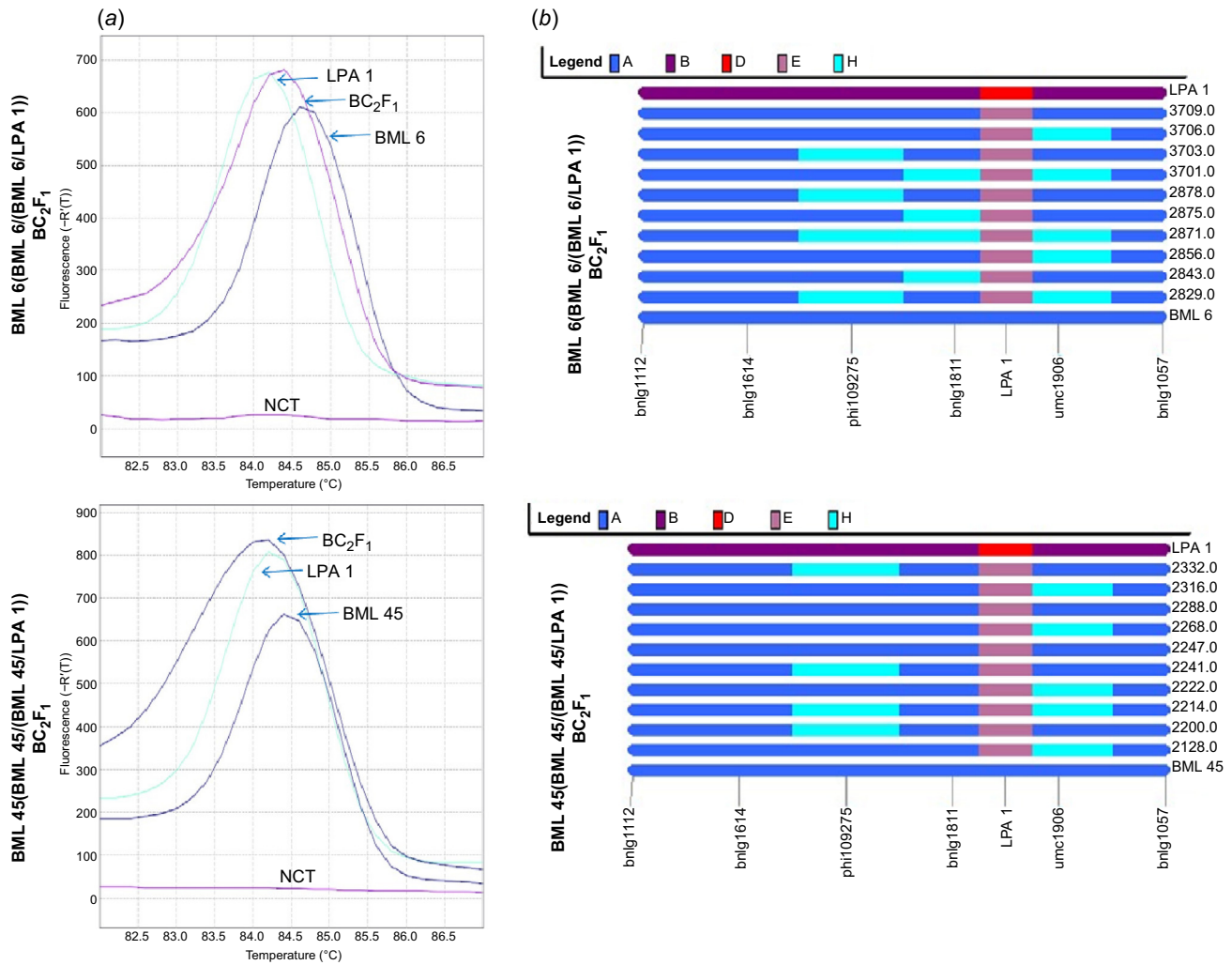
Fig. 5. Foreground and background selection in the BC_2F_1 generation for recurrent parents BML 6 and BML 45. (*a*) Foreground analysis for *lpa1* allele using the SNP marker through HRM analysis. (*b*) Recurrent parent genome recovery on chromosome 1. Donor line LPA 1 and recurrent parental lines are also shown. A, Recurrent parent specific allele; B, *LPA1* allele; D, *lpa1* in homozygous state; E, *LPA1/lpa1*, heterozygous state; H, heterozygote.

(220, BML 45*/(4, BML 45/LPA 1))) with 89.09% of BML 45 genome, were selected and selfed to develop the BC_2F_2 populations.

Molecular analysis of BC_2F_2 generation

The procedure adopted for selecting BC_2F_2 plants for foreground selection was the same as followed in BC_1F_1 and BC_2F_1 generations. Sixty plants were selected in each genetic background of BML 6 and BML 45 for foreground selection. However, plants showing melt curve peaks of 84.2°C, similar to the donor parent melt curve peak, were selected and considered homozygous for the *lpa1* allele (Fig. 6*a*). The number of plants homozygous for *lpa1* was 18 in the BML 6 genetic background and 21 in BML 45. As followed in BC_1F_1 and BC_2F_1 generations, the 10 homozygous plants with greatest resemblance to their respective recurrent

parent were selected from each BC_2F_2 population and subjected to background selection with the same set of polymorphic STMS markers. The percentage RPG recovered in the BC_2F_2 generation derived from BML 6 and BML 45 was 91.00–93.00% and 90.91–93.64%, respectively. Fig. 6b is a graphical representation of RPG recovery on chromosome 1 in BML 6 and BML 45 crosses. The BC_2F_2 plants with the highest percentage of RPG and homozygous for *lpa1* were advanced through self-pollination to generate BC_2F_3 near-isogenic lines (NILs).

Estimation of PA and P_i in BC_2F_3 NILs homozygous for *lpa1*

The donor and recurrent parental lines, as well as NILs obtained from self-pollinated BC_2F_2 plants (BC_2F_3 seeds) homozygous for *lpa1*, were analysed for PA and P_i content
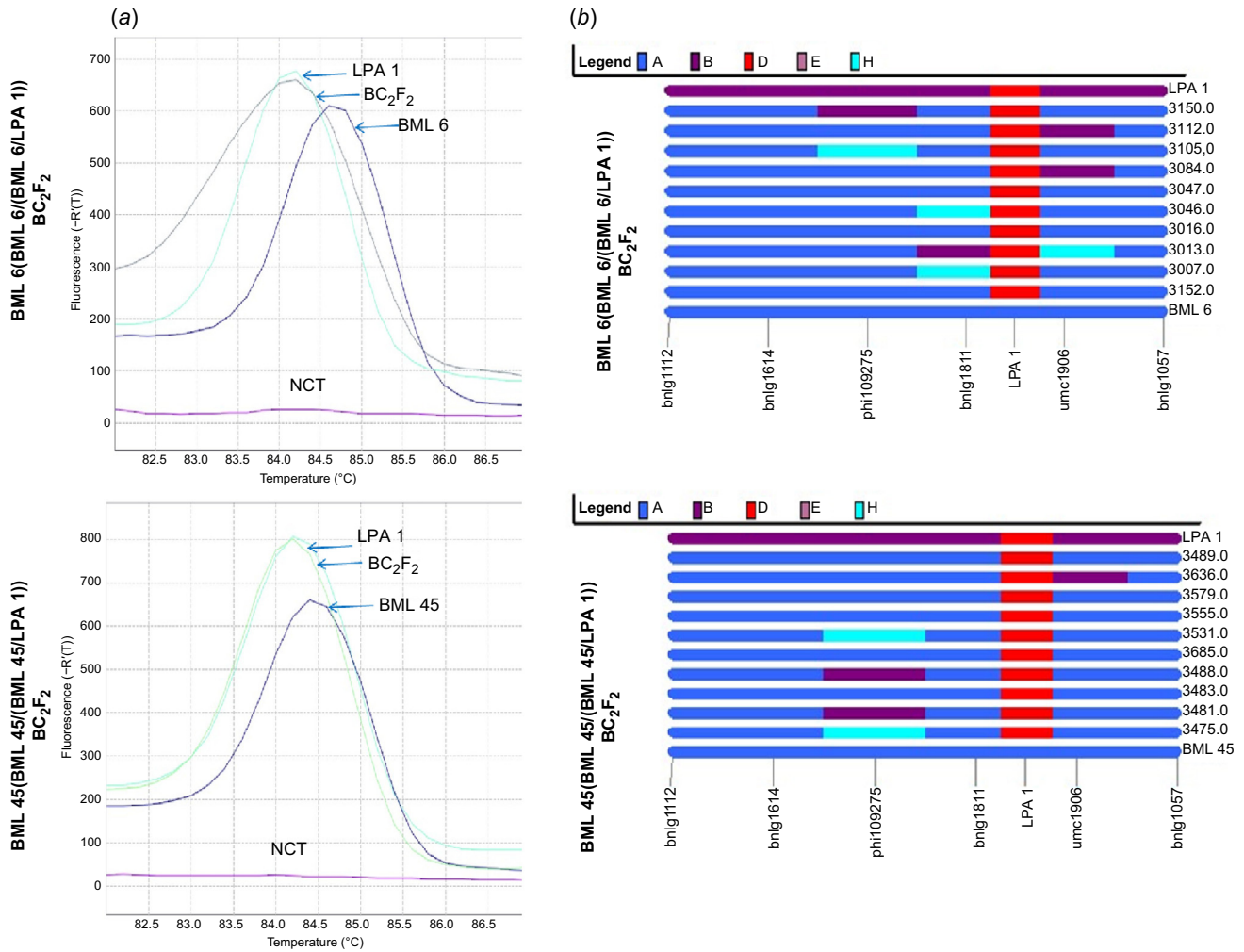
Fig. 6. Foreground and background selection in the BC_2F_2 generation for recurrent parents BML 6 and BML 45. (*a*) Foreground analysis for *lpa1* allele using the SNP marker through HRM analysis. (*b*) Recurrent parent genome recovery on chromosome 1. Donor line LPA 1 and recurrent parental lines are also shown. A, Recurrent parent specific allele; B, *LPA1* allele; D, *lpa1* in homozygous state; E, *LPA1*/*lpa1* in heterozygous state; H, heterozygote.

in order to measure the expression of *lpa1* in the genetic background of recurrent parents. Quantitative estimation of PA content showed that the recurrent parental lines contained 3.59 ± 0.12 mg/g (BML 6) and 3.16 ± 0.14 mg/g (BML 45), whereas the donor parent had the lowest PA content (1.3 ± 0.12 mg/g). Similarly, quantitative estimation of P_i showed that the recurrent parents contained 0.65 ± 0.06 mg/g (BML 6) and 0.51 ± 0.06 mg/g (BML 45), whereas the donor parent had the highest Pi content (1.42 ± 0.05 mg/g) (Table 2). PA and P_i contents in NILs varied; PA content in NILs developed in the genetic background of BML 6 varied from 1.59 ± 0.12 to 2.89 ± 0.12 mg/g and in that of BML 45 from 1.78 ± 0.14 to 3.28 ± 0.14 mg/g. Similarly, P_i content varied from 0.54 ± 0.05 to 1.45 ± 0.05 mg/g (BML 6 background) and from 0.3 ± 0.06 to 0.91 ± 0.06 mg/g (BML 45 background). The average PA content of NILs of

BML 6 and BML 45 backgrounds was significantly lower than of their respective recurrent parent, whereas P_i content was significantly higher. Further, two NILs, namely LPA1BML6-1 (plant #3152) and LPA1BML45-2 (plant #3481), showed PA (1.59 ± 0.12 mg/g and 1.78 ± 0.14 mg/g) and P_i (1.45 ± 0.05 mg/g and 0.91 ± 0.06 mg/g) contents comparable to those of the donor parent (LPA 1). Other NILs also showed considerably lower levels of PA than recurrent parents, namely LPA1BML6-2 (#3007, 2.08 ± 0.12 mg/g) and LPA1BML6-3 (#3013, 2.13 ± 0.12 mg/g) of BML 6 background, and LPA1BML45-4 (#3488, 2.33 ± 0.14 mg/g) and LPA1BML45-1 (#3475, 2.37 ± 0.14 mg/g) of BML 45 background. Therefore, application of MABB was successful in the introgression of the low-phytate allele from donor parent to recurrent parents and in developing low-phytate versions (NILs) of BML 6 and BML 45.

Table 2. Phytic acid (PA) and inorganic phosphate (P_i) content of newly developed near-isogenic lines (NILs) along with their recurrent (BML 6, BML 45) and donor (LPA 1) parents.

	Line	PA	P_i	Line	PA	P_i
1	LPA1BML6-1	1.59 ± 0.08E	1.45 ± 0.05A	LPA1BML45-1	2.37 ± 0.16E	0.66 ± 0.03DE
2	LPA1BML6-2	2.08 ± 0.06D	1.03 ± 0.04B	LPA1BML45-2	1.78 ± 0.41F	0.91 ± 0.01B
3	LPA1BML6-3	2.13 ± 0.11D	0.78 ± 0.02E	LPA1BML45-3	2.38 ± 0.07E	0.67 ± 0.02DE
4	LPA1BML6-4	2.28 ± 0.18D	0.63 ± 0.03F	LPA1BML45-4	2.33 ± 0.07E	0.73 ± 0.04CD
5	LPA1BML6-5	2.28 ± 0.08D	0.61 ± 0.06F	LPA1BML45-5	2.45 ± 0.17DE	0.78 ± 0.02C
6	LPA1BML6-6	2.57 ± 0.07C	0.54 ± 0.02F	LPA1BML45-6	2.66 ± 0.11CD	0.58 ± 0.02EF
7	LPA1BML6-7	2.54 ± 0.30C	0.90 ± 0.05CD	LPA1BML45-7	2.69 ± 0.15CD	0.47 ± 0.01F
8	LPA1BML6-8	2.89 ± 0.25B	0.80 ± 0.02DE	LPA1BML45-8	3.28 ± 0.21A	0.30 ± 0.02G
9	LPA1BML6-9	2.59 ± 0.09C	0.94 ± 0.03BC	LPA1BML45-9	3.00 ± 0.08AB	0.47 ± 0.03F
10	–	–	–	LPA1BML45-10	2.91 ± 0.15BC	0.56 ± 0.03EF
11	BML 6	3.59 ± 0.09A	0.65 ± 0.21F	BML 45	3.16 ± 0.13AB	0.51 ± 0.9F
12	LPA 1	1.30 ± 0.10F	1.42 ± 0.17A		1.30 ± 0.10G	1.42 ± 0.17A
	General Mean	2.35	0.88		2.53	0.67
	Mean SS	1.13**	0.28**		0.24**	0.96**
	P-value	<0.0001	<0.0001		<0.0001	<0.0001
	CV (%)	6.05	7.47		6.68	10.31
	s.e.d.	0.116	0.054		0.138	0.057
	l.s.d. (*P* = 0.05)	0.2423	0.1126		0.2855	0.1172

Means (±s.d.) followed by the same letter are not significantly different ($P > 0.05$) using Tukey's HSD test.
**$P < 0.01$.

Discussion

Phytic acid (myoinositol-1,2,3,4,5,6-hexakisphosphate) is considered an important plant compound associated with the seed P storage in a variety of crop plants (Raboy 1997). The presence of phytate as a seed storage compound in mature seeds of many food crop plants limits the bioavailability of mineral micronutrients such as Fe and Zn. Although phytate serves as a reservoir of P required for germinating seeds, it is considered an anti-nutritional factor for an animals. Therefore, it is desirable to reduce phytic acid content to some extent, still fulfilling plant requirements while increasing the bioavailability of essential mineral micronutrients. Breeding efforts have begun to develop low-phytate maize using LPA mutant lines as donors. The breeding method followed is the standard backcross breeding program (Ertl *et al.* 1998). Subsequently, the isolation, mapping, cloning, and characterisation of LPA mutants has led to identification of linked molecular markers and unravelling the sequence information of LPA mutant alleles (Raboy *et al.* 2000; Shi *et al.* 2003, 2005, 2007). The availability of molecular markers linked to genes determining low phytate in maize has led to the development of LPA lines or marker-assisted conversion of elite lines through backcross breeding (Naidoo *et al.* 2012; Sureshkumar *et al.* 2014a, 2014b; Tamilkumar *et al.* 2014; Yathish *et al.* 2022). Several crop improvement programs in maize are being

conducted to breed low-phytate maize crops using *lpa* mutants. Therefore, the present investigation was conducted to introgress low-phytate traits into the elite parental lines of DMH 121, a popular, widely adopted, medium-maturity normal maize hybrid, through use of MABB, and thereby develop an LPA maize hybrid.

Validation of polymorphism at *LPA1* locus between donor and recipient parents for foreground selection

Molecular markers that are linked to the gene of interest can aid in selection of the plants in the population that carry the gene of interest, segregating for the gene of interest (Singh and Singh 2015). Attempts have been made to develop gene-based and even functional molecular markers for low-phytate traits. MABB has successfully mobilised the *lpa2-2* allele into elite parental lines of popular single-cross hybrids of maize (Sureshkumar *et al.* 2014b). Both *lpa1* and *lpa2* are located on the short arm of chromosome 1, but owing to the non-availability of a co-dominant STMS marker linked to the *lpa1* allele, many researchers have instead targeted *lpa2* for successful introgression through *lpa2*-linked STMS markers into the different genetic background (Sureshkumar *et al.* 2014a, 2014b; Tamilkumar *et al.* 2014; Yathish *et al.* 2022). Thus, very few examples of successful transfer of the *lpa1*

allele through MABB in tropically adapted maize germplasm are available. However, for *lpa1-1*, a gene-based SNP marker has been developed and used for MABB (Naidoo *et al.* 2012). The co-dominant SNP marker linked to the *lpa1-1* allele was used to differentiate parental maize lines by producing different melt profiles, and the same was also validated by DNA sequencing with the C to T transition (Naidoo *et al.* 2012). Similarly, in other crops, molecular markers have been developed for genes determining low phytate (Roslinsky *et al.* 2007; Oliver *et al.* 2009). In the present study, the co-dominant SNP-based marker for *lpa1-1* developed by Naidoo *et al.* (2012) was successfully validated in the donor parent, LPA 1, and recurrent parents, BML 45 and BML 6, using HRM analysis. The SNP-based marker differentiated both recurrent parents from the donor parent by producing different melt profiles of homozygous dominant (wild type *LPA1* allele), homozygous recessive (*lpa* mutant allele).

Foreground selection for *lpa1* in the backcross progenies

The advent of molecular tools and techniques and their application has accelerated the time required to correct monogenic traits in otherwise elite lines through the transfer of mono- or oligo-genes, primarily through the backcross breeding method. The utility of molecular markers linked to the gene of interest is more pronounced in traits that are not easily measurable and are governed by recessive genes. PA is a biochemical trait, and its estimation is time-consuming and labour-intensive. Further, it can be estimated only after seed development and through a destructive method. Because low phytate is a recessive trait, it cannot express in the heterozygote condition. However, identification of heterozygotes is possible with molecular markers rather than through biochemical estimation. The transfer of genes through conventional backcross breeding is time-consuming. Thus, the *lpa1-1* gene-based SNP molecular marker was used for foreground selection in all generations, namely F_1, BC_1F_1, BC_2F_1 and BC_2F_2. Because the SNP marker is co-dominant, heterozygotes and homozygotes were successfully identified in the segregating generation. Transfer of the *lpa1-1* allele into an elite genetic background using an SNP-based marker through MABB was earlier demonstrated by Naidoo *et al.* (2012). Therefore, the availability SNP markers with the HRM technique allowed marker-assisted foreground selection for the *lpa1* allele in the present study.

Background screening for identification of RPG using SSR markers

The availability of genomic resources in the form of large number of molecular markers uniformly covering the entire genome has facilitated maize breeding immensely, especially MABB, around the world. The use of molecular markers for background selection and the selection of RPG/alleles using markers unlinked to the trait of interest aid in identifying

individual plants carrying maximum RPG. Therefore, the recovery of the RPG is considerably accelerated by the use of molecular markers for background selection. In general, conventional backcross breeding takes at least six backcrosses/generations to recover ~98% RPG. With background selection using markers, it is possible to recover the RPG much faster by identifying the individuals carrying the highest proportion of RPG in advanced backcross generations such as BC_2F_1 or even BC_1F_1 itself, given availability of a sufficiently large number of plants (Hospital and Charcosset 1997; Frisch *et al.* 1999a, 1999b). However, percentage RPG recovery also depends on other factors including number of molecular markers used in background selection, their distribution and density in the genome, other uncontrolled genetic and physiological phenomena (e.g. segregation distortion), and, finally, the number of backcrosses.

In maize, three backcrosses and 80 markers were estimated to recover 99% of the RPG (Jorboe *et al.* 1994). The number of molecular markers chosen in the present study for identifying polymorphic markers between donor and recipient parents was quite high (i.e. 450). The number of polymorphic markers found and used for background selection was also optimal (i.e. 50 and 55). In addition, the molecular markers chosen for background selection were distributed uniformly, covering the entire genome. The recovery of RPG increased as per theoretical expectations from BC_1F_1 to BC_2F_2 generation. In the present study, phenotypic selection was undertaken, and within the selected plants, foreground and background selection was applied. Here, scope was given both to exercise breeder skills and to reduce the cost of undertaking MABB. The ranges of RPG recovered in different generations were 74–79% (BC_1F_1), 85–89% (BC_2F_1) and 91–93% (BC_2F_2) in backcrosses derived from the BML 6 × LPA 1 cross, and 74–79% (BC_1F_1), 86–89% (BC_2F_1) and 90–93% (BC_2F_2) in backcrosses derived from the BML 45 × LPA 1 cross. The present study demonstrated the successful recovery of maximum RPG using molecular markers, which corroborates earlier studies (Naidoo *et al.* 2012; Sureshkumar *et al.* 2014a, 2014b; Tamilkumar *et al.* 2014). A similar RPG level was recovered in other studies transferring *lpa2* into elite parental lines. Sureshkumar *et al.* (2014b) reported 91–93% recovery of RPG in the BC_3F_2 generation, whereas Tamilkumar *et al.* (2014) reported 80.2% and Yathish *et al.* (2022) reported 88.68–91.51% recovery in the BC_2F_2 generation. The above studies involved introgression of *lpa2* from donor parent to tropical maize lines through MABB. At the same time, Naidoo *et al.* (2012) reported a recovery of 92.15% in the BC_2F_1 generation while transferring the *lpa1-1* allele through MABB.

PA and P_i content in newly developed NILs with the low-phytate trait

The expression of *lpa1* in the new genetic background of BML 6 and BML 45 was examined indirectly using PA and P_i estimates. Ideally, the NILs should contain the same PA and

P_i levels as the donor parent, but due to background effects, it is not always possible. In the original mutant lines developed through chemical mutagenesis and transposon tagging, *lpa1*, *lpa2* and *lpa3* mutant alleles reportedly reduced PA by 66%, 50% and 50%, respectively, compared with the wild-type or normal genotype where the background effect was nullified to a large extent (Raboy *et al.* 2000; Raboy 2002; Dorsch *et al.* 2003; Shi *et al.* 2005). The NILs developed in the genetic background of recurrent parents were evaluated for PA and P_i content. BC_2F_3 seeds were used for the analysis, and the PA and P_i contents were compared with those of the donor and recipient parents. The variation in PA and P_i levels in the NILs was significant, indicating the strong background effect on PA accumulation and P_i content, which indirectly shows the effect of the genetic background on the expression of *lpa1-1*. However, several NILs showed a significant reduction in PA content compared with their original (elite) parent. One NIL each in the genetic background of BML 6 and BML 45 also showed a level of PA similar to that of donor line LPA 1. The reduction in PA content in the newly developed NILs of BML 6 and BML 45 was on average 56% and 44% relative to their respective original version. Thus, the present study demonstrated that the NILs developed in the genetic background of BML 6 and BML 45 have significantly reduced PA and higher P_i, and some have PA comparable to that of the donor parent. Theoretically, when the RPGs of most of the NILs are almost comparable, it is expected that they should not differ significantly concerning PA content. The underlying genetic, physiological or any other mechanism, including biochemical, is a matter of further investigation. Reports on percentage reduction in PA content are available, and the present results agree with these (Sureshkumar *et al.* 2014a, 2014b; Tamilkumar *et al.* 2014; Yathish *et al.* 2022). As reported previously, a reduction in PA content often leads to a proportional increase in P_i content in maize seeds. In the present study, qualitative estimation of P_i content in NILs has shown a 2–3-fold increase in P_i levels. The findings of the present study also corroborate earlier reports of increased P_i levels with reduced phytate content in NILs. Among the three *lpa* genes, a higher proportional increase in P_i content was observed in *lpa1-1* than the other two genes, *lpa2* or *lpa3* (Shi *et al.* 2003; Cerino Badone *et al.* 2012).

Conclusions

Maize plays a vital role as a staple food and feed crop in many parts of the world. However, the higher PA content in maize kernel reduces its nutritional value by hindering the bioavailability of essential mineral nutrients such as iron and zinc. Reduced phytate content in maize kernels can substantially enhance its nutritional value. The present study has developed the maize NILs with reduced content of PA through transfer of the *lpa1* allele from donor LPA 1 to recurrent inbred lines BML 6 and BML 45. The study demonstrated the successful application of MABB to develop several NILs in the genetic backgrounds of BML 6 and BML 45 with reduced PA and higher P_i content through introgression of the *lpa1* allele. These newly developed NILs with low phytate content are being utilised to reconstitute the original hybrid DHM 121 with low-phytate traits. Further, the inclusion of NILs with low phytate traits in the active germplasm of maize breeding will be important in developing LPA hybrids shortly.

Supplementary material

Supplementary material is available online.

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