A rice mutant with enhanced amylose content in endosperm without affecting amylopectin structure

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The *Wx^b* gene, one of the alleles at the rice *waxy* locus, responds to environmental temperatures during seed development and, as a result, the amylose content fluctuates and affects rice quality. Seeds of a low-amylose variety ‘Snow Pearl’ could be visibly distinguished depending on whether they matured in warm or cool temperatures; based on this, we isolated a mutant candidate, namely, the enhanced amylose content (ENA) mutant, which showed enhanced amylose content in the endosperm. The amylose contents of ENA mutants that matured at 20°C and 26°C were 6–7% higher than those of Snow Pearl. As the chain length distributions of amylopectin were almost identical between ENA and Snow Pearl, the mutation was not the *amylose-extender* mutation, but a new mutation that enhanced amylose content in the rice endosperm. Furthermore, ENA and Snow Pearl had a single nucleotide polymorphism on the third exon of the *Wx* gene and a single base change at the 5′ splice junction of the first intron specific to the *Wx^b* gene. Genetic analysis revealed that enhanced amylose content is controlled by 2 recessive genes. Thus, we isolated a rice mutant with enhanced amylose content in the endosperm without affecting the amylopectin structure. As global warming or higher maturation temperatures decrease the amylose content in rice endosperms, the mutant obtained in this study would be useful in breeding programs to diversify grain quality and to understand the genetic and molecular mechanisms of amylose synthesis.

Key Words: enhanced amylose content, global warming, net amylose, rice, warm temperatures, *Wx* gene.

Introduction

The amylose content of rice endosperm starch is an important characteristic that determines the consumption and cooking qualities of rice (Juliano 1992, Chikubu 1995), although different countries have prefer rice with varying amylose contents. The amylose content is genetically controlled by a major gene (*Wx* gene, Nelson and Pan 1995) and is also affected by several modifying genes. In cultivated rice, 2 wild-type alleles, namely *Wx^a* and *Wx^b*, predominate at the *waxy* locus, which encodes the granule-bound starch synthase that is responsible for amylose synthesis in the endosperm. Rice strains having the *Wx^b* gene contain 15–20% amylose, whereas rice strains having the *Wx^a* gene contain 20–25% amylose (Sano 1984). Moreover, the levels of *Wx* gene expression and amylose synthesis are controlled by

other loci such as the *du* loci (Okuno *et al.* 1983). Mutation at the *du* loci results in decreased production of *Wx* protein, lowering amylose content in the endosperm (Yano *et al.* 1988, Hirano 1993).

In addition to genetic regulation, the environmental temperature during seed development also affects amylose synthesis in rice strains carrying the *Wx^b* allele. The levels of *Wx* protein decrease at higher temperatures, resulting in lower amylose content in mature seeds (Inatsu 1979, Asaoka *et al.* 1984, Sano *et al.* 1985). The amylose content thus varies with the year and site of cultivation, even for the same variety (Inatsu 1979, Asaoka *et al.* 1984). It is therefore important to evaluate the variation of starch characteristics in response to environmental temperatures and to develop rice varieties that do not alter their amylose content with environmental temperature.

The seeds of a low-amylose mutant can be visibly distinguished depending on whether they mature at cool or normal temperatures; based on this, we isolated a rice mutant insensitive to cool temperatures on amylose synthesis

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(Suzuki *et al.* 2002). Since global warming is a severe problem and higher temperatures affect rice quality and amylose content (Peng *et al.* 2004, Morita 2005), this method of using phenotypic changes could also be applied to isolate a rice mutant that has matured at a high temperature. In this paper, we describe the isolation and characterization of a rice mutant with enhanced amylose content in the endosperm without affecting the amylopectin structure.

Materials and Methods

Plant materials and mutant screening

To screen mutants with enhanced amylose content, we used a low-amylose rice variety 'Snow Pearl' (Higashi *et al.* 1999). Dry seeds of Snow Pearl rice were treated with 1 mM sodium azide in 0.1 M sodium phosphate buffer (pH 3.0) for 6 h at room temperature, followed by washing with tap water; they were then sown to grow M₁ plants. The harvested M₂ seeds were dehulled and, in certain cases, polished, and their seed transparency was compared with those of Snow Pearl and Nipponbare.

Temperature treatment

To investigate the response of the enhanced amylose content mutant (ENA) to environmental temperatures, growth chambers were used as previously described (Suzuki *et al.* 2002). After 5 days of anthesis, ENA and Snow Pearl plants were grown at 26°C and 20°C until seed maturation. To ascertain the genetic characteristics of ENA, F₂ and F₃ populations derived from a cross between ENA and another variety were matured at 26°C as described above. Mature seeds were polished, and F₂ and F₃ populations were segregated based on rice transparency.

Amylose content, amylopectin structure, and Wx protein determination

The amylose content of the samples was analyzed using an iodine colorimetric assay (Juliano 1971) with slight modifications (Suzuki *et al.* 2002). After starch was prepared from polished rice, the amylopectin chain length distribution in rice endosperm starch was analyzed by high-performance anion-exchange chromatography-pulsed amperometric detection (Dionex, Sunnyvale, CA, USA; Nagamine and Komae 1996, Suzuki *et al.* 2003). To estimate the amount of Wx protein, SDS-polyacrylamide gel electrophoresis and immunoblotting with antiserum against Wx protein were performed (Suzuki *et al.* 2002).

DNA analysis

Whole genomic DNA was extracted from young seedling leaves according to the cetyl trimethyl ammonium bromide (CTAB) method (Murray and Thompson 1980). The Wx gene sequence was amplified using primers described elsewhere (Bligh *et al.* 1998, Hirano *et al.* 1998, Sato and Nishio 2003). For the part of the Wx gene containing single nucleotide polymorphisms (SNPs) of Snow Pearl, gene-

specific forward (5'-TGGCTGTAAGCACACACAAACT TCG-3') and reverse (5'-ACAGGTACGAGAGGGTGAG GTTTT-3') primers were used (Nippon Suisan Kaisha 2000). The polymerase chain reaction (PCR) product was gel-purified using a QIAquick gel extraction kit (Qiagen, Tokyo, Japan), and DNA sequencing was performed using a Big Dye Terminator Cycle Sequencing kit and an ABI PRISM 3100-Avant Genetic Analyzer (Applied Biosystems, Foster City, CA).

Results

Screening of the rice mutant with enhanced amylose content

Since the amylose content profoundly affects the stickiness of cooked rice (Webb 1991, Chikubu 1995), a major objective of rice breeding is to develop rice varieties whose amylose content is not affected by temperature during seed maturation. We previously isolated a "coi" mutant insensitive to cool temperatures during seed development (Suzuki *et al.* 2002). The amylose content of coi seeds that matured at cool temperatures was almost identical to that of coi seeds at high temperatures. We crossed coi and non-glutinous varieties, but were unable to breed non-glutinous strains whose amylose content was not influenced by maturation temperatures (data not shown). Therefore, to screen a mutant that is characterized by unaltered amylose content in response to temperature changes, we used the low-amylose rice Snow Pearl as the mutagenized variety. Its amylose content was very low (approximately 5%), and the endosperm appeared chalky when seeds were matured at normal temperature (26°C) (Table 1 and Fig. 1A); however, when Snow Pearl seeds were matured at a cool temperature (20°C), the amylose content was approximately 18%, and the endosperm appeared semitranslucent. Because the amylose content of rice seed influences the degree of transparency (Suzuki *et al.* 2002), a mutant insensitive to the effect of normal (warmer) temperatures on amylose content and with (semitranslucent) seeds that mature at normal temperature should be easily distinguishable from the original variety that has low amylose (chalky) seeds; this is the basic principle for screening mutants whose amylose content remains unaltered in response to temperature changes.

The first screening was performed using M₂ seeds that matured at an average temperature of 24°C to 26°C in a

Table 1. Amylose content (%) of Snow Pearl, #14 (ENA) and Koshihikari seeds matured at different temperatures. The seeds of each plant were matured at either 20 or 26°C. The apparent amylose contents of the samples (20 mg of white rice powder) were analyzed based on an iodine colorimetric assay. n=5.

	20°C	26°C
Snow Pearl	18.3±0.8	5.2±0.5
#14 (ENA)	24.7±0.3	11.7±0.6
Koshihikari	25.1±0.8	17.0±0.3

Fig. 1. Effect of maturation temperature on seed phenotypes and the amount Wx protein in the endosperm. (A) Seed phenotypes of Snow Pearl, #14 (ENA), and Koshihikari. Rice seeds of each strain were matured at 26°C or 20°C. (B) Immunoblotting analyses of Wx protein from Snow Pearl (S), #14 (ENA) and Koshihikari (K) seeds matured at different temperatures. Seeds of each plant were matured at either 20°C or 26°C and were analyzed by immunoblotting using antiserum against rice Wx protein. N Nipponbare.

paddy field. A total of 7,200 M_1 plants were examined. Although the panicles of almost all of these plants contained exclusively chalky seeds, one of these plants contained a mixture of chalky and semitranslucent seeds; therefore, semitranslucent M_2 seeds were chosen as a mutant candidate (#14) insensitive to maturation temperature during seed development. M_2 seeds were seeded, and the amylose content of #14 (M_3 seeds) was compared with that of Snow Pearl and Koshihikari using a temperature-controlled growth chamber. The amylose content of mature seeds of #14 was approximately 12% at 26°C and 25% at 20°C (Table 1), indicating that #14 responded to maturation temperatures in a similar manner to Snow Pearl with regard to amylose content, and it was 6–7% higher in #14. Furthermore, immunoblotting analyses using antiserum against Wx protein showed that the amount of Wx protein in each strain at 20°C was higher than that at 26°C; moreover, the amount of Wx protein in #14 at each temperature was higher than that in Snow Pearl. Thus, #14 as well as the original Snow Pearl variety showed similar sensitivity to maturation temperatures with regard to amylose synthesis (Wx protein content and amylose content). On reflection, the phenotype of the endosperm in a rice mutant insensitive to higher maturation temperatures on amylose content was similar to that of a rice mutant whose amylose content increased despite its maturation temperature. Therefore, although we intended to screen a rice mutant whose amylose content did not decrease even though it matured at a higher temperature, we actually screened a mutant whose amylose content was enhanced. Hereafter, mutant #14 is designated as ENA (enhanced amylose content) mutant line.

Enhancement of net amylose content in the ENA mutant

In this experiment, amylose content in the endosperm was determined by an iodine colorimetric method, which is rapid and simple for measuring a large number of samples (Juliano 1971). This method, however, has a tendency to overestimate the net amylose content in the case of long amylopectin chain-containing seeds (Baba 1986, Takeda *et al.* 1987); therefore, the amylose content was analyzed based on the method termed “apparent amylose.” Yano *et al.* (1988) reported the *amylose-extender* (*ae*) mutant, which shows increasing levels of apparent amylose content. This increase in amylose content is caused by a dramatic reduction in the activity of the starch-branching enzyme IIb and an increase in the proportion of long amylopectin chains (Nishi *et al.* 2001). Hence, to clarify whether the amylose content in ENA was increased by the *ae* mutation, the amylopectin chain length distribution in rice endosperms was analyzed by high-performance anion-exchange chromatography (Fig. 2). The chain length distribution of amylopectin was almost identical between Snow Pearl and ENA, both of which matured at the same temperature. In addition, while the endosperm appearance of EM10 (an *ae* mutant) was usually floury (Sato *et al.* 2003), that of ENA, as well as those of Snow Pearl and Koshihikari, was not (i.e., wild type). These results indicated that the mutation in ENA was probably not the *ae* mutation reported previously (Nishi *et al.* 2001), but a new mutation that enhanced the amylose content of the rice endosperm without affecting its amylopectin structure.

Comparison of the Wx gene sequences

Snow Pearl has an SNP on the third exon of the *Wx* gene, with a mutation from G to A (Nippon Suisan Kaisha 2000) (Fig. 3A), and the SNP is one of the causative genes

Fig. 2. The relative amount (%) of α -1,4-glucan chains of α -polyglucans prepared from rice endosperms matured at either (A) 26°C or (B) 20°C. DP Degree of polymerization of the chains, SP Snow Pearl.

of low amylose content in the endosperm. If the reverse mutation, i.e., from A to G, occurred in the SNP in M_0 seeds of mutagenized Snow Pearl, a rice mutant with enhanced amylose content could be screened; therefore, part of the ENA *Wx* gene (344 base pairs) containing the SNP was compared with those of Snow Pearl and Koshihikari (Fig. 3A). The SNP of ENA was identical to that of Snow Pearl but not to that of Koshihikari, indicating that factors other than the back mutation (A to G) in the region of the *Wx* gene were responsible for the enhanced amylose content in the ENA mutant.

Second, we examined the other SNP at the 5' splice junction of the *Wx* gene's first intron because the mutation from GG to GT results in low levels of mature transcripts as well as the gene product (Bligh *et al.* 1998, Hirano *et al.* 1998, Isshiki *et al.* 1998). Since Snow Pearl contains GT, it is possible that ENA contains GG; therefore, to examine the possibility that the increased level of amylose in ENA is due to the mutation from GT to GG at the 5' splice junction, DNA fragments around the 5' splice junction of the first intron in each strain were amplified by PCR, and the sequences were compared (Fig. 3B). The resulting sequences of fragments from Snow Pearl and ENA were identical. Furthermore, the nucleotide sequences of the *wx* locus (*Wx* gene and promoter region) of Snow Pearl and ENA were identical (data not shown). These results suggested that the back mutation from GT to GG in ENA did not occur and that the mutation in ENA was not on the *wx* locus.

Inheritance mode of the enhanced amylose content characteristic

The mode of inheritance of the enhanced amylose content characteristic was analyzed by reciprocally crossing ENA with Snow Pearl. Seeds of Snow Pearl, ENA, and F_1 plants were matured at 26°C. The distribution of amylose content in F_2 seeds derived from reciprocal crosses fitted the ratio of 15 Snow Pearl-type seeds: 1 ENA-type seed (Fig. 4).

A	Snow Pearl	ACGCTTGG A ATACCAGCGTT
	ENA	ACGCTTGG A ATACCAGCGTT
	Koshihikari	ACGCTTGG G ATACCAGCGTT
B	Snow Pearl	CATCTGCAAG T TATACATAT
	ENA	CATCTGCAAG T TATACATAT
	Koshihikari	CATCTGCAAG T TATACATAT
	Lemont	CATCTGCAAG G TATACATAT

Fig. 3. Nucleotide polymorphisms on the third exon of the *Wx* gene (A) and the 5' splice junction of the first intron (B). Arrow and bold characters indicate polymorphisms in specific regions.

We also examined segregations of F_3 seeds from 85 F_2 plants derived from a cross between ENA and Nipponbare based on the endosperm phenotype (seed transparency and amylose content) (Table 2). Although some F_2 phenotypes could not be clearly distinguished, we found that at least 2 F_2 plants (04F2-3 and 04F2-66) fitted the ratio of 0 Nipponbare-type F_3 seeds: 15 Snow Pearl-type F_3 seeds: 1 ENA-type F_3 seed (Fig. 5). Type H will not be detected if it is controlled by *Wx* and 1 other gene but will be detected if it is controlled by *Wx* and 2 other genes (Table 2 and Fig. 5). Thus, the results of these F_2 and F_3 seeds from F_1 and F_2 plants, respectively, indicate that the enhanced amylose content characteristic of ENA mutants could be controlled by 2 recessive genes besides the *Wx* gene, and we isolated a new rice mutant (ENA) whose amylose content in the endosperm was enhanced, as compared with the original Snow Pearl variety.

Fig. 4. Segregation of F_2 seeds derived from reciprocal crosses of Snow Pearl and ENA based on the seed amylose content. (A and C) F_2 seeds from the cross ENA $\times$ Snow Pearl and Snow Pearl $\times$ ENA. (B and D) Seeds of Snow Pearl and ENA. Each F_1 plant was grown and matured at 26°C. F_2 seeds of each F_1 plant were segregated based on the seed amylose content, and their transparency was determined. For F_2 seeds from the cross ENA $\times$ Snow Pearl, χ^2 (15 : 1) = 0.52 and P = 0.47. For F_2 seeds from the cross Snow Pearl $\times$ ENA, χ^2 (15 : 1) = 2.39 and P = 0.12. F2-Chk chalky phenotype F_2 seeds, F2-Stl semitranslucent phenotype F_2 seeds, SP Snow Pearl.

Table 2. Expected segregation ratio of F_3 seeds derived from a cross between ENA and Nipponbare. F_3 seeds from 85 F_2 plants derived from a cross between ENA and Nipponbare are classified based on their endosperm phenotype and amylose content; that is, wild type (Koshihikari), Snow Pearl type (Snow Pearl) and ENA type (ENA) (see Table 1 and Fig. 1A, 26°C). Segregation types E and H will never be detected in a mutant controlled by 1 recessive gene other than the Wx gene but in a mutant controlled by 2 recessive genes other than the Wx gene.

Type	Segregation ratio of F_3 seeds			Expected segregation ratio of F_2 plants by additional genes besides Wx		Segregation ratio of F_2 plants		
	wild type	Snow Pearl type	ENA type	2-genes	1-gene	Observed ^a	Expected	
A	1	0	0	16	4	22	21.3	21.3
B ^b	3	1	0	14	2			
C ^b	3	0	1	2	2			
D ^b	12	3	1	8	4			
E ^b	48	15	1	8	0			
F ^c	0	1	0	7	1	26	21.3	21.3
G ^c	0	3	1	4	2			
H ^c	0	15	1	4	0			
I ^c	0	0	1	1	1			
Total				64	16	85	85.0	85.0

^a χ^2 (3 : 1) = χ^2 (15 : 1) = 1.80 (d.f. = 2; P = 0.407).

^b Types B, C, D, and E cannot be clearly distinguished.

^c Types F, G, H, and I cannot be clearly distinguished.

Discussion

In these studies, we isolated a rice mutant, ENA, whose amylose content in the endosperm was enhanced without effecting the amylopectin structure (Table 1 and Fig. 2). With respect to amylose synthesis, the suppression of Wx gene expression or the reduction of amylose content has been

studied extensively (Okuno *et al.* 1983, Sato 2002, Satoh *et al.* 2003, Suzuki 2006, Ando *et al.* 2007), but genes that enhance the amylose content in seeds have not been described. Thus far, the apparent amylose content-enhancing mutations reported were GG/GT in the 5' splicing site of the first intron in the Wx gene (Bligh *et al.* 1998, Hirano *et al.* 1998, Isshiki *et al.* 1998) and the *ae* mutation in the starch-branching

Fig. 5. Segregation of F₃ seeds from F₂ plants (04F2-3 and 04F2-66) that were derived from crosses between ENA and Nipponbare based on the amylose content. F₃ seeds are represented as 04F2-3 (A) and 04F2-66 (C). (B) and (D) indicate the results for Snow Pearl and ENA. Each F₁ plant was grown and matured at 26°C. F₃ seeds of each F₂ plant were segregated based on their amylose content. For F₃ seeds from 04F2-3, χ^2 (15 : 1) = 0.87 and P = 0.35. For F₃ seeds from 04F2-66, χ^2 (15 : 1) = 1.44 and P = 0.23. SP Snow Pearl. Because all F₃ seeds were low amylose chalky grains and enhanced amylose semitranslucent grains, seeds of Snow Pearl and ENA were used as references.

enzyme IIb (Yano *et al.* 1988, Nishi *et al.* 2001). GG, however, drastically enhances the quantity of Wx protein as well as the amylose content in the endosperm as compared with GT. In *ae* mutants, the higher apparent amylose content is not caused by an increase in net amylose content but by an increase in long amylopectin chains (Nishi *et al.* 2001). Thus, both the replacement of GT with GG and the *ae* mutation cause a significant increase in the amylose content and significantly alter consumption quality. On the other hand, the mutant ENA enhances amylose content in the rice endosperm without affecting its amylopectin structure (Table 1 and Fig. 2); therefore, the unique characteristics of ENA could be advantageous in producing a greater number of rice varieties of high yield and good quality.

To use ENA as the mother strain for breeding new varieties, we have to consider that the enhanced amylose content characteristic of ENA was regulated by 2 recessive genes, i.e., by multiple genes (Table 2, Fig. 4 and Fig. 5), other than the *Wx* genes. To breed new low amylose rice varieties with enhanced amylose content as parent lines, we could easily distinguish low amylose chalky grains from enhanced amylose semitranslucent grains on the basis of endosperm transparency (Fig. 1, at 26°C); however, we could not breed new medium- and high-amylose rice varieties with enhanced amylose content as parent lines because endosperm transparency could not be used as a simple and easy screening method for distinguishing *ena* alleles. Recently, we have been isolating some strains with 1 of the 2 recessive, multiple genes. Using these strains, it might be possible to map the *ena* character and breed new varieties with enhanced amylose content grains. At the same time, this *ena*

character would be useful for understanding the genetic and molecular mechanisms of amylose synthesis.

It should also be noted that rice quality is related to global warming. Annual mean temperatures have increased over the last few decades, and the mean temperature during the dry cropping season and the corresponding rice grain yield have been closely linked (Zakaria *et al.* 2002, Peng *et al.* 2004). In Japan, high maturation temperatures are severely affecting the quality of rice; with regard to rice appearance, the presence of a white core and milky white color, etc., have been observed (Matsumura 2005, Morita 2005). Furthermore, higher environmental temperatures during seed development are also causing a decrease in the amylose content (Asaoka *et al.* 1984, Suzuki 2006). As lower amylose content increases the stickiness of rice, the consumption quality of some varieties fluctuates; therefore, ENA could be a valuable parent strain for enhancing the amylose content of grains under warmer cultivation conditions. In addition, we should also note that other seed components of many crops are affected by warmer temperatures. As observed with regard to the amylose content of rice seeds, warmer temperatures during seed development decrease the anthocyanin content of dark purple rice (Kobayashi *et al.* 2001), the red color of apples (Sugiura 2002), and the purple color of eggplants (Nothmann *et al.* 1978). If the genes responsible for the *ena* phenotype were identified and found to resemble the genes decreasing the other seed components, *ena* genes could be effective to increase the levels of seed components like anthocyanin and thus improve the quality of these crops.

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