Recurrent Deletions of Puroindoline Genes at the Grain *Hardness* Locus in Four Independent Lineages of Polyploid Wheat[1][W][OA]

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Polyploidy is known to induce numerous genetic and epigenetic changes but little is known about their physiological bases. In wheat, grain texture is mainly determined by the *Hardness* (*Ha*) locus consisting of genes *Puroindoline a* (*Pina*) and *b* (*Pinb*). These genes are conserved in diploid progenitors but were deleted from the A and B genomes of tetraploid *Triticum turgidum* (AB). We now report the recurrent deletions of *Pina-Pinb* in other lineages of polyploid wheat. We analyzed the *Ha* haplotype structure in 90 diploid and 300 polyploid accessions of *Triticum* and *Aegilops* spp. *Pin* genes were conserved in all diploid species and deletion haplotypes were detected in all polyploid *Triticum* and most of the polyploid *Aegilops* spp. Two *Pina-Pinb* deletion haplotypes were found in hexaploid wheat (*Triticum aestivum*; ABD). *Pina* and *Pinb* were eliminated from the G genome, but maintained in the A genome of tetraploid *Triticum timopheevii* (AG). Subsequently, *Pina* and *Pinb* were deleted from the A genome but retained in the A^m genome of hexaploid *Triticum zhukovskyi* (A^mAG). Comparison of deletion breakpoints demonstrated that the *Pina-Pinb* deletion occurred independently and recurrently in the four polyploid wheat species. The implications of *Pina-Pinb* deletions for polyploid-driven evolution of gene and genome and its possible physiological significance are discussed.

For over 100 years, wheat grain has been classified into hard and soft types. Grain hardness or texture is mainly determined by the *Hardness* (*Ha*) locus. This classification forms the fundamental basis for differentiating wheat grain worldwide (for review, see Morris, 2002). Wheat speciation has been molded by polyploidy. Diploid (*Triticum monococcum*), tetraploid (*Triticum turgidum*), and hexaploid (*Triticum aestivum*) wheat species have been known since the 1920s (for review, see Gill and Friebe, 2002). Of the 600 million metric tons of wheat produced in the world in 2005 (http://faostat.fao.org), over 90% comes from hexaploid wheat, also called common or bread wheat, and the remaining from tetraploid, also called macaroni or durum wheat (http://www.fas.usda.gov/pecad/highlights/2005/10/durum_27oct2005). Bread wheat grain is either soft and used for pastries or hard and used for bread and noodles. Durum wheat grain is classified as extrahard and used for pasta. Diploid wheat grain is soft and, although it was the first domesticated wheat, is now grown as a specialty crop in isolated areas. Recently, See et al. (2004) produced a supersoft hexaploid wheat genotype by introgressing softness genes from diploid species.

Because of the pivotal importance of grain texture in determining end use quality, this trait has been intensively studied by geneticists, cereal chemists, and, more recently, by molecular biologists. In the 1970s, Mattern et al. (1973) mapped a gene with a major effect on grain texture on chromosome 5D. Later, Law et al. (1978) further localized the gene to the short arm of chromosome 5D and designated the trait as hardness with alleles *Ha* for soft and *ha* for hard. Greenwell and Schofield (1986) found that a 15-kD protein called friabilin from water-washed starch from grain was associated with the *Ha* locus. Abundant friabilin was found in soft wheat with *Ha* alleles, small amounts in hard wheat with *ha* allele, and none in extrahard durum wheat grains. Blochet et al. (1991, 1993) isolated and sequenced lipid-binding proteins and called them puroindolines because of the presence of a Trp domain. Gautier et al. (1994) isolated cDNA clones corresponding to genes *Puroindoline a* (*Pina*) and *b* (*Pinb*). A large body of research has shown that PINA, PINB, and grain softness protein (GSP) constitute a major fraction of friabilin. Functional copies of both *Pin* genes are required for soft grain texture in wheat (for review, see Morris, 2002).

Cloning of the *Pin* genes stimulated genomics research on the *Ha* locus. Gautier et al. (2000), using a PCR approach, showed that *Pina* and *Pinb* are highly

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conserved in diploid wheat and the Triticeae and closely related cereals, such as rye (*Secale cereale*), barley (*Hordeum vulgare*), and oats (*Avena sativa*), but were absent in sorghum (*Sorghum bicolor*), maize (*Zea mays*), and rice (*Oryza sativa*). They were reported absent in tetraploid wheat species *T. turgidum* and *Triticum timopheevii* and reintroduced into hexaploid wheat as it arose from the hybridization of tetraploid *T. turgidum* (lacking *Pin* genes) and *Aegilops tauschii* Coss. (*Pin* genes present). Null mutations of PINA protein in hexaploid wheat cultivars of more recent origin have been reported (for review, see Morris, 2002).

Sequencing the *Ha* loci of *T. monococcum* (Chantret et al., 2004) and *A. tauschii* (Chantret et al., 2005) showed that *Gsp* (*Gene2*), *Pina* (*Gene4*), and *Pinb* (*Gene6*) are located in an interval of approximately 70 kb. A hypothetical gene (*Gene3*) was found between *Gsp* and *Pina* and a function-unknown gene (*Gene5*) between *Pina* and *Pinb*. In the D genome of *A. tauschii* and *T. aestivum*, a 5′ untranslated region and a 5′ coding sequence of *Pinb* were duplicated downstream of the functional *Pinb*. Upstream of *Gsp*, *BGGP* (*Gene1*), coding for a β-1-3-galactosyl-*O*-glycosyl-glycoprotein, delimits the 5′ boundary of *Ha*. Downstream of *Pinb*, a cluster of *ATPase* genes (*Gene7*) coding for AAA-type ATPase and an unknown gene (*Gene8*) delimit the 3′ boundary of *Ha*. Compared to *Ha-D*, deletions of the genomic block containing *Gene3*, *Pina*, *Gene5*, and *Pinb* occurred at *Ha-A* and *Ha-B* of *T. turgidum* and *T. aestivum* (Chantret et al., 2005).

During routine mapping of the tetraploid *T. timopheevii* genome, we detected one copy of the *Pin* genes in its genome. This was a surprising result in view of the previous report of Gautier et al. (2000) and could be due to polymorphism for this locus in this species. A large and more comprehensive polymorphism survey of *Pin* genes in polyploid wheat species was initiated. To more fully characterize the nature of deletions for this region in polyploid wheat, we undertook sequencing of the *Ha* region of *Aegilops speltoides* Tausch., of which the S genome has highest affinity among the diploid species to the B and G genomes of polyploid wheat. Because two of the three genomes of polyploid wheat were donated by *Aegilops* spp., we also analyzed haplotype structure at the *Ha* locus of a small number of diploid and polyploid *Aegilops* spp. These results and their implications for polyploidy-driven mechanisms of gene and genome evolution and speciation are reported and their possible physiological significance is discussed.

RESULTS

Phylogeny of Polyploid Wheat and Genetic Nomenclature of Loci

To interpret the *Ha* deletion haplotype survey results, it is important to briefly introduce understanding of the phylogeny of polyploid wheat. Two lineages of tetraploid wheat, emmer (*T. turgidum*, $2n = 4x = 28$; AB) and Timopheevi (*T. timopheevii* Zhuk., $2n = 4x = 28$; AG), originated less than 0.5 million years ago (Huang et al., 2002) from two separate hybridization events between *A. speltoides* ($2n = 2x = 14$; S), as the female parent, and *Triticum urartu* Tumanian *ex* Gandilyan ($2n = 2x = 14$; A), as the male parent (Tsunewaki, 1993; Dvorak, 1998; Kilian et al., 2007). More recent hybridizations with two additional diploid species gave rise to hexaploid wheat lineages. Common wheat (*T. aestivum*; ABD) originated approximately 8,000 years ago (Nesbitt and Samuel, 1996) from hybridization between *T. turgidum* and *A. tauschii* in cultivated fields and does not exist in the wild (Kihara, 1944; McFadden and Sears, 1946). *Triticum zhukovskyi* Menabde and Ericzjan ($2n = 6x = 42$; AA^mG) arose in cultivation from hybridization of *T. timopheevii* sp. *timopheevii* with *T. monococcum* L. sp. *monococcum* ($2n = 2x = 14$; A^m; Upadhya and Swaminathan, 1963; Dvorak et al., 1993). The A, B, and D diploid donors of polyploid wheat diverged from a common ancestor approximately 3 million years ago (Huang et al., 2002). In polyploid wheat, the *Ha-A*, *Ha-B*, and *Ha-D* genetic nomenclature conveys both locus and genomic origin of *Ha* homoeoloci. Results summarizing the haplotype structure at the *Ha* locus in relation to the phylogeny of polyploid wheat are shown in Figure 1.

Sequence Analysis of the *Ha-S* Genomic Region of *A. speltoides*

Of the three diploid ancestors of polyploid wheat, bacterial artificial chromosome (BAC) sequences of the *Ha* genomic region of A- and D-genome ancestors were reported previously (Chantret et al., 2004, 2005). We undertook sequence analysis of a BAC from the S genome of *A. speltoides*. We screened a BAC library of *A. speltoides* (Akhunov et al., 2005) with *Gsp* and *Pina* probes, each of which identified three BACs. *Gsp*-containing BACs did not overlap with *Pina*-containing BACs. We estimate that the *Ha-S* genomic region in *A. speltoides* is 3 times the size of *Ha-*A^m in *T. monococcum* and of *Ha-D* in *A. tauschii* and 5 times the size of *Ha-D* of *T. aestivum*. A *Pina* BAC, 197O23, was shotgun sequenced at 8× coverage and assembled into 13 contigs after prefinishing, totaling 212,510 bp. Four nontransposable element (TE) protein-coding genes, *Pina*, *Pinb*, and two *ATPases*, were found in this BAC, are located in the contig at the 3′ end in the same orientation, and span 28,848 bp (Fig. 2). *Gene5*, previously reported to be present in the collinear region between *Pina* and *Pinb* of *T. monococcum* and *A. tauschii*, was not found in *A. speltoides*. Based on sequence homology and collinearity, the two *ATPase* genes in BAC 197O23 correspond to *ATPase-4* and *ATPase-5* at the *Ha-D* locus and are orthologous to two truncated *ATPase* genes upstream of *Gene8* at the *Ha-B* locus (Chantret et al., 2005). The rest of BAC 197O23 is gene free and mainly occupied by TEs and tandem repeats, a typical feature of large genomes, where genes are clustered into is-

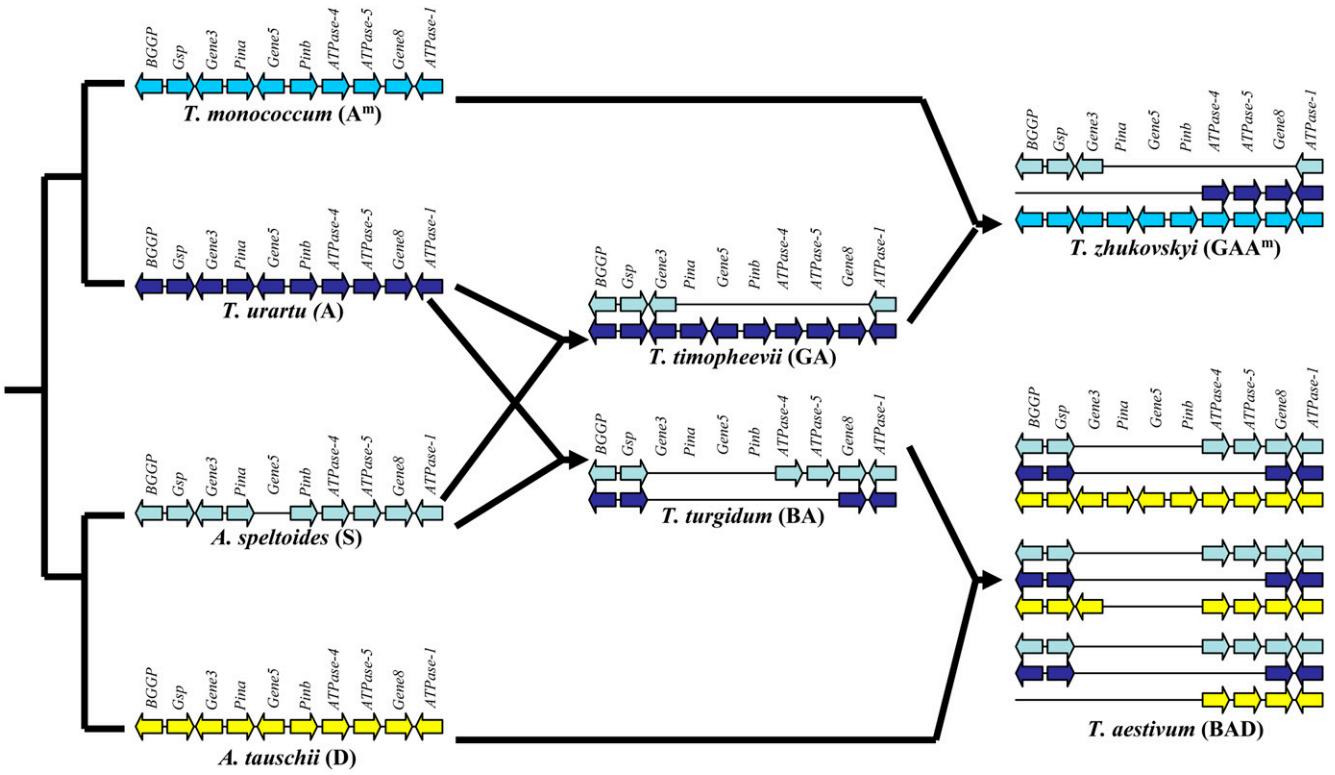


Figure 1. Scheme for polyploid wheat phylogeny and changes in the *Ha* haplotype structure during their evolution. Solid arrows represent genes and their orientation, with symbols above. Species and their genome formulae are indicated underneath. Arrow size and spacing are not proportional to gene size or intergenic interval.

lands and separated by nested TEs (Wicker et al., 2001). *Gene8*, *ATPase-1*, *ATPase-2*, and *ATPase-3* were located in a separate BAC.

Survey of the Haplotype Structure at the *Ha* Locus in *Aegilops* and *Triticum*

We determined the haplotype structure at the *Ha* locus by Southern analysis of tester DNA digested with restriction enzymes *Eco*RI, *Hin*dIII, or *Bam*HI using *Pina* and *Pinb* gene probes. We estimated the copy number of *Pina* or *Pinb* genes in tester species by counting the number of fragments detected by Southern hybridization. The data were tabulated to determine whether the haplotype structure was conserved or there were null haplotypes for either one or both the *Pin* genes at the *Ha* locus (Table I; Supplemental Table S1). Null haplotypes were further characterized according to the size of the deletion, either by Southern analysis using additional gene probes that mark the *Ha* locus (see Fig. 1), or by sequencing as described below.

Diploid Species

We randomly selected at least two accessions from each of the 12 diploid species of *Aegilops* and *Triticum* (a total of 90 accessions; Table I; Supplemental Table S1) for the haplotype survey. In all cases, Southern hybridization detected a single band or, rarely, multiple bands for *Pina* and *Pinb* gene probes, indicating that haplotype structure at the *Ha* locus is conserved in the diploid species. A single copy of *Pina* and *Pinb* was detected in A- and D-genome donor species of polyploid wheat (Table I). Five *Aegilops* species share the S genome and all except *A. speltoides* are self-pollinated. All self-pollinated S-genome species had one copy of *Pina* and *Pinb*. Most accessions of *A. speltoides* also carry one copy of the *Pin* genes and the observed multiple Southern hybridization fragments in some accessions (Supplemental Table S1) may be due to either heterozygosity, because it is a cross-pollinated species, or, rarely, the presence of intragenic restriction sites or gene duplication. All the C-, M-, U-, and N-genome species also had one copy of the *Pin* genes, except for one accession of *Aegilops comosa*, where Southern analysis indicated multiple gene copies.

Tetraploid Species

The above-mentioned diploid species have contributed genomes to tetraploid *Triticum* and *Aegilops* spp. and two copies each of *Pina* and *Pinb* genes are expected in the genomes of these tetraploid species (Table I). The tetraploid wheat species *T. turgidum* (AB) and *T. timopheevii* (AG) form the A-genome cluster. We

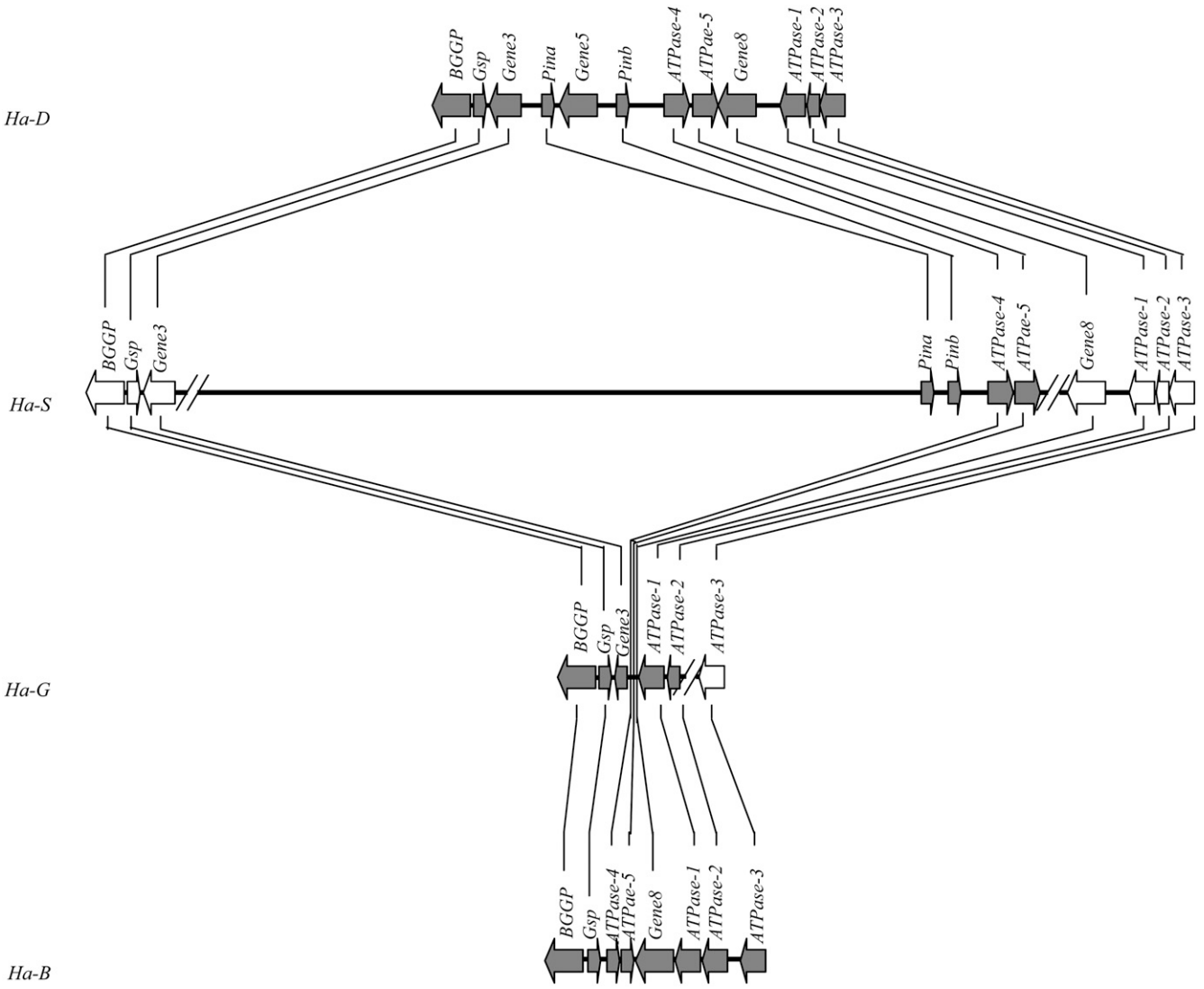


Figure 2. Comparison of haplotype structure at the *Ha* loci: *Ha-D* of *A. tauschii* and *T. aestivum*, *Ha-S* of *A. speltoides*, *Ha-G* of *T. timopheevii*, and *Ha-B* of *T. turgidum* and *T. aestivum*. Solid arrows represent genes and their orientation with the gene symbols above them. *Gene3* and *Gene8* are hypothetical and *Gene5* is coding for an unknown protein. The *Ha-S* haplotype spans three separate BACs as indicated by slashes. White arrows in *Ha-S* and *Ha-G* were not sequenced; they are deduced based on collinearity between the *Ha-B* and *Ha-D* loci. The lines connect the orthologs. The *Ha-B* and *Ha-D* haplotypes are after Chantret et al. (2005).

screened 92 accessions of *T. turgidum*, including eight subspecies representing the range of wild and domesticated forms. All showed the null haplotype for the *Pin* genes (Table I; Supplemental Table S1; Fig. 3), confirming that *Pina* and *Pinb* have been deleted in this species (Gautier et al., 2000; Dvorak et al., 2004).

We screened 65 accessions of *T. timopheevii*, including two subspecies representing the range of wild and domesticated forms (Supplemental Table S1). All carried only one copy of the *Pina* and *Pinb* genes, indicating null haplotype at the *Ha* locus for one of its genomes (Supplemental Table S1). *Gene5*, which lies between *Pina* and *Pinb*, was, as expected, present in one copy. The *Gsp* probe detected two copies, indicating that one of the breakpoints that produced the null haplotype is located between *Gsp* and *Pina* (Fig. 3).

Nine tetraploid *Aegilops* spp. are grouped into the U- and D-genome clusters. In the U-genome cluster, one accession each was analyzed for *Aegilops columnaris* Zhuk. ($U^{co}M^{co}$) and *Aegilops peregrina* (Hack. in J. Fraser) Marie and Weiller (US^{s}), two accessions for *Aegilops biuncialis* Vis. (UM), and nine accessions for *Aegilops kotschyii* Boiss. (US^{s}); all showed conserved haplotype structure for the *Pin* genes in both of their genomes (Table I). The other three U-genome cluster species, *Aegilops geniculata* Roth ($U^{g}M^{g}$), *Aegilops neglecta* Req. ex Bertol. (UM), and *Aegilops triuncialis* L. ($U^{t}C^{t}$), where more than one accession was analyzed,

Table I. *Summary of plant materials used, their ploidy levels, and their Pina-Pinb haplotypes*
For *Aegilops* polyploids, genome locations of *Pina-Pinb* haplotypes were not determined.

Species	Genome	No. of Accessions	*Pina-Pinb* Haplotype[a]		
Diploid *Triticum*			A	B/G	A^m/D
T. urartu	A	20	+ +		
T. monococcum	A^m	3			+ +
Tetraploid *Triticum*					
T. turgidum	AB	93	− −	− −	
T. timopheevii	AG	71	+ +	− −	
Hexaploid *Triticum*					
T. aestivum	ABD	37	− −	− −	+ +
		3	− −	− −	− −
T. zhukovskyi	AGA^m	3	− −	− −	+ +
Diploid *Aegilops*			Genome 1	Genome 2	Genome 3
A. tauschii	D	4	+ +		
A. speltoides	S (≈B or G)	45	+ +		
A. bicornis	S^b	2	+ +		
A. longissima	S^l	2	+ +		
A. searsii	S^s	2	+ +		
A. sharonensis	S^{sh}	3	+ +		
A. caudata	C	2	+ +		
A. comosa	M	3	+ +		
A. umbellulata	U	2	+ +		
A. uniaristata	N	2	+ +		
Tetraploid *Aegilops*					
A. crassa	$D^{cr}X$	11	+ +	+ +	
		1	+ +	− +	
A. cylindrica	D^cC^c	3	+ +	+ +	
A. ventricosa	D^vN	15	+ +	+ +	
A. biuncialis	UM	2	+ +	+ +	
A. neglecta	UM	11	+ +	+ +	
		16	+ +	− +	
A. columnaris	$U^{co}M^{co}$	1	+ +	+ +	
A. geniculata	U^gM^g	2	+ +	+ +	
		1	+ +	− +	
A. kotschyii	US^s	9	+ +	+ +	
A. peregrina	US^s	1	+ +	+ +	
A. triuncialis	U^tC^t	1	+ +	+ +	
		1	+ +	− +	
Hexaploid *Aegilops*					
A. crassa	$DD^{cr}X$	3	+ +	+ +	− +
		2[b]	+ +	+ +	− −
A. juvenalis	$D^{cr}XU$	4	+ +	+ +	+ −
A. vavilovii	$D^{cr}XS^s$	2	+ +	+ +	− +
		1[b]	+ +	+ +	− −
A. neglecta	$U^nM^nN^n$	6	+ +	+ +	− +
		4	+ +	− +	− +
		3[b]	+ +	− +	− −

[a] + indicates presence and − indicates deletion; + +, both *Pina* and *Pinb* are present; − −, both *Pina* and *Pinb* are deleted; − +, *Pina* is deleted but *Pinb* is present; + −, vice versa. [b] Genomes containing the *Pina* deletion were not determined relative to the *Pinb* deletion. − − does not necessarily indicate a haplotype where both *Pina* and *Pinb* were deleted from the same genome.

were polymorphic in *Pina* copy number. One or more accessions in each species had a conserved haplotype structure for the *Pin* genes and the other accessions for the same species showed unique haplotypes in one of their genomes where the *Pina* gene was deleted, but the *Pinb* gene was retained. Therefore, it is possible that, if a larger survey of the U-genome cluster species is undertaken, all may turn out to be polymorphic for *Ha* haplotype structure.

The D-genome cluster species *Aegilops crassa* Boiss. (DX), *Aegilops cylindrica* Host (D^cC^c), and *Aegilops ventricosa* Tausch (D^vN^v) showed a conserved haplo-

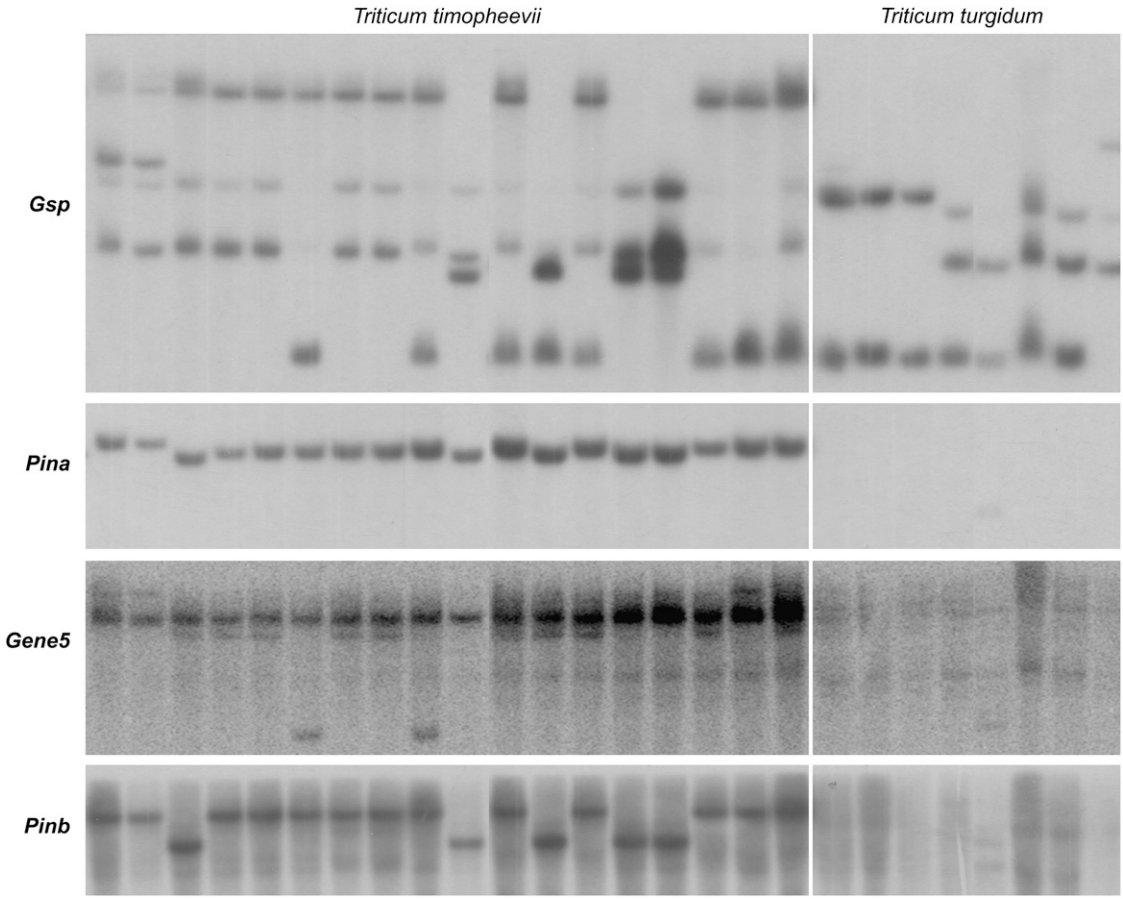


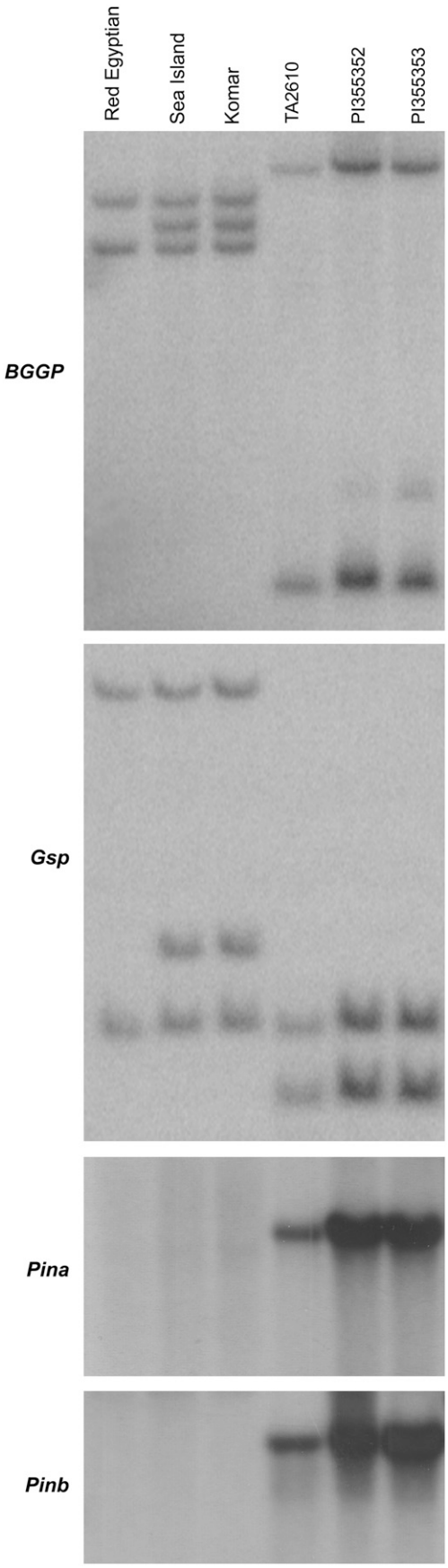
Figure 3. The autoradiogram of a Southern hybridization. Genes *Gsp*, *Pina*, *Gene5*, and *Pinb* are indicated at the left and species at the top. *Gsp* detected two major bands in both *T. timopheevii* and *T. turgidum*. *Pina*, *Gene5*, and *Pinb* detected a single fragment in *T. timopheevii* and none in *T. turgidum*.

type structure for the *Pin* genes, except one of the 12 accessions of *A. crassa* was null for *Pina* in one of its genomes.

Hexaploid Species

Some of the tetraploid species of *Triticum* and *Aegilops* mentioned above have undergone a second round of hybridization with various diploid species to form hexaploid species. The A-genome cluster species *T. aestivum* (ABD) is expected to have one copy each of the *Pina* and *Pinb* genes contributed by *A. tauschii* (D). We surveyed 40 accessions of *T. aestivum*, including all six subspecies and 18 historical hard wheat cultivars of *T. aestivum* sp. *aestivum*. Of the 40 *T. aestivum* accessions, 37 showed a conserved haplotype structure for *Pina* and *Pinb* in the D genome (*Ha-D*) and, as expected, a null haplotype for the A and B genomes. Of the 18 hard wheat cultivars that do not express the PINA protein (Morris et al., 2001), 'Red Egyptian', 'Sea Island', and 'Komar' showed the null haplotype at *Ha-D*. Further probing with *BGGP* and *Gsp* detected three copies in 'Sea Island' and 'Komar', but only two in 'Red Egyptian' (Fig. 4). RFLP analysis of the 'Chinese Spring'/'Red Egyptian' chromosome substitution lines 5A, 5B, and 5D confirmed that *BGGP*, *Gsp*, *Pina*, and *Pinb* were deleted from the *Ha-D* locus on chromosome 5D of 'Red Egyptian'. Therefore, at least two independent deletion events occurred at the *Ha-D* locus in common wheat, one with the distal breakpoints between *Gsp* and *Pina* in 'Sea Island' and 'Komar', and another haplotype with distal breakpoint beyond *BGGP* in 'Red Egyptian'.

We surveyed three accessions of *T. zhukovskyi* (AA^mG), the second A-genome cluster hexaploid species. It is expected to have two copies of *Pin* genes, one from *T. timopheevii* and the second from *T. monococcum*. However, only one copy of the *Pin* genes was detected, indicating the presence of a second null haplotype at the *Ha* locus in one of its genomes (Fig. 4). Because *T. zhukovskyi* is autoallohexaploid, the loss of a *Ha* locus could be due to either recombination between A and A^m genomes or a deletion event. This question can be resolved based on *BGGP* and *Gsp* hybridization patterns: (1) if each detects three bands, *Pina* and *Pinb* were deleted from one genome; (2) if two bands with similar



intensity are observed, all *BGGP*, *Gsp*, *Pina*, and *Pinb* were deleted from the A or A^m genome; and (3) if the two bands differ significantly in intensity, A-A^m recombination, instead of deletion, occurred. Our results support the second scenario (Fig. 4); i.e. *BGGP*, *Gsp*, *Pina*, and *Pinb* were deleted from the A or A^m genome, similar to the *Ha-D* haplotype in 'Red Egyptian'.

We surveyed three D-genome cluster and one U-genome cluster hexaploid *Aegilops* spp. and all are expected to have two to three copies of *Pina* and three copies of *Pinb*, depending upon the genotype of the tetraploid parent (see above). *A. crassa* (DDX) and *Aegilops vavilovii* (Zhuk.) Chennav. (DXS^s) were polymorphic; three accessions of *A. crassa* and two accessions of *A. vavilovii* had two copies of *Pina* and three copies of *Pinb*, and the rest had two copies of both *Pina* and *Pinb*. *Aegilops juvenalis* (Thell.) Eig (DXU) had three copies of *Pina* and two copies of *Pinb* in their genomes.

The U-genome cluster hexaploid *A. neglecta* ($U^nM^nN^n$) was polymorphic; six accessions had two copies of *Pina* and three of *Pinb*, four accessions had one copy of *Pina* and three copies of *Pinb*, and the remaining three accessions had one copy of *Pina* and two copies of *Pinb*. Because tetraploid *A. neglecta* (UM) was polymorphic for *Pina* (one or two copies), the data suggest recurrent deletion of *Pina* at tetraploid and hexaploid levels in this species. Compared to *Pina*, *Pinb* deletion was only detected at the hexaploid level in the polyploid *Aegilops* spp.

Sequence Analyses of Unique Haplotypes

A sequence analysis of deletion haplotypes detected in polyploid wheat species was used to further characterize and allocate their genomic origin. These results are summarized in Figure 1.

The Ha-A Haplotype of T. timopheevii

Detection of the conserved *Ha* haplotype in the Timopheevi lineage of polyploid wheat triggered an immediate effort to determine its genomic origin. We designed A- and G-genome-specific primers based on *Pina* and *Pinb* sequences from diploid progenitors *T. urartu* and *A. speltoides*, respectively. A-genome-specific primers amplified strong fragments approximately 650 bp for *Pina* and 780 bp for *Pinb*. G-genome-specific primers produced very weak amplicons from *T. timopheevii*. Cloning and sequencing of

Figure 4. Autoradiogram of Southern hybridization. Genes *BBGP*, *Gsp*, *Pina*, and *Pinb* as indicated at the left and accession numbers at the top. 'Red Egyptian', 'Sea Island', and 'Komar' are hard red cultivars of common wheat (*T. aestivum*); accessions TA2610, PI355352, and PI355353 belong to *T. zhukovskyi*. *BGGP* and *Gsp* each detected two fragments in 'Red Egyptian' and three in 'Sea Island' and 'Komar', whereas *Pina* and *Pinb* did not detect any signal in all three wheat cultivars. In *T. zhukovskyi*, *BGGP* and *Gsp* detected two bands with similar intensity and *Pina* and *Pinb* detected a single fragment.

the PCR products from six *T. timopheevii* accessions indicated that A-genome amplicons showed highest homology to *Pin* genes from *T. urartu* at the DNA and protein sequence levels, but G-genome amplicons showed no homology to the *Pin* genes, but weak similarities to various TEs. This clearly indicated that the A-genome haplotype (*Pina-A* and *Pinb-A*) was maintained and the G-genome copy was deleted in *T. timopheevii*.

The promoters and coding regions of *Pina* and *Pinb* are highly conserved among the *timopheevii* accessions and between *timopheevii* and *urartu*, except for a 1-bp insertion in the promoter region of *Pina* in one accession and an A-to-C transversion at position 181 of *Pinb* in another (Supplemental Figs. S1 and S2). In the 3′ region of *Pinb*, an 88-bp fragment, spanning the stop codon and polyadenylation signal, was found in triplicate in *Pinb-A* of *T. timopheevii* compared to its ancestor *T. urartu*. The repeat members are identical, except for a single-nucleotide polymorphism (Supplemental Fig. S2). A PCR assay showed that the 88-bp triple repeat is fixed at the species level (Fig. 5). A 7-bp sequence (CAACATG) was found at the beginning of each repeat member and immediately after the triple repeat in *T. timopheevii* and flanking the 88-bp sequence in *T. urartu*, suggesting that it originated by replication slippage after polyploidization (Supplemental Fig. S2). The alignments of nucleotide sequences of the *Pina* and *Pinb* genes and amino acid sequences of the PINA and PINB proteins between *T. timopheevii* and *T. urartu* are shown in Supplemental Figures S1, S2, S3, and S4.

The Ha-G Haplotype of T. timopheevii

To determine the fine structure of *Ha-G*, particularly the deletion breakpoints, we isolated *Ha-G* from a fosmid library constructed from accession Tim01 of *T. timopheevii*. The *Gsp*-containing fosmid clone, 1E05, was sequenced at 8 times coverage and assembled into five contigs after prefinishing, totaling 41,262 bp. The largest contig, 23,807 bp, contains three non-TE protein-coding genes within 10,919 bp, *BGGP*, *Gsp*, and *ATPase* (Fig. 2). In addition, a 311-bp sequence, corresponding to the 5′ portion of *Gene3* was found 1,806 bp downstream of *Gsp* and 2,749 bp upstream of *ATPase*, and a partial coding sequence of another copy of the *ATPase* gene was found 778 bp downstream of the intact *ATPase* at the 3′ end of the fosmid clone. At the nucleotide sequence level, *Gsp-G* is highly homologous to *Gsp-S* of *A. speltoides* and *Gsp-B* of *T. turgidum* and *T. aestivum*. *ATPase-G* showed highest identity (98%) to *ATPase-1* (*Gene7-1*, pseudogene) from chromosome 5B of *T. turgidum* and *T. aestivum*. *BGGP-G* has high homology to *BGGP-D* of *A. tauschii* and *T. aestivum* and to *BGGP-B* of *T. turgidum* and *T. aestivum*. As expected, *GSP-G* is encoded by the plus strand, and *BGGP-G* and *ATPase-G* are encoded by the minus strand. The *Ha-G* haplotype lost *Gene8* along with *Pina*, *Pinb*, *ATPase-4*, and *ATPase-5*, and has a distal breakpoint between *Gene3* and *Pina* and a proximal breakpoint between *Gene8* and *ATPase-1*. Compared to the *Ha-S* locus, more than 200 kb of sequence was deleted from the *Ha-G* and *Ha-B* loci during polyploid wheat evolution (Fig. 2).

The Ha-A^m Haplotype of T. zhukovskyi

Because *T. zhukovskyi* originated from a cross between *T. timopheevii* (*Ha-A*, null *Ha-G*) and *T. monococcum* (*Ha-A^m*), the conserved haplotype in this hexaploid wheat is either of *Ha-A* or *Ha-A^m* origin. The A^m genome of *T. monococcum* is highly homologous to the A genome of *T. urartu* due to their very recent divergence. Multiple sequence alignment of the *Pin* gene sequences available in the National Center for Biotechnology Information (NCBI) database from these two species and sequences of *T. timopheevii* from this research were used to identify species-specific single-nucleotide polymorphisms to unambiguously assign the genome origin of *Pina* and *Pinb* in *T. zhukovskyi*. A *monococcum*-specific synonymous point mutation was identified at position 384 within the open reading frame, where three accessions of *T. monococcum* carried an A, the mutant allele, and all accessions of *T. urartu*, *T. timopheevii*, and the remaining *T. monococcum* accessions carried a G, the wild-type allele (Supplemental Fig. S5). Similarly, a species-specific synonymous mutation was found at position 75 in the open reading frame of *Pinb*, where all the *T. urartu* and *T. timopheevii* accessions carry a T and all the accessions of *T. monococcum* carry a C (Supplemental

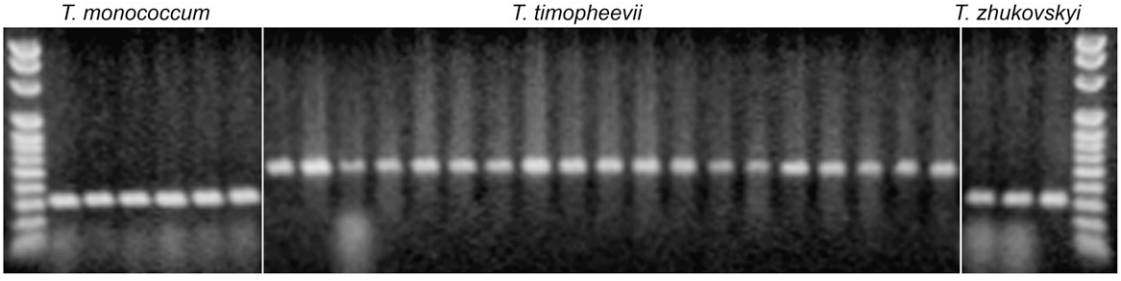


Figure 5. PCR assay of the 88-bp triple repeat at the 3′ end of *Pinb-A*. The 100-bp ladder is on either side and species are indicated at the top. Amplicons of *T. urartu* and *T. zhukovskyi* are 308 bp and those of *T. timopheevii* are 484 bp in length.

Fig. S6). All three accessions of *T. zhukovskyi* carry *monococcum*-specific alleles, indicating that the *Pina-A* and *Pinb-A* from *T. timopheevii* were deleted and are survived by *Pina-A*^{m} and *Pinb-A*^{m} from *T. monococcum* (Supplemental Figs. S5 and S6). Furthermore, the 88-bp triple repeat was not detected in *Pinb* of *T. zhukovskyi* by either sequencing or PCR assay (Fig. 5), providing additional evidence that *Pinb-A* was deleted in this hexaploid wheat species.

The *Ha* Loci of Synthetic Tetraploids

The fixation of *Ha* deletion patterns in tetraploid wheat suggested that *Pin* genes may have been eliminated immediately upon polyploidization similar to the low-copy sequences demonstrated by Feldman et al. (1997), Liu et al. (1998), and Ozkan et al. (2001). We tested this hypothesis on tetraploid AASS- and $A^mA^mS^{sh}S^{sh}$-genome amphiploids. The AASS amphiploid was produced in our laboratory by crossing diploid species *T. urartu* (A) with *A. speltoides* (S), followed by chromosome doubling. The $A^mA^mS^{sh}S^{sh}$ was produced from *T. monococcum* (A^m) and *Aegilops sharonenesis* Eig (S^{sh}) by Ozkan et al. (2001). We used genome-specific primers for both *Pina* and *Pinb* to produce amplicons for sequencing from the AASS and $A^mA^mS^{sh}S^{sh}$ amphiploids and their diploid parents. *Pina* and *Pinb* haplotypes were conserved in both amphiploids (Fig. 6). The *Pina* and *Pinb* sequences of the A and A^m genomes between the amphiploids and their parents were identical (Supplemental Figs. S7, S8, S11, and S12), but up to 1% variation was found in the *Pina* and *Pinb* sequences of the S and S^{sh} genomes of the amphiploids compared to their *Aegilops* parents (Supplemental Figs. S9, S10, S13, and S14). More striking was a 21-bp deletion detected in *Pinb-S*^{sh} of the amphiploid $A^mA^mS^{sh}S^{sh}$ compared to its *A. sharonensis* parent. Six clones from that ligation were sequenced and the wild-type allele was not found. *Pinb* sequences of the S-genome species, including *Aegilops bicornis* (Forssk.) Jaub. and Spach (S^b), *Aegilops longissima* Schweinf. and Muschl. (S^l), *Aegilops searsii* Feldman and Kislev ex Hammer (S^sS^s), *A. sharonensis* (S^{sh}), and *A. speltoides* (S) contain two 11-bp direct repeats (GAAGTTGGCGG) separated by a 10-bp spacer (CTGGTACAAT). This 21-bp deletion was obviously caused by unequal crossing over between the 11-bp direct repeats (Supplemental Fig. S10) and led to a loss of seven amino acids (WYNEVGG) in the PINB protein. The $A^mA^mS^{sh}S^{sh}$ amphiploid used is from the S2 generation; the unequal crossover either occurred during female meiosis of the *A. sharonensis* parent TMB02 or happened and was rapidly fixed after polyploidization.

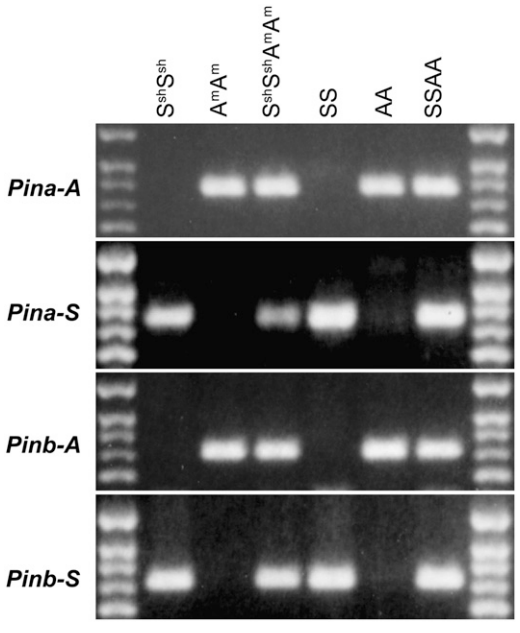


Figure 6. PCR assay of *Pina* and *Pinb* in amphiploids and their parents. The 100-bp ladder is on either side and the lowest band is 500 bp. The genome formula is indicated at the top and gene symbols are at the left of the picture.

DISCUSSION

The most remarkable observation on the structure and evolution of the *Ha* locus in wheat and the Triticeae is the absolute conservation of the locus in diploid species reported here and in previous articles (Gautier et al., 2000; Lillemo et al., 2002; Massa et al., 2004; Chen et al., 2005; Simeone et al., 2006) and recurrent and independent deletions in the polyploid *Triticum* and *Aegilops* spp. To date, more than 200 accessions from the two diploid *Triticum* and 10 diploid *Aegilops* spp. have been analyzed and not a single case of deletion polymorphism at the *Ha* locus has been reported. Especially, no deletion polymorphisms have been detected in a diverse sample of more than 130 accessions of the A-, B-, and D-genome donor species of polyploid wheat. This is in contrast to frequent deletion haplotype polymorphisms for a defense-gene cluster in the D-genome diploid, *A. tauschii* (Brooks et al., 2006). Against this high rate of deletion polymorphism in polyploid species, not a single case of insertion-deletion polymorphism was documented in a sample of *Pina* and *Pinb* sequences from 50 accessions of diploid *A. tauschii*, representing its geographical diversity (Massa et al., 2004). All polyploid wheat and most polyploid *Aegilops* spp. harbored deletion haplotypes of independent origin at the *Ha* locus. So how does a gene that is essential in a diploid suddenly become deleterious so that it must be deleted in a polyploid? To begin to answer this question, some discussion about the nature of *Pin* genes, their function, the nature of gene action in polyploids and the mechanisms of polyploid genome evolution and speciation that promote expression and evolution of novel traits is needed.

Amino acid sequence analysis has shown that numerous storage proteins, including low-molecular-

weight glutenin, α/β gliadins, lipid transfer proteins, trypsin inhibitor, α-amylase/trypsin inhibitor, GSP, PINA, and PINB, belong to the α-amylase inhibitors (AAIs) and seed storage (SS) protein subfamily because they have an AAI-SS domain. AAIs play an important role in the natural defense of plants against insects and pathogens mainly by inhibiting α-amylases and proteases. Puroindolines have bactericidal (Jing et al., 2003) and fungicidal activities (Krishnamurthy et al., 2001). PINA and PINB proteins directly bind to the surface of starch granules in the endosperm cells and form a friabilin complex. Using isogenic lines, Swan et al. (2006) showed that puroindolines seem to protect starch from microbial digestion and increased expression of PIN proteins decreased the starch digestibility of wheat in the rumen by up to 30%. α-Amylase is an important enzyme in starch metabolism and is induced in the aleurone by GA_3 from the embryo during germination. Conceivably, wheat starch can be protected from α-amylase digestion by AAI activity of the PIN proteins. Therefore, because of the important role of PIN proteins in plant defense and seed physiology, *Pina* and *Pinb* genes may be under strong selection pressure and are maintained in all the diploid species.

One of the consequences of polyploidy is doubling and tripling of gene copy number and, thus, the amount of proteins may be doubled or tripled for some of these genes. This dosage response has been demonstrated for the *Pin* genes and a supersoft hexaploid wheat genotype has been created (See et al., 2004). Because wheat starch is protected from α-amylase digestion by AAI activity of the PIN proteins, we hypothesize that the sudden dosage-driven increase in expression levels of *Pin* genes in polyploid would impede the embryos from obtaining nutrition from the endosperm. The situation may be more severe when polyploid plants are under abiotic stress, such as heat and drought during grain filling, which adversely affects endosperm development. Point mutations in *Pinb* can liberate the PIN proteins from binding to the starch granule surface and cause significant difference in grain texture (Giroux and Morris, 1997, 1998; Morris et al., 2001); however, the PIN proteins with the AAI activity remain in the endosperm cells. Therefore, deletion of *Pina-Pinb* genes provides the most efficient mechanism to reduce AAI activity and is the least detrimental because they are structural genes. The reduction in PIN proteins, in turn, enhances the exposure of starch granules to α-amylases during germination to provide sufficient nutrition for new polyploid seedlings to compete in stand establishment with surrounding diploid populations. Thus, the deleterious action of a single gene may determine the fate of a new polyploid species and, therefore, we consider it a bottleneck speciation event.

To test our hypothesis of the deleterious effect of a high dose of *Pin* genes on seed physiology, a preliminary germination experiment was performed using seeds from hexaploid 'Chinese Spring' wheat plants with two (background control), four, and six doses of the *Pina-Pinb* genes (See et al., 2004). Seeds from each genotype were placed in petri dishes on wet filter paper at room temperature. After 24 h, the check cultivar 'Chinese Spring' (with two doses) sprouted and radicle and coleoptile were visible. The genotype with four doses showed sprouting activity, but the radicle and coleoptile were invisible. The genotype with six doses did not sprout until 48 h after imbibition and radicle and coleoptile were visible only after 96 h of imbibition. The negative correlation between the *Pina-Pinb* dosages and the rate of germination and growth was consistently observed in subsequent days. Although this experiment appears to demonstrate the deleterious effects of higher doses of *Pin* genes on seed germination and stand establishment, more rigorous experiments need to be conducted, including assaying AAI activity of PIN proteins and evaluating germination, growth, and vigor of seedlings of the isogenic lines with different doses of *Pin* genes under field conditions. But what are the chances of occurrence and fixation of such a rare deletion mutation in a small founder polyploid species population and what genes might compensate for this missing function?

Because at least one copy of the *Pin* genes is maintained in the vast majority of polyploid species, the loss and fixation of null haplotype copies of both *Pin* genes in tetraploid *T. turgidum* require explanation. As mentioned earlier, a number of proteins have AAI activity and higher doses of their expression may partially compensate for the loss of function of the *Pin* genes. *Gsp* was not involved in most deletion haplotype polymorphisms and may have some compensatory functional role in defending against microbe attacks. Because extensive resetting of gene expression patterns follows polyploidy, it is possible that other genes may have been recruited for the same role. In fact, quantitative trait loci for hardness phenotype have been mapped in other regions of the genome besides the 5DS locus (Breseghello et al., 2005; Narasimhamoorthy et al., 2006).

Accumulating evidence suggests that polyploidization is accompanied by significant genome restructuring and resetting of gene expression patterns (for review, see Chen and Ni, 2006; Chen, 2007). Numerous genetic and epigenetic changes have been observed in neopolyploids (amphiploids), including sequence elimination (Song et al., 1995; Feldman et al., 1997; Liu et al., 1998; Ozkan et al., 2001), chromosome rearrangements (Pontes et al., 2004), changes in methylation (Shaked et al., 2001) and gene expression patterns (Adams et al., 2003; He et al., 2003), reactivation of TEs (Kashkush et al., 2003), and microRNA expression (Tian et al., 2006). The rate of DNA sequence divergence is much higher in polyploid wheat compared to diploid relatives (for review, see Dubcovsky and Dvorak, 2007). The great abundance of repetitive sequences, especially retroelements, in the wheat genome (Li et al., 2004; Devos et al., 2005) seems to promote gene deletion/duplication events. Chantret et al. (2005) reported that the loss of a block of genes,

including the *Pina* and *Pinb* genes from A and B genomes of tetraploid *T. turgidum*, occurred independently by illegitimate recombination among retroelements bordering the *Ha* locus. Such events appear to be common at the *Ha* locus because Chantret et al. (2005) documented two additional such events in polyploid wheat genotypes. The loss of a large gene block, including *Ha-G* in *T. timopheevii* as reported here, may have been caused by a similar mechanism. We also documented replication slippage as a mechanism leading to triple repeat of an 88-bp sequence at the *Ha-A* locus of *T. timopheevii*. Unequal crossing over led to the deletion of a 21-bp sequence in the *Pinb-S*^{sh} gene in the $A^mA^mS^{sh}S^{sh}$ synthetic amphiploid. Although *Pina* and *Pinb* were conserved in a number of tetraploid *Aegilops* spp., they may not be transcribed as documented by Chen et al. (2005). Others were polymorphic for the *Pina* and/or *Pinb* deletion haplotype and none of the hexaploid species tolerated three doses of *Pin* genes (Table I). Although there is a high rate of deletion in polyploid *Triticum* and *Aegilops* spp., polyploidy per se does not cause *Pin* gene deletions as indicated by our data on synthetic amphiploids. The high mutation rate at the *Ha* locus in polyploid species, coupled with high gametic transmission of deletion haplotypes due to polyploid buffering, and the high fitness cost of higher doses of *Pin* gene expression on seed physiology led to the fixation of deletion haplotypes at the *Ha* locus in the founder populations during polyploid wheat speciation.

Polyploidization leads to both additive and nonadditive gene expression patterns (for review, see Chen, 2007). Documentation of recurrent deletions at the *Ha* locus suggests certain rules for the fate of loci that show dosage-sensitive expression following polyploidization. If the additive gene expression has a deleterious effect on the organism, then it will be rapidly deleted. Conversely, if the additive gene expression has a beneficial effect, then it will be conserved. If the effect is neutral, then the duplicate loci may undergo subfunctionalization or mutation to assume new functions (Adams et al., 2003). Comparative genomics is generating large databases of gene duplications and deletions following whole-genome polyploidization. The challenge of the postgenomics era will be to determine the physiological bases of such duplication/deletion events.

MATERIALS AND METHODS

Plant Materials

Plant materials are summarized in Table I regarding the species, ploidy levels, genome formula, and number of accessions. The details of individual accessions are listed in Supplemental Table S1 with their *Pina* and *Pinb* scores. Accessions prefixed with TA are maintained by the Wheat Genetic and Genomic Resources Center, Kansas State University (Manhattan, KS); accessions prefixed with CItr, PI, and PVP were obtained from Dr. Harold Bockelman at the U.S. Department of Agriculture Small Grains Collection (Aberdeen, ID). *Triticum timopheevii* accession Tim01, the TH02/TMB02 amphiploid (A^mA^mSS), the *Aegilops sharonensis* (TH02), and *Triticum monococcum* (TMB02) were provided by Dr. Moshe Feldman, Weizmann Institute of Science (Rehovot, Israel). The amphiploid (TA3438, AASS) was derived from a cross made at the Wheat Genetic and Genomic Resources Center between *Aegilops speltoides* accession TA1785 and *Triticum urartu* accession TA831. All lines were grown in the greenhouse. The ploidy levels of *Aegilops crassa* and *Aegilops neglecta* accessions were determined by Badaeva et al. (1998, 2001) and this research. Critical accessions were identified by C banding (Gill et al., 1991) when necessary.

Clones and Primers

Wheat (*Triticum aestivum*) cDNA clones TMA9 (*Pina*) and TMA10 (*Pinb*) were provided by Dr. Marie-Françoise Gautier (Unité de Biochimie et Biologie Moléculaire des Céréales, Institut National de la Recherche Agronomique, France). EST BU100707 homologous to *Gene1* was obtained from the Arizona Genomics Institute (Tucson, AZ). The BAC clone 197O23 of *A. speltoides* was provided by Dr. Jan Dvorak (University of California, Davis, CA). All PCR primers for amplification of *Gsp*, *Pina*, *Gene5*, and *Pinb* and annealing temperatures are given in Supplemental Table S2.

RFLP Analysis to Detect Deletions

The copy numbers of *Pina* and *Pinb* were determined based on the number of fragments detected in Southern blots. Approximately 100 mg of leaf tissue were collected from each accession, lyophilized in a 2-mL microcentrifuge tube, and disrupted by shaking with metal beads. The procedures for DNA extraction, digestion, electrophoresis, and Southern hybridization generally followed those of Faris et al. (2000). For most accessions, *Eco*RI, *Hin*dIII, or *Bam*HI were used for digestion. The search of the coding sequences of *Pina* and *Pinb* deposited in the NCBI did not reveal restriction sites *Bam*HI and *Eco*RI, and the *Hin*dIII restriction site was only found at position 24 of *Pinb* from the S-genome species. In some cases, more bands were observed than expected, possibly due to heterozygosity, intragenic restriction site, or duplication, and they were ignored because the focus of this work was on the detection of the *Pin* gene deletions.

Molecular Cloning

PCR products of genes *Gsp*, *Pina*, *Gene5*, and *Pinb* were separated by agarose gel electrophoresis, eluted from gel, ligated to T-easy vector (Promega), and transformed into *Escherichia coli* strain DH10B.

To clone the *Ha* locus from *T. timopheevii*, we constructed a fosmid library of accession Tim01. Briefly, total genomic DNA of Tim01 was sheared by 120 cycles of freezing in liquid nitrogen and thawing in a 65°C water bath, and separated by CHEF gel electrophoresis. Fragments of 30 to 50 kb were excised, eluted, end repaired with an End-It kit (Epicentre Biotech), and ligated to the CopyControl pCC1FOS. The ligation was packaged with MaxPlax Lambda Packaging Extracts (Epicentre Biotech), diluted 100-fold, and used to infect *E. coli* strain PE1300 following the manufacturer's instructions. An aliquot of 70 μL infected bacteria (approximately 70 clones) was distributed and maintained in 384-well plates. The library first was pooled by plate and screened by PCR using *Gsp-S*-specific primers. Positive plates were pooled by rows and columns. Once a positive well was identified, the culture from that well was spread onto Luria-Bertani agar plates containing chlorophenicol (12.5 μg/mL) and colonies were picked, arrayed in 96-well plates, and screened individually by PCR. To isolate the *Ha* locus from the G-genome donor species, we hybridized the *Pina* and *Gsp* to the macroarray filters of an *A. speltoides* BAC library (Akhunov et al., 2005).

Sequence Analysis

To design genome-specific primers, nucleotide sequences for *Gsp*, *Pina*, and *Pinb* of diploid species of *Triticum* and *Aegilops* were retrieved from NCBI (http://www.ncbi.nlm.nih.gov) and subjected to multiple sequence alignment with ClustalW software at Baylor College of Medicine (http://searchlauncher.bcm.tmc.edu/multi-align/multi-align.html) and formatted by BOXSHADE 3.21 (http://www.ch.embnet.org/software/BOX_form.html). For the *Pina* and *Pinb* genes cloned from *T. timopheevii*, *Triticum zhukovskyi*, amphiploids, and their parents, eight clones from a ligation were sequenced, and the sequences were assembled with CAP3 program (Huang and Medan, 1999; http://pbil.univ-lyon1.fr/cap3.php), with all the parameters set to default. Sequence contigs were used as queries for BLASTn searches against the NCBI non-

redundant database. A fosmid and a BAC were shotgun sequenced at 8 times equivalents and assembled with the program Consed (Gordon et al., 1998). Protein-coding genes were predicted using the program FGENESH (http://sun1.softberry.com/berry.phtml?topic=fgenesh&group=programs&subgroup=gfind) with the organism set as monocot. Repeated sequences were identified by searching the Triticeae Repeat Sequence database (Wicker et al., 2002) by BLASTn and BLASTp (http://wheat.pw.usda.gov/ITMI/Repeats/blastrepeats3.html).

Gene Nomenclature

Gene designations followed the rules of nomenclature as listed in the Wheat Gene Symbol Catalogue (McIntosh et al., 1998). The *Ha* locus is triplicated in common wheat and orthologous loci of A, B, and D genome origin are designated as *Ha-A*, *Ha-B*, and *Ha-D*, respectively. For hypothetical and function-unknown genes at the *Ha* locus, the names designated by Chantret et al. (2005) are adopted to avoid any confusion.

Sequence data for this article can be found in the GenBank/EMBL data libraries under accession numbers EU267678, EU267679, and EU268462 to EU268495.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Alignment of *Pina* sequences from the A genomes of wheat.

Supplemental Figure S2. Alignment of *Pinb* sequences from the A genomes of wheat.

Supplemental Figure S3. Alignment of PINA sequences from the A genomes of wheat.

Supplemental Figure S4. Alignment of PINB sequences from the A genomes of wheat.

Supplemental Figure S5. Alignment of *Pina* sequences from the A and A^m genomes of wheat.

Supplemental Figure S6. Alignment of *Pinb* sequences from the A and A^m genomes of wheat.

Supplemental Figure S7. Alignment of *Pina-*A^m between the amphiploid and its parent.

Supplemental Figure S8. Alignment of *Pina-*S^{sh} between the amphiploid and its parent.

Supplemental Figure S9. Alignment of *Pinb-*A^m between the amphiploid and its parent.

Supplemental Figure S10. Alignment of *Pinb-*S^{sh} between the amphiploid and its parent.

Supplemental Figure S11. Alignment of *Pina-A* between the amphiploid and its parent.

Supplemental Figure S12. Alignment of *Pina-S* between the amphiploid and its parent.

Supplemental Figure S13. Alignment of *Pinb-A* between the amphiploid and its parent.

Supplemental Figure S14. Alignment of *Pinb-S* between the amphiploid and its parent.

Supplemental Table S1. Plant materials and their scores on *Pina* and *Pinb*.

Supplemental Table S2. PCR primers for *Gsp*, *Pina*, *Pinb*, and *Gene5*.

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