A Large Insertion in bHLH Transcription Factor *BrTT8* Resulting in Yellow Seed Coat in *Brassica rapa*

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Abstract

Yellow seed is a desirable quality trait of the *Brassica* oilseed species. Previously, several seed coat color genes have been mapped in the *Brassica* species, but the molecular mechanism is still unknown. In the present investigation, map-based cloning method was used to identify a seed coat color gene, located on A9 in *B. rapa*. Blast analysis with the *Arabidopsis* genome showed that there were 22 *Arabidopsis* genes in this region including *at4g09880* to *at4g10620*. Functional complementation test exhibited a phenotype reversion in the *Arabidopsis thaliana* tt8-1 mutant and yellow-seeded plant. These results suggested that the candidate gene was a homolog of TRANSPARENT TESTA8 (TT8) locus. *BrTT8* regulated the accumulation of proanthocyanidins (PAs) in the seed coat. Sequence analysis of two alleles revealed a large insertion of a new class of transposable elements, Helitron in yellow sarson. In addition, no mRNA expression of *BrTT8* was detected in the yellow-seeded line. It indicated that the natural transposon might have caused the loss in function of *BrTT8*. *BrTT8* encodes a basic/helix-loop-helix (bHLH) protein that shares a high degree of similarity with other bHLH proteins in the *Brassica*. Further expression analysis also revealed that *BrTT8* was involved in controlling the late biosynthetic genes (LBGs) of the flavonoid pathway. Our present findings provided with further studies could assist in understanding the molecular mechanism involved in seed coat color formation in *Brassica* species, which is an important oil yielding quality trait.

Introduction

*B. rapa* (AA) is an important crop and a model plant for studying *Brassica* genome evolution. Yellow seed is a desirable quality trait of the *Brassica* oilseed species. Compared with their dark-seeded counterparts, yellow seeds of *Brassica* have inherent advantages, such as higher oil content [1–3]. *Yellow sarson* (*Brassica rapa* var. *trilocularis*) is a valuable yellow-seeded variety of *B. rapa* in India. Moreover, *Yellow sarson* is a major yellow-seeded germplasm; used to create several artificial synthetic yellow-seeded varieties of *B. rapa* in India [4–5]. Presently, it has been documented that there are two genes involved in controlling the seed color in *B. rapa* [6–8]. Seed color genes have been mapped and cloned in *B. rapa* during the past decade [9–11], but the molecular mechanism of the seed color formation has not been elaborated in *B. rapa*.

Map-based cloning has been performed on several genes in the *Brassica* species [12–13], but few reports describe the cloning of seed color genes. Additionally, the physiological functions of seed color gene in the *Brassica* species are still largely unknown. However, many seed color genes from other plant species such as maize, *Arabidopsis* and rice have been successfully cloned for genetic and molecular analyses. These seed color genes mainly correspond to enzymes and regulatory factors that participate in the flavonoid biosynthesis [14–18]. The main enzymes that are involved in the flavonoid synthesis pathway include chalcone synthase (CHS), chalcone isomerase (CHI), flavonoid 3-hydroxylase (F3H), flavonoid 3’-hydroxylase (F3’H), dihydrolflavonol 4-reductase (DFR), flavonol synthase (FLS), leucoanthocyanidin reductase (LAR), leucocyanidin dioxygenase (LDOX), anthocyanidin reductase (ANR), peroxidase (POD), and polyphenol oxidase (PPO).

To date, it has been established that the transcriptional regulation of the structural genes for flavonoid biosynthesis is controlled by MYB and basic helix–loop–helix (bHLH) transcription factors, together with WD40 proteins. Recently, the bHLH transcription factors are found to be involved in the transcriptional regulation of the flavonoid pathway [19–20]. The first bHLH transcription factor regulating the flavonoid pathway is identified in maize which regulates the anthocyanin biosynthesis pathway in seed [21]. The bHLH transcription factors *Rc/Re* specifically control proanthocyanidins (PAs) synthesis in the grain pericarp in rice [22]. bHLH proteins in *Arabidopsis* such as TT8 control both anthocyanin and PA pathways [18], and is required for normal expression of the flavonoid “late” biosynthetic genes (LBGs) including *DIHYDROFLAVONOL 4-REDUCTASE* (DFR) and *BANYULS* (BAN). DFR is the first enzyme leading to the production of anthocyanidin, and the BAN encoded ANR, the
core enzyme in PA biosynthesis [23–24]. In addition, through the yeast two- and three-hybrid experiments, TT8 (bHLH), TT2 (MYB), and TTG1 (WD40) can form a ternary complex which is involved in the regulation of BAN expression, and the expression of TT8 itself is also controlled by different combinations of MYB and bHLH factors in the seed coat of Arabidopsis thaliana [25–26]. These data clearly indicate that bHLH transcription factors can regulate one or more branches of the flavonoid pathway, and the structural and functional similarities of transcription factors may slightly differ depending on its species. Using the ethyl methanesulfonate (EMS) treatment or T-DNA insertion, a series of seed coat color mutants in Arabidopsis thaliana have been produced. However, in natural mutations, seed coat color change is due to a variety of factors: for example, changes in the length of the sequences that contain the insertion/deletion mutations in flavonoid biosynthetic genes, such as the Rc in rice and TTG1 homolog in B. rapa [9,16]. Another factor is endogenous RNA interference (RNAi), which occurs in the GHS genes in soybean [27]. In addition, it is well known that the transposable elements affect the seed coat color in maize. The transposable elements participated in pigment metabolism have also been reported in other plant species [28–29].

In this study, we reported the isolation and functional characterization of the BrTT8. BrTT8 regulated the accumulation of pigment in the seed coat. The sequence analysis of two alleles showed that a transposable element could affect the seed coat color. BrTT8 encodes a protein exhibiting strong similarity with other bHLH proteins in Brassica. Additional experiments also demonstrated that BrTT8 modulated the expression of flavonoid “LBGs”. These results have provided useful information which could assist in the current understanding of the molecular mechanism of seed coat color formation in yellow-seeded Brassica crops.

Results
Phenotypic and genetic characterization of seed color in B. rapa BC5 population

The population BC5 was developed by backcrossing 3H219 (black-seeded parent) as a donor to the Yellow sarson (yellow-seeded parent). The black-seeded plant exhibited brown color seeds (Figure 1A), but the yellow-seeded individuals showed bright yellow color seeds (Figure 1D). Histological analysis of the immature seeds showed that there were three inner integument cell layers (i1, i2, i3) in the black seed coat (Figure 1C), but the PA-accumulating cell layer (i3) was completely absent in yellow seed coats, and there were some unknown fragments present (Figure 1F) when stained with Safranine O and Fast Green treatment. This shown that the absence of PAs was responsible for yellow color of the seeds.

The genetic analysis showed that the yellow seed trait was recessive, monogenic, and maternal. A total of 1183 individuals were obtained from the BC5 black-seeded individuals, of which 565 plants were yellow-seeded and 618 plants were black-seeded. This segregation of yellow and black was consistent with the expected Mendelian ratio of 1:1 ($\chi^2 = 2.28$, $\chi^2_{0.05} = 3.84$), confirming that only one seed color gene is present in the BC5 population.

Identification of candidate gene for seed coat color trait

AFLP markers were screened in the BC3 population, and the markers that tightly linked to the seed color gene were selected for sequencing. Blast analysis suggested that the molecular marker EA11MG10 shared a very high sequence similarity with the BAC KBrB072E02, which is located in the linkage Group A9 (e-171). Therefore, BAC sequences within the target region were collected from the publicly available B. rapa genomic sequences (http://www.brasicarapaplants.org/BRGP/index.jsp) and BRAD (http://brassicadb.org/brad/). Based on the BAC sequences, SSR primers were designed using the SSR finder tool. Using 22 SSR markers from the A9 BAC sequence of B. rapa (Table S1), the seed coat color gene was located on linkage group 9 corresponding with the two molecular markers bts126 and bts1 that are located at 1.3 cM and 2.7 cM, respectively (Figure 2A). In addition, we developed co-separation SSR molecular markers that linked to the seed coat color gene from the Scaffold000135 sequence on A9. Blast analysis using the Arabidopsis genome showed that the sequence was similar to a region of chromosome 4 (Figure 2B). There are 22 Arabidopsis genes in the region including atkg90620 to atkg90629. It has been well established that atkg90620 (TT8) is involved in the regulation of flavonoid biosynthesis in Arabidopsis, and t8 mutations possess transparent testa [18]. Moreover, the T8 Brassica homolog showed no recombination with the seed coat color gene. These results suggested that the candidate gene was likely a TT8 homolog.

Large insertion in the yellow-seeded line

The molecular data allowed us to design specific primers for the cloning of the full-length TT8 ortholog by PCR amplification. For this purpose, we used the primers Tu-L and Tu-R (Table S2), which were designed from the predicted homologous sequence of atkg90620 in the Scaffold000135. The amplification of the corresponding genomic sequence from the black-seeded line resulted in a 5420-bp fragment (Figure S1) that spanned the entire 3555-bp (Figure S2) putative TT8 homolog open reading frame (Figure 3A). However, using the same primers, no amplification was observed in the yellow-seeded line. The primers TL1 and TR1 (Table S2), based on the B. rapa sequences showed high homology with the first and fifth exon sequences of Arabidopsis, respectively were used to amplify a fragment of that gene in yellow-seeded. Surprisingly, a fragment was amplified that was much larger in length than anticipated (Figure 3B). It was speculated that there was a large insertion in this region.

Due to its complex secondary structure, the insertion sequence was acquired through restriction enzyme digestion and sequencing. The sequence (Figure S3) analysis showed that the inserted fragment contained the structural characteristics of a recently discovered class of transposable elements in eukaryotes, termed Helitron. The insertion was 4320 bp, starting with 5’ TC, ending with 3’ CTAG, and containing two short palindromic sequences that were possibly formed by the 17-bp hairpin that was located near its 3’ terminus (Figure 3A).

For further verification, we again designed the primer TL2 in the second exon and the primer YCR1 (Table S2) in the insertion sequence. For the three genotypes, the same fragment was amplified in the homozygous yellow-seeded line and heterozygous black-seeded line (Figure 3C1, 2), but no fragment was amplified in the homozygous black-seeded line (Figure 3C3). We determined the level of mRNA expression in the seeds of the yellow-seeded line in comparison with that of the black-seeded line, to detect the expression changes of the Brassica TT8 orthologue due to the insertion. There was no mRNA detected in the immature seeds of yellow-seeded line (Figure 3D). This indicated that the inserted fragment disturbed the normal transcription of BrTT8 in the yellow-seeded line.
BrTT8 encodes a bHLH-Domain protein

The BrTT8 gene encodes a putative bHLH protein consisting of 520 amino acids (Figure S4) with a predicted molecular weight of 59.5 kD, and a pI of 5.45 (http://www.expasy.org/tools/protparam/). The BrTT8 protein sequence contains a typical bHLH signature near the C terminus that corresponds with a putative binding domain (http://www.expasy.ch/tools/scanprosite/). The bHLH structure and most of its invariant amino acid residues are conserved, consisting of a basic region (14 amino acids) and two α-helices separated by a loop of variable length (Figure 4A).

Distance analysis of bHLH sequences was performed (Figure 4B), which suggested the existence of 4 groups. BrTT8 encodes the bHLH protein belonging to group 3, which occurs in five species of Brassicaceae: AEA03281, containing the bHLH...
BrTT8 regulated flavonoid gene expression in *B. rapa*

Expression analysis was conducted to compare the effects of *BrTT8* on flavonoid metabolism in *B. rapa* with those of *TT8* in *Arabidopsis thaliana,* both of which modify seed pigmentation pattern. The expression level of five flavonoid genes, from two group EBGs and LBGs, were analyzed during the development of seeds in yellow-seed line and black-seed line, respectively (Figure 6). Quantitative Real-time PCR (QRT-PCR) analysis revealed that the yellow-seed line contained similar amounts of mRNA as the wild-type for *BrTT6* and *BrTT7* which encoded the flavonoid 3'-hydroxylase (F3H) and flavonoid 3'-hydroxylase (F3'5'H), respectively in the flavonoid metabolism pathway, and have been classified as flavonoid EBGs. Conversely, transcripts of three flavonoid LBGs, *BrDFR, BrBAN* and *BrLDOX* (encoded leucoecyanidin dioxygenase) were statistically insignificant in the yellow-seeded line. Therefore, our results indicated that *BrTT8* is also involved in the genetic control of flavonoid late metabolism in the developing seed.

**Discussion**

**BrTT8 is essential for formation of normal seed coat color in *B. rapa***

Seed color formation is due to the accumulation of flavonoids, mainly consists of flavonols, anthocyanins, phlobaphenes, isoflavones, and proanthocyanidins [30]. The brown to dark color of the mature seeds were due to the PAs oxidations during seed desiccation that accumulate as colorless compounds in the seed coat [31–32]. At present, 20 genes involved in the PA biosynthesis pathway have been identified in the *Arabidopsis thaliana* [30]. However, only a few genes such as *Bna.BAN* and *BrTT2* involved in proanthocyanidin biosynthesis have been identified through genetic and molecular studies in *Brassica* [33–34]. *Bna.BAN* genes were located on oilseed rape genetic maps and co-localised with a potential seed color quantitative trait loci. *ProBna.BAN* was activated in proanthocyanidin-accumulating cells, namely the innermost layer of the inner integument [33].

In our study, histological analysis showed that the black color of seeds was also due to the PAs accumulation in the plant endothelium layers of immature seeds (Figure 1B). However, there was no pigment present in the seed coats of the yellow-seeded plants (Figure 1E). Compared with the black-seeded plants, there was no accumulation of red granules in the endothelium layers of yellow seed coat after Safranine O and Fast Green treatments (Figure 1E). In addition, *BrTT8* was necessary for the expression of flavonoid LBGs, such as *LDOX* and *BAN* in the developing seeds (Figure 6), similar to *TT8* in *Arabidopsis* [18,24]. These results indicated that *BrTT8* was involved in the PA biosynthesis pathway, and the mutation of the seed color in *B. rapa* was due to impairment of the PAs accumulation in the seed coat.

Furthermore, in the yellow seeds of the *B. rapa*, because of the insertion mutation of *BrTT8*, histological analysis showed that the ii layer was completely absent (Figure 1F). To our present knowledge, the mechanism of the structural change was still unknown in *B. rapa*. Recently, research has shown that epidermal cell fate, seed-coat development and flavonoid biosynthesis are linked in *Arabidopsis* [35]. Moreover, the bHLH transcription factors have multiple functions in different biosynthetic pathway in plant species [36], so we speculated that the change of cell layer

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**Figure 3. Differences in DNA and mRNA expression.** (A) The insertion location is shown in gray rectangles in the ORF of the *BrTT8*.

The black rectangles represent the exons, and TL1, TL2, TR1 and YCR1 are the primers that were developed from the corresponding exon sequences and insert sequence. The arrows are used to indicate the directions of the primers. The insertion sequences and flanking sequences and insert sequence. The black rectangles represent the exons, and TL1, TL2, TR1 and YCR1 amplified the genomic DNA from the three genotypes: (1) the homozygous yellow-seeded, (2) heterozygous black-seeded, and (3) homozygous black-seeded plants. (D) mRNA levels in the developing seed.

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**Protein synthesis in *Brassica rapa***

protein found in *Brassica rapa*, ABY59772.1, with the bHLH protein from *Brassica napus*, ADP76654.1, with the bHLH protein from *Brassica oleracea*, AEO53065.1, with the bHLH protein from *Raphanus sativus*, and NP 192720.2 (*BrTT8*), containing the *TT8* protein that is found in *Arabidopsis thaliana*. In addition, within the large family of plant bHLH-domain proteins, the four protein sequences of the *Brassica* shared the highest similarity with the *BrTT8*.
structure in yellow seed coat might be due to BrTT8 and it also influence the development of PAs accumulation cell layer in the seed coat.

A natural insertion leads to the mutation of BrTT8 in B. rapa

Yellow sarson is not only a valuable yellow-seeded variety of B. rapa but also a major yellow-seeded germplasm, used to produce several artificial yellow-seeded varieties in B. rapa [4–5]. Several seed coat color genes have been mapped, but few have been cloned by map-based cloning. Using BC3 population, a seed coat color gene was isolated and found to be located in Scaffold000135 on A9 of B. rapa which contains a homologous of TT8 gene (at4g09820). TT8 gene (at4g09820) is involved in the regulation of flavonoid biosynthesis in A. thaliana.

Interestingly, the sequence analysis of alleles revealed that the seed coat color mutation in B. rapa, similar to the tt8-3 mutation in A. thaliana, was caused through insertion in the second intron (Figure 3B and 3C). In A. thaliana, no TT8 mRNA expression was detected in siliques of tt8-3 [18]. Accordingly, we investigated the expression of BrTT8 in B. rapa and found that no mRNA expression was detected in the immature seeds of the yellow-seeded line (Figure 3D). These data demonstrated that the disturbance in the transcription was caused by the insertion of the second intron.

The sequence analysis showed that the inserted fragment was a recently discovered class of transposable elements in eukaryotes, termed Helitron. Helitrons are quite large, ranging from approximately 5 to 15 kb. They lack terminal repeats, do not duplicate host insertion sites, and insert consistently between the nucleotides A and T. The Helitron structure consists of 5′ TC and 3′ CTRR termini, and a 16- to 20-bp predicted hairpin structure located 10 to 12 bp upstream of the 3′ end [37]. The insertion was 4320 bp in our study, starting with 5′ TC, ending with 3′ CTAG, and containing two short palindromic sequences that were possibly formed by the 17-bp hairpin located near 3′ terminus (Figure 3A). This shown that the new transposable element is, in all probability, responsible for the changes in seed coat colour in B. rapa.

Although, there is no report of Helitron transposon in Brassica, and Helitrons have been found in seven additional flowering plant species [37]. Moreover, Helitrons transposable elements participated in pigment metabolism has been reported in morning glory [29]. The extensive research on its presence has been done in maize. Researchers have found that the Helitron transposon often results in exon shuffling or the duplication of gene sequences [38–39]. Moreover, Helitrons also participate in the rearrangement and duplication of genomic regions, contributing to the evolution of novel eukaryotic genomic functions [40–41].

BrTT8 is a highly conserved gene

BrTT8 in B. rapa displayed the typical features of a transcription factor with a bHLH signature at its C terminus. The bHLH domain consists of 50–60 amino acids that form two distinct segments: the basic region which contains 10–15 predominantly basic amino acids, and the helix-loop-helix region which form two predicted amphipathic a-helices that are separated by a loop of variable length [42]. The retrieved amino acid sequences were aligned with those of five other plant species in the conserved bHLH region and found that the key amino acid residues were highly conserved in the bHLH domain (Figure 4A). Moreover, the sequences of BrTT8 and other bHLH proteins in Brassica species were almost completely identical.

The coding sequence of BrTT8 from the black-seeded line of B. rapa was used to complement the Arabidopsis tt8 mutant. BrTT8 completely restored the wild-type phenotype of the tt8 mutant, suggesting that BrTT8 has a conserved function (Figure 5A). In addition, BLAST analysis showed that BrTT8 is a single-copy gene in the B. rapa genome. Thus, the analytical results demonstrated that BrTT8 was highly conserved gene in B. rapa.

Materials and Methods

Plant material

Two backcross populations, BC1 and BC2, were developed by backcrossing 3H219 (black-seeded parent) as a donor to Yellow sarson (yellow-seeded parent). Markers linked with the target gene were used to select black-seeded individuals in each generation of backcrossing. The BC1 population, containing 202 individuals,
was used to identify the amplified fragment length polymorphisms (AFLP) and simple sequence repeats (SSR) markers that are linked to the seed coat color gene. The BC₂ population, containing a total of 1183 individuals, was used for molecular mapping of the seed coat color gene.

**Markers development**

DNA was extracted using a CTAB modified method according to Lei et al. (2007) [43]. AFLP analysis was conducted as described by Liu et al. (2005) with minor modifications [44]. The two bulks along with the two parental lines were subjected to AFLP analysis. The AFLP fragments cloning and sequence analyses were performed as described by Lei et al. (2007) [43]. The AFLP sequences were analyzed using BLAST of the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/). The primers for the RT and Q-PCR tests were designed using the web-based SSR finder tool (http://www.geboc.org/index/). A local linkage map of the region on the chromosome surrounding the gene was drawn using MapDraw V2.5 [45].

**Comparison analysis and candidate gene cloning**

The sequences that contained the co-segregate SSR markers were aligned with the *A. thaliana* genome sequences from the TAIR database (http://www.arabidopsis.org/). The sequences with a threshold value of E<10⁻³⁰⁰ were regarded as homologous loci of the *A. thaliana* genome, and located on a physical map. According to the conserved co-linearity with *A. thaliana*, we developed other co-segregate markers, and finally identified the candidate gene. Based on the BAC sequence that contained the seed coat color gene, we designed the primers and cloned the gene. The comparative Ct method was used to calculate the levels of transcripts relative to black-seeded plant. (“B” in the legend is the black seed and “Y” is the yellow seed).

**Expression analysis**

Black and yellow developing seeds were harvested from 5 plants each for studying the expression analysis. We tagged individual flowers on the primary inflorescence on the first day of flower pollination and again every 10 days. Seeds were removed from the siliques at each stage, including 10_DAP, 20_DAP, and 30_DAP (DAP: days after pollination). The tissue samples were stored in liquid nitrogen, and total RNA was extracted with the highly efficient hot CTAB-LiCl synthesis [46]. 0.4 g of grated seeds was mixed with 4 ml 65°C CTAB in a 10 ml centrifuge tube, and incubated in a 65°C water bath heat treatment for 30 min. The supernatant was then collected by centrifugation after mixing with an equal volume of chloroform/isoamyl alcohol (24:1) at 4°C. After the addition of 1/4 volume of 4 M LiCl, the samples were incubated at −20°C for 2 hours. They were eluted with 1.35 mL diethyl pyrocarbonate-treated water, and then 150 μl NaAc (pH 5.2, 3 M) and 3.75 mL 70% ethanol (freezing-treated) were added. Finally, the RNA was dried and dissolved in DEPC water. The RNA extracts were converted to first-strand cDNA by using MMLV reverse transcriptase (MBI Fermentas, USA) according to the manufacturer’s instruction and the products were diluted 100-fold with sterilized ddH₂O for subsequent PCR reactions. The RT-PCR was performed in 20 μL reactions using 2 μL of diluted first-strand cDNA as the template. The PCR was conducted using the following settings: 35 cycles with 94°C for 30 sec (3 min for the first cycle), 94°C for 30 s, 58°C for 30 s, and 72°C for 40 s, followed by an extension at 72°C for 5 min, after which samples were held at 4°C. The primers for the RT and Q-PCR tests are listed in the Table S3.
Construct preparation

A pCAMBIA2300 vector [47] was digested with the EcoRI and HindIII restriction enzymes (NEB, USA). The seed coat color gene was amplified from the black-seeded lines using high fidelity polymerase (NEB, USA) with the primers Tu-L and Tu-R, and suitable restriction enzyme cleavage sites were added for use with the pC2300 vector. The PCR products from the coding sequences were treated with T4 ligase and mixed with the digested vector. Chemical transformation was used to introduce the mixture of PCR fragments and vector DNA into chemically competent E. coli DH5a (Invitrogen, USA). Positive clones were selected through PCR and the insert was confirmed by sequencing.

Microscopy

Immature seed samples were harvested after 20 days of flowering. Seeds were directly fixed in FAA (Formalin 10 ml, Acetic acid 3 ml, 50% Ethanol 87 ml) for 24 hours. After fixation, the material was dehydrated through a series of graded ethanol solutions (50, 70, 90, 100%). Then the material transferred to the graded chloroform-ethanol solutions (25, 50, 75, and 100%) for transparent processing. Finally, the material was paraffin-embedded after infiltration of graded paraffin solutions at 42°C, 56°C, 60°C each one hour later. The embedded samples were sectioned to a thickness of 8 μm using an automatic microtome (Microm HM 360, Thermo). Selected sections were stained for fast green and counterstain with safranine. The vanillin test [48] was performed by direct incubation of immature siliques samples in a freshly prepared solution of 1% (w/w) vanillin (methanol) in 6 N HCl for 30 min at room temperature.

Supporting Information

Figure S1 Genomic sequence of the BrTT8. (TIF)
Figure S2 ORF sequence of BrTT8. (TIF)
Figure S3 DNA sequence of the insertion. (TIF)
Figure S4 The amino acid sequence of BrTT8. (TIF)
Table S1 The SSR markers were developed in the study. (DOC)
Table S2 The primers were used in the study. (DOC)
Table S3 The primers were used for Q-PCR. (DOC)

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Author Contributions

Conceived and designed the experiments: JT. Performed the experiments: XL, LC MH YZ FZ. Analyzed the data: XL. Contributed reagents/materials/analysis tools: JW BY CM JS TF. Wrote the paper: XL.
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