Australian Journal of Crop Science AJCS
AJCS 8(8):1224-1231 (2014) ISSN:1835-2707

Introgression of low phytic acid locus *(lpa2-2)* into an elite Maize (*Zea mays* L.) inbred through marker assisted backcross breeding

Paramasivam Tamilkumar[1], *Natesan Senthil[2], Subbaraj Sureshkumar[2], Arumugam U. Thangavelu[2], Pothiraj Nagarajan[2], Sampathrajan Vellaikumar[2], Kalipatty N. Ganesan[3], Nagappan Natarajan[1], Ramachandran Balagopal[4], Thirunavugarasu Nepolean[5], and Muthurajan Raveendran[1].

[1]Department of Seed Science and Technology, Tamil Nadu Agricultural University, Coimbatore, India
[2]Centre for Plant Molecular Biology and Biotechnology, Tamil Nadu Agricultural University, Coimbatore, India
[3]Department of Millets, Tamil Nadu Agricultural University, Coimbatore, India
[4]Department of veterinary and animal sciences, Tamil Nadu Agricultural University, Coimbatore, India
[5]Division of Genetics, Lalbahadur Shastri Centre for Biotechnology, Indian Agricultural Research Institute, New Delhi, India

***Corresponding author: senthil_natesan@tnau.ac.in**

Abstract

Phytic Acid (PA) in maize kernel is an anti-nutritional factor; it chelates mineral cations in human gut and causes mineral deficiency in humans. Therefore, development of low-PA maize is required. Here, we have transferred the low-PA locus (i.e., *lpa2-2* allele that confers low-PA trait) by crossing low-PA line "EC 659418" (donor line) with an elite inbred "UMI 285" (recipient line) using Marker Assisted Backcross Breeding (MABB). In our MABB program, for "foreground selection" (i.e., screening for plants with introgressed *lpa2-2* locus) small sequence repeat (SSR) marker "umc2230" was used, and for "background selection" (i.e., screening for introgressed lines whose genetic background are similar to that of the recurrent parent) 47 SSR markers were used. As a result, we have developed four lines with both reduced-PA trait similar to that of donor parent, and agronomical traits (i.e., days to 50% tasseling, days to 50% silking, plant height, ear height, 100 seed weight, grain yield per plant, germination and seed vigor) similar to that of the recurrent parent.

Keywords: Background selection; Foreground selection; Maize; Marker assisted selection; *lpa2*; Phytic acid.
Abbreviations: cM_centi-morgan; HIP_high inorganic phosphate assay; MABB_marker assisted backcross breeding; MAS_marker assisted selection, PA_phytic acid; *lpa*-low phytic acid; SSR_simple sequence repeats; UMI_university maize inbred.

Introduction

Maize (*Zea mays* L.) is an important human food, livestock feed, and an industrial raw material. PA is a major storage form of phosphate in plant seeds (Raboy, 2007). PA in maize seed is an anti-nutrient (Raboy, 2007). In humans, PA chelates gut mineral nutrients (such as calcium, manganese, iron, magnesium, zinc etc.) and reduces their absorbability and cause micronutrient malnutrition (Brinch-Pedersen et al., 2002). Since the monogastric animals (such as swine, poultry and fish), lacks phytase required for separating phosphorous from PA molecule, phosphorus in PA is not bioavailable as nutrients, and additionally, the phosphorus released from undigested PA, excreted by monogastric animals, causes environmental pollution such as eutrophication. Therefore, development of new maize cultivars with "Low-PA/ High available Phosphorus" maize cultivars is desirable for potentially reducing the PA related problems (Ertl et al., 1998; Mendoza et al., 1998). Several low-PA mutants have been developed successfully in soybean, barley, maize, and rice by disrupting PA biosynthesis pathway through mutagenesis breeding (Larson et al., 2000; Raboy et al., 2000; Wilcox et al., 2000) and these mutant lines were used in genetic breeding as donors for the development of low-PA lines (Raboy et al., 2001). In maize, three low phytic acid (*lpa*) mutants have been isolated, *viz., lpa1, lpa2, and lpa3.* These mutant lines are important genetic resources for the development of low-PA maize crops. The *lpa1* mutation is caused by a mutation in a gene that encodes transmembrane transporter protein (ZmMRP4), which is hypothesized to load phytic acid into protein storage vacuoles of maize seed. The *lpa2* mutation is caused by a mutation in inositol phosphate kinase gene (ZmIpk4), which along with other kinases leads to phytic acid synthesis. The *lpa2-1* mutation is caused by genomic sequence rearrangement in the ZmIpk and *lpa2-2* is caused by a nucleotide mutation, (*i.e*., C to T at nucleotide position 158), that generated a stop codon in the N-terminal region of ZmIpk open reading frame (Shi et al., 2003). The *lpa3* mutation is caused by a mutation in a gene that encodes *myo*-Ins kinase, which catalyzes the production of $Ins(3)P_1$ in maize seed. Compared with wild-type kernels, the *lpa 1, lpa2-1, lpa3* mutations achieved 66%, 50%, and 50% reduction in phytic acid content, respectively (Raboy et al., 2000; Shi et al., 2005) . The *lpa2-2* mutation achieved a 30% reduction in phytic acid content and a three-fold increase in inorganic phosphate (Shi et al., 2003). Therefore, it is desirable to introgress the *lpa* locus from low-PA mutant lines into locally adapted agronomically superior lines to improve their nutritional benefit. In this study, we aimed to develop low-PA maize lines. Here, we have transferred the

low-PA locus (i.e., *lpa2-2* allele that confers low-PA trait) from low-PA line "EC 659418" (donor line) to our regionally adapted and high-yielding inbred "UMI 285" (recipient line) using Marker Assisted Backcross Breeding (MABB). In our MABB, we used one crossing step (for introgressing the low-PA trait from the donor to recipient parent), two backcrossing steps (for recovering the recurrent parent genome), and one selfing step (for obtaining the plants that possesses *lpa2-2* allele in homozygous condition) steps. In our previous study (Sureshkumar et al., 2014), we have found that a short sequence repeat marker (SSR) "umc2230", present in the short arm of chromosome one of maize genome, is in proximity (0.4 cM downstream) with *lpa2*, and co-segregate in a Mendelian fashion with low-PA trait. Hence, in this study we made use of umc2230 for efficient, fast, and cost effective selection of plants possessing *lpa2-2* allele. As a result, we have developed four lines that contain both low-PA trait similar to that of donor parent, and agronomical traits similar to that of the recurrent parent.

Results

MABB for developing low-PA lines

We obtained the sequence of SSR marker "umc2230" from the maize database (www.maizegdb.org) and studied the polymorphism between the EC 659418 (*lpa2-2* mutant/low-PA trait donor) and UMI 285 (high-PA genotype). We have previously validated, using backcross and selfed progenies, that umc2230 located just 0.4cM distance away from the *lpa2* region on the short arm of chromosome 1 in maize genome, cosegregate with low phytic acid trait in a Mendelian fashion, (Sureshkumar et al., 2014). The SSR marker umc2230 was found to polymorphic for parents and hybrid, i.e., PCR amplification of the marker resulted in 175bp/175bp (scored as AA), 175bp/180bp (scored as AB), and 180bp/180bp fragments (scored as BB) in UMI 285, F_1, and EC 659418 respectively (Fig 1). Therefore, we used this marker for our MABB for the development of low-PA maize. At each breeding step, we analyzed the banding pattern of umc2230 (i.e., foreground screening) for distinguishing plant types; 1) low-PA lines homozygous for *lpa2* locus (i.e., *lpa2-2*/ *lpa2-2* indicated by 180bp/180bp banding pattern), 2) lines heterozygous for *lpa2* locus (i.e., *lpa2-2*/ *LPA2-2* indicated by 180bp/175bp banding pattern), 3) High-PA lines homozygous for *LPA2* locus (i.e., *LPA2-2*/ *LPA2-2* indicated by 175bp/175bp banding pattern). The General schema of MABB used in this study for the development of low-PA lines is shown in Table 1. Firstly, we crossed UMI 285 X EC 659418 and developed F_1s. Then, 34 F_1s were raised in the field. Out of 34 F_1s, 18 true F_1s confirmed by foreground screening and morphological traits (such as leaf venation, leaf angle and kernel color), were used as female parents to backcross with recurrent male parent UMI 285 and backcross generation BC_1F_1 were developed. Then, we raised 362 BC_1F_1 progenies and analyzed them by foreground screening to select "180bp/175bp" genotypes and exclude "175bp/175bp" genotypes. 82 BC_1F_1s with "180bp/175bp" genotypes were selected and backcrossed with UMI 285 to develop BC_2F_1 generation. Then, we raised 412 BC_2F_1 progenies and analyzed by foreground screening to select "180bp/175bp" genotypes and exclude "175bp/175bp" genotypes. 80 BC_2F_1s with "180bp/175bp" genotypes were selected and selfed to develop BC_2F_2 generation. Next, we raised 350 BC_2F_2 progenies and analyzed them by foreground screening to select "180bp/180bp" (low-PA trait) genotypes and exclude "180bp/175bp" and "175bp/175bp" genotypes. Four low-PA "180bp/180bp" BC_2F_2 lines, homozygous for *lpa2-2* alleles (*viz.*, S1-28-9, S1-28-44, S1-31-16 and S1-31-26), were selected and selfed to obtain BC_2F_3 lines. Free inorganic phosphorous assay (i.e., using HIP assay) and PA estimation (i.e., Direct PA assay) were done in these 4 lines to confirm the introgression of *lpa2* locus (Table 2). These analyses indicated that the values of inorganic phosphorous and PA in four low-PA lines are almost equal to that of the *lpa2-2* mutant line EC 659418, suggesting that the low-PA trait has been introgressed successfully from EC 659418. Background screening (i.e., profiling the banding pattern of alleles of recovered recurrent parent using SSR markers) was done in the selected four low-PA lines in order to estimate and confirm the recovery of the genome of recurrent parent. For this background survey, the polymorphic SSR marker survey was carried out using 52 SSR markers (Supplementary table 1) obtained from maizegdb database (www.maizegdb.org). These markers were located among the ten chromosomes of maize genome. Out of the 52 SSR markers used to screen the parents UMI 285 and EC 659418, 47 markers were found to be polymorphic. Therefore, these 47 markers were used to survey the 4 low-PA lines (Supplementary table 2) for estimating the recovery of the background UMI 285 genotype. The PCR products were screened at 3.5 per cent agarose gel and scored for either recovery of alleles of UMI 285. A plant was scored as AA if it contains alleles of UMI 285, scored as BB if it contains alleles of EC 659418, and scored as AB if it contains both UMI 285 and EC 659418 alleles. For example, the amplicons produced by "umc1318", "umc2089", and "umc 1704" are shown in Fig 2. The recovery of recurrent parent genome was calculated based on the percentage of the markers that shows similarity with that of recurrent parent is used for calculating using Graphical Genotypes (GGT) ver. 2.0. The genome of the first (i.e., S1-28-9), second (i.e., S1-28-44), third (i.e., S1-31-16), and fourth (i.e., S1-31-26) low-PA lines are 80.2%, 73.4%, 79.9%, and 79.6% identical to the recurrent parent respectively (Fig 3, Supplementary Table 2).

Analysis of seed quality characters of the developed four low-PA lines

Analysis of germination percentages of fresh seeds of the low-PA lines (Fig 4) revealed that the mean germination percentages observed in low-PA lines (i.e., 78.37%, 78.01%, 77.65% and 78.19% for S1-28-9, S1-28-44, S1-31-16, and S1-31-16 respectively) are comparable to that of UMI 285 (78.46%). Also, the analysis of germination percentages of aged (i.e., 4 days accelerated aged) seeds of the low-PA lines (Fig 4) revealed that the mean germination percentage observed in low-PA lines (i.e., 64.45 %, 63.23 %, 64.15 % and 63.80% for S1-28-9, S1-28-44, S1-31-16, and S1-31-16 respectively) are comparable to that of UMI 285 (64.16 %), indicating that even the loss of germination due to artificial ageing in low-PA lines are comparable to that of the recurrent parent. These results indicate that the germination behavior of seeds of the developed low-PA lines is similar to that of the recurrent UMI 285 line. Analysis of vigor index of fresh seeds of the low-PA lines (Fig 5) revealed that the mean vigor indices observed in low-PA lines (i.e., 3661.22, 3450.92, 3518.08, and 3674.94 for S1-28-9, S1-28-44, S1-31-16, and S1-31-16 respectively) are comparable to that of UMI 285 (3639.36). Also, the analysis of vigor index of aged (i.e., 4 days accelerated aged) seeds of the low-PA lines (Fig 4) revealed that the mean vigor index observed in low-PA lines (i.e., 2492.06, 2680.56, 2692.95, and 2544.01 for S1-28-9, S1-28-44, S1-31-16, and S1-31-16 respectively) are comparable to that of UMI 285 (2669.76), indicating that

Table 1. General schema of MABB for the development of low-PA lines

SEASON	BACK CROSS BREEDING PROGRAMME	METHOD OF SCREENING
1	UMI 285 X EC 659418	
	↓	✸ Parental polymorphism survey was done in *lpa2-2* region using SSR marker "umc2230"
2	F_1 X UMI 285 (Recurrent parent)	
	Backcross 1 ↓	✸ 34 F_1s were raised and Marker Assisted Foreground Selection done to confirm that F1 are "180bp/175bp" genotypes. ✸ 18 true F_1s selected and backcrossed
3	BC_1F_1 X UMI 285	
	Backcross 2 ↓	✸ 362 BC_1F_1 progenies were raised and Marker Assisted Foreground Selection done to select "180bp/175bp" genotypes and exclude "175bp/175bp" genotypes ✸ 82 BC_1F_1s selected and backcrossed
4	BC_2F_1	
	Selfing 1 ↓	✸ 412 BC_2F_1 progenies raised and Marker Assisted Foreground Selection done to select "180bp/175bp" genotypes and exclude "175bp/175bp" genotypes. ✸ 80 BC_2F_1s selected and selfed
5	BC_2F_2	
	Selfing 2 ↓ BC_2F_3	✸ 350 BC_2F_2 progenies raised and Marker Assisted Foreground Selection done to select "180bp/180bp" genotypes and exclude "180bp/175bp" and "175bp/175bp" genotypes ✸ 4 BC_2F_2s selected (*viz.*, S1-28-9, S1-28-44, S1-31-16 and S1-31-26) and selfed ✸ Phenotypic screening for low-PA trait (using HIP and PA assay) ✸ Marker Assisted Background Selection to confirm the recovery of recurrent parent genome ✸ Analysis of Seed quality characters of selected low-PA lines.

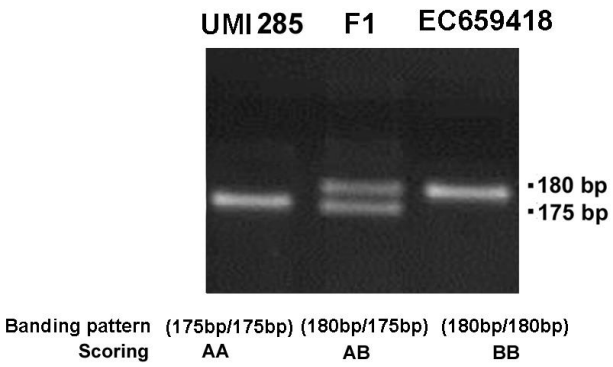

Fig 1. Profiling of alleles of SSR marker "umc2230" in F1 along with the parents. The figure shows that PCR amplification of a co-dominant SSR marker "umc2230", which is linked to *lpa2-2* locus, resulted in the amplicons 180bp/175bp (i.e., heterozygous) in F_1, 175bp/175bp (i.e., homozygous for 175 bp) in high PA parent UMI 285 and 180bp/180bp (i.e., homozygous for 180 bp) in low-PA EC 659418 line.

even the loss of vigor index due to artificial ageing in low-PA lines are comparable to that of the recurrent parent. These results indicate that the vigor of seeds of the developed low-PA lines is similar to that of the recurrent UMI 285 line.

Analysis of agronomical traits of four low-PA lines

The agronomical traits of four low-PA lines (S1-28-9, S1-28-44, S1-31-16, S1-31-26) were analyzed to verify whether these lines behave agronomically similar to the recurrent parent (UMI 285) (Fig 6). The mean values for the agronomical traits [i.e. plant height, days to 50% tasselling, days to 50% silking (days), ear height (cm), 100 grain weight (g), yield per plant (g)] were found to be almost similar to that of the recurrent parent, indicating that the low-PA lines have recovered most of the genome of the recurrent parent (Fig 6).

Discussion

The problem of micronutrient malnutrition in poor families, including children and women, in the developing countries can be addressed by biofortification of maize. In this study, we aimed to develop low-PA lines using MABB. DNA-based molecular markers have been developed and used extensively in crop improvement using MABB (McCouch et al., 1997). MABB involves introgression of one or more genes from a donor into the background of an elite variety (recurrent or recipient parent) and to recover the recurrent parent genome as rapidly as possible. MABB combines 'Foreground' selection of donor alleles linked to markers and 'Background' selection of recurrent parent alleles in the later generation for efficient introgression of desirable traits. The availability of large number of SSR markers in maize facilitates the selection of desirable traits in MABB. The advantages of using MABB in maize improvement have been well documented (Babu et al., 2005; Zhang et al., 2012). Recently, researchers (Naidoo et al., 2012; , Naidoo et al., 2013) have successfully developed and applied the *lpa1-1* SNP marker for foreground selection in a backcross-breeding programme. In this MABB study, we have transferred the low-PA trait from EC 659418 to UMI 285 and have developed four lines, which possessed low-PA trait similar to that of donor parent, along with agronomical and seed quality characters similar to that of the recurrent parent. Similar studies have been carried out in soybean for the introgression of two low-PA mutant genes in to the locally adapted soybean cultivar (Shu, 2009). Naidoo et al., 2012, studied marker-assisted selection for low-PA (conferred by *lpa1-1*) with single nucleotide polymorphism (SNP) marker and Amplified Fragment Length Polymorphisms (AFLP) in a maize backcross-breeding programme. They demonstrated that the use of foreground and background selection in MABB greatly increased the efficiency of detection of the homozygous recessive (99.58%) and heterozygous (99.59%) genotypes as well as improving the recovery of the recurrent parent (92.15%) in the BC_2F_1 generation of the maize. Marker assisted background selection for the identification of backcrossed plants that have recovered the maximum amount of genome of recurrent parent with good agronomical type was proposed by Young and Tanskley and later termed as background selection (Charcosset, 1997). Background screening aid in reducing the number of generations to produce individuals with desired gene combinations but isogenic to recurrent parent. The background recovery percentage depends on the number of markers applied in screening, their equal distribution over the chromosomes, the backcross generations and number of individual plants in the population. Naidoo et al., (2012), achieved 92.15% recovery of the recurrent parent in the BC_2F_1 generation in a maize backcross-breeding program for low-PA (*lpa1-1*) with SNP marker using marker-assisted selection for foreground and background selection. In our study, our background screening experiments with 47 makers have revealed that the four low-PA lines have sufficiently recovered the genome of recurrent parent genome and it can be evidenced from the that fact that the agronomical traits of the four low-PA lines developed were found to be almost similar to that of the recurrent parent.High and rapid germination determine better establishment of the plant and higher yields from the plant (Matthews, 1978). Reduced phytic acid content in seeds is a desired goal for genetic improvement of micronutrient in several crops. There are controversial opinions on the effects of "reducing PA content" on seed quality reported in various low-PA traits in various crops. The experiments by Raboy et al. (1985) showed that reduced PA content did not adversely affected germination of soybean seeds. The *lpa* mutant studies by Raboy (2002) showed that low-PA plants did not affect germination or yield. However, Oltmann *et al*., (Oltmans et al., 2005) as well as Anderson and Fehr (2008) cited reduced seedling emergence in low-PA lines. In some cases, seedling emergence in low-PA lines was only 45% (Oltmans et al., 2005). However, Naidoo et al., (2012) showed that the many of the low PA-maize lines did not show any reduction in germination or vigor. Our experiments on germination and vigor index revealed that the germination behavior of both fresh and aged seeds of the four low-PA were comparable to that of the recurrent UMI 285 line, indicating that the reduction of PA in the low-PA lines has not compromised their the agronomic performances.

Materials and Methods

Plant materials and development of F_1 lines

We aimed to introgress *lpa2-2* locus from a *lpa2-2* mutant line EC 659418 (source of *lpa2-2 allele* that confers low-PA trait) obtained from Victor Raboy, USDA, into UMI 285 line (a popular well-adapted agronomically superior and high PA line). The EC 659418 was used as male and donor parent.

Table 2. Phytic acid and phosphorous estimation in parent and BC_2F_3 low-PA lines.

Genotype	Inorganic Phosphorous level*	Phytic acid** (mg/g) ±SE
UMI 285	Standard 2	2.8±0.24
EC 659418	More than Standard 5 level	1.8±0.13
Low-PA line "S1-28-9"	Ranges from Standard 4 to 5 and above level	1.90±0.10
Low-PA line "S1-28-44"	Ranges from Standard 4 to 5 and above level	1.94±0.11
Low-PA line "S1-31-16"	Ranges from Standard 4 to 5 and above level	1.92±0.07
Low-PA line "S1-31-26"	Ranges from Standard 4 to 5 and above level	1.98±0.11

* In HIP assay, five level of standards used are; Standard 1: 0.00 µg P (no blue color), Standard 2: 0.15 µg P (very light blue color), Standard 3: 0.46 µg P (intermediate blue color), Standard 4: 0.93 µg P (dark blue color) and Standard 5: 1.39 µg P (very dark blue color). **Mean value of 3 replications. SE refers to standard error of mean.

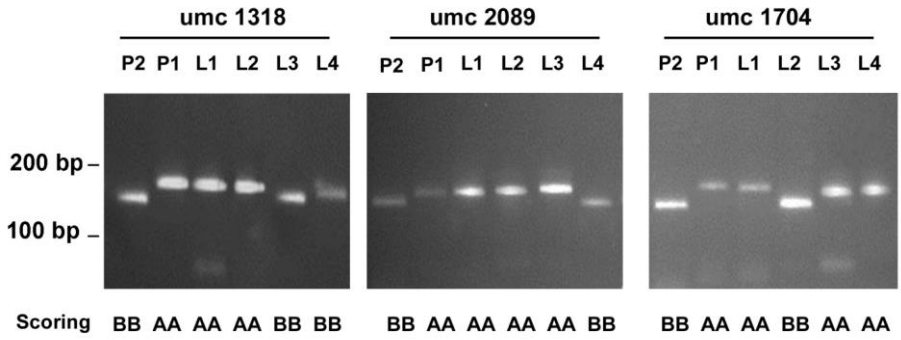

Fig 2. Background screening in 4 low-PA lines developed. The banding pattern produced by the amplification of polymorphic SSR markers "umc1318", "umc2089", and "umc 1704" in lines UMI 285 (P_1), EC 659418 (P_2), and the four PA lines developed (L_1, L_2, L_3 and L_4) in this study are shown. The plant was scored as AA if it contains UMI 285 allele and scored as BB if it contains EC 659418 allele, and scored as AB if it contains both UMI 285 and EC 659418 alleles. P1 and P2 refer to UMI 285 and EC 659418.

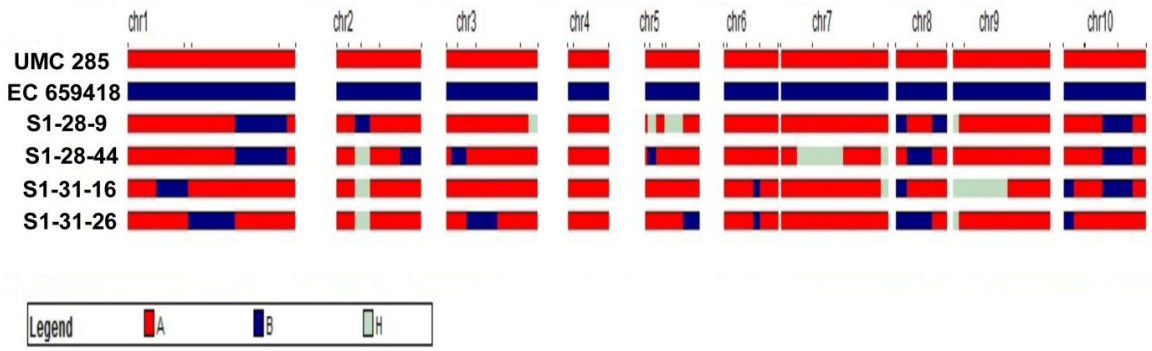

Fig 3. Schematic representation of background screening in 4 low-PA lines developed. The Profiling of alleles of all the 47 SSR markers located across all the 10 chromosomes of the 4 low-PA BC_2F_2 lines, along with the parents were graphically represented. The map provides a view of the percentage of recurrent parent genome recovery both at the level of individual chromosome and genome. In the legend A and B refer to the genome recovery from UMI 285 and EC 659418 respectively and "H" refers to heterozygous region.

UMI 285 was used as female and recurrent parent. The parents UMI 285 and EC 659418 were raised in two staggered sowings at 3 days interval at the Wheat Research Station, Indian Agricultural Research Institute, Wellington, Ooty in order to achieve programmed pollination. These two lines were hybridized to generate F_1 lines. The Development of BC_1F_1, BC_2F_1, BC_2F_2 lines, and selection of four low-PA lines are described in Table 1.

Foreground screening

A modified CTAB Method (Dellaporta et al., 1983) was used for DNA extraction. Foreground screening, i.e., profiling of co-dominant alleles of SSR marker "umc2230" linked to *lpa2-2* locus, was done to confirm the introgression of *lpa2-2* locus conferring the low-PA trait in recombinant lines. For profiling, PCR amplification of umc2230, which encompasses the core repeat (AGC)5**,** was done using the forward primer 5' –AACGCGACGACTTCCACAAG- 3' and the reverse primer 5' – ACACGTAATGTCCCTACG-GTCG- 3'. PCR was performed as follows; First initial hold was at 95^0C for 3 minutes. The second hold starting with denaturation step at 94^0C for 30 seconds, annealing at 63^0C up to 57^0C for 30 seconds (12 cycles, reducing 0.5^0C per cycle) and the extension step at 72^0C for 45 seconds. The third hold starting at denaturation temperature of 94^0C

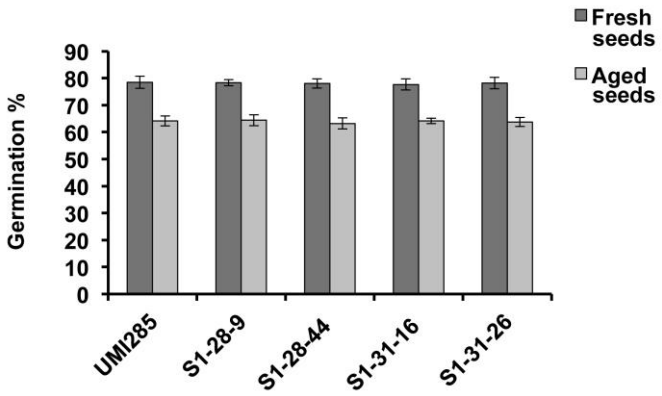

Fig 4. Comparison of the germination of fresh and aged seeds of four low-PA lines (S1-28-9, S1-28-44, S1-31-16, S1-31-26) with that of recurrent parent (UMI 285). SE refers to standard error. Based on the combined analysis of critical difference, there are no significant differences between the mean values of germination percentage of the four low-PA lines and that of the control "recurrent parent".

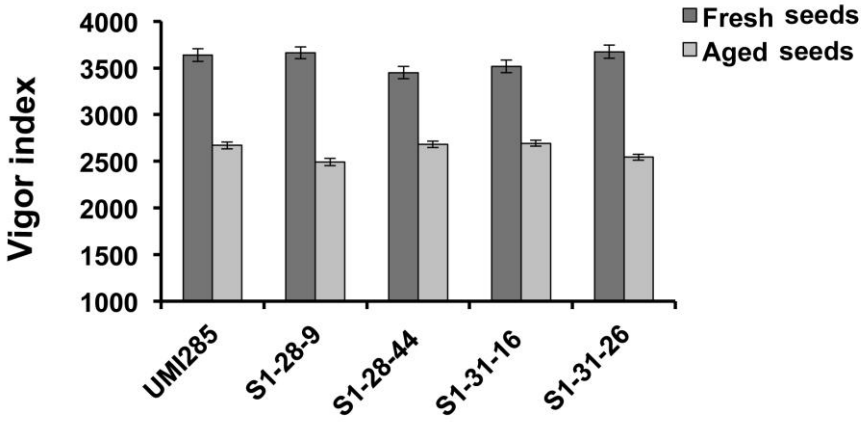

Fig 5. Comparison of the vigor index of fresh and aged seeds of four low-PA lines (S1-28-9, S1-28-44, S1-31-16, S1-31-26) with that of recurrent parent (UMI 285). SE refers to standard error. Based on the combined analysis of critical difference, there are no significant differences between the mean values of vigor index of the four low-PA lines and that of the control "recurrent parent".

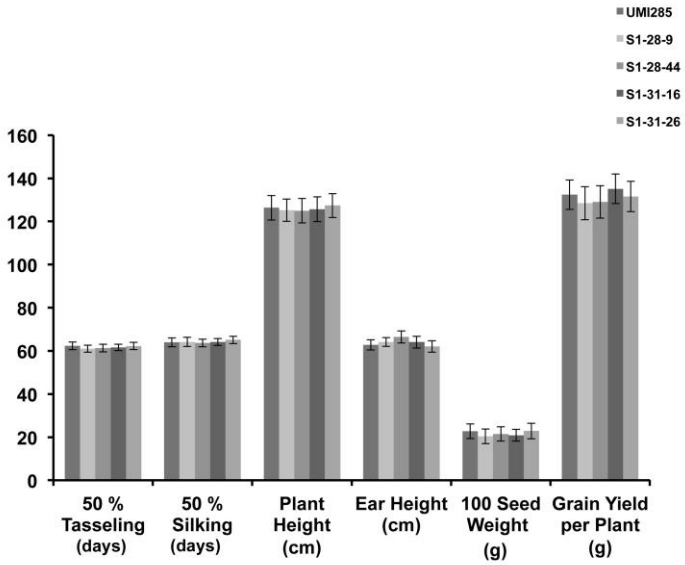

Fig 6. Comparison of agronomical traits of four low-PA lines (S1-28-9, S1-28-44, S1-31-16, S1-31-26) with that of recurrent parent line. SE refers to standard error. Based on the combined analysis of critical difference, there are no significant differences between the mean values of different agronomical traits of the four low-PA lines and that of the control "recurrent parent".

for 30 seconds, annealing at 59^0C for 30 seconds and extension at 72^0C for 45 seconds for 45 cycles). The final extension step was done at 72^0 C for 10 minutes. The amplified fragments were resolved in a 3% agarose gel for analyzing the amplicons. PCR amplification of 175bp/175bp, 175bp/180bp, and 180bp/180bp fragments are expected in UMI 285, F_1 (i.e., UMI 285 X EC 659418), and EC 659418 respectively.

Background screening

Background screening (i.e., selection of recurrent parent alleles using several molecular markers) was done to confirm the recovery of recurrent parent genome in the recombinant lines. Background screening was done using 52 markers (i.e., around 5 markers for each chromosome) to analyze the 10 chromosomes of the maize genome. The background screening data for all the 10 chromosomes showing intervals in cM were graphically represented using the software, Graphical Genotypes (GGT) ver. 2.0.

PA phenotyping

PA phenotyping (i.e., the analysis of PA trait in kernels) was done to confirm the introgression of *lpa2-2* locus in recombinant lines using single seed High Inorganic Phosphorous (HIP) assay and direct PA assay. These assays help us confirm whether plants with banding pattern 180bp/180bp (or rather *lpa2-2/ lpa2-2*) produces only low-PA kernels, that with 180bp/175bp (*lpa2-2/LPA2-2*) pattern produces mixture of high and intermediate PA kernels, and that with 175bp/175bp (*LPA2-2/ LPA2-2*) pattern produces only high PA kernels. Wild-type kernels contain increased levels of PA and decreased levels of free inorganic phosphorus. However, low-PA kernels (of *lpa* mutant plants) contain reduced levels of PA- phosphorus, and increased level of free inorganic phosphorus. Therefore, the estimation of free inorganic phosphorus provides an indirect estimation of phytic acid phosphorus of the seed. Using single seed HIP assay, it is possible to indirectly analyze the PA trait of individual seeds quickly, easily, accurately and inexpensively. Here the seeds obtained were crushed and assayed for free inorganic phosphorous using a microtiter based colorimetric assay (Raboy et al., 2000; Chen et al., 1956). The free inorganic phosphorous content in kernels was visually classified as High (i.e., high free phosphorous content produces dark blue color), Low (i.e., low free phosphorous content produces light blue color), Intermediate (i.e., intermediate free phosphorous content produces medium blue color) based on the intensity of blue color developed, which is proportional to the amount of free inorganic phosphorous content in the kernel. In order to ascertain the association between umc2230 marker and PA trait, PA phenotyping of 12 randomly selected seeds, obtained from the cobs of plants selected by foreground screening, was done using HIP assay. For HIP assay, individual seeds were weighed (in order to select seeds of almost uniform weight to get normalized HIP assay results), crushed and extracted overnight in 1ml of 0.4M hydrochloric acid per mg (approximate seed weight) at 4^0C. Samples (10 µl) of these extracts were mixed with 90 µl distilled water and 100 µl of colorimetric reagent [(one volume of 3 M Sulphuric acid, one volume 2.5% (w/v) ammonium molybdate, one volume 10% (v/v) ascorbic acid, and two volumes distilled water]. The assays were incubated at room temperature for approximately one hour. Individual seed extracts were visually scored for the presence of HIP based the development of Prussian blue color (Sundberg and Broman, 1955).

Direct PA assay

PA was estimated by the method described by Davies and Reid (1979). One gram of ground seed material was extracted with HNO3 and filtered. To 1.4 ml of the filtrate, 1 ml of ferric ammonium sulphate (21.6 mg in 100 ml water) was added, mixed and placed in a boiling water bath for 20 min. The contents were cooled and 5 ml of isoamyl alcohol was added and mixed. To this, 0.1 ml ammonia solution was added, shaken thoroughly and centrifuged at 3000 rpm for 10 min. The alcoholic layer was separated and the color intensity was read at 465 nm against amyl alcohol blank after 15 min. Sodium phytate standards were run along with the sample. The results were expressed as mg PA g dry wt $^{-1}$

Germination (%)

Germination of the seeds was tested in quadruplicate using 100 seeds (i.e., 4 sub replicates of 25 seeds each) in paper medium. The test conditions of 25 ± 2^0C and 95 ± 3 percent RH were maintained in a germination room. At the end of the germination period on 7^{th} day, the germination % of seedlings was evaluated. Based on the mean number of normal seedlings developed, the mean germination was calculated and expressed in percentage.

Vigor index

Vigor index (Abdul-Baki and Anderson, 1973) values were computed using the following formula and the mean values were expressed in whole number.
Vigor index = Germination (%) x Total seedling length (cm).

Artificial aging

For accelerated aging experiments, maize kernels were packed in perforated butter paper bags and placed in an aging jar maintained at 98 ± 2% relative humidity. Subsequently, the aging jar was kept inside a hot air oven maintained at 40 ± 1° C. After four days, the aged seeds were collected and shade dried and subjected to germination test for seed vigor status. Fresh seeds were used as control.

Statistical analysis

The data obtained from different experiments were analyzed by 'F' test of significance. The data were tested for statistical significance by calculating critical differences (CD) values. Wherever necessary, the percent values were transformed to arc-sine values before analysis. The CDs were calculated at 1 percent ($P \leq 0.01$) probability level.

Conclusion

In short, MABB has helped to develop 4 low-PA lines, which are not only genotypically homozygous for the *lpa2-2* allele and possess lower PA level that is comparable to that of donor genotype, but also have recovered almost all of the genetic background and hence agronomical and seed quality traits of recurrent parent, indicating that the MABB has successfully introgressed the low-PA trait from the donor line into agronomically superior line. In future, these lines can be used in hybridization programs to obtain high-yielding low-PA hybrids.

Acknowledgements

The financial support by Department of Biotechnology, Government of India, sponsored project on "Support for Research and Development in Agricultural Biotechnology" is kindly acknowledged. The *lpa2-2* mutant line supplied by Dr. Victor Raboy, USDA, is kindly acknowledged.

References

Anderson BP, Fehr WR (2008) Seed source affects field emergence of low-phytate soybean lines. Crop Sci. 48: 929-932.

Babu R, Nair S, Kumar A, Venkatesh S, Sekhar J, Singh N, Srinivasan G, Gupta H (2005) Two-generation marker-aided backcrossing for rapid conversion of normal maize lines to quality protein maize (QPM). Theor Appl Genet. 111: 888-897.

Brinch-Pedersen H, Sørensen LD, Holm PB (2002) Engineering crop plants: getting a handle on phosphate. Trends Plant Sci. 7: 118-125.

Charcosset A (1997) Marker-assisted introgression of quantitative trait loci. Genetics. 147: 1469-1485.

Chen Jr P, Toribara T, Warner H (1956) Microdetermination of phosphorus. Analyt Chem. 28: 1756-1758.

Davies N, Reid H (1979) An evaluation of the phytate, zinc, copper, iron and manganese contents of, and Zn availability from, soya-based textured-vegetable-protein meat-substitutes or meat-extenders. British J Nutr. 41: 579-589.

Dellaporta SL, Wood J, Hicks JB (1983) A plant DNA minipreparation: version II. Plant molecular biology reporter 1:P 19-21.

Ertl D, Young K, Raboy V (1998) Plant genetic approaches to phosphorus management in agricultural production. J Environ Qual. 27: 299-304.

Larson SR, Rutger JN, Young KA, Raboy V (2000) Isolation and genetic mapping of a non-lethal rice (L.) mutation. Crop Sci. 40: 1397-1405.

Matthews S (1978) Controlled deterioration: a new vigour test for crop seeds. Proceedings-Easter School in Agricultural Science, University of Nottingham 1978.

McCouch SR, Chen X, Panaud O, Temnykh S, Xu Y, Cho YG, Huang N, Ishii T, Blair M (1997) Microsatellite marker development, mapping and applications in rice genetics and breeding. Plant Mol Biol. 35: 89-99.

Mendoza C, Viteri FE, Lönnerdal B, Young KA, Raboy V, Brown KH (1998) Effect of genetically modified, low-phytic acid maize on absorption of iron from tortillas. The Am J Clin Nutr. 68: 1123-1127.

Naidoo R, Tongoona P, Derera J, Laing M, Watson G (2012) Combining ability of low phytic acid (lpa1-1) and quality protein maize (QPM) lines for seed germination and vigour under stress and non-stress conditions. Euphytica. 185: 529-541.

Naidoo R, Watson G, Derera J, Tongoona P, Laing M (2012) Marker-assisted selection for low phytic acid (lpa1-1) with single nucleotide polymorphism marker and amplified fragment length polymorphisms for background selection in a maize backcross breeding programme. Mol Breed. 30: 1207-1217.

Naidoo R, Watson G, Tongoona P, Derera J, Laing M (2013) Development of a single nucleotide polymorphism (SNP) marker for detection of the low phytic acid (lpa1-1) gene used during maize breeding. Afr J Biotech. 12: 892-900.

Oltmans SE, Fehr WR, Welke GA, Raboy V, Peterson KL (2005) Agronomic and seed traits of soybean lines with low–phytate phosphorus. Crop Sci. 45: 593-598.

Raboy V (2002) Progress in breeding low phytate crops. J Nutr. 132: 503S-505S.

Raboy V (2007) The ABCs of low-phytate crops. Nat Biotech. 25: 874-875.

Raboy V, Gerbasi PF, Young KA, Stoneberg SD, Pickett SG, Bauman AT, Murthy PP, Sheridan WF, Ertl DS (2000) Origin and seed phenotype of maize low phytic acid 1-1 and low phytic acid 2-1. Plant Physiol. 124: 355-368.

Raboy V, Hudson SJ, Dickson DB (1985) Reduced phytic acid content does not have an adverse effect on germination of soybean seeds. Plant Physiol. 79: 323-325.

Raboy V, Young KA, Dorsch JA, Cook A (2001) Genetics and breeding of seed phosphorus and phytic acid. J Plant Physiol. 158: 489-497.

Shi J, Wang H, Hazebroek J, Ertl DS, Harp T (2005) The maize low-phytic acid 3 encodes a myo-inositol kinase that plays a role in phytic acid biosynthesis in developing seeds. Plant J. 42: 708-719.

Shi J, Wang H, Wu Y, Hazebroek J, Meeley RB, Ertl DS (2003) The maize low-phytic acid mutant lpa2 is caused by mutation in an inositol phosphate kinase gene. Plant Physiol. 131: 507-515.

Shu QY (2009) Induced plant mutations in the genomics era. Proceedings of an International Joint FAO/IAEA Symposium, Vienna, Austria, 2008, in Induced plant mutations in the genomics era. Proceedings of an International Joint FAO/IAEA Symposium, Vienna, Austria, 2008., Joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture, International Atomic Energy Agency.

Sundberg RD, Broman H (1955) The application of the Prussian blue stain to previously stained films of blood and bone marrow. Blood. 10: 160-166.

Sureshkumar S, Tamilkumar P, Senthil N, Nagarajan P, Thangavelu AU, Raveendran M, Vellaikumar S, Ganesan KN, Balagopal R, Vijayalakshmi G, Shobana V (2014) Marker assisted selection of low phytic acid trait in maize (Zea mays L.). Hereditas. 151: 20–27.

Wilcox JR, Premachandra GS, Young KA, Raboy V (2000) Isolation of high seed inorganic P, low-phytate soybean mutants. Crop Sci. 40: 1601-1605.

Zhang X, Pfeiffer WH, Palacios-Rojas N, Babu R, Bouis H, Wang J (2012) Probability of success of breeding strategies for improving pro-vitamin A content in maize. Theor Appl Genet. 125: 235-246.