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*Full Length Research Paper*

# Production of high-amylose maize lines using RNA interference in *sbe2a*

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**To regulate the biosynthetic process of maize starch and produce high-amylose transgenic maize, RNA interference was used to inhibit the starch branching enzyme gene *Sbe2*. A construct with a 562 bp segment of *Sbe2* (pRSBE2a) was cloned as inverted repeats. Highly efficient RNAi vector pRSBE2a was transferred to inbred maize line *H99* by *Agrobacterium tumefaciens* mediated transformation. Two transgenic plants of the $T_1$ generation were obtained with a single or double T-DNA copy number, and the interference segments were observed in transgenic $T_2$ plants. The transcription of *sbe2a* and the activity of starch branching enzyme (SBE) were severely inhibited in transgenic plants, and SBE activity was decreased by up to 67.8%. The total starch content had no significant difference between the transgenic and control (wild type) plants, but the percentage of amylose was increased to approximately 66.8% versus the control. In conclusion, RNAi to silence endogenous gene *sbe2a* could produce high-amylose maize lines with a low T-DNA copy number, demonstrating that RNAi is an efficient method for the production of high-amylose maize lines.**

**Key words:** Maize, high-amylose, RNA interference, starch branching enzyme gene *sbe2a.*

## INTRODUCTION

Maize amylose, which is characterized by a high degree of polymerization and good film formation, is far superior to other amyloses in the areas of support films, foods, medical treatments, textiles, paper making, packaging, petroleum, environmental protection, optical fibers, printed circuit boards, and electronic chips. Maize high amylose is the best material for the manufacture of photo-dissociative plastics and could potentially help to control serious "white pollution" (Guo et al., 2008; Visser and Jacobsen, 1993; Smith et al., 1997). Amylose extracted from normal maize is costly. Therefore, breeding of high-amylose maize varieties can significantly expand the application of maize starch and promote the development of the maize industry, improving economic benefits (Chai et al., 2005; Casey et al., 2000). Starches, including amylose and amylopectin, are produced by a synthetic process that is regulated by a series of enzymes.

Starch branching enzyme (SBE) is a key enzyme in the process of starch biosynthesis, forming the branched structure by catalysis of glucose monomer binding through α-1,6 bonds (Clarke et al., 1999; Denyer and Johnson, 2001). SBE is composed of two families, SBE (A) and SBE (B), and the maize SBE has three isozymes, SBEI, SBEIIb, and SBEIIa. SBEI and SBEIIb are mainly present in the endosperm and SBEIIa is mainly present in the embryo, endosperm, leaves, and other nutritive tissues. Together, these three enzymes participate in the synthesis of amylopectin (Blauth et al., 2001, 2002). In addition, corn SBEIIa can directly participate in the synthesis of short chains of amylopectin, and has much higher activity than SBEIIb, indicating that the functions of SBEIIa and SBEIIb cannot be complemented by each of them (Guan et al., 1994). RNA interference (RNAi) is an effective and specific method of gene silencing, capable of post-transcriptionally regulating gene expression through the activity of double-stranded RNA molecules.

This method has been widely applied in the research of functional genomics and metabolic pathways in both plants and animals (Waterhouse and Helliwell, 2002; Louisa, 2004), specifically in the area of improvement of

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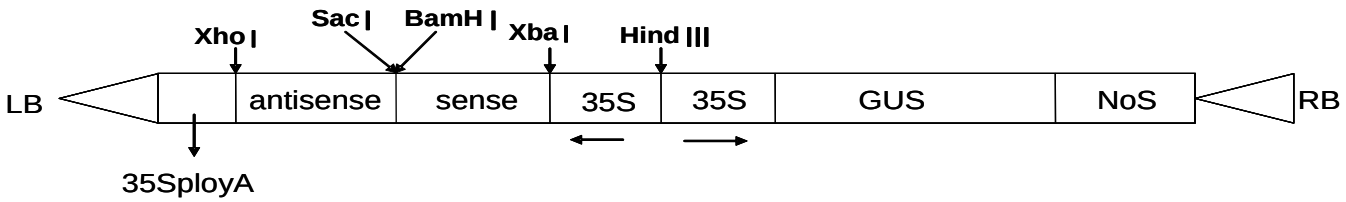


**Figure 1.** The structure map of T-DNA region in RNAi vector pRSBE2a. PCR products of *sbe2a* were excised with *Xba*I + *BamH*I and *Sac*I + *Xho*I producing specific 562 bp fragments and ligated to modified pCAMBIA1301 vector as the sense and antisense segments, respectively.

corn starch quantity and quality (Ogita et al., 2003; Kusaba et al., 2003; Wang et al., 2000). However, there are few reports of corn starch composition changes as a result of RNAi of *sbe2a*, the gene for SBEIIa enzyme (Koga et al., 2006; Zhang et al., 2006). Therefore, the aim of this study was to clone the corn SBE gene, *sbe2a*, and construct an efficient RNAi vector, pRSBE2a. The pRSBE2a construct was transferred into inbred maize line *H99* by *Agrobacterium*-mediated transformation, in order to achieve inhibition of the starch branching enzyme (*Sbe*) gene to produce high amylose. This study also investigated the genetic effects of gene interference, as well as potential applications of RNAi technology in crop improvement.

## MATERIALS AND METHODS

Waxy maize inbred line "W1" was a gift from Prof Yu-Lan Wang, Jilin Agricultural University, China. The embryogenic callus of maize inbred line *H99, Agrobacterium tumefaciens* EHA101 and EHA105, and modified pCAMBIA1301 plasmid were preserved by the laboratory. Concer$^{TM}$ Plant RNA Extract kit and reverse transcript kit were purchased from Invitrogen Shanghai, China; pMD18-T vector, PCR Amplification kit, restriction enzymes, and *Escherichia coli* DH5α were purchased from TaKaRa, Dalian, China. $T_4$ DNA ligase and DNA markers were purchased from Promega China (Beijing); nylon membranes and digoxin (DIG) labeling and testing kit were purchased from Roche China (Shanghai), amylose and amylopectin standard samples were purchased from Sigma Company, and other reagents of analytic grade were obtained from China.

### Cloning and sequencing of maize *sbe2a* gene

The 562 bp of the total coding sequence of *sbe2a* was used to design primers (implying that the entire coding sequence was larger than 562 bp) (GenBank accession no.: U65948), using Primer Premier 5.0 software (Premier, Canada) (Rychlik and Rhoads, 1989). Primer sequences were as follows (restriction sites such as *Sac* I, *Xho* I, *Xba* I and *BamH* I are underlined): P1 upstream primer: 5′-TCTTG AGCTC ATAGG CGAGA ATCCC ACAT-3′ and downstream primer: 5′-TAACC TCGAG CGTGT AAAGA TACGG ATGGA-3′; P2 upstream primer: 5′-TTTGT CGACC GTGT AAAGA TACGG ATGGA C-3′, and downstream primer: 5′-TTTGG ATTCA TAGG C GAGAA TCCC ACAT-3′.

The total RNA was extracted from waxy maize inbred line "W1" using the plant RNA extract kit, reverse transcription was performed to obtain cDNA, and PCR amplification was performed using the cDNA template. PCR products were separated by electrophoresis, recycled, and ligated to pMD18-T vector, and the construct was transferred to *E. coli* DH5α. Positive white colonies were selected and cultured, plasmid DNA was extracted using the alkali lysis method, and DNA was identified via restriction enzymes and gene sequencing (Dalian TaKaRa Company). Nucleic acid sequences were analyzed using DNASIS (Medprobe, UK) software.

### RNAi vector constructs

The RNAi construct pRSBE2a, consisting of maize *sbe2a* gene, is shown in Figure 1. The pMD18-T-SBEIIa construct containing *sbe2a* gene was excised with *Xba*I and *BamH*I. The modified pCAMBIA1301 vector was also opened by the same endonucleases. The retrieved fragments containing the *sbe2a* gene were ligated into the pCAMBIA1301 vector and the construct was transferred to *E. coli* DH5*α*, screened on LB medium supplemented with kanamycin (50 µg/ml).

Two positive colonies containing reconstructs pRSBE2a were obtained after screening. To verify the reconstructs, plasmid DNA of positive colonies were extracted and digested. Plasmid was extracted using the mini DNA extraction kit, PCR amplification was performed using P1, P2 primers and plasmid templates, and PCR products were identified by restriction enzyme digestion and DNA electrophoresis.

### *Agrobacterium*-mediated transformation with RNAi vector

Young female ears of *H99* were collected 10 to 14 days after pollination and the outermost bracteal leaves were removed, retaining three to five bracteal leaves. The portion of the young ear without kernels was removed, and the remaining ears were immersed in 70% alcohol for 5 to 10 min. All bracteal leaves and filaments were removed from the ear, and then the ears were immersed in 5% sodium hypochlorite solution for 15 to 20 min. Following three to four washes in sterile water, the young embryos with a long axis of approximately 1.5 mm were used to inoculate the callus induction medium, keeping the scutellum upwards. Each treatment was repeated three times and the samples were cultured at 26°C in darkness. The shoot and root-like tissues were removed to form embryogenic callus as explants for transformation. The pRSBE2a construct (containing the RNAi segment) was transferred to *Agrobacterium*, and then the *Agrobacterium* was allowed to invade maize callus. The infected callus samples were directly transferred to the differentiation medium for shoots and root growth, and after this point the plantlets were transplanted to agricultural lands.

### Detection of transgenic plants

DNA was extracted using a modified CTAB method (Wang and

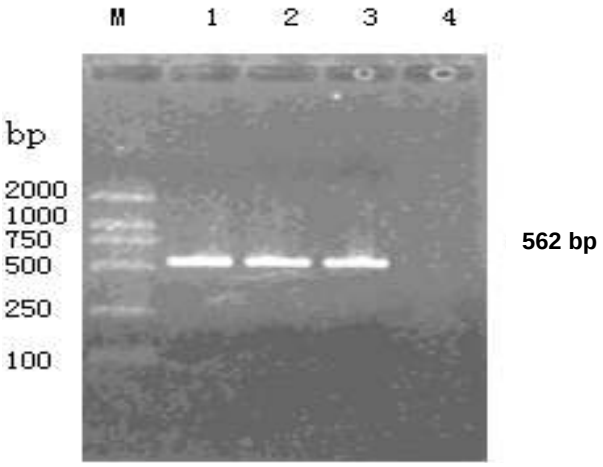


**Figure 2.** Isolation of starch branching enzyme *sbe2a* gene. The 562 bp *sbe2a* fragment was obtained from RT-PCR products of maize W1. M, DNA Marker DL-2000; 1-3, PCR products; 4, negative control.

Fang, 2002). Primers were designed for the GUS gene carried on the RNAi vector, as follows: P3 upstream, 5'-GTGAATCCG CACCTCT-3' and P3 downstream, 5'-ATCGCCGCTTTGGACATA-3'. PCR amplification was performed for primary screening of transgenic lines. Genomic DNA was extracted from PCR-positive plants, excised with *BamH* I, separated on 0.8% agarose, transferred to nylon membranes in 20 x SSC solutions, fixed for 2 h at 80°C, and hybridized with GUS probes. The 836 bp promoter segment on the RNAi vector was amplified by PCR and labeled with DIG as the hybrid probe. The DIG labeling and testing kit was used for probe labeling, hybridization, and color development.

#### RT-PCR analysis of transgenic plants

Using the RNA extract kit, total RNA was extracted from the kernels of transgenic plants from the $T_1$ generation 20 days after pollination; using RNase-free DNAase, trace genomic DNA was removed in the RNA extracts. RNA was accurately quantified using an ultraviolet spectrophotometer. In order to detect *sbe2a* mRNA accumulation in transgenic plants, first-strand cDNA synthesis was performed with the reverse transcript kit using a non-transgenic plant as the control and maize *EF-1a* gene as the inner control.

PCR amplification of *sbe2a* was conducted using the first strand of cDNA as template. The *sbe2a* gene primer sequences were as follows: 5'-CGTGTAAAGATACGGATGGAC-3' (upstream) and 5'-ATAGGCGAGAATCCCACAT-3' (downstream), with an expected product size of 562 bp. The *EF-1*a primer sequences were as follows: 5'-GCTTCACGTCCCAGGTCATC-3' (upstream) and 5'-TAGGCTTGGTGGGTATCATC-3' (downstream), with an expected product size of 213 bp (Kirchberger et al., 2007). Cycling conditions were 94°C for 5 min, 28 cycles at 94°C for 30 s, 54°C for 30 s, and 72°C for 1 min, and then final extension at 72°C for 10 min.

#### SBE activity assay in transgenic plants

*SBE* activity assay was performed according to Li et al. (1997) described with slight modification. Five kernels of each transgenic maize 20, 25, and 30 days after pollination (15 kernels total), were harvested, weighed, and soaked in 0.05 mol/L citric acid buffer solution (pH 7.0), ground and homogenized in an ice bath (22 ml in total). Homogenates were then precipitated by centrifugation at 18,000 rpm for 20 min. The gross enzyme extracts were contained in the supernatant.

1 ml of gross enzyme extracts was mixed with 1 ml of 0.2 mol/L citric acid buffer (pH 7.0) and 0.5 ml of 0.1 mol/L EDTA. This solution was oscillated to inactivate the amylase and then supplemented with 0.5 ml of 0.75% soluble starch and incubated for 40 min at 37°C. 10% TCA (trichloroacetic acid, 4 ml) were added to cease the reaction; then the mixture was precipitated by centrifugation at 3000 rpm for 8 min. Iodic fluid (0.3 ml) was added to the supernatant for color development, and optical density (OD) was measured after 10 min at 660 nm. The SBE activity of non-transgenic plant served as control. The percent of SBE activity decrease was calculated as follows:

$\Delta OD_{660}\% = (\text{control } OD_{660} - \text{experimental } OD_{660})/\text{control } OD_{660} \times 100\%$.

#### Starch content of transgenic plants

The amylose and amylopectin contents were determined using a dual wavelength method as reference (Hovenkamp-Hermelink et al., 1988). For amylose content determination, the major and contrast wavelengths were 620 and 480 nm, respectively, and for amylopectin, the major and contrast wavelengths were 550 and 760 nm respectively. Each treatment was repeated three times. Total starch was the sum of amylopectin and amylose.

#### Genetic analysis on transgenic $T_1$ and $T_2$ plants

Maize cob of transgenic $T_1$ plant was covered with a plastic bag to allow inbreeding, and 20 random seeds were harvested from one single plant. DNA was extracted from the single plant for PCR detection. SBE activity and starch content were determined as previously described. Genetic analysis was conducted on the exogenous genes in transgenic $T_2$ plants.

## RESULTS

### Cloning and sequencing of *sbe2a* fragment

As shown in Figure 2, specific and pure segments of approximately 560 bp were obtained as expected. After recycling, ligation into the vector, and transfer into *E. coli*, five positive colonies were obtained after screening. An insert segment of approximately 560 bp was obtained via restriction digestion, suggesting that the target segment had been inserted into the vector (Figure 3). Sequence analysis showed that the 562 bp fragment differed by one base from the sequence reported in GeneBank (GenBank accession number: U65948), with homology reaching 99.8%. This confirmed that the cloned sequence was the same as the corn *sbe2a* fragment. The sequences (562 bp) were as follows:

ATAGGCGAGA ATCCCACATC CAATGATGGC CTCGTGGACC ACCATGGAAG TAAT
GTGTACGGTGCCATC GAAACCATTC AAACCATCCA AGGTATTATT TGATGAATGA CTA
TGAACAA TATCCATAAG CACTAGCAAG

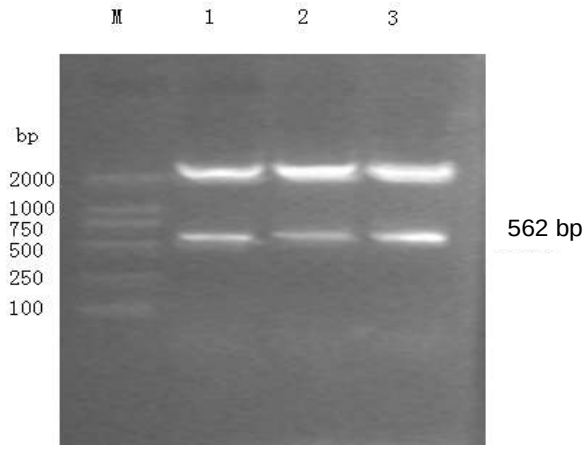


**Figure 3.** Restriction enzyme analysis of the recombinant clone. The plasmid DNA of positive colonies was extracted and digested by *Sac* I+ *Xho* I. Then DNA electrophoresis was performed. M, DNA marker DL-2000; 1,2,3, pMD18-T-SBEIIa/*Sac* I+ *Xho* I.

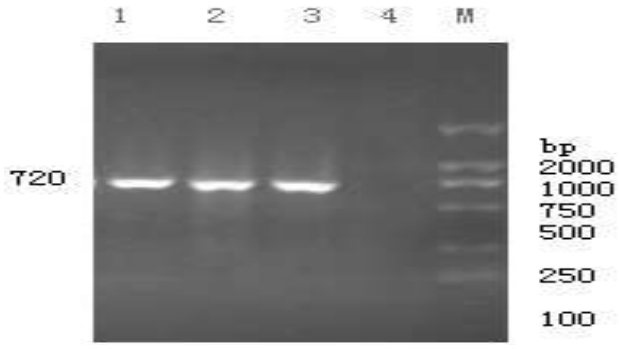


**Figure 4.** PCR analysis of the transgenic plants. The genomic DNA was extracted from regenerative plants. P3' primers (partial GUS segment of pRSBE2a) were designed and PCR amplification was conducted using genomic DNA template. Plasmid p35S-1301 and non-transgenic plant genomic DNA served as the positive and negative controls, respectively. The segments of approximately 720 bp were obtained from two transgenic plants, M, DNA marker DL-2000; 1, 2, transgenic plants; 3, positive control; 4, non-transgenic plant.


**Figure 5.** Southern blot analysis of transgenic plants. DNA was extracted from leaves of each PCR-positive transgenic plant, and digested with *BamH*I. Southern blotting was performed using the DIG-labeled *GUS* segment (720 bp) as probe. Hybrid signals were appeared for the positive plants. 1, 3, Transgenic plants (H99-1, H99-2); 2, 4, non-transgenic plants; 5, positive control.

CCAAGCTCAT GCGCTTTATC AATAAGAGAT T
TTAGGTCCT CTGGAGTCCC AAAACGGCTA
CTTGGGGCAA AAAAATTCGT AACATGGTA
C CCAAAGCTTG CATAATAAGA GTGTTCCTGG
ATTGCCATTA TCTGTACTGC ATTGTATC
CA AGCTTTTTAATTCTTGGAAG CACCTCATCT
CTGAAGTTAG CATATGTATT TATCTTTG
GT TCCGGGCTAC TCATTCCAAC ATGTGATTCA
TATATCCGCA GTGACTTGGG CCGCTTA
GGT TGAGGGTGTT TGAATACATA TTTCTCCTCT
TCAGGTGGGT CATAATATAT ACCGTT
GTAT GGTATTTCAC CTGGAGCCTG CACAGAAAAC
TTGATCCAGG CAGGAATGGAATCC
TTAACA CCAGATGGTGTGTCCATCCG TATCTTTACA

## Construction of *pRSBE2a*

PCR amplification was performed using P1 and P2 primers and *pRSBE2a* plasmid templates. PCR products were excised with *Xba*I + *BamH*I and *Sac*I + *Xho*I to identify the sense and antisense segments, respectively, producing specific 560 bp fragments for all. Excision with *Xba*I + *Xho*I to identify the interference segments produced a specific 1,110 bp fragments. This demonstrates that the RNAi vector was constructed successfully.

## PCR detection of transgenic plants

Maize calli by *Agrobacterium*-mediated transformation directly differentiated and grew into 42 regenerative plants. Of these, 18 survived and were identified by PCR. Using SDS method, genomic DNA was extracted from regenerative plants. P3' primers (partial GUS segment of pRSBE2a) were designed and PCR amplification was conducted using genomic DNA template. Expression plasmid p35S-1301 and non-transgenic plant genomic DNA served as the positive and negative controls, respectively. Evident segments of approximately 720 bp, consistent with the segment size of the positive control, were obtained from two transgenic plants, but not from the negative control (Figure 4). Two transgenic plants (H99-1, H99-2) were primarily verified, in which one plant had contained some seeds.

## Southern blot analyses of transgenic $T_1$ plants

DNA was extracted from the leaves of each PCR-positive transgenic plant, and digested with *BamH*I. Southern blotting was performed using DIG-labeled *GUS* segment (720 bp) as probe. As shown in Figure 5, hybrid signals appeared for the positive plant in accordance with the results of PCR detection, whereas the signal was not present for the non-transgenic plant. This result indicate that the exogenous gene had been integrated into the

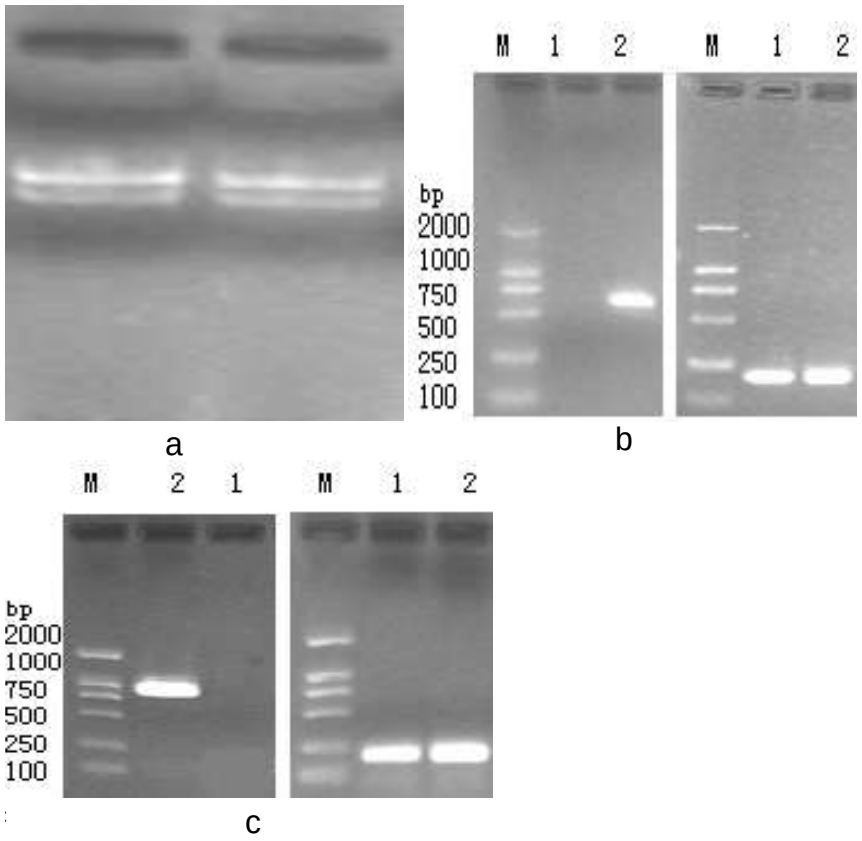


**Figure 6.** RT–PCR analysis of transgenic plants. The endogenous SBE mRNA content was analyzed in transgenic plants. (a) Total RNA extracts from maize kernels; (b) reaction for 28 cycles; (c) reaction for 30 cycles; M, DNA marker DL-2000; 1, transgenic plant (H99-1); 2, non-transgenic plant; *Sbe2a* segment, 562 bp; *EF-1* a, 213 bp.

genome of the transgenic plant. In addition, analysis of the different signal sizes of hybrids suggested that the integration sites of the exogenous gene were different in transgenic plants. The number of hybrid signals was also different, implying that the exogenous gene may integrate into the transgenic maize genome at a copy number of one to two.

### RT-PCR analyses of transgenic $T_1$ plants

The results of RT-PCR on transgenic $T_1$ maize kernels are shown in Figure 6. The endogenous SBE mRNA content was decreased obviously in transgenic plants.

### SBE activity and starch content in transgenic $T_1$ kernels

The SBE activity of transgenic maize kernels was significantly lower than that of the control, suggesting that the translation of endogenous SBE mRNA was inhibited by RNAi vector to some extent and also decreased the SBE activity. The enzyme activity for transgenic plant *H99-1* was 0.04415 U (n = 3), and 0.12721U for the control (Figure 7a); the SBE activity was reduced by 65.3% ($P < 0.01$). The total starch content of transgenic *H99-1* was 650 mg/g DW (kernels) for all, similar to that of the control. However, the percent content of amylose was up to 34.2% in transgenic plants, significantly higher than 21.9% for the non-transgenic plant ($P < 0.01$; Figure 7b).

### PCR analysis of transgenic $T_2$ plant

PCR results for transgenic $T_2$ plants are shown in Table 1. The results demonstrate that the exogenous gene could be passed on to $T_2$ generation, and the segregation ratio agreed to Mendel inheritance.

### SBE activity and starch content in $T_2$ plant

SBE activity and starch content were analyzed on H99-1-1 and H99-1-2 plants from $T_1$ H99-1 kernels. The SBE

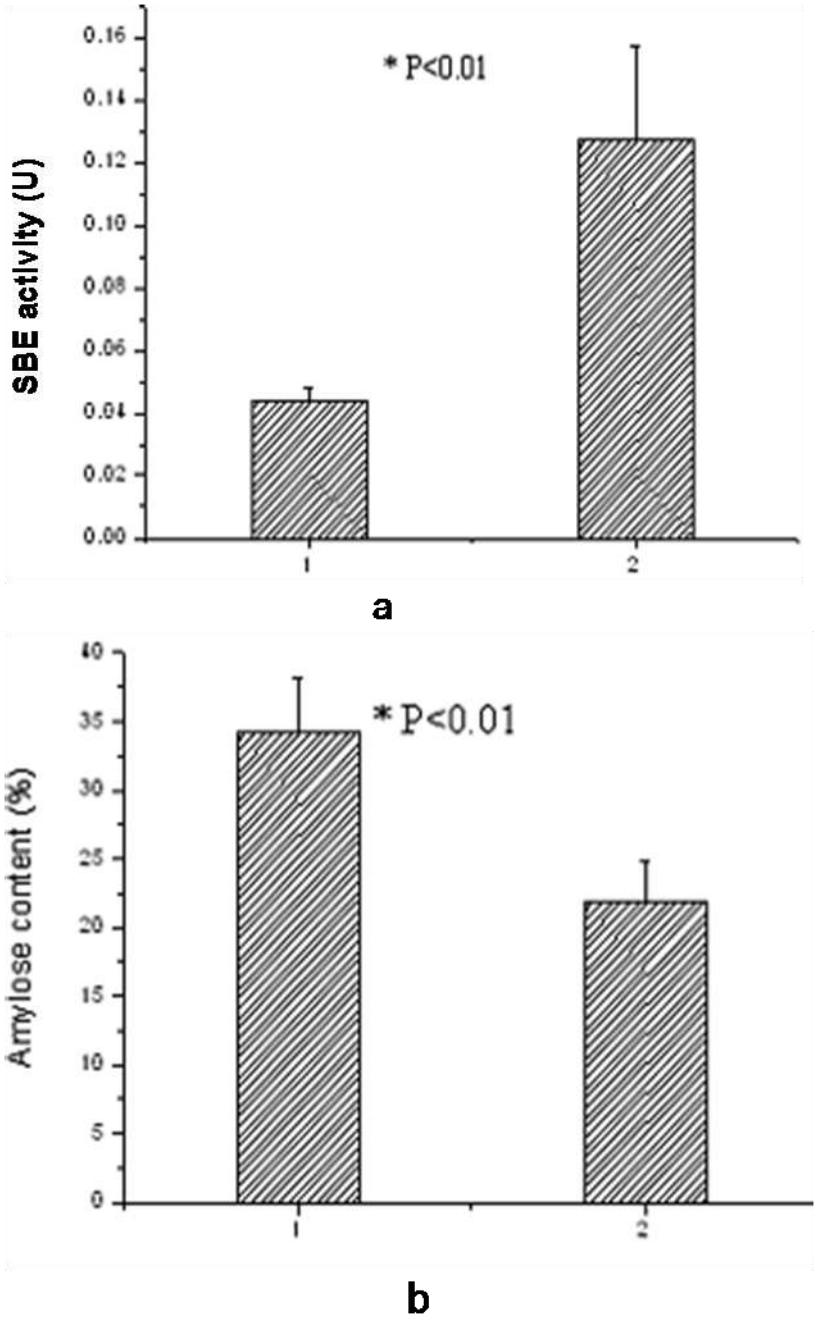


**Figure 7.** SBE activity and amylose percent contents in kernels and seeds of $T_1$ transgenic plants. SBE activity assay was performed in kernels according to Li et al. (1997) with slight modification. The starch contents were determined using a dual wavelength method as reference (Hovenkamp-Hermelink et al., 1988). Each treatment was repeated three times. Total starch was the sum of amylopectin and amylose. (a) SBE activity; (b) amylose percent content; 1, transgenic plant (H99-1); 2, non-transgenic plant.

**Table 1.** PCR analysis of transgenic T2 plants.

| $T_2$ line | Number of seed | Number of seedling | Number of positive plant | Number of negative plant | Positive/Negative |
|---|---|---|---|---|---|
| Line 1 | 20 | 17 | 13 | 4 | 3.2/1 |

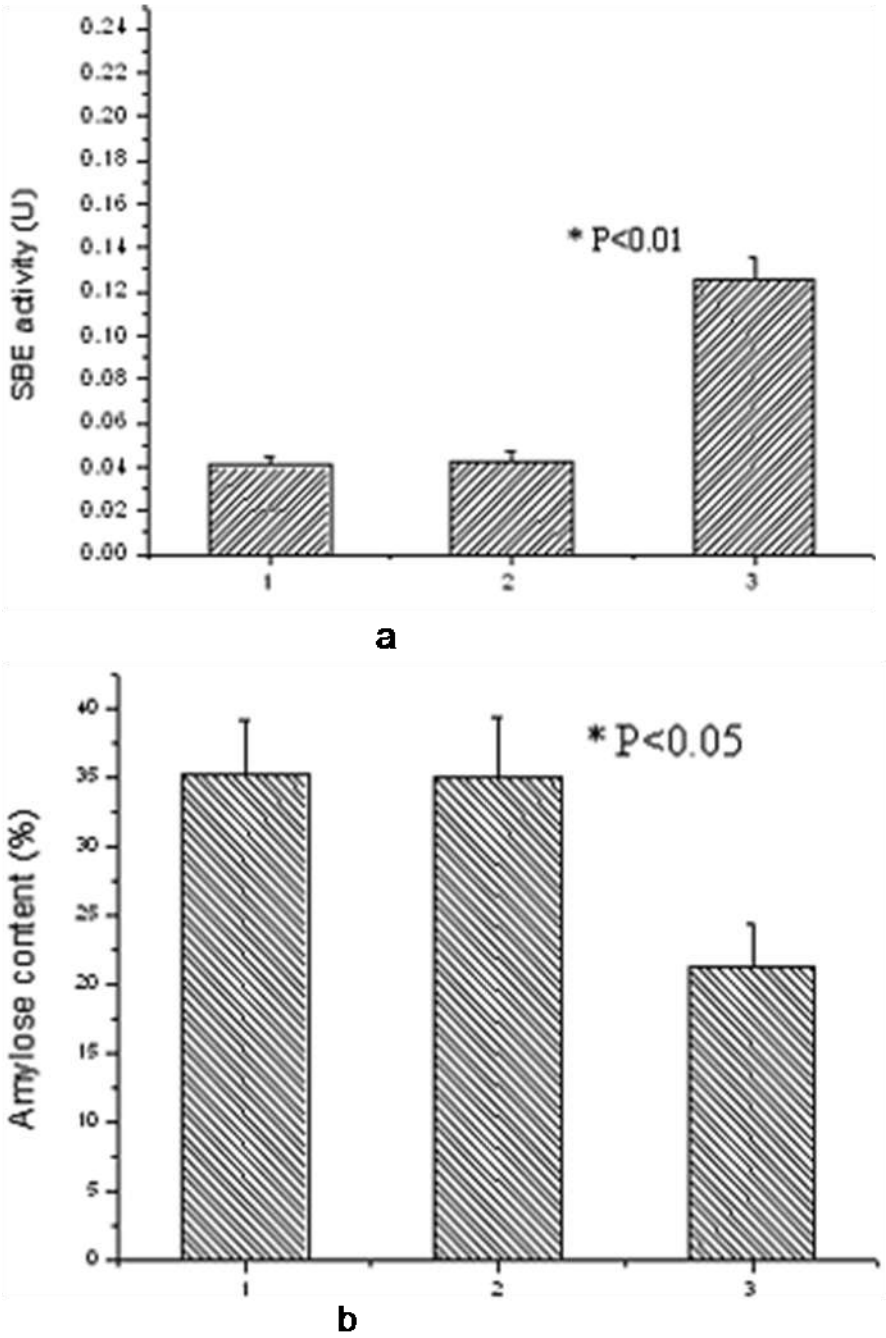


**Figure 8.** SBE activity and amylase percent contents in seeds of $T_2$ transgenic plants. SBE activity assay was performed in kernels according to Li et al. (1997) with slight modification. The starch contents were determined using a dual wavelength method as reference (Hovenkamp-Hermelink et al., 1988). Each treatment was repeated three times. Total starch was the sum of amylopectin and amylose. (a) SBE activity; (b) amylose content; 1, 2, transgenic plants (H99-1-1, H99-1-2); 3, non-transgenic plant.

activities of $T_2$ plant were 0.04048 and 0.04215 U respectively, and 0.12561 U for the control (Figure 8a), ($P < 0.01$). Furthermore, the SBE activity was reduced by 67.8 and 66.4%, respectively (mean 67.1%). For transgenic H99-1-1 and H99-1-2, the total starch content was approximately 650 and 640 mg/g DW (kernels), respectively; the percent content of amylose was 35.2 and 34.9%, respectively, and 21.1% for non-transgenic plant (Figure 8b). The amylose content was significantly increased to 66.1% (mean value) compared to that of the

control ($P < 0.05$).

## DISCUSSION

Corn starch, especially amylose, is an industrially important material. Many techniques have been developed to improve the quantity and quality of amylose. Slattery et al. (2000) reviewed the engineering of starch for increased quantity and quality, and indicated that the genetic manipulation on SBE is an efficient way to regulate the starch biosynthetic pathway. Safford et al. (1998) studied antisense RNA inhibition of SBE activity on potato starch, and the results showed that tuber SBE activity was reduced by between 5 and 98% of the control values. However, the use of antisense technology for gene inhibition yielded a low frequency of generation of high-amylose lines that was mostly correlated with high copy numbers of integrated T-DNA (Hofvander et al., 2004). RNAi can lead to double-strand RNA-mediated posttranscriptional gene silencing (Hamilton and Baulcombe, 1999). This technology has been applied in plant genetic engineering, introducing the potential for increasing the quality of maize. RNAi can regulate gene expression without destroying the target gene, making it a new way for understanding gene function, also increasing the potential for animal and plant quality improvement and treatment of diseases through genetic engineering.

Generally, RNAi as a focus issue in molecular biology and genetic engineering opens up a new field in fundamental and applied research. Schwall et al. (2000) produced a very high-amylose potato starch by the inhibition of SBE A and B. Andersson et al. (2006) used RNAi to inhibit *sbe1* and *sbe2* and produce high-amylose potato lines, yielding low copy-number T-DNA inserts with an average of 83% of backbone-free transgenic lines being single-copy events. Guo et al. (2009) transferred the RNAi vector of the starch branching enzyme gene to potato, resulting in an increase in the content of amylose in the transgenic potato. This paper cloned the *sbe2a* gene and constructed the corresponding RNAi vector. Using *Agrobacterium*-mediated transformation, the RNAi vector was successfully introduced into inbred maize lines.

The results show that SBE activity was significantly decreased (by 65.3, 67.8, and 66.4%) in transgenic $T_1$ and $T_2$ plants compared to the control, suggesting that the endogenous *Sbe2a* mRNA translation was effectively inhibited by the RNAi vector, meanwhile, the percent content of amylose was significantly increased to 66.8%, indicating that RNAi technology could effectively regulate maize starch synthesis, inhibiting the synthesis of amylopectin and greatly enhancing the content of amylose under the condition of invariable total starch content. Stable transgenic plants will become inbred maize lines to produce high-amylose with one to two integrated T-DNA copies. High-amylose transgenic plants together with amylose-extender (ae) inbred lines may breed high-amylose maize hybrid materials, providing a new way to develop high amylase in maize.

### Conclusion

In this study, an RNAi vector for *sbe2a* gene silencing was successfully constructed, leading to the specific degradation of homologous mRNA after transcription, thereby, *sbe2a* gene expression was inhibited. The amylose content was increased up to 35.5% in the transgenic plants obtained. RNAi as an effective means for gene silencing can regulate the metabolic pathway of maize starch and inhibit target gene expression. By inhibiting *sbe2a* gene expression, the RNAi vector can effectively improve the maize amylose content and thus produce new high-amylose maize germplasm.

## ACKNOWLEDGEMENTS

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