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Diversity of *waxy* gene alleles in the wild rice species of the *Oryza* genus

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ABSTRACT. Amylose content and granule-bound starch synthase activity were measured for wild rice species (*Oryza rufipogon*, *Oryza officinalis*, *Oryza meyeriana*) and four widely cultivated rice varieties. The result indicates that the activity of GBSS in all rice species is positively correlated with the amylose content. The *waxy* gene alleles and their transcripts were cloned from three wild rice species using PCR amplification from genomic DNA or RT-PCR amplification from mRNAs of immature seeds. The *waxy* gene alleles of the three wild rice species and four cultivated rice varieties had different genomic DNA sequence sizes, intron-exon structures, and amino acid sequences. The predicted secondary structures of *waxy* proteins from the wild rice and cultivated species differed significantly. The *waxy* allele of *O. rufipogon* was most closely related to those of the cultivated rice varieties, with *O. meyeriana* less closely related and *O. officinalis* most distantly related. All of these results suggest that the allelic diversity of the *waxy* gene in *Oryza* genus is very rich. There might be different regulation mechanisms controlling amylose content by *waxy* genes in the wild rice species compared with the cultivated rice varieties (*Oryza sativa* L.).

Keyword: Cloning and analysis; Gbss enzyme; *Waxy* gene allele; Wild rice.

INTRODUCTION

Rice is one of the most important crops in the world. It is a staple food for more than half of the world's population. The demand for rice with better quality has been growing in the global market (Tang et al., 1989). The challenge now is to produce high quality cultivated varieties of *O. sativa* to satisfy this demand. Amylose content and proportion are important standards in appraising rice quality (Tan and Corke, 2002; Lim et al., 1995). Low and intermediate amylose content is considered an important factor that good quality rice should have in China and many other Asian countries like Japan and Korea. Granule-bound starch synthase (GBSS), an enzyme encoded by the *waxy* gene and responsible for the synthesis of amylose in rice endosperm (Denyer et al., 2001; Nakamura and Yuki, 1992; Nakamura et al., 1989) has been investigated in cultivated crops such as *O. sativa* indica or japonica rice varieties (Li et al., 2001; Ahmadi and Baker, 2001; Hirano and Yoshio, 1991; Geng et al., 2005; Cai et al., 2000), wheat (Kumar and Simgh, 1980), corn (Doehlert, 1993), and potato, but not in wild rice species.

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A few *waxy* gene alleles in different cultivated rice varieties (*Oryza sativa* L. AA genome 2n=24) with high (25-30%) or medium amylose contents (15-20%) have been reported (Cai et al., 2000). It is found that in general, the medium amylose contents in some cultivated rice varieties are mainly caused by the G to T change at the 5' splice junction of the first intron in the *waxy* genes, which alters GBSS activity (Cai et al., 2000). The G to T point mutation in the first intron of the *waxy* gene alleles interrupts the formation of mature mRNA which causes low synthesis of amylose in the cultivated rice varieties studied (Zhu et al., 2004a; Zhu et al., 2004b; Li et al., 2005). The other introns and exons of the *waxy* genes were found to be quite conservative in the cultivated rice varieties of *O. sativa* (Zhu et al., 2004a,b; Bligh, 1995). These previous studies have showed that, the genetic diversity of the *waxy* genes of *O. sativa* rice varieties is not rich (Frances et al., 1998). Whether other *Oryza* species including some wild rice species also have conservative DNA sequences of *waxy* alleles is unknown.

Three wild rice species—*Oryza rufipogon* Griff (A'A' genome, 2n=24), the *Oryza officinalis* Wall (CC genome, 2n=24), and the *Oryza meyeriana* Baill (GG genome, 2n=24)—originating in China (Zhong and Cheng, 2000) were recently found to have relatively lower amylose content (10-14%) compared with most of the cultivated rice

varieties of *O. sativa* (Cheng et al., 2005). If we want to use the characteristics of relatively low amylase contents of these wild rice species to decrease amylose content of the cultivated rice varieties for better quality, many aspects of the GBSS activity and *waxy* alleles of the wild rice species require investigation. Namely, it is necessary to investigate whether they have conservative DNA sequences of *waxy* alleles.

In our study we investigate the relationship among the amylose content, GBSS activity, and the DNA sequences of the *waxy* genes in three wild rice species: *O. rufipogon*, *O. officinalis*, and *O. meyeriana*. We investigate first whether GBSS activity is closely related to the amylase content of wild rice species and secondly whether the exons and introns of the *waxy* genes of wild rice are as highly conservative as those of the *O. sativa* cultivated varieties. This study should also help further understanding the genetic relationship among species in the *Oryza* genus through greater knowledge of *waxy* genes. To our knowledge, this is the first report examining the enzyme activity and *waxy* gene alleles in wild rice species, and this should be useful in improving future rice quality.

MATERIALS AND METHODS

Materials

Three species of wild rice, *O. rufipogon*, *O. officinalis*, and *O. meyeriana,* were used in this study. (In China, some people mistake *O. meyeriana* for *Oryza granulata.*) All the three wild rice species originated in China (Cheng et al., 2005). Four widely cultivated rice varieties—Zhan 6, Dian-chao 6, He-xi 41 and Hou736/KM670—were used as controls. All the rice plants were grown in the same field according to the method of Jeng et al. (2007).

Amylose analysis

Amylose content was measured by a standard method developed in the previous studies reported by Zhu et al. (2004a, b).

Enzyme activity assays for GBSS

Immature seeds 10, 20 or 30 DAA were collected from the field for the extraction of enzymes (Jahan et al., 2002). A known number of dehulled grains was homogenized at 0°C in a mortar and pestled with 10 mM trismaleate buffer (pH 7) containing 1 mM dithiothreitol. The homogenate was centrifuged at 30,000 *g* for 30 min at 0°C, and the supernatant was decanted. The pellet was then washed three times with the extracting buffer. Starch synthetase bound to starch granules was prepared from the washed pellet according to the method of Cathie et al. (1995). Preliminary enzyme assays were carried out to determine conditions under which linear rates corresponding to time and substrate concentrations could be obtained. GBSS was determined with the method reported by Jahan et al. (2002). The enzyme activity was expressed in nanomoles per min per gram of rice grain.

Cloning of *waxy* genes

Extraction of genomic DNA. Rice leaves were used to extract genomic DNA with the CTAB method (Luo and Shi, 2003).

Primers design and PCR amplification. According to the conservative sequences of the reported *waxy* genes of rice (Hirano et al., 1998; Cai et al., 2000), three pairs of overlapping primers were designed. A group of TAIL-PCR primers were also designed to get the first intron. All primers were synthesized by Shanghai Sangon Biological Engineering Technology and Service Co. Ltd.

PCR primer:

A1: 5'-ATGTCGGCTCTCACCACGTC-3'

A2: 5'-TCGACTCCACGCTTGTAGCA-3'

B1: 5'-AAGGTTGCAGACAGGTACGA-3'

B2: 5'-GATGAGAT GAGCAAGCGGCG-3'

C1: 5'-CTCAAGAGCAT GGAGGAGAA-3'

C2: 5'-AGCACACCCAGAAGAGTACAA-3'

C3: 5'-CAGCATCAGACTTATTAGCC-3'

Two of these primers were chosen based on purpose for a certain *waxy* fragment of each PCR amplification. The total volume for PCR amplification was 20 µl, containing 50-100 ng of genomic DNA, 50 mM KCl, 10 mM Tris-HCl pH 9, 1.25 mM $MgCl_2$, 0.2 mM of each dNTP, 0.6 µM of each primer, and 5U of Taq DNA polymerase. The PCR amplification procedure was done as described by Luo et al. (2003) (Luo and Shi, 2003).

Extraction of total RNA and RT-PCR. The young seeds were taken 20 days after anthesis (DAA). The isothiocyanate method was used to extract total RNA (Zhang et al., 2001).

cDNAs of *waxy* genes were synthesized through reverse transcription (RT) using mRNAs as templates and two pairs of primers (A1 and A2 or B1 and P1). The AMV transcriptase and the reaction conditions were according to the manufacturer's instructions (TOYOBO Corporation). The Oligo d(T)18-CTGATCTAGAGGTACCGGATCC (P1) anchor primer was used in the 3'RACE to synthesize the cDNA. The PCR amplification was carried out by B1 primer and the anchor primer, which did not include the Oligo d(T) primer. The A2 primer was used to synthesize the cDNA in the second part. Then two parts of the cDNA were used as template for PCR with the B1 and A1 primer. PCR reactions were carried out at 94°C pre-denatured for 5min, followed by 35 cycles of 1 min at 94°C, 45 s at 50°C, 60s-90 s at 72°C and a final 7-min extension step at 72°C.

Finally, the PCR products of the *waxy* genes were ligated to a pGM-T vector, and *E. coli* was transformed with plasmid. The positive clones were chosen for sequencing by Shanghai Sangon Biological Engineening Technology and Service Co. Ltd.

Bioinformatic analysis. The sequences of the *waxy* alleles of three wild rice species were identified on the

basis of homology by using the BLAST algorithm in GenBank. Cladistic analyses of amino acid sequences were performed using the DNASIS and DNASSIST programs (Ayres et al., 1997).

RESULTS

GBSS activity assays

The results showed that the GBSS enzyme activity varied at different seed filling stages. For all the rice materials, the highest enzyme activity was around 20 days after anthesis (Figure 1). At all filling stages, GBSS activity in the three wild rice species was significantly lower than in most *O. sativa* rice varieties. *Oryza rufipogon* had the highest amylose content; *O. meyeriana* had a moderate content; and *O. officinalis* had the lowest content. Similarly, *O. rufipogon* had the highest GBSS activity; *O. meyeriana* had moderate activity; and *O. officinalis* had the lowest activity (Figure 1). The lower GBSS activity of the three wild rice species is closely related to the lower amylose content in their seeds compared with the cultivated rice varieties.

Even though the other enzymes, including ADPGP, SSS, and Q-enzyme, had some effect on amylose synthesis and content percentage, they had no correlation with amylose content of wild rice seeds (data are not shown here). Only GBSS activity had the closest correlation with amylose content. This indicated that the amylose content was also controlled by the GBSS in the three wild rice species. Therefore, cloning the *waxy* genes of wild rice species *O. rufipogon, O. officinalis*, and *O. meyeriana* is valuable.

Waxy alleles from three wild rice species

The *waxy* gene sequences were cloned from the genomic DNA of the three wild rice species. The 3,494 bp DNA sequence of the *waxy* gene was obtained from *O. rufipogon*, which included the sequence from the start codon to the downstream. The 3,213 bp DNA sequence of

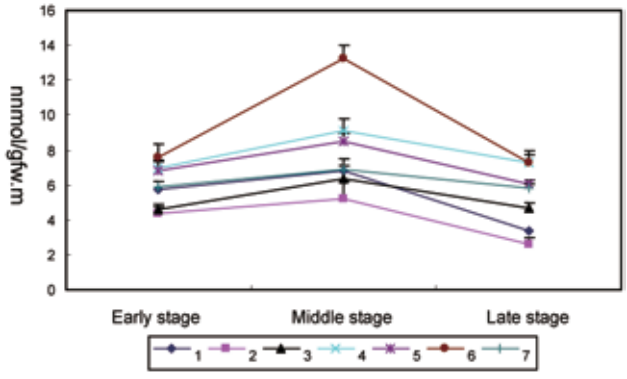

Figure 1. The GBSS activity of different wild rice species and *O. sativa* varieties at seed filling stages. AC of each accession or variety is shown in the brackets. 1: *O. rufipogon* (11.99%); 2: *O. officinalis* (9.70%) *s*; 3: *O. meyeriana* (11.28%); 4: Zhan 6 (18.00%); 5: Dian-chao 6 (16.90%); 6: He-xi 41 (19.23%); 7: Hou736/KM670 (15.50%).

Figure 2. The introns and exons (coding sequences) of the *waxy* genes from three wild rice species.

the *waxy* gene was obtained from *O. officinalis*. The 3,342 bp DNA sequence of the *waxy* gene was obtained from *O. meyeriana*. The *waxy* genes sequences of *O. rufipogon*, *O. officinalis*, *O. meyeriana* were submitted in GenBank with accession numbers GU977196, GU977195, and GU977194, respectively.

The coding region of the *waxy* gene in *O. rufipogon*. The coding sequence of *waxy* gene cloned from *O. rufipogon* was 1,410 bp. The *waxy* gene from *O. rufipogon* has 14 exons and 14 introns (as shown in Figure 2). The final terminator TAA was located at position 1569-1571. Nineteen terminators such as TAG, TGA, and TAA were also located at the 3' end of the gene, and the tail signal AATAAA in the coding region was also located at position 1535-1539. The tail signal in front of the final terminator may have some special functions and merits further study. Finally, the amino acid sequence was deduced from the *waxy* gene of *O. rufipogon* (Figure 3). There were 470 amino acids in *waxy*. The relative molecular weight of this protein was 52.07 kDa. Its isoelectric point was 6.77.

The coding region of the *waxy* gene in *O. officinalis*. The whole transcript region was obtained from the start codon to the polyA tail. The *waxy* gene of *O. officinalis* has 14 exons and 13 introns as shown in Figure 2. Its coding sequence was 1,827 bp. The terminator TGA was located at position 1,827-1,829, and the tail signal AATAAA in the coding region was also located at position 2025-2029. Compared with the *waxy* gene of *O. rufipogon*, the ternimator in *O. officinalis* was TGA instead of TAA. The tail signal AATAAA in *O. officinalis* was 194 bp upstream of the terminator TGA. This is quite different from that of the *waxy* gene of *O. rufipogon*. Finally, the deduced amino acid sequence is shown in Figure 3. There were 609 amino acids in *waxy*. The relative molecular weight of this protein was 66.48 kDa. Its isoelectric point was 8.37.

The coding region of the *waxy* gene of *O. meyeriana*. 2,043 bp of *waxy* gene transcript region was also obtained from the first codon ATG to the polyA tail. After comparison, 13 exons and 13 introns were identified in the *waxy* gene of *O. meyeriana* as shown in Figure 2. The coding sequence of the *waxy* gene was 1,827 bp in *O. meyeriana*. The terminator TGA was located in the position 1827-1829 in the *waxy* gene of *O. meyeriana*. Moreover, more than five TGA termination codons were located behind the terminator TGA at the 3' end. Whether these terminator codons make a stronger termination to transcription of the *waxy* gene is unknown. The tail signal AATAAA in the coding region was located at position 2006-2010. The amino acid sequence deduced by the coding regions of the

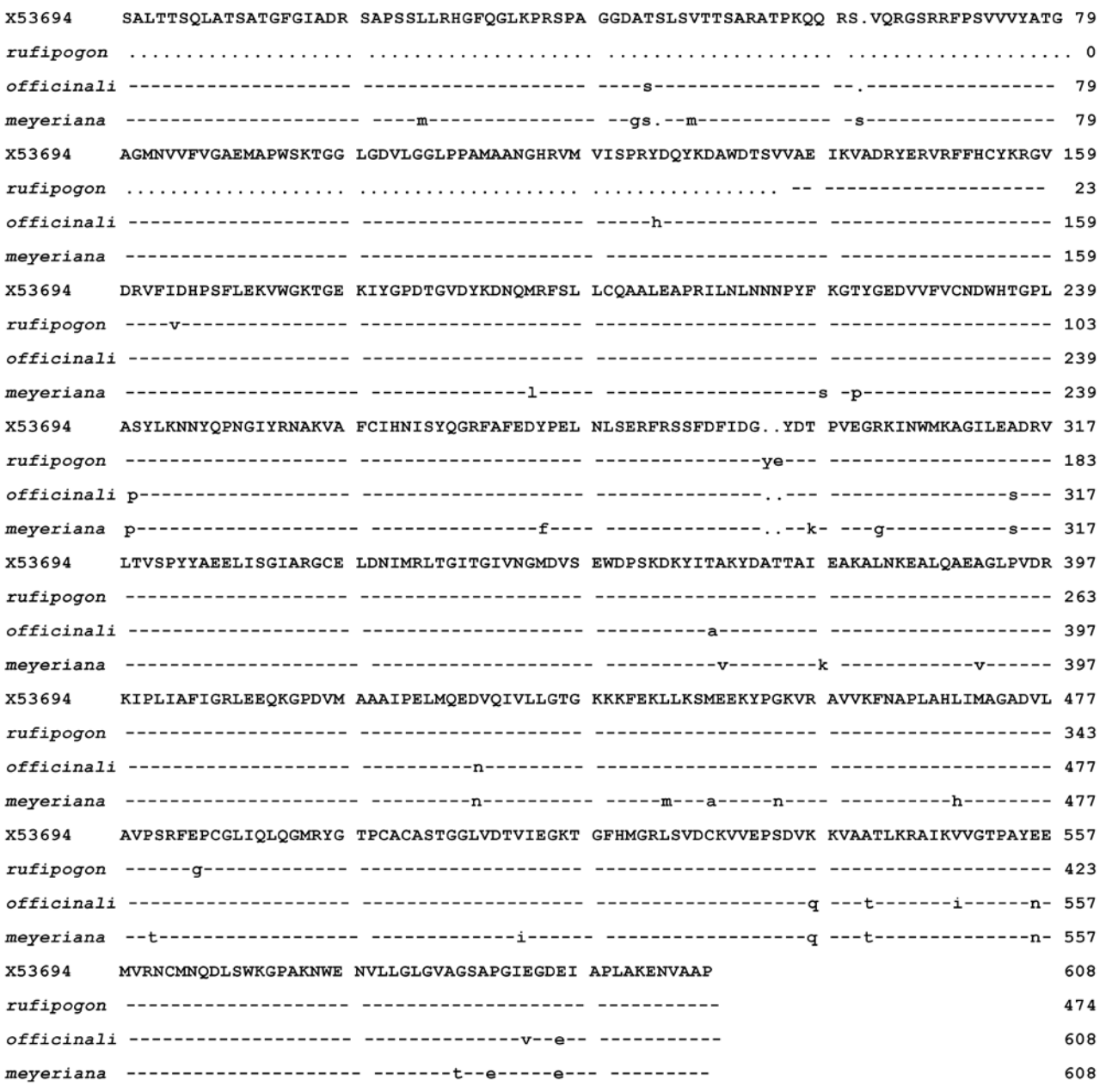

Figure 3. The amino acid sequences coded by the *waxy* genes of three wild rice species and the *O. sativa* varieties, respectively.

waxy gene was shown in Figure 3. The amino acid number of the *waxy* of *O. meyeriana* was 609. The relative molecular weight of this protein was 66.63 kDa. Its isoelectric point was 8.37.

Homogeny assay of the *waxy* gene in the three wild rice species

Multiple analysis of the coding sequences. Cluster analysis of the whole coding regions of *O. meyeriana*, *O. officinalis, O. rufipogon*, and of the *waxy* genes X53694 (Japonica variety), D10472 (African rice), and AF141955 (Indica variety) was performed using the DNAMAN software (Figure 4A). The homology matrix reached 99.4% between *O. rufipogon* and the cultivated rice varieties. However, the homology matrix between *O. meyeriana*, *O. officinalis*, and the cultivated rice varieties was lower. The distance matrix between the *O. sativa* varieties and *O. rufipogon* was the shortest, but the distance matrix between the cultivated rice varieties and *O. meyeriana* was the longest. In addition, the distance matrix between *O. rufipogon* and *O. officinalis* or *O. meyeriana* was longer than the distance matrix between *O. rufipogon* and the cultivated rice varieties. Therefore, in the cluster map, *O. sativa* rice varieties and *O. rufipogon* were clustered in a group while *O. officinalis* and *O. meyeriana* were clustered in the other two groups.

Multiple alignment of the coding amino acid sequences. The cluster analysis of the whole amino acid sequences deduced, respectively, from the coding regions of *O. meyeriana*, *O. officinalis*, and *O. rufipogon*, as well as from the *O. sativa* varieties Japonica variety (X53694), *O. glaberrima* variety (D10472), and Indica variety (AF141955) was shown in Figure 4B.

Five amino acid differences appeared among the 470 amino acids deduced from the *waxy* gene of *O. rufipogon* compared with those of the three cultivated rice varieties. In *O. officinalis*, the waxy gene sported 13 extra amino acids, but that was two amino acids less than held by the waxy gene of the *O. sativa* varieties. In the deduced GBSS of *O. meyeriana* and *O. officinalis* there were seven identical amino acid insertions compared with the GBSS of the three cultivated rice varieties (shown in Table 1). Between the three wild rice species and the cultivated rice varieties, there were six different amino acids within amino acid positions 1-65 of the sequences. There were more than six different amino acids within the positions of 275-319, 445-485 and 512-559. At position 588-597, four of the amino acids were different. These results suggest some difference exists between the three wild rice species and the *O. sativa* varieties in the amino acid sequences coded by the *waxy* gene, even though the amino acids were highly conservative in the cultivated rice varieties.

According to the cluster map of the amino acid sequences deduced from the *waxy* alleles, the shortest genetic distance was between the cultivated rice varieties and *O. rufipogon*. The distance between the *O. officinalis* and *O. sativa* varieties was moderate, and the longest distance was between the *O. meyeriana* and *O. sativa* varieties. The predicted secondary structures of the *waxy* proteins of the three wild rice species differed significantly from each other and from those of the cultivated rice varieties (X53694) (shown in Figure 5).

Multiple alignment of intron sequences. The cluster analysis on the 13 intron regions of the *waxy* genes from *O. officinalis*, *O. rufipogon*, and *O. meyeriana* and the reported sequences X53694 (Japonica variety), D10472 (African rice variety), and AF141955 (Indica variety) is shown in Figure 4C. The intron homology matrix of the *waxy* gene was generally low. The intron homology matrix was 91% between *O. rufipogon* and the cultivated rice varieties and 71% between the *O. sativa* cultivated varieties and *O. meyeriana* or *O. officinalis*. Compared with the homology matrix of the exon sequences, the intron homology matrix was obviously lower. According to the cluster analysis, the cultivated rice varieties and *O. rufipogon* were still clustered in a group. *Oryza meyeriana* and *O. officinalis* were clustered in another group. This was the same as the result of the cluster analysis of the exons and of the amino acid sequences.

Nucleotide insertion, deletion and transversion among the introns of the waxy gene sequences. Compared with X53694 and AF141955, there were in total 53 inserted nucleotides, mostly one nucleotide insertion at one site,

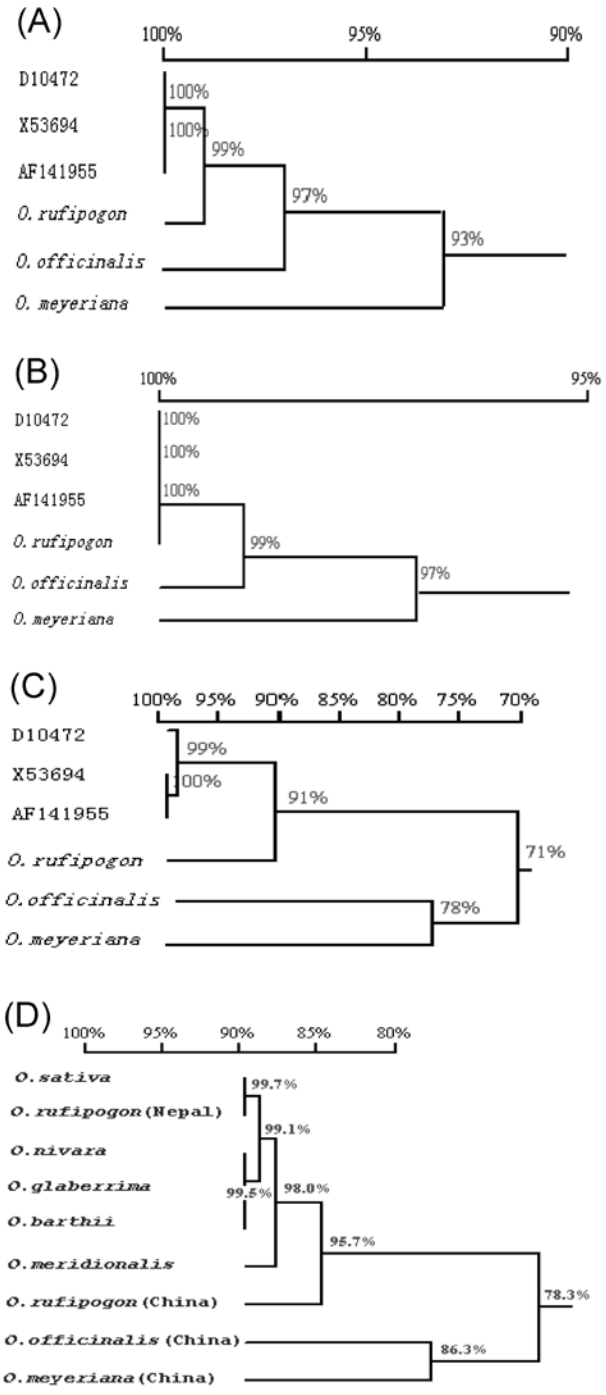

Figure 4. Cluster analysis of the 4 EST sequences (A), the coded amino acid sequences by the *waxy* genes (B), the intron sequences (C) and the homology tree (D) of the *waxy* genes from three wild rice species and the *O. sativa* varieties, respectively.

Table 1. Amino acid comparison among the sequences from the *O. officinalis*, *O. meyeriana*, and the *O. sativa* varieties.

O. officinalis and *O. meyeriana*	P	A	N	Q	T	N	E
The cultivated variety	A	S	D	K	A	E	D
Amino acid position	241	315	429	538	542	557	596

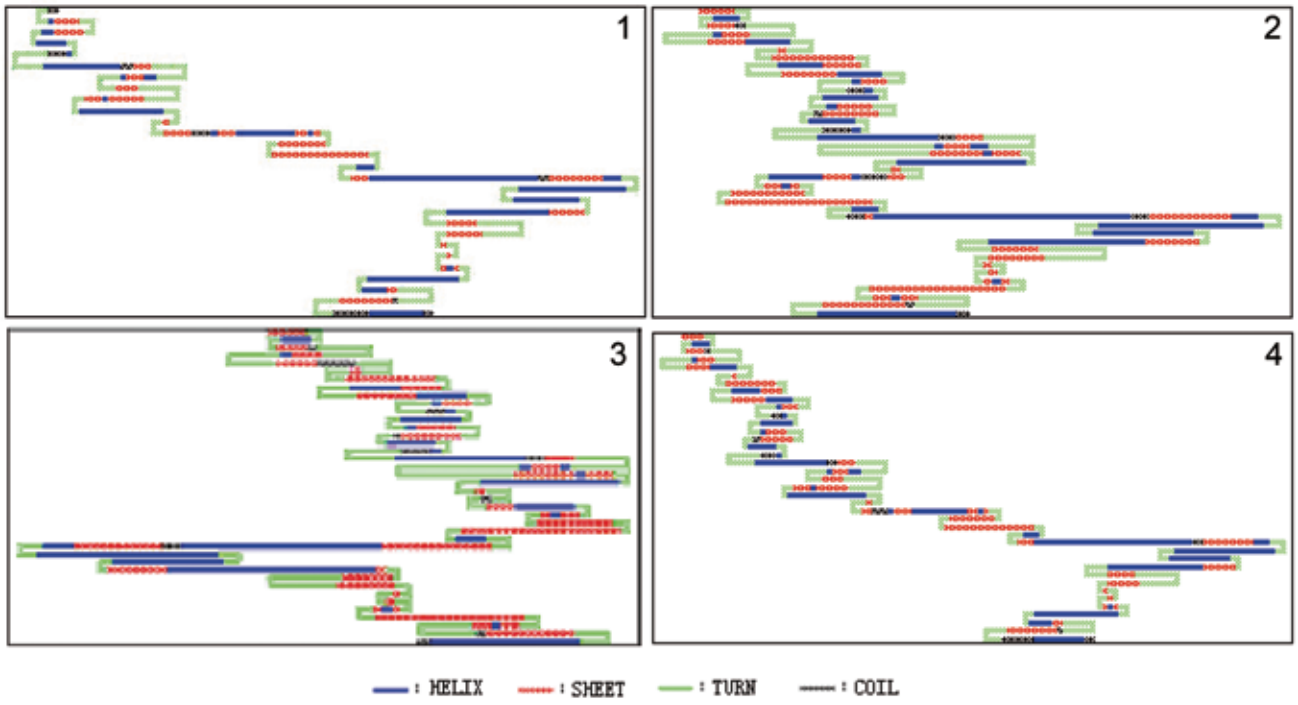

Figure 5. The predicted secondary protein structures of the *waxy* of three wild rice species and X53694. 1: *O. rufipogon*; 2: *O. officinalis*; 3: *O. meyeriana*; 4: X53694.

in the *waxy* gene from *O. rufipogon*. An insert of nine nucleotides, encoding three amino acids, was observed in an exon of the *waxy* gene from *O. rufipogon*. There were 264 nucleotides deleted in the *waxy* gene in *O. rufipogon*. One deletion of 139 nucleotides was found in an intron of the *waxy* gene from *O. rufipogon*. Most deletions happened within the introns. In the *waxy* gene sequence of *O. rufipogon*, there were more nucleotide transitions among purine nucleotides or among pyrimidine nucleotides than nucleotide transversions between purine and pyrimidine nucleotides. Interestingly, the number of nucleotide transitions or transversions in introns was the same as in exons.

All together, 300 nucleotides were deleted in the *waxy* gene from *O. officinalis*. Most of the deletions occurred in the intron regions. The longest deletion was of 139 nucleotides in an intron. Interestingly, all of the 300 deletions were located in the introns. Among the *waxy* gene sequence of *O. officinalis*, there were many nucleotide transitions or transversions, and the number in introns was the same as in exons. Compared with the *waxy* gene sequence of *O. rufipogon*, the proportion of the nucleotide insertions, transversions, and transitions was higher in the *waxy* gene of *O. officinalis*.

In total, 33 inserted nucleotides were in the *waxy* gene of *O. meyeriana*. The longest insertion was of eleven nucleotides located in an intron. Meanwhile, the number of insertions among the exons was similar to that among the introns. This phenomenon in *O. meyeriana* is very special compared with that in *O. rufipogon* and *O. officinalis*. There were 275 nucleotides deleted among the *waxy* gene in *O. meyeriana*. The longest deletion was of 139 nucleotides in the intron. In the *waxy* gene sequence of *O. meyeriana*, there were also many nucleotide transitions or transversions. Nearly two-thirds of nucleotide transitions and transversions occurred inside the intron regions. It is extraordinary that more than 1/10 of the total *waxy* gene nucleotides transited or transversed in *O. meyeriana*, which was much higher than in other wild rice species.

Specific primers of three wild rice species were designed for marker-associated selection (MAS)

Since the *waxy* alleles of the three wild rice species had a lot of different DNA sequences, three pairs of specific primers were designed according to these different sequences. Every pair of specific primers was tested by PCR in the three wild rice species and the cultivated rice varieties, respectively. (The data will be shown in another paper.) Therefore, these primers can be used for MAS in progenies of wild rice and *O. sativa* varieties after hybridization.

O. rufipogon upstream primer: ATTATGCTCACAGGTGGCCA

downstream primer: ATTAGTAAGCGGCGCGTTGA

O. officinalis upstream primer: ATGATATCATCTCCGGCATC

downstream primer: CGCCGATCAGATTATACATC

O. meyeriana upstream primer: TTCCTGGAGAAGGTGCTTGA

downstream primer: CGGATGCAGAAAGCAACCTA

DISCUSSION

The *waxy* transposon-like sequences of *waxy* genes in the wild rice species

The *waxy* alleles of the three wild rice species have transposon-like sequences. The sequence ATTATATAG is identical to the key part of transposon (Tn#7) and was the IHF binding site (Gregorio et al., 2006; Kimberly et al., 2007). The sequence ATTATATAG was located at the position 4,239-4,248 bp in the *waxy* gene of *O. rufipogon.* In addition, in the *waxy* gene of *O. rufipogon*, sequences AGCCCT, GGCGCT, and CCAACC are also transposon-like target sites located, respectively, at the positions 3,306-3,312 bp, 2,744-2,750 bp, and 112-118 bp. Similarly, in the *waxy* gene of *O. officinalis* sequences AGCCCT, GGCGCT, and GGTTGG are also transposon-like target sites located respectively at the positions 2,725-2,731 bp, 2,322-2,328 bp, and 1,329-1,335 bp. In the *waxy* gene of *O. meyeriana*, the sequences GTGGGG, AGCCCT, and GGCGCT are also transposon-like target sites located at the positions 1,939-1,945 bp, 2,302-2,308 bp, and 1,908-1,914 bp.

The transposons may be important factors contributing to the changes of the *waxy* alleles in the *Oryza* genus and may be responsible for the rich genetic diversity of the *waxy* alleles of the wild rice species. This needs to be confirmed by further study.

The homology comparison of *Oryza* species

The homology tree shows that the *waxy* alleles of *O. sativa*, *O. nivara*, *O. glaberrima, O. barthii, O. meridional*, and *O. rufipogon* (Nepal) reported in GenBank had close genetic relationships (as shown in Figure 4 D). The homology ratios among these *waxy* alleles are higher than 99.1%. However, the *waxy* gene of *O. rufipogon* (China) used in our study is different from that of *O. rufipogon* (Nepal) (Hirano et al., 1998; Kennet et al., 2002). It also indicates that the *O. rufipogon* accessions originating from different countries may have different functional genes. The *waxy* genes of *O. officinalis* and *O. meyeriana* used in our study have less genetic relationship with the *waxy* alleles of other species. For example, the homology ratios of the *waxy* genes of *O. officinalis* and *O. meyeriana* with other species were less than 86.3% and 78.3%, respectively.

There is rich genetic diversity in wild rice *waxy* gene alleles

The *Oryza* genus contains 20 wild rice species and two cultivated species. In the past a lot of investigations have shown *waxy* gene homology in the different cultivated rice varieties of *O. sativa* L. It is significant that the *waxy* gene sequences are quite conservative in many exon and intron regions among the cultivated rice varieties. However, in our study, many nucleotide inserts, deletions, transversions, and transitions occurred in many exons and introns of the *waxy* genes of the wild rice species compared with the cultivated rice varieties. In addition, the *waxy* gene from *O. rufipogon* and *O. officinalis* had one more exon than the *O. sativa* cultivated varieties. Nucleotide changes cause genetic diversity. Even though the genetic diversity of the *waxy* gene is not rich in *O. sativa* cultivated varieties, the genetic diversity among the *Oryza* genus is very rich because of several wild rice species.

Waxy genes of wild rice species used in this study may have a different mechanism for controlling amylose content

Many studies have found that the first intron of the *waxy* genes played an important role in amylose levels in rice *O. sativa* varieties. For example, Cai had discovered that the G to T mutation in the first intron would cause the decline of mature mRNA, which eventually leads to the decline of the amylose content (Cai et al., 2000). The DNA sequence of other introns or exons in the *waxy* gene of *O. sativa* varieties has less effect on amylose content because they are very conservative (Tan and Zhang, 2001).

Our study found no nucleotide change (G to T) in the first intron of the *waxy* gene alleles in *O. rufipogon*, *O. officinalis*, or *O. meyeriana* even though the amylsoe content of all three wild rice species was low (9.7%-11.99%) (Cheng et al., 2005). In the exon DNA sequence and the deduced amino acid sequence of the wild rice *waxy* genes, various differences existed among the wild rice species, and between them and the *O. sativa* varieties. These amino acid changes also cause significant change in protein structure according to the predicted secondary structure of GBSS in wild rice species. The incising sites of the signal peptide in GBSS in many monocotyledonous plants were SXVVX↓A (with X representing the changed amino acid) (Patrick and William, 2003). However, in the wild rice species *O. officinalis* and *O. meyeriana* they were SVVVY↓ while in *O. rufipogon* there was no such conservative amino acid sequence. An extra short exon was in the *waxy* genes of the wild rice species. All these imply the possible existence of a different mechanism in controlling amylose content.

The *waxy* gene of wild rice species *O. rufipogon* caused low amylose content in the progeny line of wild rice and cultivated rice variety

In our study, we got one line of BC_2F_9 from the progeny lines between *O. rufipogon* (with an amylase content of 11.99%) and a cultivated rice variety He-xi 41 (with amylase content of 19.23%). The line (BC_2F_9) contains a *waxy* gene of *O. rufipogon* confirmed by PCR (data were not shown here). The line (BC_2F_9) had an amylose content of 12.50% in endosperm—quite close to that of *O. rufipogon*. This is evidence that the *waxy* gene of *O. rufipogon* might have a different mechanism for maintaining a relatively low amylose content.

CONCLUSION

(1) The GBSS activity was positively correlated with the amylose content among the three wild rice species. (2) The *waxy* alleles cloned from *O. rufipogon*, *O. officinalis*, and *O. meyeriana* are quite different from those of *O. sativa* varieties. Therefore, the genetic diversity of the *waxy* alleles in the *Oryza* genus is very rich. (3) Wild rice species may use mechanisms different from the cultivated varieties to control amylose content, using *waxy* alleles. (4) It is important to research and utilize the *waxy* gene which has been isolated from the three wild rice species. They are useful for improving the quality of *O. sativa* rice varieties.

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野生稻 *waxy* 基因等位元資源顯示稻屬基因的多樣性

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為了揭示野生稻較低直鏈澱粉含量特點的原因，本研究測定了中國 3 種野生稻 (*Oryza rufipogon, Oryza officinalis, Oryza meyeriana*) 和 4 個栽培稻品種種子灌漿期間澱粉合成關鍵酶的活性大小和變化特點，發現顆粒澱粉合成酶（GBSS 酶) 和野生稻種子直鏈澱粉含量才密切相關，說明 GBSS 酶及其編碼基因 *waxy* 在控制直鏈澱粉含量上起了很重要作用。進一步按照已報導栽培稻的 *waxy* 基因設計引物或兼併引物，用 3 種野生稻的基因組 DNA 為範本以 PCR 分離 *waxy* 全長基因序列，用灌漿期種子的 mRNA 經過 RT-PCR 分離編碼序列，DNA 序列比較發現，3 種野生稻 *waxy* 同源基因 DNA 序列、長度、內含子和外顯子數目、推導的氨基酸序列和蛋白質二級結構差異很大，和栽培稻的差異也很明顯，不象栽培稻中那樣保守。相比之下，普通野生稻與栽培稻的 *waxy* 同源性稍高一些，而疣粒野生稻與之最低。並且 3 種野生稻第 1 內含子中關鍵城基並沒有 G → T 的變化。所有這些都說明野生稻中 *waxy* 基因變化多，豐富了稻屬該基因資源，並且暗示了野生稻中 *waxy* 基因在控制低直鏈澱粉含量可能不同於栽培稻中的機制，其發掘利用價值大。本研究還設計檢驗了 3 種野生稻 *waxy* 基因的特異引物，可以用於遠緣雜交後的分子輔助育種。本研究為今後利用野生稻 *waxy* 基因適當降低栽培稻種子直鏈澱粉含量提高稻米品質提供了理論參考。

關鍵詞：野生稻；顆粒結合澱粉合成酶；*waxy* 等位基因；分離和序列分析。