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ORIGINAL PAPER

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Identification of SNPs in the *waxy* gene among glutinous rice cultivars and their evolutionary significance during the domestication process of rice

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Abstract Common non-waxy (*Wx*) rice cultivars contain two different alleles at the *waxy* locus, designated Wx^a and Wx^b, which encode different levels of granule-bound starch synthases and are hence involved in the control of endosperm amylose content. The Wx^a allele was predominant in non-waxy *indica* cultivars, whereas the Wx^b allele was common to the non-waxy *japonica* variety. Recently, some of the molecular mechanisms underlying the differentiation of Wx^a from Wx^b have been characterized. One structural difference between these two alleles was shown to be due to alternative splicing caused by a single-base substitution (AG*G*T to AG*T*T) at a donor site of the first intron within the *Wx* gene. In the case of waxy (*wx*) rice, it was not possible to distinguish whether the each *wx* allele was derived from Wx^a or Wx^b alleles by phenotypic analysis. However, we succeeded in developing a derived cleaved amplified polymorphic sequence (dCAPS) marker for the detection of the one-base splicing mutation without the need for sequencing. A mismatch primer was used to generate a restriction site in the Wx^a allele (AGGT) but not in the Wx^b allele (AGTT). Three hundred fifty-three waxy rice strains that are widely found in Asia were then employed for analysis using this dCAPS marker. Our findings suggested that waxy rice strains have both Wx^a- and Wx^b-derived alleles, but that the Wx^b-derived allele was predominant, and its distribution was independent of *indica-japonica* differentiation. The wild relatives of cultivated rice all possessed the AGGT allele. It was concluded that the waxy mutations, and the corresponding rice cultivation, originated from *japonica* during the evolution and domestication process of rice and was preferentially selected by most Asian peoples.

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Introduction

Spontaneous waxy mutants of different cereals have been specifically found and are distributed throughout Asia (Sakamoto 1982). In the specific case of rice (*Oryza sativa* L.), there exists a "Glutinous Rice Zone" where people have been cultivating and utilizing mainly waxy (glutinous) rice cultivars in their daily diet (Watabe 1967). This Glutinous Rice Zone is distributed throughout the mountainous regions of the Indochina Peninsula in Southeast Asia. It is hypothesized that for both cultural and ethnic reasons, people in these areas showed a preference for waxy (glutinous) starch and, therefore, selected these rice strains during the process of cultivation.

Both waxy and non-waxy phenotypes are, in fact, controlled from a single locus, the *waxy* locus. The *waxy* gene has a tissue-specific expression pattern and controls amylose synthesis in both the endosperm and pollen of cereal crops (e.g., Brink and MacGillivray 1924; Demerec 1924). In rice, the amylose content in endosperm has been considered one of the most important culinary breeding traits (Juliano 1981, 1982). Because of this, there have been many studies concerning both the physical properties and the genetic profiles of seed starch in non-waxy (non-glutinous) rice strains (Okuno 1978; Sano 1984; Sano et al. 1985, 1986; Wang et al. 1995; Ayres et al. 1997; Hirano and Sano 1998, 2000).

In the case of amylose content, it was reported that there are two functional alleles in non-waxy (*Wx*) rice, Wx^a and Wx^b, originally defined by the different levels of Wx protein (Sano 1984). It was also reported that Wx^a and

Wx^b were *indica*- and *japonica*-specific, respectively (Sano et al. 1986). Furthermore, molecular studies revealed that the differentiation between these two alleles originated from a splicing mutation during post-transcriptional processing (Wang et al. 1995). Recently, sequence data of *Wx* alleles show that Wx^a has an AG*G*T sequence within a consensus donor site in the first intron, whereas Wx^b has an alternative AG*T*T sequence. This one-base substitution was attributed to a decreased splicing efficiency and lower amount of amylose in Wx^b cultivars (Cai et al. 1998; Hirano et al. 1998; Issiki et al. 1998). In this paper, we designate this one-base substitution as "G-T polymorphism."

It is noteworthy that there has so far been little information uncovered regarding the *waxy* (*wx*) allele (Bao et al. 2002; Olsen and Purugganan 2002), and the phylogenetic origins of waxy rice are still poorly defined. As mentioned above, ethnobotanical factors in waxy rice cultivars have been studied, but genetic analyses of the evolution of waxy rice strains have not been undertaken to any great extent. Taking account into the fact that *wx* alleles had been derived from *Wx* alleles and applying for the G-T polymorphisms in waxy strains, it is possible to estimate whether the waxy alleles derived from the non-waxy alleles of Wx^a or Wx^b, namely, derived from non-waxy *indica* or *japonica*. To elucidate further both the diversity and differentiation of the splicing G-T polymorphism among waxy cultivars, we developed a derived cleaved amplified polymorphic sequence (dCAPS) marker using a mismatch PCR primer which selectively generates a new restriction site (Michaels and Amasino 1998; Neff et al. 1998). We then analyzed waxy strains collected from various countries and areas in Asia using this dCAPS marker. This enabled us to predict the phylogenetic origin of waxy rice via successful detection of single nucleotide polymorphisms (SNPs) without having to rely on laborious sequencing procedures.

Materials and methods

Plant materials and DNA extraction

Three non-waxy strains, Taichung 65 (T65: temperate *japonica*), Ac. 130 (*indica*) and Ac. 419 (*indica*), and 353 waxy strains collected from various regions of Asia were examined in this study (Table 1). Waxy endosperms were confirmed by iodine-potassium iodide (I/KI) staining, and the results were confirmed by comparison with previous reports (e.g., Morishima et al. 1984). All strains used were from the property collections of Shizuoka University, Japan. Based on our own observation studies, these collections were traditionally cultivated in the field (e.g., Sato 1994; Ishikawa et al. 2002; Yamanaka et al. 2002), and thus, are considered to be appropriate materials to estimate genetic diversity or to trace evolutionary pathways during domestication processes. In the case of wild relatives of cultivated rice, 23 strains each of *O. nivara* and *O. rufipogon,* which have been proven to be the direct ancestors of *indica* and *japonica*, respectively (Yamanaka et al. 2003), were used. These wild rice strains were collected mainly from the Mekong Basin of the Indochina Peninsula in Southeast Asia (Yamanaka et al. 2003).

Total DNA was extracted from 100 mg of fresh leaf from each strain by the method of Dellaporta et al. (1983).

Table 1 Number of waxy rice strains used in this study

Origin	No. of strains
Japan	100
China	10
Taiwan	1
Philippines	10
Indonesia	20
Cambodia	2
Thailand	61
Laos	142
Myanmar	4
India	3
Total	353

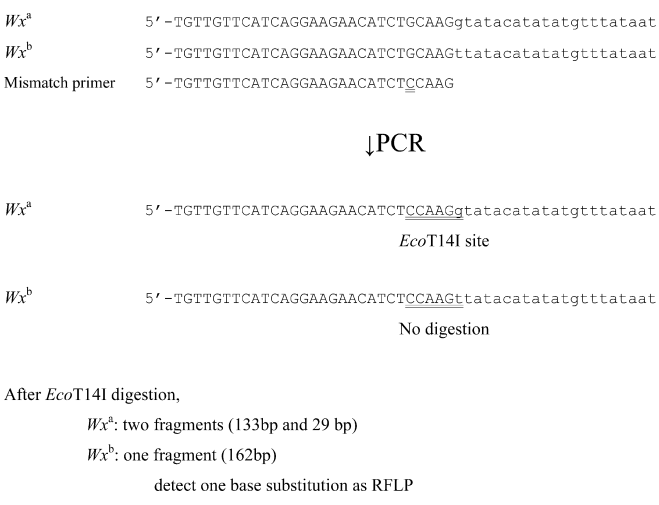

Fig. 1 Derived cleaved amplified polymorphic sequence (dCAPS) marker for detection of the one-base substitution at 5′ splice junction of the first intron in the *waxy* gene. *Capital letters* of sequences indicate the first exon (waxy promoter) and *small letters* indicate the first intron

Sequencing analysis

To determine sequence alignments of interest, we constructed a PCR primer set, WP-A2 (5′-GCT TCA CTT CTC TGC TTG TG-3′) and WP-B (5′-TTA ATT TCC AGC CCA ACA CC-3′), which amplifies the fragment containing the first exon-intron junction of the *Waxy* gene. PCR amplification was performed using 1 ng of extracted DNA in a total volume of 25 µl containing 1×*LA Taq* GC buffer I (Takara), 0.4 mM dNTPs, 1 µM of each primer, and 1.25 U of *LA Taq* polymerase (Takara). A total reaction of 35 cycles was programmed for 30 s at 94°C, 30 s at 55°C, and 1 min at 72°C in a Thermal Cycler (Perkin-Elmer Cetus). PCR products were purified using MicroSpin S-300 HR Columns (Amersham Pharmacia). PCR products were directly sequenced from both strands using the same primer set, WP-A2 and WP-B, with a BigDye Terminator Cycle Sequencing Kit using an ABI 377 sequencer (Applied Biosystems).

dCAPS analysis

To detect a one-base substitution by dCAPS analysis, a mismatch primer WP-CAPS (5′-TGT TGT TCA TCA GGA AGA ACA TCT CCA AG-3′) that generates an *Eco*T14I site specifically in the Wx^a allele was constructed (Fig. 1). PCR amplification using the primer set WP-CAPS and WP-B was performed under the same conditions used for sequencing analysis. Five microliters of each PCR product

was digested with *Eco*T14I in a total volume of 20 µl at 37°C overnight. After digestion, 1.5 µl of each digest was electrophoresed in an 8.0% polyacrylamide gel (mono:bis = 29:1).

Results

Comparison between sequencing and dCAPS analyses

Total DNA was isolated from the leaves of three *Wx* (non-waxy) and nine *wx* (waxy) cultivars, and DNA fragments around the splice donor site of the first intron were amplified by PCR. PCR fragments were then purified and analyzed by direct sequencing. The results of sequencing analysis for the splicing site are shown in Table 2. In the case of non-waxy cultivars, Wx^a (Ac. 130 and 419: *indica*) has the AGGT sequence and Wx^b (T65: temperate *japonica*) has the AGTT sequence, consistent with previous reports. Analysis of waxy strains showed that both AGGT and AGTT polymorphisms were observed and were independent of *indica-japonica* classification.

The same results were obtained by dCAPS analysis, which proved the robustness and accuracy of the procedure in detecting a SNP successfully without sequencing analysis. Strains having the AGGT sequence display a short fragment as a result of digestion with *Eco*T14I, while strains carrying AGTT are represented by a longer undigested fragment (Fig. 2). One cultivar, Th8, contained both fragments, suggesting that this strain was heterozygous for the alleles of both AGGT and AGTT. Thus, dCAPS analysis could also detect heterozygotes that would not be revealed by sequencing analysis (Fig. 2).

Differentiation of the G-T polymorphism among waxy strains and wild relatives of rice

We took advantage of the fact that the dCAPS marker was an accurate and successful tool for analyzing large numbers of samples quickly, without the need for direct sequencing determination. To clarify the relationship between *indica-japonica* classifications and the G-T polymorphism in waxy cultivars, 89 different samples, designated as either *indica* or *japonica* by the methods of Oka (1953) and Sato (1991), were used. The results, shown in Table 3, were as follows: in 54 *japonica* waxy cultivars, 49 (91%) have the AGTT sequence with only five containing AGGT. In the case of 35 *indica* cultivars, 29 (83%) contained the AGTT with six cultivar samples having AGGT. These results suggested that the Wx^b-derived AGTT allele was predominantly distributed in waxy cultivars, and that this was not dependent upon their *indica-japonica* classification. To detect the G-T polymorphism among a larger pool of waxy cultivars, we performed dCAPS analysis for a further 353 samples that were collected from different regions of Asia. Within this larger set, 342 cultivars (97%) contained AGTT, whilst only 11 cultivars had the AGGT sequence (Table 4). This result further suggested that the *wx* allele derived from Wx^b is the predominant one amongst waxy rice cultivars. Within the smaller, Wx^a-derived, allele-containing sample set, it was found that these cultivars belonged to either the *indica* or tropical *japonica* varieties, and their geographical distribution was localized mainly to tropical regions (Table 5).

Of the 46 wild-relative strains that we analyzed (Table 4), 23 each were of the *O. nivara* and *O. rufipogon* variety, which are considered to be direct ancestors of the *indica* and *japonica* strains of *O. sativa*, respectively (Yamanaka et al. 2003). In these analyses, only the AGGT allele was detected, suggesting that the AGTT-SNP has been selected for during human domestication and cultivation of rice.

Table 2 Sequencing analysis for the first intron donor site in the *waxy* locus

Strain		Allele	*indica-japonica*	Sequence
Non-waxy	T65	Wx^b	Temperate *japonica*	AGTT
	Ac. 130	Wx^a	*Iidica*	AGGT
	Ac. 419	Wx^a	*indica*	AGGT
Waxy	T65*wx*	*wx*	Temperate *japonica*	AGTT
	Ac. 221	*wx*	Tropical *japonica*	AGGT
	Th8	*wx*	Tropical *japonica*	AGGT
	NN74b	*wx*	*Iidica*	AGGT
	LH5-7	*wx*	Tropical *japonica*	AGGT
	P76	*wx*	*indica*	AGGT
	Ch7	*wx*	Temperate *japonica*	AGTT
	Is107	*wx*	*indica*	AGGT
	J177	*wx*	Tropical *japonica*	AGTT

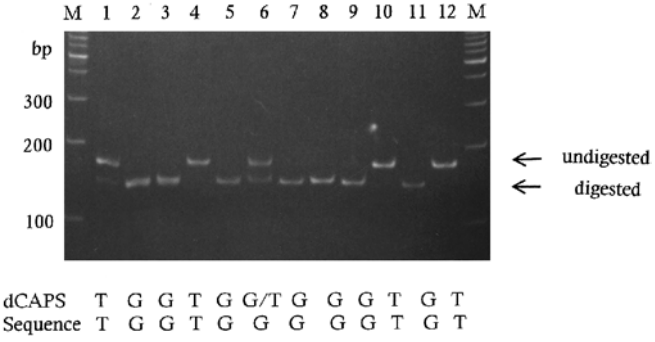

Fig. 2 Comparison between dCAPS and sequencing analysis for detection of G-T polymorphism. *M* 100 bp ladder, *1* T65, *2* Ac. 130, *3* Ac. 419, *4* T65*wx*, *5* Ac. 221, *6* Th8, *7* NN74b, *8* LH5-7, *9* P76, *10* Ch7, *11* Is107, *12* J177

Table 3 Apparent *indica-japonica* classification and G-T polymorphism

	AGTT	AGGT	Total
japonica[a]	49	5	54
indica[a]	29	6	35
Total	78	11	89

[a] Classified based on the original methods of Oka (1953) and Sato (1991)

Table 4 Results of derived CAPS analysis for waxy rice cultivars and wild relatives

Species	Origin	No. of strains	
		AGGT	AGTT
Oryza sativa	(See Table 1)	11	342
O. nivara[a]	Vietnam	1	0
	Cambodia	2	0
	Thailand	18	0
	Laos	2	0
	Total	23	0
O. rufipogon[a]	Vietnam	11	0
	Cambodia	2	0
	Thailand	7	0
	Laos	2	0
	China	1	0
	Total	23	0

[a] Details were described in the previous study (Yamanaka et al. 2003)

Table 5 *O. sativa* waxy strains having AGGT sequence

Strains	*indica-japonica*	Origin
Ch80	*indica*	China
Ac. 221	Tropical *japonica*	Philippines
P10	*indica*	Philippines
P17	Tropical *japonica*	Philippines
P23	*indica*	Philippines
P76	*indica*	Philippines
Is107	*indica*	Indonesia
Th8	Tropical *japonica*	Thailand
NN74b	*indica*	Thailand
NN79i	Tropical *japonica*	Thailand
LH5-7	Tropical *japonica*	Laos

Discussion

Since the two non-waxy alleles, Wx^a and Wx^b, correspond to *indica* and *japonica* varietal groups of non-waxy cultivars, respectively (Sano et al. 1986), and are regulated by a G-T polymorphism (Hirano et al. 1998; Issiki et al. 1998), it was possible to estimate whether individual *wx* alleles were derived from either Wx^a or Wx^b, (namely, non-waxy *indica* or *japonica*, respectively). We therefore decided to investigate the incidence of this G-T polymorphism in waxy rice cultivars. Firstly, we performed direct sequencing of PCR products, and our results indicated that there are two *waxy* alleles containing either an AGGT or an AGTT sequence at a splicing site. It was considered that these alternative waxy alleles had different origins, one being AGGT-derived from *indica* and another, AGTT-derived from *japonica*. We then investigated the incidence and distribution of this polymorphism among a large pool of waxy cultivars that were collected from different regions. Because direct sequencing would prove to be too labor intensive to analyze a large sample set, we developed a new marker to detect this SNP via a simpler, PCR-based method. CAPS (PCR-RFLP) methods rely on the presence of a restriction site at the SNP for analytical detection, but, as there was no convenient restriction site at the waxy allele SNP, we employed a derived CAPS (dCAPS) method (Michaels and Amasino 1998; Neff et al. 1998). This involved the PCR generation of an *Eco*T14I restriction site at the allele having only the AGGT sequence. Results shown in Fig. 2 indicated that this dCAPS marker detected the waxy allele SNPs as accurately as sequencing analysis (Table 2). We therefore showed that this marker was an effective and suitable tool for large-scale analysis.

From the results comparing the correlation between the G-T polymorphisms and the apparent *indica-japonica* differentiation, most waxy cultivars were shown to contain the AGTT sequence independently of their *indica-japonica* classification (Table 3). Furthermore, the results of dCAPS analyses of 353 waxy cultivars collected from a variety of regions indicated that the AGTT-containing allele is highly predominant (97%) in waxy cultivars, and the AGGT allele was detected in only a minority of samples (Tables 4, 5). These results strongly indicate that most of *wx* alleles from around the world are derived from Wx^b, namely *japonica* non-waxy cultivars. This suggests that the origins of waxy cultivars are less simple than previously thought, such as the differentiation of waxy *japonica* from non-waxy *japonica* or waxy *indica* from non-waxy *indica*. As for the rarity of the AGGT allele (Table 5), it should be noted that *indica* (Wx^a)-derived waxy cultivars did also exist. It is notable that in this case, the origins of most of these cultivars were outside of the Glutinous Rice Zone, suggesting that the people from this zone did not select and cultivate *indica* (Wx^a)-derived waxy cultivars, and also that *japonica* (Wx^b)-derived ones have been cultivated widely in the world.

From the result with the wild relatives, there was no detectable G-T polymorphism between *indica* and *japonica* types, although *indica-japonica* differentiation existed in wild relatives. This suggests that this polymorphism occurred in the *japonica* line during the domestication process of rice. In addition, the waxy mutation that occurs in the non-waxy *japonica* carrying AGTT was preferentially selected by different Asian peoples.

Recently, an evolutionary study of waxy rice, using this SNP and polymorphisms in other regions and genes, was presented by Olsen and Purugganan (2002). Based on their results, they adopted a refined classification for 18 different haplotypes. Our results essentially support their conclusions, at least for the SNP reported here, but they examined only a limited number of the waxy strains of *O. sativa* (37 waxy rice strains and 68 non-waxy strains) and had not taken any wild-relative strains into account. Additionally, their study incorporates a wide range of sequence diversity in the *waxy* locus. In contrast, it should be noted that our interest is mostly the phylogenetic relationship between a single G-T polymorphism at a specific region of the *waxy* gene and the designation of *indica-japonica* differentiation in non-waxy rice. There are no other reports concerning the phylogenetic origin of waxy rice cultivars that are focused on *indica-japonica* differentiation. The issue of general sequence diversity within the entire *waxy* gene region should be clearly

distinguished from our focus on a single SNP in "non-waxy" rice and its relevance to the origins of "waxy'" rice. In addition, we used both cultivated and wild rice strains that are more widely representative of rice cultivars and species within Asia. We also refer to *indica-japonica* differentiation using the "original" definition of Oka (1953) and Sato (1991), and of course, significantly more strains were analyzed in this study compared to the report by Olsen and Purugganan (2002).

It was concluded that waxy mutations and waxy rice cultivation occurred predominantly in the *japonica* line during the evolutionary process of domestic rice cultivation, and the *wx* allele that occurred in the *japonica* line had introgressed and dispersed in most of waxy strains. Because of limited interest, there have been few recent studies focusing on waxy strains, while this polymorphism among non-waxy strains has been well analyzed with respect to amylose content (Ayres et al. 1997). However, we present evidence here that genetic analysis of waxy rice gives us an insight and poses interesting questions concerning the evolution of cultivated rice. Also, this is the first report of the phylogenetic relationship among waxy rice cultivars based on both analysis of polymorphisms at the *waxy* locus and *indica-japonica* differentiation. We also suggest here that cultivated plants are not simply the result of natural factors, such as mutation and natural selection, but also due to human activities such as selection and management. This SNP is not only of interest for the molecular basis of *waxy* gene function, but is also a good marker to trace human impact upon the selection of the waxy mutation and waxy rice cultivars.

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