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Morphological, Molecular Structural and Physicochemical Characterization of Starch Granules Formed in Endosperm of Rice with Ectopic Overexpression of α-Amylase

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Abstract: The objective of this study was to characterize the endosperm starch in rice that ectopically overexpressed the α-amylase. Transgenic rice plants, transformed with cauliflower mosaic virus 35S promoter driven AmyI-1 (35S::AmyI-1) and AmyII-4 (35S::AmyII-4), and 10 kDa prolamin promoter driven AmyI-1 (P10::AmyI-1), were cultivated under standard conditions (23 °C, 12 h in the dark/ 26 °C, 12 h in the light), and brown grains were subsequently harvested. Each grain displayed characteristic chalkiness, while electron microanalyzer (EPMA)-SEM images disclosed numerous small pits on the surface of the starch granules, attributable to α-amylase activity. Fluorescence labeling and capillary electrophoresis analysis of starch chain length distribution revealed no significant alterations in the starches of 35S::AmyI-1 and 35S::AmyII-4 transgenic rice compared to the wild-type. Conversely, the extremely short α-glucan chains (DP 2–8) exhibited a dramatic increase in the P10::AmyI-1 starch. Rapid visco-analyzer analysis also identified variations in the chain length distribution of P10::AmyI-1 starch, manifesting as changes in viscosity. Moreover, ^{1}H-NMR analysis uncovered dynamic modifications in the molecular structure of starch in rice grain transformed with P10::AmyI-1, which was found to possess unprecedented structural characteristics.

Key words: chain length distribution, chalky grain, ^{1}H-NMR, pasting property, rice endosperm, starch granule

INTRODUCTION

Starch, an α-polyglucan, is typically found in water-insoluble, granular forms within plant cells. In higher plants, starch metabolism is facilitated by a suite of enzymes localized in plastids, such as chloroplasts and amyloplasts. Transiently formed starch granules in chloroplasts of photosynthetic tissues are termed assimilated starch, whereas those formed in amyloplasts of storage tissues, such as seeds and rhizomes, are denominated storage starch, which boasts a highly regular, semi-crystalline structure [1,2].

The principal components of starch, amylopectin, and amylose, endow the starch with varied physical properties,

dependent upon the amylose to amylopectin ratio, chain length, and additional microstructural distinctions. Moreover, it is widely acknowledged that the physical properties of starch can differ among species, varieties, and tissues, with these disparities stemming from variances in the activity and function of enzymes implicated in starch synthesis. Amylose is synthesized by starch granule-bound starch synthase (GBSS), while amylopectin synthesis involves the coordinated action of soluble starch synthase (SS), starch branching enzyme (BE), and starch debranching enzyme (DBE) [3]. Specifically, in rice endosperm, BE I initiates branching at the cluster base, SS IIIa primarily elongates the chain, BEIIb subsequently forms internal branches, and SSI and SSIIa participate in chain elongation. DBE aids in forming the cluster structure by trimming extraneous branches and orchestrating their localization [4].

The impact of starch synthesis-related enzyme functionalities on starch structure has been rigorously investigated using various mutants. For example, GBSS-I-deficient mutant (*wx*) generates starch granules devoid of amylose, BEIIb-deficient mutant (*ae*) synthesizes amylopectin with fewer branches and lengthier chain lengths [5], and ISA1-deficient mutant (*sugary-1*) accumulates phytoglycogen [6]. Additionally, changes in starch structure, consequential to the pyramiding of SSI, SSIIa, SSIIIa, SSIVb, BEI, and PUL deficient mutants, and mutations have been meticulous-

ly analyzed [7].

α-Amylase, a ubiquitous enzyme found in both prokaryotes and eukaryotes, facilitates the hydrolysis of α-1,4-glycosidic bonds within glucan polymers, such as starch. Cereal α-amylase exhibit wide polymorphism encoded by multiple genes. In rice, at least 17 α-amylase proteoforms have been identified in tissues and in calli derived from the embryo by isoelectric focusing and polyacrylamide gel electrophoretic techniques [8,9]. Rice α-amylase genes are phylogenetically classified into the *Amy1*, *Amy2*, and *Amy3* subfamilies [10]. The relationship between genes and enzyme isoforms has been partially determined by protein sequencing, mass spectrometry, and serological epitope properties [8,9]. The major isoforms AmyI-1 and AmyII-4 correspond to *Amy1A* and *Amy3D*, respectively [11]. This enzyme is recognized for its ability to directly degrade raw, non-heat-treated starch. Specifically, cereal α-amylase plays a pivotal role in the degradation and utilization of stored starch during seed germination. Its indispensable physiological role in raw starch degradation is well-acknowledged. Conversely, the primary locus for starch metabolism in plants resides in plastids, including chloroplasts and amyloplasts. There exists a debate regarding the capacity of cereal α-amylase—a secretory enzyme—to access starch granules within living cell plastids and contribute to their degradation. Nonetheless, several experimental findings suggest the involvement of α-amylases, specifically those containing the secretory glycoprotein AmyI-1, in plastidial starch degradation: (1) Immunoelectron microscopy employing specific antibodies and the analysis of green fluorescent protein (GFP)-labeled AmyI-1 dynamics demonstrated AmyI-1 targeting from the endoplasmic reticulum-Golgi apparatus system to chloroplasts [12]. (2) Analysis with α-amylase overexpressors and suppressors revealed that cellular starch content fluctuates based on the degree of α-amylase expression [13,14].

It has been shown that increased α-amylase expression during the ripening stage is involved in affecting rice grain quality such as chalkiness [15,16]. High-temperature ripening can cause an imbalance in starch synthesis and degradation in the developing endosperm, leading to abnormalities in starch accumulation and granule formation, and could be considered the main cause of chalkiness formation [17,18]. In this investigation, we examined the granule and molecular structures of endosperm-stored starch in rice plants that exhibited ectopic overexpression of α-amylase, leading to the production of starch with novel structural properties. We employed the cauliflower mosaic virus 35S promoter (35S) and the 10kDa prolamin promoter (P10) as overexpressors. The 35S promoter has been widely used in driving constitutive expression of transgenes, elucidating the function of many plant genes and understanding plant processes [19], and the P10 promoter was reported as a useful tool for rice developing seed specific expressing of transgenes [20,21]. Previous investigations have revealed that the grain chalking of rice was occurred with the overexpression of 35S::AmyI-1[13], 35::AmyII-4 [13], and P10::AmyI-1 [22].

MATERIALS AND METHODS

Plant materials. The wild-type rice cultivar (*Oryza sativa* L. cv. Nipponbare) utilized in this research was procured from Shiga Prefecture Agricultural Technology Promotion Center. The wild-type and the previously established transgenic rice plants transformed with pTN1-35S-AmyI-1 [13], pTN1-35S-AmyII-4 [13], pZH2B [22] or pZH2B-Pro10-Amy1A (AmyI-1) [22] were cultivated and harvested in artificial soil (Kumiai gousei baido 3-gou, Honen Agri Co., Ltd., Nagaoka, Japan) at 26 °C/23 °C (12 h, 20,000 lux light/12 h, dark) under 70 % humidity in a growth chamber.

Scanning electron microscopic imaging. Brown rice grain was cleaved with a razor blade. The exposed surface was then coated with a palladium gold alloy using an ion sputtering device (IC-50; Shimadzu, Kyoto, Japan), and subsequently assessed for ultrastructure with an electron probe microanalyzer (EPMA1601; Shimadzu) fitted with scanning electron microscopy observation capabilities. Observation conditions were as follows: acceleration voltage, 12 kV; magnification, 100 and 5,000 [23].

Measurement of chain-length distribution of starch. The methodology for ascertaining the chain-length distribution of starch was consistent with the procedure outlined by Fujita *et al*. [24] Starchy endosperm (5 mg) was pulverized using a mortar and pestle. The resultant powder was heated with 1.5 mL of methanol for 10 min, followed by centrifugation at $3,000 \times G$ for 5 min at room temperature. The sediment was reconstituted with 1.5 mL of 90 % (v/v) methanol and centrifuged under the same conditions. After discarding the methanol, the sediment was rehydrated with 143 μL of distilled water (18 M Ω cm), combined with 7.5 μL of 5 M NaOH, and boiled for 5 min. The α-glucan sample was desalted and subjected to hydrolysis with isoamylase (0.03 U/mg of α-glucans in 40 mM acetate buffer, pH 4.4) at 37 °C for 12 h. The chain-length distributions of α-glucans were assessed using the fluorescence capillary electrophoresis (FCEP) method of Morell *et al*. [25] in a P/ACE MDQ Carbohydrate System (Beckman Coulters, CA, USA).

Evaluation of alkali solubility. Rice grains, milled to a 90 % consistency, were submerged in 5 mL of 2.0 % (0.36 M) KOH solution, correlating to 100 mg of grains, in a cell culture dish (35ϕ × 10 mm). After 27 h at room temperature of 25 °C, the solution was neutralized and mixed with 1 M HCl to pH 5. One mL of neutralized solution and 25 μL of 0.01 M iodine solution were mixed and the absorbance at 620 nm was measured.

Measurement of pasting properties of rice endosperm starch. The pasting characteristics of rice endosperm starch were determined using a rapid-visco-analyser (RVA, model Super 4; New-port Scientific Pty Ltd., Warriewood, Australia) based on a previously described heating and cooling cycle [26, 27]. Parameters such as pasting temperature, maximum viscosity (Max vis), minimum viscosity (Mini vis), final viscosity (Final vis), breakdown, setback (SB), Consistency (Cons), Max/Mini, Max/Fin, and SB/Cons, were determined. Max vis represents peak viscosity during starch gelatinization, Mini vis reflects the lowest viscosity resulting from the disruption of starch granules due to agitation, while Final vis measures the viscosity resurgence owing to the retrograded starch granules upon temperature reduction. Starch having a high Max/Mini ratio exhibits a soft and sticky texture, while starch with a pronounced Max/

Fin ratio appears sticky and displays resistance to retrogradation. SB denotes the viscosity discrepancy between the final and peak measurements, which represents the difference between retrogradation and gelatinization. Similarly, Cons depicts the viscosity variance between the final and minimal measurements, illustrating the degree of retrogradation in starch granules. A high SB/Cons ratio in rice starch indicates its propensity to retrograde easily.

NMR analysis. Approximately 1 g of rice endosperm was accurately weighed using a precision scale. The powdered sample was suspended in 4 mL of DMSO-d6 (99.9 atom % D, contains 0.03 % (v/v) TMS) solvent in a 15 mL conical tube. It was then sonicated at 40 kHz for 10 s within a water bath at room temperature and subsequently cooled in an ice bath for another 10 s. This sonication and cooling process was repeated tenfold. Afterward, the starch suspension underwent centrifugation at $10,000 \times G$ for 1 min, and the clear supernatant was transferred into 5 mm diameter Wilmad® NMR sample tubes (Merck KGaA, Darmstadt, Germany). These tubes were sealed securely and shielded with Parafilm to minimize sample evaporation and contamination risks. The dry weight of the precipitate after centrifugation and the weight of the sample powder before addition of solvent were determined on a precision balance (accuracy of 0.001 mg): the dissolved mass of WT, P10::AmyI-1, 35S::AmyI-1 and 35S::AmyII-4 were 0.45, 0.74, 0.52 and 0.55 mg, respectively. ^{1}H-NMR data for starch structures

were acquired on a Bruker Avance 700 MHz at 20 °C, and the data were processed with Mestre Nova software.

RESULTS AND DISCUSSION

Morphological characteristics of storage starch granules in α-amylase-overexpressed rice.

The wild type (WT) of Nipponbare, along with transgenic plants transformed with P10::AmyI-1, 35S::AmyI-1, 35S::AmyII-4, and pZH2B empty vector, were cultivated at 26 °C/23 °C (12 h, light/12 h, dark) under 70 % humidity in growth chamber. The appearance of rice grains harvested from transgenics overexpressing α-amylase exhibited significant alterations, with the brown grains displaying a pronounced chalky phenotype (Fig. 1). Table 1 presents the dimensions and 1,000-kernel weight of the brown rice grains harvested. Compared to the grains of Nipponbare WT (21.75 g) and vector control (20.09 g), the 1,000-kernel weight of transgenic grains such as P10::AmyI-1 (18.88 g), 35S::AmyI-1 (18.81 g), and 35S::AmyII-4 (15.41 g) decreased. As anticipated, the grain weights of transgenics with α-amylase overexpression were apparently smaller than the WT grains. The volumes (length $\times$ width $\times$ thickness) of P10::AmyI-1, 35S::AmyI-1, 35S::AmyII-4, Nipponbare WT and vector control were estimated to be 25.5, 28.6, 22.6, 28.9, and 27.4 mm^3, respectively. Thus, the grain size (volume) often varied with overexpression of α-amylase. Several studies have

Vector Control
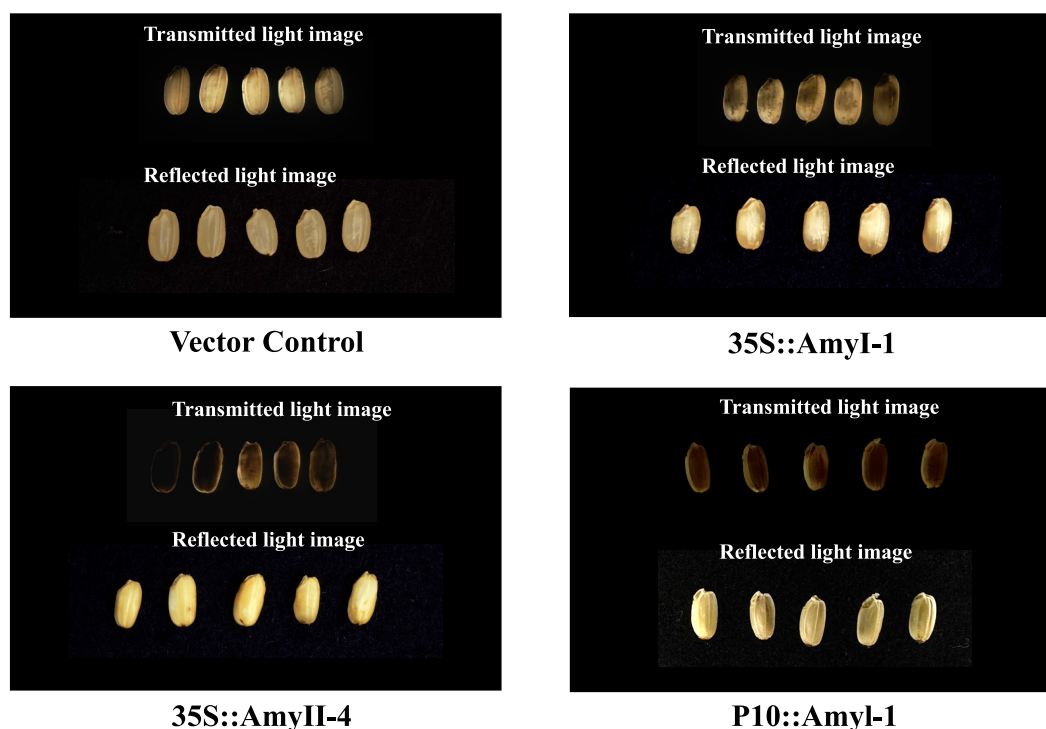
35S::AmyI-1

35S::AmyII-4

P10::AmyI-1

Fig. 1. Appearance phenotype of brown rice grain harvested in transgenic plants transformed with P10::AmyI-1, 35S::AmyI-1, 35S::AmyII-4, and pZH2B empty vector.
In transmitted light images, chalky areas are observed as shaded.

Table 1. One-thousand-kernel weight, length, width and thickness of brown rice of Nipponbare WT and transgenics.

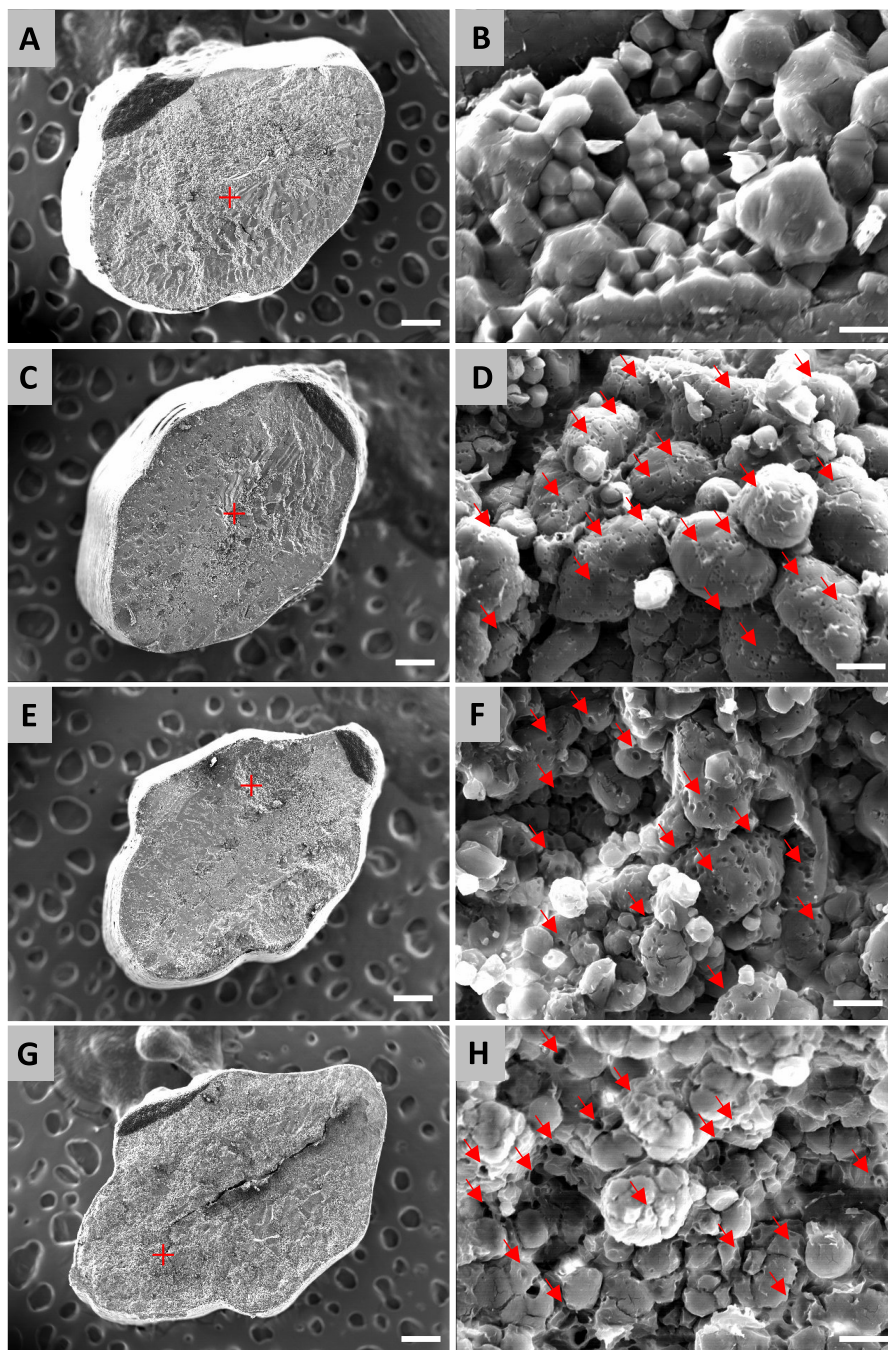|	WT	Vector control	P10::AmyI-1	35S::AmyI-1	35::AmyII-4
Weight (mg)	21.75[a] ± 0.11	20.09[b] ± 0.19	18.88[c] ± 0.16	18.81[d] ± 0.17	15.41[e] ± 0.19
Length (mm)	4.86[c] ± 0.18	5.18[a] ± 0.12	4.90[d] ± 0.17	5.00[b] ± 0.26	4.89[e] ± 0.21
Width (mm)	2.96[a] ± 0.08	2.77[c] ± 0.09	2.75[d] ± 0.18	2.89[b] ± 0.17	2.58[e] ± 0.25
Thickness (mm)	2.01[a] ± 0.03	1.91[c] ± 0.08	1.89[d] ± 0.17	1.98[b] ± 0.11	1.79[e] ± 0.09

$n = 10$; [a,b,c,d] $p < 0.05$.

Fig. 2. Scanning electron microscopic images of starch granules in brown rice with or without α-amylase overexpression.

Brown rice grains harvested in transgenic plants transformed with 35S::AmyI-1 (C,D), 35S::AmyII-4 (E,F), P10::AmyI-1 (G,H), and pZH2B empty vector (A,B) were subjected to EPMA. Arrows represent small pits and/or voids. Bars in A, C, E, and G: 200 μm; bars in B, D, F, and H: 5 μm.

shown that strengthening the action of α-amylase causes rice grain chalking [13,22,28]. Consequently, the atypically high expression of α-amylase in developing endosperm undoubtedly contributes to brown rice chalkiness, regardless of the grain size (volume).

Morphological structures of starch granules in transgenic rice grains cultivated under standard temperature conditions were examined using secondary electron imaging via EPMA (Fig. 2). Rice plants with the empty vector produced grains with typical starch granules tightly packed within endosperm cells, displaying a polygonal shape with sharp edges (Figs. 2A, B). In contrast, grains from transgenics 35S::AmyI-1 and 35S::AmyII-4 presented round starch granules exhibit-

ing multiple small pits (Figs. 2 C−F). The enzymatic degradation of starch by α-amylase is integral to cereal plant growth and development, particularly during seed germination and seedling growth. The 35S promoter facilitates pervasive gene expression in a myriad of plant tissues. However, plants transformed with 35S::AmyI-1 and 35S::AmyII-4, overexpressing α-amylase I-1 and α-amylase II-4, mirrored the WT in growth and morphology phenotype [13]. Grains with P10::AmyI-1 displayed pronounced anomalies in starch granule formation (Figs. 2G, H). The P10 promoter is designated for endosperm-specific expression, and unsurprisingly, the vegetative growth stage of rice plant with P10::AmyI-1 was typical.

The superficial pits and voids on starch granules, a consequence of α-amylase overexpression in developing endosperm, resembled the starch degradation observed in germinated cereal seeds [29,30]. Interestingly, the former represents starch degradation within living cell organelles, whereas the latter pertains to the decomposition of external storage starch in non-viable cells. Both AmyI-1 and AmyII-4 are secretory enzymes. Specifically, AmyI-1 is recognized as a prototypical secretory glycoprotein containing an N-linked oligosaccharide side chain [31]. Yet, both enzymes can target and operate within plastids such as chloroplast and amyloplast [12,32]. Based on these findings, it's clear that α-amylase is involved in the disorder of starch granule formation in endosperm cells. Chalky grains with abnormal starch granule formation, such as numerous pits and small holes, have more extensive and large voids between the starch granules. The air spaces among these abnormal starch granules refract light, making the grain appear white compared with perfect grains.

Chain-length distributions of starches from α-amylase-overexpressed rice.

Alterations in starch chain-length structure due to α-amylase overexpression were analyzed using the FCEP procedure (Fig. 3A). Differential analysis of starch chain-length distribution (Fig. 3B), revealed that the molecular structure of starches prepared from rice grains with 35S::AmyI-1 and 35S::AmyII-4 closely resembled those of Nipponbare WT and vector control. High-temperature stress during grain filling can severely impede storage starch granule formation, subsequently compromising grain quality and yield [33−35]. Starch chain-length distributions derived from both translucent and chalky portions of immaculate and chalky Koshihikari grains, grown under ripening average temperatures of 28.0 and 24.4 °C, were assessed using size-exclusion chromatography and FCEP. Results indicated negligible differences in starch molecular structures across the grains [28,36]. We infer that, in contrast to starch synthesis, the hydrolytic action of α-amylase is only on the surface of the starch granules and does not act on the majority of the starch, therefore the overall granule chain length distribution is not significantly altered. Importantly, the deformities observed in Nipponbare grains with 35S::AmyI-1 and 35S::AmyII-4 and those ripened under high temperatures in Koshihikari grains were analogous, as neither presented changes in starch chain-length distribution. In contrast, reduced starch biosynthetic enzyme activities at elevated temperatures modify the molecular structure and granule formation of rice [37−40]. Consequently, an imbalance between starch biosynthesis and degradation, stemming from atypical gene expression, is likely the primary culprit behind granule impairment and chalkiness [18].

In contrast, the chain-length distribution of P10::AmyI-1 starch exhibited an unprecedented pattern. Chains with DP 2−5, typically absent in Nipponbare WT starch, were identified (Fig. 3A). Differential analysis highlighted a considerable increase in the content of DP 2−8 chains and a decrease in DP 10−20 chains (Fig. 3B). Changes in chain-length distribution of starch have been extensively studied in starch synthesis related enzyme-deficient mutants including BEI, BEIIb, SSI, SSIIa, SSIIIa, and SSIVb, revealing that the distribution of glucan chains more than DP 6 was markedly altered by the loss-of-function of enzymes, whereas the DP 2−5 chains were scarcely changed [7]. Interestingly, the starch chain-length characteristics of P10::AmyI-1 somewhat echoed those of the *isa1* (*sugary-1*) mutant, despite the stark differences in their starch granule phenotypes [41].

A question arises as to why the respective transformations of 35S::AmyI-1 and P10::AmyI-1 result in differences in the molecular structure of starch. In the 35S::AmyI-1 transformants, α-amylase is constitutively expressed throughout the plant body, whereas in the P10::AmyI-1, the enzyme is strongly expressed in developing seed endosperm specifically. Although an indirect effect of α-amylase activation in the source tissues of 35S::AmyI-1 transformants cannot be ruled out, the strength of α-amylase expression and the degree of imbalance in starch synthesis and degradation in the ripening seed endosperm are thought to be the main causes.

Alkali solubility and pasting properties of starches from α-amylase-overexpressed rice.

Alkali solubility of rice grain starches from Nipponbare WT and transgenics was evaluated at 2 % (w/v) KOH (Table 2). The starch content in the supernatant of P10::AmyI-1 grain treated with alkali exceeded that of the WT, while the alkali-solubilized starch from 35S::AmyI-1 and 35S::AmyII-4 grains was similar to that of the WT. Alkali solubility of rice grain has been linked to the pasting temperature and retrogradation rate of starch [42]. To assess the pasting properties of starches from α-amylase-overexpressed rice, starches from Nipponbare WT and transgenic rice grains underwent RVA analysis. The viscosity of rice starches from 35S::AmyI-1 and 35S::AmyII-4 was slightly lower, but remained between those of the WT and the vector control. Conversely, the viscosity of P10::AmyI-1 rice starch was significantly reduced, dropping below the viscosity detection threshold (Fig. 4). Table 3 shows various parameters for characterizing starch properties. Notable differences were absent between WT and the two transgenics, 35S::AmyI-1 and 35S::AmyII-4, concerning pasting properties. It has been highlighted that the Set/Con and Max/Fin ratios correlate robustly with the fraction of intermediate and long chains of amylopectin (DP ≥ 13) [28]. The Set/Con values of 35S::AmyI-1 and 35S::AmyII-4 rice starches were higher than that of WT, whereas the Max/Fin values of transgenics were same or low compared with WT. These findings align with previous observations indicating minimal variation in starch chain-length distribution between WT and these two transgenics.

¹H-NMR characterization of starch molecular structure in α-amylase-overexpressed rice.

To elucidate the molecular structure of starch in α-amylase-overexpressed rice, we acquired the ¹H-NMR spectrum of starch extracted with DMSO-d6 from Nipponbare WT and transgenic rice grains using a Bruker Avance 700 MHz at 20 °C (Fig. 5). The spectra from WT (Fig. S1; see J. Appl. Glycosci. Web site), vector control, 35S::AmyI-1, and 35S::AmyII-4 rice grains were comparable across most of the magnetic field range. However, the spectra from P10::AmyI-1 showed notable differences in the 4-6 ppm range [43]. Distinctive shifts at 4.57 and 5.18 ppm were observed after P10::AmyI-1 transformation. The former
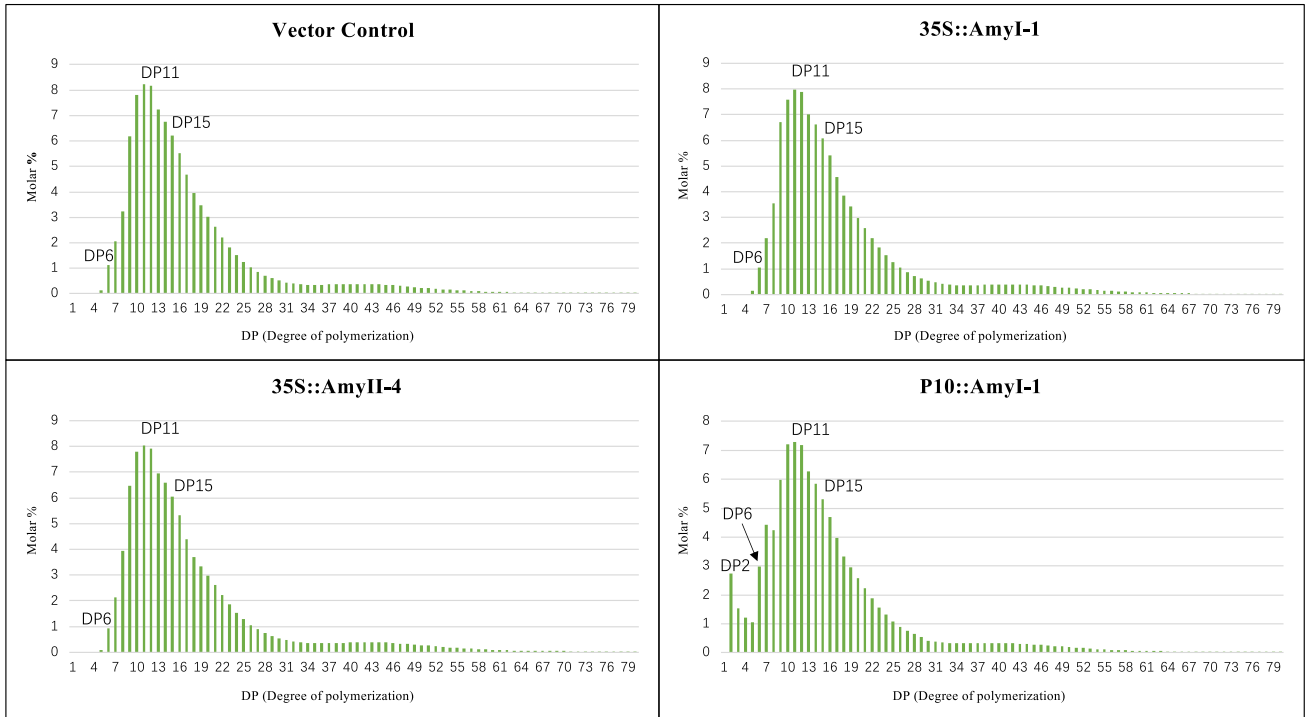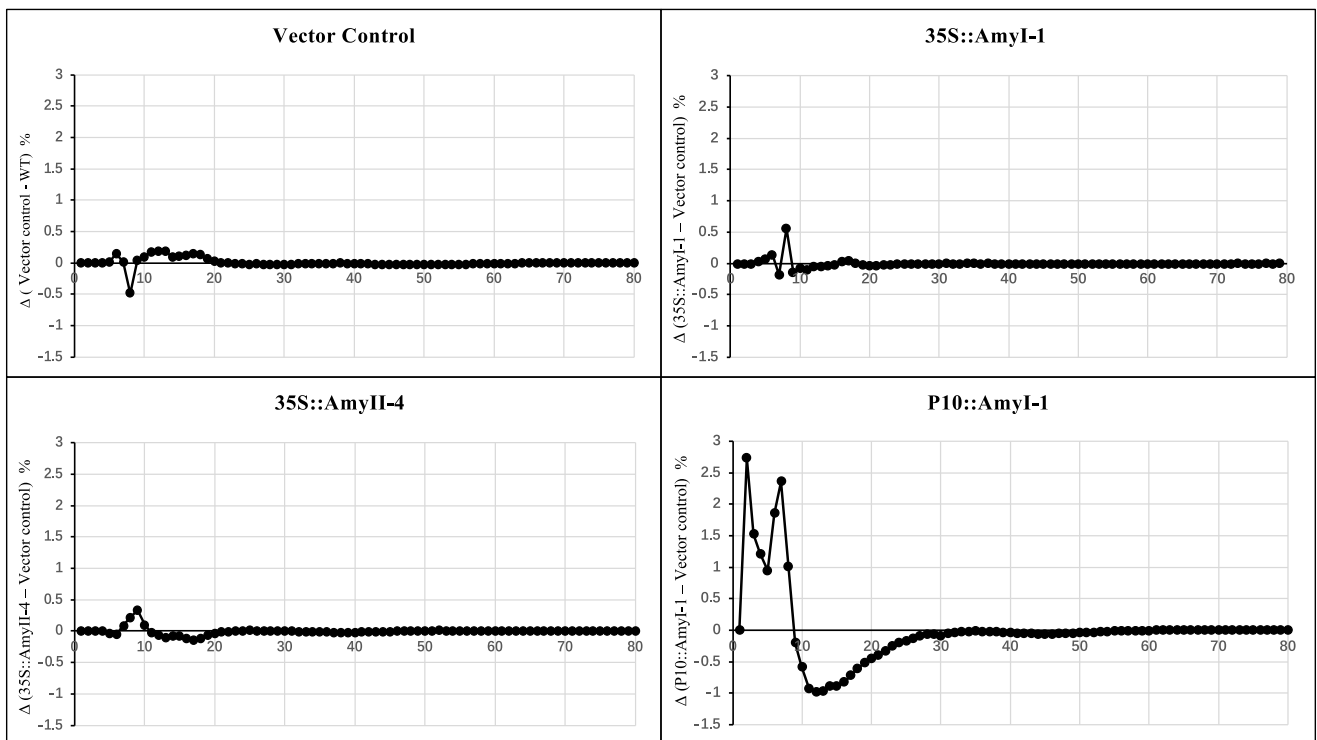
A

B

Fig. 3. Chain-length distributions of rice grain starches prepared from transgenic plants transformed with 35S::AmyI-1, 35S::AmyII-4, P10::AmyI-1, and pZH2B empty vector.

(A) Molecular % for each liberated chain to the total chains after debranching starch. (B) Rate of molar changes in chain length distribution caused by α-amylase overexpression.

Table 2. Alkali solubility of Starch from Nipponbare WT and transgenics.

	A_{620}
Hokuriku193	0.106[g] ± 0.015
Koshihikari	0.351[e] ± 0.025
Nipponbare	0.333[f] ± 0.012
35S::AmyII-4	0.366[c] ± 0.011
35S::AmyI-1	0.355[d] ± 0.011
P10::AmyI-1	0.741[a] ± 0.009
Vector control	0.438[b] ± 0.021

$n = 3$; [a,b,c,d,e] $p < 0.05$.

peak results from terminal hydrogen bonded to carbons involved in glycosidic linkage (H1) and the latter peak results from terminal hydrogen bonded to an anomeric carbon not involved in glycosidic linkage (H1α). The P10::AmyI-1 starch showed a marked increase in the 5.18 ppm peak and a decrease in the 4.57 ppm peak compared to WT starch; the behaviour of the three chemical shift peaks (5.11, 5.40 and 5.50 ppm) for the OH of α-glucan was similar to that of the 4.57 ppm peak, inferred to be relatively reduced due to increased H1α. These results indicate that α1,4-glycosidic linkages are disrupted by α-amylase activity, revealing the reducing end of the sugar (Fig. S1; see J. Appl. Glycosci. Web site), thereby providing deeper insight into the degradation mechanisms of cereal starch granules.

Several research groups have studied physicochemical alterations in various botanical storage starches during enzymatic hydrolysis *in vitro* [35,36,44−46]. SEM images of starch granules digested with α-amylase showed consistent structural deterioration with characteristic pores, depending on starch source and enzyme type. The diverse thermal and pasting properties of these porous starches have been documented [44]. Moreover, hydrolysis augmented the water retention and binding capacities of starch [45]. Potential correlations between the nuanced molecular structure and gelatinization parameters have been outlined in rice starch [46]. These findings strongly suggest that in vitro hydrolysis modifies the chain-length distribution of starch, although specific molecular details remain uncharted. In vivo starch hydrolysis necessitates meticulous regulation through the temporal and spatial expression of enzymes. The current study, which combined chain-length distribution and chemical shift analyses with RVA, offers valuable insights into starch degradation in rice endosperm under abnormal α-amylase activity enhancement. Kim and Robyt have reported that the glucoamylase-modified waxy maize starch granules can contain large amounts (10−50 %) of D-glucose inside the granule [47]. Despite the fact that α-amylase is an endo-type enzyme and glucoamylase is an exo-type enzyme, their strong activities can lead to the degradation of the interior of starch granules. As a result, the degradation products might be weakly bound to the original structure and

Table 3. Pasting properties of polished rice grains from Nipponbare WT and transgenics.

	Max.vis (RVU)	Mini.vis (RVU)	Break-down (RVU)	Fin.vis (RVU)	Setback (RVU)	Pasting Temp. (℃)	Consis-tency (RVU)	Set/Cons	Max/Fin	Max/Min
Nipponbare (WT)	261	160.3	100.7	307.5	46.5	68.7	147.2	0.3	0.8	1.6
WT:Vector control (1:1)	200.8	115.1	85.8	231.9	31.1	69.1	116.8	0.3	0.9	1.7
WT : 35S::AmyI-1 (1:1)	227.9	154.7	73.3	302.9	75	70.4	148.3	0.5	0.8	1.5
WT : 35S::AmyII-4 (1:1)	228	165.4	62.6	324.8	96.8	71.8	159.4	0.6	0.7	1.4
WT : P10::AmyI-1 (1:1)	3.2	3.1	0.1	5.5	2.3	71.4	2.4	1	0.6	1

Max vis, maximum viscosity；Mini vis, minimum viscosity; Fin vis, final viscosity; SB, setback; Cons, consistency.
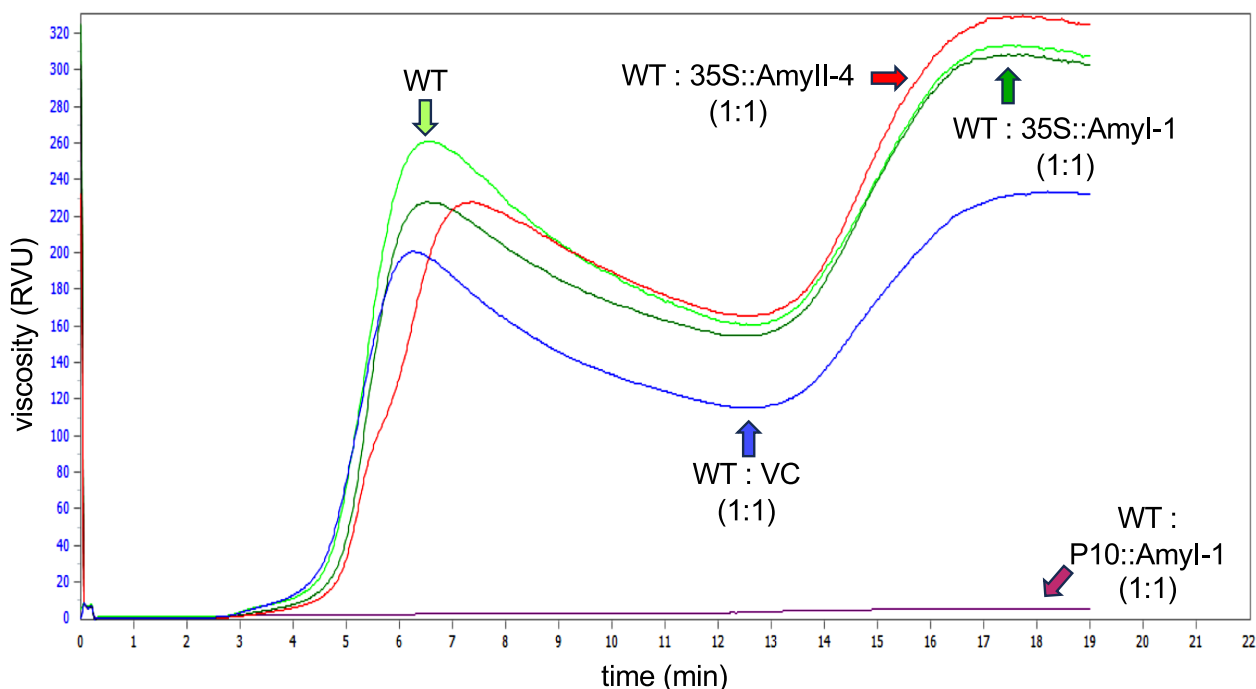
Fig. 4. Viscograms of rice grain starches prepared from Nipponbare WT and transgenic plants transformed with 35S::AmyI-1, 35S::AmyII-4, P10::AmyI-1, and pZH2B empty vector. Starch samples mixed 1:1 with WT and transgenics were evaluated with RVA.
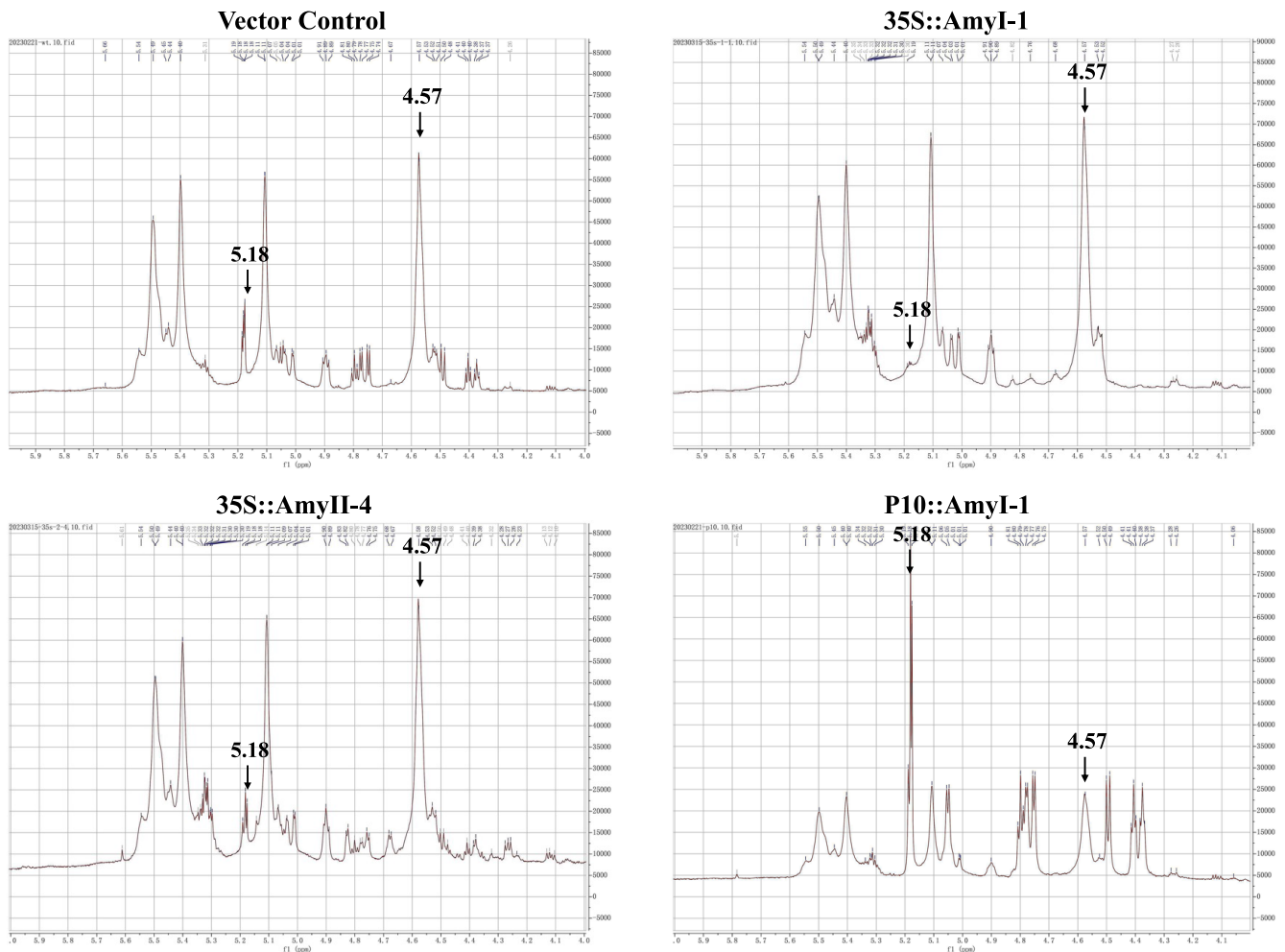
Fig. 5. ^{1}H-NMR spectra of rice grain starches prepared from transgenic plants transformed with 35S::AmyI-1, 35S::AmyII-4, P10::AmyI-1, and pZH2B empty vector.

Chemical shift peaks at 4.57 and 5.18 ppm indicate terminal hydrogen bonded to carbons involved in glycosidic bond formation (H1) and to anomeric carbon not involved in glycosidic bond formation (H1α), respectively.

retained in the granules.

CONCLUSION

We investigated the morphological, physicochemical, and molecular structural attributes of storage starch in transgenic rice seeds overexpressing α-amylases using EPMA, RVA, FCEP, and ^{1}H-NMR analyses. The transformation with 35S::AmyI-1 and 35S::AmyII-4 induced rice grain chalkiness without altering starch chain length distribution or pasting properties. Our findings strongly suggested that in the transgenic seeds, α-amylase targets starch granule surfaces for degradation without significantly impacting the starch chain length distribution. Consequently, α-amylase can induce grain chalking without modifying the overall starch molecular structure. Conversely, the P10::AmyI-1 transformation led to rice grain chalkiness and also changed the molecular and physicochemical properties of starch. The chain length distribution of starch in P10::AmyI-1 resembled that of the *isa* (*sugary*) mutant starch, with an appreciable rise in the proportion of DP 2−8 and a decline in DP 10−20. In the ^{1}H-NMR analysis, while the chemical shifts of starch in 35S::AmyI-1 and 35S::AmyII-4 transgenics were consistent with WT and vector control, the shift peak of terminal hydrogen bonded to an anomeric carbon outside the

glycosidic linkage (H1α) substantially increased in the P10::AmyI-1 starch. α-Glucan fragments cleaved by AmyI-1 appear to bind and adhere to starch granules. In the P10::AmyI-1 transgenic rice, the degradation rate of maltooligosaccharides likely exceeds that of enzyme-mediated digestion of starch granules.

CONFLICTS OF INTERESTS

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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