Full Length Research Paper

Inducement and identification of an endosperm mutant in maize

Ruo-Fei Yang[1], Wan-Chen Li[1], Jing He[1], Shu-Feng Zhou[1], Yue-Hui She[2] and Feng-Ling Fu[1]*

[1]Maize Research Institute, Sichuan Agricultural University, Chengdu, Sichuan, 611130, China.
[2]College of Agriculture, Sichuan Agricultural University, Chengdu, Sichuan, 611130, China.

To screen a workable dosage combination of gamma-ray and NaN$_3$ for mutation inducement, maize calli were treated with different dosages of ^{60}Co gamma-ray and NaN$_3$. Mutant lines were derived from the regenerated plants. The results indicate that the combination of 20 Gy of gamma-ray and 1 mmol/L of NaN$_3$ is the most effective for mutation inducement of maize calli. Three endosperm mutant lines with "super sweet" phenotype were derived from the mutated offspring. By complementation test and DNA sequence analysis, their mutation site was found in exon 14 of gene *sh2* that encodes adenosine diphosphate glucose pyrophosphorylase. For their highly consistent phenotypes of agronomic characteristics and distinguished molecular mechanism to the previous mutants of gene *sh2*, these three mutant lines are regarded as a novel multiallelic mutant of gene *sh2*.

Key words: Maize, endosperm mutant, *sh2* gene.

INTRODUCTION

Physical and chemical mutagenesis has been proven to be effective in inducement of useful mutants with novel gene alleles and increase of genetic diversity for enrichment of germplasm resources in crops (Shu, 2009; Waugh et al., 2006). However, the low mutagenesis frequency along with limited desired mutants for selection has greatly affected its application especially and mutagenesis is usually used to treat mature seeds (Nikolaya et al., 2000; Van Harten, 1998). Induced mutagenesis is a random process, and it is rather difficult to screen the desired mutants, without precise information at the molecular level for phenotypic changes (Jain, 2005; Liu et al., 2004; Shu and Lagoda, 2007).

In several decades of mutation breeding practice, a lot of efforts have been made to improve mutagenesis frequency and expand the spectrum of trait variation by the combined treatment of different physical and chemical mutagens on active organs or tissues rather than mature seeds (Gao et al., 1994; Gao and Cheng, 1991; Guo and Zhang, 1992; Jiang et al., 2002; Shu, 2009; Liu et al.,

2006; Wang et al., 1993; Wang et al., 2005; Zhao et al., 2000; Zheng et al., 1993). For example, the mutagenesis frequency of wheat calli derived from immature embryos and irradiated at 1 kR gamma-ray was three times higher than that of mature seed irradiated at 30 kR gamma-ray and the ratio of desired mutants increased as well (Gao and Cheng, 1991; Gao et al., 1994). On the other hand, the recent advances of plant molecular genetics and functional genomics have facilitated screening and identification for the desired mutants (Shu, 2009; Waugh et al., 2006).

Referring to Monille et al. (1996), MuForster (1996), Okita (1992) and Smith et al. (1997), the pathway of starch biosynthesis in maize endosperm can be summarized in Figure 1. At first, sucrose transported from leaves and bracts is catalyzed by sucrose synthase and uridine diphosphate (UDP) glucose pyrophosphorylase, and hydrolyzed into 1-phosphoglucose. Under the catalysis of adenosine diphosphate (ADP) glucose pyrophosphorylase, 1-phosphoglucose reacts with adenosine triphosphate (ATP) to form ADP-glucose, which is then transported into amyloplasts in the endosperm by adenylate transport protein and used as substrate of starch biosynthesis. Granule-bound starch synthase as well as isoenzymes of soluble starch synthase transfers

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*Corresponding author. E-mail: ffl@sicau.edu.cn. Tel: 0835-2882455. Fax: 0835-2882154.
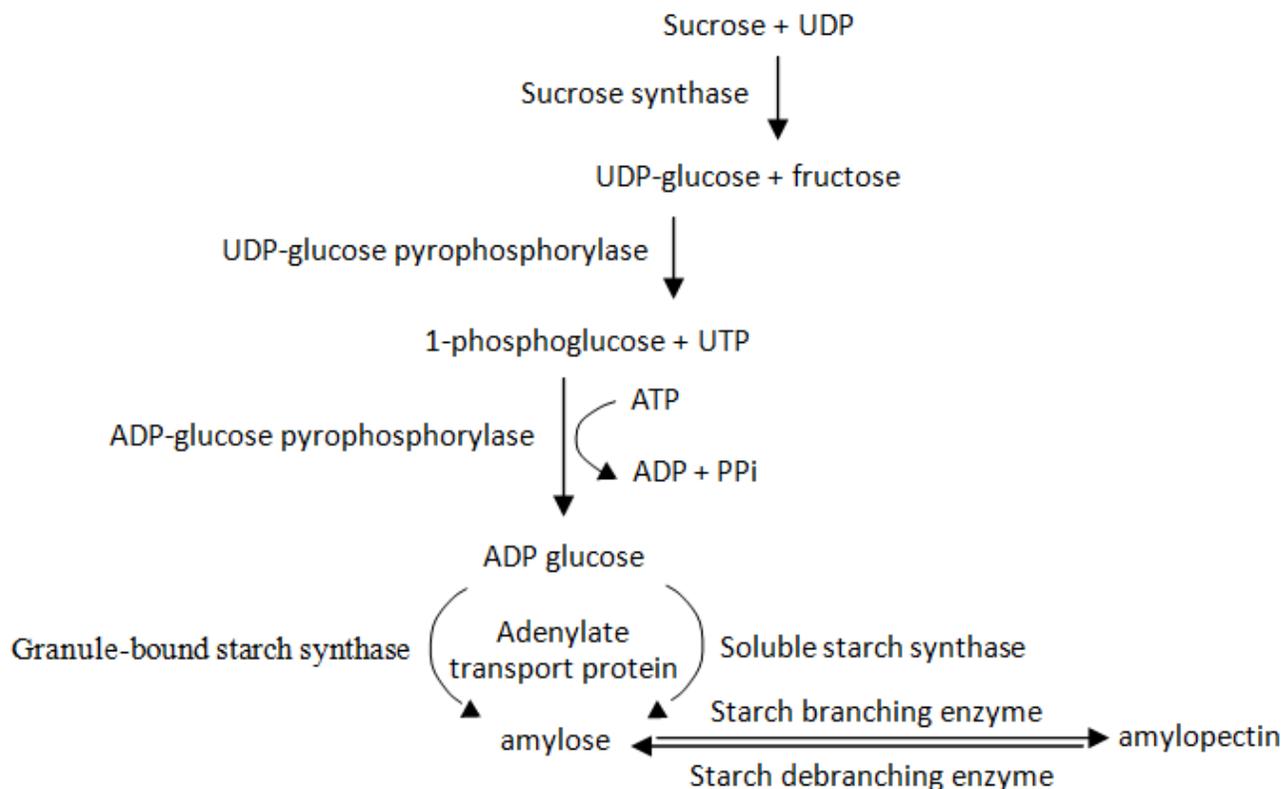
Figure 1. Pathway of starch biosynthesis in maize endosperm. UDP, Uridine diphosphate; ADP, adenosine diphosphate; ATP, adenosine triphosphate; PPi, pyrophosphatic acid; UTP, uridine triphosphate.

the glucopyranosyl from ADP-glucose to oligosaccharide precursor to form amylose linked by α-1,4 glycosidic bond. Starch branching enzyme transforms amylose into amylopectin by cleaving α-1,4 glycosidic bonds of amylose or amylose fragments of amylopectin and linking the cleaved fragments of polysaccharide by α-1,6 glycosidic bonds. Starch debranching enzyme hydrolyzes specifically α-1,6 glycosidic bonds of amylopectin and transforms amylopectin into amylose.

Sweet gene *su* is a mutant of starch debranching enzyme encoding gene. At milky ripening stage, the kernels taste sweet because of the increase of water-soluble polysaccharides. The mature kernels are wrinkled because of the decrease of starch accumulation (Dinges et al., 2001). Super sweet gene *sh2* is a mutant of the big subunit encoding gene of ADP-glucose pyrophosphorylase, resulting in significant inhibition of starch biosynthesis and dramatic accumulation of reducing sugars and sucrose (Smith et al., 1997). The mutated phenotype is recognized as 'super sweet maize'. The 'brittlely sweet' genes *bt1* and *bt2* are mutants of encoding genes of adenylate transport protein and the little subunit of ADP-glucose pyrophosphorylase, respectively (Cao et al., 1995; Sullivan and Kaneko, 1995), resulting in the obstruction of starch biosynthesis. The sweet phenotype of *bt1bt1* and *btbt2* are similar to *sh2sh2*.

In this study, workable dosage combination of gamma-ray and NaN$_3$ were screened for mutation inducement of maize calli. An endosperm mutant with "super sweet" phenotype was derived and identified by complementation test and DNA sequence analysis.

MATERIALS AND METHODS

Preparation of maize calli

According to the method introduced by Fu et al. (2005), immature embryos of 1.5 to 2.0 mm in length were sampled from inbred line 'AB' 13 days after pollination and inoculated onto the improved N6 medium for callus production. Embryonic calli (type II) identified by the standard described by Armstrong and Green (1985) were subcultured three times for three weeks each in dark at 27°C for multiplication and stability of their embryogenesis.

Mutation treatment

The embryonic calli were irradiated with ^{60}Co gamma-ray at 10, 20, 30 and 40 Gy, respectively. After recovering culture for a week, they were treated with NaN$_3$ (pH3) at 0.5, 1.0, 1.5 and 2.0 mmol/L for 4 h. After another recovering culture for two weeks, relative growth rate was recorded as [(callus weight after recovering culture - callus weight before mutation treatments) / callus weight before mutation treatments], used to determine the influence of mutation treatments with gamma-ray and NaN$_3$ on callus growth, and screened for
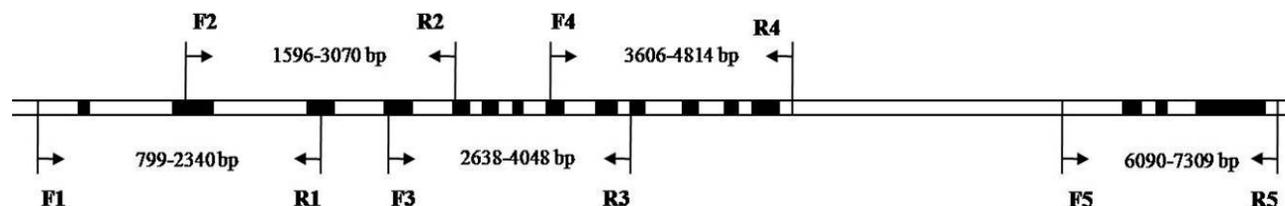
Figure 2. PCR primers to amplify all the 16 exon sequences of *sh2* gene. The solid frames represent the exons, and hollow frames stand for the introns.

workable dosage combination of gamma-ray and NaN$_3$ treatment for maize calli.

Plant regeneration and mutant screening

The survived calli were transferred to differentiation medium for regeneration. The regenerated plantlets (M$_0$) were planted in greenhouse for 2 to 3 weeks for hardening, and then transferred into field for the production of M$_1$ seed by selfing. All the M$_1$ plant lines were planted in field and screened carefully for any possible mutations. Different mutants were selfed and classified for different phenotypic evaluation. While other mutants were used for relevant researches, three endosperm mutants with obvious shrunken kernels were denotated as "sm-1", "sm-2" and "sm-3", and used for complementation test and molecular identification in this study.

Complementation test

To identify the probable mutated gene of the endosperm mutant lines by complementation test, straight and reciprocal crosses were made between the homozygous M$_5$ plant lines of mutants "sm-1", "sm-2" and "sm-3", and the inbred lines with sweet genotypes *susu* (sweet), *sh2sh2* (super sweet), *bt1bt1* (brittle sweet) and *bt2bt2* (brittle sweet), respectively. The mutated gene of the three endosperm mutants was speculated according to the phenotypic complementation of the kernels of the heterozygous F$_1$ plants (Hawley and Walker, 2003).

DNA sequence analysis

Five pairs of PCR primes (F1: 5'-GAACCTTTGTTTCTGTGCGTG-3' / R1: 5'-GGCGTAGCTCTTGTGCTTGT-3', F2: 5'-TCTTGTGAGGGTGATGGGATT-3' / R2: 5'-GCTGGCTCTTCAGGCATTTG-3', F3: 5'-GGAGGATGTTACAGGCTTAT-3' / R3: 5'-TGATCTAGTACAGCTCTTGGG-3', F4: 5'-TGAAAAACCAAAGGGTGCTG-3' / R4: 5'-AAGGCTTTGTTGCTATTGTTCTG-3' and F5: 5'-TGTCATAAGCCCTCATCAAGTC-3' / R5: 5'-AGCACCGAATTAGAAAAACACG-3') were designed according to the sequences (GenBank No: M81603) of the dominant allele of the speculated recessive mutated gene *sh2* in the complementation test (Shaw and Hannah, 1992), and used to amplify the genomic fragments including all the 16 exons piece by piece from the total DNA samples extracted from the three mutant lines, their parental line "AB" and an inbred line with genotype *sh2sh2* (Figure 2).

The 20 µL of the PCR mixture contained: 1×PCR buffer, 30 ng of the total DNA samples, 5 pmol of the primers each, 40 µmol of dNTPs and 1 U of Taq enzyme. After gradient screening of annealing temperature for each pairs of the primers, the PCR temperature cycling profile was set as: 94°C for 5 min; 32 cycles of 94°C for 30 s, annealing for 30 s at 59°C for F1/R1, at 60.0°C for F2/R2, at 58.5°C for F3/R3, at 60.5°C for F4/R4 and at 59.0°C for F5/R5; 72°C for 90 s; 72°C for 10 min. The amplified products were separated in 1% non-denatured agarose gel. The specific fragments were recovered, cloned into plasmid pMD18-T and sequenced with three replications at Invitrogen (Shanghai). The sequenced results were aligned each other by DNAMAN software (www.lynnon.com), to find out the mutation sites in mutants "sm-1", "sm-2" and "sm-3".

RESULTS

Workable dosage of gamma-ray and NaN$_3$ treatment for maize calli

Growth of calli was inhibited by combined treatments of gamma-ray and NaN$_3$ (Table 1). After recovering culture for two weeks, relative growth rate of the treated calli decreased with the increase of both gamma-ray dosage and NaN$_3$ concentration, but the decrease was much greater in the former than in the latter. A dramatic decrease of the growth rate occurred when the dose increased to 20 GY, and the calli almost stopped growing when gamma-ray increased to any higher dosage. However, the growth rate was about 60% of the check (0 Gy of gamma-ray and 0 mmol/L of NaN$_3$) when NaN$_3$ increased to the highest concentration used in this study. It is true that stronger mutagen induces more variation, but causes more injury, as well. The dosage of mutagen should be decided by the balance of variation spectrum and subject injury. In this study, the workable mutagen dosage combination for maize calli should be 20 Gy of gamma-ray and 1 mmol/L of NaN$_3$, which brought about a relative growth rate of 0.79 for the treated calli (Table 1).

During the recovering culture, most of the treated calli with gamma-ray and NaN$_3$ became brown and died. Few survived calli kept growing in dosage combination of 20 Gy of gamma-ray and 1 mmol/L of NaN$_3$, as well as in several close combinations (Table 2).

After differentiation, regeneration and transplanting, 20 fertile plants were obtained from dosage combinations of 20 Gy of gamma-ray with 1 mmol/L of NaN$_3$, and 2 fertile plants were obtained from dosage combinations of 20 Gy of gamma-ray with 0.5 mmol/L of NaN$_3$, respectively. Few plantlets regenerated from several other dosage combinations, but failed to survive afterwards due to either

Table 1. Relative growth rate of maize calli treated with different dose of gamma-ray and NaN$_3$.

NaN$_3$ concentration (mmol/L)	Gamma-ray dosage (Gy)				
	0	10	20	30	40
0	3.71 (±0.15)	3.65 (±0.16)	1.93 (±0.12)	0.37 (±0.10)	0.03 (±0.02)
0.5	3.63 (±0.12)	3.58 (±0.21)	2.11 (±0.22)	0.36 (±0.08)	0.07 (±0.02)
1.0	3.39 (±0.09)	2.03 (±0.07)	0.79 (±0.18)	0.21 (±0.11)	-0.10 (±0.07)
1.5	3.05 (±0.11)	3.15 (±0.18)	0.30 (±0.06)	0.27 (±0.05)	-0.36 (±0.04)
2.0	2.18 (±0.13)	1.79 (±0.25)	-0.02 (±0.01)	-0.42 (±0.12)	-0.69 (±0.02)

Table 2. Survival percentage of maize calli treated with different doses of gamma-ray and NaN$_3$.

NaN$_3$ concentration (mmol/L)	Gamma-ray dosage (Gy)				
	0	10	20	30	40
0	0.33 (±0.04)	0.38 (±0.11)	1.04 (±0.06)	0.00 (±0.00)	0.00 (±0.00)
0.5	0.00 (±0.00)	0.24 (±0.05)	0.78 (±0.08)	0.00 (±0.00)	0.00 (±0.00)
1.0	0.00 (±0.00)	0.07 (±0.02)	1.43 (±0.07)	0.23 (±0.01)	-
1.5	0.12 (0.01)	0.34 (±0.13)	0.32 (±0.15)	0.06 (±0.03)	-
2.0	0.00 (±0.00)	0.00 (±0.00)	-	-	-
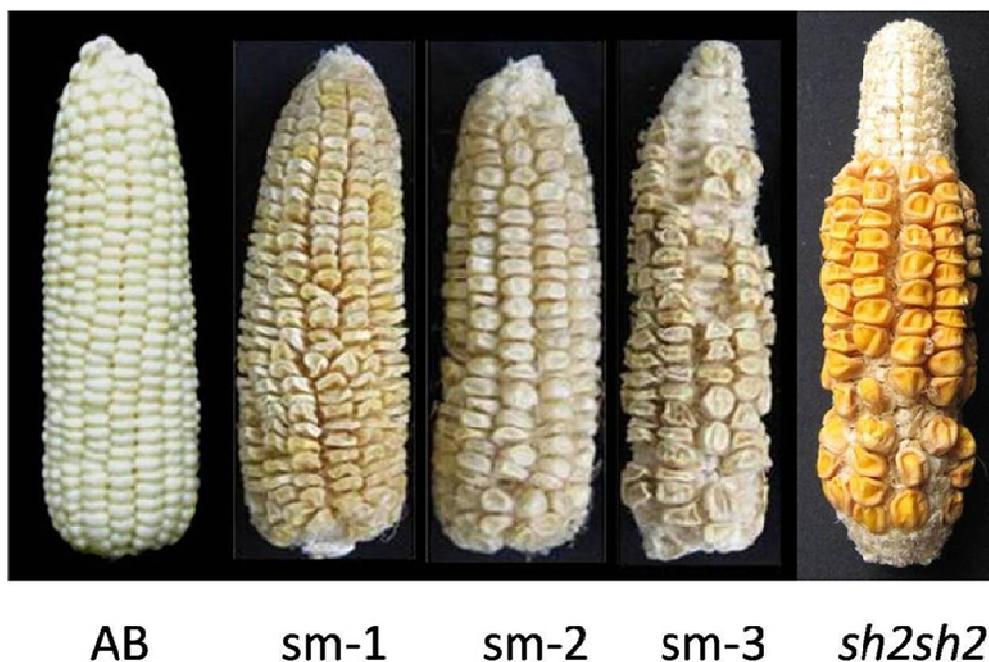
Figure 3. Shrunken kernels of endosperm mutants "sm-1", "sm-2" and "sm-3", as well as parent line "AB" and inbred line with genotype *sh2sh2*.

poor growth or female sterility of resulting plants.

Sweet mutant

During mutant screening, shrunken kernels were found from several individual plants of the M$_1$ population. From the selfed offspring of these kernels, three homozygous M$_5$ plant lines of endosperm mutants were derived, and results of the complementation test showed that the kernel phenotype of the F$_1$ hybrids of the straight and reciprocal crosses between mutants "sm-1", "sm-2" and "sm-3", and sweet genotypes *susu*, *bt1bt1* and *bt2bt2*, were plump (Figure 4), indicating that the mutation are
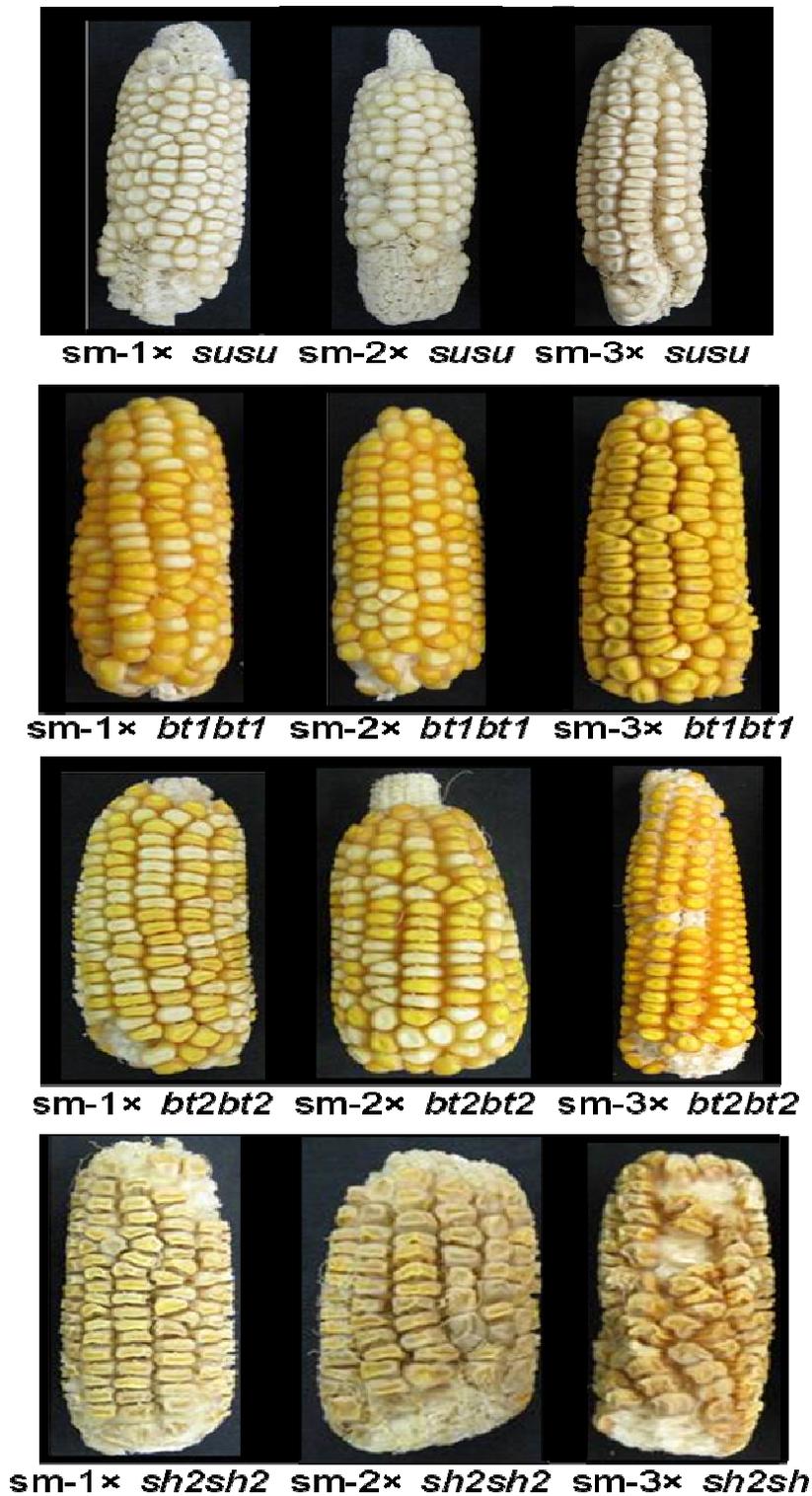
Figure 4. Kernel phenotypes of F_1 hybrids crossed between different sweet genotypes and mutants "sm-1", "sm-2" and "sm-3".

not involved in genes *su*, *bt1* and *bt2*. Only the kernel phenotype of the F_1 hybrids of the straight and reciprocal crosses between sweet genotype *sh2sh2* and mutants "sm-1", "sm-2" and "sm-3", collapsed and shrunk (Figure 3), implying that the mutations in mutants "sm-1", "sm-2" and "sm-3" are probably involved in gene *sh2*, because

Figure 5. Fragments of gene *sh2* amplified from mutants "sm-1", "sm-2" and "sm-3", parent line "AB" and inbred line with genotype *sh2sh2*.

the mutations in these mutants were not complemented by super sweet genotype *sh2sh2* (Hawley and Walker, 2003).
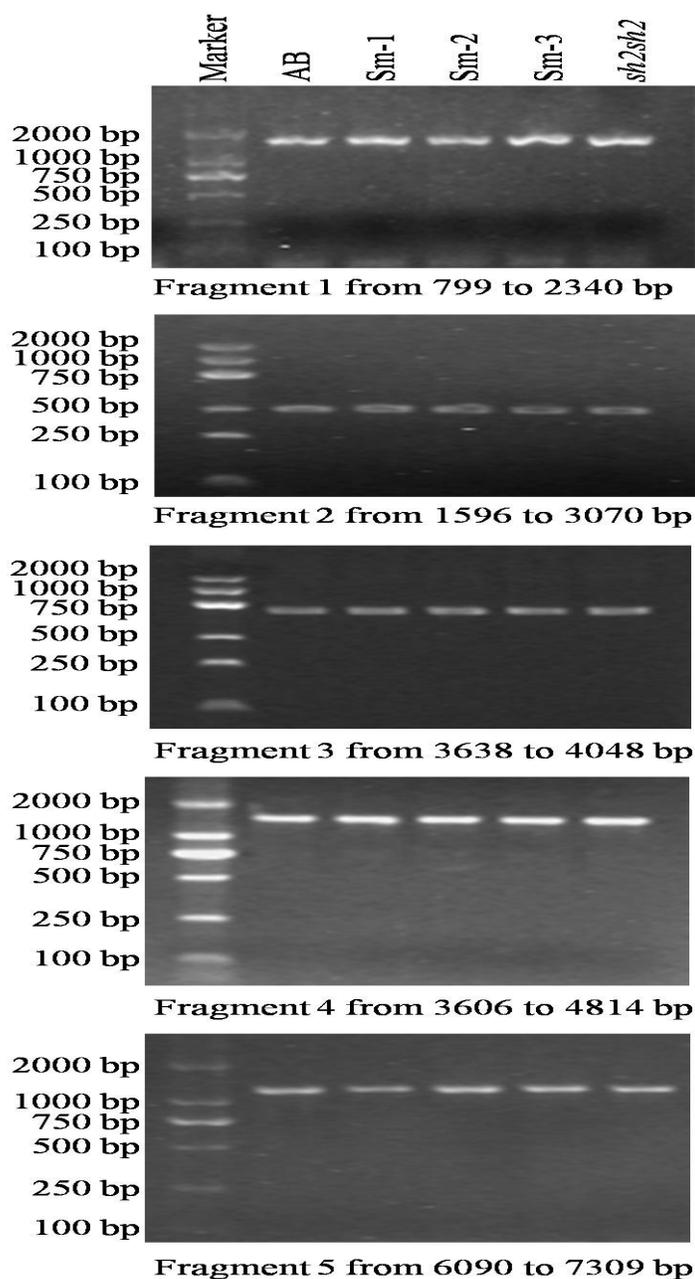
Mutation site

The fragments of gene *sh2* amplified from mutants "sm-1", "sm-2" and "sm-3", parent line "AB" and the inbred line with genotype *sh2sh2*, showed no length polymorphism

(Figure 5). A base substitution from C to T at 6571 bp in the exon 14, and two base substitutions from G to T at 7025 bp and from A to G at 7106 bp in the exon 16, and a 2-bp (GC) insertion between 7123 and 7124 bp in the exon 16 were found by sequence alignment among the three mutants, their parental line "AB" and the inbred line with genotype *sh2sh2*. The two base substitutions and the 2-bp insertion in the exon 16 were involved in the 3'end non-coding region (Figure 6). The base substitution at 6571 bp in the exon 14 was the only missense

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AB   TTATTGCGCTTTCTGATAACGACAGGACTCCGTGATGATGGGAGCGGACACCTATGAAACTGAAGAAGAAGCTTCAAAGC 6600
sm-1 ---------------------------------------------------T--------------------------- 6600
sm-2 ---------------------------------------------------T--------------------------- 6600
sm-3 ---------------------------------------------------T--------------------------- 6600
sh2  ---------------------------------------------------C--------------------------- 6600

AB   TACTGTTAGCTGGGAAGGTCCCAGTTGGAATAGGAAGGAACACAAAGATAAG 6652
sm-1 ---------------------------------------------------- 6652
sm-2 ---------------------------------------------------- 6652
sm-3 ---------------------------------------------------- 6652
sh2  ---------------------------------------------------- 6652
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Exon 14

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AB   ACCATGGGGCATGGGTTCGACTCTGTGACAGGGCATCCAAGAGGCTGATCACCCGGAAGAAGGGTACTACATAAGGTCTG 6960
sm-1 ------------------------------------------------------------------------------- 6960
sm-2 ------------------------------------------------------------------------------- 6960
sm-3 ------------------------------------------------------------------------------- 6960
sh2  ------------------------------------------------------------------------------- 6960

AB   GAATCGTGGTGATCTTGAAGAATGCAACCATCAACGATGGGTCTGTCATATAGATCGGCTGCGTGTGCGTCTACAAAACA 7040
sm-1 ----------------------------------------------------------T-------------- 7040
sm-2 ----------------------------------------------------------T-------------- 7040
sm-3 ----------------------------------------------------------T-------------- 7040
sh2  ----------------------------------------------------------G-------------- 7040

AB   AGAACCTACAATGGTATTGCATCGATGGATCGTGTAACCTTGGTATGGTAAGAGCCGCTTGACAGAAAGTCGAGCGTTCG 7120
sm-1 --------------------------------------------------------------G------------ 7120
sm-2 --------------------------------------------------------------G------------ 7120
sm-3 --------------------------------------------------------------G------------ 7120
sh2  --------------------------------------------------------------A------------ 7120

AB   GGC..AAGATGCGTAGTCTGGCATGCTGTTCCTTGACCATTTGTGCTGCTAGTATGTACTGTTATAAGCTGCCCTAGAAG 7198
sm-1 ---GC--------------------------------------------------------------------- 7200
sm-2 ---GC--------------------------------------------------------------------- 7200
sm-3 ---GC--------------------------------------------------------------------- 7200
sh2  ---..-------------------------------------------------------------------- 7198

AB   TTGCAGCAAACCTTTTTATGAACCTTTGTATTTCCATTACCTGCTTTGGATCAACTATATCTGTCATCCTATATATTACT 7278
sm-1 ------------------------------------------------------------------------------- 7280
sm-2 ------------------------------------------------------------------------------- 7280
sm-3 ------------------------------------------------------------------------------- 7280
sh2  ------------------------------------------------------------------------------- 7278

AB   AAATTTTTA 7287
sm-1 --------- 7289
sm-2 --------- 7289
sm-3 --------- 7289
sh2  --------- 7287
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Exon 16

Figure 6. Sequence alignment among the three mutants, their parental line "AB" and the inbred line with genotype *sh2sh2*.

mutation that changed the encoded amino acid at site 436 from threonine to isoleucine. Some other mutations took place in the introns, but non of them were related to the altering splicing sites.

DISCUSSION

Workable dosage combination of gamma-ray and NaN₃ treatment for maize calli

At present, information about workable dosage of gamma-ray and concentration of NaN_3 for mutation inducement of plant calli is limited. In rice, calli differentiation was enhanced with gamma-ray at less than 30 Gy, but inhibited when it is higher than 40 Gy (Wang et al., 1993). However, 1 kR equivalent to 8.7 Gy of gamma-ray was a workable dosage to treat calli in wheat (Gao et al., 1994). For rice seed, the workable dosage of gamma-ray and concentration of NaN_3 could be as high as 200 Gy and 2 mmol/L, respectively (Wang et al., 1993). In this study, a combination of 20 Gy of gamma-ray and 1 mmol/L of NaN_3 was identified to be suitable for mutation inducement of maize calli (Table 2). This gamma-ray dosage was much higher than 0.1 kR or 0.87 Gy, the dosage used to mutate wheat anthers by Zheng et al. (1993).

A novel multiallelic mutation of gene sh2

The dominant allele of mutated gene *sh2* encodes the big subunit of adenosine diphosphate (ADP) glucose pyrophosphorylase, a key enzyme to limit the rate of starch biosynthesis in endosperm (Feng et al., 2008). In previous reports, the mutations from dominant gene *Sh2* to recessive gene *sh2* were caused by transposon insertion at different sites, inhibiting the activities of ADP-glucose pyrophosphorylase in starch biosynthesis, and resulting in the phenotypes of "super sweet" shrunken kernels because of the dramatic accumulation of reducing sugars and sucrose (Smith et al., 1997). Greene and Hannah (1998) discovered a mutated *sh2* gene which encoded the big subunit with a deletion of 99 amino acids at the C'-terminal and two amino acid substitutions at sties 245 and 455, altering the interaction between the big and the little subunits. In this study, the alteration at site 436 from polar uncharged threonine to nonpolar hydrophobic isoleucine, caused by the missense mutation 6571 bp in the exon 14, was close to the mutated site (455) found by Greene and Hannah (1998). Therefore, this alteration is speculated to obstruct the interaction between the big and the little subunits of ADP-glucose pyrophosphorylase, resulting in the phenotype of "super sweet" shrunken kernels.

Among mutants "sm-1", "sm-2" and "sm-3", no sequence difference was found during the amplified fragments of gene *sh2* (Figure 5). Referring to their highly consistent phenotypes of shrunken kernels (Figure 2) and other agronomic characteristics, these three mutant lines can be speculated to be derived from a single mutagenesis event. For their distinguished molecular mechanism to the previous mutants of gene *sh2*, these three mutant lines should be regarded as a novel

multiallelic mutation of gene *sh2*.

Advantage of complementation test to identify endosperm mutation in maize

Complementation test has been widely used in microbial genetics to determine whether two recessive mutations take place in the same gene (cistron) (De Luca et al., 2003; Entcheva et al., 2002; Hawley and Walker, 2003; Yang et al., 2002). In higher plants, this simple method has been seldom used because it is usually unavailable to find appreciate mutations complementary to the mutant to be identified (Dewdney et al., 2000; Haga et al., 2005; Mulyantoro et al., 2009). For endosperm mutations in maize, however, a wide spectrum of different endosperm mutations have been collected. It is helpful to screen preliminary candidate site for random mutagenesis by complementation test before complicated molecular procedures.

ACKNOWLEDGEMENTS

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