Effects of Inhibition of Starch Branching Enzyme on *in situ* Degradation of Endosperm Starch During Rice Seedling Growth

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Abstract

Cereal endosperm with inhibition of starch branching enzyme (SBE) increases resistant starch content and has health benefit. For plants, endosperm starch is degraded to provide energy for seedling growth. However, whether the inhibition of SBE influences *in situ* degradation of starch during seedling growth is seldom reported. In this study, a normal *japonica* rice cultivar Wu-xiang 9915 (WX) and its derived transgenic rice line (WTR) with inhibition of SBE were cultivated in the dark only in deionized H_2O. The plant growth and starch property changes were investigated during seedling development. Compared with WX, WTR showed a significantly slow plant growth. The slow degradation of starch in seed restrained the plant growth. For WX, the amylopectin and amylose were simultaneously degraded, leading to that the endosperm residual starches had similar crystalline and short-range ordered structure during seedling development. However, for WTR, the amylopectin had higher resistance to *in situ* degradation than amylose, and endosperm residual starches changed from C_A- to C_B-type and its ordered structure increased during seedling development.

Keywords: *In situ* degradation; Rice; Seedling growth; Starch; Starch branching enzyme.

1. Introduction

In cereal crops, starch mainly consists of amylose and amylopectin. The former is mainly synthesized by granule-bound starch synthase I, and the latter is mainly synthesized by soluble starch synthase, starch branching enzyme (SBE), and starch debranching enzyme [1]. Suppressing or eliminating SBE activities can change amylopectin structure, decrease amylopectin synthesis, and increase the amylose content, leading to structural and functional property changes of starch [2-5]. Many studies report that cereal endosperm with inhibition of SBE increases resistant starch (RS), which cannot be digested in the upper gastrointestinal tract but functions as a substrate for bacterial fermentation in the large intestine [3, 5-7]. Foods with high RS have health benefits [6, 7]. Therefore, many high-RS cereal crops have been cultivated via inhibition of SBE or mutation of SBE genes [2-5].

For plants, endosperm starch provides energy and nutrition for grain germination and seedling growth [8-10]. Endosperm starch in maize *sbe I* mutant is resistant to amylase hydrolysis during seedling development, leading to the inhibition of plant growth [10]. A high-RS rice line derived from *indica* rice cultivar Te-qing with inhibition of SBEI/IIb has about 60% amylose and 40% amylopectin in endosperm starch [5, 11]. The amylopectin has low branching degree and long branch-chains, and exhibits C-type crystallinity, a mixture of A- and B-type crystallinities [11]. The long branch-chains and B-type crystallinity of amylopectin resist *in situ* degradation of endosperm starch during seedling development and inhibit plant growth [8]. However, a rice mutant RS4, which has about 10% RS content in the milled cooked rice, exhibits similar starch degradation to its wild type rice during seedling growth, indicating that RS has no negative impact on *in situ* degradation of endosperm starch, although RS cannot be hydrolyzed by α-amylase from human and animal *in vitro* [12]. Therefore, more studies are needed to investigate the effects of inhibition of SBE on *in situ* degradation of endosperm starch during seedling growth.

A transgenic rice line (WTR) derived from a *japonica* rice cultivar Wu-xiang (WX) has been cultivated through antisense RNA inhibition of both SBEI and SBEIIb [13]. In WTR, amylose content increases from about 16% to 24% [14]. In this study, the plant growth and starch property changes of WX and WTR were investigated during seedling development. Our objective was to reveal the effects of inhibition of SBE on *in situ* degradation of endosperm starch during seedling growth.

2. Materials and Methods

2.1. Plant Materials

The normal *japonica* rice cultivar Wu-xiang 9915 (WX) and its derived transgenic rice line WTR were used in this study. The WTR was cultivated by the antisense RNA inhibition of both SBEI and SBEIIb [13]. Protein blotting analysis showed that both SBEI and SBEIIb proteins were nearly completely inhibited in WTR. Rice materials were

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planted in the transgenic closed experimental field of Yangzhou University, Yangzhou, China. Mature grains were harvested.

2.2. Rice Grain Germination and Seedling Growth

Rice grains were imbibed in deionized H_2O in the dark at 28 ºC for 2 d. The germinated grains continued to be cultivated in the well of 96-well plate with embryo up and the lower two-third of grain immersed in H_2O. During grain germination and seedling growth, the H_2O was changed every day. The seedlings were taken out at 1, 4, 8, 12 and 16 days after imbibition (DAI), and the grains at 1 DAI were used as a control.

2.3. Measurement of Seedling Weight and Endosperm Starch Content

The seedlings were separated carefully from seeds and dried in oven at 110 °C for 3 h and 80 °C for 2 d. Their dry weight was measured. The whole seed without embryo was freeze-dried. The seeds were ground extensively into flour and filtered a 100-mesh sieve. The starch content in flour was measured using Megazyme Total Starch Assay Kit (K-TSTA), and then converted to the starch content in seed.

2.4. Isolation of Endosperm Starch

Residual starch in endosperm was isolated from seeds following the method of Pan, *et al.* [8]. Briefly, the seeds without embryos were separated carefully grains, and ground extensively in a mortar and pestle with water. The starch water slurry was filtered through 100-, 200-, and 400-mesh sieves, successively, and centrifuged at 5 000 g for 10 min. The starch precipitate was washed 3 times with deionized H_2O and 2 times with absolute ethanol, and dried at 40 °C.

2.5. Determination of Iodine Absorption Spectrum of Starch

The iodine absorption spectrum of starch was determined following the method of Lin, *et al.* [15]. Briefly, starch was dissolved in urea dimethyl sulfoxide solution and stained with iodine solution. The sample was scanned from 400 to 900 nm with a spectrophotometer.

2.6. Crystalline Structure Analysis of Starch

The crystalline structure of starch was analyzed using an X-ray powder diffractometer (D8, Bruker) as previously described [8]. The sample was exposed to the X-ray beam at 40 kV and 40 mA, and scanned from 3 to 40º 2θ with a step size of 0.02º.

2.7. Ordered Structure Analysis of Starch

The ordered structure of starch was analyzed using a Varian 7000 Fourier transform infrared (FTIR) spectrometer equipped with an attenuated total reflectance (ATR) single reflectance cell following the method of Pan, *et al.* [8].

2.8. Statistical Analysis

The data reported in all Tables were mean values and standard deviations. Analysis of variance (ANOVA) by Tukey's test was evaluated using the SPSS 16.0 Statistical Software Program.

3. Results and Discussion

3.1. Seedling Growth

In order to investigate the influence of starch degradation on the seedling growth, the grains were cultivated in the dark and in deionized H_2O. The Figure 1 shows the grain germination and seedling growth. The grain germination began from 2 DAI, and was slightly slower in WTR than in WX. The seedling growth was slower in WTR than in WX from 4 DAI. The dry weight of 30 seedling plants without seeds were quantitatively measured (Figure 2). The seedling weight was significantly lower in WTR than in WX, indicating that the WTR endosperm starch were more resistant to *in situ* degradation and provided less energy and nutrient substance for plant growth than did the WX starch. The present results agreed with previous reports that the inhibition of SBE in endosperm can restrain the seedling growth [8, 9].

3.2. Starch Content in Seed During Seedling Growth

The starch content in 30 seeds was determined, and the result is presented in Figure 3. The starch content in WX endosperm reduced rapidly from 1 DAI to 12 DAI. Though the starch content in WTR was markedly lower than that in WX at 1 DAI, the starch was degraded more slowly in WTR than in WX, leading to that the starch residual in endosperm was higher in WTR than in WX at 12 DAI. It was noteworthy that starch in endosperm was degraded very slowly after 12 DAI, indicating that the endosperm residual starch in WTR was highly resistant to hydrolysis. The above results showed that the starch in endosperm with inhibition of SBE had high resistance to *in situ* degradation during seedling growth. Similar phenomenon has been reported in *indica* rice with inhibition of SBEI/IIb [8].

3.3. Relationship between Starch Degradation and Seedling Growth

When rice grain was cultivated in the dark only in deionized H_2O, the only opportunity of germinating plantlet to obtain energy is through respiration of storage compounds in seed [9]. Starch is the main storage material. The relationship between the decreased starch weight and the seedling weight was investigated in this study (Figure 4). The decreased starch weight was significantly positively related with the seedling weight during seedling growth. The correlation coefficients were 0.980 and 0.989 for WX and WTR, respectively, indicating that the slow growth of WTR seedling resulted from the slow degradation of endosperm starch. The present results agreed with the reports of Shaik, *et al.* [9] and Pan, *et al.* [8].

3.4. Iodine Absorption Spectrum of Endosperm Residual Starch

The iodine absorption spectrum of endosperm residual starch is showed in Figure 5, and its derived parameters including maximum absorption wavelength (λ_{max}), optical density 620 nm/550 nm ratio (OD 620/550), and blue value (BV, absorbance at 680 nm), are presented in Table 1. The λ_{max} is related to the polymerization degree and average chain length of amylose and amylopectin, the OD 620/550 can indicate the relative content of longer chain segments in starch, and the BV reflects the iodine affinity of starch [15]. For WX, the λ_{max}, OD 620/550, and BV of endosperm starch had no significant difference between 1 and 4 DAI, indicating that the amylose and amylopectin in starch were simultaneously degraded. However, for WTR, the λ_{max} first slightly increased from 593.0 nm at 1 DAI to 597.5 nm at 4 DAI, and then decreased gradually to 564.3 nm at 12 DAI; the OD 620/550 first slightly increased from 1.094 at 1 DAI to 1.121 at 4 DAI, and then decreased gradually to 0.851 at 12 DAI; the BV first increased from 0.295 at 1 DAI to 0.325 at 4 DAI, and then decreased gradually to 0.246 at 12 DAI. Shaik et al. [9] measured the OD 620/550 to assess the relative contents of amylose and amylopectin during barley seedling growth. For wild type barley, the OD 620/550 was constant during seedling growth, but for AO line with inhibition of SBEs there was a decline in this ratio. Their results indicate that both amylose and amylopectin are simultaneously degraded in wild type barley but amylose was preferably degraded compared to amylopectin in AO line. In the present study, the changes in the λ_{max}, OD 620/550, and BV of endosperm residual starches during WTR seedling growth showed that amylopectin was more resistant to *in situ* degradation than amylose.

3.5. Crystalline Structure of Endosperm Residual Starch

The X-ray diffraction (XRD) patterns of endosperm residual starches are shown in Figure 6. Starches can be divided into A-, B- and C-types according to their XRD patterns. A-type starch has strong diffraction peaks at about 15° and 23° 2θ and an unresolved doublet at around 17° and 18° 2θ. B-type starch has strong diffraction peak at 17° 2θ, a few small peaks at around 15°, 22° and 24° 2θ, and a characteristic peak at about 5.6° 2θ. However, C-type starch contains A- and B-type crystallinities, and shows a mixed spectrum of A- and B-type starch. According to the proportion of A- and B-type crystallinity, the C-type starch can further divided into C_A- (close to A-type), C_C- (typical C-type) and C_B-type (close to B-type). The C_C-type starch has strong diffraction peaks at about 17° and 23° 2θ and a few small peaks at around 5.6° and 15° 2θ. Compared with C_C-type starch, the C_A-type starch has one shoulder peak at 18° 2θ, and the C_B-type starch has two shoulder peaks at 22° and 24° 2θ [16, 17]. In the present study, the starches from WX endosperm showed A-type XRD patterns during seedling growth. However, the WTR endosperm at 1 DAI had C_A-type starch with obvious shoulder peak at 18° 2θ, and this shoulder peak vanished and the peak at 23° 2θ became wide at 8 DAI, exhibiting a C_C-type starch. At 12 and 16 DAI, endosperm starch had C_B-type spectrum with two shoulder peaks at 22° and 24° 2θ. The above spectrum changes of WTR endosperm residual starches indicated that A-type crystallinity was preferentially degraded or degraded faster than B-type crystallinity, leading to starch change from C_A-type to C_B-type. Similar results have been reported in *indica* rice with inhibition of SBEI/IIb [8].

3.6. Ordered Structure of Endosperm Residual Starch

The short-range ordered structure of starch can be detected using ATR-FTIR, and the spectra of endosperm residual starches during seedling growth are shown in Figure 7. For infrared spectrum, the band at 1045 cm^{-1} is linked with order/crystalline regions in starch, the band at 1022 cm^{-1} is sensitive to amorphous structure, and the band at 995 cm^{-1} results from bonding in hydrated carbohydrate helices [18]. For WX, the FTIR spectrum did not significantly change during seedling growth. Whereas for WTR, the FTIR spectrum was similar except that the 1022 cm^{-1} absorbance band vanished after 8 DAI. The results showed that the ordered and amorphous structures were simultaneously degraded in WX endosperm starch, but the amorphous structure was degraded faster than the crystalline structure in WTR endosperm starch, leading to the increase of ordered structure. The present study indicated that the amylopectin, not the amylose, was resistant to *in situ* degradation in WTR. Similar phenomenon has been reported in *indica* rice with inhibition of SBEI/IIb [8].

4. Conclusion

Compared with WX, WTR showed a significantly slow seedling growth. The slow degradation of endosperm starch in WTR restrained the seedling growth. During seedling growth, the amylopectin and amylose in WX starch were simultaneously degraded, and endosperm residual starches had the same A-type crystallinity and similar ordered structure. However, for WTR, the amylopectin was more resistant to *in situ* degradation than the amylose, and the crystallinity of endosperm residual starch changed from C_A-type to C_B-type and the ordered structure

increased. Therefore, the B-type crystallinity of amylopectin in cereal endosperm with inhibition of SBE had high resistance to *in situ* degradation and restrained seedling growth.

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Table-1. Iodine absorption parameters of endosperm residual starches during seedling growth

	WX			WTR		
	λ_{max} (nm)	OD 620/550	BV (OD680)	λ_{max} (nm)	OD 620/550	BV (OD680)
1 DAI	599.8±3.2a	1.135±0.035a	0.236±0.018a	593.0±1.4c	1.094±0.014c	0.295±0.011b
4 DAI	604.5±4.9a	1.166±0.027a	0.253±0.013a	597.5±0.0d	1.121±0.002d	0.325±0.006c
8 DAI	–	–	–	575.0±0.7b	0.938±0.003b	0.296±0.002b
12 DAI	–	–	–	564.3±0.4a	0.851±0.001a	0.246±0.001a
16 DAI	–	–	–	563.0±0.0a	0.834±0.016a	0.217±0.013a

Data are means ± standard deviations from 3 replicates. Values in the same column with different letters are significantly different ($p < 0.05$).
– Data are not detected.

Figure-1. Photographs of rice seedlings
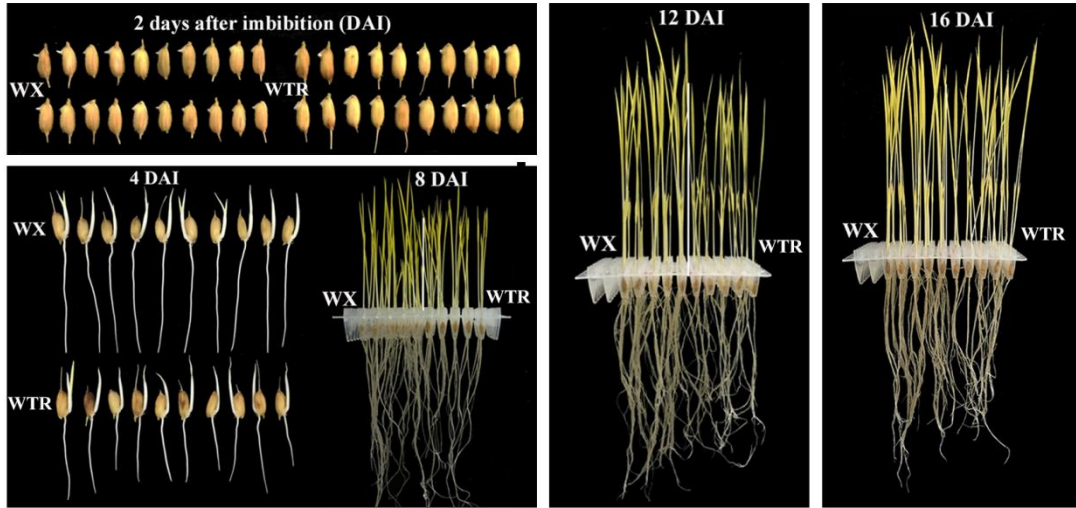

Figure-2. Dry weight of seedling without seed. Values are means ± standard deviations from 3 replicates. Asterisks (*) highlight significant difference between WX and WTR by Student's test (** $P < 0.01$)
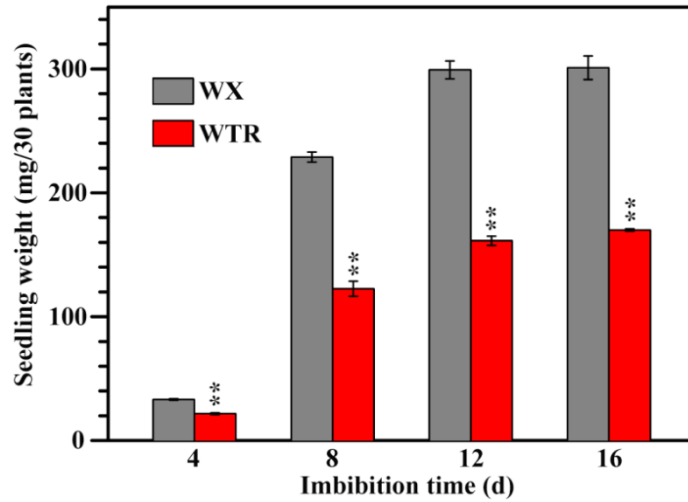

Figure-3. Dry weight of starch in seed. Values are means ± standard deviations from 3 replicates. Asterisks (*) highlight significant difference between WX and WTR by Student's test (*** $P < 0.001$)
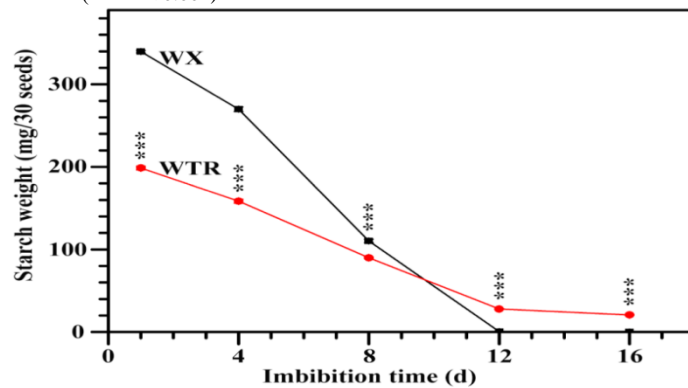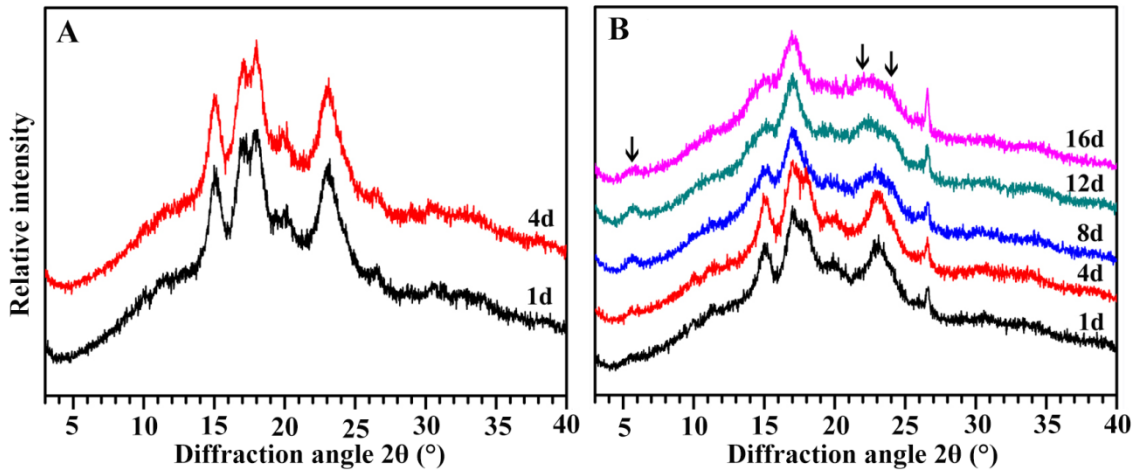
Figure-4. The relationship between decreased starch weight and seedling weight. The "R" value indicates the regression coefficient.
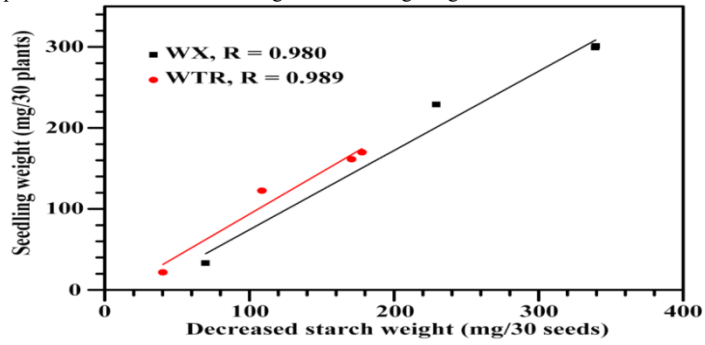
Figure-5. Iodine absorbance spectra of endosperm residual starches during seedling growth. (A) WX, (B) WTR
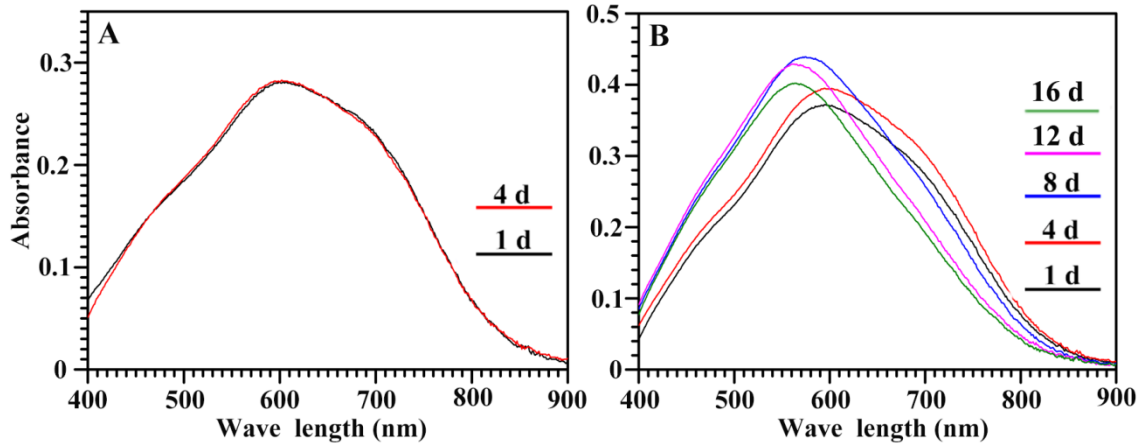
Figure-6. XRD patterns of endosperm residual starches during seedling growth. (A) WX, (B) WTR

Figure-7. ATR-FTIR spectra of endosperm residual starches during seedling growth. (A) WX, (B) WTR