

Interactions Among *Wx* Genes and Effects of a Mutated *Wx-D1e* Allele on Starch Properties in Wheat

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ABSTRACT

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The waxy mutant wheat Tanikei A6599-4 contains little amylose and exhibits a stable hot paste viscosity. It has null alleles at the *Wx-A1* and *Wx-B1* loci and a mutated allele at the *Wx-D1* locus (*Wx-D1e*). From the cross-combination of Kanto 123 (normal amylose line) and Tanikei A6599-4, 51 DH (doubled haploid) lines were produced and their genotypes were determined by SDS-PAGE and a derived cleaved amplified polymorphic sequence (dCAPS) analysis. Eight genotypes were obtained at the expected ratio. Using a Rapid Visco Analyser (RVA), all the lines

with the same genotype as Tanikei A6599-4 showed a stable hot paste viscosity. The other lines did not show a stable hot paste viscosity regardless of the presence of the *Wx-D1e* allele. When two genotypes with the same *Wx-A1* and *Wx-B1* alleles were compared, the one with *Wx-D1e* contained less amylose and exhibited a lower final viscosity and a lower setback with RVA. Although the *Wx-D1e* allele produces an almost inactive *Wx* protein, these findings suggest that this allele contributes at some level to starch synthesis.

Amylose synthesis in the cereal grain endosperm is controlled primarily by the granule-bound starch synthase I (GBSS I), also known as the *Wx* protein (Echt and Schwartz 1981; Sano 1984; Rohde et al 1988). In hexaploid wheat (*Triticum aestivum* L.), there are three copies of the *Wx* gene, and normal wheat has three active *Wx* alleles, termed *Wx-A1a*, *Wx-B1a*, and *Wx-D1a*, which code *Wx* protein isoforms, *Wx-A1*, *Wx-B1*, and *Wx-D1*, respectively (Chao et al 1989; Nakamura et al 1993). Waxy (amylose-free) wheat lines with null alleles at all three *Wx* loci, and which do not express any of the *Wx* protein, have been produced recently (Nakamura et al 1995; Kiribuchi-Otobe et al 1997; Yasui et al 1997).

A wheat mutant, Tanikei A6599-4, which, while not amylose-free, expresses only small amounts of amylose, is a different type of waxy wheat (Kiribuchi-Otobe et al 1998). This line has been shown to have null alleles at the *Wx-A1* and *Wx-B1* loci (*Wx-A1b*, *Wx-B1b*) and a mutated allele at the *Wx-D1* locus (*Wx-D1e*) (Kiribuchi-Otobe et al 2001; Yanagisawa et al 2001). While the amount of *Wx-D1* protein coded by the *Wx-D1e* gene is equal to that in its original line, Tanikei A6099 (a low-amylose line), almost all of the protein present in Tanikei A6599-4 is enzymatically inactive due to a point mutation that generates a single amino acid substitution from alanine to threonine (Yanagisawa et al 2001). Tanikei A6599-4 exhibited a unique pasting curve with stable hot paste viscosity as measured by the Rapid Visco Analyser (RVA). Our previous genetic analysis on this strain showed that both waxy character and stable hot paste viscosity are controlled by the same mutated *Wx-D1e* allele (Kiribuchi-Otobe et al 2001). However, the effect of *Wx-D1e* in the presence of active *Wx-A1a* and *Wx-B1a* genes remained unclear. We have therefore analyzed the interactions among the three *Wx* genes, as well as the effects of *Wx-D1e* on amylose content and starch pasting properties.

MATERIALS AND METHODS

Plant Materials

A normal amylose line 'Kanto 123' [*Wx-A1a Wx-B1a Wx-D1a*] and the mutant line 'Tanikei A6599-4' [*Wx-A1b Wx-B1b Wx-D1e*]

were crossed in 1997. From the F₁ hybrids, 51 homozygous doubled haploid (DH) lines were obtained by the haploid breeding method using the improved maize technique (Suenaga and Nakajima 1989; Ushiyama et al 1991). The second-generation doubled haploid (DH₂) lines were sown in a field in October 1999 and harvested in June 2000. The third-generation doubled haploids (DH₃) were sown in October of the same year and harvested in June 2001.

Amylose Content

Grains of each DH₃ and parental line were milled into flour using a Brabender Quadrumat Jr. laboratory mill (Brabender OHG, Duisburg, Germany), and the starch was isolated by a water wash and centrifugation. The amylose content of the starch was colorimetrically determined using an AutoAnalyser II (Bran+ Luebbe, Norderstedt, Germany), as described previously (Oda et al 1992). A regression curve was generated using wheat starch whose amylose content had been already measured by amperometric titration (Norin 61, 31.8%; Kanto 107, 20.9%; Tanikei A6099, 17.7%; Tanikei A6599-4, 1.6%; Tanikei H1881, 0.4%) (Kiribuchi-Otobe et al 1998). All measurements were made in duplicate.

Starch Paste Viscosity

Starch paste viscosity was measured using the Rapid Visco Analyser (RVA) (Newport Scientific, Warriewood, Australia). Starch (3 g, 13.5% mb) was mixed with 25 mL of distilled water, and the suspension was heated from 34 to 94°C at a rate of 5°C/min, held at 94°C for 5 min, then cooled to 34°C at a rate of 5°C/min.

SDS-PAGE

Wx proteins were extracted and subjected to the modified SDS-PAGE according to the method of Zhao and Sharp (1996). After electrophoresis, the gel was stained with a silver staining kit (Wako Pure Chemicals Industries, Osaka, Japan).

dCAPS Analysis

Genomic DNA was extracted from young leaves using an Isoplant kit (Nippon Gene, Tokyo, Japan) and subjected to a polymerase chain reaction (PCR) using the Gene Amp PCR System 2400 (Applied Biosystems Japan, Tokyo, Japan) as described by Yanagisawa et al (2003). The first PCR step was conducted using the *Wx-D1* gene specific primers F1 (CAATGGCATCTACAGGCCGCA) and R1 (GACGGCGAGGAAGCTTGCTCTGG). The second (nested) PCR was conducted using primers F2 (CTTCTCCTTCGACGACTTCG) and R2 (GTCGAGCTCGCAGCCACCGG). R2 is a dCAPS (derived cleaved amplified polymorphic sequence) primer that was designed to create an *Age* I site (ACCGGT) in the *Wx-D1e* gene (Fig. 1).

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RESULTS

The modified SDS-PAGE separated the three Wx protein isoforms (Wx-A1, Wx-B1, Wx-D1) into three distinct gel bands. Because *Wx-A1b* and *Wx-B1b* are null alleles that produce no protein bands, it was easy to determine the genotype of the *Wx-A1* and *Wx-B1* loci. However, it was difficult to distinguish *Wx-D1a* from *Wx-D1e*, because both alleles produce a band with the same mobility. Therefore, we utilized a dCAPS marker to determine the genotype of the *Wx-D1* locus. When nested PCR products (208 bp) were treated with *Age I*, the *Wx-D1e* allele was digested, yielding a 192-bp fragment, while the *Wx-D1a* allele was not digested. By combining SDS-PAGE and dCAPS analysis, we determined the genotypes of all the DH lines. As shown in Table I, eight genotypes were observed, and their segregation ratio fit the expected ratio of 1:1:1:1:1:1:1:1 ($\chi^2 = 5.00$; $0.5 < P < 0.75$).

When we performed RVA analysis, we found that all nine lines with the same genotype as Tanikei A6599-4 [*Wx-A1b Wx-B1b Wx-D1e*] possessed stable hot paste viscosity. The other lines did not show stable hot paste viscosity regardless of the presence of the mutant *Wx-D1e* gene (Fig. 2). Peak temperature in RVA also showed a significant difference only between the [*Wx-A1b Wx-B1b Wx-D1e*] genotype and other genotypes (Table II). When two genotypes with the same *Wx-A1* and *Wx-B1* alleles (e.g., [*Wx-A1a Wx-B1b Wx-D1a*] and [*Wx-A1a Wx-B1b Wx-D1e*]) were compared, the genotype with *Wx-D1e* usually contained less amylose and exhibited a significantly lower final viscosity and a lower setback than the genotype with *Wx-D1a*. There were, however, a few exceptions—two cases had no difference (final viscosity of [*Wx-A1a Wx-B1a Wx-D1a*] and [*Wx-A1a Wx-B1a Wx-D1e*], with a setback of [*Wx-A1a Wx-B1b Wx-D1a*] and [*Wx-A1a Wx-B1b Wx-D1e*]). These findings suggest that the *Wx-D1e* gene affects amylose content, final viscosity, and setback. With respect to peak viscosity and breakdown, a significant effect of *Wx-D1e* was only detected between [*Wx-A1b Wx-B1b Wx-D1a*] and [*Wx-A1b Wx-B1b Wx-D1e*].

DISCUSSION

An unusual feature of Tanikei A6599-4 is the stable hot paste viscosity of its starch. Because the simulated starch mixture (1.6% amylose) of waxy (amylose-free) wheat and nonwaxy wheat did not show this stability (Kiribuchi-Otobe 1998), we hypothesized that the presence of small amounts of amylose is not the primary reason for this stability. Although it was reported that the amylopectin structures of waxy (amylose-free) wheats and their

nonwaxy parents were identical (Yasui et al 1996; Miura et al 2002), there was a possibility that *Wx-D1e* of Tanikei A6599-4 affected the amylopectin structure and caused the stable hot paste viscosity. If so, it was possible that all lines containing *Wx-D1e* may have the same changed amylopectin structure and show certain amounts of stability. Therefore, we compared pasting curves of the genotypes with *Wx-D1e* with those with *Wx-D1a*. Stable hot paste viscosity was not observed in the lines with *Wx-D1e*, except for those with the same genotype as Tanikei A6599-4 [*Wx-A1b Wx-B1b Wx-D1e*]. Thus, it is unclear whether *Wx-D1e* affects amylopectin structure.

Yamamori and Quynh (2000) crossed each of two low-amylose lines, 'Kanto 107' and 'Saikai 173' [*Wx-A1b Wx-B1b Wx-D1a*], to the 'Bai-Huo' strain [*Wx-A1a Wx-B1e Wx-D1b*], which contains a variant Wx-B1 protein, obtaining eight genotypes. They reported that the amylose content was influenced the most by the change from *Wx-B1a* to *Wx-B1b* (Wx-B1 protein deficiency), followed by the change from *Wx-D1a* to *Wx-D1b* (Wx-D1 protein deficiency), and least by the change from *Wx-A1a* to *Wx-A1b* (Wx-A1 protein deficiency). Miura et al (2002) showed almost the same results using near-isogenic lines with a common genetic background of 'Chinese Spring', but statistically no significant difference was detected between Wx-B1 deficiency and Wx-D1 deficiency. In both cases, the effect of Wx-D1 protein deficiency was larger than that of Wx-A1 protein deficiency. On the other hand, the amylose content of our materials was influenced the most by the change from *Wx-B1a* to *Wx-B1b* (Wx-B1 protein deficiency), followed by the change from *Wx-A1a* to *Wx-A1b* (Wx-A1 protein deficiency), and least by the change from *Wx-D1a* to *Wx-D1e* (production of almost inactive Wx-D1 protein). The reversed order of the influence of the *Wx-A1* and *Wx-D1* loci suggests that *Wx-D1e* contributes to starch synthesis to some extent but we cannot conclude that it contributes to amylose synthesis. Because we have not clarified the effect of the *Wx-D1e* gene on amylopectin synthesis,

TABLE I
Number of Lines of Each Genotype

<i>Wx-A1</i>	<i>Wx-B1</i>	<i>Wx-D1</i>	No. of Lines
a	a	a	4
a	a	e	8
b	a	a	5
b	a	e	9
a	b	a	4
a	b	e	7
b	b	a	5
b	b	e	9

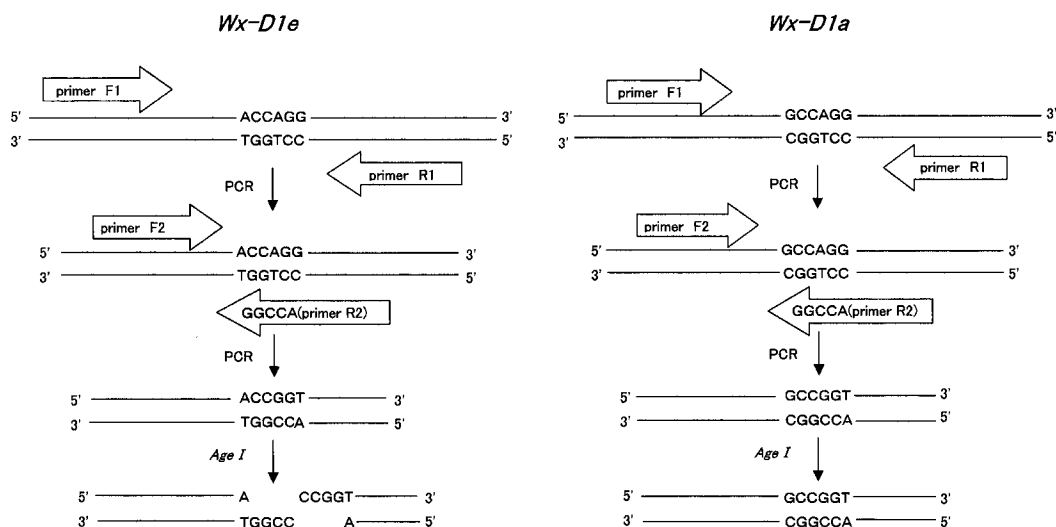


Fig. 1. Detection of single nucleotide polymorphism (SNP) by dCAPS analysis.

TABLE II
Amylose Content and Starch Pasting Properties of Eight Genotypes

Genotype			Amylose Content	Peak Viscosity	Peak Temp.	Breakdown	Final Viscosity	Setback
<i>Wx-A1</i>	<i>Wx-B1</i>	<i>Wx-D1</i>						
a	a	a	33.0 ± 0.5 ^a	266 ± 19a	93.9 ± 0.1a	109 ± 12a	441 ± 28a	176 ± 17a
a	a	e	30.1 ± 2.1b	295 ± 26ab	93.9 ± 0.1a	133 ± 22ab	415 ± 25a	119 ± 31b
b	a	a	29.6 ± 0.7bc	295 ± 18ab	93.8 ± 0.1a	133 ± 21ab	408 ± 31a	114 ± 44b
b	a	e	26.9 ± 1.6d	301 ± 24ab	93.9 ± 0.1a	152 ± 24bc	358 ± 25b	57 ± 38c
a	b	a	27.7 ± 4.0cd	321 ± 19b	93.8 ± 0.0a	183 ± 15c	340 ± 26b	19 ± 25d
a	b	e	20.4 ± 1.6e	313 ± 22b	93.8 ± 0.1a	174 ± 14c	292 ± 26c	-21 ± 18d
b	b	a	21.9 ± 1.7e	302 ± 19ab	93.9 ± 0.1a	171 ± 12c	287 ± 28c	-15 ± 11d
b	b	e	2.1 ± 0.7f	428 ± 25c	81.9 ± 5.6b	308 ± 21d	170 ± 6d	-258 ± 20e

^a Means ± standard deviation. Values followed by the same letter in the same column are not significantly different ($P < 0.05$) by Newman-Keuls method.

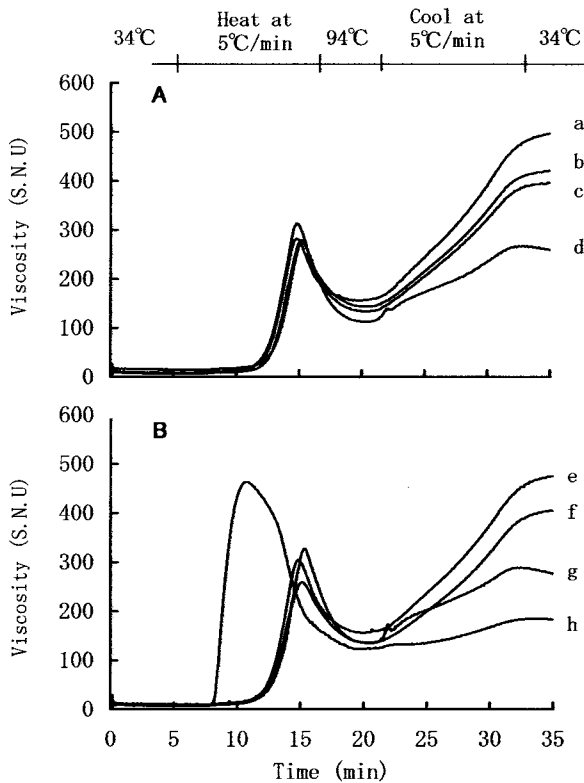


Fig. 2. Pasting curves of eight genotypes. **A**, Lines with *Wx-D1a* allele: a, Tanikei H3675 [*Wx-A1a Wx-B1a Wx-D1a*]; b, Tanikei H3654 [*Wx-A1b Wx-B1a Wx-D1a*]; c, Tanikei H3660 [*Wx-A1a Wx-B1b Wx-D1a*]; d, Tanikei H3700 [*Wx-A1b Wx-B1b Wx-D1a*]. **B**, Lines with *Wx-D1e* allele: e, Tanikei H3670 [*Wx-A1a Wx-B1a Wx-D1e*]; f, Tanikei H3677 [*Wx-A1b Wx-B1a Wx-D1e*]; g, Tanikei H3689 [*Wx-A1a Wx-B1b Wx-D1e*]; h, Tanikei H3656 [*Wx-A1b Wx-B1b Wx-D1e*].

it is possible that the amylopectin of Tanikei A6599-4 has long external chains that form complexes with iodine. If such complex formation does occur, it would explain the increase in apparent amylose content determined from iodine affinity.

CONCLUSIONS

We studied the interactions among the three *Wx* genes and the effects of *Wx-D1e* on amylose content and starch pasting properties. Although stable hot paste viscosity was observed only in lines with the same genotype as Tanikei A6599-4, it was clear that *Wx-D1e* gene affected the final viscosity and setback. Starch with novel properties has latent value in the food industry (Mauro 1996). There may be a special *Wx* genotype whose flour or starch is suitable for special processed foodstuffs.

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