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Research Paper

Identification of stress defensive proteins in common wheat-*Thinopyron intermedium* translocation line YW642 developing grains via comparative proteome analysis

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Thinopyrum intermedium (2n = 6x = 42, $E_1E_1E_2E_2XX$) serves as an important gene source of desirable traits for genetic improvement of wheat cultivars resistant to stresses. This study used the comparative proteomic approach to identify stress defense related proteins in the developing grains of common wheat (Zhongmai 8601)-*Thinopyron intermedium* 7XL/7DS translocation line YW642 and to explore their potential values for improving wheat stress resistance. Two-dimensional electrophoresis identified 124 differentially accumulated protein spots representing 100 unique proteins, which mainly participated in stress defense, energy metabolism, protein metabolism and folding and storage protein synthesis. Among these, 16 were unique and 35 were upregulated in YW642. The upregulated DAPs were mainly involved in biotic and abiotic stress defense. Further *cis*-elements analysis of these stress-related DAP genes revealed that phytohormone responsive elements such as ABREs, G-box, CGTCA-motif and TGACG-motif, and environment responsive element As-1 were particularly abundant, which could play important roles in response to various stressors. Transcription expression analysis by RNA-seq and qRT-PCR demonstrated a large part of the stress-related DAP genes showed an upregulated expression in the early-to-middle stages of grain development. Our results proved that *Thinopyron intermedium* contains abundant stress responsive proteins that have potential values for the genetic improvement of wheat stress resistance.

Key Words: 7XL/7DS translocation, grain development, proteome, stress defense, transcript expression.

Introduction

Wheat (*Triticum aestivum* L., 2n = 42, AABBDD), as an allohexaploid species, is the main grain crop widely cultivated in the world and the national staple food of many countries (Cao *et al.* 2011). Wheat grains contain albumins, globulins, gliadins and glutenins, of which albumins and globulins include various metabolic proteins such as enzymes and their inhibitors that are important for plant growth and development and stress response. These proteins also have a high level of essential amino acids such as lysine, tryptophan, and methionine, and, therefore, are relatively well balanced for human nutrition. Gliadins and glutenins are the major storage proteins, which are rapidly synthesized and accumulated at 11–20 days post-anthesis (DPA) and closely related to the viscoelastic properties of wheat dough (Yu *et al.* 2016, Zhen *et al.* 2020). Thus, wheat flour can be widely used to make bread, noodles, biscuits, cakes, steamed bread and other foods (Shewry and Halford 2002).

Wheat growth and development are generally affected by various biotic stresses (frequent diseases and pests such as powdery mildew, fusarium head blight, stripe rust, yellow dwarf disease, starscream and aphids etc.) and abiotic stresses (drought, salt, high and low temperature, and heavy metal etc.). These adverse factors often cause huge losses of wheat production and threaten the safety of food production (McIntosh 1998, Tardif *et al.* 2007). In order to deal with these stressors, plants have developed a sophisticated defense system during long-term evolutionary process. Once external stress overcomes the mechanical barriers to infection, the plant immune system can recognize enemy molecules, generate signal transduction and activate signaling pathways to drive the expression of defense-responsive genes such as salicylic acid, jasmonic acid and ethylene induction pathways (Hahn 1996). It is known that two types of proteins in plants participate in stress response: functional proteins and regulatory proteins. Functional proteins

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mainly include osmotic protective proteins, proteases for protecting cell structure, degradation proteins, repairing proteins and detoxifying proteins. Regulatory proteins play an adjustive role in signal transduction and gene expression, including phospholipase, transcription factors and protein kinases (Bray 1993, Yan *et al.* 2002, Zhu 2000).

Wheat related species generally contain abundant gene resources resistant to biotic and abiotic stresses such as *Haynaldia villosa* L. with powdery mildew resistance gene *Pm21* (Huang *et al.* 1997) and drought-resistant proteins (Wang *et al.* 2018). A large-scale proteomic analysis of three wheat wild relatives (*Triticum urartu*, *Aegilops speltoides* and *Aegilops tauschii*) revealed that considerable differentially abundant proteins in the grains were involved in disease/defense (Kim *et al.* 2010). Some stress defense proteins such as heat shock proteins and antioxidant enzymes showed an upregulated expression in the early grain developmental stages of durum wheat (Giovanna *et al.* 2016). Thus, the introgression of alien genes from related Triticeae species can improve wheat stress resistance (Friebe *et al.* 1996). *Thinopyrum intermedium* L. ($2n = 6x = 42$, $E_1E_1E_2E_2XX$, JJJ^SJ^SStSt or EeEeEbEbStSt), regarded as the sibling species of polyploid *Triticum* L. (Chen *et al.* 1998, Wang and Zhang 1996, Xin *et al.* 2001), is one of the most important perennial *Triticeae* species and a source of desirable traits for wheat improvement due to its resistance to adverse stresses, high biomass production and high crossability with various *Triticum* species (Dewey 1984). Studies showed that *Thinopyrum intermedium* possesses biotic stress resistances to wheat streak mosaic virus (WSMV), wheat curl mite (WCM) and barley yellow dwarf virus (BYDV) (Chen 2005, Fedak and Han 2005, Friebe *et al.* 1996). To date, several useful wheat germplasms and varieties containing *Th. intermedium* chromosome in their genealogies have been developed via producing partial amphiploids, alien addition lines, and chromosome substitution and translocation lines. BYDV-resistance was identified in a *Th. intermedium* and Zhong 5 translocation line by genome in situ hybridization (GISH; Tang *et al.* 2000). A *Fusarium graminearum* resistant substitution line was developed, in which a pair of *Th. intermedium* chromosomes confer excellent resistance to *Fusarium graminearum* substituted wheat chromosome 2D (Han *et al.* 2003). An addition line resistant to powdery mildew was obtained via the progeny selection of a hybrid between *Th. intermedium* and common wheat Yannong 15 (Liu and Wang 2005). A number of desirable genes transferred from *Th. intermedium* into wheat produced useful wheat germplasms that were used throughout the world for wheat improvement (Xin *et al.* 1988).

The available wheat stocks with *Th. intermedium* chromatin conferring resistance to BYDV include three groups with translocations on chromosome arm 7DL and one substitution line with chromosome 2Ai#2 replacing chromosome 2D of wheat. The common wheat Zhongmai 8601-*Th. intermedium* 7XL/7DS translocation line YW642 with BYDV resistance belongs to the former, which harbors distal segments of the long arm of *Th. intermedium* chromosome 7Ai-1 with the resistance gene *Bdv2* (Banks *et al.* 1995). It was estimated that 10% of wheat chromosome 7DL distal or the whole 7DL were replaced by *Th. Intermedium* chromatin in YW642 by GISH analysis (Ayala-Navarrete *et al.* 2009, Xin *et al.* 2001). YW642 has been regarded as a good resistance source used for wheat breeding, compared to his parental wheat cultivar Zhongmai 8601 is highly resistant to yellow dwarf virus disease (**Supplemental Table 1**). However, the proteome compositions of the stress responsive proteins in YW642 are still not clear. In this study, we used the comparative proteome approach to identify the key differentially accumulated proteins (DAPs) between common wheat cultivar Zhongmai 8601 and Zhongmai 8601-*Thinopyron intermedium* 7XL/7DS translocation line YW642. Considerable DAPs caused by 7XL/7DS translocation were found to participate in various stress defenses, which have potential application values in the genetic improvement of wheat cultivars resistant to biotic and abiotic stresses.

Materials and Methods

Plant materials

The materials used in this work included common wheat cultivar Zhongmai 8601 and Zhongmai 8601-*Thinopyron intermedium* 7XL/7DS translocation line YW642 collected from Institute of Crops Science, Chinese Academy of Agricultural Sciences (CAAS). YW642 with yellow dwarf virus resistance was developed by Institute of Crops Science, CAAS and Australian scientists by using wheat-*Thinopyron intermedium* alien addition line L1 carrying anti-yellow dwarf gene *Bdv2* as the resistance source. Chinese Spring *ph* mutant was used to induce partial chromosome recombination and pairing, and then homozygous and stable strain YW642 resistant to yellow dwarf disease was obtained through selection and breeding (Chen *et al.* 2011).

Field trial, sampling and agronomic trait measurement

Both Zhongmai 8601 and YW642 were planted in the experimental station of China Agricultural University (CAU), Beijing in 2017–2018 wheat growing season with three biological replicates (each replicate 12 m^2). The cultivation and management were same as local field cultivation and climate conditions (**Supplemental Table 2**). After anthesis, the developing grains in the middle of ear were labeled at 9:00–11:00 am and collected from five different developmental stages (15, 20, 25, 30 and 45 DPA). The collected samples were snap-frozen in liquid nitrogen, and then stored in the refrigerator (–80°C) prior to use. The main agronomic traits were measured after maturity, including growth period (day), plant height (cm), tiller number, effective spikelets, ear grain number, length of main spike (cm), grain width (mm), grain length (mm) and thousand-grain weight (g).

Protein extraction, 2-DE and determination of DAP spots

Total protein from developing grains was extracted and separated by two-dimensional electrophoresis (2-DE) based on Liu *et al.* (2012) and Wang *et al.* (2018) with minor modifications. After electrophoresis, all gels were stained with colloidal Coomassie Brilliant blue G-250 (Sigma-Aldrich, St. Louis, MO, USA) and then analyzed with Image Master 2D Platinum Software Version 7.0 (Amersham Biosciences, America). The protein spots with significant and biological reproducible changes (abundance variation >1.5-fold, Student's t-test with p value <0.05) were determined as the differentially accumulated protein (DAP) spots.

Protein identification via MALDI-TOF/TOF-MS

All the DAP spots determined by 2-DE were excised from the 2-DE gels manually, and then transferred to centrifuge tubes (2.0 cm^3) for digestion with trypsin as described by Lv *et al.* (2014). The tandem mass spectrometry spectra were obtained using an ABI 4800 Proteomics Analyzer (Applied Biosystems/MDS Sciex, USA) matrix-assisted laser desorption/ionization time-of-flight/time-of-flight mass spectrometer (MALDI-TOF/TOF-MS) operating in result-dependent acquisition mode. The MS/MS spectra were searched against Viridiplantae (green plant) sequences in the non-redundant National Center for Biotechnology Information (NCBI) database (Posted on December 23, 2016, 5147436 protein sequences, 46272342986 residues) and *Triticum* NCBI database using MASCOT software (ver. 2.1, Matrix Science, London, United Kingdom) with the following parameter settings: trypsin cleavage, one missed cleavage allowed, carbamidomethylation set as fixed modification, oxidation of methionines allowed as variable modification, peptide mass tolerance set to 100 ppm, and fragment tolerance set to ±0.3 Da. All searches were evaluated based on the significant scores obtained from MASCOT. The protein score C.I.% and total ion score C.I.% were both set to >95%, and a significance threshold of $p < 0.05$ was used.

Bioinformatic analysis

The Venn diagram was constructed by Venny 2.1 (https://bioinfogp.cnb.csic.es/tools/venny/). Protein function classification was based on the annotation from UniProt (https://www.uniprot.org/; Wang *et al.* 2015). Principal component analysis (PCA) was conducted in the R language and Environment for Statistical Computing (version 3.0.2, Auckland, New Zealand; Kristiansen *et al.* 2010, Valledor and Jorrín 2011). The 1500-bp genomic DNA sequence upstream from the start codon of the DAP genes was obtained from the wheat database (http://plants.ensembl.org/Triticum_aestivum/Info/Index). The *cis*-acting elements were determined by PlantCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) with default parameters (Doi *et al.* 2008, Lescot *et al.* 2002). The average copy number of each *cis*-acting element is calculated through dividing the total *cis*-acting element copy number of all genes by the total number of genes.

Vector construction and subcellular localization

The subcellular localization of the identified DAPs was first predicted via the integration of websites: WoLF PSORT (https://wolfpsort.hgc.jp/), Plant-mPLoc (https://www.csbio.sjtu.edu.cn/bioinf/plant-multi/) and Uni-ProtKB (https://www.uniprot.org/). Then, the subcellular localization results were further verified by using polyethylene glycol (PEG) 4000-mediated wheat mesophyll protoplast transformation. The coding sequence without a stop codon of four genes (*BADH*, *TLP*, *HSP70*, and *DHAR*) were subcloned to fuse with the 5′ end of the GFP (green fluorescent protein) coding sequence in a p*35S:GF*P vector (16318-35S-GFP vector). The transcripts of *BADH-GFP*, *TLP-GFP*, *HSP70-GFP*, and *DHAR-GFP* were also controlled using a *Cauliflower mosaic virus* (CaMV) 35S promoter. Four recombined plasmids and p*35S:GFP* control were separately introduced into wheat mesophyll protoplasts by PEG-mediated transformation (Shan *et al.* 2014). Transformed cells were incubated in W5 solution for 12–18 h at 25°C in the darkness. GFP signal was detected by Leica sp8 fluorescence confocal microscopy (Leica, Germany).

Hierarchical clustering analysis

Hierarchical clustering analysis of the DAP spots identified from Zhongmai 8601 and YW642 during five grain developmental stages was performed by Cluster 3.0 software (Eisen *et al.* 1998). The relative ratios of DAP spots were conducted after log2-transformation, and then the Euclidean distance similarity metric was used to define the similarity, and hierarchical clusters were assembled by using the complete linkage clustering method. The clustering results were visualised through TreeView software (https://sourceforge.net/projects/jtreeview/).

RNA-seq expression analysis

The publicly available RNA-seq data generated from bread wheat (var. Chinese Spring) was used to detect the expression profiling of the identified DAP genes. RNA-seq data were obtained from expVIP (http://www.wheat-expression.com/; Philippa *et al.* 2016), including grain, leaf/shoot, root and spike during developing seedling, vegetative and reproductive stages. Cluster analysis of the RNA-seq data was performed by employing the Euclidean distance method over a complete linkage dissimilarity matrix using the Cluster 3.0 and TreeView.

mRNA extraction and qRT-PCR

Total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Then reverse transcription reactions were performed using PrimeScript® RT Reagent Kit with gDNA Eraser (TaKaRa, Shiga, Japan) according to the manufacturer's instructions. Gene-specific primers were designed

using online Primer3Plus (https://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi; **Supplemental Table 3**) and their specificities were checked by observing the melting curve of the amplification products and the specific band on the agarose gel. *Ubiquitin* was used as the reference gene. Reverse transcription quantitative polymerase chain reaction (RT-qPCR) was performed in CFX96 Real Time system (Bio-Rad Laboratories) as previously described method (Bian *et al.* 2017). Three biological replicates were performed for each sample.

Results

Phenotype and agronomic trait performance of 7XL/7DS translocation line YW642

Field growth and developmental features showed that Zhongmai 8601-*Thinopyron intermedium* 7XL/7DS translocation line YW642 displayed significant changes in plant phenotype (**Fig. 1A**), ear type (**Fig. 1B**), grain development (**Fig. 1C**) and main agronomic traits (**Supplemental Table 4**) compared to Zhongmai 8601. Plant height and thousand-grain weight increased significantly while spike length and spikelet number had no marked changes. However, number of tillers, effective spikelet number and ear number decreased dramatically.

Proteome characterization during grain development of YW642

The differential proteome in the five grain developmental stages between Zhongmai 8601 and translocation line YW642 was identified by 2-DE (**Fig. 2A**, **2B**, **Supplemental Fig. 1**). The main DAP spots were distributed in the range of pH 4–10, and their molecular weights ranged from 15 to 80 kDa. In total, 367 protein spots were determined in Zhongmai 8601 and YW642 through ImageMaster 2D Platinum Software (Version 7.0, Amersham Biosciences) analysis, of which 124 were identified as differential accumulated protein (DAP) spots. Then all the DAP spots were manually excised from gels and digested by trypsin for further MALDI-TOF/TOF-MS analysis. Finally, 124 DAP spots representing 100 unique proteins in the developing grains were successfully identified (**Supplemental Tables 5**, **6**). Venn diagram shown in **Fig. 2C** indicated that 7XL/7DS translocation caused the specific expression of 16 DAP spots (15 proteins), and the significant expression changes of 105 common DAP spots (82 proteins), including upregulation of 35 DAP spots (30 proteins) and downregulation of 70 DAP spots (52 proteins). Only 3 DAP spots representing 3 unique proteins were specifically present in Zhongmai 8601. Venn diagram shown in **Fig. 2C** indicated the dynamic changes of the DAP spots in five developmental stages between Zhongmai 8601 and YW642. These DAP spots mainly occurred from 15 to 30 DPA of grain development. In particular, the specific and common DAPs in YW642 were mainly involved in biotic and abiotic stress defense (**Table 1**).

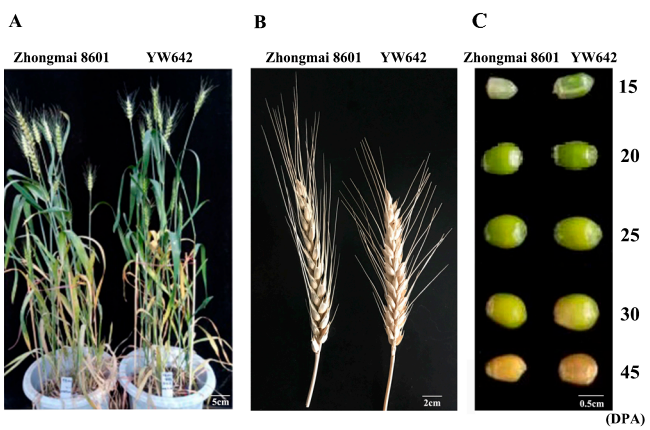

Fig. 1. Phenotype comparison between Zhongmai 8601 and Zhongmai 8601-*Thinopyron intermedium* 7XL/7DS translocation line YW642. A. Plant morphology; B. Spike morphology; C. Grain morphological characteristics during development.

Principal component analysis (PCA) of 367 protein spots indicated that both YW642 and Zhongmai 8601 showed a similar proteome change during grain development. The grain developmental stage, named PC1 accounting for 66.34%, had greater effects on grain proteome than genotype, named PC2 accounting for 14.86%. In particular, the early stages such as 15 DPA and late stages such as 45 DPA showed distinct proteome changes (**Fig. 3A**). The scatter diagram of the DAP spots indicated that six DAPs had a large amount of dispersion with other proteins (**Fig. 3B**), including disease resistance protein RPP8 (spot 20, EMS64545), glyceraldehyde-3-phosphate dehydrogenase (spot 21, CAA42901), dimeric alpha-amylase inhibitor (spot 24, AFJ45085), hydrogen peroxide isoenzyme 1 (spot 59, EMS61345), heat shock protein 70 (spot 101, AFD04130) and stress responsive protein 1 (spot 106, AFN10736). This result suggests that these DAPs had greater effects on the proteome differences between Zhongmai 8601 and translocation line YW642.

According to the identification results of tandem mass spectrometry (**Supplemental Table 5**), the DAPs were classified into 11 functional categories: stress defense (31%), storage protein (23%), energy metabolism (13%), protein metabolism and folding (12%), carbon metabolism (7%), nitrogen metabolism (2%), starch metabolism (2%), nucleic acid metabolism (1%), photosynthesis (1%), transport (2%) and other metabolic pathways (6%) (**Fig. 4A**). In particular, among the 35 upregulated and 16 specific DAP spots in YW642 as shown in **Table 1**, 21 and 5 DAP spots were, respectively, involved in stress defense, which accounted for 67% (26/39) of the stress defense proteins.

Subcellular localization prediction by three websites showed that the DAPs were distributed in seven subcellular structures, mainly including cytoplasm (35%), vacuole (21%), chloroplast (16%) and mitochondria (11%) (**Fig. 4B**). To verify the results of subcellular localization prediction, we selected four representative proteins to further

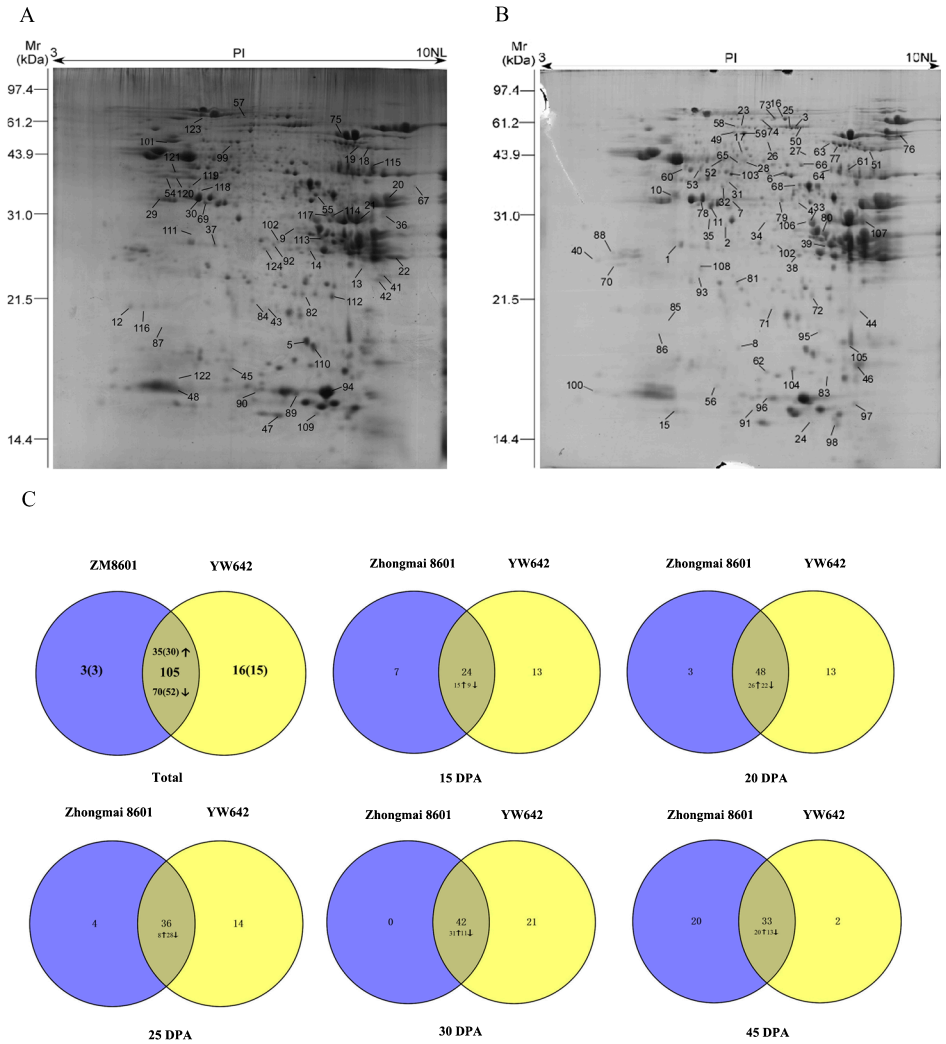

Fig. 2. Differential proteome identification of grain proteome in the Zhongmai 8601 and Zhongmai 8601-*Thinopyron intermedium* 7XL/7DS translocation line YW642. A. 2-DE images of grain proteome at 30 DPA in YW642. The 35 upregulated and 16 unique DAP spots in YW642 are numbered; B. 2-DE images of grain proteome at 30 DPA in Zhongmai 8601. The 70 downregulated DAPs in Zhongmai 8601 are numbered; C. The Venn diagram of the DAP spots between Zhongmai 8601 and YW642 and their changes at five grain developmental stages. The numbers in parenthesis indicate the number of unique DAPs; The upward arrows indicate upregulation and the downward arrows indicate downregulation. DPA, days post anthesis.

conduct subcellular localization experiments via wheat mesophyll protoplast transformation, including heat shock protein 70 (HSP70, AFD04130), betaine-aldehyde dehydrogenase (BADH, AAL05264), thaumatin-like protein (TLP, AAM15877) and dehydroascorbate reductase (DHAR, AAL71854). The structures of the control vector and four recombinant plasmids for subcellular localization are shown in **Supplemental Fig. 3**. After transient expression with green fluorescent protein (GFP) fusion proteins in wheat suspension culture cells, microexamination was performed by using confocal laser microscopy. The results showed that BADH and TLP were located in chloroplast while HSP70 and DHAR were located in cytoplasm based on the strong green fluorescent signals (**Fig. 4C**), well consistent with the website prediction results.

Dynamic expression profiling of the DAPs during grain development

Hierarchical clustering was used to reveal the dynamic expression profiling of the DAPs during five grain developmental stages in YW642 and Zhongmai 8601 (**Fig. 5**). Overall, seven protein expression patterns from cluster I to VII were classified. Three clusters (I, II and IV) with 54 spots had higher expression in the middle to late stages. Among them, the proteins in cluster IV displayed a continuous upregulation during grain developmental process, including 37% (11/30) of the upregulated DAP spots and 40% (6/15) of the unique DAP spots in YW642. The clusters (III, V and VII) with 62 DAP spots contained 41% (16/39) of the stress-related proteins and these proteins generally showed a high expression level at the early stages (15–20 DPA).

Table 1. Classification of stress-related differentially accumulated proteins (DAPs) identified by MALDI-TOF/TOF-MS from Zhongmai 8601 and Zhongmai 8601-*Thinopyron intermedium* 7XL/7DS translocation line YW642

Protein Name (Spot ID)	Protein Score	Protein Score C.I.%	Total Ion C.I.%	*p*-value	Subcellular location prediction	Expression ratio (ZmX/ZM)				
						15 DPA	20 DPA	25 DPA	30 DPA	45 DPA
Biotic stress										
Translationally-controlled tumor protein (12)	552	100	100	0.037	Cyto	1:0.8	1:0.9	1:1.4	1:2.4	1:1.6
Basic endochitinase C (13)	125	100	100	0.009	Vacu	1:0	1:0.7	1:1.5	1:1.9	1:1.9
Disease resistance protein RPP8 (20)	48	100	100	0.019	Cyto	1:1	1:1.9	1:1.5	1:1.6	1:1.2
Class II chitinase (22)	128	100	100	0.034	Vacu	1:2.6	1:2.5	1:2.6	1:0.8	1:0.8
Disease resistance protein RPM1 (34)	53	100	100	0.026	Cyto	0:0	0.1:0	1:0.7	1:1.3	1:1.2
Translationally controlled tumor protein (37)	264	100	100	0.034	Cyto	0:0	1:1.9	1:1.5	1:1.8	1:1.2
Thaumatin-like protein (42)	344	100	100	0.004	Chlo*	1:2.8	1:2.1	1:1.2	1:2.0	0.4:0
Disease resistance protein RPM1 (45)	49	100	100	0.035	Cyto	1:1.6	1:1.6	1:0.7	1:2.1	1:1.0
Basic endochitinase C (55)	130	100	100	0.037	Vacu	0:0	0:0.3	0:0.8	1:2.2	0:0
Class II chitinase (57)	133	100	100	0.048	Vacu	1:0.9	1:1.6	1:0.9	1:2.0	1:1.5
PR-4, partial (109)	57	100	100	0.009	Wall	0:0	0:0	0:0	0:0.2	0:0
Detoxification										
Lactoylglutathione lyase (1)	129	100	100	0.031	Nucl	1:0.7	1:0.1	1:0.6	1:1.9	1:1.4
Peroxidase 1 (36)	340	100	100	0.021	Extr	1:1.6	1:1.8	1:1.5	1:1.1	1:1.2
Glucose and ribitol Dehydrogenase-like protein (38)	264	100	100	0.009	Cyto	0:0.1	1:1.5	1:0.9	1:1.2	0.2:0
Glucose and ribitol Dehydrogenase-like protein (39)	264	100	100	0.009	Cyto	0:0	1:0.8	1:0.4	1:0.3	1:0.4
Lactoylglutathione lyase (40)	382	100	100	0.024	Cyto	1:1.2	1:0.9	0.1:0	1:1.8	0.1:0
Glutathione S-transferase (43)	200	100	100	0.028	Cyto	0:0	0:0.1	1:0.8	1:1.2	1:1.5
Catalase isozyme 1 (59)	426	100	100	0.048	Pero	1:0.9	1:0.5	1:0.5	1:0.8	1:1.2
Betaine-aldehyde dehydrogenase (60)	433	100	100	0.035	Chlo*	1:1.7	1:0.7	0:0	0:0	1:0.7
Aldose reductase (80)	1030	100	100	0.047	Cyto	0:0	1:1.2	1:1.1	1:0.7	1:0.3
Peroxidase 1 (82)	228	100	100	0.032	Extr	1:1.6	1:0.6	1:1.1	1:1.6	1:1.9
Dehydroascorbate reductase (84)	812	100	100	0.037	Cyto*	1:1.6	1:1.6	1:1.9	1:1.4	1:0.3
Oxalate oxidase 2 (85)	97	100	100	0.036	Cyto	0:0	1:0.7	0:0	0:0	1:0
Peroxiredoxin-2C (87)	118	100	95	0.047	Cyto	1:1.6	0:0	0:0	0:0.2	1:1.6
Superoxide dismutase (90)	124	100	100	0.011	Mito	0:0	1:1.8	1:0.9	1:1.9	1:1.5
Glucose and ribitol Dehydrogenase-like protein (92)	225	100	100	0.021	Cyto	1:1	1:1.8	1:0.7	1:1.6	1:6
Stress responsive protein 1 (106)	379	100	100	0.039	Cyto	1:1.4	1:1.7	1:0.6	1:0.7	1:0.3
Thiol-specific antioxidant protein (116)	427	100	100	0.013	Nucl	0:0.1	0:0.1	0:0.1	0:0.1	0:0.1
Protein degradation										
Formate dehydrogenase (7)	196	100	100	0.023	Cyto	1:1.2	1:0.8	1:0.7	1:1	1:0.2
ATP-DAP sendent zinc metalloprotease FTSH 2 (14)	53	100	100	0.039	Chlo	0:0.1	1:2.7	1:2.2	1:1.8	1:2.4
20S proteasome alpha subunit D (41)	99	100	100	0.029	Cyto	1:10.0	1:8.0	1:8.8	1:5.0	1:3.4
20S proteasome beta 5 subunit (112)	100	100	100	0.026	Cyto	0:0	0:0	0:0.2	0:0.3	0:0
26S proteasome non-ATPase regulatory subunit 14 (124)	182	100	100	0.034	Cyto	0:0.1	0:0	0:0	0:0.1	0:0
Protein repairation										
Serpin-N3.2 (10)	278	100	100	0.047	Extr	0:0	1:0.6	1:0.3	1:0.5	1:0.1
Heat shock protein 101 (11)	741	100	100	0.028	Cyto	1:0.6	1:0.6	1:0.6	1:0.9	1:2.6
Heat shock protein 101 (16)	916	100	100	0.018	Cyto	1:1.3	1:0.2	1:0.3	1:1.1	1:0.6
Serpin-Z2B (29)	79	100	98	0.03	Mito	0:0	1:1.6	1:0.9	1:1.8	1:1.5
Serpin 1 (30)	490	100	100	0.019	Mito	0:0	1:0.6	1:0.3	1:0.6	1:0.4
Serpin-Z2B (32)	712	100	100	0.034	Chlo	1:0.6	1:0.3	1:0.7	1:0.7	1:0.9
Serpin-N3.2 (54)	867	100	100	0.031	Chlo	1:2.2	1:3.0	0:0	0:0.1	1:2.0
Serpin-N3.2 (76)	385	100	100	0.018	Chlo	0.1:0	0:0	0:0	0:0.1	1.1
Serpin 1 (78)	500	100	100	0.048	Mito	1:0.6	1:0.6	1:0.6	1:0.6	1:0.2
Heat shock protein 70 (101)	531	100	100	0.032	Cyto*	1:1.8	1:1.7	1:1.1	1:1.4	1:2.1
Serpin-N3.2 (103)	620	100	100	0.016	Chlo	1:0.8	1:0.6	1:0.6	1:1.1	0.1:0
Serpin 1 (118)	582	100	100	0.012	Mito	0:0.1	0:0.1	0:0	0:0.1	0:0

Red represents the upregulated DAPs in YW642.
*represents the subcellular localization of these proteins verified by wheat protoplast cells.
ZmX, wheat-*Thinopyron intermedium* 7XL/7DS translocation line YW642; ZM, Zhongmai 8601.

Analysis of the cis-acting elements in the promoter region of the stress responsive DAP genes

The promoter region generally contains various *cis*-acting elements that regulate expression of genes in response to environmental stimuli. We selected 16 upregulated and 5 specifically expressed stress-related DAP genes in YW642 to identify *cis*-acting elements in the promoter region by using PlantCARE server. In total, 14 *cis*-acting elements were identified in 21 DAP genes, including 8 phytohormone responsive elements and 6 environmental

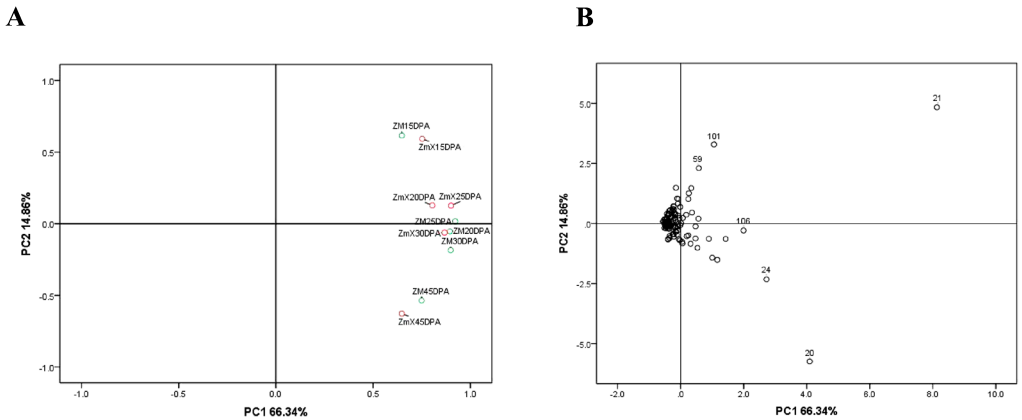

Fig. 3. Principal component analysis (PCA) of protein samples and DAP spots. A. PCA of individual protein samples in Zhongmai 8601 and YW642; B. Scatter diagram of 367 protein spots separated in Zhongmai 8601 and YW642. ZmX, wheat-*Thinopyron intermedium* 7XL/7DS translocation line YW642; ZM, Zhongmai 8601.

stress-related elements (**Table 2**). These *cis*-acting elements were unevenly distributed in the promoter region of different genes. The frequent presence of these elements could be an indication that the genes involve in various signaling pathway of phytohormones and stress defense to respond to biotic and abiotic stress. Phytohormone responsive elements are mainly involved in response to gibberellin, salicylic acid (SA), auxin, abscisic acid (ABA) and methyl jasmonic acid (MeJA). Among them, the response element ABRE (21%), G-box (16%), CGTCA-motif (11%) and MeJA-response elements TGACG-motif (10%) were frequently present with an average copy number of 3.19, 2.43, 1.62 and 1.43, respectively. The oxidative stress-responsive element As-1 also occurred at a higher frequency with average 1.62 copies while three others, low-temperature responsive element LRT, drought-inducible element MBS and antioxidant response element ARE, only had 0.76 copies on average.

RNA-seq expression analysis in different tissues and organs

The publicly available RNA-seq data generated from bread wheat (var. Chinese Spring) was used to study the expression profiling of 31 stress-related DAP genes in five different organs (root, leaf, shoot, spike and grain) and different development stages (**Fig. 6**). The results showed that these genes displayed different expression patterns in different organs and development stages. In general, all stress-related genes were expressed in grains, and some genes had tissue/organ expression specificity or expression preference. In general, 31 DAP genes were divided into three distinct expression patterns (Cluster A–C). Cluster A contained 7 genes, five of which had the highest expression in leaves, higher expression in spikes and lower expression in grain such as catalase isozyme and lactoylglutathione lyase genes. Twelve DAP genes in Cluster B were highly expressed in roots and spikes such as translationally controlled tumor protein and superoxide dismutase genes. Cluster C with 12 genes generally showed a high expression level in grain, and five genes were specifically expressed in grain, including class II chitinase, serpin-Z2B, serpin-N3.2, basic endochitinase and serpin 1 genes.

Dynamic transcriptional expression profiling of the DAP genes during grain development by qRT-PCR

Dynamic transcriptional expression profiles of seven representative DAP genes during grain development were detected by *qRT-PCR*, including ADP-glucose pyrophosphorylase large subunit (*AGPL*, spot 6), *serpin-N3.2* (spot 10), thaumatin-like protein (*TLP*, spot 42), ribulose-1,5-bisphosphate carboxylase/oxygenase (*rbcS*, spot 48), *enolase* (spot 53), betaine-aldehyde dehydrogenase (*BADH*, spot 60) and dehydroascorbate reductase (*DHAR*, spot 84). As shown in **Fig. 7**, these DAP genes generally showed similar expression patterns and only BADH seems to be negatively correlated during grain development in both Zhongmai 8601 and YW642. Except for *rbcS* with a low expression level, the remaining six DAP genes generally had a high expression during grain development (**Fig. 7**), generally consistent with RNA-seq data (**Fig. 6**). Comparison between transcriptional and translational levels found that three DAP genes (*AGPL*, *serpin-N3.2* and *TLP*) displayed a better consistence, two genes (*enolase* and *DHAR*) basically had a similar trend, but two genes (*rbcS* and *BADH*) were poorly consistent (**Supplemental Fig. 2**).

Discussion

This study identified 30 upregulated and 15 unique DAPs in the developing grains of wheat-*Thinopyron intermedium* 7XL/7DS translocation line YW642 via a comparative proteomic approach. These DAPs were mainly involved in the biotic and abiotic stress defense. Herein, we further discuss their potential functions and application values for wheat genetic improvement.

Wheat growth and development would confront various

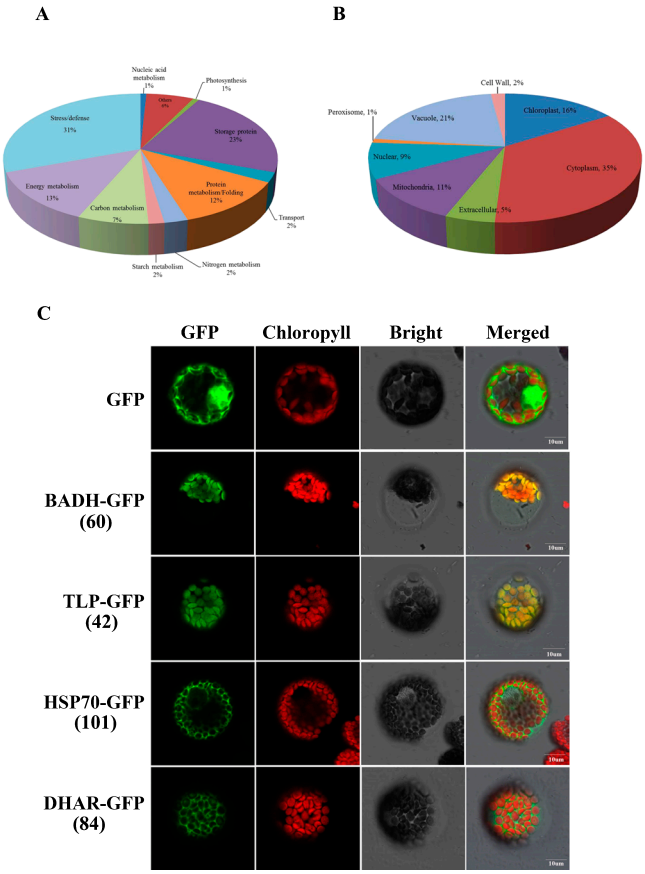

Fig. 4. Functional classification and subcellular localization of the identified DAPs. A. Functional classification of the DAPs in Zhongmai 8601 and YW642; B. Subcellular localization prediction of DAPs in Zhongmai 8601 and YW642; C. Subcellular localization assay via wheat protoplast cells. GFP: GFP fluorescence signal. Green fluorescence indicates the location of DAPs; Chlorophyll: chlorophyll autofluorescence signal; Red fluorescent signal indicates the location of chloroplasts in protoplasts; Bright light: field of bright light; Merged: emergence of the GFP fluorescence signal, chlorophyll autofluorescence signal and bright light field; Control: 16318-35S-GFP empty vector. Scale bar = 10 μm. BADH (betaine-aldehyde dehydrogenase), TLP (thaumatin-like protein), HSP70 (heat shock protein 70) and DHAR (dehydroascorbate reductase).

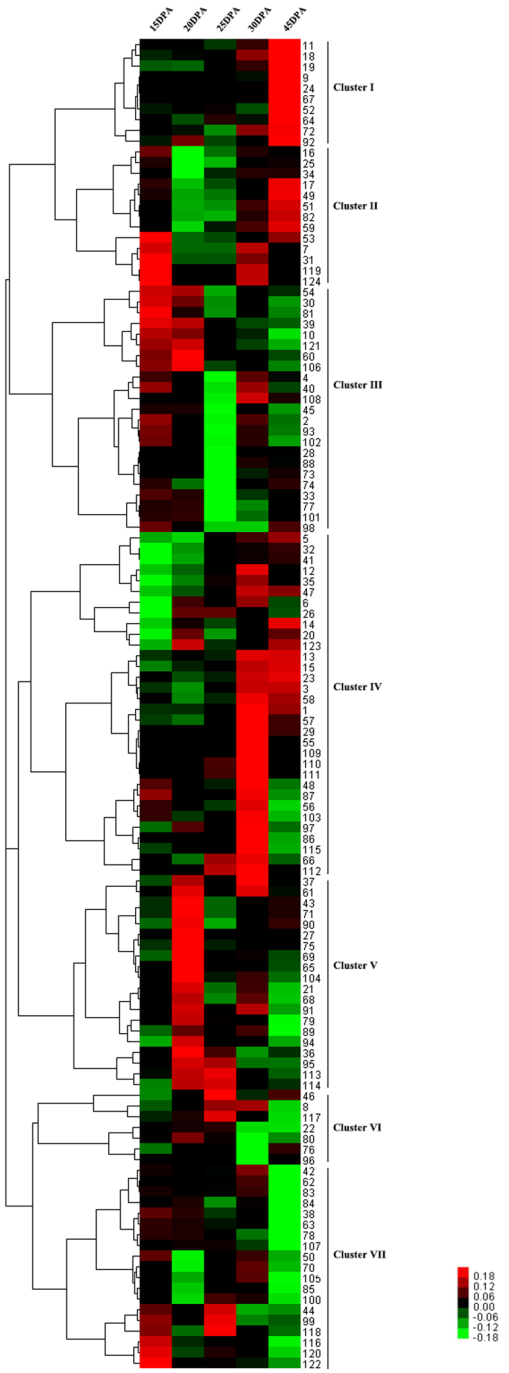

Fig. 5. Protein expression clustering analysis of DAPs in Zhongmai 8601 and YW642. The numbers are protein spot IDs listed in **Supplemental Table 5**.

biotic and abiotic stressors, particularly at the early-middle stages of grain development. Pathogenesis-related (PR) proteins are very important for plant abiotic stress resistance such as basic endochitinase C (spot 55) and class II chitinases (spot 57) with an upregulation at three grain developmental stages (**Table 1**). In addition, the promoter of the gene encoding basic endochitinase C has 20 *cis*-acting elements, including 6 G-box and 4 ABA-responsive elements (ABREs) (**Table 2**). Plant chitinases can hydrolyze the β-(1, 4) linkages of chitin, degrade fungal cell walls (Wargo 1975) and release inducers of defense responses (Hadwiger and Beckman 1980). Chitin is an unbranched homopolymer of 1,4-β-linked N-acetyl-D-glucosamine (GlcNAc) and a major component of fungal cell walls (Majeti 2000). Expressing chitinase genes were able to increase tolerance of *B. napus* var. *Oleifera* L. to infection of *Cylindrosporium concentricum*, *Phoma lingam* and *Sclerotinia sclerotiorum* (Grison *et al.* 1996). The expression of barley chitinase increased the resistance of wheat to powdery mildew (Oldach *et al.* 2001). This finding suggests that chitinases play a pivotal role in plant defense system for abiotic stress.

Translationally-controlled tumor protein (TCTP) is highly

Table 2. Composition of the *cis*-acting elements in the 1500 bp upstream promoter regions of the DAP genes

DAP genes*	Phytohormone responsive elements								Environmental stress-related elements						Total
	P-box	GARE-motif	TGA-element	ERE	TGACG-motif	CGTCA-motif	G-box	ABRE	LTR	As-1	GC-motif	ARE	MBS	TC-rich repeats	
ATP-DAP sendent zinc metalloprotease FTSH 2 (14)	0	1	1	0	3	3	0	6	4	0	2	0	1	1	22
Disease resistance protein RPP8 (20)	0	0	0	0	0	0	3	1	0	0	0	1	0	0	5
Class II chitinase (22)	1	0	0	1	0	2	2	9	0	1	0	0	1	0	17
Serpin-Z2B (29)	0	1	0	0	0	0	0	2	0	1	0	2	3	3	12
Peroxidase 1 (36)	1	2	1	2	2	2	0	4	0	1	0	0	1	0	16
Translationally controlled tumor protein (37)	0	1	2	0	1	1	2	3	3	2	1	0	0	0	16
20S proteasome alpha subunit D (41)	0	0	0	1	1	1	0	9	4	1	0	1	2	0	20
Thaumatin-like protein (42)	0	0	1	1	1	1	3	0	0	0	0	0	1	0	8
Disease resistance protein RPM1 (45)	1	0	0	0	2	2	3	2	0	2	0	0	1	0	13
Serpin-N3.2 (54)	1	1	0	0	1	1	0	1	0	2	0	0	1	2	10
Basic endochitinase C (55)	0	0	1	0	2	2	6	4	0	3	0	2	0	0	20
Dehydroascorbate reductase (84)	0	0	0	0	0	0	2	3	0	3	0	0	0	1	9
Peroxiredoxin-2C (87)	0	0	1	0	2	2	4	2	1	0	0	1	0	1	14
Superoxide dismutase (90)	0	1	0	0	2	2	2	1	2	3	1	1	0	1	16
Glucose and ribitol Dehydrogenase-like protein (92)	0	0	0	0	3	3	6	4	0	2	1	0	0	0	19
Heat shock protein 70 (101)	0	0	1	4	0	0	0	2	1	1	0	0	0	0	9
PR-4, partial (109)	0	0	0	0	3	3	0	4	1	2	0	0	1	0	14
20S proteasome beta 5 subunit (112)	1	2	0	0	0	2	3	6	0	4	0	1	1	3	23
Thiol-specific antioxidant protein (116)	0	1	1	0	1	1	4	3	0	2	0	0	1	0	14
Serpin 1 (118)	1	0	0	0	2	2	7	1	0	2	0	1	1	1	18
26S proteasome non-ATPase regulatory subunit 14 (124)	1	1	1	0	4	4	4	0	0	2	0	6	1	0	24
Total	7	11	10	9	30	34	51	67	16	34	5	16	16	13	319

* Numbers in brackets indicate the DAP spots corresponding to **Supplemental Table 5**.

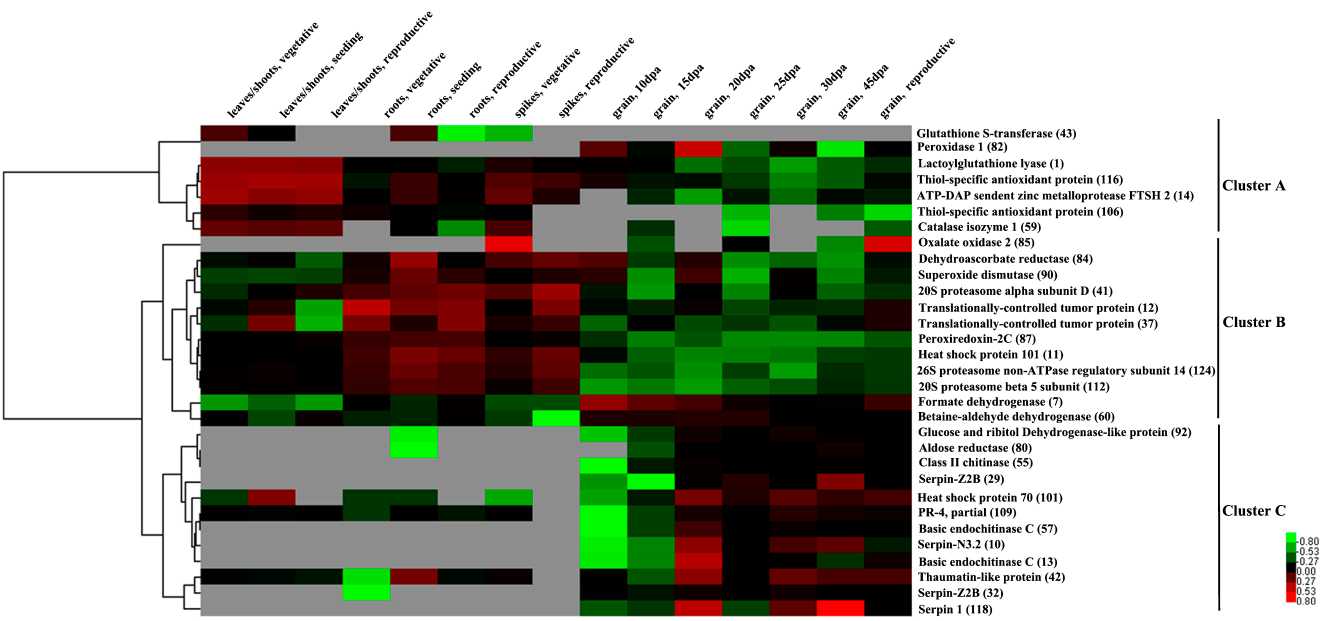

Fig. 6. Expression profiles of the stress-related and upregulated DAP genes in different tissues, organs and periods by RNA-seq analysis.

conservative in the eukaryotic cells (Bommer and Thiele 2004), and the expression of TCTP mRNA in plants was closely related to mitosis (Sage-Ono *et al.* 1998). In *Arabidopsis thaliana*, TCTP serves as a positive regulator of mitotic growth and regulates the timing of cell cycle (Bommer and Thiele 2004). The overexpression of *AtTCTP* significantly inhibited the programmed cell death (PCD) response of tobacco leaves (Hoepflinger *et al.* 2013). TCTP also participates in the response of the host to the pathogens infection. Wheat TCTP affected the infection of *Blumeria graminis* at the transcription level and played an important role in wheat-*Bgt* interaction. Silenced TCTP gene caused increased proportion of resistant structure and rapid accumulation of H_2O_2 (Zheng *et al.* 2014). Study also showed

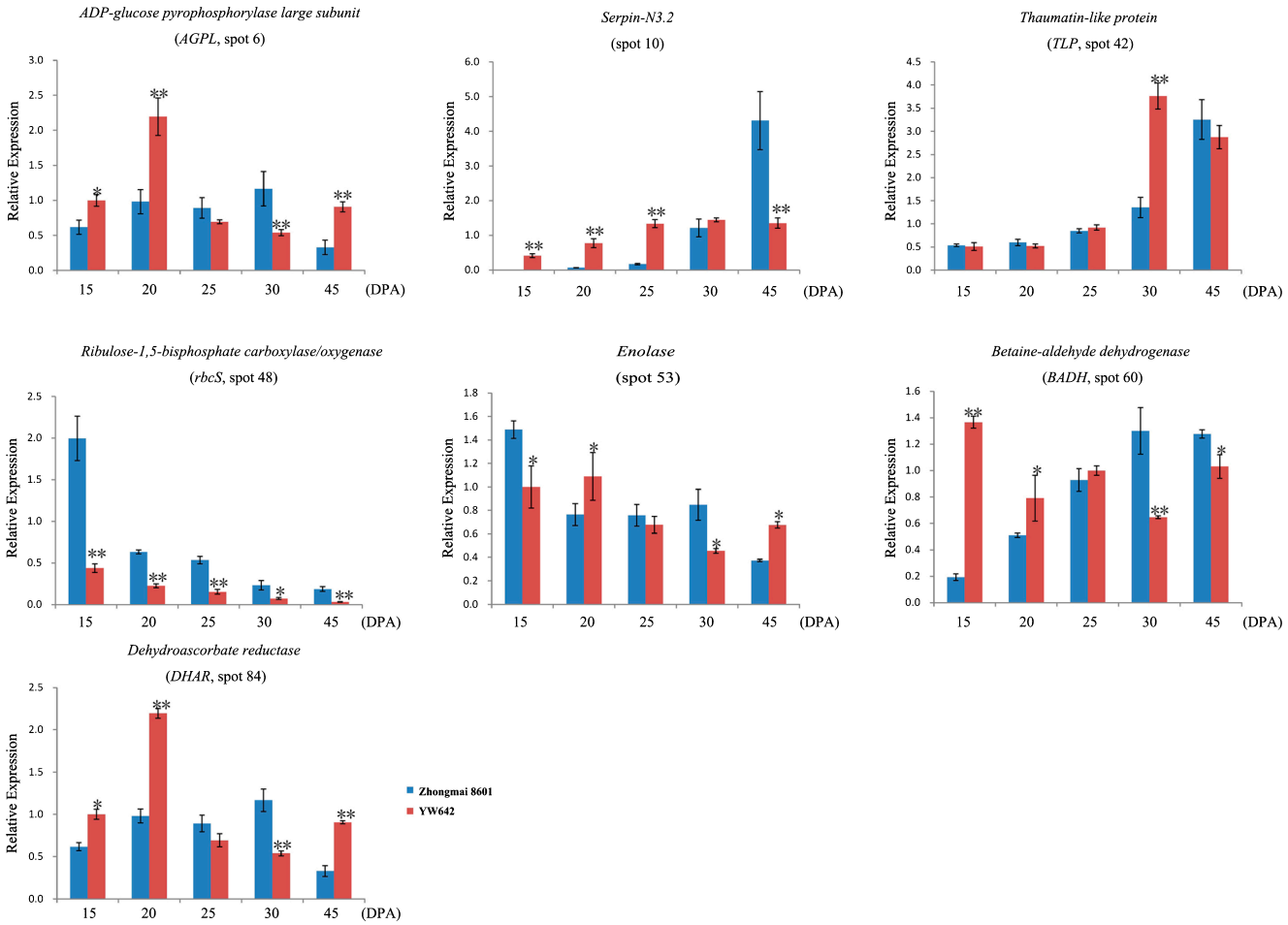

Fig. 7. qRT-PCR analysis of seven representative DAP genes during grain development of Zhongmai 8601 and YW642. *agpl*, *ADP-glucose pyrophosphorylase large subunit*; *TLP*, *thaumatin-like protein*; *rbcS*, *ribulose-1,5-bisphosphate carboxylase/oxygenase small subunit*; *BADH*, *betaine-aldehyde dehydrogenase*; *DHAR*, *dehydroascorbate reductase*. Statistically significant differences are calculated based on an independent Student's t-tests: * $p < 0.05$; ** $p < 0.01$.

that TCTP might belong to a novel small molecular weight heat shock protein involved in stress defense (Gnanasekar *et al.* 2009). We found that TCTPs (spots 12 and 37) were upregulated at 20–30 DPA in YW642 (**Table 1**), which could benefit to enhance wheat biotic stress resistance.

Previous studies showed that when affected by pathogens, thaumatin-like protein (TLP) expressed and accumulated in the plants such as tobacco, maize, barley, oat and wheat, which has a significant anti-fungal effect by altering the osmosis of the fungal membrane (Andersen *et al.* 2018). In addition, serine protease inhibitor superfamily has a function of anti-microbes and insects through inhibiting the protease activity of pathogenic microorganisms and exerting active defense roles in the process of exogenous infection (Roberts and Hejgaard 2008). Our results showed that TLP (spot 42), serpin-N3.2 (spots 54 and 10) and serpin 1 (spot 118) were upregulated at early grain developmental stage (**Table 1**). Their encoding genes also showed a high transcription expression level (**Figs. 6**, **7**). Wheat-*Haynaldia villosa* 6VS/6AL translocation also caused upregulation of three serpins under drought stress (Wang *et al.* 2018), indicating that both 7XL and 6VS chromosomes carry similar stress defense proteins. The upregulation of these proteins could promote wheat resistance ability for various stressors frequently occurred at grain developmental stages.

Reactive oxygen species (ROS) and H_2O_2 function as versatile signalling molecules of many biological processes, including stress responses, hormone signaling, cell growth and development (Fujita *et al.* 2006, Mittler *et al.* 2004). To maintain the dynamic equilibrium of ROS, plants constantly produce ROS along with continuous clearing to keep an appropriate level. Stressors generally cause radical accumulation in plants and result in the membrane lipid oxidation and membrane permeability loss, causing a series of physiological and biochemical changes and the metabolic disorder. In response to these events, plants would accumulate a large amount of antioxidant metabolites such as ascorbate, glutathione and tocopherol, and construct a large enzymatic antioxidants system (Mittler *et al.* 2004). Studies

showed that superoxide dismutases (SOD) can catalyze the dismutation of superoxide anion radical (O_2^-) to H_2O_2 and O_2 and enhance plant stress tolerance through its overexpression (Smirnoff 1993). H_2O_2 is required for the ABA pathway via modulating the expression of stress-responsive genes, which is removed through the ascorbate-glutathione (AsA-GSH) cycle (Noctor and Foyer 1998). In this study, 7XL/7DS translocation caused a significant upregulation of some detoxifying proteins during grain development, including peroxidase 1 (POD 1, spot 82), dehydroascorbate reductase (DHAR, spot 84) and SOD (spot 90). The promoter regions of these protein-encoding genes also contained 3 stress responsive As-1 elements (**Table 2**), which could play important roles in counteracting the toxicity of ROS, maintaining the AsA-GSH dynamic balance and enhancing plant antioxidant stress capacity (Virginia *et al.* 2002).

Under stress conditions, unfolded or misfolded proteins in the cytosol have a high tendency to form cytotoxic, life-threatening and nonspecific aggregates if accumulated to a high level (Kabashi and Durham 2006, Meredith 2005). The heat shock protein (Hsp) family contains Hsp60s, Hsp70s, Hsp90s etc, collectively called molecular chaperons. The highly conserved HSPs are essential components of plant defense signalling pathways, expressed in cells subjected to elevated temperatures or other forms of environmental stress to stabilize and facilitate the refolding of the denatured proteins (Cooper 2000). They are also required for folding of nascent proteins and intracellular transportation in addition to stress responses. We found that Hsp70 (spot 101) displayed an upregulation at both transcription and translation expression level during grain development (**Fig. 6**, **Table 1**), suggesting its important roles in stress defense and grain protein synthesis.

In contrast to Hsp70, the ubiquitin/proteasome system (UPS) removes unfolded or misfolded proteins through protein degradation (Esser *et al.* 2004). The 26S proteasome is composed of 20S proteasome and two 19S regulatory complexes that bind to ubiquitinated substrates (Rabl *et al.* 2008). Oxidation causes chemical modifications to proteins, which result in conformational changes and subsequent exposure of hydrophobic residues (Carrard *et al.* 2002). These hydrophobic patches on the surface of oxidized proteins are then recognized by 20S proteasomes and can promote the opening of the α-rings (Kisselev *et al.* 2002) and enhance selective degradation within the β-rings (Giulivi *et al.* 1994, Pacifici *et al.* 1993). Our results showed that 7XL/7DS translocation induced an upregulation of three proteasome proteins (spots 41, 112 and 124), of which 20S proteasome alpha subunit D (spot 41) dramatically increased at all grain developmental stages, particularly up to 10 folds at 15 DAP (**Table 1**). 20S proteasome participates in the ubiquitin/26S proteasome pathway and plays an important role in the physiological and biochemical processes such as embryonic development, pathogen defense, and hormone response (Wang and Huang 1999). In addition, the promoter of the gene encoding 20S proteasome alpha subunit D has 20 *cis*-acting elements, including 9 ABA-responsive elements (ABREs) (**Table 2**). It is known that ABREs regulate gene response to ABA, drought or salt signals (Li *et al.* 2012). Thus, the significant upregulation of 20S proteasome protein in the developing grains could improve the degradation of oxidatively damaged proteins in cells and enhance plant stress resistance.

Common wheat Zhongmai 8601-*Thinopyron intermedium* 7XL/7DS translocation caused significant grain proteome changes. Among 124 DAP spots identified, 38 spots were mainly involved in biotic and abiotic stress defense. Some key stress-defensive proteins such as serpin, chitinase, TCTP, POD, SOD and DHAR, showed clearly upregulated expression in the translocation line YW642, particularly at the early grain developmental stages. The promoters of these DAP genes generally contain abundant stress responsive elements (**Table 2**) that could play important roles in resistance to various biotic and abiotic stressors. In addition, 28 storage protein spots were also identified, and 7XL/7DS translocation led to upregulation of 18 protein spots representing 1 glutenin, 5 gliadins and 12 globulins at the early stages of grain development (**Fig. 5**, **Supplemental Table 5**). It is known that starch and protein accumulate rapidly in the grain-filling periods of 14–28 DPA (Shewry *et al.* 2012, Zhen *et al.* 2020), which are important stages in determining the final yield and quality of wheat (Gelang *et al.* 2008, Nass and Reiser 1975). Thus, the upregulation of stress defense proteins and storage proteins could improve the plant resistance as well as yield and quality formation. In conclusion, our results demonstrated that *Thinopyron intermedium* genus carries abundant gene resources that have potential application values for the genetic improvement of wheat cultivars.

Author Contribution Statement

YL and JW performed experiments and data analysis, and wrote the paper. RW performed a part of experiments and data analysis. YY designed and conducted experiments.

Acknowledgments

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Literature Cited

Andersen, E.J., S. Ali, E. Byamukama, Y. Yen and M.P. Nepal (2018) Disease resistance mechanisms in plants. Genes (Basel) 9: 339.

Ayala-Navarrete, L., E. Tourton, A.A. Mechanicos and P.J. Larkin (2009) Comparison of *Thinopyrum intermedium* derivatives

carrying barley yellow dwarf virus resistance in wheat. Genome 52: 537–546.

Banks, P.M., P.J. Larkin, H.S. Bariana, E.S. Lagudah, R. Appels, P.M. Waterhouse, R.I. Brettell, X. Chen, H.J. Xu, Z.Y. Xin *et al.* (1995) The use of cell culture for subchromosomal introgressions of barley yellow dwarf virus resistance from *Thinopyrum intermedium* to wheat. Genome 38: 395–405.

Bian, Y.W., X. Deng, X. Yan, J.X. Zhou, L.L. Yuan and Y.M. Yan (2017) Integrated proteomic analysis of *Brachypodium distachyon* roots and leaves reveals a synergistic network in the response to drought stress and recovery. Sci. Rep. 7: 46183.

Bommer, U.A. and B.J. Thiele (2004) The translationally controlled tumour protein (TCTP). Int. J. Biochem. Cell Biol. 36: 379–385.

Bray, E.A. (1993) Molecular responses to water deficit. Plant Physiol. 103: 1035–1040.

Cao, A., L. Xing, X. Wang, X. Yang, W. Wang, Y. Sun, C. Qian, J. Ni, Y. Chen, D. Liu *et al.* (2011) Serine/threonine kinase gene *Stpk-V*, a key member of powdery mildew resistance gene *Pm21*, confers powdery mildew resistance in wheat. Proc. Natl. Acad. Sci. USA 108: 7727–7732.

Carrard, G., A.L. Bulteau, I. Petropoulos and B. Friguet (2002) Impairment of proteasome structure and function in aging. Int. J. Biochem. Cell Biol. 34: 1461–1474.

Chen, Q., R.L. Conner, A. Laroche and J.B. Thomas (1998) Genome analysis of *Thinopyrum intermedium* and *Thinopyrum ponticum* using genomic *in situ* hybridization. Genome 41: 580–586.

Chen, Q. (2005) Detection of alien chromatin introgression from *Thinopyrum* into wheat using S genomic DNA as a probe—A landmark approach for *Thinopyrum* genome research. Cytogenet. Genome Res. 109: 350–359.

Chen, Y., L.Y. Gao, Y.M. Shao and Z.Y. Zhang (2011) Phenotypic identification and molecular analysis of a heat line mutant YW642 induced by EMS. J. Nucl. Agric. Sci. 25: 617–621.

Cooper, G.M. (2000) The Cell: A Molecular Approach, 2nd edn. ASM Press, Washington, p. 246.

Dewey, D.R. (1984) The genomic system of classification as a guide to intergeneric hybridization with the perennial *Triticeae*. Gene Manipulation in Plant Improvement pp. 209–279.

Doi, K., A. Hosaka, T. Nagata, K. Satoh, K. Suzuki, R. Mauleon, M.J. Mendoza, R. Bruskiewich and S. Kikuchi (2008) Development of a novel data mining tool to find *cis*-elements in rice gene promoter regions. BMC Plant Biol. 8: 20.

Eisen, M.B., P.T. Spellman, P.O. Brown and D. Botstein (1998) Cluster analysis and display of genome-wide expression patterns. Proc. Natl. Acad. Sci. USA 95: 14863–14868.

Esser, C., S. Alberti and J. Höhfeld (2004) Cooperation of molecular chaperones with the ubiquitin/proteasome system. Biochim. Biophys. Acta 1695: 171–188.

Fedak, G. and F. Han (2005) Characterization of derivatives from wheat-*Thinopyrum* wide crosses. Cytogenet. Genome Res. 109: 360–367.

Friebe, B., J.M. Jiang, W.J. Raupp, R.A. McIntosh and B.S. Gill (1996) Characterization of wheat-alien translocations conferring resistance to diseases and pests: current status. Euphytica 91: 59–87.

Fujita, M., Y. Fujita, Y. Noutoshi, F. Takahashi, Y. Narusaka and K. Yamaguchi-Shinozaki (2006) Crosstalk between abiotic and biotic stress responses: a current view from the points of convergence in the stress signaling networks. Curr. Opin. Plant Biol. 9: 436–442.

Gelang, J., H. Pleijel, E. Sild, H. Danielsson and G. Selldén (2008) Rate and duration of grain filling in relation to flag leaf senescence and grain yield in spring wheat (*Triticum aestivum*) exposed to different concentrations of ozone. Physiol. Plant. 110: 366–375.

Giovanna, V., G. Angelica, S. Fabio, U. Bonas, S. Speca, A. Faccini, M. Pisante and N. Marmiroli (2016) Proteomics of durum wheat grain during transition to conservation agriculture. PLoS ONE 11: e0156007.

Giulivi, C., R.E. Pacifici and K.J. Davies (1994) Exposure of hydrophobic moieties promotes the selective degradation of hydrogen peroxide-modified hemoglobin by the multicatalytic proteinase complex, proteasome. Arch. Biochem. Biophys. 311: 329–341.

Gnanasekar, M., G. Dakshinamoorthy and K. Ramaswamy (2009) Translationally controlled tumor protein is a novel heat shock protein with chaperone-like activity. Biochem. Biophys. Res. Commun. 386: 333–337.

Grison, R., B. Grezes-Besset, M. Schneider, N. Lucante, L. Olsen, J.J. Leguay and A. Toppan (1996) Field tolerance to fungal pathogens of *Brassica napus* constitutively expressing a chimeric chitinase gene. Nat. Biotechnol. 4: 643–646.

Hadwiger, L.A. and J.M. Beckman (1980) Chitosan as a component of pea-*Fusarium solani* interactions. Plant Physiol. 66: 205–211.

Hahn, M.G. (1996) Microbial elicitors and their receptors in plants. Annu. Rev. Phytopathol. 34: 387–412.

Han, F.P., G. Fedak, A. Benabdelmouna, K.C. Armstrong and T. Ouellet (2003) Characterization of six wheat × *Thinopyrum intermedium* derivatives by GISH, RFLP and multicolor GISH. Genome 46: 490–495.

Hoepflinger, M.C., J. Reitsamer, A.M. Geretschlaeger, N. Mehlmer and R. Tenhaken (2013) The effect of translationally controlled tumour protein (TCTP) on programmed cell death in plants. BMC Plant Biol. 13: 135.

Huang, X.Q., S.L.K. Hsam and F.J. Zeller (1997) Identification of powdery mildew resistance genes in common wheat (*Triticum aestivum* L. em Thell.). IX. Cultivars, land races and breeding lines grown in China. Plant Breed. 116: 233–238.

Kabashi, E. and H.D. Durham (2006) Failure of protein quality control in amyotrophic lateral sclerosis. Biochim. Biophys. Acta 1762: 1038–1050.

Kim, K.H., A.H.M. Kamal, K.H. Shin, J.S. Choi, H.Y. Heo and S.H. Woo (2010) Large-scale proteome investigation in wild relatives (A, B, and D genomes) of wheat. Acta Biochim. Biophys. Sin. (Shanghai) 42: 709–716.

Kisselev, A.F., D. Kaganovich and A.L. Goldberg (2002) Binding of hydrophobic peptides to several non-catalytic sites promotes peptide hydrolysis by all active sites of 20 S proteasomes. Evidence for peptide-induced channel opening in the alpha-rings. J. Biol. Chem. 277: 22260–22270.

Kristiansen, L.C., S. Jacobsen, F. Jessen and B.M. Jørgensen (2010) Using a cross-model loadings plot to identify protein spots causing 2-DE gels to become outliers in PCA. Proteomics 10: 1721–1723.

Lescot, M., P. Déhais, G. Thijs, K. Marchal, Y. Moreau, Y. Van de Peer, P. Rouzé and S. Rombauts (2002) PlantCARE, a database of plant *cis*-acting regulatory elements and a portal to tools for in silico analysis of promoter sequences. Nucleic Acids Res. 30: 325–327.

Li, W., X. Cui, Z. Meng, X. Huang, Q. Xie, H. Wu, H. Jin, D. Zhang and W. Liang (2012) Transcriptional regulation of Arabidopsis *MIR168a* and *ARGONAUTE1* homeostasis in abscisic acid and abiotic stress responses. Plant Physiol. 158: 1279–1292.

Liu, S.B. and H.G. Wang (2005) Characterization of a wheat-*Thinopyron intermedium* substitution line with resistance to powdery mildew. Euphytica 143: 229–233.

Liu, W., Y.Z. Zhang, X. Gao, K. Wang, S.L. Wang, Y. Zhang, Z.H. He, W.J. Ma and Y.M. Yan (2012) Comparative proteome analysis of glutenin synthesis and accumulation in developing grains between superior and poor quality bread wheat cultivars. J. Sci. Food Agric. 92: 106–115.

Lv, D.W., S. Subburaj, M. Cao, X. Yan, X. Li, R. Appels, D.F. Sun, W.J. Ma and Y.M. Yan (2014) Proteome and phosphoproteome characterization reveals new response and defense mechanisms of *Brachypodium distachyon* leaves under salt stress. Mol. Cell. Proteomics 13: 632–652.

Ma, J., T. Chen, S.F. Wu, C.Y. Yang, M.Z. Bai, K.X. Shu, K.L. Li, G.Q. Zhang, Z. Jin, F.C. He *et al.* (2019) iProX: an integrated proteome resource. Nucleic Acids Res. 47: D1211–D1217.

Majeti, N.V.R.K. (2000) A review of chitin and chitosan applications. React. Funct. Polym. 46: 1–27.

McIntosh, R.A. (1998) Breeding wheat for resistance to biotic stresses. Euphytica 100: 19–34.

Meredith, S.C. (2005) Protein denaturation and aggregation: Cellular responses to denatured and aggregated proteins. Ann. N. Y. Acad. Sci. 1066: 181–221.

Mittler, R., S. Vanderauwera, M. Gollery and F.V. Breusegem (2004) Reactive oxygen gene network of plants. Trends Plant Sci. 9: 490–498.

Nass, H.G. and B. Reiser (1975) Grain filling period and grain yield relationships in spring wheat. Can. J. Plant Sci. 55: 673–678.

Noctor, G. and C.H. Foyer (1998) Ascorbate and glutathione: keeping active oxygen under control. Annu. Rev. Plant Physiol. Plant Mol. Biol. 49: 249–279.

Oldach, K.H., D. Becker and H. Lörz (2001) Heterologous expression of the genes mediating enhanced fungal resistance in transgenic wheat. Mol. Plant Microbe Interact. 14: 832–838.

Pacifici, R.E., Y. Kono and K.J. Davies (1993) Hydrophobicity as the signal for selective degradation of hydroxyl radical-modified hemoglobin by the multicatalytic proteinase complex proteasome. J. Biol. Chem. 268: 15405–15411.

Philippa, B., R.G. Ricardo and U. Cristobal (2016) expVIP: a customizable RNA-seq data analysis and visualization platform. Plant Physiol. 170: 2172–2186.

Rabl, J., D.M. Smith, Y.D. Yu, S.C. Chang, A.L. Goldberg and Y.F. Cheng (2008) Mechanism of gate opening in the 20S proteasome by the proteasomal ATPases. Mol. Cell 30: 360–368.

Roberts, T.H. and J. Hejgaard (2008) Serpins in plants and green algae. Funct. Integr. Genomics 8: 1–27.

Sage-Ono, K., M. Ono, H. Harada and H. Kamada (1998) Dark-induced accumulation of mRNA for a homolog of translationally controlled tumor protein (TCTP) in *Pharbitis*. Plant Cell Physiol. 39: 357–360.

Shan, Q.W., Y.P. Wang, J. Li and C.X. Gao (2014) Genome editing in rice and wheat using the CRISPR/Cas system. Nat. Protoc. 9: 2395–2410.

Shewry, P.R. and N.G. Halford (2002) Cereal seed storage proteins: structures, properties and role in grain utilization. J. Exp. Bot. 53: 947–958.

Shewry, P.R., R.A.C. Mitchell, P. Tosi, Y. Wan, C. Underwood, A. Lovegrove, J. Freeman, G.A. Toole, E.N.C. Mills and J.L. Ward (2012) An integrated study of grain development of wheat (cv. *Hereward*). J. Cereal Sci. 56: 21–30.

Smirnoff, N. (1993) The role of active oxygen in the response of plants to water deficit and desiccation. New Phytol. 125: 27–58.

Tang, S., Z. Li and X. Jia (2000) Genomic in situ hybridization (GISH) analyses of *Thinopyrum intermedium*, its partial amphiploid Zhong 5, and disease-resistant derivatives in wheat. Theor. Appl. Genet. 100: 344–352.

Tardif, G., N.A. Kane, H. Adam, L. Labrie, G. Major, P. Gulick, F. Sarhan and J.F. Laliberté (2007) Interaction network of proteins associated with abiotic stress response and development in wheat. Plant Mol. Biol. 63: 703–718.

Valledor, L. and J. Jorrín (2011) Back to the basics: maximizing the information obtained by quantitative two dimensional gel electrophoresis analyses by an appropriate experimental design and statistical analyses. J. Proteomics 74: 1–18.

Virginia, G., J. Carpinelli, X. Jordana and L. Holuigue (2002) The as-1 promoter element is an oxidative stress-responsive element and salicylic acid activates it via oxidative species. Plant Physiol. 130: 1516–1526.

Wang, G.H. and J.C. Huang (1999) Selective degradation of proteins. Life Sci. 11: 24–30.

Wang, H., K. Wu, Y. Liu, Y. Wu and X. Wang (2015) Integrative proteomics to understand the transmission mechanism of *Barley yellow dwarf virus-GPV* by its insect vector *Rhopalosiphum padi*. Sci. Rep. 5: 10971.

Wang, R.M., J.S. Wu, X. Deng, D.M. Liu and Y.M. Yan (2018) Drought-responsive protein identification in developing grains of a wheat-*Haynaldia villosa* 6VS/6AL translocation line. Crop Pasture Sci. 69: 1182–1196.

Wang, R.R.C. and X.Y. Zhang (1996) Characterization of the translocated chromosome using fluorescence in situ hybridization and random amplified polymorphic DNA on two *Triticum aestivum-Thinopyrum intermedium* translocation lines resistant to wheat streak mosaic or barley yellow dwarf virus. Chromosome Res. 4: 583–587.

Wargo, P.M. (1975) Lysis of the cell wall of *Armillaria mellea* by enzymes from forest trees. Physiol. Plant Pathol. 5: 99–105.

Xin, Z.Y., R.I.S. Brettell, E.M. Cheng, P.M. Waterhouse, R. Appels, P.M. Banks, G.H. Zhou, X. Chen and P.J. Larkin (1988) Characterization of a potential source of barley yellow dwarf virus resistance for wheat. Genome 30: 250–257.

Xin, Z.Y., Z.Y. Zhang, X. Chen, Z.S. Lin, Y.Z. Ma, H.J. Xu, P.M. Banks and P.J. Larkin (2001) Development and characterization of common wheat-*Thinopyrum intermedium* translocation lines with resistance to barley yellow dwarf virus. Euphytica 119: 163–167.

Yan, H., L.H. Jia and G.X. Wang (2002) Advances in studies on proteins induced by water stress in plants. Life Chem. 2: 165–168.

Yu, Y.L., D. Zhu, C.Y. Ma, H. Cao, Y.P. Wang, Y.H. Xu, W.Y. Zhang and Y.M. Yan (2016) Transcriptome analysis reveals key differentially expressed genes involved in wheat grain development. Crop J. 4: 92–106.

Zhen, S.M., X. Deng, X.X. Xu, N.N. Liu, D. Zhu, Z.M. Wang and Y.M. Yan (2020) Effect of high–nitrogen fertilizer on gliadin and glutenin subproteomes during kernel development in wheat (*Triticum aestivum* L.). Crop J. 8: 38–52.

Zheng, S.Y., Y.H. Wang and Y. Xiao (2014) Analysis of TCTP gene expression in susceptible wheat Jing 411 induced by powdery mildew. Biotechnol. Bull. 9: 72–77.

Zhu, J.K. (2000) Genetic analysis of plant salt tolerance using Arabidopsis. Plant Physiol. 124: 941–948.