Molecular evolution of Wcor15 gene enhanced our understanding of the origin of A, B and D genomes in *Triticum aestivum*

Fangfang Liu¹,²,*, Hongqi Si¹,²,*, Chengcheng Wang¹,², Genlou Sun³, Erting Zhou¹, Can Chen¹ & Chuanxi Ma¹,²,⁴,⁵

The allohexaploid bread wheat originally derived from three closely related species with A, B and D genome. Although numerous studies were performed to elucidate its origin and phylogeny, no consensus conclusion has reached. In this study, we cloned and sequenced the genes Wcor15-2A, Wcor15-2B and Wcor15-2D in 23 diploid, 10 tetraploid and 106 hexaploid wheat varieties and analyzed their molecular evolution to reveal the origin of the A, B and D genome in *Triticum aestivum*. Comparative analyses of sequences in diploid, tetraploid and hexaploid wheats suggest that *T. urartu*, *Ae. speltoides* and *Ae. tauschii* subsp. *strangulata* are most likely the donors of the Wcor15-2A, Wcor15-2B and Wcor15-2D locus in common wheat, respectively. The Wcor15 genes from subgenomes A and D were very conservative without insertion and deletion of bases during evolution of diploid, tetraploid and hexaploid. Non-coding region of Wcor15-2B gene from B genome might mutate during the first polyploidization from *Ae. speltoides* to tetraploid wheat, however, no change has occurred for this gene during the second allopolyploidization from tetraploid to hexaploid. Comparison of the Wcor15 gene shed light on understanding of the origin of the A, B and D genome of common wheat.

Wheat (*Triticum aestivum* L.) is an annual species in the tribe *Triticeae* of the grass family *Poaceae*. It is the most widely cultivated food crop followed by rice and maize, and is the primary cereal in the temperate region, serving as a staple food for about 40% of the world’s population (http://faostat.fao.org)¹. Common wheat is one of the earliest domesticated crop plants in the Pre-Pottery Neolithic Near East²,³. Polyploidization played an important role in the evolution of eukaryotes, and is one of the important mechanisms for creating genetic variation, and major evolutionary factor affecting genome size and gene copy number⁴–⁷. Polyploids can be formed via the duplication of genomes, either of the same genomes (autopolyploid) or of diverged genomes with homoeologous relationships (allopolyploid)⁸–⁹. *Triticum aestivum* (AABBDD) as a good example of allopolyploid is derived from the three homologous genomes, A, B, and D, each of which contributes 7 pairs of chromosomes to the wheat’s total genome (2n = 6x = 42)¹⁰ with an approximate genome size of 16–17 Gb¹¹–¹³. It was suggested that the origin of allohexaploid wheat (*Triticum aestivum* L.) involved two sequential allopolyploidization events¹⁴,¹⁵. The first wheat allotetraploidization involved diploid AA genome species and diploid BB species to form tetraploid AABB approximately 0.36 to 0.5 million years ago¹⁶,¹⁷. The second polyploidization between diploid goat grass species (DD, *Aegilops tauschii* Coss) and the tetraploid (AABB) emmer wheat (closely related to *Triticum turgidum* subsp. *durum*, genome AABB) led to the formation of common wheat (AABBDD) approximately 8,000 years ago¹⁶,¹⁸. The progenitor of the A genome of the tetraploid and hexaploid wheat species contains *Triticum urartu* Thum ex Gand (genome A¹)¹⁹ and *Triticum monococcum* Linn (genome A²) including two subspecies: the wild *T. monococcum* subsp. *boeoticum* (*T. m. boeoticum*) ²⁰ and its domesticated form *T. monococcum* subsp. *boeoticum* Boiss. (*T. m. boeoticum*)²⁰ and its domesticated form *T. monococcum* subsp. *boeoticum* Boiss. (*T. m. boeoticum*)²⁰

¹School of Agronomy, Anhui Agricultural University, Hefei 230036, China. ²Key Laboratory of Wheat Biology and Genetic Improvement on South Yellow & Huai River Valley, Ministry of Agriculture, Hefei 230036, China. ³Biology Department, Saint Mary’s University, Halifax, NS, B3H 3C3 Canada. ⁴National United Engineering Laboratory for Crop Stress Resistance Breeding, Hefei 230036, China. ⁵Anhui Key Laboratory of Crop Biology, Hefei 230036, China. ⁶These authors contributed equally to this work. Correspondence and requests for materials should be addressed to H.S. (email: sihq2002@163.com) or G.S. (email: genlou.sun@smu.ca) or C.M. (email: machuanxi@ahau.edu.cn)

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and hexaploid wheat species23,24. In polyploid wheat, the origin of the B genome is still under debate. Diploid wheat from the Fertile Crescent region has long been considered as the A-genome donor to tetraploid (SlSl, 2n = 14) progenitor of T. aestivum donor remains unknown. There has been little debate on T. aestivum, and it was concluded that the B genome species they investigated is a close relative of the B genome in common wheat. It has been reported that the B genome is closely related to S. rupifaciens (SS, 2n = 2x = 14), Ae. longissima (SSb, 2n = 2x = 14) and Ae. searsii (SSb, 2n = 2x = 14), and that Ae. speltoides (SS, 2n = 2x = 14) is phylogenetically distinct from the other species in the Sitopsis section. Ae. speltoides (S genome) has been suggested as the most likely progenitor of the B genome26,27. However, Huang et al.24 and Haider29 reported that none of the five Sitopsis species they investigated is a close relative of the B genome in T. aestivum, and concluded that the B genome donor remains unknown. There has been little debate on Ae. tauschii Coss (genomic DD) as the D genome progenitor of T. aestivum24.

There has been great interest in the determination of ancestral diploid genome donors of hexaploid wheat10,29. Understanding the origin of hexaploid wheat not only enhances its genetic improvement, but also is important in the development of artificial synthetic forms20,34, because genome progenitors of common wheat are very important genetic resources to improve the economical traits of modern cultivars35,36. However, so far, the direct experimental evidence for clear understanding of the phylogenetic history among the three A, B, and D genome lineages are still challenging. Maybe, this debate can be greatly simplified by analyzing the molecular evolution of a conservative gene among diploid, tetraploid and hexaploid wheat species.

Wcor15 (GenBank: AB095006), a member of the wheat cold-responsive gene family, which could encode the chloroplast-targeted protein when exposed to low temperature, plays an important role in the cold hardiness of wheat37. Based on our sequencing data, we found that the Wcor15 gene was very conservative in the hexaploid genome in common wheat, and compared their evolution among diploid, tetraploid and hexaploid wheats.

Wcor15 gene (GenBank: AB095006) was used as a probe. The ORF sequence of CBTL0110083500 on 2AL was 563 bp. The ORF sequence of CBTL0111257031 on 2BL was 565 bp. The ORF sequence of CBTL0110522649 on 2DL was 566 bp.

Table 1. Three homoelogous Wcor15 sequences obtained from the ENA. The ORF sequence (563 bp) of Wcor15 gene (GenBank: AB095006) was used as a probe. The ORF sequence of CBTL0110083500 on 2AL was 563 bp. The ORF sequence of CBTL0111257031 on 2BL was 565 bp. The ORF sequence of CBTL0110522649 on 2DL was 566 bp.

| Primer set | Primer sequence (5’-3’) | Amplified target | Size of PCR product (bp) |
|------------|-------------------------|------------------|------------------------|
| Wcor15A | CCTTCTCATCATCATGAGTC | 2AL genome-specific | 1840 |
| Wcor15B | CCATCCAGTTGAAAGGT | 2BL genome-specific | 2400 |
| Wcor15D | CAGAATCTAGTTACGAGA | 2DL genome-specific | 2012 |
| Wcor15s | CCGTTCCGTCATGCCCTGT | Coding region of Wcor15 | 570 |

The three homoelogous Wcor15 sequences were identified using the ORF sequence (including the intron, 563 bp) of Wcor15 gene (GenBank: AB095006) as probe to screen the nucleotides databases of EBI (EBI: http://www.ebi.ac.uk/ena/)38, and sequences were found from the wheat genome A, B and D, respectively (Table 1). The specific PCR primers named Wcor15A, Wcor15B and Wcor15D (Table 2) for amplifying three homoelogous Wcor15 sequences which contained intact ORFs were designed, based on the highly variation region of accession CBTL0110083500 (2AL), CBTL0111257031 (2BL) and CBTL0110522649 (2DL).

The primer pairs were used to amplify genomic DNA of hexaploid wheat cultivar Annong 0822. Each primer pair generated single-band amplicon with the expected size. The genes were designated as Wcor15-2A.
(KT264885), Wcor15-2B (KT264957) and Wcor15-2D (KT265022) respectively, which contained the 5′ upstream region, two exons, one intron and 3′ downstream region. Further analysis demonstrated that these three sequences are very similar with a few nucleotide insertions, deletions, and substitutions (Supplementary Fig. S1).

The Wcor15-2A sequence from A genome is exactly the same to the sequence of AB095006 previously reported by Takumi et al. 37, suggesting that the Wcor15-2A and Wcor15 (GenBank: AB095006) is the same gene. After RT-PCR using RNA templates from Annong 0822, all of the three homoeologous Wcor15 genes were specifically induced by low temperature (data not shown), suggesting the three homoeologous Wcor15 genes are the cold-responsive gene.

In order to further confirm the location of the gene, one set of nulli-tetrasomic lines of cv. Chinese Spring was used. Wcor15-2B was found in the lines except nullisomic 2B–tetrasomic 2D (N2B–T2D). This indicates that the Wcor15-2B is located on chromosome 2B. In turn, Wcor15-2A and Wcor15-2D were assigned to chromosome 2A, and 2D, respectively (Fig. 1).

Each Wcor15 cDNA clone contained an ORF of 441 nucleotides that putatively encoded a polypeptide with 147 amino acid residues (Fig. 2). They shared common characteristics such as a sorting signal that is predicted to target them to the chloroplast 37. The properties of the N-terminal end of the Wcor15-2A, Wcor15-2B and Wcor15-2D polypeptides were determined. They have the conserved regions coding for the putative chloroplast signal peptides and the putative cleavage site of the signal peptide (Fig. 2), and shared the common site of an intron insertion and 14-3-3 protein recognition motif that could interact with the 14-3-3 proteins. The binding of the proteins to the signal peptides is essential for the chloroplast precursor proteins to be efficiently transported into chloroplasts 39,40. We also uncovered evidence that WCOR15-2A, WCOR15-2B and WCOR15-2D contained 11-mer amino acid motifs and α-helix structures characterizing LEA Group341. Together these findings suggested that WCOR15-2A, WCOR15-2B and WCOR15-2D might belong to the chloroplast-targeted LEA3 protein.

Sequence analysis of the Wcor15-2A, Wcor15-2B and Wcor15-2D genes in hexaploid wheats (AABBDD, T. aestivum and T. spelta). The Wcor15A primer was used to amplify the Wcor15-2A among individual 106 hexaploid wheats including winter wheats, spring wheats and T. spelta from different geographical regions (Table 3). All the studied hexaploid wheats yielded an expected PCR product of approximately 1.8 kb. To further analyze Wcor15-2A, we randomly sequenced 100 samples (Supplementary Table S1). All sequences were
identical and were exactly same to the \textit{Wcor15-2A} sequence of Annong 0822 (Supplementary Table S2), suggesting that \textit{Wcor15-2A} gene was highly conservative in hexaploid wheat.

The complete sequence of \textit{Wcor15-2B} gene was also amplified from these 106 hexaploid wheats using \textit{Wcor15B} primer. The PCR products from 54 wheats were sequenced (Supplementary Table S1). The \textit{Wcor15-2B} sequences were highly conserved in the 54 hexaploid wheats (Supplementary Table S3). Fifteen substitutions (13 in the 5' upstream, 2 in the 3' downstream) and 2 insertion and deletion (one in the 5' upstream, another in the intron) were occurred in the untranslatable region, however, no significant differences were found in the two exons among the 54 sequences of \textit{Wcor15-2B} (Supplementary Fig. S2). They shared 100% identities in the deduced amino acid sequences.

The \textit{Wcor15-2D} in these 106 hexaploid wheat accessions was also characterized. All of the samples yielded PCR products of ~2 kb. The PCR products from 33 wheat varieties were sequenced (Supplementary Table S1). No variation was found among 33 hexaploid wheat varieties (Supplementary Table S4), indicating highly conservative of \textit{Wcor15-2D} gene in hexaploid wheat.

Our results indicated that the three genes \textit{Wcor15-2A}, \textit{Wcor15-2B} and \textit{Wcor15-2D} derived from the three homoeologous 2A, 2B and 2D chromosomes were highly conserved among hexaploid wheat varieties from different geographical regions.

### Table 3. The names of diploid, tetraploid and hexaploid wheat accessions.

| Accessions          | Genome   | Nu. of accessions |
|---------------------|----------|-------------------|
| Common wheat        |          |                   |
| WWRNC               | AABBDD   | 4                 |
| NCPSR               | AABBDD   | 24                |
| NHRPSR              | AABBDD   | 28                |
| WUSR                | AABBDD   | 7                 |
| JUSR                | AABBDD   | 3                 |
| WWR                 | AABBDD   | 11                |
| SWWR                | AABBDD   | 7                 |
| SWRNC               | AABBDD   | 5                 |
| IWVF                | AABBDD   | 13                |
| Spelt wheat         | AABBDD   | 4                 |
| T. dicoccoides      | AABB     | 3                 |
| T. dicoccum         | AABB     | 3                 |
| T. durum            | AABB     | 3                 |
| T. carthlicum       | AABB     | 1                 |
| T. antartica        | A’A’     | 3                 |
| T. boesticum        | A”A”     | 1                 |
| T. monoccocum       | A”A”     | 2                 |
| Ae. speltoides      | SS       | 3                 |
| Ae. longissima      | S’S’     | 1                 |
| Ae. bicornis        | S’S’     | 1                 |
| Ae. sharoenensis    | S’S’S’   | 3                 |
| Ae. searsii         | S’S’     | 3                 |
| Ae. tauschii ssp. tauschii | DD | 3 |
| Ae. tauschii ssp. strangulata | DD | 3 |

Our results indicated that the three genes \textit{Wcor15-2A}, \textit{Wcor15-2B} and \textit{Wcor15-2D} derived from the three homoeologous 2A, 2B and 2D chromosomes were highly conserved among hexaploid wheat varieties from different geographical regions.

### Sequence analysis of the \textit{Wcor15-2A}, \textit{Wcor15-2B} and \textit{Wcor15-2D} genes in tetraploid species (AABB).

The DNA from 10 tetraploid materials including three \textit{T. dicoccoides}, three \textit{T. dicoccum}, three \textit{T. durum} and one \textit{T. carthlicum} (Table 3) were amplified using the primer pairs \textit{Wcor15A}, \textit{Wcor15B} and \textit{Wcor15D} (Table 2). As expected, only the \textit{Wcor15A} and \textit{Wcor15B} amplified the PCR products with expected size (Fig. 3a). The \textit{Wcor15D} primer did not give rise to any amplification products (Fig. 3a), confirming absence of \textit{Wcor15-2D} in the tetraploid wheat genome.

The \textit{Wcor15-2A} sequences from A genome in 10 tetraploid species (AABB) (Table 4) are exactly the same with the sequence of \textit{Wcor15-2A} from hexaploid wheats (Supplementary Table S5), suggesting that \textit{Wcor15-2A} gene is highly conserved within tetraploid wheats, and between tetraploid and hexaploid wheats.

Alignment of the 10 \textit{Wcor15-2B} sequences from tetraploid wheat showed a number of single nucleotide substitutions among these sequences whose situation was the same to \textit{Wcor15-2B} in the 54 hexaploid varieties
SS and DD).

Sequence analysis of the hexaploids during and after the second polyploidization. (Supplementary Fig. S2), suggesting that diversification of Wcor15-2B did not occur between tetraploids and hexaploids during and after the second polyploidization.

**Figure 3. PCR amplification of the Wcor15A Wcor15B and Wcor15D primers in diploid and tetraploid accessions.** (a) PCR amplification with Wcor15A Wcor15B and Wcor15D primers in tetraploid species. As829, As836 and As839 belong to *T. dicocoides*. PI272527, PI193873 and PI221401 belong to *T. dicocoides*. Club57, Simeto-2 and Dr8 belong to *T. durum*. Tc belongs to *T. carthlicum*. (b) PCR amplification with Wcor15A Wcor15B and Wcor15D primers in *T. urartu*, *T. monococcum* and *T. boeoticum*. PI428222, PI428260 and PI428266 belong to *T. urartu*. Bo8 belongs to *T. boeoticum*. Mo4 and TL belong to *T. monococcum*. (c) PCR amplification with Wcor15A primers in *T. monococcum* and *T. boeoticum* and eleven species of the *Sitopsis* section. Bo8 belongs to *T. boeoticum*. Mo4 and TL belong to *T. monococcum*. PI542276, PI369663 and PI369624 belong to *Ae. speltoides*. Q03-004 belongs to *Ae. longissima*. Q03-021 belongs to *Ae. bicornis*. PI584395, PI584408 and PI584406 belong to *Ae. sharonensis*. PI599142, PI599124 and PI599126 belong to *Ae. searsii*. (d) PCR amplification with Wcor15A Wcor15B and Wcor15D primers in eleven species of the *Sitopsis* section. PI542276, PI369663 and PI369624 belong to *Ae. speltoides*. Q03-004 belongs to *Ae. longissima*. Q03-021 belongs to *Ae. bicornis*. PI584395, PI584408 and PI584406 belong to *Ae. sharonensis*. PI599142, PI599124 and PI599126 belong to *Ae. searsii*. (e) PCR amplification with Wcor15A Wcor15B and Wcor15D primers in *Ae. tauschii* species. As77, As80 and As2392 belong to *Ae. sp. tauschii*. As2386, As2387 and As2388 belong to *Ae. sp. strangulata*.

(Supplementary Fig. S2), suggesting that diversification of Wcor15-2B did not occur between tetraploids and hexaploids during and after the second polyploidization.

**Sequence analysis of the Wcor15-2A, Wcor15-2B and Wcor15-2D genes in diploid species (AA, SS and DD).** In order to compare if Wcor15-2A, Wcor15-2B and Wcor15-2D genes have changed between diploid and polyploid, we sequenced these genes in a set of diploid wild relatives with genome AA, SS and DD, respectively (Table 4).

In all the three varieties of *T. urartu* (genome A*′ A*) surveyed, the primer Wcor15B and Wcor15D did not generate any amplification products (Fig. 3b), suggesting that the Wcor15-2B and Wcor15-2D sequence is absent in *T. urartu*. Amplicons were obtained from all three *T. urartu* with the primer Wcor15A. The three exactly same sequences (designated as Wcor15-2A1) showed 100% identity with the Wcor15-2A sequences from tetraploid and hexaploid wheats (Supplementary Table S5). Wcor15A, Wcor15B and Wcor15D primers failed to amplify the DNA from *T. monococcum* and *T. boeoticum* (Fig. 3b). In order to obtain the Wcor15 gene from the *T. monococcum* and *T. boeoticum*, we redesigned a pair of Wcor15s primers which located at near the coding region based on the previously reported Wcor15 gene (GenBank: AB095006). Three Wcor15 sequences were obtained (Fig. 3c) and are identical which was designated as Wcor15-2A2 containing a complete encoding region. The identity between Wcor15-2A2 and Wcor15-2A was 97.87% at the DNA level (Supplementary Fig. S3 and Table S5).

In all eleven accessions of the *Sitopsis* species (1 *Ae. bicornis* S*S*, 1 *Ae. longissima* S*S*, 3 *Ae. sharonensis* S*S*S*, 3 *Ae. searsii* S*S* and 3 *Ae. speltoides* SS) (Table 3) surveyed, the primer Wcor15A, Wcor15B and Wcor15D did not generate any amplification products (Fig. 3d). In order to obtain the Wcor15 gene from the *Sitopsis* section, we again employed the primer Wcor15s which only amplified the coding region of Wcor15 genes without the 5′ upstream sequence (>1 Kb). Eleven Wcor15 sequences were obtained (Fig. 3c). Sequences analysis showed that all the three *Ae. speltoides* shared the two same exons of Wcor15-2B with tetraploid and hexaploid wheats. However, the intron of Wcor15-2B had two haplotypes in tetraploid and hexaploid wheats, one with a G deletion, the other with G insertion at the same location, while all the three *Ae. speltoides* only had one haplotype, a G deletion in the intron (Supplementary Fig. S4). The gene Wcor15-2B from *Ae. bicornis* (Q03-021), *Ae. longissima* (Q03-004), *Ae. sharonensis* (PI584395, PI584408 and PI584406), and *Ae. searsii* (PI599142, PI599124 and PI599126) showed 100% identity with each other, nevertheless, besides the difference of base G indel mentioned above, there were still many base differences compared with the gene from *Ae. speltoides*, 2 located in the first exon, 7 in the intron, and 5 in the second exon (Supplementary Fig. S4). These results suggested that *Ae. speltoides* is the most likely gene donor of Wcor15-2B, and diversification of the gene occurred during the first polyploidization.
From diploid *Ae. tauschii* (As 80, As 77, As 2392, As 2386, As 2387 and As 2388), six *Wcor15-2D* were cloned with the primer Wcor15D (Table 4). The six *Wcor15-2D* sequences were divided into two types: (I) As 2386, As 2387 and As 2388 with 100% identity, (II) As 80, As 77 and As 2392 with only a base substitution in the upstream non-coding regions. However, the *Wcor15-2D* from As 2386, As 2387 and As 2388 which belong to *Ae. tauschii* subsp. *strangulata* showed 100% identity with the *Wcor15-2D* from hexaploid wheat varieties (Supplementary Table S6). The coding region sequences from *Ae. bicornis* (Q03-021), *Ae. longissima* (Q03-004), *Ae. sharonensis* (PI584395, PI584408 and PI584406), and *Ae. searsii* (PI599142, PI599124 and PI599126) are same to the sequences from As 80, As 77 and As 2392 of *Ae. tauschii* subsp. *tauschii*. The primer Wcor15A and Wcor15B failed to amplify a product from these species (Fig. 3e). The results suggested that *Ae. tauschii* subsp. *strangulata* is the donor to the gene *Wcor15-2D* in hexaploid wheat.

**Discussion**

The hexaploid bread wheat is believed to have originated through one or more hybridization events. The study on origin of A, B and D genomes of bread wheat has been a hot topic. Understanding the origin of hexaploid wheat would benefit not only the genetic diversity but also expand the genetic basis for wheat breeding. Previous studies have demonstrated that the sequence data of conserved gene can be used to study the evolution of...
of gene families from different species. In this study, we reported the utility of the Wcor15 sequence to identify the progenitors of the tetraploid and hexaploid wheats and to define the evolution of their close relatives. Wcor15 is the member of the Cor gene family, which could encode the chloroplast-targeted protein when exposed to low temperature, and play an important role in the cold hardiness of wheat. Based on the previous research on Wcor15 (GenBank: AB095006) gene, it was found that the gene of AB095006 located on chromosome 2AL, and we named it Wcor15-2A, in addition to this gene, we cloned the other two homoeologous Wcor15 sequences (Wcor15-2B and Wcor15-2D) from the wheat genome 2BL and 2DL, respectively. Gene characterization analyzing showed that the three homoeologous Wcor15 genes may belong to the chloroplast-targeted LEA3 protein, which is consistent with previous studies about characterization of Wcor15-2A.

To see whether Wcor15-2A, Wcor15-2B and Wcor15-2D genes are a conserved gene or not, the Wcor15-2A, Wcor15-2B and Wcor15-2D genes were cloned from 106 hexaploid wheat varieties from different geographical areas, 10 tetraploid species and 23 diploid species. Comparative analyses indicated that the Wcor15-2A (Supplementary Fig. S5), Wcor15-2B (Supplementary Fig. S4) and Wcor15-2D (Supplementary Fig. S6) genes were highly conservative during wheat evolution. Moreover, the three genes kept invariable during the second allopolyploidization from tetraploid to hexaploid (Fig. 4).

The Wcor15 gene is a good candidate gene for investigating the donor of A-, B- and D-genome. The three homoeologous Wcor15 sequences from the wheat genome A, B and D, respectively (Table 1) were different (Supplementary Fig. S1). Each of the three sequences was highly conservative in respective diploid (Supplementary Figs S4–S6), tetraploid (Supplementary Figs S4 and S5), and hexaploid (Supplementary Figs S7–S10). Wcor15-2A and Wcor15-2B on the A- and B-genome were very stable from diploid (AA, BB) to tetraploid (AABB) (Supplementary Figs S4 and S5), and from tetraploid (AABB) to hexaploid (AABBDD) (Supplementary Figs S4 and S5). Wcor15-2D is also highly conserved from diploid (DD) to hexaploid (AABBDD) (Supplementary Fig. S6). Comparison of the conserved Wcor15 gene can provide some evidences on the origin of the A, B and D genome of common wheat.

The diploid wheats carrying A-genome included T. urartu (genome Au), T. monococcum (genome Am) and T. boeoticum (genome Am). To investigate the evolutionary relationships of Wcor15-2A genes from diploid and polyploid wheats, the sequences from T. urartu, T. monococcum, T. boeoticum, tetraploid and hexaploid wheats were compared. The six genes in diploid wheats (genome AA) were classified into two types (Supplementary Fig. S11). The three T. urartu (PI428222, PI428260 and PI428266) were type I (Wcor15-2A1). The two T. monococcum (Mo4 and TL) and one T. boeoticum (Bo8) were type II (Wcor15-2A2). Compared to the Wcor15-2A1 sequence from T. urartu, the Wcor15-2A2 sequence showed much higher identity (100%) with the Wcor15-2A sequences from tetraploid and hexaploid wheats, suggesting that the T. urartu might be the direct donor of the Wcor15-2A in common wheat and that Wcor15-2A gene from A genome has no mutation during two sequential allopolyploidization events from T. urartu to tetraploid and hexaploid wheats. The result is consistency with the previous studies.
However, taking into consideration of no amplicon from T. monococcum and T. boeoticum when using Wcor15A primer, it suggested that non-coding regions of Wcor15-2A1 were obviously different from Wcor15-2A2. Coding regions alignments also revealed variation between Wcor15-2A2 and Wcor15-2A1 from T. urartu (Supplementary Fig. S3).

Many researchers have suggested that the B genome is closely related to the S genome of the Sitopsis section which was comprised of five diploid species: Ae. speltoides, Ae. longissima, Ae. sharonensis, Ae. searsii, and Ae. bicornis. To validate which species is the potential donor of B genome, eleven accessions of the Sitopsis species were amplified using the primers pair Wcor15A, Wcor15B and Wcor15D, but no PCR product was obtained. However, the primer Wcor15S successfully amplified the eleven accessions of the Sitopsis species, their sequences were classified into two types: (I) Ae. speltoides (PI542276, PI369663 and PI369624), and (II) Ae. bicornis (Q03-021), Ae. longissima (Q03-004), Ae. sharonensis (P1584395, P1584408 and P1584406), and Ae. searsii (P1599142, P1599124 and P1599126) (Supplementary Fig. S12). Our results showed that Ae. speltoides is distinct from the other species in the Sitopsis section, supporting the previous reports.

In terms of coding region, Wcor15-2B sequences from different tetraploid and hexaploid wheats were divided into two groups by the insertion and deletion of a nucleotide G in the intron. All three Ae. speltoides sequences shared 100% identity, are different from tetraploid and hexaploid wheats with only a G deletion in the intron. On the other hand, no amplicon obtained from Ae. speltoides when using Wcor15B primer, suggested that non-coding regions of Wcor15-2B might be obvious differences between Ae. speltoides and tetraploid and hexaploid wheats. Our results suggested that Ae. speltoides might be the direct donor of the Wcor15-2B in tetraploid and hexaploid wheat varieties, non-coding region of Wcor15-2B gene from B genome might mutate during the first polyploidization from Ae. speltoides to tetraploid wheat, however, no change has occurred for this gene during the second polyploidization from tetraploid to hexaploid.

The Wcor15-2D sequences of D-genome were highly conservative among 106 hexaploid wheats. However, Wcor15-2D genes from six accessions of Ae. tauschii (Table 4) were divided into two allelic groups (Supplementary Fig. S13), suggesting variations in diploid wheats. Our results supported that subsp. strangulata may be the D-genome donor of common wheat suggested by previous studies.

The Wcor15 coding region of Ae. tauschii subsp. tauschii is same to the sequences from the S genome species, Ae. bicornis, Ae. longissima, Ae. sharonensis and Ae. searsii. Mayer et al. reported that Ae. sharonensis was much closer to Ae. tauschii than to Ae. speltoides. The analysis of the multispecies coalescent species tree for Aegilops and Trititicum diploid suggested that Ae. bicornis, Ae. longissima, Ae. sharonensis and Ae. searsii are more closely related to Ae. tauschii ssp. tauschii than to Ae. speltoides. However, no amplicon obtained from Ae. bicornis, Ae. longissima, Ae. sharonensis and Ae. searsii when Wcor15D primer was used, indicating that non-coding region of Wcor15-2D from Ae. bicornis, Ae. longissima, Ae. sharonensis and Ae. searsii were obviously different from that of Ae. tauschii ssp. tauschii.

This paper examined the evolutionary relationship of the Wcor15 in diploid, tetraploid and hexaploid wheats during wheat allopolyploidization (Fig. 4). Trititicum urartu, Ae. speltoides and Ae. tauschii subsp. strangulata are most likely the donors of the Wcor15-2A, Wcor15-2B and Wcor15-2D loci in common wheat, respectively. The Wcor15 genes from subgenomes A and D were very conservative without insertion and deletion of bases during evolution of diploid, tetraploid and hexaploid. However, the Wcor15-2B genes mutated only during the first allopolyploidization event.

Materials and Methods

Wheat germplasm. One hundred and six hexaploid wheat (genome AABBDD) were used in this study, including 4 varieties from Winter wheat region of North China (WWRNC), 24 varieties from North China plain sub-region of Yellow & Huai river winter wheat region (NCPSR), 28 varieties from North Huai river plain sub-region of Yellow & Huai river winter wheat region (NHRRPSR), 7 varieties from West upland sub-region of Yellow & Huai river winter wheat region (WUSR), 3 varieties from Jiaodong upland sub-region of Yellow & Huai river winter wheat region (JUSR), 11 varieties from Winter wheat region of middle and lower reaches of the Yangtze river (WW), 7 varieties from Southwest winter wheat region (SWWR), 13 varieties from Introduced wheat variety of foreign (IWVF), 5 spring wheat region of North China (SWRNC) and 4 T. spelta, 10 tetraploid species (AABB), and 23 diploid species (AA, BB and DD) (Table 3).

DNA extraction, primer design, PCR and sequencing. Genomic DNA was extracted from young leaves of ten days seedlings using the Easypure Plant Genomic DNA Kit (Sangon Biotech, Shanghai, China). Genome-specific primers were designed for each of the homoeologous Wcor15 genes (Table 2) using the software Primer Premier Version 5.0, and were synthesized by Shanghai Sangon Biological Technology Company.

PCR reaction were performed in total volumes of 20 μl, containing 12.8 μl ddH₂O, 10 × PCR buffer (with Mg²⁺), 2.0 μl, dNTPs (2.5 mM) 2.0 μl, 0.5 μl of each primer (10 mM), 2.0 μl genomic DNA and Taq DNA polymerase (5 U/μl) 0.2 μl. Amplifications were performed using a standard touchdown PCR protocol with the appropriate annealing temperature. Each PCR was done five repeats up to a total of 100 μl.

All PCR products were directly sequenced. Each of 50 μl PCR products were sequenced by Shanghai Sangon Biological Technology Company, and the other 50 μl PCR products were sequenced by Huada Biotech Company in Beijing. To guarantee sequence accuracy, DNA sequencing was repeated three times.

Sequence analysis and characterization were performed using DNAman software at default settings (http://www.lynnonly.com). The three homoeologous Wcor15 sequences were identified at EBI web site (http://www.ebi.ac.uk/ena) and All of the sequences of the AA, BB, DD, AABB and AABBDD genome homoeologs of Wcor15-2A, Wcor15-2B and Wcor15-2D were submitted to the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/) (Table 4 and Supplementary Table S1).
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