Gene expressions at the *waxy*¹⁾ locus in *Oryza glaberrima* STEUD.

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To investigate the expression of a *waxy* gene in *O. glaberrima*, chemical and physical properties of the endosperm starch were examined employing *waxy* and *non-waxy* cytoplasmic substitution lines that have the nucleus of *O. glaberrima* and the cytoplasm of *O. sativa*, a *waxy* *O. glaberrima* line having a *waxy* gene derived from *O. sativa* and an induced *waxy* mutant of *O. glaberrima*. The endosperm starch lacks amylose in three lines tested, namely the *waxy* *O. glaberrima* line having a *waxy* gene derived from the Japanese cultivar of *O. sativa*, the induced *waxy* mutant of *O. glaberrima* and the *waxy* *O. glaberrima* line with both the cytoplasm and *waxy* gene derived from the Japanese cultivar of *O. sativa*. However, the ratio of the amount of short to long chain in the amylopectin seems to be affected by the nuclear genes, with the exception of a *waxy* gene. A *waxy* gene seems to have no effect on the gelatinization characteristics of the starch. Thus, these characteristics of the starch seem to be determined by nuclear genetic background except for a *waxy* gene, whereas cytoplasm seems to have no effect on both elution patterns of debranched starch and gelatinization characteristics of endosperm starch. A *waxy* gene seems simply to reduce the amylose content of endosperm starch without affecting other characteristics of starch.

KEY WORDS : *Oryza glaberrima*, *waxy* gene, amylose content, amylopectin, gelatinization characteristics.

Introduction

Oryza glaberrima is cultivated in a restricted region of West Africa, and is important as a genetic resource for rice breeding. *O. sativa* and *O. glaberrima* have the same chromosome number ($2n=24$) and their F_1 hybrid shows the normal chromosome pairing during the meiosis. And the F_1 hybrid is highly male sterile (MORINAGA and KURIYAMA 1960).

Waxy cultivars of *O. sativa* are important in East Asia, whereas no spontaneous *waxy* line has been known in *O. glaberrima*. A *wx* gene belongs to linkage group I or chromosome 6, in *O. sativa* (IWATA and OMURA 1971), and controls the amylose content of endosperm and pollen starches; *non-waxy* starch contains both amylose and amylopectin whereas *waxy* starch contains only amylopectin. YABUNO (1981) has produced a *waxy* *O. glaberrima* line by means of continuous back-crossings of a Japanese *waxy* cultivar of *O. sativa*, 'Iwai-mochi', to a *O. glaberrima* using the fertility restoring gene derived from *O. sativa*, 'Akebono'. SANO (1978) obtained for the first time a *waxy* mutant induced by ethyl methane sulfonate (EMS) treatment in *O. glaberrima*.

The detailed characteristics of *waxy* *O. glaberrima* starch are little known. This study investigated the chemical structural properties and gelatinization characteristics of *waxy* *O. glaberrima* starch under different nuclear and cytoplasmic backgrounds in relation to the expression of the *waxy* gene.

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¹⁾ 'Waxy' means 'glutinous' phenotype of endosperm and pollen starch.

Materials and Methods

1. Materials

The *non-waxy* and *waxy* cytoplasmic substitution lines, (Iwai-mochi)-W 0440, *Wx/Wx* and (Iwai-mochi)-W 0440, *wx/wx* have been produced by repeated backcrosses (seven times) of 'Iwai-mochi' to a *non-waxy* *O. glaberrima* line, W 0440. Two *waxy* *O. glaberrima* lines, (W 0440)-W 0440, *wx/wx* and GMS were also used. The (W 0440)-W 0440, *wx/wx* line has a *waxy* gene derived from 'Iwai-mochi' while the GMS line has the *waxy* gene induced by EMS treatments of a *non-waxy* *O. glaberrima* line, GMS 87. (Table 1).

All the lines were cultivated under paddy conditions and were self-fertilized in 1984 and 1985. To obtain seeds of *O. glaberrima* heterozygous for a *waxy* gene, *non-waxy* and *waxy* *O. glaberrima* lines, GMS 87 and GMS were crossed reciprocally. Seeds were gathered at maturity.

2. Preparation of starch granules

Stach granules were prepared by the cold and dilute alkali method according to YAMAMOTO *et al.* (1973, 1981). About ten grams of selfed seeds and about two grams of crossed seeds heterozygous for *waxy* alleles were used for preparation of starch granules. Selfed and crossed seeds were hulled, and selfed seeds were polished. Crossed seeds were not polished. Hulled or polished rice were crushed and the crushed rice was submerged with about 15 ml of cold 0.2% sodium hydroxide solution per gram of sample and kept at 0-4°C overnight. The swollen crushed rice was ground with a pestle and then passed through a 200-mesh sieve. The pulp was suspended in about 30 ml of cold 0.2% sodium hydroxide per gram of sample, the mixture was stirred at 0-4°C for 3 hours and the starch granules were separated by spontaneous sedimentation. The supernatant is discarded, the pulp was resuspended in cold distilled water, the starch suspension centrifuged at 1,600×g for ten minutes and supernatant discarded. This washing process was repeated until the supernatant became completely neutral. After drying under reduced pressure, the starch was passed through a 145-mesh sieve.

3. Measurement of iodine absorption spectra

Absorption curves of starch-iodine complexes were recorded with a Japan Spectroscopic Co. UVLDE C-660 spectrophotometer on mixtures containing 1 mg of starch, 2 mg of

Table 1. Materials used in this study.

Lines	Cytoplasm	Nucleus	Alleles for <i>waxy</i> locus
<i>O. glaberrima</i>			
W 0440			<i>Wx</i>
GMS 87			<i>Wx</i>
GMS			<i>wx</i> (induced by EMS)
(W 0440)-W 0440, <i>wx/wx</i>	W 0440	W 0440	<i>wx</i> (from Iwai-mochi)
<i>O. sativa</i>			
Iwai-mochi			<i>wx</i>
Cytoplasmic substitution lines			
(Iwai-mochi)-W 0440, <i>Wx/Wx</i>	Iwai-mochi	W 0440	<i>Wx</i> (from W 0440)
(Iwai-mochi)-W 0440, <i>wx/wx</i>	Iwai-mochi	W 0440	<i>wx</i> (from Iwai-mochi)

iodine and 20 mg of potassium iodide per 25 ml (FUJIMOTO *et al.* 1971). From absorption curves, the wavelength of absorption maximum (λ max.) of the spectra and the blue value (B.V.), the O.D. value at 680 nm, were obtained. Measurements were made at least two times, and the means of each measurement are shown (Table 2).

2. Debranching of starch and fractionation of debranched materials

Starches were debranched and gel-filtered by the method of IKAWA *et al.* (1981). Carbohydrate content in each tube was measured by the phenol-sulfuric acid method (DUBOIS *et al.* 1956). According to the λ max. of absorbance spectra of iodine-carbohydrate complexes, the fractions were divided as follows; Fr. I: λ max. ≥ 620 nm, intermediate fraction: $620 \text{ nm} > \lambda \text{ max.} \geq 600$ nm, Fr. II: $600 \text{ nm} > \lambda \text{ max.} \geq 540$ nm, Fr. III: $540 \text{ nm} > \lambda \text{ max.}$

Experiment was replicated twice in the 'Iwai-mochi' and GMS lines, and three times in the other lines.

5. Differential scanning calorimetry

Starch and distilled water were sealed in an aluminium DSC pan in a 1:2 dry starch/water ratio. Therograms of starches were recorded on Rigaku Denki CN 8059 L1 DSC and a Rigaku Denki DSC-8240 by heating with recorder sensitivity of ± 1 mcal/sec (Table 4, Fig. 2) and 0.5 mcal/sec (Table 5, Fig. 3) respectively. To clarify the differences among GMS, GMS 87 and their F_1 hybrids, high sensitivity value, 0.5 mcal/sec, was used (Table 5, Fig. 3). In these lines, four replications were done. In the six lines shown in Table 4, at least two replications were done.

Heat of gelatinization (ΔH) was obtained from the area of the endotherm (peak of heat flow). A baseline was constructed as a smooth line from beginning to end of the endotherm, approximating the thermogram if the endotherm was absent. The area between the baseline and the endotherm was measured by means of chart weight. The calibration coefficient for the ΔH calculation was derived from the known heats of fusion for indium. The onset temperatures (T_o), the peak temperatures (T_p), and the conclusion temperatures (T_c) of endotherms were determined as shown in Fig. 2.

Results

1. Absorption curves of starch-iodine complexes

Normal corn starch was used as the standard.

Starches of two *non-waxy* *O. glaberrima* line, W 0440 and GMS 87, and a *non-waxy* cytoplasmic substitution line, (Iwai-mochi)-W 0440, *Wx/Wx*, revealed typical *non-waxy* absorption curves. The λ max. value ranged from 595 nm to 599 nm and B.V. values ranged from 0.47 to 0.48 (Table 2). Starches of 'Iwai-mochi', the *waxy* cytoplasmic substitution line, (Iwai-mochi)-W 0440, *wx/wx* and GMS, showed *waxy* type absorption curves; λ max values ranged from 525 to 527 nm and B.V. values from 0.18 to 0.21. Absorption spectra of the F_1 hybrids between GMS 87 and GMS were intermediate between those of parents (Table 2). The hybrid with the one *waxy* gene (GMS 87 $\times$ GMS, *Wx/Wx/wx*) revealed a longer wavelength at λ max. and a larger B.V. value than those of the hybrid with two *waxy* genes (GMS $\times$ GMS 87, *Wx/wx/wx*).

2. Elution patterns of isoamylase-debranched starch

The elution of debranched starch fractions yielded Fraction I in W 0440 and in a *non-waxy* cytoplasmic substitution line, (Iwai-mochi)-W 0440, *Wx/Wx* (Table 3, Fig.1). This fraction corresponded to the amylose of the original starch. Amylose contents of W 0440 and the *non-waxy* cytoplasmic substitution line were 23.7% and 24.2%, respectively.

Fraction I was absent in 'Iwai-mochi', a *waxy* cytoplasmic substitution line, (Iwai-mochi)-W 0440, *wx/wx*, and two *waxy O. glaberrima* line, (W 0440)-W 0440, *wx/wx* and GMS. Fractions II and III, derived from the long and short chains of the amylopectin of the original starch, respectively, were recognized in *non-waxy* and *waxy* lines. The ratio of Fr. III to Fr. II is considered to be one of the characteristics of the starch. *Non-waxy* and *waxy* cytoplasmic substitution lines, (Iwai-mochi)-W 0440, *Wx/Wx* and (Iwai-mochi)-W 0440, *wx/wx*, and a *waxy O. glaberrima* line with the *waxy* gene of 'Iwai-mochi', (W 0440)-W 0440, *wx/wx*, were similar to W 0440 in the ratios of Fr. III to Fr. II. The elution patterns of debranched starch in the *non-waxy* cytoplasmic substitution line was similar to a *waxy O. glaberrima* line having a nuclear background of W 0440.

Differential Scanning Calorimetry (DSC)

The DSC characteristics of (Iwai-mochi)-W 0440, *Wx/Wx*, (Iwai-mochi)-W 0440, *wx/wx* and (W 0440)-W 0440, *wx/wx* were similar to those of W 0440 whether they have a

Table 2. Absorption spectra of starch-iodine complexes of *non-waxy* and *waxy* rice lines, F_1 hybrids and n-corn.

Lines	λ max. (nm)	B. V. (O. D.)
n-corn	570	0.44
W 0440	595	0.47
Iwai-mochi	526	0.18
(Iwai-mochi)-W 0440, <i>Wx/Wx</i>	596	0.47
(Iwai-mochi)-W 0440, <i>wx/wx</i>	525	0.20
(W 0440)-W 0440, <i>wx/wx</i>	525	0.21
GMS	527	0.21
GMS 87	599	0.48
F_1 hybrids		
GMS $\times$ GMS 87	568	0.35
GMS 87 $\times$ GMS	583	0.36

Table 3. Properties of isoamylase-debranched components of *non-waxy* and *waxy* rice lines.

Lines	Fr. I ¹⁾ (%)	Intermediate (%)	Fr. II (%)	Fr. III (%)	Ratio of Fr. III/Fr. II
W 0440	23.7 $\pm$ 1.62	4.3 $\pm$ 0.42	17.7 $\pm$ 1.18	54.5 $\pm$ 1.21	3.1 $\pm$ 0.15
Iwai-mochi	—	1.0	25.8	73.2	2.7
(Iwai-mochi)-W 0440, <i>Wx/Wx</i>	24.2 $\pm$ 2.03	8.1 $\pm$ 6.88	16.2 $\pm$ 0.63	51.3 $\pm$ 8.68	3.2 $\pm$ 0.07
(Iwai-mochi)-W 0440, <i>wx/wx</i>	—	3.6 $\pm$ 2.43	24.6 $\pm$ 1.32	71.8 $\pm$ 1.23	2.9 $\pm$ 0.11
(W 0440)-W 0440, <i>wx/wx</i>	—	0.6 $\pm$ 1.19	23.5 $\pm$ 1.09	74.9 $\pm$ 1.90	3.2 $\pm$ 0.21
GMS	—	4.5	25.1	70.4	2.8

¹⁾ Each fraction was divided according to λ max. of carbohydrate-iodine complexes as follows : Fr. I : λ max. $\geq$ 620 nm, Intermediate : 620 nm $>$ λ max. $\geq$ 600 nm, Fr. II : 600 nm $>$ λ max. $\geq$ 540 nm, Fr. III : λ max $>$ 540 nm.

Fig. 1. Elution patterns of Isoamylase-debranched endosperm starches of *non-waxy* and *waxy* rice lines. a: W 0440 b: (Iwai-mochi)-W 0440, W_x/W_x , c: GMS, d: Iwai-mochi, e: (Iwai-mochi)-W 0440, w_x/w_x , f: (W 0440)-W 0440, w_x/w_x

Table 4. DSC characteristics of *non-waxy* and *waxy* rice lines.

Lines	To (°C)	Tp (°C)	Tc (°C)	ΔH (cal/g)
W 0440	70.3	75.0	82.0	2.2
Iwai-mochi	62.7	70.0	80.9	3.1
(Iwai-mochi)-W 0440, W_x/W_x	71.1	75.6	83.0	2.3
(Iwai-mochi)-W 0440, w_x/w_x	71.0	76.7	85.9	2.3
(W 0440)-W 0440, w_x/w_x	72.4	78.2	85.4	2.1
GMS	74.5	80.3	90.3	3.6

Table 5. DSC characteristics of *waxy* and *non-waxy* African rices and their F_1 hybrids.

Lines	To (°C)	Tp (°C)	Tc (°C)	ΔH (cal/g)
GMS	71.8±0.64a ¹⁾	78.9±0.53 a	90.3±1.22 a	4.1±0.18 a
GMS 87	67.6±0.32 c	73.8±0.72 d	81.3±0.23 c	2.8±1.38 b
F_1 hybrids				
GMS×GMS 87	70.3±0.28 b	76.1±0.10 b	83.9±0.71 b	3.2±0.31 b
GMS 87×GMS	69.5±0.63 b	75.1±0.37 c	83.2±0.74 b	3.3±0.14 b

¹⁾ Average values with the same letters are not significantly different at the 5% level.

Fig. 2. DSC thermograms of endosperm starches of various rice lines, a : W0440, b : Iwai-mochi c : (Iwai-mochi)-W0440, Wx/Wx , d : (Iwai-mochi)-W0440, wx/wx , e : (W0440)-W0440, wx/wx , f : GMS

Fig. 3. DSC thermograms of endosperm starches of *non-waxy* and *waxy* African rices and their F_1 hybrids. a : GMS ($wx/wx/wx$), b : GMS $\times$ GMS 87 F_1 ($Wx/wx/wx$), c : GMS 87 $\times$ GMS F_1 ($Wx/Wx/wx$), d : GMS 87 ($Wx/Wx/Wx$)

waxy gene or not (Table 4, Fig. 2). The DSC characteristics of 'Iwai-mochi' were different from those of the lines carrying a *waxy* gene of 'Iwai-mochi', (Iwai-mochi)-W0440, wx/wx and (W0440), wx/wx . Thus, gelatinization characteristics of starches seem to be determined by the genetic background of the nucleus in these lines. However, the starch of GMS showed a higher gelatinization temperature and larger ΔH value than that of GMS 87. The gelatinization temperature and the ΔH value of F_1 hybrids were intermediate between the both parents (Table 5, Fig. 3).

Discussion

In double mutants of maize, the *waxy* gene acts epistatically to other endosperm genes such as *sugary-1* or *dull* (KRIMER *et al.* 1958). Also in *O. sativa*, the *waxy* gene is epistatic to the *dull* gene in reducing the amylose content (OKUNO *et al.* 1983). However, in maize with both *waxy* and *dull* or *amylose-extender* genes, a *waxy* gene shows no effect on the Fraction III to Fraction II debranched starch elution patterns (IKAWA *et al.* 1981). *Waxy* cereals are considered to be incapable of producing amylose molecules in the endosperm and pollen because of the lack of granule-bound ADPG-starch glucosyltransferase, and a *waxy* locus is probably the structural locus of the enzyme (NELSON and

RINES 1962, MURATA *et al.* 1964, TSAI 1974). Therefore, the *waxy* gene seems to reduce amylose content but not to affect the ratio of the short to the long chain of the amylopectin. In this study, the *waxy* cytoplasmic substitution line and the *waxy* *O. glaberrima* line with a *waxy* gene derived from 'Iwai-mochi' contained absolutely no amylose (Table 3, Fig.1). Also, the ratios of Fraction III to Fraction II of these *waxy* lines were similar to that of W 0440 (Fig.2). This result is consistent with the hypothesis that the *waxy* locus in 'Iwai-mochi' is the structural locus of granule-bound ADPG-starch glucosyltransferase. In *O. sativa*, a *waxy* gene shows apparent dosage effect on the amylose content of starch (OKUNO 1978). In this study, a *waxy* gene induced by EMS treatment showed dosage effect (Table 2).

Gelatinization characteristics of starches of the *waxy* line of *O. sativa* are similar to those of the *non-waxy* counterparts except for the conclusion temperature and the heat of gelatinization; *waxy* starches have higher conclusion temperatures and higher heats of gelatinization than *non-waxy* starches (ASAOKA *et al.* 1985). In this study, a series of lines produced by continuous backcrossings of W 0440 show DSC characteristics similar to those of W 0440 whether the line is *waxy* or not (Table 4, Fig.2), whereas the DSC characteristics of 'Iwai-mochi' are different from the *waxy* lines which have the *waxy* gene of 'Iwai-mochi'. Therefore, in *O. glaberrima*, DSC characteristics of starches seem to be determined by nuclear gene(s), except for a *wx* gene. The GMS line shows, however, higher gelatinization temperatures and a higher heat of gelatinization than its *non-waxy* counterpart, GMS 87. Because the DSC characteristics of F₁ hybrids of these lines are intermediate between the parents, GMS and GMS 87 seem not to be isogenic at the loci that determine the gelatinization characteristics of the starch. These result may be caused by pleiotropy of *waxy* gene. But this assumption is inconsistent with the result in W 0440 and (W 0440)-W 0440, *wx/wx* (Table 4, Fig.2). Therefore, the effects may be explained by genetic heterozygosity of GMS 87 lines or mutation during the process of producing the GMS line from GMS 87. GMS is also different from GMS 87 in several morphological characteristics such as the shape of seeds and leaf blades. Thus, this result is probably caused by genetic heterozygosity of GMS 87. The cytoplasm of 'Iwai-mochi' shows no effect on the expression of the *waxy* or *non-waxy* gene; the ratio of the long to short chain of the amylopectin, the amylose content of starch, and gelatinization characteristics of starches seem to be determined by nuclear genes. Both *waxy* genes derived from 'Iwai-mochi' and induced by EMS treatment expressed under the genetic background of *O. glaberrima*, and a *waxy* gene of 'Iwai-mochi' seems to reduce the amylose content of starch without affecting the ratio of the short to the long chain of amylopectin and gelatinization characteristics. The production of an isogenic *waxy* line of GMS 87 will clarify the gene(s) determining gelatinization characteristics of starch in GMS line.

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アフリカイネ (*Oryza glaberrima*) におけるモチ遺伝子座の形質発現

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本研究では, 異なる細胞質をもつモチ性およびウルチ性の *O. glaberrima* を用い, *O. glaberrima* の遺伝的背景のもとでのモチ遺伝子の発現と, その発現に細胞質が及ぼす影響について検討した. 供試系統として (1) *O. sativa* のモチ性日本品種祝糯の細胞質とウルチ性 *O. glaberrima*, W 0440 の核をもつウルチ性細胞質置換系統 (Iwai-mochi)-W 0440, Wx/Wx , (2) 祝糯の細胞質と W 0440 の核をもち, 祝糯由来のモチ遺伝子をもつ細胞質置換系統 (Iwai-mochi)-W 0440, wx/wx , (3) W 0440 の細胞質と核をもち, 祝糯由来のモチ遺伝子をもつ系統 (W 0440)-W 0440, wx/wx , (4) ウルチ性 *O. glaberrima*, GMS 87 の EMS 処理により誘発されたモチ性突然変異系統 GMS の 4 系統および対照として W 0440, 祝糯, GMS 87 の 3 系統, 計 7 系統を用い (Table 1), 胚乳澱粉を調製してヨウ素吸収曲線, 澱粉の枝切りによるアミロース含量とアミロペクチンの鎖長分布, 示差走査熱量計 (DSC) による糊化特性について調べた. また, GMS 87 と GMS を相互交配した F_1 を用い, モチ遺伝子の量的効果について検討した.

ヨード吸収曲線のパターンと枝切り澱粉のゲル濾過実験により, モチ遺伝子をもつ供試系統の澱粉はすべてアミロースを含んでいないことが明らかとなった (Table 2, Fig. 1, Table 3). したがって日本品種祝糯由来のモチ遺伝子および誘発突然変異による *O. glaberrima* のモチ遺伝子のいずれも *O. glaberrima* の遺伝的背景のもとで正常に発現されていると考えられた. しかし (Iwai-mochi)-W 0440, Wx/Wx , (Iwai-mochi)-W 0440, wx/wx , および (W 0440)-W 0440, wx/wx の 3 系統では, アミロペクチンの鎖長分布を示す一つの指標となる値であるアミロースの長鎖と短鎖の比が, 祝糯における値よりも W 0440 の値に近く, モチ遺伝子以外の核遺伝子が澱粉のアミロペクチンの鎖長分布に影響を及ぼしている可能性が考えられた. また, GMS 87 と GMS のあいだの F_1 で, 澱粉のアミロース含量に関してモチ遺伝子の量的効果が起こっていることが示唆された (Table 2). (Iwai-mochi)-W 0440, Wx/Wx , (Iwai-mochi)-W 0440, wx/wx , および (W 0440)-W 0440, wx/wx の 3 系統ではモチ遺伝子の有無にかかわらず, 澱粉の糊化特性には大きな差異がなかった (Table 4, Fig. 2). 一方, EMS で誘発されたモチ性の *O. glaberrima* GMS は, ウルチ性 *O. glaberrima* GMS 87 に比べて糊化温度が高く, 糊化熱も大きかった. これらの系統間の F_1 の糊化特性は両親の中間となった (Table 5, Fig. 3). 澱粉のアミロース含量, 糊化特性のいずれにも細胞質は影響を与えていなかった.