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Original scientific article

MOLECULAR CHARACTERIZATION AND PHYLOGENETIC ANALYSIS OF NOVEL *Wx* GENES FROM *Psathyrostachys juncea* AND *Eremopyrum bonaepartis*

Lijuan WU[2], Zaijun YANG[1], Junqiang WANG[1,3], Jundong HE[1,3], Jin JIANG[4], Fang FANG[4], Shian SHEN[1,3], Xinkun HU[1,3*]

[1]Key Laboratory of Southwest China Wildlife Resources Conservation of the Ministry of Education, China West Normal University, Nanchong, Sichuan, China
[2]College of Agronomy, Sichuan Agricultural University, Chengdu, Sichuan, China
[3]Institute of Ecology, China West Normal University, Nanchong, Sichuan, China
[4]Nanchong Academy of Agricultural Sciences, Nanchong, Sichuan, China

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The ratio of amylose/amylopectin can greatly influence the end-use quality of wheat products, and amylose synthesis only needs the granule-bound starch synthase I that is encoded by the *Waxy* (*Wx*) gene. Hence, the *Wx* allele variability is a vital determinant of amylose synthesis. In this study, four novel *Wx* genes were isolated from two diploid Triticeae species. Their sequence variations were analyzed, which showed that the polymorphism among the *Wx-F1* alleles was low; only seven single nucleotide polymorphisms (SNPs) and one amino acid (AA) residue change (Arg541 → Cys) were detected in the exon regions, and this change in *Wx-F1b* was predicted to have deleterious effects on protein function. The two *Wx-Ns1* alleles in this study were different from two published alleles obtained using three overlapping primer sets. The polymorphism among the four *Wx-Ns1* alleles was high, and 37 SNPs and ten AA residue changes were found, while all of those substitutions were neutral. However, one substitution (Leu237 → Pro)

Corresponding author: Xinkun Hu, Key Laboratory of Southwest China Wildlife Resources Conservation of the Ministry of Education, China West Normal University and Institute of Ecology, China West Normal University, Nanchong, Sichuan, China, Phone: +86-0817-2260685, E-mail: huxinkun123@163.com

between Wx-H1 and Wx-Ns1 was predicted that would probably affect the protein function. Divergence time analysis showed that *Wx-Ns1a* diverged from *Wx-Ns1b* about 1.06 MYAs. Phylogenetic analysis indicated that *Wx-F1* were more closely related to *Wx-O1* from *Henrardia persica*, *Wx-R1* from *Secale cereale* ssp. *cereale*, and *Wx-Xe1* from *Eremopyrum triticeum*, while *Wx-Ns1* were more closely related to *Wx-H1* from *Hordeum vulgare* ssp. vulgare and *Wx-H^{ch}1* from *Hordeum chilense*. These genes may be important candidates for wheat quality improvement.

Keywords: Amylose; *Waxy* gene; *Psathyrostachys*; *Eremopyrum*; Phylogenetic relationships

INTRODUCTION

Wheat is one of the staple food crops consumed by human beings worldwide, and its flour can be made into various nutritious and appealing foods due to its special properties. Three main traits, i.e., grain hardness, gluten quality, and starch, determine wheat processing quality and its end-use (RAM and MISHRA, 2008). Starch is the main component of wheat endosperm, and its proportion reaches 65%–75% of the wheat grain dry weight. Starch is made up of two categories of glucose polymers, linear amylose (22%–35%) and branched amylopectin (65%–78%). The ratio of amylose to amylopectin is a key determinant of starch physicochemical properties, which can influence the gelatinization, pasting and gelation of starch, and finally affect the quality of the end-products (FREDRIKSSON *et al*., 1998), such as the nutritional benefits (BIRD *et al*., 2010) and shelf-life (HAYAKAWA *et al*., 2004) of the wheat end-products.

Starch synthesis occurs within the amyloplast and involves a series of enzymes, such as five starch synthases, i.e., starch synthase I (SS I or SGP 3; 80 kDa), starch synthase II (SS II or SGP 1; 100–115 kDa), starch synthase III (SS III), starch synthase IV (SS IV), and granule-bound starch synthase I (GBSS I or waxy protein; 59 or 61 kDa), and several enzymes in charge of starch branching and debranching (ORTEGA *et al*., 2014a). Amylopectin synthesis is more complex than amylose synthesis and involves at least three starch synthases (SS I, SS II and SS III) and several branching (SBE I, SBE IIa and SBE IIb) and debranching enzymes (GUZMÁN *et al*., 2016). However, amylose synthesis needs only the sole GBSS I or waxy protein in storage tissues, which was verified by the creation of the first waxy wheat (NAKAMURA *et al*., 1995).

In bread wheat (*Triticum aestivum* ssp. *aestivum*; 2n=6x=42, BBAADD), there are three GBSS-I or *Waxy* (*Wx*) genes, *Wx-B1* (2,794 bp; 56.7 kDa), *Wx-A1* (2,781 bp; 62.8 kDa), and *Wx-D1* (2,862 bp; 58.7 kDa), located on chromosomes 4AL (translocated from 7BS), 7AS, and 7DS, respectively (CHAO *et al*., 1989; AINSWORTH *et al*., 1993), each consisting of 11 exons and 10 introns (MURAI *et al*., 1999). Previous efforts focused mainly on identifying null *Waxy* alleles on each locus. For example, 179 and 159 cultivars that lacked the respective Wx-A1 and Wx-B1 proteins were identified from 1,960 cultivars, while only one Chinese cv., Bai Huo, lacked the Wx-D1 protein (YAMAMORI *et al*., 1994). Two cultivars, Ike and Kanto 107, carrying null alleles on both *Wx-A1* and *Wx-B1* loci, were identified from USA and Japan germplasm, respectively (GRAYBOSCH *et al*., 1998). The detection of null alleles on each locus provides an opportunity to develop partial waxy wheat with only one or two active waxy genes or to even develop waxy wheat with three null alleles. In fact, waxy bread wheat or durum wheat, carrying no amylose or its content ≤ 1%, has been developed by crossing cv. Kanto 107 with cv. Bai Huo

or durum cv. Aldura since 1995, respectively (NAKAMURA *et al*., 1995). However, partial waxy wheat often had amylose contents ≥ 20%, and only triple null wheat had a content ≤ 1% (YAMAMORI *et al*., 1994). However, there are exceptions: when combining two null *Wx* alleles with one reduced activity or expression allele, the resultant wheat lines will have amylose contents between 1% and 20% (YAMAMORI, 2009; YAMAMORI and YAMAMOTO, 2011). Hence, searching for novel waxy protein variants with different expression levels and enzymatic activities is meaningful for manipulating the ratio of amylose/amylopectin and finally modifying the wheat flour quality. Moreover, the complete or partial sequences of the *Wx* gene have been frequently used to analyze the phylogenetic relationships among Triticeae species (YAN *et al*., 2000; YAN and BHAVE, 2001; GUZMÁN and ALVAREZ, 2012).

Hitherto, dozens of *Wx* genes have been identified from diploid, tetraploid, and hexaploid wheat (GUZMÁN *et al*., 2011, 2012a, b; GUZMÁN and ALVAREZ, 2012; ORTEGA *et al*., 2014a; GUZMÁN and ALVAREZ, 2016; ZHANG *et al*., 2017), and from species of other Triticeae genera, such as *Aegilops*, *Hordeum*, *Secale*, *Taeniatherum*, *Henrardia*, *Eremopyrum*, and *Psathyrostachys* (ORTEGA *et al*., 2014b; LI *et al*., 2014; MENG *et al*., 2014; DAI *et al*., 2016, 2021). *Eremopyrum* is a special annual genus, for example, two y-type High-molecular-weight glutenin subunit (HMW-GS) encoding genes, *1Fy1.5* and *1Fy4.0*, were identified from one accession of *Eremopyrum distans*, and the 1Fy4.0 subunit was the largest y-type HMW-GS currently reported, but no x-type HMW-GS was identified from this species. Moreover, 1Fy1.5 and 1Xey2.6 (isolated from *Eremopyrum triticeum*) were neither purely x-type nor purely y-type HMW-GS because they exhibit hybrid properties of both x-type and y-type subunits, and the x-type subunit (1Xex1.7) encoded by the *Glu-1Xe* locus was smaller than the y-type subunit (1Xey2.6) (DAI *et al*., 2013; 2018). Besides, *Psathyrostachys juncea* is a perennial cross-pollinated forage grass in the *Psathyrostachys* genus (WANG *et al*., 2002). Although the *Wx* genes of *Eremopyrum* and *Psathyrostachys* have been identified using three overlapping primer sets method (DAI *et al*., 2021), we have also cloned the *Wx* gene from above two genera using only one pair of primers (see the method described by ORTEGA *et al*. (2014a)). Finally, four novel *Wx* genes were obtained, and some new sequence variations were identified, and their phylogenetic relationships with orthologous genes from other Triticeae genera were analyzed.

MATERIALS AND METHODS

Plant materials

Two accessions of *Eremopyrum bonaepartis* (PI 203442 and PI 219964 from Turkey and Afghanistan, respectively) and two accessions of *Psathyrostachys juncea* (PI 565068 and PI 565068, both from Kazakhstan) were used in this study. All materials were kindly supplied by the USDA-ARS germplasm bank (http://www.ars-grin.gov).

DNA isolation and PCR amplification

Genomic DNA was extracted from 100 mg of young leaf tissue using the CTAB extraction method (DOYLE and DOYLE, 1990). DNA extraction quality was tested by using 0.8% agarose gel electrophoresis. One primer set Wx-F and Wx-R (Table 1) was used to amplify the

complete *Wx* gene sequences from materials mentioned above. PCR was performed in total volumes of 50 μl, containing 100-ng template DNA, 10 μM of each primer, and the remainder complemented with Golden Star T6 Super PCR Mix (1.1x) (TSE101, Tsingke Biotechnology Co., Ltd., Beijing, China). PCR amplifications were performed in a GeneAmp 9700 Thermo Cycler (Applied Biosystems, Foster City, CA, USA), and PCR conditions included a pre-denaturation at 98°C for 2 min followed by 30 cycles of 98°C for 10 s, 66°C for 30 s and 72°C for 2 min, and a final extension at 72°C for 5 min.

Table 1. PCR primers used in this study

Primer name	Sequence	Annealing (°C)
Amplification primers		
Wx-F	5′-TTGCTGCAGGTAGCCACACC-3′	66
Wx-R	5′-TTCTCTCTTCAGGGAGCGGC-3′	
Sequencing primers		
pJET1.2F	5′-CGACTCACTATAGGGAGAGCGGC-3′	60
pJET1.2R	5′-AAGAACATCGATTTTCCATGGCAG-3′	60
Wx-Ns1-F1	5′-ACTGCTACAAGCGTGGAGTG-3′	60
Wx-Ns1-R1	5′-CCATCATCTGGTGAGCCAGC-3′	60
Wx-F1-F1	5′-CTGACAAGTACGAGAGGGTGAG-3′	60
Wx-F1-R1	5′-CCATCATCTGGTGAGCCAGC-3′	60

PCR products were separated on 0.8% agarose gels, and the target fragments were purified using a gel extraction kit (D2500-01, Omega, Beijing, China). The purified target bands were ligated into the plasmid vector pJET1.2 using a CloneJET PCR Cloning Kit (K1231, Thermo Scientific, Shanghai, China) and were then transformed into *Escherichia coli Trans*5α chemically-competent cells (CD201-01, TransGen Biotech, Beijing, China). Three individual positive clones for each fragment were selected for sequencing at Sangon Biotech Co. Ltd. (Shanghai, China), and the full-length gene sequences were obtained by the primer walking strategy (Table 1). Four short sequences of every clone, each containing an ~800 bp nucleotide sequence and ~100 bp overlaps, were assembled into the full-length sequence using DNAMAN 8.0 software.

Data analysis

Gene sequences were submitted to the NCBI public database (https://blast. ncbi. nlm. nih. gov/Blast. cgi) for BLAST search. Sequence data were analyzed using the DNAsp 6.0.70 software (ROZAS *et al*., 2017). Parameters included the number of variable sites (*s*), total number of mutations (η), and average number of nucleotide differences (k). Nucleotide diversity was assessed using two indexes, namely, pi (π, Nei's nucleotide diversities per site between two sequences) (NEI, 1987) and theta (θ, Watterson's estimate) (WATTERSON, 1975). Neutrality tests of mutation were accomplished using Tajima's *D* statistic (TAJIMA, 1989). The ratio (*Ka*/*Ks*) of non-synonymous (*Ka*) substitution rates to synonymous (*Ks*) substitution rates was also calculated using DNAsp 6.0.70, and the average divergence times of 2.7 million years ago

(MYAs) between wheat A and D genomes was used to estimate the divergence times between *Wx* genes from various species (DVOŘÀK and AKHUNOV, 2005). The effects of amino acid substitutions between the deduced proteins of the *Wx* genes identified in this study and reference proteins were predicted using the PROVEAN PROTEIN tool of the PROVEAN web server (http://provean.jcvi.org/index.php) (CHOI and CHAN, 2015).

Phylogenetic analysis

The complete coding sequences of the four *Wx* genes obtained in this study and 37 published *Wx* genes were used to construct the phylogenetic tree. The published *Wx* genes came from eight *Aegilops* species (C (JX679009, JX679010), D (AF1103735), M (JX402790, JX679003), U (JX679008, JX679006), S^s (JX679011, JX679012), S^{sp} (JX679013, JX679014), S^{sh} (JX679016, JX679017), and S^L (JX679018, JX679012) genomes), four *Triticum* species (namely, *Triticum urartu* (KF612973), *Triticum monococcum* ssp. *monococcum* (KF612977), *Triticum turgidum* ssp. *dicoccoides* (AB029061, AB029062), and *Triticum aestivum* ssp. *aestivum* (AB016922-AB019624)), two *Eremopyrum* species (*Eremopyrum triticeum* (MT112900, MT112901) and *Eremopyrum bonaepartis* (MT112900, MT112901)), two *Hordeum* species (*Hordeum chilense* (MK045501, MK045502) and *Hordeum Vulgare* ssp. *vulgare* (AB087716, AB088761)), three *Taeniatherum* species (*Taeniatherum caput-medusae* ssp. *crinitum* (KT878862), *Taeniatherum caput-medusae* ssp. *asperum* (KT878864), and *Taeniatherum caput-medusae* ssp. *caput-medusae* (KT878869)) and one species each from *Secale* (*Secale cereale* ssp. *cereale*, KC572701 and KC572704) and *Henrardia* (*Henrardia persica*, MT112904 and MT112905). Multiple sequence alignments of all sequences involved were performed using the ClustalW 1.83 program (CHENNA *et al*., 2003). A neighbor-joining tree was generated using the maximum composite likelihood method of the MEGA X 10.0.2 software (KUMAR *et al*., 2018), and each bootstrap support value was estimated based on 1,000 replicates.

RESULTS

PCR amplification of Wx genes

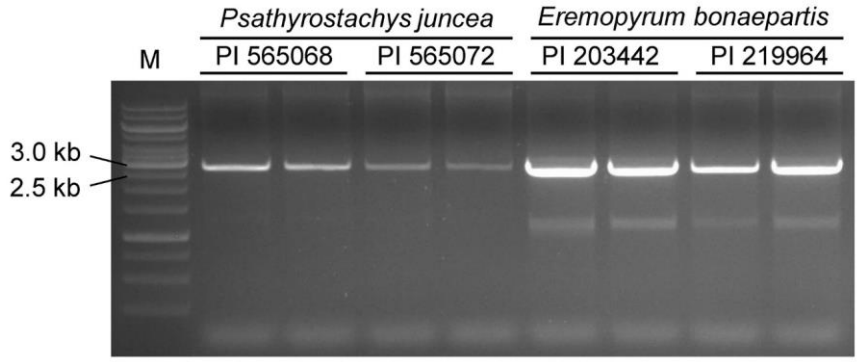

Fig 1. PCR amplification of *Wx* genes from two diploid species of genera *Psathyrostachys* and *Eremopyrum*. M is the DNA molecular weight marker.

Only one single band, with a size between 2.5 kb and 3.0 kb, was amplified from two diploid Triticeae species *Psathyrostachys juncea* and *Eremopyrum bonaepartis*, except for a smear present in the lane of two *Eremopyrum bonaepartis* accessions (Fig. 1). After sequencing with the primer walking strategy, the complete *Wx* gene sequences from four accessions used were assembled, and three individual clones for each *Wx* gene were sequenced to exclude sequencing errors. Then, four different *Wx* alleles were obtained, and their sizes ranged from 2,815 bp to 2,873 bp, each containing 31 bp upstream and 8 bp downstream of the coding region. All those sequences were deposited in the GenBank database under accession numbers MW802246 to MW802249.

Characterization of the DNA sequence of the four novel Wx alleles

The four *Wx* alleles obtained here were BLAST identified in the GenBank database, which showed that they had not yet been reported. All of them contained eleven exons and 10 introns as reported in other Triticeae species. Two *Wx* alleles isolated from *Psathyrostachys juncea* (accession numbers MW802246 and MW802247) showed a sequence similarity of 97.53% to each other and exhibited 98.65% and 98.94% similarities to *Wx-Ns1* (MT112902) and 97.53% and 99.68% similarities to *Wx-Ns2* (MT112903), respectively. Multiple sequence alignments of the four *Wx* alleles from *Psathyrostachys juncea* revealed that the first (first to third exon) and the second (third to sixth exon) fragments of MT112902 were more similar to those of MW802247, while its third (sixth to eleventh exon) fragment was more similar to that of MW802246. The second and the third fragments of MT112903 were more similar to those of MW802247, while its first fragment was not similar to MW802246 or MW802247. The second fragment of MW802246 was different from the three other *Wx-Ns* alleles (Fig. 2). Hence, the two *Wx* alleles from *Psathyrostachys juncea* were two novel genes, and they were named *Wx-Ns1a* and *Wx-Ns1b* according to the international nomenclature (MCINTOSH *et al*., 2014). Based on a comparison of the two *Wx* alleles of *Eremopyrum bonaepartis* (accession numbers MW802248 and MW802249), 10 SNPs were identified between them, and MW802248 had five SNPs in comparison with *Wx-Fs1* (MT112898) or *Wx-Fs2* (MT112899), while MW802249 had nine SNPs in comparison with *Wx-Fs1* or *Wx-Fs2*. Similarly, they were two novel *Wx* alleles, named *Wx-F1a* and *Wx-F1b*.

The DNA polymorphisms of *Wx-Ns1* and *Wx-F1* alleles were evaluated using the complete nucleotides sequence (exons plus introns) and only the open reading frame (ORF) sequence (only exons). The result showed that the polymorphism of the four *Wx-F1* alleles was low; the polymorphism of the four *Wx-Ns1* alleles was much higher than that of the four *Wx-F1* alleles. In the four *Wx-Ns1* alleles, 57 and 37 polymorphic sites were identified using the complete nucleotide and only the ORF sequences, respectively. However, only fourteen and seven polymorphic sites were found in the above corresponding regions of the four *Wx-F1* alleles, with an average number of nucleotide differences (k) of 3.50. In both cases, more polymorphisms were evaluated using the complete sequences compared with using only the ORF sequences. The nucleotide diversity, estimated by π (NEI, 1987) and θ (WATTERSON, 1975), was much higher between the four *Wx-Ns1* alleles than among the four *Wx-F1* alleles (Table 2). However, Tajima's *D* test was not significant among the four *Wx-F1* alleles, and also among the

four *Wx-Ns1* alleles; the result indicated the polymorphism level fitted a neutral equilibrium model.

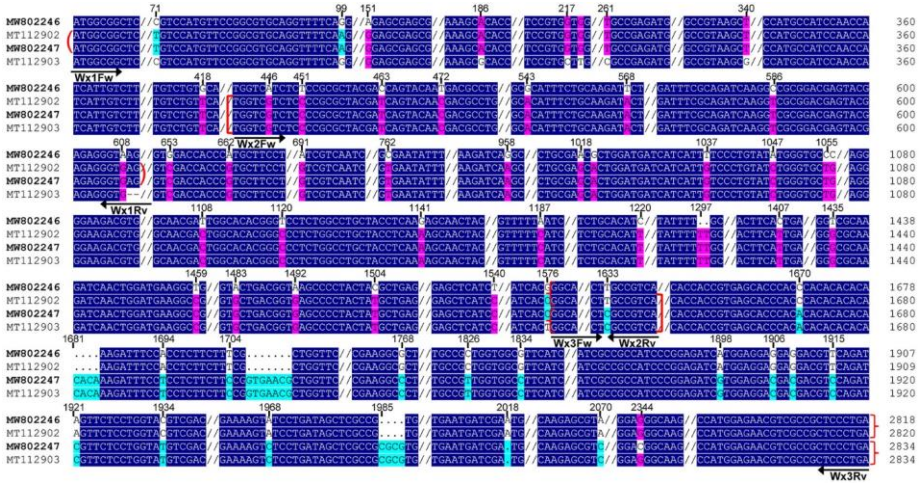

Fig 2. Multiple sequence alignments of four *Wx* alleles from *Psathyrostachys juncea*. MW802246 and MW802247 are from this study and shown in bold. MT112902 and MT112903 are from Dai et al. (2021). The arrowheads show the general sites of three overlapping primer sets. The parentheses, brackets and braces indicate the most similar parts among four genes. Double slashes denote the remove of the consensus sequence among four genes.

Table 2. DNA polymorphisms and test statistics for Wx alleles from Psathyrostachys juncea and Eremopyrum bonapatis

Gene	*Psathyrostachys juncea*		*Eremopyrum bonapatis*	
	Complete nucleotides	ORFs	Complete nucleotides	ORFs
n	4	4	4	4
s	57	37	14	7
η	58	38	14	7
h	4	4	4	4
$\pi \times 10^{-3}$	11.24	11.40	2.52	1.93
$\theta \times 10^{-3}$	11.23	11.34	2.75	2.10
k	31.667	20.833	7.000	3.500
D	0.00998 n.s.	0.05309 n.s.	-0.84532 n.s.	-0.81734 n.s.

n: number of sequences; *s*: number of variable sites; *η*: total number of mutations; *h*: number of haplotypes; *π*: nucleotide diversity (per site); *θ*: Watterson's estimate; *k*: average number of nucleotide differences; *D*: Tajima's estimate *D* test; n.s.: not significant.

Analysis of deduced amino acid sequences

Four different Wx proteins were deduced from the ORFs of the four novel *Wx* genes.

Table 3. Amino acid comparison between Wx-H1 and Wx-Ns1 (Change 1), and between Wx-R1 and Wx-F1 (Change 2)

Change 1	P*sathyrostachys juncea*		Change 2	E*remopyrum bonaepartis*	
	MW802246	MW802247		MW802248	MW802249
Ala5 → Val	+	+	Phe27 → Ile	+	+
Val18 → Ile	+	+	Ser34 → Asn	+	+
Pro24 → Leu	-	+	Met42 → Val	+	+
His29 → Arg	+	+	Gly54 → Ser	+	+
Lys39 → Asn	+	+	Ala57 → Gly	+	+
Gly43 → Ala	+	+	Met67 → Val	+	+
Thr44 → Ala	+	+	Val131 → Ile	+	+
Phe45 → Leu	+	+	Glu221 → Asp	+	+
Gly46 → Ala	+	+	Ser363 → Ala	+	+
Val50 → Ile	+	+	Lys421 → Gln	+	+
Gly51 → Arg	+	-	Val428 → Ile	+	+
Gly65 → Glu	+	+	Leu437 → Phe	+	+
Asn66 → Ser	+	+	Lys439 → Arg	+	+
Val120 → Ile	+	-	His441 → Leu	+	+
Lys128 → Asn	+	+	Ile444 → Ala	+	+
Ile136 → Val	+	+	Lys447 → Arg	+	+
Val141 → Ala	+	-	Ser450 → Gly	+	+
Phe150 → Tyr	+	+	Val536 → Ala	+	+
Ile163 → Val	+	+	**Arg541 → Cys**	-	+
Trp167 → Cys	+	+	Ile589 → Val	+	+
Asn213 → Asp	+	+			
Phe218 → Tyr	+	+			
Leu237 → Pro	+	+			
Ile291 → Thr	+	-			
Gln312 → Glu	+	+			
Glu331 → Val	+	+			
Ile372 → Ala	+	+			
Leu424 → Met	+	-			
Leu424 → Val	-	+			
Lys425 → Glu	+	+			
Glu426 → Asp	-	+			
Glu427 → Asp	-	+			
Ile432 → Val	+	+			
Lys443 → Arg	+	+			
Met448 → Val	+	+			
Leu476 → Val	+	+			
Val501 → Ala	+	+			
Glu514 → Asp	-	+			
Glu586 → Ala	+	+			
Val593→ Ile	+	+			

The Wx-Ns1 from *Psathyrostachys juncea* and Wx-F1 from *Eremopyrum bonaepartis* contained a total of 608 and 604 amino acid (AA) residues, and the first 75 and 70 respective AA residues in the N-terminals were transit peptides. Although there were seven polymorphic sites in the exon regions of the four *Wx-F1* alleles, only one AA residue change was identified in the Wx-F1b protein (Arg541 → Cys; PROVEAN score = −3.809), and PROVEAN analysis indicated this substitution had potential deleterious effects on the protein function. There were 38 SNPs among the ORFs of four *Wx-Ns1* alleles, and a total of ten AA residue changes were found, while all of those substitutions were neutral. The four Wx proteins deduced here were BLAST identified in the GenBank database, which showed that Wx-Ns1 from *Psathyrostachys juncea* was more similar to Wx-H1 from *Hordeum vulgare* ssp. *vulgare*, while Wx-F1 from *Eremopyrum bonaepartis* was more similar to Wx-R1 from *Secale cereale* ssp. *cereale*. The effects of the AA residue substitution between Wx-H1 and Wx-Ns1 and between Wx-R1 and Wx-F1 were analyzed using the PROVEAN web tool. Wx-H1 (accession number BAC41202) and Wx-R1 (accession number AHA58701) were used as respective references. The results revealed that there was a total of 40 AA residue changes between Wx-H1 and Wx-Ns1, and only one AA residue substitution (Leu237 → Pro; PROVEAN score = −3.596) was predicted to have potential deleterious effects on protein function. In addition, 20 AA residue substitutions were discovered between Wx-R1 and Wx-F1, and only one AA residues substitution (Arg541 → Cys; PROVEAN score = −3.809) was predicted to potentially affect protein activity (Table 3).

Phylogenetic analysis of Wx genes

A maximum likelihood phylogenetic tree was generated based on the complete sequences of *Wx* genes from this study together with some published *Wx* alleles from *Aegilops*, *Triticum*, *Henrardia*, *Eremopyrum*, *Secale* and *Hordeum* species. All of these *Wx* genes were classified into six clades. Clade I was formed by the alleles from *Aegilops longissimi* (*Wx-S^L1*) and *Aegilops sharonensis* (*Wx-S^{sh}1*). Clade II consisted of *Wx-A^m1* from *Triticum monococcum* ssp. *monococcum* and *Wx-A^u1* from *Triticum urartu*. Clade III was composed of *Wx-B1* from *Triticum turgidum* ssp. *dicoccoides* and *Triticum aestivum* ssp. *aestivum*, *Wx-S^{sp}1* from *Aegilops speltoides*, and *Wx-S^s1* from *Aegilops searsii*. Clade IV was formed by the alleles from *Aegilops* species such as *Aegilops markgrafii* (*Wx-C1*), *Aegilops tauschii* (*Wx-D1*), *Aegilops comosa* (*Wx-M1*), *Aegilops umbellulate* (*Wx-U1*), and from *Taeniatherum* species (*Wx-Ta1*) such as *Taeniatherum caput-medusae* ssp. *crinitum*, *Taeniatherum caput-medusae* ssp. *asperum*, and *Taeniatherum caput-medusae* ssp. *caput-medusae*, and by *Wx-D1* from *Triticum aestivum* ssp. *aestivum*. Clade V was composed of *Wx-Xe1* from *Eremopyrum triticeum*, *Wx-R1* from *Secale cereale* ssp. *cereale*, *Wx-O1* from *Henrardia*. *persica*, and *Wx-F1* from *Eremopyrum bonaepartis*. Clade VI was made up of *Wx-A1* from *Triticum turgidum* ssp. *dicoccoides* and *Triticum aestivum* ssp. *aestivum*, *Wx-H1* from *Hordeum vulgare* ssp. *vulgare*, *Wx-H^{ch}1* from *Hordeum chilense*, and *Wx-Ns1* from *Psathyrostachys juncea* (Fig. 3).

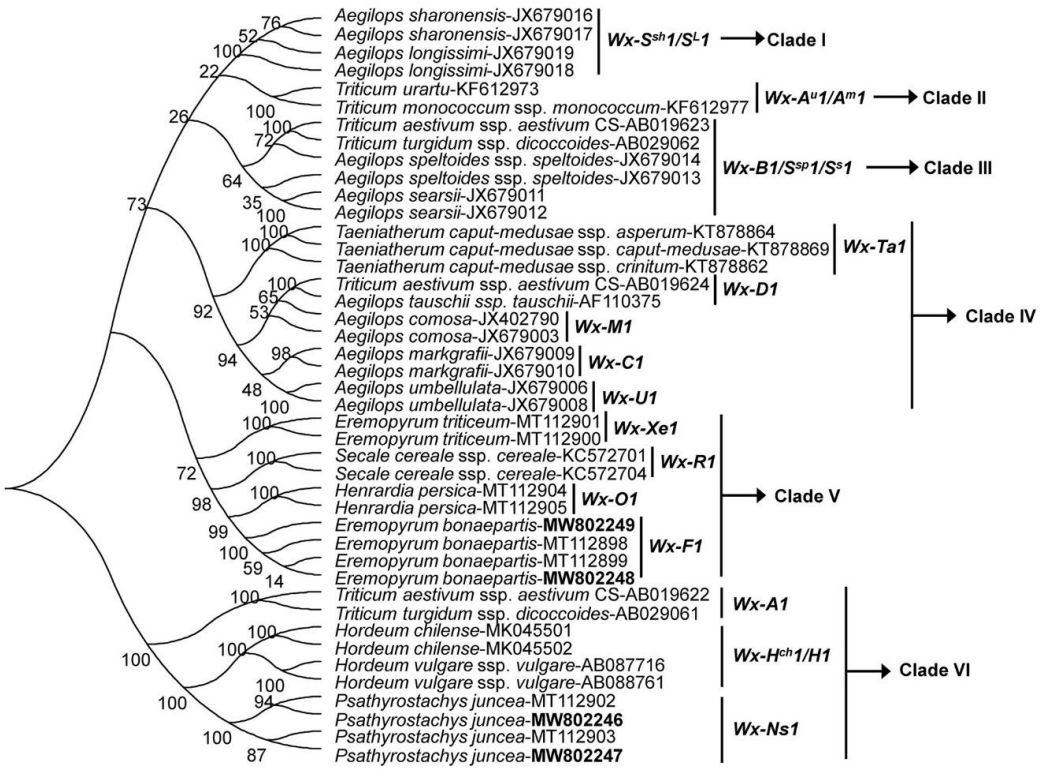

Fig 3. Phylogenetic analysis of *Wx* genes from various Triticeae species based on the complete gene sequences. Bootstrap values are set as 1,000. The four *Wx* genes from this study are shown in bold.

Divergence time analysis

The average divergence times (2.7 MYAs) between wheat A and D genomes and the *Ks* value (0.144) between *Wx-A1* and *Wx-D1* genes were used to compute a divergence rate (r = 0.0533 synonymous substitution/MYA) between the A and D genomes (DVOŘÀK and AKHUNOV, 2005). Then, the divergence times (t) among *Wx* genes were calculated by linear regression of the *Ks* value (*Ks*) and divergence rate (r) based on equation $t = Ks/0.0533$ (MYAs). The results showed that *Wx-F1a* (MW802248) and *Wx-F1b* (MW802249) from *Eremopyrum bonaepartis* had nearly equal divergence times with *Wx* genes from other Triticeae species. However, *Wx-Ns1a* (MW802246) and *Wx-Ns1b* (MW802247) from *Psathyrostachys juncea* diverged, and the divergence time between them was 1.06 MYAs. In addition, the divergence time of *Wx-Ns1a* was 0.52 MYAs earlier than that of *Wx-Ns1b* (Table 4).

Table 4. Variations and divergence time analysis of Wx genes from various species

Compared with	Parameters	P.*juncea Wx-Ns1*		E.*bonaepartis Wx-F1*	
		MW802246	MW802247	MW802248	MW802249
Wx-A1 (AB019622)	*Ks*	0.2026	0.1688	0.1616	0.1617
	Ka	0.0327	0.0327	0.0225	0.0233
	Ka/*Ks*	0.1614	0.1937	0.1392	0.1441
	Ks/0.0533 (MYA)	3.80	3.17	3.03	3.03
Wx-B1 (AB019623)	*Ks*	0.1663	0.1381	0.1630	0.1603
	Ka	0.0245	0.0253	0.0229	0.0236
	Ka/*Ks*	0.1473	0.1832	0.1405	0.1472
	Ks/0.0533 (MYA)	3.12	2.59	3.06	3.01
Wx-D1 (AB019624)	*Ks*	0.2074	0.1821	0.1814	0.1800
	Ka	0.0238	0.0245	0.0199	0.0207
	Ka/*Ks*	0.1148	0.1345	0.1097	0.1150
	Ks/0.0533 (MYA)	3.89	3.42	3.40	3.38
Wx-R1 (KC572701)	*Ks*	0.1858	0.1596	0.1394	0.1385
	Ka	0.0272	0.0272	0.0151	0.0158
	Ka/*Ks*	0.1464	0.1704	0.1083	0.1141
	Ks/0.0533 (MYA)	3.49	2.99	2.62	2.60
Wx-O1 (MT112904)	*Ks*	0.1885	0.1637	0.1243	0.1243
	Ka	0.0215	0.0215	0.0110	0.0117
	Ka/*Ks*	0.1141	0.1313	0.0885	0.0941
	Ks/0.0533 (MYA)	3.54	3.07	2.33	2.33
Wx-Xe1 (MT112900)	*Ks*	0.1864	0.1660	0.1797	0.1740
	Ka	0.0215	0.0208	0.0207	0.0214
	Ka/*Ks*	0.1153	0.1253	0.1152	0.1230
	Ks/0.0533 (MYA)	3.50	3.11	3.37	3.26
Wx-H1 (AB087716)	*Ks*	0.1927	0.1594	0.1828	0.1786
	Ka	0.0283	0.0279	0.0259	0.0270
	Ka/*Ks*	0.1469	0.1750	0.1417	0.1512
	Ks/0.0533 (MYA)	3.62	2.99	3.43	3.35
Wx-H^{ch}1 (MK045501)	*Ks*	0.1900	0.1625	0.1745	0.1731
	Ka	0.0218	0.0200	0.0229	0.0237
	Ka/*Ks*	0.1147	0.1231	0.1312	0.1369
	Ks/0.0533 (MYA)	3.56	3.05	3.27	3.25
Wx-Ns1a (MW802246)	*Ks*	-	0.0564	0.2079	0.2050
	Ka	-	0.0065	0.0245	0.0253
	Ka/*Ks*	-	0.1152	0.1178	0.1234
	Ks/0.0533 (MYA)	-	1.06	3.90	3.85
Wx-Ns1b (MW802247)	*Ks*	0.0564	-	0.1752	0.1724
	Ka	0.0065	-	0.0245	0.0253
	Ka/*Ks*	0.1152	-	0.1398	0.1468
	Ks/0.0533 (MYA)	1.06	-	3.29	3.23

DISCUSSION

Starch, one of the major components of wheat endosperm, accounts for 65%–70% of the dry matter weight and is composed of two biomacromolecules, amylose (22%–35%) and amylopectin (65%–78%). The amylose/amylopectin ratio is an important determinant of starch properties (ZENG *et al*., 1997). Amylose synthesis is catalyzed by a sole enzyme GBSS I, which is encoded by the *Waxy* gene. The genetic variation of *Wx* alleles can affect the catalytic activity of GBSS I and can regulate the synthesis of amylose, as well as further change the ratio of amylose to amylopectin and the starch properties (GUZMÁN and ALVAREZ, 2016). In bread wheat, reduced expression of the Wx-A1 protein was detected in some Spanish spelt accessions (CABALLERO *et al*., 2008; GUZMÁN *et al*., 2012b), and a low expression of the Wx-B1 protein was identified in a Canadian cv. Reward (DEMEKE *et al*., 1997). The low or reduced expression variants provide an opportunity for developing "quasi-waxy" wheat by combining with double null alleles of the two other *Wx* genes (GUZMÁN and ALVAREZ, 2016).

Previous studies showed few polymorphisms of *Wx* genes from tetraploid and hexaploid wheat (GUZMÁN *et al*., 2009; GUZMÁN and ALVAREZ, 2012), while the diversities of *Wx* genes from some ancient wheat species and their wild relatives were reported to be high (CABALLERO *et al*., 2008). Therefore, in order to modify wheat starch properties, great efforts have been placed on seeking novel *Wx* genes from diploid donor species of wheat genomes and wheat-related species (GUZMÁN and ALVAREZ, 2012; ORTEGA *et al*., 2014a, b; LI *et al*., 2014; MENG *et al*., 2014; GUZMÁN and ALVAREZ, 2016; DAI *et al*., 2016; 2021). In this study, four novel *Wx* genes were identified from four accessions of two wild related species, *Psathyrostachys juncea* and *Eremopyrum bonaepartis*. Sequence analysis showed that the two *Wx* alleles from *Psathyrostachys juncea* were different from each other, and their similarity was 97.53%. Multiple sequence alignments of *Wx-Ns1* alleles from the present study and a previous study revealed that *Wx-Ns1* (MT112902) appeared to be the chimera of *Wx-Ns1a* (MW802246) and *Wx-Ns1b* (MW802247), while *Wx-Ns2* (MT112903) appeared to be the chimera of *Wx-Ns1b* (MW802247) and another unknown *Wx-Ns1* allele. Additionally, the middle part of *Wx-Ns1a* was distinct from other *Wx-Ns1* alleles (Fig. 2). The results suggested that the method described by DAI *et al*. (2021), i.e., three overlapping primer sets, might not be suitable for amplifying *Wx-Ns1a* genes from *Psathyrostachys juncea*.

DNA polymorphism analysis showed that 57 and 37 SNPs were separately detected from the complete nucleotide sequence and ORFs of the four *Wx-Ns1* alleles, and fourteen and seven SNPs were observed in the corresponding region of the four *Wx-F1* alleles, indicating that the polymorphism level of the *Wx-Ns1* alleles was higher than that of the *Wx-F1* alleles. Similarly, the nucleotide diversity identified from the 5′ untranslated regions (LI *et al*., 2012) or translated regions (ORTEGA *et al*., 2014b) of *Wx* genes in *Aegilops* species was higher than that detected in polyploid wheat (GUZMÁN *et al*., 2012b). The nucleotide diversity of *Wx* alleles in *Psathyrostachys juncea* and *Aegilops speltoides* ssp. *speltoides* was much higher than that of the *Wx* alleles in *Taeniatherum* (DAI *et al*., 2016), which is a strictly self-pollinating species. The higher nucleotide diversity in *Psathyrostachys juncea* and *Aegilops speltoides* ssp. *speltoides* might arise from their cross-pollinating property because a previous study showed that the DNA variation level of cross-pollinating species was higher than that of self-pollinating species (DVOŘÀK *et al*., 1998).

The nucleotide variations could lead to protein sequence changes. However, the seven SNPs among the four *Wx-F1* alleles caused only one AA residue change (Arg541 → Cys) in the Wx-F1b protein, and this change could affect the protein function based on PROVEAN analysis (CHOI and CHAN, 2015). The effects of AA residue substitution between Wx-R1 and Wx-F1 were analyzed: a total of 20 AA changes were identified, and the substitution (Arg541 → Cys) mentioned above probably influenced the protein function. Similarly, 37 SNPs and ten AA changes were found between four *Wx-Ns1* alleles, but all of them were neutral. Forty AA changes were detected when comparing Wx-Ns1 with Wx-H1, and one substitution (Leu237 → Pro) was associated with adverse effects on *Wx* gene functions. Previous research did not detect any deleterious effect between Wx-R1 (KC572701) and Wx-F1 from *Eremopyrum bonaepartis*, or between Wx-H1 (KT356856) and Wx-Ns1 from *Psathyrostachys juncea* (DAI *et al*., 2021). The former case could be interpreted as the finding of a novel Wx-F1b protein in this study, which carried the substitution Arg541 → Cys compared with Wx-R1 or other Wx-F1 proteins. The latter case might be caused by skipping over the substitution (Leu237 → Pro) when PROVEAN analysis was performed by DAI *et al*. (2021) because this substitution truly existed between Wx-H1 and Wx-Ns1. In previous studies, four substitutions (Val5 → Ala, Val138 → Ala, Gln190 → Leu and Asp574 → His) between Wx-A1 from wheat and Wx-A^m1 from *Triticum monococcum* ssp. *monococcum* or Wx-A^u1 from *Triticum urartu* (ORTEGA *et al*., 2014a), two substitutions (Arg250 → Met and Thr538 → Ile) between Wx-B1a from wheat and Wx-S^S1 from *Aegilops searsii* (ORTEGA *et al*., 2014b), two substitutions (Gly330 → Ser and Ile444 → Thr) between Wx-D1 from wheat and Wx-Ta1 from *Taeniatherum caput-medusae* ssp. *crinitum* (DAI *et al*., 2016), and three substitutions (Phe81 → Leu, Leu233 → Pro, and Ala304 → Ser) between Wx-D1 from wheat and Wx-Xe1 from *Eremopyrum triticeum* (DAI *et al*., 2021) were predicted to have deleterious effects on waxy protein functions. Meanwhile, previous work indicated that the synthesis ability of amylose will change if a single AA substitution takes place in a pivotal position of the waxy protein (YAMAMORI and GUZMÁN, 2013). However, the expression and effect of these novel *Wx* alleles in the genetic background of modern wheat would need to be assessed by transgenesis strategy or distant hybridization.

The *Wx* gene sequences are frequently used for studying the phylogenetic relationships among Triticeae species (YAN *et al*., 2000; YAN and BHAVE, 2001; GUZMÁN and ALVAREZ, 2012). For example, the phylogenetic relationships among species of *Aegilops* and *Triticum* were analyzed using the complete coding regions (ORTEGA *et al*., 2014b) or the 5′-untranslated regions (LI *et al*., 2012). In the present research, the phylogenetic relationships among *Aegilops*, *Triticum*, *Eremopyrum*, *Hordeum*, *Taeniatherum*, *Secale* and *Henrardia* were studied using the complete coding regions of the *Wx* genes. The dendrogram constructed here was similar to that of a previous study (DAI *et al*., 2021), excepted for some differences, e.g., *Wx-Xe1* from *Eremopyrum triticeum* was clustered with *Wx-R1*, *Wx-O1* and *Wx-F1* and formed Clade V, while it was independent from above three alleles and formed a separate clade in previous study (DAI *et al*., 2021). Additionally, *Wx-A1* from *Triticum turgidum* ssp. *dicoccoides* and *Triticum aestivum* ssp. *aestivum* was clustered with *Wx-Ns1*, *Wx-H1* and *Wx-*H^{ch}*1*, and formed Clade VI, which was also different from the research of DAI *et al*. (2021). The differences of dendrogram in two separate studies could be explained by the different methods used for constructing phylogenetic tree. It was strange that *Wx-A1* from *Triticum turgidum* ssp. *dicoccoides* and *Triticum aestivum*

ssp. *aestivum* didn't clustered with *Wx-A^{m}1* from *Triticum monococcum* ssp. *monococcum* and *Wx-A^{u}1* from *Triticum urartu*, indicated that the *Wx* genes of them existed great variation, hence the *Wx-A^{m}1* and *Wx-A^{u}1* might be good candidate variants for improving the starch quality of common wheat (ORTEGA *et al*., 2014a). The close relationship between *Psathyrostachys* and *Hordeum* species was also illustrated by the isolation of novel D-hordeins from *Psathyrostachys* species (HU *et al*., 2018), while the D-hordeins were first identified in *Hordeum* species (PISTÓN *et al*., 2007).

CONCLUSIONS

In summary, four novel *Wx* genes—two *Wx-Ns1* and two *Wx-F1* alleles—were characterized from *Psathyrostachys juncea* and *Eremopyrum bonapartis*. The polymorphisms of the *Wx-Ns1* alleles from the cross-pollinating species *Psathyrostachys juncea* were higher than those of *Wx-F1* alleles from the self-pollinating species *Eremopyrum bonapartis*. Divergence time analysis revealed that *Wx-Ns1a* diverged from *Wx-Ns1b* about 1.06 MYAs, and it diverged on average 0.52 MYAs earlier than *Wx-Ns1b* from other species. PROVEAN analysis showed only one substitution (Arg541 → Cys) in the Wx-F1b protein that was predicted to have adverse effects on protein activity compared to Wx-R1 from *Secale cereale* ssp. *cereale* or other Wx-F1 proteins, while one substitution (Leu237 → Pro) between Wx-Ns1 and Wx-H^{ch}1 from *Hordeum chilense* or Wx-H1 from *Hordeum vulgare* ssp. *vulgare* was identified as a deleterious effect alteration. The genetic effect and expression of these new *Wx* variants in the modern wheat background need to be verified by genetic transformation or distant hybridization, so that they can be used for modification of the amylose/amylopectin ratio and the breeding of wheat varieties to meet the needs of the modern food industry.

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MOLEKULARNA KARAKTERIZACIJA I FILOGENETSKA ANALIZA NOVIH *Wx* GENA IZ *Psathyrostachys juncea* I *Eremopyrum bonaepartis*

Lijuan WU[2], Zaijun YANG[1], Junqiang WANG[1,3], Jundong HE[1,3], Shian SHEN[1,3], Jin JIANG[4], Fang FANG[4], Xinkun HU[1,3*]

[1]Glavna laboratorija Jugozapadne Kine za konzervaciju divljih resursa, Ministarstvo za obrazovanje, Kineski Zapadni Normal Univerzitet, Nanchong, Sichuan, Kina
[2]Koledž za agronomiju, Sichuan poljoprivredni univerzitet, Chengdu, Sichuan, Kina
[3]Institut za ekologiju, Kineski Zapadni Normal Univerzitet, Nanchong, Sichuan, Kina
[4]Nanchong Akademija za poljoprivredne nauke, Nanchong, Sichuan, Kina

Izvod

Odnos amiloza/amilopenektin može u velikoj meri da utiče na kvalitet krajnjih proizvoda od pšenice, a za sintezu amiloze samo su potrebne vezane granule skrobne sintetaze I koja je kodiran *Wx* genom. Dakle, varijabilnost alela *Wx* je vitalna determinanta sinteze amiloze. U ovoj studiji, četiri nova *Wx* gena su izolovana iz dve diploidne vrste Triticeae. Analizirane su njihove varijacije sekvenci, što je pokazalo da je polimorfizam među alelima *Wx* -F1 nizak; detektovano je samo sedam SNP-ova i jedna promena ostatka aminokiseline (AA) (Arg541 → Cis) u regionima egzona, a predviđalo se da će ova promena u *Wx* -F1b imati štetne efekte na funkciju proteina. Dva alela *Wx-Ns1* u ovoj studiji bila su različita od dva objavljena alela dobijena korišćenjem tri seta prajmera koji se preklapaju. Polimorfizam između četiri alela *Wx-Ns1* je bio visok i pronađeno je 37 SNP-ova i deset promena AA ostataka, dok su sve te supstitucije bile neutralne. Međutim, predviđena je jedna zamena (Leu237 → Pro) između *Wx-H1* i *Wx-Ns1* koja će verovatno uticati na funkciju proteina. Analiza vremena divergencije pokazala je da se *Wx-Ns1a* odvaja od *Wx-Ns1b* oko 1,06 MIA. Filogenetska analiza je pokazala da su *Wx-F1* bliže povezani sa *Wx-O1* iz *Henrardia persica*, *Wx-R1* iz *Secale cereale* ssp. *cereale*, i *Wx-Xe1* iz *Eremopirum triticeum*, dok su *Wx-Ns1* bili bliže povezani sa *Wx-H1* iz *Hordeum vulgare* ssp. *vulgare* i *Wx-H^{ch}1*iz *Hordeum chilense*. Ovi geni mogu biti važni kandidati za poboljšanje kvaliteta pšenice.

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