Identification of three gene families coordinating the conversion between fructose-6-phosphate and fructose-1,6-bisphosphate in wheat

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Abstract

Saccharides are a direct energy source for most organisms and the primary components in grains of common wheat (Triticum aestivum L., 2n = 6x = 42, AABBDD). However, genes involved in the metabolism of primary saccharides such as glucose and fructose have not been fully characterized in wheat, which limits our understanding of how these genes influence wheat growth. In this study, genes coding ATP-dependent phosphofructokinase (PFK), fructose-1,6-bisphosphatase (FBP), and pyrophosphate-dependent fructose-6-phosphate 1-phosphotransferase (PFP), which participate in the conversion between fructose-6-phosphate (F-6-P) and fructose-1,6-bisphosphate (F-1,6-P2), were identified at the genome-wide level. A total of 24, 13, and 12 genes were found encoding TaPFK, TaFBP, and TaPFP, respectively. All predicted peptides of these genes exhibited conserved substrate-binding domain, suggesting they are active enzymes in vivo. Transcriptome data ranked the gene levels as follows: TacFBP-1 > TacpFBP-1 > TaPFPa-2 ≈ TaPFK-1 ≈ TaPFK-5 >> all remaining genes at different developmental stages of wheat. In the three tapfp-a, b, and d knockout lines, there was a decrease in the plant height, anther length, and thousand-grain mass, while the percentage of abnormal pollen increased compared to that of wild type cv. Huapei3 (HP3). During germination, tapfp-f-a exhibited a lower germination rate, shorter coleoptile and primary root length, and higher fructose content than HP3, tapfp-f-b, and tapfp-f-d lines. Expressions were ranked as follows: TaPFK-5 ≈ TaPFPa-2 >> TaPFPa-1 = TaPFKβ > TacFBK-1 = TaPFK-7, 9 in HP3. All these genes were downregulated during the 24 - 96 h germinating process in three mutant lines. Collectively, main TaPFK, TaFBP, and TaPFP members cooperated during wheat growth, while TaPFKβ knockout decreased wheat vitality. Results from this study can aid more systematic studies of the physiological and molecular functions of TaPFK, TaFBP, and TaPFP.

Keywords: ATP-dependent phosphofructokinase, fructose-1,6-bisphosphatase, germination, pyrophosphate-dependent fructose-6-phosphate 1-phosphotransferase, transcriptome, Triticum aestivum.

Introduction

In crop seeds, such as wheat, rice, and maize, activated glucose-1-phosphate (G-1-P) is a substrate for sucrose and starch synthesis (Pfister and Zeeman 2016). During germination of the crop seeds, starch breaks down
to G-1-P to afford ATP, NADPH, and intermediate metabolites through glycolysis, the tricarboxylic acid (TCA) cycle, and pentose phosphate pathway (PPP) (Fig. 1 Suppl.) (Fernie et al. 2004). In the glycolysis and gluconeogenesis pathway, G-1-P is converted to fructose-6-phosphate (F-6-P) through two reversible steps, while the conversion between F-6-P and fructose-1,6-bisphosphate (F-1,6-P2) is irreversibly catalyzed by ATP-dependent phosphofructokinase (PFK) in the glycolysis pathway and fructose-1,6-bisphosphatase (FBP) in gluconeogenesis pathway, respectively (Fig. 1 Suppl.), or reversibly converted by a pyrophosphate-dependent fructose-6-phosphate 1-phosphotransferase (PFP) (Fig. 1 Suppl.) (Ap Rees et al. 1985, Montavon and Kruger 1992, Plaxton and Podestá 2007). Although previous studies have investigated PFK, FBP, and PFP enzymes individually in plants, limited studies have investigated their cooperation during plant growth.

Studies on Arabidopsis thaliana, Oryza sativa, Saccharum species, Spinacia oleracea, and Pyrus bretschneideri have shown that isoforms of PFKs are encoded by a small gene family comprising three sub-class (PFK-A, -B, and -C, respectively) (Knowles 1990, Winkler et al. 2007, Mustroph et al. 2007, 2013, Zhu et al. 2013, Lü et al. 2019). Functional studies have shown that plant PFKs may be involved in several processes. For example, OsPFK-Bs are downregulated, while OsPFK-Cs are induced under anoxia stress (Mustroph et al. 2013). Transient transformation of plastid PhPFK1 in pear fruits significantly increased fructose and sorbitol content (Lü et al. 2019). Furthermore, PFK members in a plant are differently expressed; for example, 3 out of 10 Saccharum ScPFKs are highly expressed in sugarcane (Zhu et al. 2013).

FBP dephosphorylates F-1,6-P2 (Fig. 1 Suppl.). Plants have two isoforms of FBP, namely cytosolic FBP (cyFBP) and chloroplast FBP (cpFBP), which exhibit approximately 50% sequence identity and are derived from a gene duplication event during early eukaryotic evolution (Martin et al. 1996, Jiang et al. 2012, Gulte et al. 2016). The cyFBP is primarily involved in gluconeogenesis and sucrose biosynthesis, whereas cpFBP participates in photosynthesis (Rojas-Gonzalez et al. 2015). The disruption of OscyFBP1 hinders growth due to a reduction in photosynthetic rate and chlorophyll content (Lee et al. 2008). Loss of AtcyFBP has no significant effect on the phenotype, but increases chloroplast starch content, whereas AtcpFBP knockout plants exhibit a smaller rosette size, increased superoxide dismutase activity, lower photosynthesis rate, soluble sugar content, and starch accumulation. AtcpFBP and AtcyFBP double mutants exhibit a dwarf phenotype (Rojas-Gonzalez et al. 2015, Soto-Suarez et al. 2016). The above data from rice and Arabidopsis suggested cooperation between cy- and cpFBP.

Among the three enzymes (PFK, FBP, and PFP), PFP has been extensively studied. PFP exists as a heterotetramer with two regulatory alpha-subunits (FFPA) and two catalytic beta-subunits (FFPB), and its activity is tightly regulated by fructose-2,6-bisphosphate (F-2,6-P2) (Tripodi and Podesta 1997, Fernie et al. 2001, 2002, Nielsen et al. 2004). FFPα is encoded by several genes, whereas FFPβ is encoded by a single gene in monocots (Mustroph et al. 2013, Lü et al. 2019). Functional studies have illustrated that PFP is not only involved in glycolysis/ gluconeogenesis, but also in pyrophosphate (PPI) generation for hydrogen proton pumping in tonoplast and stress responses to N and P limitations and hypoxia (Costa dos Santos et al. 2003, Basson et al. 2011, Lin et al. 2013, Mustroph et al. 2013, Duan et al. 2016). Arabidopsis PFP-knockout plants have growth retardation phenotype (Lim et al. 2009), while frame-shift mutation of OsPFPβ causes opaque, brittle, and floury grains, but no visible abnormal phenotype during the seedling and tillering stages (Duan et al. 2016, Chen et al. 2020a). These results indicate that PFP may function differently in different plants. Whether disturbing one of these three genes' expression affects the other two remains to be determined.

Common wheat is a hexaploid species (2n = 6x = 42, AABBDD) derived from three diploid progenitors in two rounds of natural hybridization (Marcussen et al. 2014), and most of its genes have three homoeologs. As the second largest crop, wheat yield is important for global food security (Senapati and Semenov 2020, Shew et al. 2020). Therefore, to determine the relationship between carbon metabolism and wheat yield, we identified TaPFK, TaPFP, and TaFPP genes in the wheat genome, determined their expression in different tissues using publicly available RNA-seq data to characterize the main functional members during different developmental stages (Brenchley et al. 2012, Jia et al. 2013, Ling et al. 2013, International Wheat Genome Sequencing Consortium 2014, 2018). To better understand the function of TaPFP and investigate the cooperation between PFP, PFK, and FBP genes in wheat, phenotype [plant height, thousand-grain mass (TGM), anther morphology, pollen fertility] of the three tapfpβ homoeologous gene knock-out mutant lines was observed, TaPFK, TaPFP, and TaFPP gene expression during the germination process were also determined in this study.

Materials and methods

Plants and reagents: Triticum aestivum L. cv. Huapei 3 (HP3) and a fast neutron-induced mutant population of the HP3 genetic background (4 500 lines from 8 000 seeds treated with 1 × 1011 n.cm-2 fast neutrons, obtained from the Institute of Genetic and Developmental Biology, Chinese Academy of Sciences) were used in this study. Common wheat cultivar Chinese Spring (CS) and its null-tetrasomic lines were used as references in genotype screening. Common wheat cultivar Huapei 3 (HP3) and a fast neutron-induced mutant population of the HP3 genetic background (4 500 lines from 8 000 seeds treated with 1 × 1011 n.cm-2 fast neutrons, obtained from the Institute of Genetic and Developmental Biology, Chinese Academy of Sciences) were used in this study. Common wheat cultivar Chinese Spring (CS) and its null-tetrasomic lines were used as references in genotype screening. Common wheat cultivar Chinese Spring (CS) and its null-tetrasomic (NT) lines were used as references in genotype screening. Common wheat cultivar Chinese Spring (CS) and its null-tetrasomic (NT) lines were used as references in genotype screening.
**Phylogenetic analysis and prediction of protein features:** The latest versions of the genome sequences of *A. thaliana*, *Oryza sativa*, *Aegilops tauschii* (2n = 14, DD), and wheat (*Triticum urartu*, 2n = 14, AA, *Triticum dicoccoides*, 2n = 24, AABB, and *T. aestivum*, 2n = 42, AABBDD) were downloaded from the *Ensemble* database ([http://plants.ensembl.org/](http://plants.ensembl.org/)); and those of *Brachypodium distachyon* (Bd21) and *Hordeum vulgare* (2n = 14, HH) were downloaded from *Phytozome* ([https://phytozome.jgi.doe.gov/pz/portal.html](https://phytozome.jgi.doe.gov/pz/portal.html)). Seed files of PFK (PF00365) and FBP (PF00136) domains were downloaded from the Pfam website ([http://pfam.xfam.org/](http://pfam.xfam.org/)) and used to search the plant FBPs (PF00136) domains were downloaded from the *Phytozome* ([https://phytozome.jgi.doe.gov/pz/portal.html](https://phytozome.jgi.doe.gov/pz/portal.html)). Seed files of PFK (PF00365) and FBP (PF00136) domains were downloaded from the Pfam website ([http://pfam.xfam.org/](http://pfam.xfam.org/)) and used to search the plant peptide databases mentioned above using *HMMER*. The e-value cut-off was 10⁻²⁰ and SMART ([http://smart.embl-heidelberg.de](http://smart.embl-heidelberg.de)) and CDD ([https://www.ncbi.nlm.nih.gov/cdd](https://www.ncbi.nlm.nih.gov/cdd)) were used to confirm the sequences obtained. Only one peptide sequence of every gene was kept for further phylogenetic and gene structural analysis, and sequences that were too long or too short were manually excluded. Multiple alignments of PFK, FPF, and FBP peptide sequences were generated using *ClustalW*, and phylogenic trees of PFK, PFP, and FBP were constructed using the neighbor-joining method with 1 000 bootstrap replicates in *MEGA7* ([Saitou and Nei 1987, Kumar et al. 2016]). Newick format files of phylogenic trees of *TaPFK*, *TaPFP*, and *TaFBP* genes were saved for further use. The isoelectric point (pI) and relative molecular mass (Mr) of proteins were predicted using the *ExPaSy* server ([https://web.expasy.org/compute_pi/](https://web.expasy.org/compute_pi/)) and the sublocalization of these proteins was predicted on the website of *TargetP* ([http://www.cbs.dtu.dk/services/TargetP/](http://www.cbs.dtu.dk/services/TargetP/)). Nomenclature of the *TaPFK*, *TaPFP*, and *TaFBP* was based on gene name of *Arabidopsis* and rice ([Martin et al. 1996, Mustroph et al. 2007, 2013, Lee et al. 2008, Serrato et al. 2009, Jiang et al. 2012, Gutle et al. 2016]).

**Gene feature analyses:** Conserved motifs in PFK, PFP, and FBP were analyzed using *MEME* ([http://meme-suite.org/](http://meme-suite.org/)), with parameter settings according to Lü et al. (2019): expected motif sites: zero or one per sequence; maximum number of motifs: 15; minimum motif width: 6; maximum motif width: 50; and maximum number of sites: 300. Results were exported in XML format. A figure combining the phylogenetic tree, conserved motifs, and gene structure was generated by submitting gene IDs of *TaPFKs*, *TaPFKs*, and *TaPFPs* (*Table 1*), a phylogenetic tree file (*.*xml), and the *Triticum aestivum* IWGSC.44.gff3 file to the graphics tools of TBtools ([Chen et al. 2020b]). The conserved motifs of *TaPFK/PFPs* were compared to *Escherichia coli* PFK (PDB1PFK) ([Ronimus and Morgan 2001]), while the *TaFBPs* were compared to the *AtFBPs* ([Chueca et al. 2002, Serrato et al. 2009, Gutle et al. 2016]).

**Mining transcriptions through RNA-seq database:** Expression data of all three gene families at different developmental stages of Chinese Spring (CS) were downloaded from the *Triticaceae Multi-omics Center* ([http://202.194.139.32/](http://202.194.139.32/)) according to Borrill et al. (2016). An expression heatmap was drawn using the *HeatMap* tool in TBtools ([Chen et al. 2020b]).

**TaPFPβ mutant screening and phenotypic analysis:** In total, 4 500 lines of a fast neutron-induced mutant population of HP3 were subjected to genotype identification using a pair of conserved primers specific to three *TaPFP* genes (*Table 1 Suppl.*). PCR products were separated by 6 % undenatured polyacrylamide gel electrophoresis (PAGE) and visualized using silver staining according to Merril (1990). Gene-specific primers (*Table 1 Suppl.*) were designed to detect the expression of the three homologous genes in leaves, to verify the mutants.

Seeds of mutant lines were surface-sterilized once using 70 % (v/v) ethanol and 0.1 % (m/v) Tween-20 for 10 min, washed with double-distilled water for at least five times, and germinated in Petri dishes (diameter 12 cm) on wet filter paper at 18 °C (night) and 22 °C (day) in the dark. At least 100 seeds and three repeats were used to analyze the germination rate. The lengths of primary roots and coleoptile were measured after 112 h of germination. Plant height was measured after full heading in the field during the growth season (*n* > 50, 2018 - 2019). The thousand-grain mass (TGM) was measured in three repeats after the seeds were dried to a constant mass.

Spikelets of HP3 and three mutant lines, 2 - 3 d before flowering, were fixed in formalin-aceto-alcohol solution (85 cm³ of 95 % ethanol, 5 cm³ of acetic acid, 5 cm³ of formalin, 5 cm³ of glycerol in a 100 cm³ solution). Paraffin sections were prepared according to the methods described by Cao et al. (2012). Sections were observed and imaged under Zeiss Axioscope 1 microscope (Oberkochen, Germany). All data were collected from at least three biological repeats.

Pollen was collected during flowering and stained by iodine-potassium iodide solution (1 %KI, 0.5 g iodine, and 1 g potassium iodide in 100 cm³ dDH2O) to observe the phenotypes.

**Real-time qPCR analysis of PFK, FBP, and PFP expression during germination:** Total RNA was extracted from the grain after 24 - 96 h of germination and used for first-strand cDNA synthesis. Gene-specific primers were designed for the three homologous genes (*Table 1 Suppl.*). An *ABI 7500* instrument with software version 2.3.1 ([Applied Biosystems, Foster City, CA, USA]) was used for qPCR analysis; wheat 26S rRNA gene was used as an internal control ([Ali-Benali et al. 2005]). qPCR mixture composition and PCR conditions were as per...
domains were searched in the genomes of, Genes containing PFK (PF00365) and FBP (PF00136)

Results and discussion

−ΔΔCt was calculated using the 2 method for at least three technical and three biological repeats (pooled).

Data analysis: Data were illustrated using graphs with SigmaPlot 9.0 and presented as the mean ± standard deviation (SD), which were calculated in Excel. Means were compared using Student’s t-test in SPSS17 (IBM, Armonk, USA). P < 0.05 was considered as significant.

Results and discussion

Genes containing PFK (PF00365) and FBP (PF00136) domains were searched in the genomes of Arabidopsis, rice, Brachypodium, barley, T. urartu, Ae. tauschii, T. dicoccoides, and T. aestivum. We found 49 genes encoding TaPFK, TaPFP, and TaFBP, and they were scattered over all 21 wheat chromosomes (Table 1).

In a phylogenetic tree of PFKs/PFPs constructed using the neighbor-joining method, PFKs and PFPs clearly formed separate clusters (Fig. 2 Suppl.). According to the evolutionary analysis of AtPFKs and OsPFKs, the nomenclatures for TaPFK followed the OsPFKs (Mustroph et al. 2007, 2013) (Table 1, Fig. 2 Suppl.). Fig. 2 Suppl. shows the homologous relationships of PFKs/PFPs in grass species. Loss of gene members was found in the PFK_A and PFP_ALPHA sub-groups. Five members of PFK_A were found in rice and Brachypodium, whereas only four members were identified in barley, T. urartu, Ae. tauschii, T. dicoccoides, and T. aestivum (Table 1, Fig. 2 Suppl.). This indicated that an event of gene loss happened after the divergence of the Brachypodium/ Triticaceae species. The phylogenetic analysis also showed that one member of PFP_ALPHA was lost between the divergence of rice and the ancestor of Brachypodium and Triticaceae, before the loss of the PFK_A member.

When using sedoheptulose-1,7-bisphosphatase (SBP) as an outgroup, FBP_A in the phylogenetic tree were clustered in four subgroups (Fig. 3 Suppl.), and nomenclature for TaFBPs was according to the “inherited” or α4β4 hetero-octamer in different plants (Ronimus and Morgan 2001), the F-6-P binding sites were mutually exclusive. Based on our findings, we believe the “inherited” model may be more suitable to explain the phylogenetic relationships of PFKs, PFPs, and FBPs among the three wheat subgenomes and their progenitors, since the chromosome locations and gene copies did not vary in the wheat subgenomes (Tables 1, Fig. 2 Suppl. and Fig. 3 Suppl.). The phylogenetic relationship of the wheat PFK, FPF, and FBP, and other plants, found herein, can be used to compare the function among orthologs in a plant.

Most homoeologous TaPFK, TaPFP, and TaFBP proteins have similar peptide lengths, theoretical pl, Mr, sublocalization, and genetic exon-intron structure (Table 1, Figs. 1 and 2). However, the pls of the homoeologous members, including TaPFK-6, TaPFK-8, and TaccFBP1, differed more than 0.5 pH unit. Interestingly, the pls of seven PFKs (TaPFK-1, -2, -5, -6, -7, -8, and -10) and two cpFBPs (TaccFBP-1 and -2) differed more than 1 pH unit.

Analysis of the conserved motifs showed that all TaPFKs/PFPs have five conserved motifs, 1 - 4 and 9, which were organized in the same order (Fig. 1). When TaPFK/ PFPs was aligned to Escherichia coli PFK (PDB1PFK) (Ronimus and Morgan 2001), the F-6-P binding sites were in motifs 1 - 4 and 9, and the binding site for ATP, which is the specific substrate for PFKs, was in motif 8 (Fig. 1). PFP forms a heterotetramer (α2β2), α3β3 heterohexamer, or α4β4 hetero-octamer in different plants (Theodorou and Plaxton 1996, Turner and Plaxton 2003, Mucut et al. 2019). Nine PFPA and three PFPβs members were found in wheat (Table 1, Fig. 1). However, how α and β subunits organize into a whole enzyme in wheat requires further research. More interestingly, how the nine α subunits and
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Table 1. Characteristics of TaPFKs, TaPFPs, and TaFBPs. **Traces** - *Triticum aestivum*; **CS** - Chinese Spring; **a.a.** - number of amino acids; **NE** - number of exons (UTRs excluded). **Grey shade** indicates a pI difference larger than 0.5 pH units among the three homoeologous proteins. **Cy** - cytoplasmic; **Cp** - chloroplast. A, B, and D in the gene ID stands for the subgenome. The number before subgenome is the group.

| Subclass | T. aestivum | Gene name | Length of peptide [a.a.] | pI / Mr [kDa] | Sublocalization | NE |
|----------|-------------|-----------|--------------------------|---------------|-----------------|----|
| PFK_A    | TaeCS3A02G107600 | TaPFK-A1  | 535                      | 7.21 / 58.212 | Cy              | 11 |
|          | TaeCS3B02G126400 | TaPFK-B1  | 535                      | 7.21 / 58.055 | Cy              | 11 |
|          | TaeCS3D02G109600 | TaPFK-D1  | 535                      | 7.21 / 57.998 | Cy              | 11 |
|          | TaeCS3A02G277700 | TaPFK-A2  | 609                      | 5.85 / 66.620 | Cy              | 15 |
|          | TaeCS3B02G311900 | TaPFK-B2  | 608                      | 5.97 / 66.606 | Cy              | 15 |
|          | TaeCS3D02G278000 | TaPFK-D2  | 609                      | 5.88 / 66.672 | Cy              | 15 |
|          | TaeCS1A02G344000 | TaPFK-A5  | 569                      | 6.61 / 62.242 | Cy              | 14 |
|          | TaeCS1B02G357000 | TaPFK-B5  | 566                      | 6.61 / 62.044 | Cy              | 14 |
|          | TaeCS1D02G346200 | TaPFK-D5  | 569                      | 6.58 / 62.287 | Cy              | 14 |
|          | TaeCS7A02G106800 | TaPFK-A6  | 494                      | 7.21 / 53.844 | Cy              | 13 |
|          | TaeCS7B02G005500 | TaPFK-B6  | 488                      | 6.68 / 53.466 | Cy              | 13 |
|          | TaeCS7D02G101300 | TaPFK-B7  | 494                      | 6.37 / 53.902 | Cy              | 13 |
| PFK_B    | TaeCS2A02G331700 | TaPFK-A9  | 469                      | 6.05 / 50.735 | Cy              | 2  |
|          | TaeCS2B02G350500 | TaPFK-B9  | 469                      | 5.97 / 50.796 | Cy              | 2  |
|          | TaeCS2D02G331200 | TaPFK-D9  | 469                      | 6.15 / 50.732 | Cy              | 2  |
| PFK_C    | TaeCS7A02G088100 | TaPFK-A7  | 529                      | 8.68 / 58.117 | Cy/Cp           | 13 |
|          | TaeCS4A02G387000 | TaPFK-B7  | 529                      | 8.73 / 58.093 | Cy/Cp           | 13 |
|          | TaeCS7D02G083200 | TaPFK-D7  | 529                      | 8.60 / 58.199 | Cy/Cp           | 13 |
|          | TaeCS5A02G226600 | TaPFK-A8  | 530                      | 7.59 / 58.097 | Cy/Cp           | 13 |
|          | TaeCS5B02G225200 | TaPFK-B8  | 530                      | 7.28 / 58.123 | Cy/Cp           | 13 |
|          | TaeCS5D02G234000 | TaPFK-D8  | 530                      | 7.05 / 57.840 | Cy/Cp           | 13 |
|          | TaeCS5A02G092600 | TaPFK-A10 | 420                      | 6.50 / 45.752 | Cy/Cp           | 13 |
|          | TaeCS5B02G098700 | TaPFK-B10 | 412                      | 6.12 / 44.926 | Cy/Cp           | 13 |
|          | TaeCS5D02G104900 | TaPFK-D10 | 420                      | 6.36 / 45.718 | Cy/Cp           | 13 |
| PFP_ALPHA| TaeCS5A02G072700 | TaPFPa-A1 | 621                      | 6.83 / 67.597 | Cy              | 19 |
|          | TaeCS5B02G087300 | TaPFPa-B1 | 621                      | 6.83 / 67.885 | Cy              | 19 |
|          | TaeCS5D02G085500 | TaPFPa-D1 | 621                      | 6.85 / 67.692 | Cy              | 19 |
|          | TaeCS6A02G279100 | TaPFPa-A2 | 581                      | 6.33 / 63.118 | Cy              | 17 |
|          | TaeCS6B02G307200 | TaPFPa-B2 | 616                      | 6.76 / 67.059 | Cy              | 17 |
|          | TaeCS6D02G259900 | TaPFPa-D2 | 616                      | 6.76 / 67.144 | Cy              | 17 |
|          | TaeCS7A02G226600 | TaPFPa-A3 | 611                      | 6.27 / 66.818 | Cy              | 18 |
|          | TaeCS7B02G193600 | TaPFPa-B3 | 611                      | 6.35 / 66.821 | Cy              | 18 |
|          | TaeCS7D02G228500 | TaPFPa-D3 | 611                      | 6.35 / 66.853 | Cy              | 18 |
| PFP_BETA | TaeCS7A02G198200 | TaPFPb-A  | 560                      | 5.83 / 60.558 | Cy              | 16 |
|          | TaeCS7B02G104400 | TaPFPb-B  | 560                      | 5.83 / 60.589 | Cy              | 16 |
|          | TaeCS7D02G200800 | TaPFPb-D  | 560                      | 5.83 / 60.558 | Cy              | 16 |
| cpFBP    | TaeCS7A02G484200 | TacPFB-A1 | 391                      | 8.27 / 42.406 | Cp              | 3  |
|          | TaeCS7B02G387900 | TacPFB-B1 | 398                      | 8.95 / 42.782 | Cp              | 3  |
|          | TaeCS7D02G471600 | TacPFB-D1 | 397                      | 8.57 / 42.764 | Cp              | 3  |
|          | TaeCS4A02G093100 | TacPFB-A2 | 409                      | 5.14 / 44.218 | Cp              | 4  |
|          | TaeCS4B02G211400 | TacPFB-B2 | 409                      | 5.07 / 44.276 | Cp              | 4  |
|          | TaeCS4D02G212000 | TacPFB-D2 | 409                      | 5.07 / 44.300 | Cp              | 4  |
| cyFBP    | TaeCS3A02G377600 | TacyFBP-A1| 341                      | 5.48 / 37.148 | Cy              | 11 |
|          | TaeCS3B02G410400 | TacyFBP-B1| 341                      | 5.38 / 37.206 | Cy              | 11 |
|          | TaeCS3D02G370700 | TacyFBP-D1| 341                      | 5.48 / 37.148 | Cy              | 11 |
|          | TaeCS1A02G273900 | TacyFBP-A2| 341                      | 5.70 / 37.113 | Cy              | 10 |
|          | TaeCS1B02G283600 | TacyFBP-B2| 341                      | 5.58 / 37.036 | Cy              | 10 |
|          | TaeCS1D02G274000 | TacyFBP-D2.1| 341                    | 5.87 / 37.067 | Cy              | 10 |
|          | TaeCS1D02G274200 | TacyFBP-D2.2| 341                    | 5.46 / 36.999 | Cy              | 10 |
three β isoforms in allohexaploid wheat interact with each other and regulate carbon efflux, remains to be elucidated.

Since signal peptides are present in the N-termini of cpFBPs, their peptide lengths are longer than those of cyFBPs (Table 1). All TaFBPs have nine conserved motifs (Fig. 2), except cpFBP1, which has an extra motif 9. We next aligned the TaFBPs to AtcyFBP (Fig. 4 Suppl.), which showed that substrate- or magnesium ion-binding sites are generally conserved in the FBP family. Compared to cpFBP1, cpFBP2 displayed some differences: two conserved motifs in the F-1,6-P 2-binding site, RYxG (AtcyFBP 246-249) and KLRL (AtcyFBP 277-280) were substituted by RYxC and HLRL, respectively. Moreover, a six-amino-acid deletion was found right before the KLRL conserved motif. Gutle et al. (2016), Serrato (2009), and Chueca et al. (2002) showed that these mutations (two amino acid substitutions and the deletion) are common in plant cpFBP2s (Fig. 4 Suppl.).

We assessed the expression of all members of the three gene families at different developmental stages of cv. CS. Fig. 3A shows that homoeologs of the same gene in the A, B, and D subgenome usually shared similar expression patterns. For example, TaPFK-A1, B1, and D1 were more strongly expressed in the roots than in the stem. Of the eight TaPFKs (24 members in total), only TaPFK-1 and 5 were highly expressed (more than 60% of the total cytosol TaPFKs), whereas others were expressed at low levels or specifically in certain tissues. Among the three PFPαs, homoeologous PFPα2s were highly expressed in root, stem, and spikelet, but at relatively lower levels in leaves and developing grains. PFPβ expression patterns were very similar to those of PFPα2s (Fig. 3A). Among four FBPαs, cpFBP1s and cyFBP1s were highly expressed in green tissues (80 - 150 transcripts per million, TPM), but rarely expressed in roots and developing grains (< 1 TPM). The expression of cyFBP-D2.1 was lower than...
that of cyFBP-D2.2, and its expression pattern did not resemble that of cyFBP-A2, which is phylogenetically closely related (Fig. 2 and Fig. 3 Suppl.). Comparing all members of the three gene families, the expressions were as follows: TacyFBP-1s > TacyFBP-2s ≈ TaPFPA-2s > TaPFPA-2s ≈ TaPFPA-1s > TaPKF-1s > TaPKF-5s ≈ the remaining genes in most of the tissues (Fig. 3A). Interestingly, in green tissues such as coleoptile, shoot, leaf blade, the number of total transcripts of cyFBPs and cpFBPs were higher (> 200 TPM) than those of PFPA and PFPAβ (about 50–70 TPM), which was vice versa in sink tissue such as root, spikelets, and grain where PFPA and PFPAβ were higher than that of cyFBPs and cpFBPs. Furthermore, in green tissues, cyFBPs were higher than cpFBPs. PKFs were relatively stable at different development stages (Fig. 3B).

From the expression patterns, we concluded that products of photosynthesis (glyceraldehyde-3-phosphate and dihydroxyacetone phosphate) were actively converted to sucrose (in the cytoplasm) and starch (in the chloroplast) and that due to sucrose transport to sink tissues, the expression of cyFBPs was higher than that of cpFBPs in these tissues.

In summary, the highly expressed PKFs, PFPA, and FBPs in wheat were revealed through mining public RNA-Seq data. The expression of these members was more than 60 % of the total transcripts in most tissues (Fig. 3). As the RNA-Seq data have large sample numbers (Fig. 3), we believe these results may be close to the real situation in vivo. Furthermore, the regulation of gene activity could happen after translation (post-translational regulation). It was reported that pea cyFBP1 (Pisum sativum) is redox-regulated through thioldisulfide switch (Serrato et al. 2018). Therefore, from the results inferred from Arabidopsis, rice, and this study (Lee et al. 2008, Rojas-Gonzalez et al. 2015, Soto-Suarez et al. 2016), future studies should target these main members to disclose their function in wheat not only at the transcriptional level.

The above results showed that TaPFPA is induced in sink tissue (Fig. 3B); to analyze TaPFPA functions, we identified mutant lines in a fast neutron-induced mutant population of common wheat cv. HP3. A pair of conserved primers was used to detect mutations in all three TaPFPAβs simultaneously (Table 1 Suppl.). We observed that the bands corresponding to TaPFPAβ-A, -B, and -D were absent in the NT lines of CS (Fig. 4A), indicating that TaPFPAβ-A, -B, and -D were located on chromosomes 7A, 7B, and 7D, respectively. The genotyping results showed that a few single plants lacked the band corresponding to TaPFPAβ-A, whereas most plants harbored all three copies of TaPFPAβ in line 686. In addition, we obtained a TaPFPAβ-B mutant. Since we found only one heterozygote plant at the TaPFPAβ-D locus in M2 plants, we further self-hybridized these plants to obtain a homozygous βTaPFPA-D mutant in the M3 plants (data not shown). All mutant lines were further identified at the RNA level. Gene-specific primers were designed to detect the expression of TaPFPAβs. As expected, TaPFPAβ-A, -B, and -D transcripts were absent in lines 686 (or 419), 557, and 785 respectively (Fig. 4B). Thus, we obtained knockout mutant lines of all three TaPFPAβs.

Based on field data collected over 2 years (2018 - 2019), we found that plant height and TGM of all mutant lines were decreased, especially in lines 668 (tapfpβ-a) and 557 (tapfpβ-b) (Fig. 5A,B), although all lines had normal grains. This phenotype is reminiscent of that of an AtPFPAβ RNAi line with smaller rosette leaves (Lim et al. 2009). Since we could not establish tapfpβ-a/b, tapfpβ-a/d, and tapfpβ-b/d double-mutant lines by hybridization, we further observed the phenotype of anther and pollen (Fig. 5C,D, Fig. 5 Suppl.). Pollen grains of HP3 (WT) plants were round and well-developed, with only 4 % pale, collapsed, or irregularly shaped pollen (Fig. 5C, Fig. 5 Suppl., Fig. 6 Suppl.). However, in the mutant lines, especially, tapfpβ-a and -d, 12 - 13 % of the pollen grains

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**Fig. 2.** Phylogenetic tree, conserved motifs, and gene structure of FBP. The tree was constructed by the neighbor-jointing method using amino acid sequences in MEGA7.0. Motifs and exons are indicated as rectangles, other sequences as lines. CDS - coding sequence; FBP - fructose-1,6-bisphosphatase; Ta - Triticum aestivum; UTR - untranslated region.
showed abnormalities, including being stuck to each other (Fig. 5C, Fig. 5 Suppl. Fig. 6 Suppl.). These phenotypic findings may explain why we could not establish double or triple mutants. Thus, the lack of one copy of TaPFPβ decreased wheat vitality. Based on these findings, we concluded that the three homologs of TaPFPβ regulate plant growth additively in common wheat. The hydrolysis of stored starch to glucose and the formation of ATP or other products through glycolysis and the TCA cycle are very important during seed germination (Fig. 1 Suppl.). Thus, we further observed the germination process in HP3 and tapfpβ mutant lines and found that, except for line 686 (tapfpβ-a mutant), which had a decreased germination rate, lengths of coleoptile, and...
primary root (Fig. 6A - C), other lines had no differences ($P < 0.05$). Fructose and glucose contents were slightly increased during germination in all lines (Fig. 6D,E) ($P < 0.05$). Sucrose content exhibited a decrease-increase-decrease dynamic trend during 24 - 96 h of germination (Fig. 6F). Interestingly, fructose contents were higher in all mutant lines than in the HP3 at 24 h, whereas the increase in fructose was relatively smaller in mutant lines at 24 - 72 h of germination. These findings indicated that fructose dynamics were affected by the loss of TaPFPβ. Moreover, in line 686 (tapfpβ-a mutant), fructose content was 2 - 3 times higher than those in other lines at the end of germination. A high fructose content may indicate that the metabolism downstream of fructose was hampered (Fig. 1 Suppl.). This would cause a shortage of ATP and other materials needed for DNA, RNA, and protein biosynthesis, which may have resulted in a lower germination rate or slower growth of young seedlings in line 686 than in the other lines (Fig. 6A - C).

To elucidate the mechanism underlying the differences in fructose content among the mutant lines and the HP3, we detected the expression of genes related to the F-6-P and F-1,6-P₂ balance. Gene-specific primers were designed based on the differences among the gene
families (Table I Suppl.). The expression data revealed that: 1) only a few members of TaPFKs, TaPFPs, and TaFBPs were expressed during germination; for example, homologous TacyFBP-1s were the only TaFBPs expressed during germination (Fig. 7); 2) only TaPFK-5s and TaPFPα-2s were highly expressed in HP3, whereas the other genes were poorly expressed; 3) in mutant lines, expressions of TaPFK-5s, TaPFPα-2s, and TaPFPβs were lower than those in the HP3; and 4) fructose content was negatively correlated with expressions of total TaPFPs ($R^2 = -0.6077$ to $-0.7787$), but not with those of TaPFKs (Figs. 6D and 7). The negative relationship between TaPFK expression and fructose content was consistent with a previous finding, which showed that the downregulation of PFP activity in sugarcane elevated sucrose and hexose-phosphate content (Van der Merwe et al. 2010).

In higher plants, photosynthesis products are converted to sucrose and then transported to sink tissues (e.g. roots) or stored as starch (e.g. in the grains) for further use. In the membranes of root cells, the abundant nitrogen, phosphorus, and mineral transporters consume large amounts of ATP (Léran et al. 2014). In developing spikelets and during seed germination, proteins, lipids, RNA, and DNA are very actively biosynthesized. In these tissues, stored sucrose or starches are metabolized through glycolysis and then shuttled to the tricarboxylic acid cycle to generate ATP and carbon skeletons for further biosynthesis; hence, the glycolysis pathway may prevail over the gluconeogenesis pathway in these tissues. To meet demands, organisms may upregulate the expression of enzymes in the glycolysis pathway. In contrast, in tissues such as leaves, sucrose and starch are stored for further use and thus, the glycolysis pathway may be downregulated to save energy. As shown in Fig. 3, transcriptions of TaPFPα and PFPβ were relatively higher in sink tissues such as the roots, spikelet, and grain, with transcript amounts of most TaPFKs lower in leaves than in roots or grains. During germination, TaPFK-5 and PFPα2 were more strongly expressed than cyFBP-1, indicating upregulation of the genes involved in the glycolysis pathway (Fig. 7).
Notably, F-2,6-P₂ is an activator of PFP, an inhibitor of FBP, but has no effect on ATP-PFK in plants (Muchut et al. 2019, Plaxton and Podestá 2007, Theodorou and Kruger 2001). F-2,6-P₂ content is consistently high in sink tissues but significantly varies in the leaf cytosol (Nielsen et al. 2004). This implies that high PFP activity is maintained in non-photosynthetic tissues. Nielsen and Stitt (2001) found that PFP expression and PFP enzyme activity at the base and tip of young growing leaves were decreased in tobacco. This validates our findings to a certain degree as the downregulation of Tapfpβs affected germination (or young seedling growth) and pollen development (Fig. 6, Figs 5 and 6 Suppl.). Based on the expression patterns, phenotypes of tapfpβs mutants, and previous findings, we conclude that TaPFP is essential in sink tissues.
Conclusions

In this study, we identified three gene families namely TaPFK, TaPF, and TaFBP, which are involved in the balance between F-6-P and F-1,6-P2. From the phylogenetic analysis, we found that gene loss and duplication occurred at different time points during grass genome evolution and shaped the gene variability in the three gene families in grass species. Based on expression profiling of these genes and phenotypic analyses (at the morphological, cellular, and physiological levels) of tapfpβ mutant lines, we found that TaPF functions dominate those of TaPFKs during seed germination, seedling growth, and pollen grain development in wheat. For further studies, modern CRISPR/Cas technology should be used to obtain homologous TaPFK- and TaFBP-knockout lines (main expressed members) and to unravel how these genes are involved in wheat development and yield production.

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