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Molecular marker-assisted selection of the *ae* alleles in maize

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ABSTRACT. The *ae* (amylose extender) recessive mutant alleles in maize are an important genetic resource for the development of high-amylose cultivars. On the basis of *ae* allele sequences (from the National Center for Biotechnology Information), the *ae* mutant alleles were cloned from high-amylose maize and the allelic *Ae* gene from common maize luyuan92 inbred lines. Five pairs of primers were designed to screen for a molecular marker of *ae* alleles, yielding a dominant molecular marker, *ae*474. We used 53 types of high-amylose maize and common maize inbred lines and their hybrid and backcross offspring for verification and analysis. The *ae* dominant molecular marker was effective in selecting for the *ae* alleles and for biological materials with a high-amylose genotype. Presence and absence of the marker in the offspring conformed to the expected Mendelian ratios. Using this marker, we were able to detect the *ae* alleles in a backcross and its second generation more efficiently (53.3 and 73.3%, respectively) than was possible without marker selection. These data indicate that the marker can be used as a tool to improve selection efficiency and accelerate the cultivation of new varieties of high-amylose maize.

Key words: Maize; Starch branching enzyme; High amylase; Marker-assisted selection

INTRODUCTION

Maize grain is high in starch, amounting to 70% of the total weight of grain (Wu et al., 2009). Common maize starch is a mixture of approximately 28% amylose and 72% amylopectin. There are a large number of industrial uses for amylose, including food, medicines, textiles, paper, and environmental protection (Rutenberg and Solarek, 1984; Smith et al., 1997; Sun et al., 1998; Nishi et al., 2001; Seetharaman et al., 2001; Leterrier et al., 2008).

Separation of high-quality amylose from common maize is expensive, thus limiting its industrial use and significantly influencing the cost of products. Although most high-amylose maize production occurs in the United States, the actual amount cultivated in the US is low (Whistler, 1958; Fergason, 1994). Because of the substantial commercial demand for amylose, the development of high-amylose maize cultivars is an important research goal. Discovery of the *ae* (amylose extender) mutant alleles was a very important step in developing high-amylose plants. Amylose content is much higher in maize endosperm possessing the *ae* alleles, which are single recessive endosperm mutant alleles (Fisher et al., 1996; Kim et al., 1998). Wu et al. (2009) found that the amylose content of a maize *ae* homozygote, possessing the modified gene, was elevated by 50-80%, relative to a cultivar without the modified gene.

The traditional method of cultivating high-amylose maize cultivars is screening by phenotypic selection using backcross and alternate selfing, such that a backcross can be acquired every two generations. The method, however, is inefficient and the breeding cycle is long. The development of a stable molecular marker for the *ae* alleles would facilitate the identification of promising phenotypes and would accelerate the breeding of high-amylose cultivars. The goal of this study was to clone the *ae* and *Ae* alleles in maize, to analyze their sequences and to develop an *ae* allele molecular marker. We studied the characteristics and reliability of the *ae* alleles and analyzed the effect and efficiency of molecular marker-assisted selection in a high-amylose phenotype backcross.

MATERIAL AND METHODS

Maize and bacterial supplies

Fifty-three test lines, including 38 inbred lines (14 high-amylose maize inbred lines with the *ae* alleles and 24 common maize inbred lines), were used. Among them, high-amylose maize inbred lines, ae-1 and ae-2, were introduced from the US, and W64, B37, A619, and W23 were purchased from the market. The remaining lines were bred and maintained in the laboratory. We used 15 kinds of combination groups of high-amylose maize and common maize, derived from three initial crossings: We_{-4-2} x chang98, B37 x qi478, and $S_{4-25-2-1}$ x chang72. In each group, with chang98, qi478 and chang72 as recurrent parents, we were able to obtain seven groups: F_1, F_2, BC_1F_1, BC_1F_2, BC_2F_1, BC_2F_2, and BC_3F_1, each constructed in our laboratory. We used *Escherichia coli* DH5α, a standard strain maintained in our laboratory.

DNA extraction

We extracted genomic DNA from maize leaves using the CTAB method described by Saghai Maroof et al. (1994). To extract genomic DNA from half seeds, the endosperms were

first removed and then crushed in 200 µL chloroform in a 1.5-mL tube. The crushed samples were mixed with 300 µL DNA extraction liquid (100 mM Tris-HCl, 100 mM EDTA-Na_2, 500 mM NaCl, 1.5% SDS), followed by centrifugation at 12,000 rpm for 2 min at room temperature. The supernatant was transferred to a 1.5-mL tube with 500 µL cold isopropanol to precipitate the DNA. To further concentrate the DNA, the solution was centrifuged at 12,000 rpm for 2 min. The resulting supernatant was discarded and the pellet dried at room temperature before being dissolved in 200 µL TE buffer. DNA quality was confirmed by electrophoresis and UV spectrophotometry. The DNA was stored at -20°C.

Determination of amylose content

Maize amylose content was measured by colorimetry (Morrison and Laignet, 1983; Martinez and Prodolliet, 1996) and near infrared reflectance spectroscopy (Orman and Schumann Jr., 1991; Ciurczak, 1995; Campbell et al., 1997).

Cloning and sequence analysis of the *ae* and *Ae* alleles

Using the sequence of the *ae* alleles of maize (GenBank: AF072725), nine pairs of sequencing primers were designed using Primer Designer. The primers were synthesized by the Shanghai Sangon Company (Shanghai, China). The *ae* and *Ae* alleles were amplified from the leaf-derived genomic DNA of high-amylose inbred lines ae-1 and common maize inbred lines luyuan92, respectively. After electrophoresis detection, recycling and connection to carriers, we obtained full-length sequences of the two alleles, which illustrated their differences.

Screening, verification and analysis of the *ae* allele molecular marker selection

Based on the differences between the *ae* and *Ae* alleles, five pairs of primers were designed. Three high-amylose maize inbred lines (ae-1, ae-2, and $S_{3\text{-}4\text{-}5}$) and three common maize inbred lines (luyuan92, qi478, chang72) were polymerase chain reaction (PCR)-amplified to search for *ae* allele-specific molecular marker bands. Using the *ae* allele specific molecular marker primers, DNA from inbred lines and the combination groups was amplified to determine the utility of the *ae* allele molecular marker in marker-assisted selection (MAS). Reaction conditions for the degenerate primer pairs were initial denaturation for 5 min at 94°C, followed by 35 cycles at 94°C for 45 s, 53°C for 45 s, an extension of 72°C for 90 s, and a final extension at 72°C for 10 min. PCR analysis was performed on a Bio-Rad PTC-100 type PCR instrument.

RESULTS

Identifying an *ae* alleles molecular marker

Cloning and sequence analysis

After sequencing and splicing, we obtained full-length sequences of the *ae* and *Ae* alleles, which were analyzed for differences. The two alleles are homologous; the main differ-

ences are single nucleotide polymorphisms, a single-base insertion/deletion, and three locations where the consecutive bases have been deleted (Figure 1). At bases 453-456 of this sequence, corresponding to the region between exon 9 and exon 10, the *ae* allele has a four-base insertion compared to the *Ae* allele.

```
ae-1    CTCATAGCATTATATAACATTTCAAAACAATATTCATATGTTGCTCATGAATGGTCATCA   360
ly92    CTCATAGCATTATATAACATTTCAAAACAATATTCATATGTTGCTCATGAATGGTCATCA   360
Consensctcatagcattatataacatttcaaaacaatattcatatgttgctcatgaatggtcatca

ae-1    ATATGTTATATTTCTTCTACTTTAGTTCATACATTTTGCTTAATATTTAAAATTAAATTC   420
ly92    ATATGTTATATTTCTTCTACTTTAGTTCGTACATTTTGCTTAATATTTAAAATTAAATTC   420
Consensatatgttatatttcttctactttagttg tacattttgcttaatatttaaaattaaattc

ae-1    ATATTTCCCACAATTACTTTGAGTTAAAAAAAATAAACATCGCCATAGCACAGCCACAGT   480
ly92    ATATTTCCCACAATTACTTTGAGTTAAAAAAAA....CATAGCCATAGCACAGCCACAGT   476
Consensatatttcccacaattactttgagttaaaaaaaa    cat gccatagcacagccacagt

ae-1    ATTGTTACTGTCACCCTACATTTGTTTCTAATTGCCATACGATTTTACATACATCTTCTT   540
ly92    ATTGTTACTGTCACCCTACATTTGTTTCTAATTGCCATACGATTTTACATACATCTTCTT   536
Consensattgttactgtcaccctacatttgtttctaattgccatacgattttacatacatgttctt
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Figure 1. Partial sequence alignment between the *ae* and *Ae* alleles.

Screening for molecular markers of the *ae* alleles

Five pairs of primers were used to amplify fragments (Table 1). Only primer 1 (upstream primer: 5'-TCATCTTCTCACATTGGTCTTCC-3', downstream primer: 5'-GCTGTGCTATG GCCATGTTTAT-3') showed a polymorphism (Figure 2). The amplified regions of the *ae* and *Ae* alleles were at bases 6971-7467. We were unable to amplify a fragment from the common maize inbred lines, but we were able to amplify a 474-bp fragment from the high-amylose maize inbred lines. Consequently, we named this marker *ae*474.

Table 1. Primer sequences used in this study.

No.	Upstream 5'	Downstream 3'
1	CCTCTTCTTAACTCGTAATGATC	TGCCTCTATATTGTCTGGCTAAC
2	CTTCATAGTGTTGCTGGAAGGTC	GTACTTGATCCAGGCTGGAATTG
3	TCATCTTCTCACATTGGTCTTCC	GCTGTGCTATGGCCATGTTTAT
4	CACAGGCAAAGTGATGAAAC	TTATACACCCCAGGCTTTC
5	TTCATGACATCTGATCACC	ATATAGAGAGGACAACGCAGC

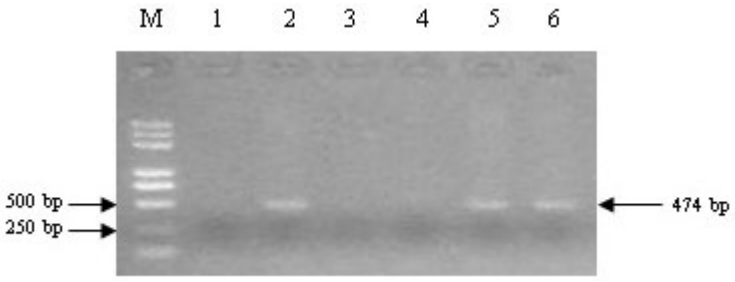

Figure 2. Amplification of high-amylose content maize and common maize with markers. M = DNA marker (DL2000 plus). *Lane 1* = luyuan92; *lane 2* = ae-1; *lane 3* = qi478; *lane 4* = chang72; *lane 5* = ae-2; *lane 6* = $S_{3\text{-}4\text{-}5}$.

Verification of the maize *ae* allele molecular marker and analysis of amylose content

In 14 high-amylose maize inbred lines with the *ae* alleles, the specific *ae*474 band was clearly amplified (Figure 3A). However, in 24 common maize inbred lines without the *ae* alleles, no band was amplified (Figure 3B). Because the *ae*474 marker is stable and generates a clear band, it can be used as a dominant molecular marker linked to the *ae* alleles. The amylose content in 38 maize lines varied from 19.0% in cultivar 8129 to 58.2% in $S_{4\text{-}25\text{-}2\text{-}1}$, which were negative and positive, respectively, for the *ae*474 marker (Table 2). All cultivars that were positive for *ae*474 had substantially higher levels of amylose.

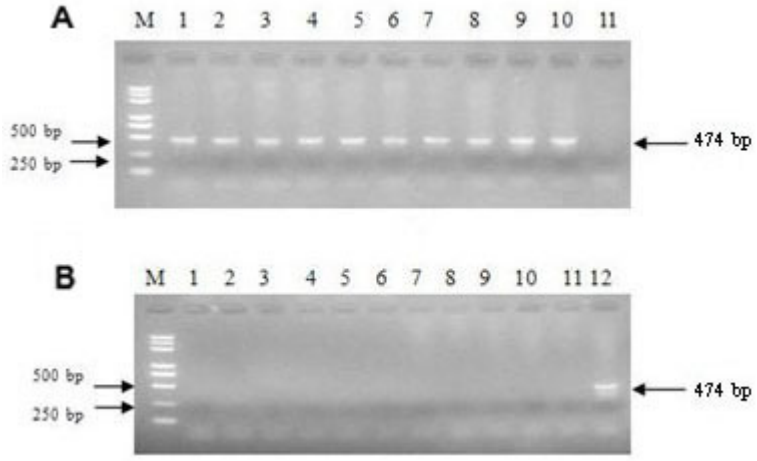

Figure 3. PCR amplification of maize inbred lines with markers. **A.** High-amylose content maize with markers. *Lanes 1-10* = high-amylose maize; *lane 11* = CK(-); M = DNA marker (DL2000 plus). **B.** Common maize with markers. *Lanes 1-11* = common maize; *lane 12* = CK(+); M = DNA marker (DL 2000 plus).

Table 2. Amylose content in 38 maize cultivars.

No.	Cultivar	Amylose molecular marker	No.	Cultivar	Amylose molecular marker	No.	Cultivar	Amylose molecular marker
1	ae-1	57.8 +	14	W23	53.4 +	27	qi478	22.2 -
2	ae-2	56.5 +	15	luyuan92	24.2 -	28	dan340di	23.8 -
3	$S_{2\text{-}9\text{-}4}$	50.2 +	16	8129	19.0 -	29	chang72	24.0 -
4	$S_{4\text{-}15\text{-}3\text{-}2}$	52.0 +	17	w-3	21.5 -	30	dan599	21.7 -
5	$S_{2\text{-}9\text{-}2\text{-}1}$	53.4 +	18	lian87	19.8 -	31	ye478	21.1 -
6	$S_{3\text{-}4\text{-}6\text{-}2}$	49.7 +	19	qi319	23.2 -	32	78599xuan	20.5 -
7	$S_{3\text{-}4\text{-}5}$	51.8 +	20	zi06281	21.2 -	33	liao88	19.5 -
8	$S_{4\text{-}21\text{-}4}$	54.2 +	21	danyou	24.6 -	34	P138	24.0 -
9	$S_{4\text{-}25\text{-}2\text{-}1}$	58.2 +	22	sb-16	20.5 -	35	zheng58	21.8 -
10	$We_{4\text{-}2}$	56.0 +	23	danyu133	23.1 -	36	K14	23.0 -
11	W64	50.7 +	24	H178	24.9 -	37	chang98	25.2 -
12	B37	53.1 +	25	shen137	20.7 -	38	chang94-2	22.7 -
13	A619	49.7 +	26	414	27.0 -			

Nos. 1-14 are high-amylose maize cultivars of inbred lines; Nos. 15-38 are common maize cultivars of inbred lines; +: plant with *ae*474 marker; -: plant without *ae*474 marker.

Segregation of the *ae* allele molecular marker in offspring

We randomly selected 90 F_1 plants, 150 BC_1F_1 plants and 150 F_2 plants from three crosses in each generation (30, 50 and 50 plants per cross, respectively) between high-amylose and common maize cultivars. Offspring from all F_1 crosses were positive for the *ae* marker (Table 3). The *ae*474 marker was present in the other two offspring types according to expected Mendelian ratios, as shown by the chi-square analysis ($P < 0.05$) (Table 3, Figure 4). These data show that, using MAS, it is possible to distinguish between the *AeAe* genotype and the *Aeae*/*aeae* genotypes.

Table 3. Segregation of the *ae* molecular marker in the offspring.

Generation	Combination	Total plants	Positive plants	Negative plants	Expected pos:neg ratio	χ^2 test
F_1	$We_{4\text{-}2}$ × chang98	30	30	0		
	B37 × qi478	30	30	0		
	$S_{4\text{-}25\text{-}2\text{-}1}$ × chang72	30	30	0		
F_2	$We_{4\text{-}2}$ × chang98	50	33	17	3:1	$\chi^2 = 2.16$, $P < 0.05$
	B37 × qi478	50	32	18	3:1	$\chi^2 = 3.22$, $P < 0.05$
	$S_{4\text{-}25\text{-}2\text{-}1}$ × chang72	50	35	15	3:1	$\chi^2 = 0.67$, $P < 0.05$
BC_1F_1	$We_{4\text{-}2}$/chang98 × chang98	50	21	29	1:1	$\chi^2 = 1.28$, $P < 0.05$
	B37/qi478 × qi478	50	21	29	1:1	$\chi^2 = 1.28$, $P < 0.05$
	$S_{4\text{-}25\text{-}2\text{-}1}$/chang72 × chang72	50	19	31	1:1	$\chi^2 = 2.88$, $P < 0.05$

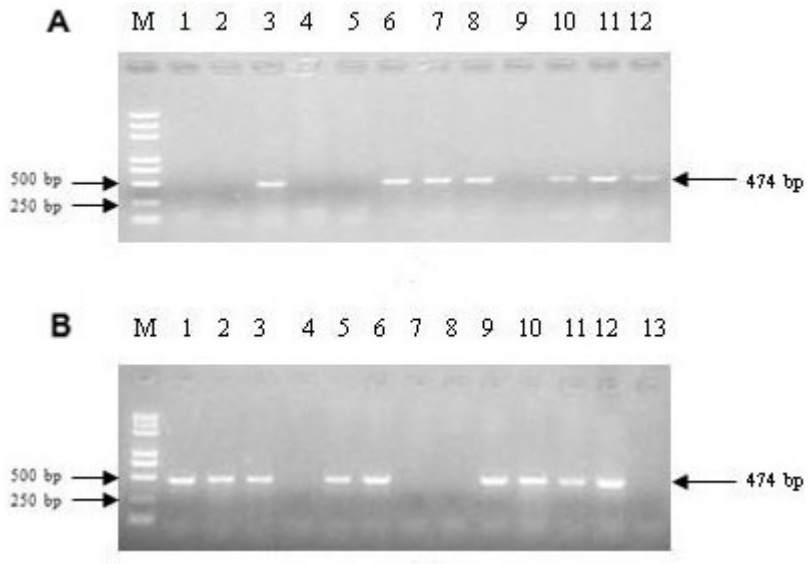

Figure 4. PCR amplification in partial BC_1F_1 and F_2 generations with *ae*474 molecular markers. **A.** PCR amplification of a partial BC_1F_1 generation with *ae*474 molecular markers. *Lanes 1-12* = BC_1F_1 generation plants; M = DNA marker (DL2000 plus). **B.** PCR amplification in partial generation with *ae*474 molecular markers. *Lanes 1-13* = PCR amplification of the genome from F_2 generations; M = DNA marker (DL 2000 plus).

Relationship between the *ae* allele molecular marker and amylose content

We randomly selected 100 seeds from the We_{-4-2} x chang98 combination for DNA extraction from the endosperm; an analysis was carried out for the presence of the *ae*474 marker and amylose content. Maize grains with ≥32.1% amylose amplified the *ae*474 band, while those with less than 29.5% did not amplify the *ae*474 band (Table 4). These statistics show that, with molecular MAS, we could obtain *aeae*, high-amylose genotypic materials. Only *aeae* homozygous seeds have an exceptionally high amylose content. While some of the seeds with less than 21.1% amylose were *ae*474-positive, those seeds are likely to have an *Aeae* genotype. We identified nine seeds of the F_2 generation with >38% amylose; they were all homozygous for *ae*.

Table 4. Relationship between the amylose content of F_2 grain and the presence of the *ae* molecular marker.

Amylose content (%)	Positive for *ae*	Negative for *ae*
<20.0	0	6
20.1-23.0	26	4
23.1-26.0	31	4
26.1-29.0	15	1
29.1-32.0	1	1
32.1-35.0	0	0
35.1-38.0	2	0
38.1-41.0	3	0
41.1-44.0	2	0
>44.0	4	0
Total	84	16

Analysis of the *ae* allele molecular MAS in BC_1F_1 and BC_2F_1 generations

We selected nine plants from the BC_1F_1 and BC_2F_1 generations of the We_{-4-2} x chang98 cross, of which five plants yielded *ae*474 bands and four did not. After self-crossing, we obtained BC_1F_2 and BC_2F_2 generations, from each of which we chose 100 plants for *ae* dominant molecular marker detection (Figure 5, Table 5).

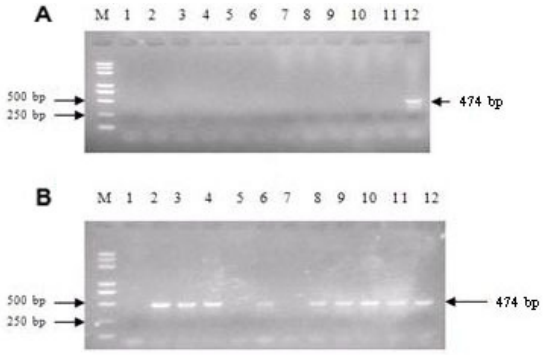

Figure 5. PCR amplification in BC_2F_2 of the BC_1F_1 generation with *ae*474 markers. **A.** PCR amplification in BC_2F_2 of the marked BC_1F_1 generation with *ae*474 markers. *Lanes 1-11* = BC_1F_2 generation plant; *lane 12* = CK(+); M = DNA marker (DL2000 plus). **B.** PCR amplification in BC_2F_2 generation of unmarked BC_1F_1 generation with *ae*474 markers. *Lanes 1-12* = BC_1F_2 generation plant; M = DNA marker (DL2000 plus).

Table 5. Performance of BC_2F_1 and BC_2F_2 maize with the molecular marker.

Generation	Type	No.	Generation	Total plants	No. of positive	No. of negative	Expected pos:neg ratio	χ^2 test
BC_1F_1	Negative plants	1	BC_1F_2	100	0	100		
		2		100	0	100		
		3		100	0	100		
		4		100	0	100		
	Positive plants	5		100	70	30	3:1	$\chi^2 = 1.33$, $P < 0.05$
		6		100	67	33	3:1	$\chi^2 = 3.41$, $P < 0.05$
		7		100	67	33	3:1	$\chi^2 = 3.41$, $P < 0.05$
		8		100	69	31	3:1	$\chi^2 = 1.92$, $P < 0.05$
		9		100	68	32	3:1	$\chi^2 = 2.61$, $P < 0.05$
BC_2F_1	Negative plants	1	BC_2F_2	100	0	100		
		2		100	0	100		
		3		100	0	100		
		4		100	0	100		
	Positive plants	5		100	70	30	3:1	$\chi^2 = 1.33$, $P < 0.05$
		6		100	67	33	3:1	$\chi^2 = 3.41$, $P < 0.05$
		7		100	67	33	3:1	$\chi^2 = 3.41$, $P < 0.05$
		8		100	69	31	3:1	$\chi^2 = 1.92$, $P < 0.05$
		9		100	68	32	3:1	$\chi^2 = 2.61$, $P < 0.05$

From the BC_1F_1 and BC_2F_1 plants that were positive for the marker, we detected *ae*474 bands in their BC_1F_2 and BC_2F_2 offspring. For those plant sets, which demonstrated some positive *ae*474 bands, the ratio of positive to negative plants was 3:1. No *ae*474 bands were detected in the BC_1F_2 and BC_2F_2 offspring derived from the BC_1F_1 and BC_2F_1 plants that did not originally display the marker. These data confirm that we can obtain single plants with the *ae* alleles from offspring using MAS.

Selection efficiency analysis of the *ae* allele molecular marker in backcross offspring

Molecular MAS and non-marking random selection were respectively performed in the BC_1F_1 and BC_2F_1 generations of the We_{-4-2} x chang98 cross. The frequency of molecular marker detection and the selection efficiency of the *ae* alleles in the backcross offspring were observed and calculated. We chose 30 plants for molecular marker detection from each BC_2F_1 and BC_3F_1 generation (Table 6).

Table 6. Efficiency analysis of *ae* allele molecular marker-assisted selection in backcross generations.

Parameter	Marker selected	Non-marker selected
Selective plants in BC_1F_1 generation	30	30
No. of plants with marker in BC_2F_1 generation	30	14
Selection efficiency (%)	100	46.7
Efficiency improvement		53.3
Selective plants in BC_2F_1 generation	30	30
No. of plants with marker in BC_3F_1 generation	30	8
Selection efficiency (%)	100	26.7
Efficiency improvement		73.3

When MAS was used to identify the presence of the marker, the selection efficiency was 100% for both BC_2F_1 and BC_3F_1, a 53.3 and 73.3% improvement, respectively, over the efficiency of non-marker selection. These results confirm that the use of MAS in a backcross can significantly improve efficiency.

Rapid detection technology of the *ae* allele molecular marker

To establish a quick and easy PCR detection method for the *ae* allele molecular marker, seedling leaves were used for rapid extraction of DNA. By adding DNA-staining fluorescent dyes to the tube containing DNA for PCR amplification, we could directly observe the development of PCR products under UV light. Using this method, we could accurately identify positive and negative samples at an efficiency that was comparable to that of electrophoretic analysis. It is possible, therefore, to omit the electrophoretic analysis step, which will simplify MAS procedures and improve efficiency.

DISCUSSION

Selection of the most appropriate plants with desirable characteristics is an important step in breeding new crop cultivars (Van Berloo and Stam, 2001). Individual, or direct, selection, which focuses on agronomic traits that meet breeding objectives, is a phenotypic rather than genotypic selection technique (Ribaut and Betrán, 1999; Van Berloo and Stam, 2001; Francia et al., 2005). For the target gene, molecular marker-assisted breeding technology is a rapid and accurate method, providing a very effective tool for backcross breeding (Ribaut and Betrán, 1999; Frisch and Melchinger, 2005; Collard and Mackill, 2008). Several complex factors influence MAS efficiency, such as the distance between the marker and the target gene, where the marker is not part of the target gene. In our study, the marker is part of the target gene, thus eliminating the main disadvantage of MAS; the problems of linkage and exchange are also avoided (Lande and Thompson, 1990). Because this method can be used at the seedling stage, and DNA quality required for PCR is not high, it can significantly shorten the breeding cycle and improve selection efficiency. The *ae* alleles are recessive mutant alleles that promote a decrease in the quantity of starch converted from sugar. The resulting maize endosperm is dull and the grain shows a certain degree of shrinkage. These phenotypic traits are distinctive for plants that are homozygous for *ae*. Its stability, however, is low and therefore using this homozygous phenotype to select the *ae* alleles is not efficient.

In this study, an *ae* allele-specific molecular marker was acquired that contained a 4-bp nucleotide deletion, lying in an intron between exons 9 and 10 of the *ae* and *Ae* alleles, making it completely linked to the *ae* alleles. Although the marker is dominant, it cannot distinguish between the *Aeae* and *aeae* genotypes. The marker has high reliability and efficiency, and the PCR product can be identified visually in a micro-tube; therefore, it is simple and rapid. With the aid of this marker, high-amylose materials can be selected at a variety of life stages, from harvest to seedlings. This is particularly true when the target is the *Aeae* genotype in backcross breeding. The high amylose maize backcross breeding process can therefore be accelerated.

Studies have shown that the interaction(s) between *ae* and other starch mutant alleles (e.g., du and su) may significantly alter amylose content (Yun and Matheson, 1993; Wang et al., 1993). Vineyard et al. (1958) reported that the level of amylose in hybrid offspring ranged from 36.5 to 64.9% in cross tests between 135 different inbred lines and a common *ae* donor. The substantial variability in amylose content of hybrid offspring was likely due to interactions between different "modified genes" of the germplasm and the *ae* alleles. The presence of the *ae* alleles itself, therefore, is no guarantee of exceptionally elevated amylose since the presence of other genes may cause amylose to vary from 50% to as much as 85%.

The combination of *ae* and modified genes should be taken into consideration in the breeding process. More modified genes are needed if a consistent amylose content of 75% or more is to be achieved. Only in *ae* homozygous loci does the accumulation of modified genes have a selective effect.

In the process of high-amylose maize backcross breeding, the initial step is to choose the desired *ae* genotype - *Aeae* - using the *ae*474 molecular marker. Self-crossing is conducted every two or three generations, which greatly reduces costs and improves the selection efficiency. Using half seed PCR and rapid amylose measurement, it is possible to screen high-amylose seeds for *ae*-modified genes. Using cross-breeding, we can directly select high-amylose seeds from the identified *Aeae* and *aeae* genotypes, to obtain *aeae* plants and additional modified genes.

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