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An Assessment of Alkali Degradation, Waxy Protein and Their Relation to Amylose Content in Thai Rice Cultivars

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ABSTRACT: In this study, the amount of waxy protein and starch properties including amylose content and alkali digestibility, and their correlation were investigated in a collection of Thai rice cultivars. We found that glutinous rice lacked Wx protein, whereas nonglutinous rice showed a positive correlation between amylose content and the amount of Wx protein ($r = 0.97$, $p<0.01$). Starch granules of glutinous rice varieties showed high levels of disintegration in alkali (KOH) solution, while starch granules of nonglutinous rice cultivars were significantly more resistant to alkali digestibility ($t = 12.2$, $p< 0.01$).

KEYWORDS: *Oryza sativa,* physicochemical properties, Waxy protein, amylose content.

INTRODUCTION

The rice *waxy* gene encodes a granule-bound starch synthase enzyme (GBSS, or commonly called waxy protein), a key enzyme that is responsible for synthesis of amylose in endosperm tissue. In 1984, Sano[1] first studied rice waxy protein accumulation in seeds by SDS-PAGE and illustrated that the protein has a molecular weight of 60 kDa. Based on the Wx protein abundance, the rice waxy gene was further classified into two functional alleles, Wx^a and Wx^b. In nonwaxy rice cultivars, the Wx protein produced from the Wx^a allele is approximately 10 fold higher than that produced by the Wx^b allele.

In Thailand, there are many rice varieties, including glutinous rice (or waxy rice) and nonglutinous rice with variation in the apparent amylose content. Amylose content is a key determinant of the cooking and processing quality of rice (*Oryza sativa*). Low amylose levels are usually associated with tender, cohesive, glossy cooked rice, while higher amylose levels tend to cook dry, and have fluffy, separated grains.

Since amylose level is one of the most important breeding traits in the development of new rice cultivars, characterization of the relation between physicochemical properties, Wx protein and apparent amylose content may be useful in breeding programs to improve cooking quality. Thus, this report presents the investigation of the alkali disintegration susceptability and Wx protein level in Thai rice cultivars with different amylose contents.

MATERIALS AND METHODS

Rice cultivars and their physicochemical properties

Seeds of 90 accessions of rice (*Oryza sativa*) were sampled in this study, as listed in Table 1. Rice cultivars were classified as either glutinous or nonglutinous on the basis of a colorimetric assay for the glutinous phenotype, as described by Olsen and Purugganan[2]. Five to 10 seeds were cut crosswise and stained in a 1% iodine for 30 sec and then destained in dH_2O for 2-5 min and observed under a dissecting microscope. A bluish black color indicates the nonglutinous phenotype, whereas the glutinous phenotype is indicated by a redish brown color (Fig 1). In addition,

Fig 1. Glutinous (left) and non-glutinous (right) rice endosperms stained with IKI_2 solution.

the apparent amylose content in the endosperm of each cultivar was determined by using the spectrophotometric method[3].

The disintegration of the starch granules of the rice cultivar in alkali (KOH) solution was determined using a modification of the previously described procedure[4]. The seeds were hulled and halved vertically with a razor. The disintegration of starch granules were detected by standing three-halved grains of each cultivar in 1 mL of 1.7 % (w/v) KOH solution for 23 h at 30ºC. The degree of disintegration was determined by comparing to Japonica rice cv. Todorokiwase, which has a high level of disintegration, and Indica rice cv. KDML 105, which shows a low level, as positive and negative standards, respectively. Variation of alkaline digestibility in Thai rice cultivars was quantified with alkali spreading scores of 1, 2 and 3 for low, intermediate and high digestibility, respectively (Fig 2). The differences between the level of alkali digestibility and type of rice endosperm (glutinous and nonglutinous) were statistically tested, as described below.

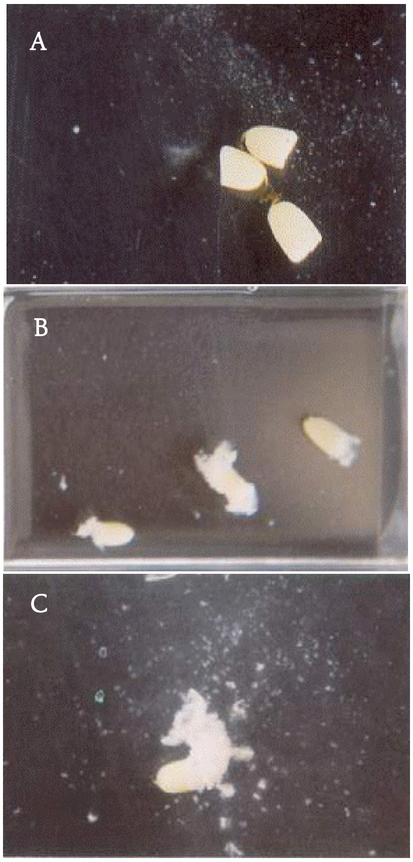

Fig 2. Degrees of alkali digestibility of starch granules in Thai rice cultivars.
A, Low
B, Intermediate
C, High

Waxy protein analysis

Starch granules from dried grains of glutinous rice, low- , intermediate- and high-amylose cultivars were isolated according to Echt and Schwartz[5] and Sano *et al.*[6]. The embryos were removed from 12 h-presoaked grains and then ten grains were homogenized in 1 mL of SDS buffer (0.055 M Tris-HCl, pH 6.8, 2.8% SDS, 5% β-mercaptoethanol, 10% glycerol) with a mortar and pestle. The suspension was filtered through a layer of Miracloth and centrifuged at 11000 *g* for 1 min. The pellet was washed three times in 1 mL of SDS buffer and twice in 1 mL of acetone. The starch granules were then dried in vacuo. For SDS-PAGE analysis, 5 mg of the granules were mixed with 50 ml of SDS buffer and heated in a boiling water bath for 5 min. The solution was cooled and an additional 100 mL of SDS buffer was added with gentle stirring. The slurry was centrifuged at 11000 *g* for 4 min and the protein concentration was determined using the Lowry assay with bovine serum albumin (BSA) as a standard. The total protein concentration was adjusted to 100 µg/mL. Then 15 µL of each sample was loaded on a 10% SDS-PAGE gel. The staining intensity of the Wx protein (60 kDa) was observed and compared among the cultivars (and accessions) tested. The intensities of the Wx protein bands were measured with a GDS 8000 Gel Documentation System, GDS8000 (UVP Inc., CA, USA).

Statistical analysis

The Pearson correlation coefficients for the relationship between amylose content and the level of waxy protein, and the differences of alkali digestibility between glutinous and nonglutinous rice were calculated using SPSS/PC^{+} version 4.

Results and Discussion

Physicochemical properties: alkali disintegration and amylose content

Starch gelatinization, the disruption of the molecular order within starch granules when they are heated in the presence of water, is one of the most important rheological indicators of the cooking quality and processing characteristics of rice starch. Juliano *et al.*[7] found a significant correlation between the disintegration of rice starch granules in alkali (KOH) solution and gelatinization temperature (GT) of milled rice. Rice with low GT disintegrates completely in 1.7 % KOH solution, whereas rice with intermediate GT shows partial disintegration. Rice with high GT remains largely unaffected in alkali solution. In addition, the disintegration of rice starch granules is affected by the fine structure of amylopectin[8]. Umemoto *et al.*[4] reported that the amylopectin chain-length profiles of disintegrated-starch granule cultivars were clearly

Table 1. Thai rice accessions examined for alkali digestibility, waxy protein and amylose contents.

Name	Locality (Province)	Disintegration in 1.7% KOH	Endosperm type[a]	Amylose content
Daw Gaset	Mukdahan	High	Glutinous	6.9
Daw Nahng Nuan	Kalasin	Intermediate	Glutinous	6.9
Daw Nahng Nuan Lai	Sakhon Nakhon	High	Glutinous	8.2
Daw Yuan	Mukdahan	High	Glutinous	7.2
Dok Gian	Kalasin	High	Glutinous	7.5
E-Dam	Kalasin	High	Glutinous	8.5
E-Dam	Sakon Nakhon	High	Glutinous	7.6
E-Khao	Kalasin	Intermediate	Glutinous	8
E-Khao Yai	Sakon Nakhaon	High	Glutinous	7.9
E-Nawn	Yasothon	High	Glutinous	6.8
E-Non	Yasothon	High	Glutinous	8.5
E-Pae	Mukdahan	High	Glutinous	7.8
E-Pon	Mukdahan	High	Glutinous	7.6
E-Pon	Kalasin	High	Glutinous	6.9
E-Pua	Mukdahan	High	Glutinous	7.1
E-Tia	Sakon Nakhon	High	Glutinous	8.8
Gai Ngaw	Sakon Nakhon	High	Glutinous	7.6
Gam	Mukdahan	Intermediate	Glutinous	9.4
Gam Pun	Mukdahan	High	Glutinous	8.9
Gra Dook Ngoo	Kalasin	High	Glutinous	7.5
Hahng Yi 71	Mukdahan	High	Glutinous	7.2
Hao' Kaen Doo	Mukdahan	High	Glutinous	8.7
Hao' Ma Phai	Mukdahan	Intermediate	Glutinous	6.2
Hawm Pae	Yasothon	High	Glutinous	8.2
Ka Saen	Mukdahan	High	Glutinous	7.5
Khao Gung	Mahasarakham	High	Glutinous	6.5
Khao Gung	Kalasin	High	Glutinous	6.5
Khao Yai	Kalasin	Intermediate	Glutinous	8.5
Khao' Ngan	Kalasin	Intermediate	Glutinous	8.4
Khi Tom Hahng Nahk	Sakon Nakhon	High	Glutinous	6.8
Khi Tom Yai	Sakon Nakhon	High	Glutinous	8.5
Khi Tom Yai	Kalasin	High	Glutinous	7.8
Leuang Bun Mah	Mahasarakham	High	Glutinous	7.2
Ma Yom	Mukdahan	High	Glutinous	7.4
Mae Hahng	Kalasin	High	Glutinous	6.8
Nahm Man Nghua	Sakon Nakhon	High	Glutinous	6.8
Nahng Ni	Sakon Nakhon	High	Glutinous	7.2
Nahng Nuan	Kalasin	Intermediate	Glutinous	7.8
Nahng Nuan	Mahasarakham	High	Glutinous	6.5
Nam Ang	Sakon Nakhon	High	Glutinous	6.5
Ngan Khao	Kalasin	High	Glutinous	8.5
Pa Sew	Sakon Nakhon	High	Glutinous	8.6
Pawng Aew	Mukdahan	High	Glutinous	6.7
Pla Sew	Kalasin	Intermediate	Glutinous	6.7
RD 6	Mahasarakham	High	Glutinous	5.9
San Huang Khao	Mukdahan	High	Glutinous	6.3
San Pah Tawng	Kalasin	High	Glutinous	8
San Pah Tawng Daw	Mahasarakham	High	Glutinous	7.8
U Kham	Mukdahan	High	Glutinous	6.5
Yi Pun	Kalasin	High	Glutinous	7.4
Accession 35	Phichit	Low	Nonglutinous	30.1
Accession A	Phichit	Low	Nonglutinous	30.4
Bawng Gaset	Surin	Intermediate	Nonglutinous	26.2
Chainaht 60	Pathum Thani	High	Nonglutinous	17.2
Chao' Daeng	Surin	High	Nonglutinous	29.5
Chao' Yai	Nakhon Ratchasima	Low	Nonglutinous	22.5
Chaw Lung	Phatthalung	Low	Nonglutinous	23.7
Chiang' Phatthalung	Phatthalung	Low	Nonglutinous	28.9
Gaen Jan	Phatthalung	Low	Nonglutinous	29.3
Hao Nah	Songkhla	Low	Nonglutinous	25
Hawm Janh	Phatthalung	Low	Nonglutinous	26.1

Table 1. *Cont'd.*

Name	Locality (Province)	Disintegration in 1.7% KOH	Endosperm type[a]	Amylose content
Jampah	Phatthalung	Low	Nonglutinous	28
Jaw Dam	Suphan Buri	High	Nonglutinous	16.2
Jaw Haw	Chiang Rai	High	Nonglutinous	17.5
Jaw Yai	Nakhon Ratchasima	Low	Nonglutinous	20.3
KDML 105	Yasothon	Low	Nonglutinous	14.1
KDML 105	Nakhon Phanom	Low	Nonglutinous	13.7
KDML 105	Mahasarakham	Low	Nonglutinous	12.9
KDML 105	Roi Et	Low	Nonglutinous	14.1
Khai Mod Rin	Phatthalung	Low	Nonglutinous	23.3
Khao Pitsanulok	Pitsanulok	Low	Nonglutinous	25.7
Khao Ruang Yao	Phatthalung	Low	Nonglutinous	25
Khem Tawng	Phatthalung	High	Nonglutinous	26.1
Leb Nok Pattani	Pattani	Low	Nonglutinous	23.8
Leuang 152	Phatthalung	Low	Nonglutinous	24
Leuang Pratew	Phetchaburi	Low	Nonglutinous	30.3
Mali Thawng	Pathum Thani	Low	Nonglutinous	28.8
Nahng Payah 132	Phatthalung	Low	Nonglutinous	28.3
Peuang Nam	Phatthalung	Low	Nonglutinous	30.5
Phatthalung 60	Phatthalung	Low	Nonglutinous	28.5
Pratum	Nakhon Ratchasima	Low	Nonglutinous	23.7
Puang Rai 2	Petchaburi	Low	Nonglutinous	30.5
Puang Tawng	Phatthalung	Low	Nonglutinous	27.8
Puang Thawng	Phatthalung	Low	Nonglutinous	27.8
RD 13	Pathum Thani	Low	Nonglutinous	30.2
RD 27	Phathum Thani	Low	Nonglutinous	28.5
Sang Yod	Phatthalung	Low	Nonglutinous	16.4
Srirak	Phatthalung	Low	Nonglutinous	28
Srirak	Phatthalung	Low	Nonglutinous	28
Tam Me Rai	Songkhla	Low	Nonglutinous	27.6

[a] Classified as glutinous or nonglutinous by staining with IKI_2 solution.

different from those of the unaffected-starch granule cultivars. Starch granules that had amylopectin enriched in short chains (degree of polymerization: DP 7 to 10) were more easily disintegrated in alkali solution than starch granules with amylopectin enriched in long chains (DP 12 to 21).

Figure 2 shows the variation of alkali digestibility in rice cultivars that was low (cv. KDML 105), intermediate (cv. Bong Gaset) and high digestibility (cv. Todorokiwase). Based on this standard, rice cultivars used in this study were scored as low (1), intermediate (2) and high (3) levels of disintegration because starch granules were scarcely degraded, incompletely degraded and completely degraded, respectively.

In this study, only intermediate and high alkali digestibility were observed in the glutinous cultivars, whereas all three types of alkali digestibility were found in nonglutinous cultivars (Table 1).

Based on these results, glutinous rice showed high degrees of disintegration of starch granules in alkaline solution with the average value of 2.8 on the 1-3 digestibility scale described above, whereas starch granules of nonglutinous rice showed a low level of disintegration (average = 1.3). Alkali digestibility was significantly different between glutinous and nonglutinous rice ($t = 12.2$, $p < 0.01$). Starch granules of the 50 glutinous cultivars used in this study were completely digested in alkali solution. In contrast, almost all of nonglutinous rice cultivars (29 out of 40 or 72.5%) with intermediate (20-25%) and high amylose content (>25%) had low digestibility of starch granules in alkali solution. In glutinous rice, it has been reported that a high level of alkali digestion was found in starch granules of rice from China and Japan[9]. Previously, Nakamura *et al.*[10] reported that no correlation was observed between the amylose content and the thermal property of rice starch. Therefore, the significance of differences in the starch properties among rice cultivars is still inconclusive. However, Harushima *et al.*[11] suggested that variation in a gene encoding an amylopectin-synthesizing enzyme in the rice varieties is responsible for the difference in physicochemical properties of starch granules through structural alteration of their amylopectin molecules.

Waxy protein level and amylose content

Glutinous rice lacks the starch amylose, which constitutes up to 30% of the total starch in nonglutinous rice endosperm[12,13]. In this study, amylose content ranged from 5.9 to 9.4 % and 12.9 to 30.5 % for glutinous ranged from 5.9 to 9.4 % and 12.9 to 30.5 % for glutinous and nonglutinous rice, respectively. This result indicated that amylose content is diverse in both Thai glutinous and nonglutinous rice cultivars.

From Table 1, rice cultivars that were classified as glutinous had 5.9 to 9.4 % amylose content. These cultivars have no waxy protein accumulating in their endosperm (data not shown), in agreement with previous results[14,15,16]. In nonglutinous rice, the intensity of the 60 kDa Wx protein bands was correlated with amylose content (Fig 3, 4). The results showed that high-amylose content varieties gave the intensity values higher than that of low-amylose content varieties. Figure 5 shows the relationship between amylose contents and intensity of the Wx protein in the 19 rice varieties (9 high- and 10 low-amylose varieties). These results clearly illustrated that amylose content in nonglutinous rice showed a positive correlation (r=0.97) with peak intensity of Wx protein.

Previously, rice scientists have suggested that Wx protein was present in different amounts in starch granules of different nonglutinous cultivars and even

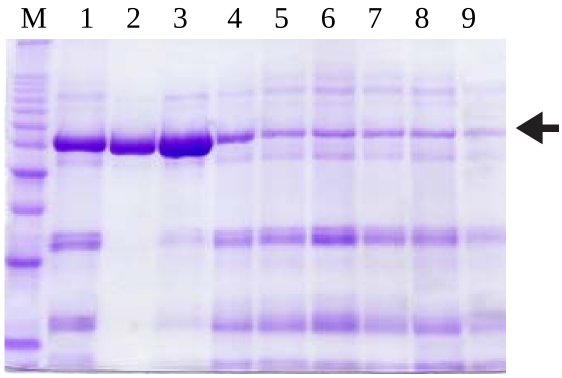

Fig 3. Waxy protein with about 60kDa (indicated by arrow) extracted from starch granules of rice cultivars with high amylose content and low amylose content. Each lane contained 100 µg/ml of total protein extracted from seeds. 1, Mali Thawng (28.8%); 2, Puang Tawng (27.8%); 3, Leuang Pratew (30.3%); 4, Jaw Haw (17.5%); 5, Jaw Dam (16.2%); 6, Mali Daeng (15.4%); 7, KDML105 (14.1%); 8, KDML105 (13.7%); 9, KDML105 (12.9%). M=10 kDa protein ladder (Gibco, BRL).

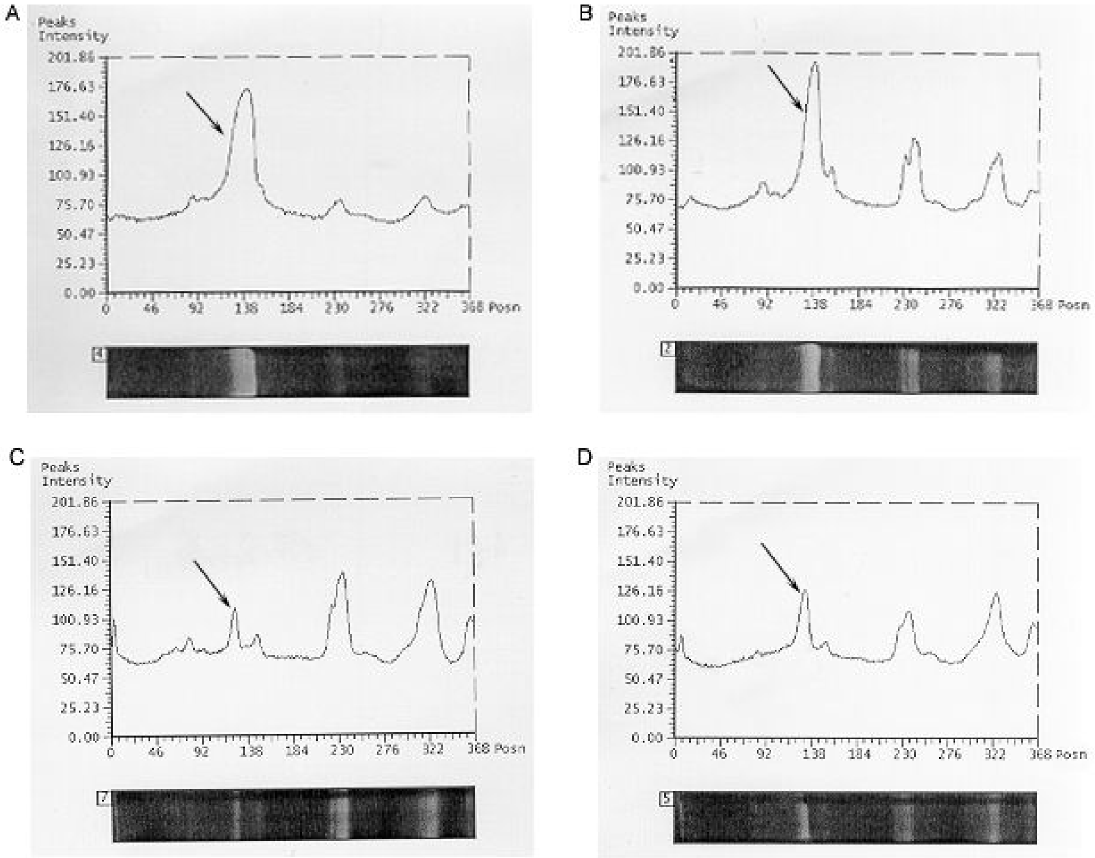

Fig 4. Comparison of the intensity of waxy protein in rice endosperm from high- and low-amylose cultivars. A, Leuang Pratew (30.3% amylose); B, Mali Thawng (28.8%); C, Jaw Haw (17.5%); D, Mali Daeng (15.4%). Arrows indicated the waxy protein (60 kDa).

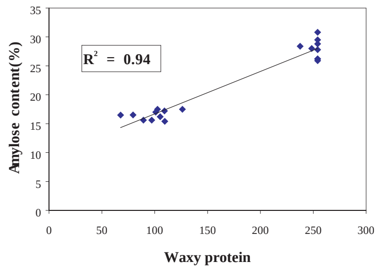

Fig 5. Relationships between amylose content and peak intensity of Wx protein in high-amylose varieties (>25%) and low-amylose varieties (<20%).

(>25 %), intermediate amylose (20-24 %) and low amylose (<20 %), the 60 kDa band was found at high, intermediate and low intensity, respectively, whereas, in glutinous rice cultivars, the Wx protein was not detected using the same method[14,15,17,18].

The glutinous phenotype arises through the disrupted expression of the amylose biosynthesis *Waxy* (*Wx*) gene, which encodes a granule-bound starch synthase (or Wx protein)[1]. Glutinous rice contains a G to T mutation at the 5' splice site of *Wx* intron 1 that leads to incomplete post-transcriptional processing of *Wx* pre-mRNA[17,18,19,20,21].

In contrast to glutinous rice, nonglutinous rice shows wide variation in amylose content. Isshiki *et al.*[18] demonstrated that the allele of rice *waxy* gene that controls the production of lower amounts of Wx protein is Wx^b (base T at the 5' splice site of *Wx* intron 1), whereas the Wx^a allele (base G at the 5' splice site of *Wx* intron 1) produces a higher level of Wx protein than Wx^b). In addition, there is a little variation in the intensity of Wx protein within cultivars with Wx^a or Wx^b alleles. Based on these results, rice scientists have concluded that the G to T mutation at the 5' splice junction of the first intron is the major reason for variation in the expression of the rice *Waxy* gene.

For Thai indica rice, Prathepha[22] reported that intermediate- and high-amylose cultivars contained G at the 5' splice site of *Wx* intron 1, whereas low-amylose cultivars and glutinous rice had T at the at the 5' splice junction of the first intron. The G to T mutation at the 5' splice junction has assessed by PCR-RFLP method[22,23].

Conclusion

In this paper, we reported that starch granules of Thai glutinous cultivars had intermediate and high digestibility in alkali solution, while nonglutinous rice cultivars with intermediate and high amylose contents were resistant to disintegration of starch granules in alkali solution. In addition, we also demonstrated that Wx protein levels of Thai rice are related to their amylose content. These results are consistent with those of previous studies. Rice cultivars with high amylose content produce large amounts of Wx protein, whereas cultivars with intermediate and low levels of Wx protein had intermediate and low amylose contents, respectively. In contrast to nonglutinous rice, glutinous rice accumulated no Wx protein. In kernels, the ratio of the two primary components of starch, amylose and amylopectin, varies from cultivar to cultivar, indica or japonica rice. The molecular mechanisms of genetic control of this trait are still unclear. This study described the expression of the *Waxy* gene at the protein level in Thai rice cultivars. It will facilitate the study of the molecular mechanism controlling the amylose synthesis of rice cultivars in Thailand.

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