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ORIGINAL PAPER

Allelic diversification at the *wx* locus in landraces of Asian rice

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Abstract To examine continuous variation of amylose levels in Asian rice (*Oryza sativa*) landraces, the five putative alleles (Wx^a, Wx^{in}, Wx^b, Wx^{op}, and *wx*) at the *wx* locus were investigated in near-isogenic lines (NILs). Apparent amylose levels ranged from 0.5 to 29.9% in the NILs, showing a positive relation with the levels of *Wx* gene product, granule-bound starch synthase (GBSS) as well as the enzymatic activity per milligram starch granule. Only opaque (Wx^{op}) accessions had an enzymatic activity per GBSS that was reduced to half the level of the others**.** Nucleotide sequences in the *Wx* gene were compared among 18 accessions harboring the five different alleles. Each of the *Wx* alleles had a unique replacement, frameshift or splice donor site mutation, suggesting that these nucleotide changes could be reflected in phenotype alterations. A molecular phylogenetic tree constructed using the *Wx* gene indicated that ssp. *japonica* forms a distinct clade, whereas ssp. *indica* forms different clades together with the wild progenitor. Unexpectedly, the *wx* allele of 160 (*indica* from Taiwan) joined the *japonica* lineage; however, comparisons using linked genes for two Taiwanese accessions revealed that the *wx* gene was the product of gene flow from *japonica* to *indica*. Therefore, the *japonica* lineage frequently included Wx^{in}, Wx^b and *wx*, while Wx^a and Wx^{op} were found in the other lineages, strongly suggesting that allelic diversification occurred after divergence of the two subspecies. The present results were discussed in relation to the maintenance of agronomically valuable genes in various landraces.

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Introduction

The *Waxy* (*Wx*) gene encodes a granule-bound starch synthase (GBSS) that plays a role in amylose synthesis in plants (Preiss 1991; Smith et al. 1997). Waxy (or glutinous) rices lack amylose, which represents up to 30% of the total starch in non-waxy rice endosperms. Amylose content is a key determinant of cooking, processing, and eating qualities in rice (Juliano 1971). The GBSS encoded by the *Wx* gene (called the Wx protein) is accumulated during the process of grain filling, and its GBSS level in the endosperm is believed to explain the amylose level in rice (Sano 1984). The functional alleles Wx^a and Wx^b, which are associated with high (22–29%) and low (12–19%) amylose content, respectively, are predominantly distributed in *Oryza sativa* ssp. *indica* and *Oryza sativa* ssp. *japonica*, respectively (Sano 1984; Sano et al. 1985; Wang et al. 1995). The lower level of expression of the Wx^b allele has been shown to result from the inefficient splicing of intron 1 due to a G-to-T mutation at the 5′ splice site (Bligh et al. 1998; Cai et al. 1998; Hirano et al. 1998; Isshiki et al. 1998). However, the existence of these two alleles itself is not sufficient to explain the wide range of variation in amylose content

observed in non-waxy (or non-glutinous) Asian rice. Inheritance of amylose content traits is often complex because of the impact of modifiers like *du* genes (McKenzie and Rutger 1983; Isshiki et al. 2000), epistasis, cytoplasmic effects, the triploid nature of the endosperm (Kumar and Khush 1986; Pooni et al. 1993), and environmental temperature (Inatsu 1979; Asaoka et al. 1987), all of which make it difficult to identify the allelic diversification at the *wx* locus. However, opaque or chalky endosperms are known to have very low amylose content (<10%) and to be controlled by the Wx^{op} allele (Heu 1986; Mikami et al. 1999). Furthermore, intermediate amylose content (20–25%) is reportedly determined by a single gene, although it is not yet certain whether the causal gene is an additional allele at the *wx* locus or another modifier (Kumar and Khush 1987). In US cultivars with intermediate amylose content (20–22%), the *Wx* gene can be divided into several haplotypes on the basis of CT repeats in the 5′-untranslated region of the *Wx* gene (Ayres et al. 1997), suggesting that unknown alleles might be present at the *wx* locus of rice. Post-transcriptional processing of the GBSS transcript is also proposed to play a role in determining the amylose content (Wang et al. 1995; Frances et al. 1998; Larkin and Park 1999), showing that trans-acting genes are also involved in the expression of the *Wx* gene (Isshiki et al. 2000), although to what extent the multiple alleles at the *wx* locus affect the enzymatic activity of GBSS remains largely unknown in rice. The *Wx* region on chromosome 6 plays a significant role for determinants of the eating quality (Tan et al. 1999; Tian et al. 2005; Wang et al. 2007); however, it is not easy to examine whether phenotypic changes are caused by the *Wx* alleles or its linking modifiers.

The waxy phenotype arises through disrupted expression of the *Wx* gene (Sano 1984). The genealogy of the *Wx* gene has been successfully examined to trace the evolutionary and geographical origins of this phenotype, with the finding that the splice donor mutation associated with waxy phenotypes has a single evolutionary origin, and that the origin of waxy rice is associated with reduced genetic variation characteristic of selection at the *wx* locus (Olsen and Purugganan 2002). However, some waxy rice landraces have no splice donor mutation, suggesting that the *wx* phenotype is not inevitably associated with the mutation (Inukai et al. 2000; Bao et al. 2002; Yamanaka et al. 2004). *Oryza sativa* comprises two subspecies, ssp. *indica* and ssp. *japonica*, and the latter is divided into *tropical japonica* and *temperate japonica* (Oka 1988; Khush 1997; Garris et al. 2005). The splice donor mutation is prevalent in *temperate japonica* rice, but rare or absent in *indica* and *tropical japonica* rice (Yamanaka et al. 2004; Olsen et al. 2006). Sequence analysis of regions linked to the *Wx* gene has revealed a strong signature indicating that a selective sweep has occurred in the *temperate japonica* landraces associated with the mutation (Olsen et al. 2006). In rice and in maize, intra-allelic recombination produces non-waxy pollen grains in some crosses between *wx* accessions (Li et al. 1968; Nelson 1968), giving a contrasting explanation of why *wx* phenotypes might have repeatedly emerged in domesticated forms of rice. Thus, the evolutionary changes in the expression at the *wx* locus are still to be clarified.

The objective of the present investigation is to examine allelic diversification at the *Wx* locus that affects amylose content in Asian rice. To minimize the genetic background effecting amylose content in the endosperm, we used near-isogenic lines (NILs) having five different *Wx* alleles affecting starch properties, including changes in the enzymatic activity. Secondly, we show that based on the genealogy of the *Wx* genes in 20 rice accessions including outgroups, these alleles are distributed in distinct lineages, with allele-specific amino acid substitutions or indels causing a frame shift. Finally, we indicate that the level of gene diversity is partly influenced through gene flow, showing that a *wx* gene in an *indica* landrace has introgressed from a *japonica* landrace.

Materials and methods

Near-isogenic lines

Five near-isogenic lines (NILs) of Taichung 65 (ssp. *japonica* from Taiwan, designated T65) were used for examining *Wx* gene expression (Table 1). To compare the effects of all the five putative alleles (Wx^a, Wx^{in}, Wx^b, Wx^{op}, and *wx*), a NIL carrying the Wx^{in} gene was developed by backcross introgression [T65Wx^{in} (219)]. The Wx^{in} allele proposed in this study is the *Wx* allele responsible for intermediate levels of amylose and GBSS relative to Wx^a and Wx^b. The other four NILs reported were used for comparison in this experiment. Allelic differentiation at the *wx* locus was originally assessed by protein analysis (Sano 1984). In the present study, allelic differentiation was assessed on the basis of phenotype, including GBSS level and endosperm amylose level. Some *japonica* accessions have intermediate levels of amylose and GBSS relative to Wx^a and Wx^b carriers (Sano et al. 1991). One of these, 219 (Garumbaley from Indonesia), was crossed with a *wx* tester (T65*wx*), and the *Wx* allele was introgressed into T65*wx* by repeated backcrossings. Given that the resultant NILs (from the BC_6 generation) had intermediate levels of amylose and GBSS, considering that the donor carried the Wx^{in} (Table 1). The five NILs used had the T65-derived *C* gene (*Chromogen* or *OsC1*, Saitoh et al. 2004) on chromosome 6. Apparent amylose content and GBSS level were measured as reported elsewhere (Sano 1984; Mikami et al. 1999). All the donor accessions are preserved in the National Institute of Genetics, Mishima, Japan.

Table 1 Near-isogenic lines of T65 used in the present study

NILs	Generation	Donor		Origin	Ref
		Accession	Subspecies		
T65 Wx^a (868)	BC_8	Patpaku	*indica*	Taiwan	Hirano et al. (1998)
T65 Wx^{in} (219)	BC_6	Garumbaley	*tropical japonica*	Indonesia	Present study
T65 Wx^{op} (6622)	BC_5	ARC6622	*indica*	India	Mikami et al. (1999)
T65 Wx^{op} (10818)	BC_5	ARC10818	*indica*	India	Mikami et al. (1999)
T65 *wx* (563)	BC_{12}	Kinoshita-mochi	*temperate japonica*	Japan	Oka (1974)
T65 (recurrent parent)[1]	–	Taichung 65	*temperate japonica*	Taiwan	

[1] The allele of T65 is Wx^b

Genetic stocks used for the genealogical study

The materials used were 16 accessions of Asian cultivated rice (*O. sativa*) and two accessions of its wild ancestor (*O. rufipogon*). Two African *Oryza* species (*O. glaberrima* and *O. barthii*) were used as outgroups. The Asian cultivated rice accessions were landraces except for T65 and Norin 8, and they were classified as ssp. *indica*, ssp. *temperate japonica* or ssp. *tropical japonica* based on morphological and physiological traits (Oka 1988). The allelic states at the *wx* locus for these accessions had been previously predicted on the basis of GBSS and amylose levels (Mikami et al. 1999; Sano et al. 1991). The genetic stocks used here are preserved in the National Institute of Genetics, Mishima, Japan. Three opaque accessions (ARC6622, ARC10818, Acc35618) and ARC12446 were gifts from Dr. G.S. Khush and Central Rice Research Institute, Cuttack, India, respectively.

GBSS enzyme assays

UDP-[^{14}C] glucose (227 mCi/mmol) and UDP-glucose were purchased from Amersham Bioscience, USA and Sigma-aldrich Inc., USA, respectively. Starch granules were purified from the triploid endosperms of immature grains (13–15 days after pollination) by using the method of Leiloir et al. (1961). The reaction mixtures each contained 5 mg of the starch granule preparation and 10^5 dpm [^{14}C] UDP-glucose in 50 µl of a 0.1 M glycosylglycine buffer solution. The mixtures were incubated at 37°C for 30 min. The enzymatic reaction was terminated by addition of 0.5 ml of methanol (70%, v/v), followed by centrifugation. The pellet was washed twice with 0.5 ml of methanol (70%, v/v), and then suspended in 0.5 ml of distilled water. The pelleted material was then trapped on a glass fiber filter, washed with 30 ml of distilled water, and emissions were assessed by using a liquid scintillation counter (LSC-5000; Aloka, Tokyo, Japan) (Nelson et al. 1978). For each genotype, four samples were assayed. *Wx* activity was measured in terms of [^{14}C]UDP-glucose incorporated per milligram of starch granules over 30 min (Weil et al. 1992).

DNA sequencing

Genomic DNA was extracted from 2-week-old seedlings by using the CTAB method as described by Murray and Thomson (1980). The *Wx* gene was amplified by PCR, and two regions, A (867 bp, aligned length) and B (3,455 bp), were sequenced (Fig. 1). For sequencing region B, primers described by Inukai et al. (2000) were used, while primers 5′-GTCCCGTTGCGTCGTCATAG-3′ and 5′-CTCAAGA CACAAATAACTGCAG-3′ were used for sequencing region A. The amplified DNA was cloned into pBSII (Stratagene), and sequenced using an automated sequencer (ABI Prism 377 DNA Sequencing System; Applied Biosystems, Foster City, CA, USA) in accordance with the method of Hirano et al. (1998). Sequences linked to the *Wx* gene were examined in three cultivars (T65, 160, and Peh-ku), those originated from Taiwan. P0541H01.20 (in AP001389), the two predicted genes (P0535G04.1 and P0535G04.24 in AP000399) and *RFT1* (AB062675) were investigated for comparison. PCR amplification was carried out using primers 5′-TGGTAGCATTTAGGGCATCC-3′ and 5′-CTCC CCGTATTTTGTAACTG-3′ for P0541H01.14, 5′-AGCT TACAGGATGCTGATCAATCG-3′ and 5′-ACCTCGC TTCAAAGGACGCAAATA-3′ for P0535G04.1, 5′-CA AGGGCATCTGCACTAACAC-3′ and 5′-ACGCAATT CTTACAGCTTTCAAC-3′ for P0535G04.24, and 5′-CT AGCTAGCAATCTCTATCGATCTGT-3′ and 5′-ATGC ATATACAGCTAGGCAGGTCT-3′ for *RFT1*. The PCR

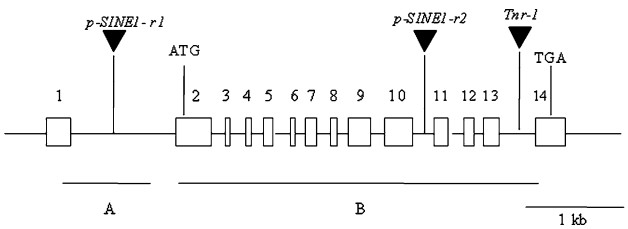

Fig. 1 The structure of the *Wx* gene of rice. The two regions (*A* and *B*) were analyzed in this study. Region *A* is 867 bp (aligned length) including the 5′ splice junction of intron 1 and *p-SINE1-r1*, and region *B* is 3,455 bp (aligned length) including the coding regions, *p-SINE1-r2* and *Tnr-1* (Umeda et al. 1991)

products were purified and directly sequenced using an ABI 377 automatic sequencer (Applied Biosystems) with a Big Dye Terminator Cycle Sequencing Kit (Applied Biosystems). The lengths of the sequences compared were 984 bp for P0541H01.14, 906 bp for P0535G04.1, 1,304 bp for P0535G04.24, and 1,512 bp for *RFT1*. For comparison of the multiple alleles at the *wx* locus, a part of the *Wx* sequences previously reported were included in this study. They were X65183 (232), AB008795 (Patpaku), X58228 (T65), and D10472 (GMS1). All new DNA sequences obtained in this study are available from DDBJ with the accession numbers AB281428–AB281474. A whole region of *Wx* gene was not compared because the amplification failed in some wild strains. In addition to the 20 accessions used in this study, the 76 sequences reported in the public data bank were also used to survey the distribution of the two amino acid substitutions specific to Wx^{op} and Wx^{in}. The 69 accessions (DQ280600-DQ280604, DQ280606-DQ280609, DQ280611-DQ280623, DQ280625-DQ280632, DQ280634-DQ280637, DQ280640-DQ280646, DQ280648-DQ280671, DQ280672-DQ280675) were from Olsen et al. (2006), and the seven accessions (AB066093, AB066094, DQ415640, X53694, AF031162, AF141954, AF141955) were from others. Classification into ssp. *tropical japonica* and ssp. *temperate japonica* is based on Olsen et al. (2006).

Data analysis

Sequence alignment was performed using CLUSTAL X software (Thompson et al. 1997) with additional minor modifications carried out by visual inspection. The two data sets (A and B regions of the *Wx* gene; Fig. 1) were combined, and phylogenetic analyses were conducted by using the maximum parsimony (MP) method using PAUP, version 4.0 (Swofford 1998). No differential weighting of the substitutions was used. The level of support for branches of the phylogenetic tree was evaluated by using bootstrap analysis (Felsenstein 1985) based on 1,000 replicates, using a full heuristic search. The two reproductively isolated species, *O. glaberrima* and *O. barthii*, were used as outgroups.

Results

Five naturally occurring *Wx* alleles

The five NILs carrying each of the five putative alleles (Wx^a, Wx^b, Wx^{in}, Wx^{op} and *wx*,) and the recurrent parent (T65) were used to examine the impacts of these alleles on starch properties against a T65 genetic background (Table 1). We found that intermediate amylose content (22.8 + 2.8%) produced by the Wx^{in} allele, was carried in an Indonesian landrace (219). Including T65, the six lines showed a wide range of variation in GBSS (0.0–8.0, the value of T65 as unity) and amylose (0.5–29.9%) levels in the endosperm, showing a positive correlation between the two parameters ($r = 0.872$, $df = 5$). GBSS activity (per milligram of starch granules) also varied greatly in the six lines, and correlated positively with amylose content ($r = 0.863$, $df = 5$). GBSS activity in the two Wx^{op} NILs, T65 Wx^{op}(6622) and T65 Wx^{op}(10818), was half than that in the other lines except for T65*wx*(563) (Table 2).

Nucleotide variations in the *Wx* gene

The putative existence of five *Wx* alleles in rice landraces studies suggests the possibility that nucleotide substitutions might exist in the *Wx* gene. Two regions of the *Wx* gene, A (867 bp) and B (3,455 bp), were sequenced and compared with 20 rice accessions harboring different *Wx* alleles (Table 3, Fig. 1). Allelic diversity at the *Wx* gene was evident for *O. sativa* accessions, whereas the wild progenitor *O. rufipogon* and the other taxa, *O. glaberrima* and *O. barthii*, carried only the Wx^a allele. Informative sites detected among the *Wx* sequences of these accessions were listed in Table 4. In rice, two short-interspersed elements (*p-SINE1-*

Table 2 Enzymatic activities of GBSS in the five NILs carrying different *Wx* alleles

NILs	Enzymatic activity[1]		Level of GBSS[2]	Amylose content (% dry wt)
	per mg starch granule	per GBSS		
T65Wx^a (868)	3495.9 (253.4)	435.9 (31.6)	8.0 (0.8)	29.9 (3.5)
T65Wx^{in} (219)	1504.7 (63.1)	428.7 (18.0)	3.5 (0.4)	22.8 (2.8)
T65Wx^b [3]	469.2 (24.4)	469.2 (24.4)	1.0 (–)	16.6 (2.4)
T65Wx^{op} (6622)	189.3 (12.3)	230.9 (15.0)	0.8 (0.4)	13.6 (1.1)
T65Wx^{op} (10818)	197.9 (6.8)	250.5 (8.7)	0.8 (0.5)	12.4 (0.9)
T65*wx* (563)	14.8 (2.1)	–	0.0 (0.0)	0.5 (0.0)

[1] Activity is based on dpm [^{14}C] UDP-glucose in corporated/mg starch granule/30 min ($n = 4$). Standard deviations are shown in parentheses

[2] The level of GBSS is shown by the relative amount to that of T65Wx^b

[3] The recurrent parent carrying Wx^b

Table 3 The *Wx* alleles carried by 20 rice accessions, showing polymorphisms of a microsatellite in the exon 1 and the 5′ splice junction of the intron 1

Accessions	Species	Subspecies	Origin	Wx allele[5]	CT repeats in exon 1	5′ splice junction
232[1]	*O. sativa*	*indica*	China	Wx^a	11	GT
Patpaku[2]	*O. sativa*	*indica*	Taiwan	Wx^a	10	GT
ARC6622	*O. sativa*	*indica*	India	Wx^{op}	10	GT
ARC10818	*O. sativa*	*indica*	India	Wx^{op}	10	GT
Acc35618	*O. sativa*	*indica*	Indonesia	Wx^{op}	10	GT
160	*O. sativa*	*indica*	Taiwan	*wx*	17	TT
219	*O. sativa*	*tropical japonica*	Indonesia	Wx^{in}	17	GT
647	*O. sativa*	*tropical japonica*	Indonesia	Wx^{in}	17	GT
ARC12446	*O. sativa*	*tropical japonica*	India	Wx^{in}	17	GT
532	*O. sativa*	*tropical japonica*	Japan	*wx*	17	TT
703	*O. sativa*	*temperate japonica*	China	Wx^{in}	17	GT
734	*O. sativa*	*temperate japonica*	China	Wx^{in}	19	GT
848	*O. sativa*	*temperate japonica*	China	Wx^{in}	16	GT
T65[3]	*O. sativa*	*temperate japonica*	Taiwan	Wx^b	17	TT
Norin 8	*O. sativa*	*temperate japonica*	Japan	Wx^b	17	TT
563	*O. sativa*	*temperate japonica*	Japan	*wx*	17	TT
W107	*O. rufipogon*	(Annual)	India	Wx^a	8	GT
W1943	*O. rufipogon*	(Perennial)	China	Wx^a	13	GT
GMS1[4]	*O. glaberrima*		Hybrid	Wx^a	10	GT
W1468	*O. barthii*		Cameroon	Wx^a	10	GT

Data from
[1] Wang et al. (1990)
[2] Hirano et al. (1998)
[3] Hirano and Sano (1991)
[4] Umeda et al. (1991)
[5] Estimated by the levels of GBSS and amylase, as well as by I_2-KI staining (Sano et al. 1991; Mikami et al. 1999)

r1 and *p-SINE1-r2*) and a miniature inverted-repeat transposable element (*Tnr-1* or *Stowaway*) resided in the *Wx* gene (Fig. 1). Out of 57 polymorphic sites detected in regions A and B in the present study, 25 were present in these elements, suggesting a higher substitution rate in the inserted elements (Table 4). Table 4 showed that ssp. *japonica* accessions (both ssp. *tropical japonica* and ssp. *temperate japonica*) differed distinctly from ssp. *indica* and wild accessions with respect to the nucleotides of the *Wx* gene. The nucleotide sequence of a waxy landrace of *indica* (160), however, was similar to those of the *japonica* landraces.

The splice site mutation of intron 1 (G to T) was found in *temperate japonica* accessions as well as in a waxy landrace of *indica* (160). Polymorphic microsatellites (comprising CT repeats) were found 55 bp upstream of the 5′-leader intron splice site in the accessions examined in this study (Table 4). A greater number of CT repeats (17 to 19) was found in *tropical japonica* and *temperate japonica* accessions than in ssp. *indica* and wild accessions, except for a waxy landrace of *indica* (160), as reported by Ayres et al. (1997).

Changes in amino acids encoded by the *Wx* genes

In the 20 accessions examined, 32 informative sites were found, distributed over almost the entire coding region (Table 4). Moreover, in addition to 25 indels, three replacements and four synonymous substitutions were also detected. All the three *wx* accessions (160, 532, and T65*wx*) had the same 23-bp duplication in exon 2 (positions 111–133 in region B), which caused a frame-shift resulting in non-functional GBSS proteins. All three opaque accessions (ARC6622, ARC10818 and Acc35618) had the same replacement in exon 4 (A to G at position 762), which changed Asp to Gly. Furthermore, all six Wx^{in} carriers (C9071, 734, 703, 848, 647, and 219) had the same substitution in exon 6 (A to C at position 1132), altering Tyr to Ser. The two replacements specific to Wx^{op} and Wx^{in} were also examined using additional 75 *Wx* sequences reported (Table 5). Out of the 95 accessions surveyed, the same replacement specific to Wx^{op} was found only in *indica* landraces (4.2%), while that specific to Wx^{in} was found in various groups of *O. sativa* (33.3%). However, the replacement specific to Wx^{in} was especially frequent in ssp. *tropical japonica* (70.4%) and an *aromatic* group of ssp. *indica* (80.0%).

Molecular phylogenetic tree of the *Wx* gene

We examined 20 accessions that were representative of the five putative *Wx* alleles. The combined data (4,322 bp) were used for constructing a maximum parsimony tree. All 10 *japonica* accessions formed a distinct clade that was supported by a high bootstrap value (100%), but *indica* accessions were not grouped into a single clade (Fig. 2). All

Table 4 Informative sites of the *Wx* genes (regions A and B) in 20 accessions of wild and cultivated rice

Region		A (867 bp)	B(3, 455 bp)
Exon		1 1	2 2 4 5 6 6 9 12
Position			1 1 1 1 1 1 2 2 2 2 2 2 2 2 2 2 3 3 3 3 3 3 3 3 3 3 3
		1 2 2 2 2 2 2 3 4 4 4 4 4 4 4 4 5 5 5 7 7 8	1 7 8 9 0 0 1 1 8 9 2 2 2 3 6 7 7 8 8 9 1 1 1 1 1 2 2 2 3 3 3
		1 6 4 3 3 3 9 9 9 8 0 1 2 5 6 7 8 9 0 1 1 3 3 6	7 1 6 8 8 6 8 3 4 5 2 6 6 9 5 9 0 1 2 6 5 6 7 7 8 9 0 0 9 0 4 9
		8 5 2 2 4 5 4 5 9 2 9 1 6 6 5 7 0 0 6 4 6 3 4 7	5 8 1 2 4 5 5 9 2 3 3 4 0 7 4 4 7 2 2 6 1 3 3 0 7 5 1 3 4 4 5 4 8
Species	Allele		S R S S R S R
O. sativa ssp. *indica*			
232	Wx^a	T *l* G - A T T n T G T C G C - T C G C T C A - C	- C - A T T C A A T C A p - - - T A C G A T T A G G C A - G A C C
Patpaku	Wx^a	. *l* . . . - . - - A C . . . . - . .	. . . . G . . . . G - . T A . . . .
ARC6622	Wx^{op}	. *l* . . . - . - - A C . . . . - . .	. . . G . . . . G . . . T . . . . G . . T A . . . .
Acc35618	Wx^{op}	. *l* . . . - . - - A C . . . . - . .	. . . G . . . . G . . . . G . . T A . . . .
ARC10818	Wx^{op}	. *l* . . . - . - - A C . . . . - . .	. . . G . . . . G . . . . G . . T A . . . .
160	*wx*	. *l* T . G . . - - A C . A . . C A T . C T . . T	. . *o* G . . . . G . . *q* . . . . T . A . . . T . . .
O. sativa ssp. *japonica*			
219	Wx^{in}	. *l* . . G . . - - A C . A . . C A T . C T . . T	. . . . C . T . . A *q* . . . . T . A . . . T . . .
647	Wx^{in}	. *l* . . G . . - - A C . A . . C A T . C T . . T	. . . . C . T . . A *q* . . . . T . A . . . T . . .
703	Wx^{in}	. *l* . . G . . - - A C . A . . C A T . C T . . T	. . . . C . T . . . *q* . . . . T . A . . . T . . .
ARC12446	Wx^{in}	. *l* . . G . . - - A C . A . . C A T . C T . . T	. . . . C . T . . . *q* . . . . T . A . . . T . . .
734	Wx^{in}	. *l* . . G . . - - A C . A . . C A T . C T . . T	. . . . C . T . . . *q* . . . . T . A . . . T . . .
848	Wx^{in}	. *l* . . G . . - - A C . A . . C A T . C T . . T	. . . . C . T . . . *q* . . . . T . A . . . T . . .
T65	Wx^b	. *l* T . G . . - - A C . A . . C A T . C T . . T	. . . . T . . . *q* . . . . T . A . . . T . . .
N8	Wx^b	. *l* T . G . . - - A C . A . . C A T . C T . . T	. . . . T . . . *q* . . . . T . A . . . T . . .
532	*wx*	. *l* T . G . . - - A C . A . . C A T . C T . . T	. . *o* . . . . T . . . *q* . . . . T . A . . . T . . .
563	*wx*	. *l* T . G . . - - A C . A . . C A T . C T . . T	. . *o* . . . . . C . T . . . *q* . . . . T . A . . A T . . .
O. rufipogon			
W107	Wx^a	. - . . . . . *n* . A . . . . - . . . . T	. . . . . . - . . T . . . . T . . . . . T . . .
W1943	Wx^a	. *l* . . G . - - . A C . . . - . . . . T	A . . . . . - . . . . . - . . . . *r* . . . - C . A . . . .
Outgroup			
GMS1	Wx^a	C *l* . *m* . - . - - . C T . T . C . . T C . . T T	. T . . C G - C . . . . - . . . A *r* *s* A . . C T . . A - . . G T -
W1468	Wx^a	C *l* . *m* . - . - - . C T . T . C . . T C . . T T	A T . . C G - C . . . . - . . . A *r* *s* A . . C T . . A - . . G T -

The types of mutations are indicated by letters (*S* synonymous; *R* replacement; - indel) below the position. Dots indicate nucleotides or indel sequences identical to the first sequence. Region A includes parts of exon 1 (1–141 bp) and intron 1 (142–867 bp). *p-SINE1-r1* is at positions 387–539. *l* shows a polymorphic microsatellite (CT repeats) in exon 1 (Table 2). *m* and *n* show 2 bp- and 3 bp-insertions. The 5′ splice junction of intron 1 is at position 142. Region B includes the coding regions. The translation initiation (ATG) is at positions 22–24. *p-SINE1-r2* and *Tnr1* is at positions 2260–2400 and 3127–3370, respectively (Umeda et al. 1991). *o* shows a 23 bp-duplication causing a frameshift mutation in the 3 *wx* accessions. *p* shows the presence of *p-SINE1-r2*. *q* shows 3 bp-insertion. *r* shows 6 bp- (W1943) and 8 bp (GMS1, W1468) deletions. *s* shows 3 bp- (W1943), 4 bp- (GMS1, W1468) deletions. Allele-specific nucleotide changes are indicated by boxes

six Wx^{in} genes were included in the *japonica* clade, and the three Wx^{op} genes were found to be closely related to the Wx^a gene of Patpaku (*indica*). These results indicated that diverse alleles have emerged in different lineages. All the 20 accessions carried *p-SINE1-r1* and *Tnr-1*, but *p-SINE1-r2* was absent in W1943 (*O. rufipogon*) and outgroups (*O. glaberrima* and *O. barthii*).

Despite that a waxy accession from Taiwan (160) belongs to ssp. *indica*, the *wx* sequence was joined into the *japonica* clade (Fig. 2). The *japonica* clade was clearly separated from the other four *indica* accessions. A possible explanation is, therefore, that the *wx* allele has transgressed from *japonica* to *indica*. To examine this possibility, we compared the nucleotide sequences around the *Wx* gene (P0541H01.20, P0535G04.1, P0535G04.24 and *RFT1*) for 160 and two typical *indica* accessions (Peh-ku) and *japonica* (T65) from Taiwan (Fig. 3). Although region A of the *Wx* gene was different in Peh-ku and T65 (11 substitutions and 3 indels), 160 had an identical sequence to T65. In contrast, the four regions linked to the *Wx* gene were almost the

Table 5 Frequencies of the two replacements specific to Wx^{op} and Wx^{in} found in cultivated and wild rice species

Species	No. of strains examined	Amino acid substitution[a]		Accessions carrying the replacement in[b]	
		Exon 4	Exon 6	Exon 4	Exon 6
O. sativa					
ssp. *indica*	28	4 (14.3)	2 (7.1)	DQ415640, AB281446, Acc35618, ARC10818	DQ280629, DQ280622
aus	5	0 (0.0)	0 (0.0)		
aromatic	5	0 (0.0)	4 (80.0)		DQ280601–DQ280604
ssp. *tropical japonica*	26	0 (0.0)	19 (73.1)		DQ280658–DQ280660, DQ280662–DQ280671, AF031162, AB281450, AB281451, AB281457, AB281452, B281453
ssp. *temperate japonica*	23	0 (0.0)	4 (17.4)		AB281454, AB281455, DQ280635, DQ280643
O. rufipogon	6	0 (0.0)	1 (16.7)		DQ280675
O. glaberrima & *O. barthii*	2	0 (0.0)	0 (0.0)		
Total	95	4 (4.2)	30 (31.6)		

[a] Wx^{op} and Wx^{in} alleles carried amino acid substitutions of Asp to Gly (in exon 4), and Tyr to Ser (in exon 6), respectively, as shown in Table 4. The percentage is shown in a parenthesis

[b] In addition to the 20 accessions used in this study, the 75 sequences reported were surveyed. The 69 accessions from Olsen et al. (2006) and the 6 accessions from others were used. Classification into ssp. *tropical japonica* and ssp. *temperate japonica* is based on Olsen et al. (2006). Underlined accessions were examined in the present study

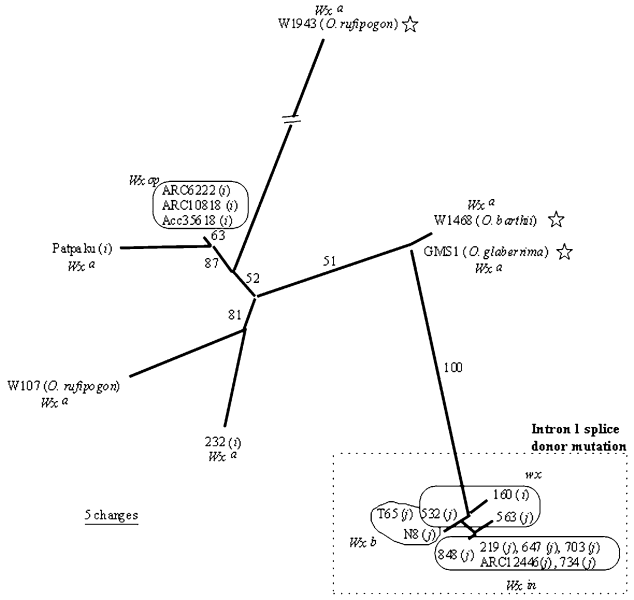

Fig. 2 Most parsimonious tree for wild and cultivated rice strains carrying different *Wx* alleles, based on the combined data sets (A and B regions). Bootstrap replicates are shown near the branches. (*i*) and (*j*) show *O. sativa* ssp. *indica* and ssp. *japonica*, respectively. *Star* shows the absence of *p-SINE1-r2* which is present in *O. sativa*. Tree length is 136. Consistency index (CI) is 0.941 (0.849 excluding uninformative characters). Retention index (RI) is 0.942

same in 160 and Peh-ku (33 out of 34 polymorphic sites). This result indicated that the *wx* allele of 160 was a result of gene flow from *japonica* to *indica* through recombination.

The physical boundaries of the transgressed fragment containing the *Wx* gene were expected to be located between P0541H01.20 and *wx* (about 1 Mb) at one extremity and between P0532G04.1 and *wx* (about 0.4 Mb) at the other, showing that a relatively small fragment (less than 1.4 Mb) has been integrated into the *indica* background.

Discussion

Allelic diversification in the *Wx* gene

There is a large degree of similarity among the mature GBSSI peptides (*Wx* gene product) of maize, wheat, barley, rice, potato and pea, showing an important role in starch synthesis (Ainsworth et al. 1993). Isoforms of GBSS (GBSSI and GBSSII) and soluble starch synthase (SSS) contribute to starch synthesis in plants (MacDonald and Preiss 1985; Umemoto and Terashima 2002; Nakamura et al. 2005). The deduced amino acid sequences of GBSSI are also very similar to those of GBSSII (Dry et al. 1992) and SSSI (Tanaka et al. 1995). In the Wx^{op} and Wx^{in} alleles, amino acid substitutions occurred at positions in which residues are conserved in the GBSSI peptides of maize, wheat, barley, potato and pea. These substitutions in the Wx^{op} and Wx^{in} alleles were also detected at positions in which the amino acids are conserved in GBSSI, GBSSII and SSSI. This agrees with the previous assumption that the mutation

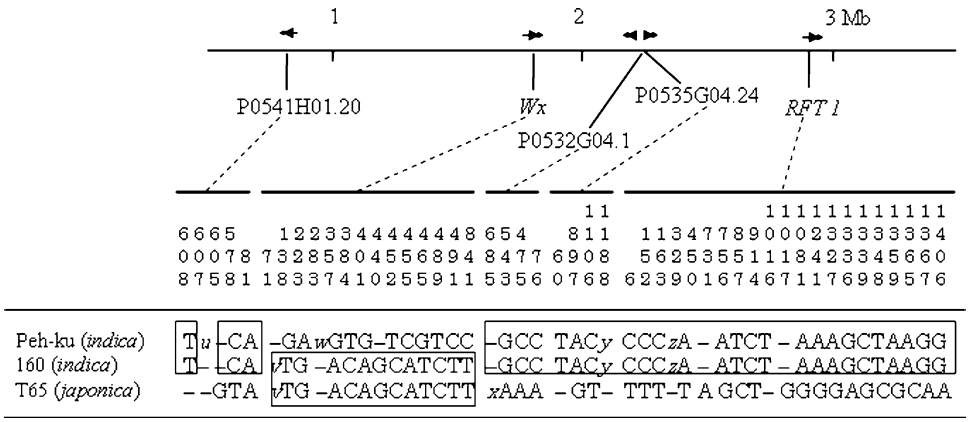

Fig. 3 A presumed introgression of the *wx* gene from *japonica* to *indica*. The three rice accessions from Taiwan were compared. 160 (*indica*) was waxy, whereas Peh-ku (*indica*) and T65 (*japonica*) were non-waxy. Notes: The three predicted genes (P0451H01.20, P0535G04.1 and P0535G04.24) and *RFT1* in AP001389 were used in addition to the region A of *Wx*. *u*, *v*, *w*, *x*, *y* and *z* are 2, 12, 4, 3, 3 and 70 bp insertions, respectively. *Boxes* show the identical nucleotides

in the Wx^{op} allele affects the enzymatic activity of GBSS, as reported in maize that *wx-m1* mutant has half of the GBSS activity of the wild allele (Wessler et al. 1986). The present results also support the previous finding that opaque accessions have a lower level of amylose in the endosperm, despite the GBSS level being similar to that in T65 carrying the Wx^{b} allele (Mikami et al. 1999).

A great variation is found in the sequences of the *wx* locus in landraces of rice, which gives an opportunity to inquire how multiple alleles affect phenotypes relating to agronomic traits. Assuming that the replacements in exon 4 and exon 6 are possibly specific to Wx^{op} and Wx^{in} alleles, respectively, their distribution was surveyed in 95 rice accessions including those in the public data bank (Table 5). The replacement of Asp to Gly in exon 4 was detected in an *indica* landrace (DQ415640: Haomuxi from Yunnan, China), in addition to the three landraces used in their study. The result suggests that the Wx^{op} allele is widely distributed in India, Nepal, Indonesia and China, despite that only ssp. *indica* possesses the Wx^{op} allele. On the other hand, accessions exhibiting intermediate amylose content have been reported in various groups of *O. sativa* (Resurreccion et al. 1994; Ayres et al. 1997; Frances et al. 1998). These accessions including Lemont (AF031162), Fortuna (DQ280658) and IR64 (DQ280622) carried the replacement specific to Wx^{in} in exon 6, while Rexmont (AF141954) and Jodon (AF141955) with high amylose content carried no replacement in exon 6. The Wx^{in} allele controlling intermediate amylose content might be frequently distributed in an *aromatic* group (Garris et al. 2005) and ssp. *tropical japonica*. However, this does not exclude a possibility that the Wx^{in} carrier shows intermediate amylose content due to the presence of modifiers such as *du* genes, as suggested by Mikami et al. (2000) and Dung et al. (2000).

In mutation analysis of rice, most waxy mutations were associated with changes in amino acid, resulting in generation of a stop codon, frame-shifts, amino acid substitutions or disrupted mRNA splicing (Umeda et al. 1991; Inukai et al. 2000; Sato and Nishio 2003). Further, a rice variety (Milky Queen) with a lower amylose content had a mutant allele (*Wx-mq*) induced with *N*-methyl-*N*-nitrosourea (MNU) at the *wx* locus. The mutant allele reportedly had two amino acid substitutions that were likely to produce lower amylose content in the endosperm (Sato et al. 2002). The amino acid substitutions found in the Wx^{op} and Wx^{in} alleles in the present study have not been reported from any mutant allele produced in previous mutation analyses, showing that the *Wx* gene has the potential to undergo various mutations affecting starch properties in rice.

Genealogy of the *Wx* gene

Genealogical approaches offer a way of determining the types of contemporary and historical processes that have influenced the current distribution of variation although lineage diversification has been difficult to analyze because of the hybridizing nature of crop species (Schall et al. 2003). Results in the *Wx* genealogy show that all the examined accessions carried *p-SINE1-r1*, and *Tnr-1*, but *p-SINE1-r2* was present in all the cultivated rice accessions and W107 (*O. rufipogon*), supporting the previous finding that *p-SINE1-r2* integrated into the *Wx* gene after *O. sativa* and *O. rufipogon* had diverged from other species with the AA genome (Hirano et al. 1994). The level of nucleotide diversity in the *Wx* gene made it possible to consider the genealogical relations of the putative *Wx* alleles. A distinct feature is that *indica* accessions predominantly possess Wx^{a}, but they rarely possess the other alleles (Sano et al. 1986). Among the five *Wx* alleles studied, only Wx^{a} was

frequently shared between the wild progenitor and the ssp. *indica*. In the phylogenetic tree constructed, a waxy *indica* accession (160) was, unexpectedly, located in the *japonica* clade. The *japonica* accessions and 160 carried a greater number of CT repeats (16–19) in region A than did the other accessions (8–11). It is unlikely that multiple mutations have occurred in the different lineages. Incongruence between the tree and the subspecific classification suggest lineage sorting and hybridization or a combination of these phenomena in a group of closely related taxa. The examination in the region linking to the *Wx* allele showed an example of introgression between the two subspecies.

Regarding the genealogy of the *Wx* gene in rice, Olsen and Purugganan (2002) concluded that the splice donor mutation has a single evolutionary origin. However, in the present study, polymorphisms were observed for the splice donor mutation and *wx* within both *indica* and *japonica* subspecies, as reported by Yamanaka et al. (2004). Recently, the splice donor mutation has been found to be associated with not only waxy landraces but also with ssp. *temperate japonica*. Further, the nucleotide diversity surrounding the *Wx* gene has revealed that a selective sweep has occurred in the *temperate japonica* landraces associated with the mutation (Olsen et al. 2006). Thus, the question of whether the *wx* mutations have occurred independently in both ssp. *indica* and ssp. *japonica* after their divergence need to be inquired using more *wx* accessions of ssp. *indica*.

Diversification during rice domestication

The five putative *Wx* alleles investigated are likely to be distributed widely in rice, given that they have been found in landraces from different geographical regions. However, opaque mutants similar to the *wx* phenotype are found in only ssp. *indica* (Heu 1986; Heu and Kim 1989; Mikami et al. 1999), and *wx* accessions are rare in ssp. *indica* (Bao et al. 2002; Yamanaka et al. 2004). The *wx* allele found in an *indica* landrace (160) in the present study may be a result of gene flow from ssp. *japonica*. Further, the amino acid substitution specific to Wx^{in} was frequent in ssp. *tropical japonica* as well as the *aromatic* group (Table 5). It is interesting to note that the *aromatic* group associated with Basmati or high-quality rice is closely related to ssp. *japonica* than to ssp. *indica* (Garris et al. 2005). These results support the hypothesis that allelic diversification at the *wx* locus occurred after the divergence of ssp. *indica* and ssp. *japonica*. Rice accessions can be divided into four groups on the basis of amylose content (very low, low, intermediate or high content), in addition to amylose-less accessions (Kumar and Khush 1987). Altered levels of amylose in the NILs could be caused by genes linked to the *Wx* gene; however, the existence of the amino acid substitutions and frame-shift mutations that are frequently found in the *Wx* genes indicates that the *Wx* alleles themselves contribute to the continuous variation of amylose content observed in Asian rice landraces.

Recently, researchers working on crop evolution have examined how genetic diversity is shaped by population bottlenecks and intense selection for agronomic traits during the domestication of crop species (Eyre-Walker et al. 1998; Vigouroux et al. 2005). Selective sweeps can dramatically reduce genetic diversity in target genes, whereas diversifying selection is encountered during geographic expansion of domesticated crops in response to regional environments and human cultural preferences. Asian rice landraces show significant loss of diversity at the *Wx* gene, indicating a selective sweep for a region spanning >250 kb (Olsen et al. 2006).

It has been reported that allelic diversification in *OsC1* causes polymorphic coloration patterns in Asian rice. The genealogy of the *OsC1* gene suggests that mutant alleles might have resulted from recent amino acid substitutions in the *japonica* lineage rather than from pre-existing alleles in other lineages (Saitoh et al. 2004). The present results imply that farmers have selected favorable mutant alleles at the *wx* locus, suggesting that diversifying selection has occurred owing to diverse cultural preferences. Therefore, it could be assumed that compared with wild relatives, landraces of rice might maintain agronomically valuable alleles that have been accumulated during their geographical expansion, even if nucleotide diversity has been reduced by a population bottleneck during domestication.

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