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Full Length Article

Virus-induced Silencing *SBEIIa* and *SSIIa*, Alone or Together, Increased the Amylose and Resistant Starch Contents in Spring Wheat

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Abstract

Foods rich in amylose and resistant starch (RS) have great potential to improve human health and lower the risk of noninfectious diseases. Common wheat (*Triticum aestivum* L.) is a major staple food crop with low RS content in the grains. The content of RS, preferentially derived from amylose, may be increased by suppressing amylopectin synthesis *via* silencing the starch branching enzyme (SBE) II a or/and starch synthase (SS) II a. In this study, *SBEIIa* and *SSIIa* were silenced separately and simultaneously using a barley stripe mosaic virus-virus-induced gene silencing (BSMV-VIGS) system. Compared with grains from control BSMV:00-inoculated spikes, grains from BSMV:SBEIIa- and BSMV:SSII a-infected spikes had fewer SBEIIa and SSIIa transcripts, together with increased amylose contents (18.62 and 24.48%, respectively) and RS contents (11.61 and 16.67%, respectively). Infection with BSMV:SBEIIa-SSII a reduced SBEII a and SSII a transcript levels and increased the amylose and RS contents (32.02 and 22.33%, respectively). Thus, BSMV-VIGS is a useful tool for the rapid silencing of single or multiple starch synthase-related genes and BSMV shows great potential to study the functions of genes involved in starch biosynthesis or other processes/traits in developing grains. We showed that the *SS*II*a* gene plays an important role in the synthesis of amylose and RS and that the effects of simultaneously silencing *SBE*II*a* and *SSIIa* on starch synthesis are greater than those of single gene silencing. Our study lays the foundation for the molecular design-based breeding of high-amylose and high-RS wheat. © 2021 Friends Science Publishers

Keywords: BSMV–VIGS; Amylose; Resistant starch; Wheat

Introduction

Starch is the most consumed carbohydrate in the human diet. Although most of the starch is degraded by specific enzymes and absorbed in the small intestine, a fraction of the starch, known as resistant starch (RS), transits through the small intestine under undigested state. Upon entering the large intestine, this RS is fermented by the microflora to produce short chain fatty acids (Topping and Clifton 2001). RS exerts the same or a similar function as dietary fiber and has been recognized as a beneficial carbohydrate by the World Health Organization (Asp *et al.* 1993). Several recent studies have indicated that RS might play a protective role against numerous diseases, including colon cancer (Hylla *et al.* 1998), colorectal neoplasia (Young and Leu 2004), diabetes (Kim *et al.* 2003), obesity (Zhou *et al.* 2009), and inflammatory bowel disease (Moreau *et al.* 2003). Evidence also indicates that RS enhances the absorption of many minerals (Lopez *et al.* 2001). These results suggest that increasing RS levels in the diet may be beneficial for human health.

Mutations in genes encoding key enzymes involved in starch biosynthesis may alter the amylose and RS contents in cereal grains. For example, mutation of the amylose extender in maize increases amylose content by 80%. This high-amylose maize may be added to increase the amount of RS in wheat products (Brown 2004). In wheat grains, the amylose and RS contents are also increased by knocking out the starch branching enzyme *SBEIIa*/*SBEIIb* genes (Regina *et al.* 2006) or edited *SBEIIa* gene (Li *et al.* 2020). Amylose content is also markedly increased in transgenic wheat lines by silencing the *SBEIIa* gene (Sestili *et al.* 2010; Hazard *et al.* 2012). In contrast, silencing the granule-bound starch synthase gene reduces the starch content in wheat (Bennypaul *et al.* 2012). A mutant wheat line lacking starch synthase (SS)IIa function results in increased amylose content (approximately 35%) over the wild type line (Yamamori *et al.* 2000). In barley, the starch structure is altered by the loss of *SSIIa*, which increases the amylose content (Morell *et al.* 2003). Loss of *SSIIa* also affects the starch structure in rice (Umemoto *et al.* 2002). In maize, SSIIa deficiency alters the structure of amylopectin, which subsequently changes the physicochemical properties of starch (Zhang *et al.* 2004). Therefore, introducing gene mutations through genetic engineering may be a useful strategy for increasing the amylose and RS contents in human diets.

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Virus-induced gene silencing (VIGS) is a rapid, efficient tool for analyzing gene function in plants (Ratcliff et al. 1997; Baulcombe 1999) that is based on plant RNA interference defense mechanism against viruses. In VIGS, a fragment of a target gene cloned in a viral vector enters the host cell *via* infection with *Agrobacterium* or a virus and is converted to long double-stranded RNA (dsRNA) by the RNA-dependent RNA polymerase. In the cytoplasm, these long dsRNAs are then cleaved by the RNase III family enzyme Dicer to yield short interfering RNAs (siRNAs; 21–25 bp) with 5' phosphates and 3' dinucleotide overhangs. These siRNAs are then loaded into an RNA-induced silencing complex (RISC) that very efficiently searches the transcriptome for target sequences. When an mRNA target sequence is recognized, the RISC cleaves it. Therefore, VIGS is a type of post-transcriptional gene silencing (Baulcombe 1999; Waterhouse *et al.* 2001; Rana 2007). VIGS systems have been widely used in dicotyledonous plant species, including tomato (Cox *et al.* 2019; Bao *et al.* 2020), tobacco (Tang *et al.* 2020), and *Arabidopsis* (Calvo-Baltanás *et al.* 2020). More recently, VIGS has been used in monocotyledonous species, including wheat (Yang *et al.* 2020), barley (Gunupuru *et al.* 2019), maize (Murphree *et al.* 2020), and rice (Purkayastha *et al.* 2010). Barley stripe mosaic virus (BSMV)-based VIGS has been developed for gene silencing in wheat, and has been reported for silencing genes related to growth and development (Gunupuru *et al.* 2019; Murphree *et al.* 2020), resistance to disease and insects (Eck *et al.* 2010; Yousaf *et al.* 2013; Yang *et al.* 2020), and resistance to drought (Kuzuoglu-Ozturk *et al.* 2012). The BSMV-VIGS system has also been used to investigate the functions of genes affecting wheat grain quality, especially starch and protein biosynthesis (Ma *et al.* 2012). However, directly improving RS content in spring wheat through manipulating key genes involved in starch biosynthesis by VIGS has not been documented yet.

Wheat is an important cereal crop and one of the sources of nourishment and energy for humankind (Yan *et al.* 2018). Increasing RS content in wheat is useful for better health of the global population. We envisioned that silencing *SBEIIa* and *SSIIa* genes through VIGS might be an alternative way to modify the starch composition of wheat to increase its RS content for human health benefits. In the present study, we separately and simultaneously silenced the *SBEIIa* and *SSIIa* genes to investigate the effect of silencing single and multiple genes on the biosynthesis of amylose and RS in spring wheat through BSMV-VIGS. Our results provide fundamental information for improving RS content in wheat as well as other cereal crops for global population health benefits.

Materials and Methods

Plant materials and growing conditions

Spring wheat (*Triticum aestivum* L. cv. Xinchun 11) seedlings were grown in a greenhouse at 22–25°C and 50–70% relative humidity under a 16/8-h light/dark cycle. Wheat spikes at the heading stage were inoculated with BSMV:*PDS*, BSMV:*SBEIIa*, BSMV:*SSIIa*, or BSMV:*SBEIIa*-*SSIIa*. For each treatment, three biological replicates with ten plants per replicate were used. The contents of amylose and RS were determined after the seeds matured.

RNA isolation and cDNA synthesis

To quantify *PDS*, *SBEIIa*, or *SSIIa* transcription, inoculated spikes were pooled for each of the three biological replicates. At 12 d after flowering, the embryos were removed from the wheat grains and immediately frozen in liquid nitrogen on harvest. Total RNA was extracted using the TRIzol reagent (TaKaRa Bio, Dalian, China) according to the manufacturer's instructions and then treated with RNase-free DNase I. First-strand cDNA was synthesized using M-MLV reverse transcriptase (TaKaRa Bio).

Construction of BSMV-derived vectors

BSMV α, βΔβ, and γ plasmids were used as previously described by Holzberg *et al.* (2002). Four recombinant γ vectors, γ:*PDS*, γ:*SBEIIa*, γ:*SSIIa*, and γ:*SBEIIa*-*SSIIa*, were constructed to silence target genes (Fig. 1, 2). RT-PCR was used to amplify a 185-bp *PDS* fragment (FJ517553.1), 178-bp *SBEIIa* fragment (AF286319.1), 171-bp *SSIIa* fragment (AF155217.2), and 349-bp *SBEIIa*-*SSIIa* fragment (including a 178-bp fragment of *SBEIIa* (AF286319.1), and a 171-bp *SSIIa* fragment (AF155217.2) using the following specifically designed primers:

PDS-F: 5′-ATATTAATTAACTGGATGAAAAAGCAGGGTGTTCC-3′,
PDS-R: 5′-TTATGCGGCCGCCTACTTTCAGGAGGATTACCATCC-3′,
SBEIIa-F: 5′-ATATTAATTAAGACTTGGCAAGTCCGGCGCAACCT-3′,
SBEIIa-R: 5′-TATGCGGCCGCCGACTAGTTCCTTAACTCCTTTGG-3′,
SSIIa-F: 5′-ATATTAATTAAAGCCGCTCCAGCCCCGCATGCGTG-3′,
SSIIa-R: 5′-TATGCGGCCGCTCTGCTACGGACCAGATCGAGATC-3′.

The PCR products were digested with *PacI* and *NotI* restriction enzymes and inserted into the γ vectors. The specific primers were designed to amplify the target gene. Then the correctness and location of the genome were verified by gene sequencing.

In vitro transcription of viral RNAs and plant inoculation

The α, βΔβ, and γ plasmids and four modified γ plasmids (γ:*PDS*, γ:*SBEIIa*, γ:*SSIIa*, and γ:*SBEIIa*-*SSIIa*) were digested to generate linear plasmids using a mono-restriction endonuclease. The linear plasmids were then used for *in vitro* transcription with the mMessage mMachine T7 using an *in vitro* transcription kit (Ambion, Austin, TX, USA) according to the manufacturer's recommendations (Ma *et al.* 2012). The α, β, and γ transcription products were

mixed in equal amounts to generate BSMV:00. Similarly, the α, βΔβ, and one of the recombinant γ plasmids (γ-*PDS*, γ:*SBEIIa*, γ:*SSIIa*, and γ:*SBEIIa-SSIIa*) transcription products were mixed to generate BSMV:*PDS*, BSMV:*SBEIIa*, BSMV:*SSIIa*, and BSMV:*SBEIIa-SSIIa*, respectively. These mixed infectious viral RNAs were added to inoculation buffer to generate inoculation solutions as described by Scofield *et al.* (2005).

Wheat spikes were inoculated at heading using the spike-rub method. Briefly, a 20-μL aliquot of the inoculation solution was applied onto each spike by gently sliding three pinched fingers from the base to the tip of the spikes five times (Ma *et al.* 2012). Spikes inoculated with BSMV:00 were used as controls. After inoculation, the spikes were misted with nuclease-free water and then covered with plastic film for 1 d.

Measurement of transcript abundance by qRT-PCR

To measure the expression levels of target genes, quantitative real-time PCR (qRT-PCR) was performed using DNA Master SYBR Green I chemistry on a Roche Light-Cycler® 480 (Roche Diagnostics, Indianapolis, IN, USA). The gene-specific primers used for qRT-PCR were as follows:

Actin-RT-F: 5′-TGTGCTTGATTCTGGTGATGGTGTG-3′,
Actin-RT-R: 5′-CGATTTCCCGCTCAGCAGTTGT-3′,
PDS-RT-F: 5′-TCGAAGGGTTCTATCTGG-3′,
PDS-RT-R: 5′-CTACAACAATGTGGCAAT-3′,
SBE IIa-RT-F: 5′-GCAGAACTGCGGTCGTGT-3′,
SBE IIa -RT-R: 5′-TCCCAGTCATGGCGCTTA -3′,
SS IIa-RT-F: 5′-TGCCGCCAAGCTCTACG-3′, and
SS IIa-RT-R: 5′-CGTCCGCTCTACTCTGCTAC-3′.

The wheat actin gene (AY423548.1) was used as an internal control for normalization in the VIGS experiments. The following cycling parameters were used: 95°C for 2 min, followed by 40 cycles at 95°C for 15 s, 55°C for 30 s, and 72°C for 30 s. Relative gene expression was calculated by the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001).

Determination of amylose and resistant starch contents

Spring wheat grains contained approximately 16% amylose. Grains of BSMV:*SBEIIa*- or BSMV:*SSIIa*- or BSMV:*SBEIIa-SSIIa*-infected wheat spikes at 15 d post inoculation (dpi) were analyzed using qRT-PCR to detect whether the endogenous target genes were silenced. Target gene-silenced spikes were collected and milled to determine the amylose and resistant starch contents. Amylose content was estimated with an iodometric assay according to the method described by Chrastil (1987), and RS content was determined using the Megazyme Resistant Starch Assay kit (Megazyme Int., Wicklow, Ireland) according to AACC method.

Data analyses

The means and standard deviations were calculated using Excel 2007 (Microsoft, Redmond, WA, USA) and S.P.S.S. 19.0 (S.P.S.S., Inc., Chicago, IL, USA). Comparisons between groups were conducted using *t*-tests with a significance level of $P < 0.01$ or $P < 0.05$. Nucleic acid sequences were analyzed using DNAMAN version 5.2.2 (Lynnon Biosoft, San Ramon, CA, USA).

Results

Establishment of a BSMV-VIGS system for wheat spikes and grains

To develop an effective BSMV-VIGS system for evaluating gene function in wheat spikes and grains, a test viral vector, BSMV:*PDS*, was constructed and inoculated on 15 wheat spikes at the heading stage and its effects on photobleaching were assessed. Photobleaching was observed on 86.7% (13/15) of the BSMV:*PDS*-inoculated wheat spikes. Photobleaching first appeared at 5–6 dpi, became distinct at 15–16 dpi, and peaked at 25–27 dpi (Fig. 3A). Photobleaching was also observed on grains collected from BSMV:*PDS*-inoculated spikes at 25 dpi (Fig. 3B). No photobleaching was observed on BSMV:00-inoculated spikes.

We then measured the *PDS* transcript abundance to confirm that the photo bleaching of spikes and grains was caused by silencing of the endogenous *PDS* gene. A decrease in *PDS* transcript abundance was observed in BSMV:*PDS*-inoculated spikes at 3 dpi. The *PDS* transcript abundance was the lowest at 15 dpi, and *PDS* gene expression was suppressed until 25 dpi (Fig. 3C). Similarly, the *PDS* transcript abundance was much lower in grains from BSMV:*PDS*-inoculated spikes than in grains from BSMV:00-inoculated spikes (Fig. 3D).Collectively, these results suggest that the BSMV-VIGS vector silenced the *PDS* gene in wheat spikes and grains.

Silencing of the *SBEIIa* and *SSIIa* genes separately in wheat grains

The BSMV-VIGS system was used to evaluate the function of two genes, *SBEIIa* and *SSIIa*, involved in the biosynthesis of amylose and RS in wheat grains. To silence *SBEIIa* or *SSIIa*, two recombinant BSMV vectors, carrying either a 178-bp *SBEIIa* fragment (BSMV:*SBEIIa*) or a 171-bp *SSIIa* fragment (BSMV:*SSIIa*) were constructed. Then, ten spikes each were inoculated with either BSMV:*SBEIIa* or BSMV:*SSIIa* at heading stage. The grains in the middle of the inoculated spikes were collected at 6, 9, 12, 15, 18 and 21 dpi for RNA extraction to examine the changes in the abundance of *SBEIIa* and *SSIIa* transcripts through qRT-PCR. The *SBEIIa* transcript abundance decreased approximately 58% at 6 dpi, 84% at 12 dpi, and 88% at 21

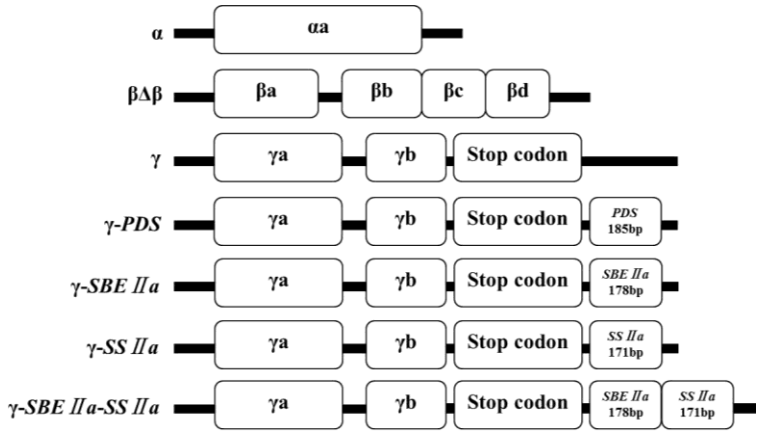

Fig. 1: Genomic organization of BSMV (α, βΔβ, and γ) and the four modified γ genomes (γ-*PDS*, γ-*SBEIIa*, γ-*SSIIa*, and γ-*SBEIIa*-*SSIIa*) used in this study. α, βΔβ, and γ are the three parts of the BSMV genome. γ-*PDS*, γ-*SBEIIa*, γ-*SSIIa*, and γ-*SBEIIa*-*SSIIa* are recombinant γ vectors carrying cloned fragments of *PDS* (185 bp), *SBEIIa* (178 bp), *SSIIa* (171 bp), and *SBEIIa*-*SSIIa* (349 bp), respectively. The *SBEIIa*-*SSIIa* fragment (349 bp) is composed of a 178-bp *SBEIIa* fragment and a 171-bp *SSIIa* fragment

Fig. 2: Gene fragment for recombinant vectors

dpi in the grains of BSMV:*SBEIIa*-infected spikes compared with the corresponding levels in BSMV:00-inoculated spikes (Fig. 4A). Similarly, the *SSIIa* transcript abundance decreased approximately 49% at 6 dpi, 92% at 12 dpi, and 92% at 21 dpi in the grains of BSMV:*SSIIa*-infected spikes compared with the corresponding levels in BSMV:00-inoculated spikes (Fig. 4B). These results indicated that the BSMV-VIGS system effectively silenced *SBEIIa* and *SSIIa* in wheat grains.

To evaluate the effect of silencing *SBE IIa* and *SS IIa* on the biosynthesis of amylose and RS, ten spikes were inoculated with either BSMV:*SBEIIa* or BSMV:*SSIIa* at heading stage. The contents of amylose and RS were determined in mature grains. The average amylose and RS contents of grains from *SBEIIa*-silenced spikes were 18.62 and 11.61% higher, respectively, than the contents in control BSMV:00-inoculated spikes (Table 1). Similarly, the amylose and RS contents in *SSIIa*-silenced spikes were 24.48 and 16.67% higher, respectively, than those in control BSMV:00-inoculated spikes (Table 1).

Co-silencing of the *SBEIIa* and *SSIIa* genes in wheat grains

After successfully silencing the *SBEIIa* and *SSIIa* genes separately, we attempted to explore the usefulness of the BSMV-VIGS system for silencing multiple target genes in wheat grains simultaneously. To co-silence *SBEIIa* and *SSIIa*, a BSMV:*SBEIIa*-*SSIIa* recombinant vector carrying a 349-bp fragment containing the 178-bp wheat *SBEIIa*

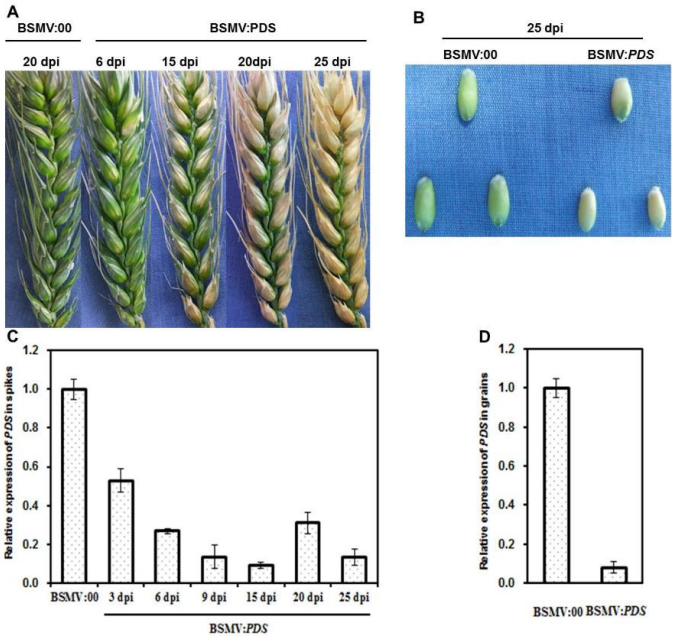

Fig. 3: Silencing of the *PDS* gene in wheat spikes and grains. **A**: The far-left spike is a control spike inoculated at heading with BSMV:00 at 20 dpi. The next four spikes show the development of photobleaching on a single spike inoculated with BSMV:*PDS* at heading. The photos were taken at 6, 15, 20, and 25 dpi. **B**: Grains were collected at 25 dpi from spikes inoculated with BSMV:00 (left) or BSMV:*PDS* (right). **C**: Relative *PDS* expression in wheat spikes inoculated with BSMV:*PDS* was detected by quantitative real-time PCR (qRT-PCR) at 3, 6, 9, 15, 20, and 25 dpi. **D**: Relative *PDS* expression in wheat grains collected at 25 dpi with BSMV:*PDS* or BSMV:00. Comparisons were made with control spikes inoculated with BSMV:00. Each column represents the mean of three samples; the error bars indicate the standard deviation

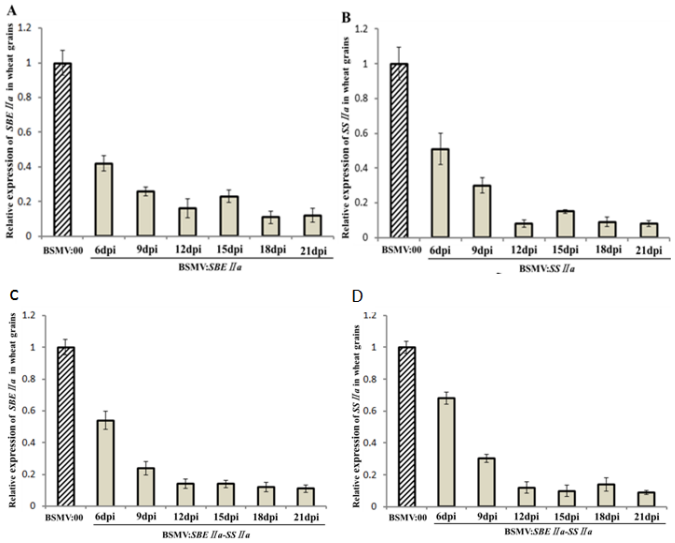

Fig. 4: Relative *SBEIIa* and *SSIIa* expression levels in wheat grains. Grains were collected from spikes inoculated with BSMV:*SBEIIa*, BSMV:*SSIIa*, and BSMV:*SBEIIa-SSIIa* at 6, 9, 12, 15, 18, and 21 dpi for RNA isolation. The relative expression levels of *SBEIIa* (A) and *SSIIa* (B) in grain samples collected from spikes inoculated with either BSMV:*SBEIIa* (**A**) and BSMV:*SSIIa* (**B**) were determined by quantitative real-time PCR (qRT-PCR). Relative expression of *SBEIIa* (**C**) and *SSIIa* (**D**) in grain samples from spikes inoculated with BSMV:*SBEIIa-SSIIa* was determined by quantitative real-time PCR (qRT-PCR)

fragment and the 171-bp wheat *SSIIa* fragment was developed. The grains in the middle of the ten BSMV:*SBEIIa-SSIIa*-inoculated wheat spikes were collected at 6, 9, 12, 15, 18 and 21 dpi for RNA isolation and qRT-PCR. The results showed that the *SBEIIa* and *SSIIa* transcripts in grains from BSMV:*SBEIIa-SSIIa*-inoculated wheat spikes were reduced approximately 46 and 32% at 6 dpi, 86 and 88% at 12 dpi, and 89 and 90% at 21 dpi,

Table 1: Average amylose and resistant starch (RS) contents in mature grains from spikes inoculated with BSMV:00, BSMV:*SBEIIa*, BSMV:*SSIIa*, and BSMV:*SBEIIa-SSIIa*

Inoculant	Amylose content (%)	Change in amylosecontent (%)	RS content (%)	Change in RS content (%)
BSMV:00	16.75 ± 0.16		2.04 ± 0.02	
BSMV:*SBEIIa*	19.87 ± 0.16**	+18.62	2.26 ± 0.02**	+11.61
BSMV:00	16.67 ± 0.28		2.04 ± 0.04	
BSMV:*SSIIa*	20.75 ± 0.23**	+24.48	2.38 ± 0.05 **	+16.67
BSMV:00	16.91 ± 0.14		2.02 ± 0.01	
BSMV:*SBEIIa- SSIIa*	22.32 ± 0.37 **	+32.02	2.47 ± 0.03 **	+22.33

Amylose content (%) and resistant starch (RS) content (%) are shown as the mean ± standard deviation. The percent change in amylose content was calculated as follows: amylose content (%) = (amylose content of BSMV:*SBEIIa*-inoculated spikes - amylose content of BSMV:00-inoculated spikes)/amylose content of BSMV:00-inoculated spikes. The formula used to calculate the change in RS content is the same as that used to calculate the change in amylose content. The average amylose and RS contents in mature grains inoculated with BSMV:*SSIIa* and BSMV:*SBEIIa* are the same as those in BSMV:*SBEIIa-SSIIa*. **Significant difference when compared to BSMV:00 at the .01 probability level

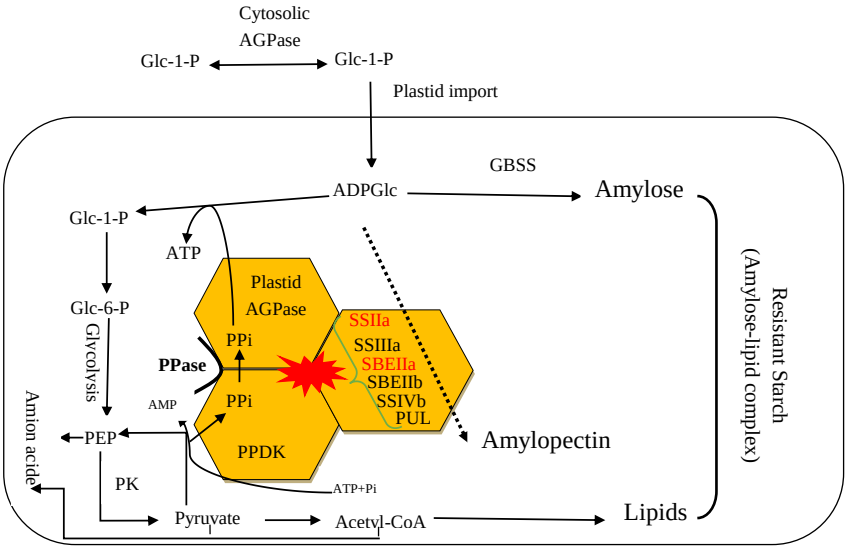

Fig. 5: A proposed starch biosynthesis and metabolic pathway in the plastid. Biosynthesis of ADPGlc is brought about primarily by cytosolic AGPase, and ADPGlc is then imported into the plastid for starch biosynthesis. Amylopectin-synthesizing enzymes, such as SSIIa, SSIIIa, SBEIIa, SBEIIb, SSIVb, and PUL can physically interact with each other to form multienzyme complexes and the complex may also contain other enzymes, such as PPDK and plastid AGPase, that are considered to function in the global regulation of carbon partitioning between starch and lipid. The amyloplast contains a high level of pyrophosphatase, which keeps the concentration of PPi in the stroma very low. PPDK could promote plastid AGPase activity by directly supplying PPi through a substrate-channeling mechanism, resulting in the smooth conversion of ADPGlc to Glc-1-P and enhancing the biosynthesis of lipids. The amylopectin biosynthetic enzymes in the complex are proposed to inhibit the activity of PPDK and AGPase. As a result, ADPGlc is more easily used as a substrate for amylose and amylopectin synthesis by GBSS and amylopectin-synthesizing enzymes, respectively. But when SSIIa and SBEIIa are defective, the interaction between PPDK/AGPase and amylopectin-synthesizing enzymes is disrupted, making AGPase free to channel more ADPGlc for the synthesis of Glc-1-P, a substrate for lipid production. At the same time, the absence of SSIIa and SBEIIa means that relatively more ADPGlc can also be consumed by the Wx protein in the biosynthesis of amylose. Mutation of SSIIa and SBEIIa also causes a defect in amylopectin biosynthesis. This process leads to an increase in amount of amylose–lipid complex or type 5 RS

respectively, compared to the corresponding levels in the grains of BSMV:00-inoculated spikes (Fig. 4C, 4D). These data were similar to those obtained in the experiments in which *SBEIIa* and *SSIIa* were silenced separately. Of the ten BSMV:*SBEIIa*-*SSIIa*-inoculated spikes, six (60%) displayed co-silencing of *SBEIIa* and *SSIIa* in wheat grains. Two of the remaining inoculated spikes (20%) only showed silencing of *SBEIIa*, and the *SSIIa* transcripts levels in these spikes were the same as those in the control BSMV:00-inoculated spikes. These results indicated that the BSMV-VIGS system was useful for co-silencing of *SBEIIa* and *SSIIa* in wheat grains.

To assess the effects of *SBEIIa* and *SSIIa* co-silencing in wheat grains on the biosynthesis of amylose and RS, ten spikes were inoculated with BSMV:*SBEIIa-SSIIa* at heading stage. Mature grains were collected from BSMV:*SBEIIa-SSIIa*-inoculated spikes to measure the amylose and RS contents. The average amylose contents were 32.03% higher, and the RS contents were 22.33% higher in grains from *SBEIIa* and *SSIIa* co-silenced spikes than in grains from control BSMV:00-inoculated spikes (Table 1). The amylose content in *SBEIIa*-silenced grains increased 18.8% and the RS content s increased 10.2% compared to the corresponding levels in the control (BSMV:00-inoculated; data not shown). These results were similar to those observed in the *SBEIIa* silencing experiments described above.

Discussion

Many traditional approaches that are used to generate mutants, including chemical methods, random mutagenesis, and T-DNA insertions, can be used to evaluate gene function. However, these approaches are technically demanding and inefficient in many plant species. In contrast, VIGS systems are highly efficient and may overcome many of the limitations of the aforementioned methods (Baulcombe 1999; Burch-Smith *et al.* 2004). Since the BSMV-VIGS system was first successfully applied for the analysis of gene function in barley (Holzberg *et al.* 2002), it has been widely used in various plants, including wheat (Yang *et al.* 2020), maize (Murphree *et al.* 2020), rice (Purkayastha *et al.* 2010), and *Haynaldia villosa* (Xing *et al.* 2018). Studies using the BSMV-VIGS system revealed that three genes, *Rar1*, *Sgt1*, and *Hsp90*, were involved in a powdery mildew resistance pathway containing Mla-13 in barley (Hein *et al.* 2005).

The BSMV-VIGS system has mainly been used in rapid functional analyses of genes in leaves, although some data on roots and flowers have been published. For example, the BSMV-VIGS system was used to explore the functional genes, powdery mildew resistance genes (Chen *et al.* 2018), yellow rust resistance genes (Yang *et al.* 2020), and aphid resistance genes (Kuzuoglu-Ozturk *et al.* 2012) in leaves. In the present study, two genes related to starch biosynthesis in wheat grains, *SBEIIa* and *SSIIa*, were separately or simultaneously silenced using the BSMV-VIGS system, which resulted in significant increases in amylose and RS contents. These findings demonstrate that the BSMV-VIGS system is a powerful tool for assessing the functions of genes in wheat grains.

In the first experiment, we used BSMV:*PDS* to silence the *PDS* gene in wheat spikes and grains. Photobleaching was observed on the wheat spikes and grains of BSMV:*PDS*-inoculated spikes. qRT-PCR revealed a reduction in *PDS* transcript levels at 3 dpi, which was 2–3 days earlier than when the photobleaching appeared on the wheat spikes. It is possible that photobleaching required a sufficient decrease in *PDS* transcripts. Differences in photobleaching on the spikes and grains were observed among the BSMV:*PDS*-inoculated plants; similar differences were detected in a previous study (Ma *et al.* 2012). The variable efficiency and unstable phenotypes of plants generated using VIGS have been previously described. These variations may not be consistent among different experiments or plants. To solve this problem, the BSMV-VIGS system may need to be optimized for each plant and tissue. In our experiments, we optimized BSMV-VIGS for wheat panicle by comparing different inoculation methods, panicle positions, inoculation times, and culture temperatures. The optimal parameters included inoculation of wheat ears by friction and incubation at 21–24°C after inoculation.

Agriculture is considered as the foundation of all food systems and primary source of all the nutrients. Malnutrition and/or disease develop if agriculture cannot supply the nutrient required for good health (Yaseen *et al.* 2018). Changes in human lifestyle and food consumption have resulted in a large increase in the incidence of type-2 diabetes, obesity, and colon disease, especially in Asia. These conditions are a threat to human health, but consumption of foods high in RS may potentially reduce their incidence. By exploiting natural and induced variation in genes of starch biosynthesis pathways, starch synthesis may be modified to increase the ratio of amylose to amylopectin and other starch properties leading to an increased proportion of resistant starch. Four types of enzymes are required for starch synthesis: starch synthases (SSs) and granule-bound starch synthases (GBSSs), which elongate glucose chains in amylopectin and amylose, respectively, starch branching enzymes (SBEs) that introduce branching points, and starch debranching enzymes (DBEs), which trim branched chains to create a structure that can crystallize to form the granule matrix (Brittany *et al.* 2020). The *SBEIIa* and *SSIIa* genes are closely related to the biosynthesis of amylose and resistant starch. Our results showed that *SBEIIa* and *SSIIa* could be silenced using the BSMV-VIGS system. Silencing of *SBEIIa* and *SSIIa* increased the amylose and RS contents in wheat grains. This result is in agreement with the findings of studies by Regina *et al.* (2015) and Sestili *et al.* (2015). However, the benefit of silencing *SSIIa* in improving amylose and RS was greater than that of silencing *SBEIIa*. We postulated that this was probably because silencing SSs would decrease amylopectin biosynthesis and result in a shift in carbon allocation toward amylose biosynthesis through GBSS, which is encoded by the Wx gene and lipid biosynthesis (Zhou *et al.* 2016).

Based on our findings in this work and published results (Ordonio and Matsuoka 2016; Zhou *et al.* 2016; Xia *et al.* 2018), an overview of the main metabolic pathways for starch biosynthesis is shown in Fig. 5. Biosynthesis of ADPGlc is brought about primarily by cytosolic AGPase, and ADPGlc is then imported into the plastid for starch biosynthesis. Amylopectin-synthesizing enzymes, such as SSIIa, SSIIIa, SBEIIa, SBEIIb, SSIVb, and PUL can physically interact with each other to form multienzyme complexes and the complex may also contain other enzymes, such as PPDK and plastid AGPase, that are considered to function in the global regulation of carbon partitioning between starch and lipid. The amyloplast contains a high level of pyrophosphatase, which keeps the concentration of PPi in the stroma very low. And PPDK could promote plastid AGPase activity by directly supplying PPi through a substrate-channeling mechanism, resulting in the smooth conversion of ADPGlc to Glc-1-P and enhancing the biosynthesis of lipids. The amylopectin biosynthetic enzymes in the complex are proposed to inhibit the activity of PPDK and AGPase. As a result, ADPGlc is more easily used as a substrate for amylose and amylopectin synthesis by GBSS and amylopectin-synthesizing enzymes, respectively. But when SSIIa and SBEIIa are defective, the

interaction between PPDK/AGPase and amylopectin-synthesizing enzymes is disrupted, making AGPase free to channel more ADPGlc for the synthesis of Glc-1-P, a substrate for lipid production. At the same time, the absence of SSIIa and SBEIIa means that relatively more ADPGlc can also be consumed by the Wx protein in the biosynthesis of amylose. Mutation of SSIIa and SBEIIa also causes a defect in amylopectin biosynthesis. This process leads to an increase in amount of amylose–lipid complex or type 5 RS. In our study the benefit of silencing *SS II*a and *SBE II* in improving amylose was greater than that of resistant starch. According to published result (Zhou *et al.* 2016), we speculated that this may be due to the low expression of Wx gene, and result in more carbon allocation toward lipid biosynthesis.

Conclusion

We showed that the BSMV-VIGS system is a powerful tool for assessing gene functions in wheat grains. We further demonstrated that the BSMV-VIGS system may be used to silence *SBEIIa* and *SSIIa* in wheat grains separately and simultaneously. The benefit of silencing *SSIIa* in improving amylose and RS was greater than that of silencing *SBEIIa*. Our results provide fundamental information for improving RS contents in wheat as well as other cereal crops for improved health benefits among the global population. In the future, high-amylose and high-RS wheat may be produced through the targeted mutagenesis of *SSIIa* by CRISPR/Cas9 or/and other breeding methods.

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Author Contributions

Zhaofeng Li performed the concepts, design, definition of intellectual content, literature search, data acquisition, data analysis, and manuscript preparation. Weihua Li and Wei Liu provided assistance for data acquisition, data analysis, and statistical analysis. Fubo Nan and Donghai Zhang reviewed the manuscript. All authors have read and approved the content of the manuscript.

Conflict of Interest

The autors declare that they have no conflict of interest.

Ethics Approval

Not applicable.

Data Availability

The data obtained in this study is available from the corresponding author upon reasonable request.

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