Molecular analysis of phosphomannomutase (PMM) genes reveals a unique PMM duplication event in diverse Triticeae species and the main PMM isozymes in bread wheat tissues

Chunmei Yu1,2,3, Yiwen Li1, Bin Li1,3, Xin Liu1, Lifang Hao1,3, Jing Chen1,3, Weiqiang Qian1,3, Shiming Li1,3, Guanfeng Wang1,3, Shiwei Bai1,3, Hua Ye1,3, Huanju Qin1, Qianhua Shen1, Liangbiao Chen1, Aimin Zhang1, Daowen Wang1,*

Abstract
Background: Phosphomannomutase (PMM) is an essential enzyme in eukaryotes. However, little is known about PMM gene and function in crop plants. Here, we report molecular evolutionary and biochemical analysis of PMM genes in bread wheat and related Triticeae species.

Results: Two sets of homoeologous PMM genes (TaPMM-1 and 2) were found in bread wheat, and two corresponding PMM genes were identified in the diploid progenitors of bread wheat and many other diploid Triticeae species. The duplication event yielding PMM-1 and 2 occurred before the radiation of diploid Triticeae genomes. The PMM gene family in wheat and relatives may evolve largely under purifying selection. Among the six TaPMM genes, the transcript levels of PMM-1 members were comparatively high and their recombinant proteins were all enzymatically active. However, PMM-2 homoeologs exhibited lower transcript levels, two of which were also inactive. TaPMM-A1, B1 and D1 were probably the main active isozymes in bread wheat tissues. The three isozymes differed from their counterparts in barley and Brachypodium distachyon in being more tolerant to elevated test temperatures.

Conclusion: Our work identified the genes encoding PMM isozymes in bread wheat and relatives, uncovered a unique PMM duplication event in diverse Triticeae species, and revealed the main active PMM isozymes in bread wheat tissues. The knowledge obtained here improves the understanding of PMM evolution in eukaryotic organisms, and may facilitate further investigations of PMM function in the temperature adaptability of bread wheat.

Background
Phosphomannomutase (PMM, EC 5.4.2.8), catalyzing the interconversion between mannose-6-phosphate and mannose-1-phosphate, is an essential and conserved enzyme in eukaryotic organisms [1,2]. Mannose-1-phosphate is necessary for synthesizing the vital cellular metabolite GDP-mannose, which plays a crucial role in the formation of polysaccharide chains required for the glycosylation of protein and lipid molecules [3,4]. In higher plants, GDP-mannose also acts as an important precursor for the biosynthesis of the key antioxidant ascorbic acid (AsA) [5,6], and the mannose containing polysaccharides are essential for the development of functional cell walls [2,4]. From available information, the copy number of functional PMM in diploid eukaryotic species varies from one (i.e., in Saccharomyces cerevisiae and Arabidopsis thaliana) to two (e.g., in human). PMM proteins from different eukaryotic species are highly similar in primary structure, with more than 50% amino acid sequence identities found among the PMMs.
from *S. cerevisiae*, *Arabidopsis* and human [6]. The crystalline structure and catalytic mechanism of mammalian PMMs are well understood [7], and there is also a wealth of information on human PMM2, whose point mutations lead to a genetically inherited disease CDG-Ia [8-11].

Two recent molecular genetic studies in *Arabidopsis* show that defect in PMM function reduces AsA biosynthesis and protein glycosylation, causing enhanced susceptibility to oxidation stress and plant death at high growth temperature (28°C) [6,12]. The requirement of a functional PMM protein for *Arabidopsis* to grow at 28°C is consistent with the pioneering finding of temperature sensitive PMM mutations that arrest *S. cerevisiae* growth at 37°C [1], suggesting that PMM may be generally important for the temperature adaptability of eukaryotes.

Compared to the above progress, little is known about PMM gene and function in crop plants, although broad temperature adaptability is vitally important for crops to achieve high yield potential under diverse environmental conditions. To improve our understanding on PMM in crop plants, we have embarked on a systematic molecular and biochemical analysis of PMM genes in bread wheat (*T. aestivum*, 2n = 6x = 42, AABBDD), a polyploid crop species cultivated in both temperate and tropical regions and with harvest possible under a wide range of temperature conditions [13,14]. Bread wheat belongs to the tribe Triticeae, which is composed of approximately 350 species, and contains both diploid (such as barley and rye) and polyploid (e.g., bread wheat) crop plants [15]. The diploid genomes in *Triticeae* species are homoeologous, and may be combined to form polyploids through natural and artificial hybridizations [16]. Bread wheat was evolved through natural hybridization between tetraploid wheat (*T. turgidum*, 2n = 4x = 28, AABB) and *Aegilops tauschii* (2n = 2x = 14, DD) about 8000 years ago [16-18]. *T. turgidum* was formed about 0.5 million years ago (MYA) through spontaneous hybridization between an unidentified *Triticeae* species containing the B genome and the wild einkorn wheat *T. urartu* (2n = 2x = 14, AA) [19-22]. The A, B and D genomes diverged from each other around 2.5 to 4.5 MYA, whereas the divergence of wheat and barley occurred around 11 MYA [19-22]. Although bread wheat and related *Triticeae* species generally contain large and complex genomes, the advent of structural and functional genomics research in model cereals (*rice, Brachypodium distachyon*) has made it possible to conduct relatively detailed molecular genetic and biochemical studies of important genes both within *Triticeae* and among rice, *Brachypodium* and *Triticeae* species. The genomic resources of rice and *B. distachyon* (e.g., molecular markers, annotated genomic information) have both been proved to be very useful in the investigations of important *Triticeae* genes, such as *Ph1* and *Lr34/Yr18* in common wheat and *Ppd-H1* and *Cly1* in barley [23-26].

From the information presented above, the main objectives of this work were to 1) determine PMM genes and loci in bread wheat and related *Triticeae* species, and 2) investigate the evolution of *Triticeae PMM* genes and the main active PMM isozymes in bread wheat tissues, with the aid of the genomic knowledge of rice and *B. distachyon*.

### Results

#### Isolation and characterization of PMM genes

Two approaches (bioinformatic analysis, molecular cloning) were used to identify and isolate the PMM genes in *B. distachyon*, bread wheat and related *Triticeae* species (barley, *T. urartu*, *T. turgidum*, *Ae. tauschii*). In total, 17 distinct PMM coding sequences were isolated (Table 1).

| Species | Gene | gDNA (bp) | cDNA (bp) | ORF | Protein (aa) |
|---------|------|-----------|-----------|-----|-------------|
| *Brachypodium distachyon* (Bd21) | BdPMM | 2696 | 750 | + | 249 |
| Barley (*Hordeum vulgare*) | HvPMM-1 | 2185 | 750 | + | 249 |
| (Betzes) | HvPMM-2 | 3263 | 771 | - | - |
| Triticum urartu | TuPMM-A1 | 2228 | 750 | + | 249 |
| (DV877 and IE29-1) | TuPMM-A2 | 2542 | 751 | - | - |
| *Aegilops tauschii* | AetPMM-D1 | 2409 | 759 | + | 252 |
| (AS67 and AS91) | AetPMM-D2 | 2348 | 756 | + | 251 |
| *Triticum aestivum* ssp. durum | TPaMM-A1 | 2227 | 750 | + | 249 |
| (Langdon) | TPaMM-A2 | 2781 | 752 | - | - |
| *Triticum turgidum* ssp. aetivum | TPaMM-B1 | 2256 | 750 | + | 249 |
| Bread wheat (*Triticum aestivum*) | TPaMM-B2 | 2500 | 747 | + | 248 |
| (Xiaoyan 54, Chinese Spring) | TPaMM-A2 | 2225 | 750 | + | 249 |
| Rice (*Oryza sativa* ssp. japonica) | OsPMM | 2466 | 747 | + | 248 |

* The genomic DNA (gDNA) and cDNA sequences include start (ATG) and stop (TGA) codons.
* Plant materials from which PMM sequences were isolated.
* ORF disrupted by premature stop codon.
* ORF interrupted by frameshift mutation.
* The chromosomal locus containing OsPMM genomic sequence is Os04g0682300.
* The GenBank accession number for OsPMM cDNA is DQ442996.
A single PMM gene was found for both rice (OsPMM) and B. distachyon (BdPMM). The cDNA of OsPMM was reported previously [6], whereas that of BdPMM was isolated in this work using RT-PCR (Table 1).

Six different PMM genes were isolated from each of the two bread wheat varieties, Xiaoyan 54 and Chinese Spring (CS). *T. urartu, Ae. tauschii* and barley were each found to contain two distinct PMM genes, while *T. turgidum* genes were each found to contribute two members to the six PMM genes in bread wheat, which were thus named as PMM-A1, PMM-B1, PMM-B2, PMM-D1, PMM-D2, respectively (Table 1). The PMM-1 genes in bread wheat and its diploid and tetraploid progenitor species and barley were homoeologous. Likewise, homoeologous relationship existed among the PMM-2 genes from these species.

The intron and exon patterns of the PMM genes from rice, *B. distachyon*, and wheat and relatives were highly similar. Except for OsPMM and BdPMM, both of which had 11 exons owing to the split of the first exon into two parts, the remaining genes all had 10 exons and 9 introns (Figure 1). Apart from PMM-A2 homoeologs and HvPMM-2, the remaining Triticeae PMM genes cloned in this work all contained intact ORF. The coding region of HvPMM-2 was disrupted by premature stop codon based on analyzing the PCR products amplified from two barley varieties (Betzes, Zaoshou 3) (Table 1, Additional file 1).

Consistent with our earlier study [6], the proteins deduced from the PMM genes of common wheat and progenitor species, barley and *B. distachyon* were more than 50% identical to the PMMs of human and *S. cerevisiae* (Additional file 2). The identities among the grass PMMs were generally above 90%, with BdPMM showing higher identity to its homologs from bread wheat and related Triticeae species than to OsPMM. The identities among the deduced PMM-1 proteins of bread wheat and its diploid and tetraploid ancestors were generally 98% or higher, with complete identity found among the deduced TaPMM-A1, TuPMM-A1, TtPMM-A1 and TaPMM-B1 proteins. The identities among the deduced PMM-B2 and D2 proteins of bread wheat and its diploid and tetraploid ancestors were generally 97%. By contrast, the identities between the deduced PMM-1 and 2 proteins were comparatively lower, being 95% between B1 and B2 proteins, and 96% between D1 and D2 proteins. The deduced PMM-B1 (or B2) proteins of tetraploid and bread wheats were 99% identical. Interestingly, TaPMM-B2 and TtPMM-B2 displayed unusual amino acid substitutions. The first one, involving the substitution of a broadly conserved glycine residue by alanine, was shared between TaPMM-B2 and TtPMM-B2 (Additional file 3). The second one was unique to TaPMM-B2, involving the replacement of a highly conserved arginine residue by cysteine. The four structural and active site motifs (motifs I to IV), identified by analyzing the crystal structure of HsPMM-1 [7], were generally conserved among the compared plant PMMs (Additional file 3).

### Determination of PMM copy number and chromosomal locations

To investigate the copy number of PMM genes, we made use of intron length polymorphisms and designed a pair of intron flanking (IF) primers (Additional file 4), which enabled the amplification of PCR fragments containing introns III to V (Figure 1). By carefully analyzing the products amplified with fluorescently labeled IF primers, six distinct fragments were found for bread wheat (varieties Xiaoyan 54 and CS), and their size equaled to that calculated from the six cloned TaPMM genomic sequences in all cases (Additional file 5). Using the same strategy, two PMM fragments were found for *T. urartu* (accessions DV877 and IE29-1), *Ae. tauschii* (accessions AS67 and AS91) and barley (Betzes), respectively, and for all three species, the size of the amplified fragments corresponded exactly to the one deduced from the cloned PMM members (Additional file 5). Four fragments were amplified from *T. turgidum* (Langdon, LDN), whose size again showed specific correspondence to that derived from each of the four cloned TtPMM members (Additional file 5). Finally, a single fragment was amplified from *B. distachyon* (Bd21) and its size agreed with the one calculated from the cloned BdPMM genomic sequence (Additional file 5).
The copy number of PMM genes in an additional set of materials (containing 12 diploid and 9 tetraploid Triticeae species) was also investigated by fragment analysis using the IF primers (see above). The results showed that the diploid Triticeae species carrying the A\textsuperscript{m}A\textsuperscript{n}, CC, MM, NN, RR, SS, S\textsuperscript{S}\textsuperscript{S}, S\textsuperscript{S}\textsuperscript{S}, S\textsuperscript{S}\textsuperscript{S}\textsuperscript{S}, S\textsuperscript{S}\textsuperscript{S}\textsuperscript{S}\textsuperscript{S} or UU genomes generally had two different PMM fragments, whereas the tetraploid Triticeae species containing the AAGG, DDCC, DDMM, DDNN or UMM genomes generally possessed four PMM specific fragments (Additional file 6).

The fluorescently labeled IF primers were further employed for investigating the chromosomal locations of wheat and barley PMM members by PCR amplifications. The fragments derived from PMM-A\textsubscript{1}, B\textsubscript{1} and D\textsubscript{1} of bread wheat were not amplified from the NT lines lacking 2A, 2B and 2D chromosomes, respectively (Additional file 7), indicating that TaPMM-1 homoeologs were located on group 2 chromosomes. This finding was confirmed by the fragment analysis data obtained with LDN substitution lines (Additional file 7). The fragments derived from PMM-A\textsubscript{2} and D\textsubscript{2} members of bread wheat were not amplified from the NT lines lacking 4A and 4D chromosomes, respectively (Additional file 7). Moreover, the fragment from TtPMM-B\textsubscript{2} was absent in the LDN substitution line lacking the 4B chromosome (Additional file 7). Together, these results indicated that TaPMM-2 homoeologs were located on group 4 chromosomes. Using a similar strategy, the two barley PMM genes were mapped to the 2H and 4H chromosomes, respectively (Additional file 8), which are homoeologous to wheat groups 2 and 4 chromosomes, respectively [27,28].

The finding of six PMM genes located on groups 2 and 4 chromosomes in bread wheat by this work is consistent with the data generated in the wheat expressed sequence tag (EST) mapping project http://wheat.pw.usda.gov/NSF/progress_mapping.html. The EST BE489906, a 421 bp cDNA exhibiting higher than 95% nucleotide sequence identity to the 5' half of the mRNAs of the six bread wheat PMM genes, has been used as a probe to hybridize with the genomic DNA samples of CS and associated NT lines digested with the restriction enzyme EcoRI. The hybridization yields 11 positive bands, which are mapped to groups 2 or 4 chromosomes. Our bioinformatic analysis suggests the presence of three to five EcoRI sites in the genomic ORF of bread wheat PMM genes, with the last EcoRI site located in the 8th exon (Additional file 9). Because the first seven exons give rise to the 5' half of PMM mRNAs upon post transcriptional processing, the DNA blot hybridization with BE489906 probe is expected to show about 11 to 12 hybridizing bands from six TaPMM gene members. The hybridization with another PMM EST probe (BE591860) yields approximately seven positive bands, which are mapped mainly to groups 2 or 4 chromosomes. Consequently, it is suggested that the number of homoeologous PMM genes in bread wheat is likely to be around six. However, we can not exclude the existence of additional and divergent PMM copies or fragments in bread wheat genome, because they may not be amplified by our PCR strategy (owing to mutations in the primer binding sites), or be distinguishable from one or more of the six completely sequenced copies during fragment analysis (because of the lack of difference in intron length).

**Duplication of PMM in diploid Triticeae species**

The foregoing experiments indicated that there existed two PMM genes (PMM-1, PMM-2) in diploid Triticeae species, and a single PMM gene in either B. distachyon or rice. These findings raised an important question on how and when the two PMM gene members in diploid Triticeae species were evolved. To investigate this question, phylogenetic analysis was conducted using the genomic DNA sequences of PMM genes. Two distinct clades, one composed of Triticeae PMM-2 homoeologs and the other of the PMM-1 homoeologs from common wheat and progenitors, were consistently found in the phylogenetic trees constructed using different tree building methods (Figure 2). HvPMM-1, BdPMM and OsPMM fell outside of the two clades, with HvPMM-1 showing a closer association with both clades than BdPMM and OsPMM (Figure 2). These observations, plus the established synteny between barley chromosome 2 (harboring HvPMM-1) and rice chromosome 4 (on which OsPMM resides) [27-29], indicated that the Triticeae PMM-1 genes were most likely the orthologs of BdPMM and OsPMM, and that the Triticeae PMM-2 member may be derived from PMM-1 through duplication. Trees with topology highly similar to that displayed in Figure 2 were also obtained when the deduced amino acid sequences of the PMM genes were used for phylogenetic analysis, but they could not reveal the phylogenetic relationships of PMM-A2 homoeologs to other PMM genes because TaPMM-A2, TtPMM-A2 and TuPMM-A2 could not yield intact proteins upon conceptual translation (Table 1).

To find the approximate timing at which the duplication of Triticeae PMM occurred, we first calculated the number of substitutions per synonymous site for the grass PMM genes characterized in this work. The values of synonymous substitutions calculated with PMM-1 genes were generally lower compared to those obtained with PMM-2 members (Table 2). This prompted us to calculate the molecular clocks for grass PMM-1 and 2 genes, respectively. Based on the averaged synonymous substitutions between OsPMM
and wheat PMM-1 or 2 genes (Table 2), and assuming that rice and wheat diverged about 60 MYA [22], the average nucleotide substitution rate for PMM-1 genes was estimated to be $3.7 \times 10^{-9}$ per site per year, whereas that for PMM-2 genes was $4.2 \times 10^{-9}$ per site per year. BdPMM and wheat PMM-1 genes diverged about $40.2 \pm 6.6$ MYA (Table 3). The divergence time between wheat PMM-1 and 2 genes was estimated to be $18.9 \pm 4.0$ or $16.6 \pm 3.6$ MYA, depending on whether the clock rates of PMM-1 or 2 genes were used for the calculation (Table 3).

**Analysis of evolutionary rate and potential positive selection**

The cDNA sequence of 11 PMM genes (including seven PMM-1 and four PMM-2 members from bread wheat and its progenitor species) were aligned (Additional file 10), and the resultant alignment was used to construct a NJ tree (Additional file 10). The non-synonymous/synonymous substitution rate ratios ($\omega = d_N/d_S$) were calculated for analyzing evolution rate and potential positive selection on amino acid sites by appropriate models (Table 4). The analysis with one-ratio model (M0) produced an averaged \(\omega\) value of 0.15, indicating that purifying selection dominated the evolution of the examined PMM gene members (Table 4). The analysis with two-ratio model revealed that the mean \(\omega\) value of PMM-2 genes was larger than that of PMM-1 members, though this difference was not statistically significant by the likelihood-ratio test (LRT) comparing one-ratio with two-ratio models (Table 4). The analysis with six site specific models, M1a, M2a, M3 (\(k = 2\)), M3 (\(k = 3\)), M7 and M8, suggested that more than 90% of codon sites were under strong purifying selection (with \(p\) ranging from 0.91 to 0.99, Table 4). Two candidate sites (10 and 96) were found to be under positive selection based on naive empirical Bayes (NEB) posterior probability, one of which (site 10) was significant (Table 4, Additional file 10). However, the positive selection on site 10 was not significant according to Bayes empirical Bayes (BEB) posterior probability, and neither was it supported by LRT (Table 4).

**Transcriptional patterns of six TaPMM genes**

Semiquantitative RT-PCR experiments, using member specific primers, were conducted to compare the transcriptional patterns of six PMM genes in Xiaoyan 54. In general, the transcripts of the six PMM members were detectable in both vegetative (root, culm, leaf) and reproductive (immature spikes) organs, with relatively higher transcript levels detected in the seedling and flag leaves (Figure 3). The transcript levels of TaPMM-1 members (A1, B1, D1) were generally and relatively higher than those of TaPMM-2 members (A2, B2, D2) (Figure 3). The transcriptional profiles of the three TaPMM-1 genes were basically similar, except that the transcript level of D1 in the immature spikes was much lower relative to those of A1 or B1 (Figure 3). Among the three TaPMM-2 members, B2 and D2 showed a highly similar transcriptional profile (Figure 3). The transcript levels of A2 in the roots, culms and immature spikes were substantially lower than those of B2 and D2 in the same set of organs (Figure 3).

**Biochemical activity of TaPMM proteins**

First, yeast complementation assays were carried out to test if the proteins expressed from TaPMM-A1, B1, D1, B2 or D2 may complement the temperature sensitivity of the sec53-6 mutant of S. cerevisiae. TaPMM-A2 was not included because it did not possess an intact ORF (Table 1). From Figure 4, it is clear that the expression of TaPMM-A1, B1, D1 or D2 restored the growth of sec53-6 cells at 30 or 37°C, and to a
similar degree. By contrast, the expression of TaPMM-B2 complemented PMM deficiency of sec53-6 only weakly at 30°C, with no complementation observed at 37°C (Figure 4).

Subsequently, TaPMM-A1, B1, B2, D1 and D2 were individually expressed in the bacterial cells, and the resultant recombinant proteins were purified using nickel affinity chromatography (Additional file 11). The recombinant proteins of A1, B2, D1 and D2 were examined for catalytic activity using mannose-1-phosphate as the substrate. The B1 recombinant protein was not included in this analysis because its deduced amino acid sequence was identical to that of A1 (Additional file 2). In three independent experiments, B2 did not show detectable catalytic activity. By contrast, the recombinant A1, D1 and D2 proteins were all catalytically active. The average $K_m$ (mM, mannose-1-phosphate) values calculated for recombinant A1, D1 and D2 proteins were $0.46 \pm 0.02$, $0.40 \pm 0.02$ and $0.48 \pm 0.02$, respectively.

Temperature-activity profiles of the recombinant PMMs of rice, B. distachyon, barley and bread wheat OsPMM, BdPMM and HvPMM-1 were expressed in the bacterial cells, and the resultant recombinant proteins were purified as described above. The main temperature-activity characteristics of the three PMMs and TaPMM-A1, D1 and D2 were compared using 1 mM mannose-1-phosphate as the substrate and at three test temperatures (24, 30 and 37°C). From Figure 5, it is evident that the activity level of OsPMM was significantly higher at 30 and 37°C than at 24°C. Moreover, increasing the test temperature from 30 and 37°C did not decrease OsPMM activity significantly (Figure 5). BdPMM was most active at 30°C, but its activity level was reduced by nearly 95% as the temperature increased from 30 to 37°C (Figure 5). HvPMM-1 was most active at 24°C, and its activity level also decreased sharply (by more than 90%) as the temperature shifted from 30 to 37°C (Figure 5). The three bread wheat PMMs exhibited different temperature-activity characteristics.

### Table 2 Relative synonymous substitutions of PMM genes from rice, B. distachyon, bread wheat, and barley

| PMM genes compared | Number of substitutions per synonymous site (dS) | Averaged dS          |
|--------------------|-----------------------------------------------|----------------------|
| OsPMM/BdPMM        | 0.4231 ± 0.0646                                |                      |
| OsPMM/HvPMM-1      | 0.4807 ± 0.0723                                |                      |
| OsPMM/TaPMM-A1     | 0.4324 ± 0.0656                                | 0.4385 ± 0.0669 (OsPMM/TaPMM1 genes) |
| OsPMM/TaPMM-B1     | 0.4573 ± 0.0701                                |                      |
| OsPMM/TaPMM-D1     | 0.4259 ± 0.0651                                |                      |
| OsPMM/TaPMM-B2     | 0.5150 ± 0.0791                                | 0.4997 ± 0.0763 (OsPMM/TaPMM2 genes) |
| OsPMM/TaPMM-D2     | 0.4844 ± 0.0735                                |                      |
| BdPMM/HvPMM-1      | 0.3449 ± 0.0542                                |                      |
| BdPMM/TaPMM-A1     | 0.2884 ± 0.0471                                | 0.2974 ± 0.0486 (BdPMM/TaPMM1 genes) |
| BdPMM/TaPMM-B1     | 0.3121 ± 0.0508                                |                      |
| BdPMM/TaPMM-D1     | 0.2918 ± 0.0479                                |                      |
| BdPMM/TaPMM-B2     | 0.3647 ± 0.0583                                | 0.3503 ± 0.0562 (BdPMM/TaPMM2 genes) |
| BdPMM/TaPMM-D2     | 0.3358 ± 0.0541                                |                      |
| HvPMM-1/TaPMM-A1   | 0.0921 ± 0.0229                                | 0.0971 ± 0.0237 (HvPMM-1/TaPMM1 genes) |
| HvPMM-1/TaPMM-B1   | 0.1126 ± 0.0261                                |                      |
| HvPMM-1/TaPMM-D1   | 0.0867 ± 0.0222                                |                      |
| HvPMM-1/TaPMM-B2   | 0.1857 ± 0.0355                                | 0.1826 ± 0.0351 (HvPMM-1/TaPMM2 genes) |
| HvPMM-1/TaPMM-D2   | 0.1795 ± 0.0346                                |                      |
| TaPMM-B1/TaPMM-B2  | 0.1567 ± 0.0323                                | 0.1398 ± 0.0299 (TaPMM1/TaPMM2 genes) |

* The dS values and their standard deviations were calculated using PMM cDNA coding sequences. TaPMM-A2 was not included in the calculation owing to the occurrence of mutation in its coding region.

### Table 3 Divergence time estimates for PMM genes

|                     | Divergence time (MYA) | Averaged dS value used for the estimation          |
|---------------------|-----------------------|---------------------------------------------------|
| BdPMM vs TaPMM-1    | 40.2 ± 6.6 *          | 0.2974 ± 0.0486 (BdPMM/TaPMM-1 genes)             |
| TaPMM-1 vs TaPMM-2  | 18.9 ± 4.0 *          | 0.1398 ± 0.0299 (TaPMM-1/TaPMM-2 genes)           |
|                     | 166 ± 3.6 *           |                                                   |

* Specific molecular clock used for the estimation: $3.7 \times 10^{-9}$ per site per year.

* Specific molecular clock: $4.2 \times 10^{-9}$ per site per year.
Table 4 Maximum-likelihood analysis of PMM-1 and 2 genes and detection of codon sites under positive selection

| Model          | P   | Likelihood | Parameter estimate | NEB b | BEB b | 2δl    | df | P value |
|----------------|-----|------------|--------------------|-------|-------|--------|----|---------|
| M0 (one ratio) | -1433.52 | ω = 0.155 | NA                 | NA    | NA    | 0.4223 | 1  | 0.5158  |
| Two ratio      | -1433.09 | ωPMM-1 = 0.114; ωPMM-2 = 0.186 | NA | NA | | |
| M1a: nearly neutral | -1430.84 | ρ0 = 0.0917; ρ1 = 0.083; ω0 = 0.076; ω1 = 1.000 | NA | NA | | |
| M2a: positive selection | -1429.69 | ρ0 = 0.989; ρ1 = 0.000; ρ2 = 0.011; ω0 = 0.119; ω1 = 1.000; ω2 = 4.902 | 10N (0.999*); 96 H (0.793) | 10N (0.867); 96 H (0.680) | 2.2947 (M2a versus M1a) | 2 | 0.3175 |
| M3: discrete (k = 2) | -1429.69 | ρ0 = 0.989; ρ1 = 0.011; ω0 = 0.119; ω1 = 4.901 | 10N (0.999*); 96 H (0.793) | None | |
| M3: discrete (k = 3) | -1429.69 | ρ0 = 0.933; ρ1 = 0.056; ρ2 = 0.011; ω0 = 0.119; ω1 = 0.120; ω2 = 4.902 | 10N (0.999*); 96 H (0.793) | None | 7.6562 (M3 versus M0) | 4 | 0.1050 |
| M7: beta       | -1431.48 | p = 0.0489; q = 0.241 | NA | NA | | |
| M8: beta and ω | -1429.70 | ρ0 = 0.989; p = 13.583; q = 99.000; p1 = 0.011; ω = 4.909 | 10N (0.999*); 96 H (0.786) | 10N (0.944); 96 H (0.778) | 3.5539 (M7 versus M8) | 2 | 0.1692 |

a ω values are ω0/ω1, ratios. p0, ρ1 and ρ2 indicate the proportion of sites in categories 1, 2 or 3.
b The codon sites (10N, 96H) were numbered based on TaPMM-D1 cDNA (Additional file 10). Asterisk denotes statistical significance level at or above 0.95. NEB, naive empirical bayes; BEB, Bayes empirical bayes; NA, not applicable.

TaPMM-A1 was less active than BdPMM and HvPMM-1 at 24 and 30°C, but its activity level was substantially higher than those of BdPMM and HvPMM-1 at 37°C, owing to a 51% increase of its activity as the temperature changed from 30 to 37°C (Figure 5). TaPMM-D2, although less active than BdPMM and HvPMM-1 at 24 and 30°C, displayed substantially higher activity levels than BdPMM and HvPMM-1 at 37°C, because the change of test temperature from 30 to 37°C did not reduce TaPMM-D2 activity as strongly as that occurred to BdPMM and HvPMM-1 (Figure 5). The activity level of TaPMM-D1 was lower than those of BdPMM and HvPMM-1 but higher than those of TaPMM-A1 and D2 at 24°C (Figure 5). However, TaPMM-D1 was highly active at 30°C, with an activity level statistically comparable to that of OsPMM (Figure 5). The shift of assay temperature from 30 to 37°C reduced the activity of TaPMM-D1 by about 40%, but the scale of this reduction was much lower compared to that observed for BdPMM, HvPMM-1 or TaPMM-D2.

Discussion

PMM genes and chromosomal loci in Triticeae species

Through the molecular cloning experiments in this work, the following suggestions may be made regarding PMM genes and chromosome loci in Triticeae species. First, there exist two distinct PMM gene members (PMM-1 and 2) in the progenitor diploid genomes (i.e., A, B and D) of bread wheat. There are four and six PMM genes (including both active and inactive members) in tetraploid and hexaploid wheats, respectively. Second, in bread wheat and its diploid and tetraploid progenitors, the loci containing PMM-1 or 2 genes are located on homoeologous groups 2 and 4 chromosomes, respectively. Third, two PMM genes (HvPMM-1 and 2) exist in diploid barley. The loci harboring HvPMM-1 or 2 are located on the 2H and 4H chromosomes, respectively. Finally, judging from the fragment analysis data of PMM genes described in Additional file 6, it is probable that the basic organization of PMM gene family, as defined for the A, B, D and H genomes (e.g., two distinct members contained in two separate chromosomal loci), is conserved in the diploid Triticeae species possessing ABB, CC, MM, NN, RR, SS, SSS, SSS, SSS, SSS or UU genomes. However, further work is needed to verify if in the 12 Triticeae genomes the two PMM genes may also be located on homoeologous group 2 and 4 chromosomes.

The existence of two PMM genes in diploid Triticeae species contrasts with the finding of a single PMM gene for Arabidopsis, rice and B. distachyon [6,12, this work]. A search of recently accumulated genomic data deposited in the phytozone website (http://www.phytozone.net) reveals that Medicago truncatula, Vitis vinifera, Sorghum bicolor and Zea mays each possess a single PMM gene. However, both Glycine max and Populus trichocarpa harbor two PMM copies in their genomes (http://www.phytozone.net). Two functional PMM copies have also been found present in several mammals (e.g., human, mouse and rat) [30-32].
Biochemically active PMM members in bread wheat and its progenitors

From the presence of intact ORF, the results of yeast complementation experiments, and the data of biochemical assays using recombinant proteins, we deduce that bread wheat expresses four biochemically active PMMs, TaPMM-A1, B1, D1 and D2. The primary structure and basic biochemical function of the four PMMs are similar to their orthologs in humans, *S. cerevisiae* and *Arabidopsis*. In contrast to TaPMM-A1, B1, D1 and D2, TaPMM-B2 is probably not active in vivo because it did not complement PMM deficiency of yeast sec53-6 cells efficiently, and was not catalytically competent in the in vitro biochemical assay. The lack of detectable biochemical activity by TaPMM-B2 correlates with the presence of unusual amino acid substitutions in its deduced protein (Additional file 3). Further work is needed to determine the specific amino acid change(s) responsible for decreasing the biochemical activity of TaPMM-B2.

Owing to the possession of identical amino acid sequences (Additional file 2), it is likely that TuPMM-A1 (in *T. urartu*) behaves like TaPMM-A1, and AetPMM-D1 and D2 of *Ae. tauschii* may act like...
TaPMM-D1 and D2, respectively. Contrary to the above scenario, it is not possible to deduce the biochemical activity of TaPMM-B1 and B2 based on the data gathered for TaPMM-B1 and B2, because complete amino acid sequence identity did not exist between the two B1 or B2 members (Additional file 2).

**Main features of PMM evolution in diploid and polyploid Triticeae species**

Despite its vital importance in eukaryotes, the molecular evolution of PMM has not been investigated in higher plants previously, and has been studied only recently in animals. Several investigations suggest that the presence of two functional PMM copies in human and murine cells may be caused by a gene duplication event that occurred 75 to 110 MYA [30,32-34]. From the data presented in this work, the main features in the evolution of PMM genes in diploid and polyploid Triticeae species may be summarized below. First, a major event in PMM evolution in the diploid Triticeae species carrying AA, A\textsuperscript{m}A\textsuperscript{m}, BB, CC, DD, HH, MM, NN, RR, SS, S\textsuperscript{b}S\textsuperscript{b}, S\textsuperscript{h}S\textsuperscript{h}, S\textsuperscript{s}S\textsuperscript{s} or UU genomes is the duplication of PMM. This duplication event gave rise to two different PMM genes, PMM-1 and 2, located on groups 2 and 4 chromosomes, respectively. Out of the two PMM genes in diploid Triticeae species, PMM-1 is likely more ancestral. This possibility is mainly supported by 1) the phylogenetic tree shown in Figure 2, 2) the existence of a single PMM gene in rice, and 3) the established syntenic relationship between Triticeae group 2 chromosomes (on which PMM-1 homoeologs are located) and rice chromosome 4 (on which OsPMM resides) [27-29]. Second, the PMM duplication, as discussed above, occurred after the divergence between B. distachyon and wheat, but before the radiation of diploid Triticeae genomes. This is supported by not only the presence of two different PMM gene members in many diploid Triticeae genomes examined in this work, but also the estimated divergence time between TaPMM-1 and TaPMM-2 genes (Table 3). The two estimates, whether 16.6 ± 3.6 or 18.9 ± 4.0 MY, are both much larger than the divergence time between wheat and barley (about 11 MY) reported in past studies [19-22]. But they are substantially smaller than the divergence time between B. distachyon and wheat (35 to 37.8 MY) calculated by previous investigations [35,36]. Third, substantial differences exist in the evolutionary patterns of PMM-1 and 2 genes. PMM-1 genes generally had intact ORF, their transcript levels in the vegetative and reproductive organs were comparatively higher (Figure 3), and their protein products were all biochemically active (Figure 5). By contrast, multiple types of defects occurred to PMM-2 members. The PMM-A2 copies in bread wheat and its diploid and tetraploid progenitors and HvPMM-2 in barley possessed mutated ORF (Table 1). TaPMM-B2 encoded a protein with undetectable biochemical activity (Figure 4). The recombinant protein of TaPMM-D2 was significantly less active than that of the product of TaPMM-D1 (Figure 5). Moreover, the three PMM-2 members in bread wheat all exhibited lower transcript levels relative to their PMM-1 paralogs (Figure 3). Fourth, the differences of PMM-1 and 2 genes established at the diploid level (i.e., in the A\textsuperscript{a} or D genomes) are basically retained in polyploid wheats. Thus, PMM-A1 is active in diploid, tetraploid and hexaploid levels whereas the ORF of PMM-A2 is disrupted at all three ploidy levels. The two PMM genes encoded in D genome are all active in both Ae. tauschii and bread wheat. In contrast to the PMM genes in A and D genomes, the origin of the contrasting biochemical features of the two B genome PMM members in bread wheat is unclear at present, because the absence of a diploid species containing the B genome made it difficult to study the evolution of PMM-B1 and B2 members at the diploid level. Finally, from the data described in Additional file 6, the duplicated PMM genes in the G, C, N, M and U genomes are also likely retained in the tetraploid species possessing AAGG, DDCC, DDMM, DDNN or UUMM genomes, although further work is needed to study the PMM genes in these species in more detail.

Because PMM-1 and 2 genes were located on groups 2 and 4 chromosomes, respectively, in wheat and progenitors and barley, the duplication event that led to the formation of two PMM copies in the ancestral Triticeae species must have occurred interchromosomally. Strong evidence for the occurrence of interchromosomal duplications in wheat genome evolution has been obtained in recent years [37-39]. Importantly, it has been suggested that these duplications are largely conserved across different Triticeae species [39,40]. However, it is still not well understood how these events might have been accomplished in Triticeae genomes. Thus, the PMM duplication reported here may serve as a useful model for studying the molecular mechanisms underlying the interchromosomal duplications in Triticeae in the future.

The average nucleotide substitution rates of grass PMM-1 or 2 genes estimated based on the 60 MY divergence time between rice and wheat by this work are both lower than the commonly adopted mutation rate (6.5 × 10\textsuperscript{-9} per site per year) for grass genes [41-44]. This may not be unusual because evolutionary rates frequently differ among different grass genes [45]. For example, the mutation rates of ten randomly selected grass genes have been found to vary from 4.1 to 7.1 × 10\textsuperscript{-9} per site per year [45]. Furthermore, we estimated that the divergence time between BdPMM and wheat
PMM-1 genes was about 40.2 ± 6.6 MY (Table 3) using the average nucleotide substitution rate calculated for grass PMM-1 genes. This estimate is comparable to the previously reported timeline (35 to 37.8 MY) for the divergence of the two species [35,36].

Possible mechanisms involved in the evolution of PMM-1 and 2 genes
Past studies have suggested several fates in the evolution of duplicated genes [46-48]. Neofunctionalization leads to the acquisition of new function by one copy, whereas subfunctionalization renders the progenitor and the duplicated copy acquiring non-overlapping but complementary expression patterns. Non-functionalization results in pseudogenization of one of the duplicates. In barley, the duplicate copy of HvHox2 (encoding a homeodomain-leucine zipper transcription factor) acquired a new function in controlling spikelet fertility [49]. In rice, evidence for subfunctionalization has been obtained for two C-class MADS box genes (OSMADS3 and OSMADSS58) [50]. The evolutionary process of duplicated genes may be divided into two stages [51]. During the early stage, the duplicates generally evolve under purifying selection, with a phase of relaxed selective constraint immediately after the duplication [51,52]. During the later stage, the great majority of the duplicates become non-functional pseudogenes that are no longer expressed, with only a few of them being involved in neofunctionalization or subfunctionalization [51-53].

From the data in Table 4 and the main evolutionary features of PMM-1 and 2 genes discussed above, we suggest that 1) purifying selection, but not positive selection, may have dominated the evolution of PMM gene family in wheat and relatives, and 2) despite the prevalence of purifying selection, the function of PMM-2 copies is not as strongly maintained as that of PMM-1 members. The first point is consistent with the findings that the vast majority of duplicated genes in cellular organisms experience purifying selection [51,52], even though some of them are in the process of becoming pseudogenes [54]. Owing to purifying selection, the PMM-1 genes are generally and relatively stable. By contrast, TaPMM-A2 is close to becoming a non-functional pseudogene, because its ORF was mutated and its transcript level was decreased relative to PMM-1 genes (Figure 3). This scenario may also apply to PMM-A2 copies in the diploid and tetraploid progenitors of bread wheat and HvPMM-2 in barley although further work is needed to check if the transcript levels of these members may be lowered than those of PMM-1 genes. TaPMM-B2 and D2, although possessing intact ORF, also show signs of degenerative evolution, because their transcript levels were lower than corresponding PMM-1 members, and the PMM enzyme activity of their recombinant proteins was either undetectable or reduced (Figure 4 and 5). The degeneration of PMM-2 genes and the comparatively high mean evolutionary rate of these members indicate the occurrence of relaxed purifying selection in the evolution of PMM gene family in wheat and relatives, which may have affected PMM-2 copies more strongly than PMM-1 paralogs. However, further work is needed to verify this hypothesis.

In contrast to the degenerative evolution of the duplicated PMM-2 copies in diploid Triticeae species, the two mammalian PMM members, products of a duplication event occurred before the radiation of mammals (i.e., 75 to 105 MYA), all encode highly active enzymes [29-33]. However, unlike PMM2 whose mutation leads to hypoglycosylation of cellular proteins and severe disease [9,12], the deletion of PMM1 in mouse does not cause obvious abnormalities in either growth or development under normal conditions [33]. Moreover, despite their overlapping expression profiles, PMM1 does not compensate for the functional loss of PMM2 [32,33]. These findings have led to the suggestion that PMM1 may have diverged from PMM2 in physiological function [33]. This possibility has been confirmed with the recent discovery that PMM1 but not PMM2 acts as a glucose-1,6-bisphosphatase in the brain tissues [55]. Clearly, the unique PMM duplication event in Triticeae plants revealed in this work differs from the one in mammals in multiple aspects including the timing of the event, the evolutionary fates of PMM members after the duplication, and possibly the main mechanism involved in the evolution. The evolutionary knowledge generated in this work increases our understanding of PMM evolution in eukaryotes, and may be useful for future studies of PMM evolution in additional plant species such as G. max and P. trichocarpa, both of which have been found to contain two PMM genes (see above).

The main active PMM isozymes in bread wheat tissues
Among the four biochemically active TaPMMs, functional similarity may be limited to only TaPMM-A1 and B1 because their deduced proteins were identical, and the transcriptional patterns of their coding genes were very similar (Figure 3). By contrast, functional differences are likely to exist among A1(B1), D1 and D2 members because of dissimilarities in their deduced amino acid sequences and transcriptional patterns. For further analysis of the physiological function of PMM genes in bread wheat, it is necessary to find out the main active PMM isozymes in vivo. Considering the fundamental importance of PMM in the temperature adaptability of eukaryotes [1,12], the differences in the temperature-activity profiles of the four enzymatically active TaPMMs are of particular interest. From the data shown in Figure 5, it is suggested that
TaPMM-D1 may be highly active in a wider range of temperatures (from 24 to 37°C). The activity of TaPMM-A1 is less than that of TaPMM-D1, but it is unique in exhibiting the highest activity at 37°C (although this activity level was still below that of TaPMM-D1 at 37°C). The activity of TaPMM-D2 is generally lower than that of TaPMM-D1 and A1, especially at 37°C (Figure 5). This finding and the observation that the transcript levels of TaPMM-D2 were considerably lower than those of TaPMM-A1, B1 and D1 in multiple organs (Figure 3) indicate that TaPMM-A1, B1 and D1 are likely to be the main active PMM isozymes in bread wheat tissues. Moreover, judging from Figure 3, at least two main PMM isozymes are expressed in each of the five organs (root, seedling leaf, culm, flag leaf, and developing spike) examined in this work.

As displayed in Figure 5, rice, a tropical cereal and adaptable to both tropical and temperate regions [56], possesses a PMM showing very high activity levels at elevated test temperatures (30 and 37°C). By contrast, B. distachyon, barley and bread wheat, originated from temperate regions [57-59], have the PMMs exhibiting relatively high activity levels at 24 or 30°C (Figure 5). A general decrease in the activities of the PMMs of B. distachyon, barley and bread wheat at 37°C seems to correlate with the temperate origin of these species. However, compared with BdPMM and HvPMM-1, at 37°C, the magnitude of activity decrease was much smaller for TaPMM-D1 and D2, and the activity of TaPMM-A1 even increased relative to its performance at 24 and 30°C. This indicates that bread wheat PMM isozymes may be generally more stable than their counterparts in B. distachyon and barley at elevated test temperatures (such as 37°C), which correlates well with the wide temperature adaptability of the bread wheat crop [13,14].

Conclusion
This work has generated new information on PMM genes and their evolution in bread wheat and related Triticeae species. The main active PMM isozymes (e.g., TaPMM-A1, B1 and D1) in bread wheat tissues are revealed. The three isozymes may be more tolerant to elevated temperatures than their counterparts in barley and B. distachyon. The insights gained in this work have broadened the understanding of PMM evolution in eukaryotic organisms. The resources produced here may aid future investigations of the physiological function of this important gene in the temperature adaptability of bread wheat.

Methods
Plant materials, yeast strains and oligonucleotide primers
The wheat and barley varieties and the T. urartu, T. turgidum ssp. dicocoides, Ae. tauschii and B. distachyon genotypes used for cloning PMM genes are listed in Additional file 1. The NT lines of CS, the D genome substitution lines in the tetraploid wheat variety LDN background, and the barley chromosome addition lines in CS background, employed for mapping the chromosomal locations of PMM genes, are also listed in Additional file 1. The growth of wheat and related Triticeae species was accomplished as described previously [6]. B. distachyon was cultured in the greenhouse under a constant temperature (23°C) and with a 16 h light/8 h dark photoperiod. Two S. cerevisiae strains, sec53-6 (MAT a, sec53-6, leu2-3, -112, ura3-52) and its wild type progenitor RSY249 used for the yeast complementation experiment, were described previously [6]. The oligonucleotide primers used in this work are all listed in Additional file 4. The general molecular methods for handling nucleic acid and protein samples, PCR amplification, DNA cloning, and protein expression in bacterial cells were adopted from Sambrook and Russell [60]. High fidelity Taq DNA polymerases, LA-Taq (TaKaRa, China) and Advantage 2 Polymerase Mix (Clontech, USA), were used in PCR amplifications. The accuracy of the yeast or bacterial expression constructs prepared in this work was all confirmed by DNA sequencing.

Cloning of PMM cDNA and genomic DNA sequences
Total RNA samples were prepared from the desired leaf materials using RNeasy plant mini kit (Qiagen, Germany), and were converted into cDNAs using Moloney Murine leukemia virus (M-MLV) reverse transcriptase (Promega, USA). Public nucleotide and protein data banks were searched using the cDNA and deduced amino acid sequence of rice PMM (OsPMM) [6], leading to the finding of the ESTs derived from wheat and barley PMM genes. By analyzing the obtained ESTs, the oligonucleotide primers permitting the amplifications of full length cDNA and genomic DNA sequences of the PMM genes from bread wheat and related Triticeae species (T. urartu, T. turgidum, Ae. tauschii, barley) were developed (Additional file 4). With these primers and the reverse transcription products described above, the PMM cDNAs from bread wheat and relatives were amplified by RT-PCR, cloned and sequenced. The PMM cDNA of B. distachyon was cloned using a similar strategy, except that the development of the oligonucleotide primers specific for BdPMM was facilitated by the genome sequence of Bd21 (http://www.phytozome.net/bra- chy.php). Genomic DNA samples were extracted from the relevant leaf materials as detailed previously [61], and used for isolating the genomic coding sequences of the PMM genes of bread wheat and related Triticeae species and B. distachyon with the same sets of primers employed for cDNA cloning.

During the above experiments, at least 70 positive PCR clones derived from three separate cloning
experiments were sequenced for each of the plant species examined in this work. This ensured the identification of the cDNA and genomic DNA sequences corresponding to different PMM genes. The final nucleotide sequences for the cDNA and genomic DNA coding regions of a given PMM gene were each constructed from the sequencing information of at least three independent clones. The nucleotide sequences of the PMM genes cloned in this work have all been submitted to GenBank. The accession numbers are listed in Additional file 12.

### Investigation of PMM copy number and chromosomal location

The genomic PCR products amplified with the IF primers (Additional file 4, Figure 1) from Xiaoyan 54 were confirmed to be derived from PMM genes by cloning and sequencing. The amplified fragments differed in size, which was thus useful for distinguishing the different PMM genes and estimating the copy number of PMM in bread wheat, its Triticeae relatives, and *B. distachyon*. The forward IF primer was labeled with D4 fluorescence by a commercial company (TaKara, China), allowing the separation and identification of PCR products via capillary electrophoresis (CE) and fragment analysis in the CEQ/GeXP Genetics Analysis System (GAS) (Beckman, USA). The preparation of PCR products for CE and the subsequent fragment analysis in CEQ/GeXP GAS were conducted following the supplier’s instructions. Using the same strategy, PMM copy numbers in the *Triticeae* species carrying the diploid A^nA^m, CC, MM, NN, RR, SS, S^nS^m, S^hS^l, or S^gS^h genomes, or the tetraploid AAGG, DDCC, DDMM, DDNN or UUMM genomes (Additional file 6), were also investigated.

For investigating PMM chromosomal location, the PMM gene specific fragments, amplified from CS, the different NT lines of CS, LDN, and the D genome substitution lines in LDN background, were separated using CE and differentiated by fragment analysis. The IF primers and fragment analysis were also used for mapping the chromosomal locations of two barley PMM genes (*HvPMM-1* and 2). In this case, the DNA samples extracted from the barley variety Betzes and its derivative chromosome addition lines in CS background were utilized as templates for genomic PCR amplifications.

### Bioinformatic, phylogenetic and evolutionary analysis of PMM

Bioinformatic investigations of cloned PMM genes were carried out using the softwares installed in public websites, including EBI (http://www.ebi.ac.uk/Tools/), ExPASy (http://www.expasy.ch/tools/) and NCBI (http://www.ncbi.nlm.nih.gov/). Full length genomic DNA sequences of PMM genes were aligned using the Clustalw2 program (EBI website). The resulted alignment was used for constructing phylogenetic trees by MEGA4 [62]. The number of substitutions per synonymous site (dS) was calculated as described previously [63]. The average nucleotide substitution rates for PMM-1 and 2 genes were estimated by the formula $k = dS/2t$ [64], assuming a divergence time of 60 MY between rice and wheat [22]. The obtained rates were then used to estimate the timing of PMM duplication in wheat.

To investigate the potential mechanisms behind the evolution of PMM genes in bread wheat and its progenitor *Triticeae* species, two complementary evolutionary tests (one for difference in the evolutionary rates of *PMM-1* and 2 genes, and the other for the codon sites under positive selection) were carried out by the CODEML program implemented in the PAML package [65]. The NJ tree, constructed using the cDNA sequence of 11 active *PMM-1* and 2 genes, was employed for these tests. The one-ratio and two-ratio models [66,67] were used for investigating evolutionary rate, whereas the site specific models M1a, M2a, M3 ($k = 2$), M3 ($k = 3$), M7 and M8 [68,69] were deployed for testing potential diversifying selection at individual codon sites. Statistical analysis of the evolutionary data was conducted as described previously, with the confidence level set at or above 0.95 [70,71].

### Semiquantitative RT-PCR

The seeds of Xiaoyan 54 were germinated for 48 h at 25°C, followed by 4 weeks vernalization treatment at 4°C. The vernalized seedlings were then grown in the greenhouse as described above. The root and seedling leaf samples were collected after two weeks growth in the greenhouse. The stem, flag leaf, and immature spike samples were collected at the bolting stage (after 5 weeks growth in the greenhouse). The isolation of total RNA samples from the collected materials and cDNA synthesis were conducted as described above. Semiquantitative RT-PCR analysis of the relative transcript levels of 6 *TaPMM* genes in different wheat organs was performed using gene specific primers (Additional file 4), and following the method detailed previously [72]. The amplification of wheat *tubulin* transcripts served as an internal control, and the reproducibility of the transcriptional patterns revealed was verified in three independent experiments.

### Yeast complementation

The complementation assay was conducted as described previously [6]. Briefly, five complementation constructs (p181-A1, B1, D1, B2 and D2) were prepared by cloning the cDNAs of *TaPMM-A1*, *B1*, *D1*, *B2* and *D2* individually into the yeast expression vector p181AINE. The
oligonucleotide primers used for amplifying TaPMM cDNAs during the cloning are listed in Additional file 4. The five constructs, together with the empty vector control (p181-VC), were individually introduced into the sec53-6 strain whose endogenous PMM harbors a temperature sensitive mutation [1]. The recombinant strains were serially diluted and plated onto agar medium, followed by incubation at three temperatures (24, 30 and 37°C). As controls, sec53-6 and its wild type progenitor RSY249 were also included in the growth assays. Successful complementation by TaPMM expression was indicated by restoring the growth of sec53-6 cells at elevated temperatures (30 and 37°C). The complementation assay was repeated three times to verify the reproducibility of the data obtained.

Biochemical analysis of recombinant PMMs

The PMM cDNAs from bread wheat (TaPMM-A1, B1, D1, B2 and D2), barley (HvPMM-1), B. distachyon (BdPMM) and rice (OsPMM) were individually cloned into the bacterial expression vector pET-30a (Novagen, USA), in order to express recombinant PMM proteins with a C-terminal histidine tag. The oligonucleotide primers used for amplifying PMM cDNAs during preparing the bacterial expression constructs are provided in Additional file 4. The induction of PMM cDNA expression in the bacterial cells and the purification of histidine tagged PMMs through metal chelate affinity chromatography were accomplished as described previously [6]. The purified PMMs were characterized using 10% SDS-PAGE, and were confirmed to be the previously characterized TaPMMs. The resultant data were used to calculate average $K_m$ values. The temperature-activity characteristics of OsPMM, BdPMM, HvPMM-1, and TaPMM-A1, D1 and D2 were examined using 1 mmol/L mannose-1-phosphate as the substrate, and following the method detailed in our earlier work [6]. The assay was repeated three times for each of the four recombinant TaPMMs. The resultant data were used to calculate average $K_m$ values. The temperature-activity characteristics of OsPMM, BdPMM, HvPMM-1, and TaPMM-A1, D1 and D2 were examined using 1 mmol/L mannose-1-phosphate as the substrate, and following the method detailed in our earlier work [6]. The OD$_{340}$ readings were collected at 10 min after the start of the enzyme assays. At this time point, the reactions catalyzed by the six recombinant PMMs were in the linear phase. The assay was repeated three times per recombinant PMM per test temperature, with each assay consisting of three technical replicates. The OD$_{340}$ readings obtained were statistically analyzed using the software SPSS 10 for comparing the relative activity levels of the six recombinant PMMs.

Additional material

**Additional file 1: Plant materials.** The materials were used for PMM gene cloning and chromosomal localization experiments.

**Additional file 2: Amino acid sequence identities.** The PMM proteins under comparison were from wheat and related species, yeast (S. cerevisiae, SdPMM), human (HsPMM1 and 2), Arabidopsis (APMM), rice (OsPMM) and barley (BdPMM).

**Additional file 3: Alignment of the deduced amino acid sequences of the PMMs from wheat and related Triticeae species, B. distachyon (BdPMM), rice (OsPMM), and human (HsPMM1 and 2).** The four structural motifs required for PMM catalysis are boxed. The replacement of a broadly conserved arginine residue by cysteine (marked in red) was observed in both TaPMM-B2 and TdPMM-B2. Asterisks indicate identical residues. The symbols ** and *** represent conserved and semi-conserved substitutions, respectively. The Swiss-Prot accession numbers for OsPMM, HsPMM1 and HsPMM2 are Q7XPWS, Q92871 and O15305, respectively.

**Additional file 4: Oligonucleotide primers used in this work.**

**Additional file 5: Investigation of PMM copy numbers in bread wheat and related Triticeae species (T. urartu, A. tauschii, H. vulgare, T. turgidum ssp. durum) and B. distachyon by fragment analysis.** PMM gene specific peaks are indicated by arrows. The minor peaks labeled by asterisks are caused by DNA size standards. The scale on the horizontal axis indicates fragment size (number of nucleotides), which was determined using the DNA size standards co-separated with the PCR products. The specific genotypes used in this analysis are Q7XPWS, Q92871 and O15305, respectively.

**Additional file 6: Estimation of PMM copy numbers in the diploid and tetraploid species possessing additional Triticeae genomes.**

**Additional file 7: Chromosomal assignment of PMM genes in hexaploid and tetraploid wheats.** The fragments specific for individual PMM gene members were amplified by PCR, followed by separation via capillary electrophoresis. The templates for PCR were prepared from Chinese Spring (CS), the null-tetrasomic (NT) lines of CS, the durum wheat variety Langdon (LDN), and the D genome substitution lines in Chinese Spring (CS). The nulli-tetrasomic (NT) lines of CS, the durum wheat variety Langdon (LDN), and the D genome substitution lines in Chinese Spring (CS) are abbreviated as T. aestivum, N2AT2D, N2DT2A, N2BT2D, and N4DT4A. The six PMM specific fragments were all amplified in the NT lines lacking other groups (i.e., 1, 3, 5, 6 and 7) of chromosomes (data not shown). The presence of four PMM specific fragments in LDN. Note that the fragments representing PMM-A1, B1 or B2 genes were not amplified from the substitution lines 2D(2A) lacking 2A chromosome, 2D(2B) lacking 2B chromosome or 4D(4B) lacking 4B chromosome. The fragment representing PMM-D1 was amplified from 2D(2A) and 2D(2B) owing to the presence of 2D chromosome in the two lines. Similarly, the fragment representing PMM-D2 was amplifiable from 4D(4B) due to the presence of 4D chromosome in this line. The PMM-A1, B1, A2 and B2 fragments were all amplifiable in the other substitution lines in which 2A, 2B, 4A and 4B chromosomes are not involved in the substitution by D genome chromosomes (data not shown). The data displayed are typical of five independent sets of experiments.

**Additional file 8: Chromosomal locations of barley PMM genes (HvPMM-1 and 2).** The genomic DNA samples, extracted from the bread wheat variety Chinese Spring (CS), barley variety Betzes, and barley chromosome addition lines in CS background, were used for PCR amplifications with the nucleotide primers capable of recognizing PMM genes from Triticeae species. The peaks in blue represent PCR fragments amplified from specific bread wheat or barley PMM genes. The six PMM genes in CS are abbreviated as A1, B1, D1, A2, B2 and D2, respectively. HvPMM-1 and 2 were found in the two addition lines containing barley.
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Author details

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Authors’ contributions

CY participated in experimental design, cloned PMM genes, determined PMM copy number, and conducted RT-PCR, yeast complementation, protein purification and biochemical experiments. XL, BL, LH, JC, WQ, SL, GW and HQ contributed resources, reagents and greenhouse facilities to experiments. XL and HY performed evolutionary analysis. QS, LC and AZ took part in purification and biochemical experiments. YL, BL, LH, JC, WQ, SL, GW, SB and PMM contributed resources, reagents and greenhouse facilities to experiments. All authors have read and approved the final manuscript.

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