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Detection of single nucleotide polymorphism (SNP) controlling the waxy character in wheat by using a derived cleaved amplified polymorphic sequence (dCAPS) marker

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Abstract We investigated a single nucleotide polymorphism (SNP) in the *Wx-D1* gene, which was found in a mutant waxy wheat, and which expressed the Wx-D1 protein (granule-bound starch synthase I) as shown by immunoblot analysis. We also assayed starch synthase activity of granule-bound proteins. Using 22 doubled-haploid (DH) lines and 172 F_5 lines derived from the wild type × the mutant, we detected SNP via a PCR-based (dCAPS) marker. Amplified PCR products from *Wx-D1* gene-specific primers, followed by mismatched primers designed for dCAPS analysis, were digested with the appropriate restriction enzyme. The two alleles, and the heterozygote genotype were easily and rapidly discriminated by gel-electrophoresis resolution to reveal SNP. All progeny lines that have the SNP of the mutant allele were waxy. Integrating the results of dCAPS analysis, immunoblot analysis and assays of starch synthase activity of granule-bound proteins indicates that the SNP in the *Wx-D1* gene was responsible for its waxy character. This dCAPS marker is therefore useful as a marker to introduce the mutant allele into elite breeding lines.

Keywords Wheat · *Waxy* gene · dCAPS marker · SNP · Starch synthase

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Introduction

Cereal grain endosperm starch generally consists of both amylose and amylopectin. Amylose content is used as a parameter for characterizing starch and plays an important role in wheat quality because it affects starch properties. When starch is composed exclusively of amylopectin, it is called waxy. Waxy wheat expands the possibility of new flour uses in food and non-food industries.

The Waxy (Wx) protein, a *waxy* (*Wx*) gene product, is granule-bound starch synthase I (GBSSI) responsible for amylose synthesis in storage tissue (Nelson and Rines 1962; Echt and Schwarz 1981). Hexaploid wheats have three *Wx* genes, *Wx-A1, B1* and *D1*, coding for Wx protein isoforms, Wx-A1, B1 and D1 (Chao et al. 1989; Nakamura et al. 1993). 'Tanikei A6599-4 (Wheat Norin PL8),' containing 1.6% amylose content, was assumed to be a waxy mutant wheat, induced from Tanikei A6099 (amylose content 17.7%) by sodium azide mutagenesis, and exhibiting a unique starch pasting curve (Kiribuchi-Otobe et al. 1998). Tanikei A6099 was induced from Kanto 107 by ethyl methane sulfonate treatment (Oda et al. 1992). These lines do not have Wx-A1 and B1 proteins (*Wx-A1b, Wx-B1b*: null alleles in the *Wx-A1* and *B1* gene). Point mutation in the *Wx-D1* gene results in amino-acid substitution (A→T) at position 258 in the mature Wx-D1 protein in Tanikei A6599-4 (Yanagisawa et al. 2001), and this gene was assigned the allele designation *Wx-D1e*.

As point mutations or single nucleotide polymorphisms (SNPs) in genes can result in a dramatic change of phenotype in organisms, SNP detection is crucial in plant molecular genetics and breeding research for rapidly and easily identifying alleles.

PCR-based markers are widely used as a means of rapidly and reliably detecting a SNP that creates unique restriction sites that differentiate a pair of alleles. dCAPS analysis enables us to discriminate between alleles that differ by single nucleotide changes, which do not generate restriction-site differences (Michaels and Amasino 1998;

Neff et al. 1998). Amplified PCR fragments from mismatched PCR primers were used to create restriction sites digested by an appropriate restriction enzyme and resolved by gel electrophoresis.

This paper is concerned with studying the dCAPS marker to detect a SNP in the *Wx-D1e* allele responsible for GBSSI activity.The dCAPS marker is useful for identifying alleles at the molecular level and for introducing the waxy character via the *Wx-D1e* allele to elite lines.

Materials and methods

Plant materials

For analysis, we used 22 doubled-haploid (DH) lines from the cross of Kanto 118 (*Wx-D1a*) × Tanikei A6599-4 (*Wx-D1e*), and 172 F_5 lines from the cross of Kanto 119 (*Wx-D1a*) × Tanikei A6599-4 (*Wx-D1e*). DH lines were produced by the maize cross method in which F_1 plants of Kanto 118 × Tanikei A6599-4 were pollinated with maize pollen followed by immature embryo culture (Inagaki et al. 1997). Kanto 118 and Kanto 119 (*Wx-A1b, Wx-B1b, Wx-D1a*) are breeding lines whose maternal parent is Kanto 107 (*Wx-A1b, Wx-B1b, Wx-D1a*).

Immunoblot analysis

SDS polyacrylamide-gel electrophoresis (SDS-PAGE) was conducted according to Yanagisawa et al. (2001), and immunoblot analysis was conducted as described by Suzuki et al. (1993) using a polyclonal antiserum against the purified rice Wx protein (Hirano and Sano 1991). Wx protein was extracted from the starch fraction of mature and immature seeds, then electroblotted onto a PVDF membrane. Waxy rice (Kijyu-mochi), non-waxy rice (Nipponbare), non-waxy wheat (Norin 61, Kanto 107 and Saikai 168) and amylose-free waxy wheat lines (Tanikei H1881–1885; Kiribuchi-Otobe et al. 1997, 1998) were used as controls.

Starch synthase activity of granule-bound proteins

Immature seeds (Tanikei A6099, A6599-4 and H1881) were collected 15 days after anthesis by removing grains from panicles, immediately frozen in liquid nitrogen, and stored at –80 °C until processed. Starch synthase activity was measured according to Nakamura et al. (1989).

Extraction of mRNA and total DNA

mRNA was extracted from immature embryos 15 days after anthesis using an RNeasy Plant Mini Kit (Qiagen) and cDNAs were constructed using a cDNA synthesis module (Amersharm Pharmacia Biotech). Total DNA was extracted from young leaves using Isoplant (Nippon gene) in both DH lines and F_5 lines.

PCR amplification, restriction enzyme digestion and electrophoresis

PCR was conducted using a Gene Amp PCR system 2400 (Applied Biosystems; Japan). First PCR was conducted using a *Wx-D1* gene-specific primer and nested (second) PCR was conducted using a dCAPS primer. PCR amplification was conducted in a volume of 50 μl, containing Ex *Taq* buffer, 2.5 mM of the dNTP mixture, 0.4 μM of primer and a digested DNA fragment amplified by nested PCR. After an initial step at 95 °C for 5 min, 30 cycles at 95 °C for 30 s, and 60 °C for 30 s and 72 °C for 1 min, were conducted: PCR products of 22 DH lines were cloned in the pCR2.1-TOPO vector (TOPO TA Cloning Kit; Invitrogen) and analyzed using a DNA sequencer (ABI 377). PCR products were digested with appropriate restriction enzymes (5 units) by adding 10 μl of the appropriate 1× buffer, and incubated at the temperature recommended by the manufacturer's protocol for 1 h. Samples were separated by gel electrophoresis on 4% Nusieve GTG agarose gel (FMC) in 0.5× TBE for 1 h, then visualized with ethidium bromide (10 μg/ml).

KI–I2 solution analysis

Cut half-grains were immersed in KI–I_2 solution (potassium iodide 0.4%, iodine 0.02%). Non-waxy grains were stained purple and waxy grains stained dark-brown.

Results

Immunoblot analysis

Immunoblot analysis was conducted to obtain evidence that the mutant produces the Wx-D1 protein (Fig. 1). Antiserum against the rice Wx protein recognized the wheat Wx protein (Fig. 1; lanes 3–6), including the Wx-D1 protein of the *Wx-D1e* allele (lane 12), but no protein band was recognized by the antiserum in amylose-free waxy lines (Fig.1; lanes 7–11).

Starch synthase activity of granule-bound proteins

Starch synthase activities of granule-bound proteins of the mutant and wild-type were measured (Table 1). Tanikei H1881 is another waxy line, which has null alleles at three *Wx* genes (no Wx protein) (Kiribuchi-Otobe et al. 1997). The activity from immature seeds, which were harvested over the period of seed development for 15 DPA, was measured. Experiments were repeated three times and similar patterns obtained. The activity of Tanikei A6599-4 was less than half that of Tanikei A6099 and similar to that of Tanikei H1881 (amylose-free; no Wx protein).

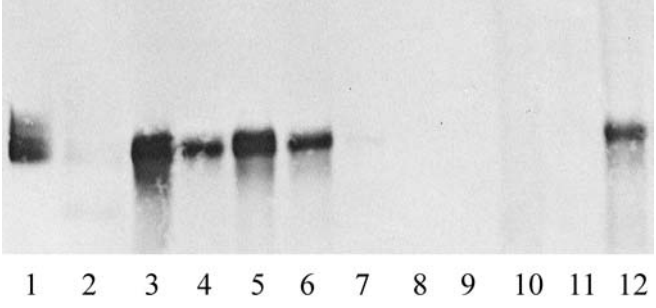

Fig. 1 Immunoblot analysis of waxy proteins. *Lane 1*: Nipponbare (rice); *lane 2*: Kijyumochi (waxy rice); *lane 3*: Norin 61; *lane 4*: Kanto 107; *lane 5*: Saikai 168; *lane 6*: Tanikei A6099; *lanes 7–11*: Tanikei H1881–1885 (amylose-free waxy wheat); *lane 12*: Tanikei A6599-4. Molecular size of detected signals are 60 kDa

Table 1 Starch synthase activity of granule-bound proteins in developing endosperm. Values represent the means ± SE of the results of three seperate experiments

Tanikei A6099 (non-waxy)	0.0718 ± 0.0086 (nmol min^{-1} $grain^{-1}$)
Tanikei A6599-4 (Waxy)	0.0286 ± 0.0020
Tanikei H1881 (Waxy: no Wx protein)	0.0324 ± 0.0030

Table 2 PCR primers used in this experiment. Sequences underlined are mismatches with template DNA

*Age*I digestion	1st primer		Forward 1	CAATGGCATCTACAGGGCCGCA
			Reverse 1	GACGGCGAGGAACTTGTCCTTGG
	Nested primer		Forward 2	CTTCTCCTTCGACGACTTCG
		dCAPS	Reverse 2	GTCGAGCTCGCAGCCACCGG
*Dde*I digestion	1st primer		Forward 3	GCTGACGGTGAGCCCCTACTAC
			Reverse 3	CCCTCACCTTGCTCGGGAAT
	Nested primer	dCAPS	Forward 4	CGGAGGAGCTCATCTCTGGCTAA
			Reverse 4	TGCAGCGCCTCCTTGTTCAG

CAATGGCATCTACAGGGCCGCAAAGgttttgcatcttcttctcaaactatatatcctctctgcattcatatgcatgcatatcttgc
F1
tcttcattctgaaacaggcatatcaattttgcggttcattctggcctgaattttacattgcaacttcatttcatggccagGTGGCA
TTCTGCATCCACAACATCTCGTACCAGGGCCGCTTCTCCTTCGACGACTTCGCGCAGCTCAACCTGCCCGACAGGTTCAAGTCGTC
F2
CTTCGACTTCATCGACGGCTACGACAAGCCGGTGGAGGGGCGCAAGATCAACTGGATGAAGGCCGGGATCCTGCAGGCCGACAAGG
TGCTGACGGTGAGCCCCTACTACGCGGAGGAGCTCATCTCTGGCGAA*CCAGGGGCTGCGAGCTCGACAACATCATGCGCCTCACT
F3 F4 ----------T--
R2 --G-T----------
GGGATCACCGGCATCGTCAACGGCATGGATGTTAGCGAGTGGGACCCCACCAAGGACAAGTTCCTCGCCGTCAACTACGACATCAC
R1
CACCgtgagcaaccacacaaagatttcttcctcttcttccggtgatcgctggttctgggtgggttctcacgaacgaggcaaagtga
cagGCGTTGGAGGGGAAGGCGCTGAACAAGGAGGCGCTGCAGGCCGAGGTGGGGCTGCCGGTGGACCGGAAGGTGCCCCTGGTGGC
R4
GTTCATCGGCAGGCTGGAGGAGCAGAAGGGCCCCGACGTGATGATCGCCGCCATCCCGGAGATCCTGAAGGAGGAGGACGTCCAGA
TCGTTCTCCTGgtacatcatcgagcccgcaacccgaccgccattgctgaaacttcgatcaagcagacctaaggaatgatcgaatgc
attgcagGGCACCGGGAAGAAGAAGTTCGAGCGGCTACTCAAGAGCATTGAGGAGAAATTCCCGAGCAAGGTGAGGG
R3

Fig. 2 DNA sequence of the *Wx-D1* gene (partial). Intron sequences are given in small letters. The asterisk indicates SNP-G(*Wx-D1a*), A(*Wx-D1e*). Identities between sequences are indicated by "–"

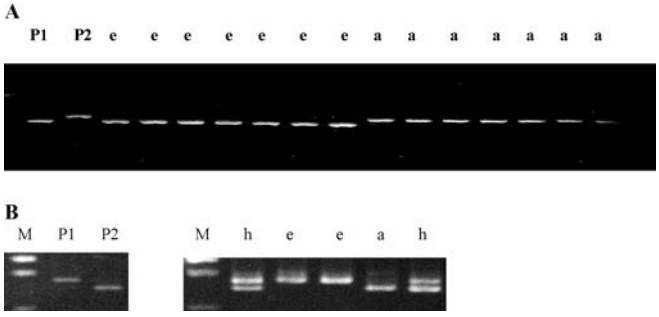

Fig. 3A, B Electrophoresis of dCAPS products **A**: DH lines from the cross Kanto 118 (*Wx-D1a*) × Tanikei A6599-4 (*Wx-D1e*); PCR products were amplified by nested PCR (F2 and R2) and digested with *Age*I. *P1*: Tanikei A6599-4 (*Wx-D1e*), *P2*: Kanto 118 (*Wx-D1a*), *e*: waxy progeny, *a*: non-waxy progeny. Molecular size of DNA fragments: 192 bp (*P1* and *e*), 208 bp (*P2* and *a*). **B**: F_5 lines from the cross of Kanto 119 (*Wx-D1a*) × Tanikei A6599-4 (*Wx-D1e*); PCR products were amplified by nested PCR (F4 and R4) and digested with *Dde*I. *M*: molecular marker ϕX-*Hinc*II digest, *P1*: Tanikei A6599-4 (*Wx-D1e*) (274 bp), *P2*: Kanto119 (*Wx-D1a*) (256 bp), *h*: heterozygous progeny, *e*: waxy progeny, *a*: non-waxy progeny

Starch synthase activity of soluble fractions of Tanikei A6599-4 and A6099 were similar (data not shown).

dCAPS analysis

Using genomic DNA of parental lines of DH lines (Kanto 118; *Wx-D1a* and Tanikei A6599-4; *Wx-D1e*) as a template, we conducted PCR amplification. After amplification with primers F1 and R1 (Table 2), nested PCR was conducted using primers F2 and R2 (Table 2). Since this SNP was not involved in restriction sites of any enzyme, dCAPS primer 'R2' was designed to create an *Age*I site (ACCGGT) including two mismatches, instead of ACCAGG (Table 2; Fig. 2). The molecular size of amplified products by F2 and R2 was 208 bp. All DNA fragments amplified by nested PCR were treated with *Age*I. The wild-type allele (*Wx-D1a*) was not digested, but the mutant allele (*Wx-D1e*) was (Fig. 3A; P1, P2).

*Age*I digestion of the 208-bp product resulted in a 192-bp restriction fragment in the mutant allele (*Wx-D1e*). These types were clearly identified by the difference in molecular size. Nested PCR fragments from 11 of the 22 DH lines were digested, but the other 11 lines were not. In the KI-I_2 staining test, 11 lines that produced 192-bp fragments (Fig. 3A; e) were waxy and the other 11 lines that produced 208-bp fragments (Fig. 3A; a) were non-waxy. To confirm the dCAPS analysis results, we analyzed the DNA sequence of the *Wx-D1* gene in all DH lines (data not shown). The sequence data agreed with dCAPS analysis.

After designing another dCAPS primer (Table 2; F4), which creates a *Dde*I site (CTNAG), DNA fragments were also successfully amplified by F4 and R4 sets (Table 2) after amplification of F3 and R3 sets (Table 2). *Dde*I digestion of 274-bp products resulted in a 256-bp restriction fragment in the wild-type allele (*Wx-D1a*) (Fig. 3B). In 172 F_5 lines, 274-bp, 256-bp, and both fragments from *Dde*I digestion were produced from 83,

79 and 10 lines. All lines that produce 274-bp fragments were waxy as indicated by KI-I_2 staining. Heterozygous plants (*Wx-D1a*/*Wx-D1e*) were clearly identified (Fig. 3B; h) because the dCAPS marker is codominant. Heterozygous plants (*Wx-D1a*/*Wx-D1e*) and non-waxy plants (*Wx-D1a*/*Wx-D1a*) were not, however, discriminated by observing seeds by KI–I_2 staining. In F_5 seeds, 1/16 of the seeds were theoretically heterozygous. This number (10/172) was fitted to the expected ratio by chi-square analysis and results summarizing these dCAPS markers enabled us to efficiently identify the *Wx-D1e* allele.

Discussion

Immunoblot analysis showed that the mutated Wx-D1 protein from the *Wx-D1e* allele is expressed in original lines and non-waxy wheat lines. GBSSI (Wx protein) is a major starch synthase bound to starch granules. Three distinct granule-bound isoforms of starch synthase are identified in the developing endosperm of wheat (Denyer et al. 1995), so we cannot rule out the possibility that the difference in starch synthase activity other than GBSSI was detected. We believe, however, that this possibility is very low because the starch synthase activity of granule-bound proteins of Tanikei A6599-4 is similar to that of Tanikei H1881 (amylose-free line; no Wx protein). Nakamura et al. (1998) indicated a significant difference in granule-bound starch synthase activity between waxy and non-waxy endosperms in wheat. Fujita et al. (1998) indicated that no granule-bound starch synthase activity was detected in the *waxy* mutant (no Wx protein) in *Triticum monococcum,* so that there was little activity of other starch synthases than GBSSI bound to starch granules. This view is supported by our genetic analysis showing that the mutant allele (*Wx-D1e*) and waxy character did not segregate genetically. This mutation directly influenced starch synthase activity because the SNP is within the coding region of the Wx-D1 protein. We thus conclude that the SNP within the *Wx-D1* gene is responsible for the lack of enzyme activity of GBSSI from the *Wx-D1e* allele.

In analyzing SNP, PCR-based methods were simple and rapid, and reagents and equipment are commonly available in most molecular-biology laboratories. dCAPS analysis is useful because an appropriate primer introduces a restriction enzyme recognition-site for analysis. At first, it was planned to design dCAPS primers that created a *Hind*III site (AAGCTT), because *Hind*III costs approximately 1/100 as much per unit as *Age*I. However, amplification under different conditions such as lowering the annealing temperature produced no DNA fragments because of the effect of mismatched primers. Complete genomic sequences of the *Wx-A1a, Wx-B1a* and *Wx-D1a* genes were examined in the wheat cultivar 'Chinese Spring' (Murai et al. 1999a), and the three genes were mutually highly homologous. To easily discriminate the size of the two bands after digestion by the enzyme, the preferable size of nested PCR products is about 200–300 kb, but we could not find the *Wx-D1* gene-specific region upstream of the mutated region. We therefore conducted nested PCR using the first PCR products amplified from the *Wx-D1* gene. Both first forward (F1 and F3) and reverse (R1 and R3) primers were designed to include *Wx-D1* gene-specific sequences. PCR products were confirmed from the *Wx-D1* gene, so nested PCR is useful for efficiently amplifying fragments from the 'true' *Wx-D1* gene. Each amplified product should include the intron sequence, if possible, to prevent amplifying from 'false' fragments. The first PCR products and nested products (F4 and R4) include the intron to amplify from the 'true' *Wx-D1* gene. Intron sequences from all samples tested were the same as the sequence of 'Chinese Spring' (Fig. 1). Heterozygous plants can be also rapidly discriminated using this method because of the codominant nature of dCAPS. In KI-I_2 staining, the heterozygous plant was not distinguished by staining. This dCAPS marker using nested PCR enabled us to easily select the *Wx-D1e* allele. In this research, the mutant allele was directly related to the waxy phenotype because both parental lines have *Wx-A1b* and *Wx-B1b*. We identified *Wx-D1* using our breeding (DH) lines from a combination of Tanikei A-6599-4(*Wx-A1b, Wx-B1b, Wx-D1e*) × Kankei w421 (*Wx-A1a, Wx-B1a, Wx-D1a*), and easily identified the *Wx-D1e* allele (Kurita et al. 2002). dCAPS is thus vital in identifying alleles and selecting lines having the *Wx-D1e* allele. The dCAPS marker linking the *Vrn-B1* gene in wheat was detected using near-isogenic lines (Iwaki et al. 2002). The dCAPS marker is thus also applicable for fine genetic mapping.

In *Gramineae* species, such as wheat (Ainsworth et al. 1993; Murai et al. 1999a), *Triticum turgidum, Triticum durum* (Murai et al. 1999b), *Triticum monococcum, Aegilops speltoides, Aegilops tauschii* (Yan et al. 2000), rice (Hirano and Sano 1991; Wang et al. 1990), *Oryza glaberrima* (Umeda et al. 1991), barley (Rohde et al. 1988), sorghum (Hsieh et al. 1996), maize (Klösgen et al. 1986) and foxtail millet (Fukunaga et al. 2002), alanine was conserved in this region of GBSSI. Assuming the predicted secondary structure of the protein by the PredictProtein server (http://www.embl-heidelberg.de/predictprotein/predictprotein.html), it is possible that the position of a helix and coil is changed by the single amino-acid change. The precise structural difference by SNP is unclear, but SNP may be in an active site of the enzyme.

The starch-pasting curve of Tanikei A6599-4 by the Rapid Visco Analyzer was unique, and hot-paste viscosity was more stable than that of amylose-free wheat (Tanikei A6099) (Kiribuchi-Otobe et al. 1998). The waxy character and stable hot-paste viscosity are controlled by the same mutated *Wx-D1* gene (Kiribuchi-Otobe et al. 2001), suggesting that this SNP in the *Wx-D1* gene affected amylose synthesis and other properties of the starch. Considering that the starch properties are mostly dependent on amylopectin, it is possible that the *Wx-D1e* gene is involved in amylopectin synthesis.

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