

RESEARCH ARTICLE

A proteomic study of spike development inhibition in bread wheat

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Spike development in wheat is a complicated development process and determines the wheat propagation and survival. We report herein a proteomic study on the bread wheat mutant strain 5660M underlying spike development inhibition. A total of 121 differentially expressed proteins, which were involved in cold stress response, protein folding and assembly, cell-cycle regulation, scavenging of ROS, and the autonomous pathway were identified using MS/MS and database searching. We found that cold responsive proteins were highly expressed in the mutant in contrast to those expressed in the wild-type line. Particularly, the autonomous pathway protein FVE, which modulates flowering, was dramatically downregulated and closely related to the spike development inhibition phenotype of 5660M. A quantitative RT-PCR study demonstrated that the transcription of the FVE and other six genes in the autonomous pathway and downstream flowering regulators were all markedly downregulated. The results indicate that spike development of 5660M cannot complete the floral transition. FVE might play an important role in the spikes development of the wheat. Our results provide the theory basis for studying floral development and transition in the reproductive growth period, and further analysis of wheat yield formation.

Received: January 16, 2013

Revised: June 17, 2013

Accepted: June 26, 2013

Keywords:

2DE / Autonomous pathway / Bread wheat / Cold responses / Plant proteomics / Spike-development inhibition



Additional supporting information may be found in the online version of this article at the publisher's web-site

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Abbreviations: DEPs, differentially expressed proteins; **DR-S**, double ridge stage; **M-D-D**, serving as the sample of dead tillers of 5660M at double ridge stage; **M-I-D**, serving as the sample of development-inhibited tillers of 5660M at double ridge stage; **M-S**, mature stage; **M-T**, the sample of 5660M at tillering stage; **N-D**, the sample of the 5660 at double ridge stage; **NIL**, near isogenic line; **N-T**, the sample of 5600 at the Tillering stage; **SE-S**, stem-elongation stage; **T-S**, Tillering stage

1 Introduction

Spike number in wheat (*Triticum aestivum* L.) is one of the three grain yield factors and is closely related to the wheat grain yield, accounting for 87% of the variation in yield [1]. Spikes development in cereal crops, e.g. rice and wheat, is a two-stage process, i.e. formation of axillary buds and subsequent outgrowth of the buds [2]. Li et al. [3] characterized the rice *mo1* mutant, which has only a culm and is without tillers. *MOC1*, which controls tillering and the formation of tiller buds, has been mapped to the long arm of chromosome

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6 and encodes a plant-specific GRAS transcription factor. In bread wheat, the recessive gene *tin* inhibits tiller formation and has been mapped to the short arm of chromosome 1AS [4]. A dominant gene *tin2* controlling the oligo-culm phenotype was located on 2A by the genetic analysis of Chinese Spring monosomic series [5]. Vasu and Shilpa (2007) isolated a wheat mutant that produced one main culm and lost tillering capability using ethyl methanesulphonate-based mutagenesis in diploid wheat. The mutant single recessive gene *tin3* was positioned to 3A long arm and then closely linked with *Xpsr1205* [6].

The development of the wheat tillers into spikes depends critically on its ability to precisely regulate the transition from vegetative to reproductive growth. The transition is a complex development process in plant that requires an integrated response to multiple environmental cues and endogenous signals [7]. For this report, we characterized the wheat mutant line 5660M, which has normal tillering capacity, but a spike development inhibition phenotype. The spike development inhibition phenotype of 5660M is controlled by a single recessive gene termed fertile tiller inhibition gene, which was mapped to 1AS and linked closely to markers *Xcfa2153* with 1.0 cM genetic distance [8]. The mutant single recessive gene caused the tillers to be unable to complete the floral transition and form spikes. In order to analyze the changes of the key proteins and genes related to spikes development inhibition phenotype, we examined the proteomes of the bread wheat line 5660M and its near isogenic line (NIL) 5660 and tried to isolate and identify the key proteins and metabolic pathways by 2DE technology and nano-scale LC–high-definition MS/MS. Based on the proteomic data, we measured the transcription levels of the spike development genes in wheat homologous to those in *Arabidopsis thaliana* and controlled the floral transition of the wheat. Our results increase our understanding of the spike development inhibition and provide a genetic basis to study the spike development of the common wheat.

2 Materials and methods

The wheat mutant line 5660M (*T. aestivum* L., $2n = 6x = 42$, AABBDD) and NIL 5660 (*T. aestivum* L., $2n = 6x = 42$, AABBDD) were generous gifts from the Institute of Crop Science, Beijing, China. The wheat NIL 5660 was the sixth-generation progenies of a cross between the wheat 4783 and YouLa (*T. aestivum* L., $2n = 6x = 42$, AABBDD), both of them were the wheat–*Agropyron cristatum* (L.) Gaertn. derived lines from the cross between Fukuhokomugi (*T. aestivum* L., $2n = 6x = 42$, AABBDD) and Z559 (*A. cristatum* (L.) Gaertn., $2n = 4x = 28$, PPPP; National Gene Bank accession number Z559). The wheat mutant line 5660M was spontaneously generated from one individual plant of the NIL 5660 at the seventh progenies during the generations of inbreeding. Then, 5660M and 5660 were self-crossed respectively at three generations by single-seed descent. The seeds of 5660M

and NIL5660 were planted on the six plots in Beijing on October 1, 2008 in autumn at above 20°C (Fig. 1A) that each plot contained 24 rows, 2 m in length, and separated by 0.25 m. The individuals from five plots were used to extract the protein and RNA. The individuals from one plot were used to count the number of tillers and spikes. Crop management was according to the local practice. Both of them would go through a long winter, as the temperature usually falls below zero here. In addition, their seeds were also planted in six rows in another plot respectively in Beijing on March 1, 2009 in spring so that the temperature is kept above zero (Fig. 1A).

2.1 Morphological and anatomical characterization

Wheat growth is divided into ten physiological stages: germination, emergence, tillering, double ridge, terminal spikelet, stem elongation, boot, spike emergence, anthesis, and maturity [9, 10]. At least 30 shoot apices of the 5660M and 5660 were obtained and counted at the tillering stage (T-S) which is the critical period of tillers formation and most of the shoot apices keep at the vegetative growth, double ridge stage (DR-S) which is the stage of the floral initiation and the two ridges of the floral primordium emerge at the shoot apex, stem-elongation stage (SE-S), and mature stage. The tillers from 5660M and 5660 were dissected and examined by stereomicroscopy (SZX2-ILLT, OLYMPUS) to assess the physiological stages of their shoot apices. It is identified as the T-S that most of the shoot apices keep at vegetative growth by the continuous observation of the NIL 5660 shoot apex's morphological anatomy. It is identified as the DR-S that the two ridges of the floral primordium obviously emerge at the most of the NIL 5660's shoot apices.

2.2 Protein extraction and quantification

Proteins were extracted according to [11] with minor modifications. Five different plots were used as biologists. One protein sample per plot was obtained from approximately 500 shoot apices that were collected on 100 individual plants from five different rows. The shoot apices of the samples of M-T, N-T, M-D-D, M-I-D, and N-D were harvested from the tillers of the 5660M and 5660 at the T-S (on Mar. 8, 2009, Fig. 1A) and DR-S (on Mar. 15, Fig. 1A). The apices were individually ground into fine powders in liquid nitrogen, and the powders were each suspended overnight in 30 mL of a 10% w/v TCA/acetone solution that contained 0.07% v/v mercaptoethanol at 20°C. After centrifugation at $40\,000 \times g$ for 1 h at 4°C, the pellets were each washed with 30 mL of ice-cold acetone. After vacuum drying, pellets were dissolved in 7 M urea, 2 M thiourea, 4% w/v CHAPS, 65 mM DTT, and incubated for an additional 2 h at 4°C. The suspensions

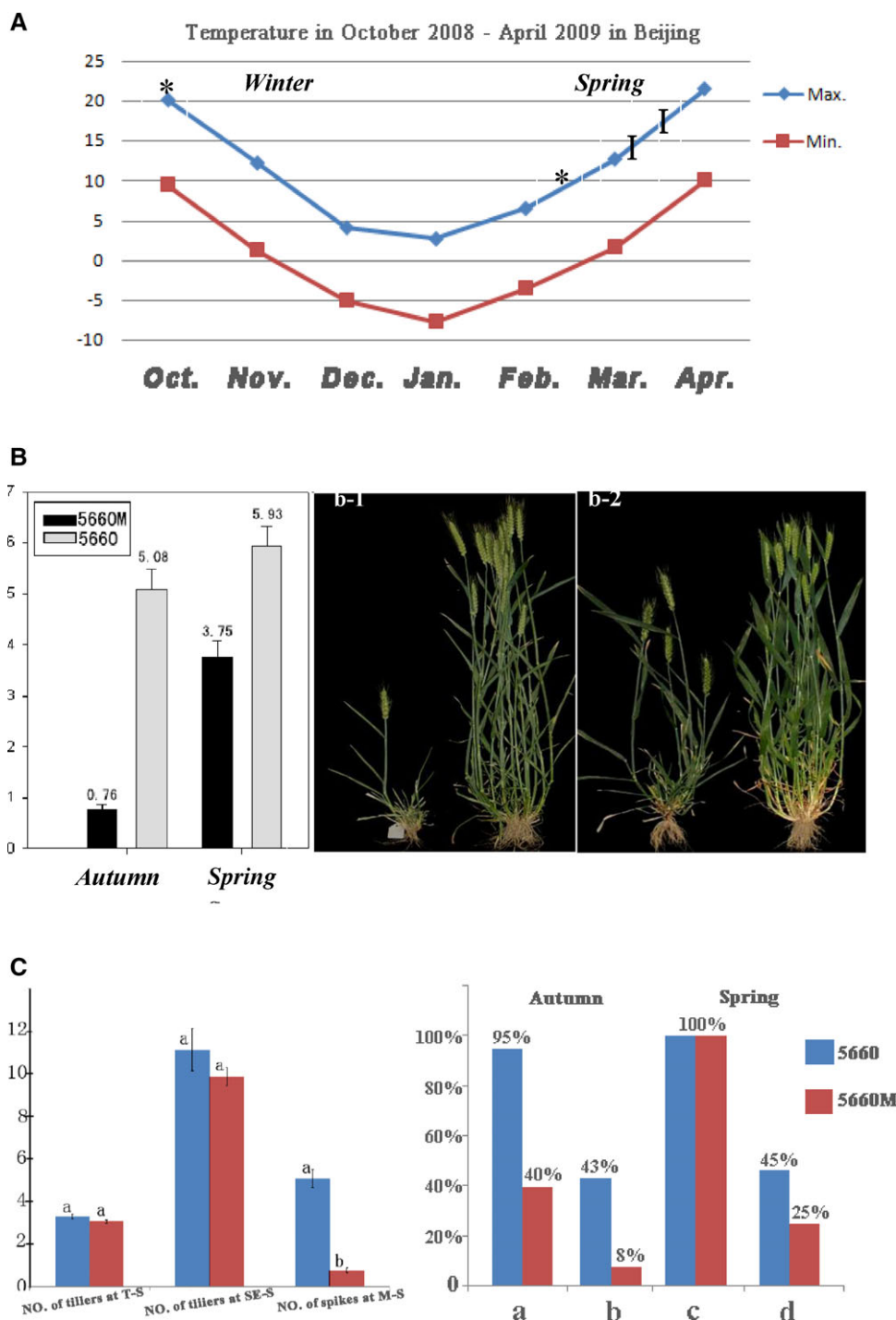


Figure 1. Temperature curve and statistical analysis of the number of spikes, tillering capacity, and degree of tillering inhibition for 5660M and 5660. (A) Temperature curve during the 5660M and 5660 development process. * indicates the planting dates of 5660 and 5660M in autumn and spring. I indicates the harvest date of the shoot apices. (B) Comparison of the number of spikes in 5660 and 5660M between autumn and spring ($p < 0.05$). b-1, Mature morphology of 5660M and 5660 that were planted in autumn; b-2, Mature morphology of 5660M and 5660 that were planted in spring. (C) Tillering capacity, number of spikes, and degree of tillering inhibition for 5660M and 5660. a, percentage of deformed apices of 5660 and 5660M; b, percentage of earbearing tiller of 5660 and 5660M in autumn; c, percentage of deformed apices of 5660 and 5660M in spring; d, percentage of earbearing tiller of 5660 and 5660M in spring.

were each centrifuged at $120\,000 \times g$ for 1 h at 4°C to remove insoluble material. Protein was quantified using 2D Quant kit reagents (GE Healthcare; Little Chalfont, UK) with BSA as the calibration standard. Then, the samples were immediately frozen in liquid nitrogen and stored at -80°C .

2.3 2DE, image analysis, and in-gel digestion

For IEF, we used an IPGphor IEF system and reagents (GE Healthcare), the extract concentration was $1800\,\mu\text{g}$ protein/mL in 8 M urea, 2% w/v CHAPS, 20 mM DTT, 0.5% IPG buffer. Protein ($800\,\mu\text{g}$) was loaded onto a commercial precast

IPG strip (24-cm length, pH 4–7 gradient), and actively rehydrated at 30 V for 8 h and 50 V for 4 h at 20°C. The focusing program was: 100 V for 1 h, 300 V for 1 h, 500 V for 1 h, 1000 V for 1 h, and 8000 V for 12 h for a total of 90 000 V-h. The strips were then equilibrated for 15 min in 20 mL of 50 mM Tris-HCl, pH 8.8, 6 M urea, 30% v/v glycerol, 2% w/v SDS, 1% w/v DTT, and then in a similar buffer that contained 2.5% w/v iodoacetamide but no DTT for 15 min. The strips were each placed on top of a vertical 12.5% SDS-PAGE gel in an Ettan DALT system (GE Healthcare), and the samples were electrophoresed for 1 h at 1 W/gel and then at 8 W/gel until the dye fronts reached the gel bottoms. Protein spots were detected using Blue silver, a modified colloidal CBB R-350 stain (GE Healthcare). The gels were fixed in 40% v/v ethanol/10% v/v acetic acid for 30 min and then swollen in 10% v/v acetic acid for 20 min. Next, the gels were stained for 10 min at 90–100°C in dye liquor (one piece of R-350 dissolved in 1.6 L of 10% acetic acid) and then rinsed with 10% v/v acetic acid until the gel spots were distinct from the background. Gels were stored in distilled water. Gel images were acquired using a Labscan 3.0 densitometer (GE Healthcare; resolution, 300 dots/inch). Image processing used ImageMaster 2D Platinum v6.0 software (GE Healthcare). Five Coomassie-stained biological replicated gels were acquired for each sample. Spots were detected automatically with the parameter settings: smooth, 2; minimum area, 54; and saliency, 12. Spots in the gels were manually edited for spot detection, and for split and merged spots. The gel pattern for each sample was first automatically matched with those of the two corresponding reference gels and then edited to correct for mismatched and unmatched spots. The volume of each spot was normalized to the sum of the spot volumes per gel and reported as a percentage (vol%). After averaging over the five gels for each spot, differences in vol% between gels for different samples were considered significant when their mean vol% differed by more than ± 1.5 -fold (Student's *t*-test, $p < 0.01$); [12]. To identify spot similarities between the 5660M and 5660 gels, 2D scatter plots of vol% versus vol% were prepared using ImageMaster 2D Platinum v6.0 software. Spot-by-spot comparisons of the reference and replicated gels were quantified as 2D scatter plots [13, 14]. The protein spots that had significant vol% differences were subjected to tryptic digestion according to [15] with minor modifications. The gel particles were excised and destained with 50 μ L of 25 mM NH_4HCO_3 in 50% ACN for 20 min. After that, the particles were dehydrated with 200 μ L of 100% ACN and then dried under vacuum for 10 min. Proteins in the particles were digested with 8 μ L of 25 mM NH_4HCO_3 , 5 ng/mL trypsin (Promega) and 12 μ L 25 mM NH_4HCO_3 for 12–14 h at 37°C. Then, the digested solutions were used in the MS/MS analysis.

2.4 Protein identification by nano LC-HDMS MS/MS and database searching

Nano LC–high-definition MS (HDMS)/MS/MS was performed using a nanoAcquity UPLC system (Waters Corp.,

Milford, USA) and a Synapt HDMS with a nanospray ion source (Waters), which was operated in the data-dependent mode for the MS/MS scans (2 s per scan). Tandem mass spectra were acquired for the two most intense peaks from each MS scan (1 s per scan) in the positive ion mode. The voltage of the noncoated capillary was 2300 V. Glu-fibrinopeptide served as the calibration standard for the MS/MS mode. Peak lists for each LC-MS/MS run were generated using PLGS 2.2 software (Waters) and automatically combined into a single PKL file. MS/MS-ion database searches were carried out by Mascot on line in the web (<http://www.matrixscience.com>). All tandem mass spectra were deconvoluted and transferred to a PKL file prior to a Mascot search. The mass tolerance for the precursor and fragment ion was 0.2 Da. Other search parameters were: one missed tryptic cleavage was allowed, carboxyamidomethylation of cysteine and methionine oxidation were allowed but not required, and the parent and fragment mass types were monoisotopic. The Viridiplantae database (green plants, 719 459 sequences) from NCBI was searched, and an ions score > 44 indicated sequence identity or extensive similarity between a sample sequence and one in the database ($p < 0.05$). Generally, if the protein spot showed multiple members of a protein family or was identified as multiple functional proteins, the highest score of that protein was selected to analyze its functions.

2.5 Transcription assay of flowering genes and C-repeat-binding factors (CBFs) by quantitative RT-PCR

About 150 shoot apices in 20 individuals from the five plots were used as one biological replicate to extract the total RNA. Total RNA was isolated from the five apex samples using the TRIZOL reagent and treated with DNase I (Promega) to remove contaminating genomic DNA. Total RNA (2 μ g) was used for reverse transcription with 25 μ L of the Moloney murine leukemia virus reverse transcriptase (Promega). RT-PCR was conducted using the reagents of a SYBR Premix Ex Taq kit (Takara) according to the manufacturer's recommendations. Relative transcript abundance was normalized to that of the 18S rRNA transcript. Specific RT-PCR primers and the mRNA sequences of the wheat-flowering genes were used to amplify the (CBFs) and flowering genes. The relative expression levels were calculated using the relative $2^{-\Delta\Delta C_t}$ method. The RT-PCR experiments used three replicates.

3 Results

3.1 Morphological and anatomical characterization of the spike development inhibition phenotype

Whether it is autumn or winter planting, all of 5660M have shown the spike development inhibition phenotype (Fig. 1B). It is noteworthy that the spike number of 5660M was

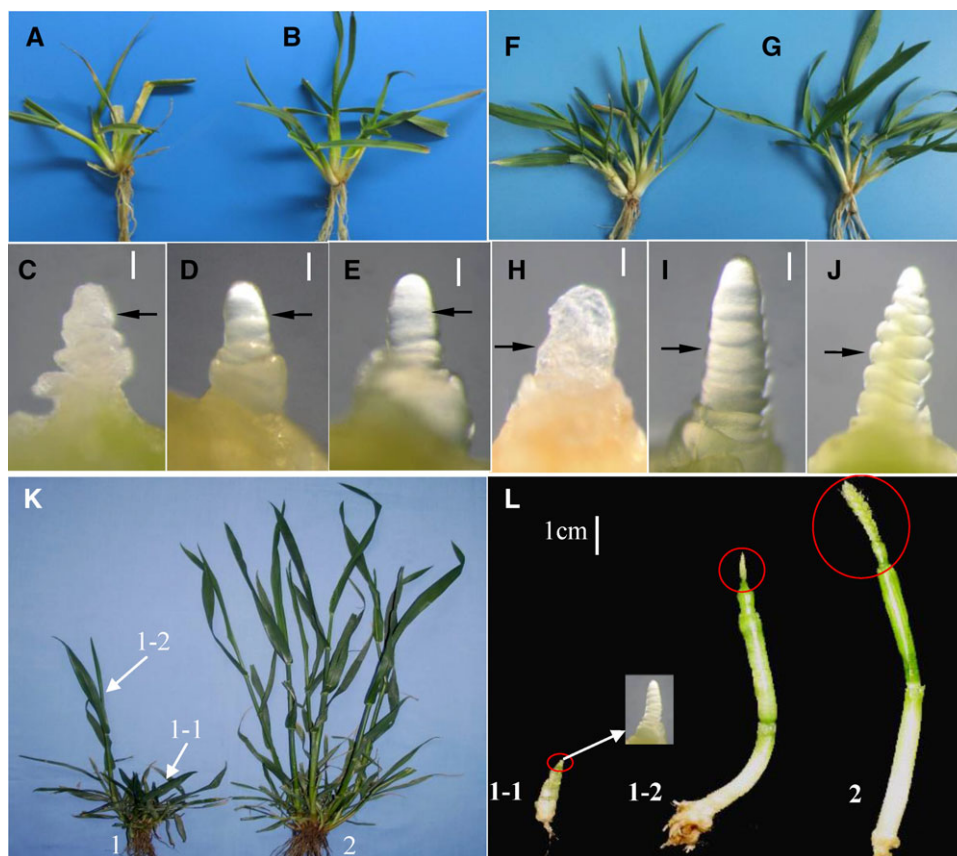


Figure 2. Morphology and anatomy of 5660M and 5660 at the T-S and DR-S. (A) Seedling morphology of 5660M at T-S. (B) Seedling morphology of 5660 at T-S. (C) The dead shoot apex of 5660M. (D) The inhibited shoot apex of 5660M. (E) The normal shoot apex of 5660. (F) Seedling morphology of 5660M at DR-S. (G) Seedling morphology of 5660 at DR-S. (H) The dead shoot apex of 5660M at DR-S. (I) The inhibited shoot apex of 5660M at DR-S. (J) The normal shoot apex of 5660 at DR-S. (K) Seedling morphology of 5660M and 5660 at the SE-S. (L) 5660 and 5660M tiller anatomy at the SE-S: 1-1, growth inhibited 5660M tiller. 1-2, growth inhibited 5660M spike (circled).

significantly increased when 5660M was planted in spring. The number of spikes when 5660M was grown in the spring was above 50% of that for 5660; the number of spikes when 5660M was planted in autumn and grown through the winter was much lower (Fig. 1A and B) while the spike number of 5660 showed no significant differences between the autumn and spring sowing (Fig. 1D). We further analyzed temperature trends during the development process of the 5660M and found 5660M grown through a long winter with a freezing temperature (Fig. 1A), while the temperatures have basically kept above freezing when the 5660M were planted in spring.

The 5660M had the normal tillering capacity. The number of tillers present at the T-S and the maximum number of tillers present at the SE-S were not significantly different for the 5660M and 5660 both of which were planted in autumn (Fig. 1B); the average number of tillers was 3.02 and 3.04 at the tillering stage and 9.86 and 11.06 at the SE-S for 5660M and 5660, respectively. However, the spike number of 5660M was significantly less than that for 5660 (0.76 and 5.08, respectively, $p < 0.01$, Fig. 1B). Anatomical examination indicated that 60% of the shoot apices in 5660M planted in autumn were deformed, lusterless at the T-S (Figs. 1C and 2C), and did not survive to the DR-S (Fig. 2H); the remaining shoot apices (40%) showed no significant difference in comparison to those in 5660 at the T-S (Fig. 2D and E), but developed significantly slower at the DR-S (Fig. 2I and J). Even

after the 5660 apices had transited from the vegetative to the reproductive stage and their floret primordia had developed (Fig. 2J), the 5660M apices remained at the vegetative with the two ridges barely detectable (Fig. 2I). The difference was that the shoot apices in 5660M planted in spring were completely normal at the T-S and not died at the DR-S. Finally, among the 40% of the 5660M tillers, only 8% of their apices developed into spikes (Fig. 1C, bar b), and these spikes grew more slowly than those of 5660 (Fig. 2K and L); the remaining 32% did not complete the floral transition and remained at the vegetative phase for the rest of their lives (Fig. 2K and L).

3.2 2DE mapping and protein spot identification

To delineate the expression changes in protein level underlying the phenotypic differences between lines 5660M and 5660, 2DE and MS/MS peptide sequencing was performed. At the T-S, there was no obvious difference between the dead and inhibited shoot apices of the 5660M. So the shoot apices of the 5660M were not been classed into two samples. At the DR-S, the dead and development inhibited shoot apices of 5660M were obviously classed into two samples. Then, the shoot apices were harvested and samples of the M-T were classed (Fig. 2C and D), N-T (Fig. 2E), M-D-D (Fig. 2H), M-I-D (Fig. 2I), and N-D (Fig. 2J). A total of 2000 ± 50 spots

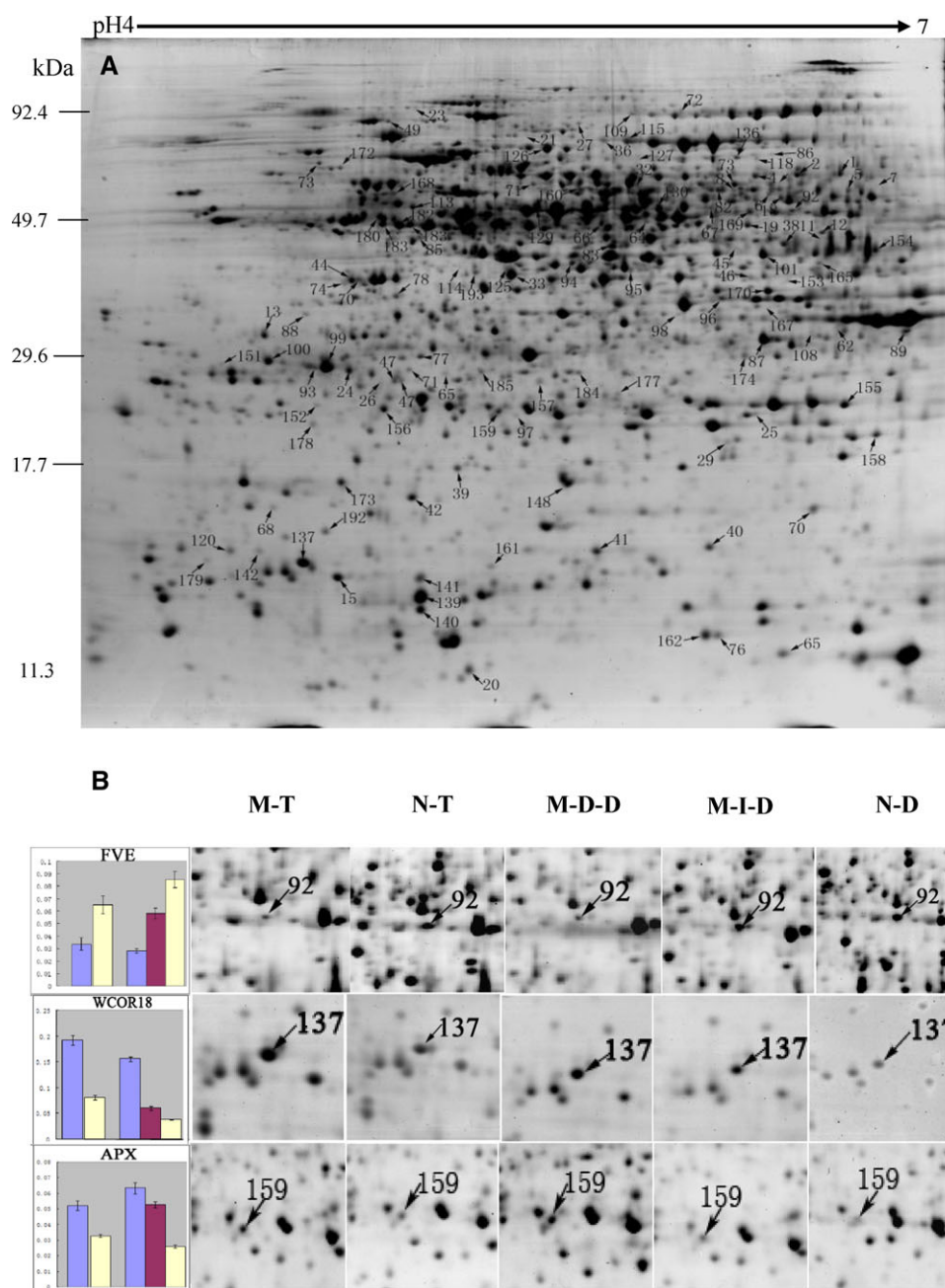


Figure 3. 2DE reference maps of apex proteomes. (A) 2DE map for the M-T apex proteome (5660M, T-S). (B) The expression pattern of the key identified DEPs.

with molecular masses between 10 and 110 kDa and pI values from 4 to 7 were reproducibly detected (Fig. 3A). There was a significant correlation between each of the replicate gels for each of the samples with respect to the vol% ($p < 0.0001$, Supporting Information Fig. 1). For the 5660M spots, 121 had significantly altered intensities compared with the corresponding 5660 spots, and almost all of the intensities for these spots increased by more than 1.5-fold in at least one sample, i.e. M-T, M-D-D, or M-I-D ($p < 0.01$). Spots having an altered intensity were excised and digested with trypsin, and the tryptic peptides were subjected to MS/MS. The identities of all

121 spots were determined using Nano LC-HDMS MS/MS and database searching (Table 1 and Supporting Information Table 1).

3.3 Functional classification of the differentially expressed proteins

Among the 121 differentially expressed proteins (DEPs), the sequences for 48 were found in the *T. aestivum* L. database.

Table 1. DEPs in the apexes of the mutant 5660M and wild-type wheat lines at the tillering and double-ridge stages

Spot number ^{a)}	Protein name	NCBI accession number	Species	T _{pl} /E _{pl} ^{b)}	T _{mw} /E _{mw} ^{c)}	Coverage ^{d)} (%)	Score	Average change ^{e)}		
								T-S		
								M-T/N-T	M-D/D/N-D	M-I-D/N-D
Protein folding and transport										
1	T-complex protein 1 subunit zeta	ACF80750	<i>Zea mays</i>	6.23/6.24	59.08/52.60	25	585	−1.93**	−1.32**	1.47**
2	T-complex protein 1 subunit gamma	NP_001152322	<i>Zea mays</i>	6.27/6.13	60.71/51.77	22	791	−2.2**	−1.82**	ns
4	T-complex protein 1 subunit gamma	XP_002325286	<i>Populus</i>	6.05/6.03	60.53/51.33	20	659	−1.82**	−1.77**	ns
5	T-complex protein 1 subunit ETA	NP_187789	<i>Arabidopsis thaliana</i>	6.03/6.26	59.74/50.37	28	955	−1.91**	−1.21*	ns
6	T-complex protein 1 subunit beta	NP_001050672	<i>Oryza sativa subsp. japonica</i>	5.56/6.06	57.19/48.87	28	828	−2.1**	−1.89**	1.35**
7	T-complex protein 1 subunit theta	BAD45605	<i>Oryza sativa Japonica Group</i>	6.16/6.51	60.23/59.13	9	103	−2.06**	−3.12**	−1.43**
8	Delta-coat protein 1	NP_001055159	<i>Oryza sativa subsp. japonica</i>	5.56/5.97	57.47/49.15	13	262	−1.86**	ns	1.5*
15	Cyclophilin A-1	AAK49426	<i>Triticum aestivum</i>	8.53/4.98	18.38/18.32	15	153	1.7**	3.34**	2.8**
16	Heat shock 70 kDa protein	ACR38468	<i>Zea mays</i>	5.62/5.27	72.70/62.49	20	668	−1.67**	−1.6**	1.4*
Protein catabolic process										
19	26S proteasome subunit 7	NP_001057037	<i>Oryza sativa subsp. japonica</i>	6.03/6.01	47.65/45.00	61	1401	−1.66**	−1.54**	ns
25	Proteasome subunit beta type-1	NP_001063603	<i>Oryza sativa japonica</i>	6.43/6.01	24.27/28.34	12	160	−1.44**	−1.69**	−1.6**
20	Cysteine proteinase inhibitor	ACM43995	<i>Triticum aestivum</i>	5.4/5.31	11.33/14.02	71	395	1.6**	2.32**	1.54**
21	Oligopeptidase B	BAE97779	<i>Triticum aestivum</i>	5.2/5.45	73.30/57.04	15	493	−1.93**	−2.51**	−
23	Presequence protease 1	XP_002452870	<i>Sorghum bicolor</i>	5.31/5.12	126.60/59.9	5	239	+	ns	1.2*
24	Aspartic proteinase	BAE20413	<i>Triticum aestivum</i>	5.14/5.01	54.28/33.56	18	397	1.3*	1.72**	1.27*
26	Papain-like cysteine proteinase	CAQ00103	<i>Hordeum vulgare subsp. vulgare</i>	5.28/5.09	38.63/32.08	6	84	1.27*	1.76**	ns
27	Chaperone protein ClpC2	NP_001066442	<i>Oryza sativa</i>	6.62/5.58	101.95/69.2	13	437	1.85**	4.7**	2.62**
Biosynthetic process										
32	Dihydroxy-acid dehydratase	NP_001062503	<i>Oryza sativa subsp. japonica</i>	7.12/5.73	63.61/49.58	25	444	−1.55**	ns	1.3*
33	Glutamine synthetase	AAZ30057	<i>Triticum aestivum</i>	5.41/5.41	39.17/39.35	27	700	1.76**	5.37**	2.08**
36	Methionine synthase	BAD34660	<i>Hordeum vulgare</i>	5.68/5.71	84.45/56.14	18	541	3.39**	−1.54*	1.84**
38	Tryptophan synthase	P43283	<i>Zea mays</i>	6.1/6.10	42.49/42.93	6	136	−1.68**	−1.94**	−1.69**
39	ID11 protein	BAB61039	<i>Hordeum vulgare</i>	5.23/5.28	23.45/23.62	27	268	1.45*	2.2**	1.57*
40	EIF5A2	AAZ95172	<i>Triticum aestivum</i>	5.7/5.63	17.35/19.16	56	643	ns	−2.25**	−2.88**
41	EIF5A2	AAZ95172	<i>Triticum aestivum</i>	5.7/5.63	17.35/19.16	56	643	1.8**	ns	2.94**

Table 1. Continued

Spot number ^{a)}	Protein name	NCBI accession number	Species	Tp/Ep ^{b)}	Tmw/Emw ^{c)}	Coverage ^{d)} (%)	Score	Average change ^{e)}		
								T-S	DR-S	M-I-D/N-D
								M-T/N-T	M-D-D/N-D	M-I-D/N-D
42	40S ribosomal protein S5	O65731	<i>Cicer arietinum</i>	9.91/5.16	22.01/22.23	11	105	1.84**	6.95**	2.84**
44	RPSAb	ACF85147	<i>Zea mays</i>	4.94/4.51	33.62/30.51	34	540	ns	1.83**	1.88**
45	AGD2	AAR01225	<i>Oryza sativa subsp. japonica</i>	8.3/5.98	49.84/42.05	21	527	–	–1.3*	–1.24**
46	cp-wap13	AAB61672	<i>Vigna unguiculata</i>	6.24/6.02	39.40/38.95	25	276	ns	ns	1.52**
47	CCoAMT	ABX60824	<i>Triticum monococcum</i>	4.67/5.12	17.90/29.38	28	221	1.39**	3.4**	1.79*
48	CCoAMT	ABX60824	<i>Triticum monococcum</i>	4.67/5.14	17.90/29.38	16	64	1.53**	2.74**	1.3*
60	EF-1-beta	P29546	<i>Triticum aestivum</i>	4.54/4.80	23.07/21.76	6	47	–2.25**	–1.46**	–
64	EF-1-alpha	Q03033	<i>Triticum aestivum</i>	9.2/5.76	49.14/51.14	12	189	–1.32**	–2.87**	–1.25**
62	Sterol methyltransferase	AAB49338	<i>Triticum aestivum</i>	7.53/6.21	41.61/37.03	28	541	–1.26**	–1.73**	–1.81**
63	RGPI protein	CAA09469	<i>Oryza sativa</i>	8.21/5.57	39.45/42.77	10	132	2.53**	+	ns
65	NHP2-like protein 1	NP_001049514	<i>Oryza sativa</i>	6.56/6.09	13.92/14.15	34	159	–1.3**	ns	1.76**
Transcription										
66	RH15	NP_001141599	<i>Zea mays</i>	5.52/5.64	48.49/50.95	40	635	ns	–1.73**	ns
18	MAC protein 3B	NP_001150622	<i>Zea mays</i>	6/6.09	57/48.87	22	661	–1.95**	–1.58**	ns
67	Tyrosyl-tRNA synthetase	NP_001148699	<i>Zea mays</i>	6.62/6.07	42.78/49.82	5	87	–1.39*	ns	1.56**
68	mRNA transport factor	AAV84882	<i>Triticum aestivum</i>	5.1/5.08	22.82/26.48	14	78	–1.24*	1.48*	2.68**
70	Transcription factor BTF3	NP_001064883	<i>Oryza sativa subsp. japonica</i>	6.62/6.21	17.62/16.23	41	236	–1.35*	–1.22*	–2.1**
71	Zinc finger protein-like	NP_001058435	<i>Oryza sativa</i>	5.27/5.49	45.12/56.45	6	140	1.32**	+	+
11	Plasminogen activator inhibitor	XP_002440366	<i>Sorghum bicolor</i>	5.74/6.20	40.64/44.17	6	149	–	–	–
12	Plasminogen activator inhibitor	XP_002440366	<i>Sorghum bicolor</i>	5.74/6.27	40.64/44.93	6	92	–1.49**	–2.88**	–2.39**
DNA replication										
72	MCM family protein	NP_001054989	<i>Oryza sativa Japonica Group</i>	5.55/5.23	92.48/50.46	24	823	–1.2*	–1.45**	1.27*
13	PCNA	NP_001048438	<i>O. sativa subsp. japonica</i>	4.62/4.52	29.25/28.12	36	487	–1.25*	–2.03**	–1.2**
Purine ribonucleoside salvage										
70	Adenosine kinase	BAC02723	<i>Oryza sativa</i>	5.29/5.27	32.17/26.56	30	457	+	+	+
Calvin cycle										
73	RuBisCO large subunit	NP_114267	<i>Triticum aestivum</i>	6.22/5.98	52.82/50.15	36	990	–1.9**	–2.4**	–1.4**
75	RuBisCO large subunit	NP_114267	<i>Triticum aestivum</i>	6.22/5.42	52.82/33.71	19	461	2.67**	1.53**	1.64**
76	RuBisCO small subunit	Q40004	<i>Hordeum vulgare</i>	8.98/5.91	19.41/15.48	44	256	3.13**	2.3**	1.3*
77	OEE1	ABQ52657	<i>Leymus chinensis</i>	6.08/5.18	34.49/31.45	40	783	1.96**	1.55**	–1.58**
78	Xantha-h	AAA99720	<i>Hordeum vulgare subsp. vulgare</i>	4.89/5.13	36.42/41.49	12	203	ns	2.04**	1.3*

Table 1. Continued

Spot number ^{a)}	Protein name	NCBI accession number	Species	T _p /E _p ^{b)}	T _m w/E _m w ^{c)}	Coverage ^{d)} (%)	Score	Average change ^{e)}		
								T-S		
								DR-S		
M-T/N-T										
M-D-D/N-D										
M-I-D/N-D										
Pentose phosphate pathway										
82	g6pdh	BAA97663	<i>Triticum aestivum</i>	5.92/5.93	58.29/49.58	27	656	2.02**	1.84**	1.93**
83	6-phosphogluconate dehydrogenase	NP_001056586	<i>Oryza sativa subsp. japonica</i>	5.85/5.65	52.69/43.16	11	399	1.69**	1.34*	1.2**
84	Fructokinase-2	NP_001060837	<i>Oryza sativa subsp. japonica</i>	5.02/5.35	35.49/35.61	18	290	-	-	-
85	Transaldolase 2	NP_001150674	<i>Zea mays</i>	6.38/5.18	46.10/43.22	12	328	3.14**	3.92**	3.03**
86	Transketolase	CAO49711	<i>Vitis vinifera</i>	5.95/6.04	67.14/53.76	5	169	1.55*	-1.4**	ns
Signal transduction										
87	GPB-LR	NP_001043910	<i>Oryza sativa subsp. japonica</i>	5.97/6.05	36.21/33.18	14	201	-1.71**	ns	ns
88	C2 domain-containing protein	NP_001062521	<i>Oryza sativa subsp. japonica</i>	4.77/4.91	30.33/35.49	7	82	+	+	ns
90	GID1L2	NP_001150025	<i>Zea mays</i>	5.64/5.31	35.28/33.57	3	75	2.37**	1.51**	ns
89	annexin2	NP_001105475	<i>Zea mays</i>	6.82/6.39	35.24/36.61	22	335	ns	-1.43**	1.36**
92	OSFV	NP_001044036	<i>Oryza sativa</i>	5.87/6.11	50.08/52.66	15	379	-1.93**	-3.61**	-1.56**
93	14-3-3B	Q43470	<i>Hordeum vulgare</i>	4.67/4.93	29.67/32.95	46	676	1.42**	2.34**	4.1**
100	14-3-3B	Q43470	<i>Hordeum vulgare subsp. vulgare</i>	4.67/4.86	29.67/33.63	17	193	1.28**	ns	ns
99	14-3-3A	CAA44259	<i>Hordeum vulgare subsp. vulgare</i>	4.83/4.95	29.25/32.84	41	644	1.28**	2.33**	2.81**
Glycolysis										
94	Phosphoglycerate kinase	P12783	<i>Triticum aestivum</i>	5.64/5.56	42.10/40.32	11	240	2.96**	2.77**	ns
95	Phosphoglycerate kinase	P12783	<i>Triticum aestivum</i>	5.64/5.71	42.10/39.12	27	451	3.08**	+	+
96	Fructose-bisphosphate aldolase	ACO44684	<i>Triticum aestivum</i>	6.85/5.93	38.77/37.03	14	208	1.6**	1.41**	1.29*
97	Triosephosphate isomerase	CAC14917	<i>Triticum aestivum</i>	5.38/5.42	26.79/27.35	29	387	+	+	+
Tricarboxylic acid cycle										
101	Isocitrate dehydrogenase	NP_001043749	<i>Oryza sativa subsp. japonica</i>	6.34/6.05	46.01/41.44	35	941	-1.63**	ns	1.5**
108	Malate dehydrogenase	EAO7096	<i>Oryza sativa subsp. indica</i>	7.66/6.17	41.55/34.25	19	340	-1.5**	-1.9**	-1.54**
109	Aconitate hydratase	XP_002445174	<i>Sorghum bicolor</i>	6.41/5.73	107.87/2.66	12	539	ns	-2.28**	1.2*
ATP synthesis										
1113	ATP synthase subunit beta	NP_114266	<i>Triticum aestivum</i>	5.06/5.16	53.82/47.00	60	1530	1.56**	1.55**	1.39**
1114	ATPB	P17614	<i>Nicotiana plumbaginifolia</i>	5.95/5.28	59.82/40.03	6	166	-3.4**	-1.35**	ns

Table 1. Continued

Spot number ^{a)}	Protein name	NCBI accession number	Species	T _{pl} /E _p ^{b)}	T _{mw} /E _{mw} ^{c)}	Coverage ^{d)} (%)	Score	Average change ^{e)}		
								T-S	DR-S	M-I-D/N-D
116	ATP synthase	AAT36616	<i>Triticum aestivum</i>	7.71/5.59	27.09/29.29	60	842	ns	ns	2.37**
117	ATP synthase D	XP_002459794	<i>Sorghum bicolor</i>	6.02/5.8	19.74/19.01	23	249	−1.23*	1.25*	1.92**
115	Cl−75kD	ACG43573	<i>Zea mays</i>	6.1/5.78	80.59/64.36	20	836	1.87**	−1.77**	2.03**
120	Metal ion transport cch	CAE51321	<i>Hordeum vulgare</i> var. <i>distichum</i>	4.67/4.71	11.24/19.38	41	328	1.5**	2.55**	1.72**
126	Transketolase	AAO33154	<i>Oryza sativa Japonica</i> Group	6.12/5.51	79.98/63.73	12	433	ns	2.82**	1.48**
127	ARA-I	AAK38481	<i>Hordeum vulgare</i>	5.59/5.79	81.94/61.09	8	221	ns	−1.62**	1.92**
129	Xylose isomerase	Q40082	<i>Hordeum vulgare</i>	5.31/5.50	53.58/53.77	40	966	ns	2.19**	ns
130	Lactoylglutathione lyase	NP_001130143	<i>Zea mays</i>	6.2/5.78	38.26/52.66	12	146	ns	1.33**	−1.3*
131	Beta-1,3-glucanase	AAD28732	<i>Triticum aestivum</i>	5.69/5.52	34.86/32.29	30	490	ns	2.03**	1.34**
136	Heat shock protein STI	NP_001151932	<i>Zea mays</i>	6.26/5.98	65.51/53.91	15	453	−1.98**	−2.13**	−1.13*
137	Cold regulated protein Wcor18	BAC41494	<i>Triticum aestivum</i>	4.84/4.89	17.73/18.91	47	629	1.77**	3.31**	1.16*
139	Pathogenesis-related protein 10	ACG68733	<i>Triticum aestivum</i>	5.19/5.19	17.05/17.04	83	1005	4.03**	18.43**	3.45**
140	Pathogenesis-related protein 10	ACG68733	<i>Triticum aestivum</i>	5.19/5.18	17.05/16.48	51	465	3.06**	14.19**	2.4**
141	Pathogenesis-related protein 10	ACG68733	<i>Triticum aestivum</i>	5.19/5.18	17.05/17.98	64	679	1.8**	4.65**	1.76**
142	LEA/RAB-related COR protein	AAF68628	<i>Triticum aestivum</i>	4.84/4.78	17.15/18.79	28	204	2.88**	+	+
143	WCOR615	AAB18208	<i>Triticum aestivum</i>	4.92/4.78	17.77/18.79	29	150	2.88**	+	+
147	WCOR719	AAC49404	<i>Triticum aestivum</i>	4.43/4.67	15.76/18.81	16	84	1.51**	2.37**	1.61**
148	Cold shock domain protein 3	BAD08701	<i>Triticum aestivum</i>	5.73/5.57	21.53/24.38	74	957	1.57**	+	+
151	Chitin catabolic process	AAD28733	<i>Triticum aestivum</i>	4.69/4.68	29.01/31.09	42	366	+	+	+
150	Chitinase IV precursor	BAB82472	<i>Triticum aestivum</i>	6.06/5.51	34.14/33.01	32	410	ns	+	ns
152	Wali7 protein	AAC37416	<i>Triticum aestivum</i>	7.64/4.96	29.48/28.87	25	395	ns	1.76**	ns
185	Aluminum-induced protein	AAP12992	<i>Oryza sativa Japonica</i> Group	9.65/5.34	35.51/28.42	10	212	1.59**	+	+
153	Stearyl-Acyl-carrier protein desaturase	BAB86112	<i>Oryza sativa subsp. japonica</i>	6.39/6.10	44.85/38.78	18	472	−2.88**	−2.08**	ns
154	Alcohol dehydrogenase	ABL74258	<i>Triticum aestivum</i>	6.15/6.30	40.99/41.91	22	441	2.3**	2.13**	1.52**
155	Glutathione S transferase	NP_001048884	<i>Oryza sativa subsp. japonica</i>	6.67/6.24	25.64/29.38	28	299	−1.82**	ns	2.56**
156	Glutathione transferase	CAC94005	<i>Triticum aestivum</i>	5.01/5.09	26.40/27.23	39	590	1.72**	4.37**	2.2**

Table 1. Continued

Spot number ^{a)}	Protein name	NCBI accession number	Species	TpI/EpI ^{b)}	Tmw/Emw ^{c)}	Coverage ^{d)} (%)	Score	Average change ^{e)}		
								T-S	M-T/N-T	M-D-D/N-D
157	Glutathione S transferase 24	NP_001104988	<i>Triticum aestivum</i>	5.36/5.4	26.44/32.02	5	84	–1.22*	–1.24**	–1.75**
158	Manganese SOD	AAB68035	<i>Triticum aestivum</i>	7.9/6.34	25.26/26.06	44	525	–1.77**	1.55**	1.2**
162	Superoxide dismutase	NP_001060564	<i>Oryza sativa subsp. japonica</i>	5.92/5.90	15.07/10.39	19	120	1.38**	3.97**	2.4**
159	Ascorbate peroxidase	AAL08496	<i>Hordeum vulgare</i>	5.1/5.34	27.62/28.46	8	123	1.4*	2.5**	2.14**
160	Betaine-aldehyde hydrogenase	AAL05264	<i>Triticum aestivum</i>	5.44/5.58	54.37/49.36	23	530	1.4**	1.52**	1.35**
161	Glutathione peroxidase	EEC77777	<i>Oryza sativa subsp. indica</i>	7.63/5.36	18.47/18.53	25	298	1.48**	2.67**	1.44**
167	Aldo/keto reductase AKR	XP_002436403	<i>Sorghum bicolor</i>	6.23/6.05	37.64/36.34	14	245	–	–	–
168	Protein disulfide isomerase	AAK49425	<i>Triticum aestivum</i>	4.96/5.11	56.59/48.80	31	504	–1.74**	–1.45**	–1.76**
169	Glutathione reductase	AAQ64632	<i>Triticum monococcum</i>	5.93/6.02	52.98/42.73	34	830	–1.69**	–1.87**	ns
170	Protein disulfide isomerase	NP_001054696	<i>Oryza sativa subsp. japonica</i>	6.58/6.06	39.89/37.89	14	261	–1.45**	1.63**	1.46**
171	Protein disulfide isomerase 2	AAK49424	<i>Triticum aestivum</i>	5.03/5.09	56.41/56.45	16	247	1.83**	6.09**	3.38**
172	PDI-like protein	NP_001105407	<i>Zea mays</i>	4.8/4.98	63.32/60.38	5	126	1.64**	4.43**	2.15**
173	Thioredoxin peroxidase	AAC78473	<i>Secale cereale</i>	6.34/5.0	28.12/24.51	45	497	ns	2.07**	ns
174	PIG3	ABK23274	<i>Picea sitchensis</i>	6.09/5.98	34.63/34.37	9	87	1.17*	2.94**	1.78**
175	Pox1 protein	CAA59484	<i>Triticum aestivum</i>	6.06/5.43	33.16/37.20	4	80	ns	+	ns
177	GSTZ5	ABF95327	<i>Oryza sativa subsp. japonica</i>	8.86/5.68	33.81/28.27	7	85	+	+	+
178	Dehydroascorbate reductase 1	BAA90672	<i>Oryza sativa subsp. japonica</i>	5.0/4.93	23.54/26.73	8	48	3.9**	5.94**	1.92**
Cell structure										
179	Actin depolymerization factor	ACH69772	<i>Hordeum vulgare subsp. vulgare</i>	4.69/4.67	15.83/18.81	48	108	1.51**	2.37**	1.61**
180	Tubulin beta-5 chain	Q9ZRA8	<i>Triticum aestivum</i>	4.73/5.04	50.28/52.43	70	2194	–1.62**	–2.02**	ns
181	Alpha tubulin	AAB08791	<i>Hordeum vulgare subsp. vulgare</i>	4.92/5.01	49.58/52.25	49	1068	ns	–1.61**	1.56**
182	Alpha tubulin-4D	ABD92936	<i>Triticum aestivum</i>	4.81/5.09	49.74/50.95	48	972	ns	ns	2.03**
183	Alpha tubulin-4D	ABD92936	<i>Triticum aestivum</i>	4.81/5.13	49.74/50.61	35	786	ns	–1.3**	1.6**

a) Number of the protein spot in the gel.

b) M_r calculated using the protein sequence/ pI value as estimated by the migration of the protein through the gel.c) pI value calculated using the protein sequence/ pI value as estimated by the migration of the protein through the gel.

d) Percentage of predicated peptide sequences covered by the matched peptide sequences from the MS/MS peak list.

e) Spot abundance expressed as an average of the vol% of the protein from the 5660M samples divided by the vol% of the protein from the 5660 samples, upregulated (+) or downregulated (–), absent in 5660M (–). The changes in vol% were statistically significant (* stands for $p < 0.05$; ** stands for $p < 0.01$).

In addition, 78 DEPs sequences had homologous sequences to those in other plant databases, including those of *Oryza sativa*, *Zea mays*, and *Hordeum vulgare* (Table 1). To confirm the identities of the DEPs and to determine their functions, the amino acid sequence of each protein was used as the query to search the expressed-sequence-tag (EST) database of *T. aestivum* L. Each matched database sequence was then used to search for a homolog(s) in the Viridiplantae database (Supporting Information Table 1). By doing so, the functions of all 121 proteins were confirmed. The 121 DEPs were grouped into 18 categories according to their functional annotations in UniProtKB databases and found to be involved in many different processes. The largest group contained proteins involved in redox reactions (18.9%). Other groups of note contained proteins involved in biosynthetic processes (14.8%), protein folding and transport (7.4%), stress response (7.4%), transcription (7.4%), protein catabolic processes (6.6%), and signal transduction (6.6%, Supporting Information Fig. 2).

3.4 DEPs involved in cold-stress response and defense against oxidation stress

In the present study, most of the DEPs involved in response to the cold stress and oxidation stress were upregulated in 5660M. The intensities of five 5660M DEPs spots that involved in cold-stress response changed dramatically during the T-S and DR-S. These proteins are cold-regulated protein (WCOR18, spot 137, Fig. 3A and B), cold-responsive LEA/RAB-related COR protein (spot 142), cold-acclimation protein WCOR615 (spot 143), WCOR719 (spot 147), and cold-shock domain protein 3 (spot 148). The amounts of these proteins from 5660M were all several fold higher than their counterparts from 5660 (Table 1). In the meanwhile, the amounts of these proteins consistently decreased in both normal and mutant lines from the T-S to the DR-S as the temperature increased. The cold-responsive LEA/RAB-related COR protein, WCOR615, and cold-shock domain protein 3 were identified in the N-T sample but absent in the N-D sample. Seven DEPs were identified as redox proteins involved in ROS detoxification (Table 1), namely, manganese superoxide dismutase (spot 158), Cu/Zn superoxide dismutase 2 (spot 162), ascorbate peroxidase (spot 159, Fig. 3B), glutathione peroxidase (spot 161), glutathione reductase (spot 169), class III peroxidase (spot 175), and GSH-dependent dehydroascorbate reductase 1 (spot 178). Compared with those in 5660, the intensities of spots 159, 161, 162, 175, and 178 were significantly upregulated in the M-T, M-I-D, and M-D-D samples (Table 1). These detoxifying enzymes were upregulated or expressed at constant levels during the T-S and DR-S in 5660M, especially in M-D-D, whereas in 5660, the expression of these DEPs was significantly reduced, indicating that the antioxidant defense systems in 5660M responded to oxidative stress. The two phenotypes, death of shoot apices for M-D-D and slow spike development for M-I-D was correlated positively with the increased levels of ROS-related enzymes in 5660M.

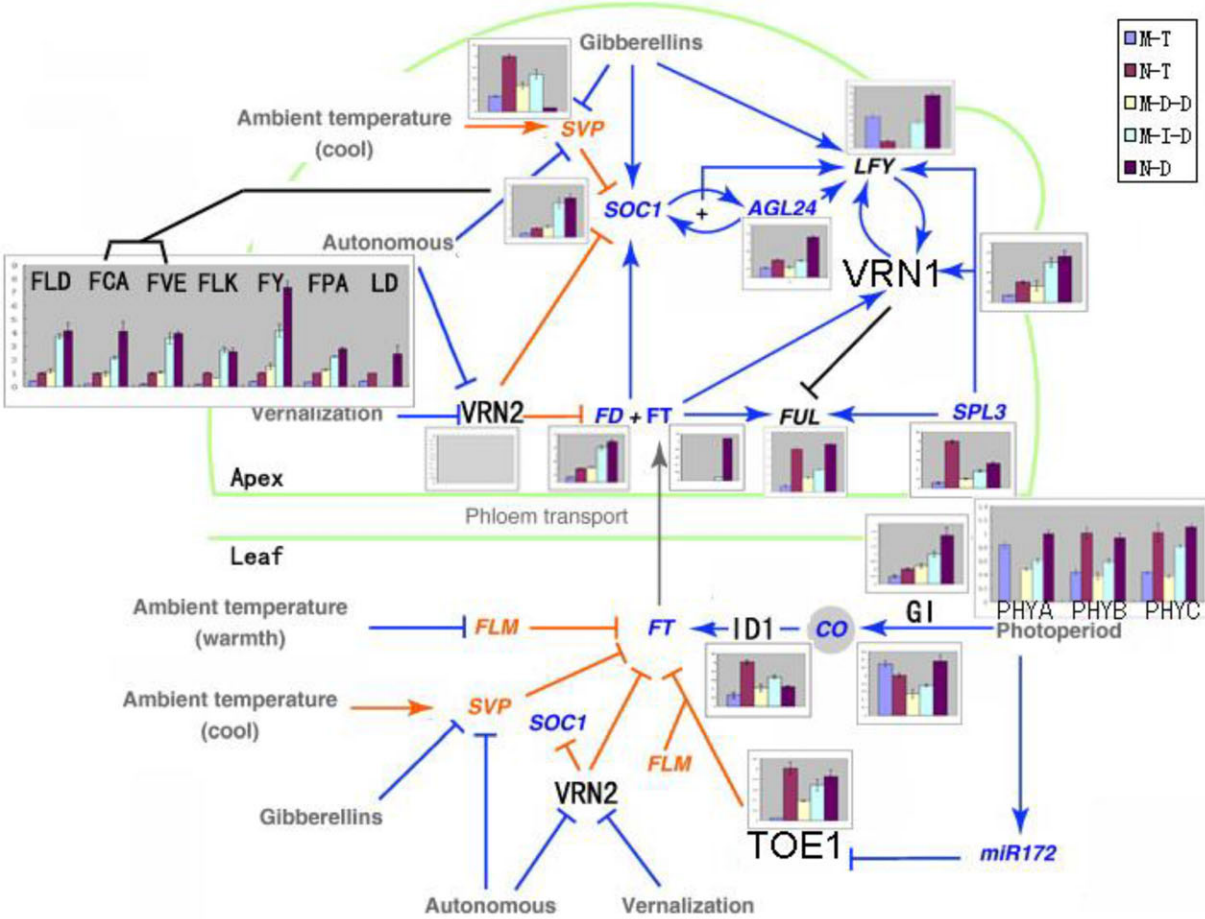
3.5 DEPs involved in protein folding and assembly

Eleven of the DEPs were identified as molecular chaperones, i.e. five of the eight subunits of T-complex protein 1 (TCP-1, spots 1–7), peptidyl-prolyl cis-trans isomerase (spot 15), heat-shock 70-kDa protein (spot 16), heat-shock protein 90 (spot 17), heat-shock protein ST1 (spot 136), chopper chaperone (spot 120), and delta-coat protein 1 (spot 8). The sequences of the five TCP-1 subunits are >80% identical to those of *A. thaliana* and 90% identical to those of *O. sativa*. These TCP-1 subunits were all substantially downregulated at the sample of M-T and M-D-D. In addition to the identified TCP-1 subunits, heat-shock protein ST1 and heat-shock protein 70 were also downregulated in 5660M. In contrast to these chaperones, the 14–3–3-like proteins A and B, which can bind many signal proteins, e.g. kinases, phosphatases, and transmembrane receptors, were all upregulated in 5660M.

3.6 The expression of FVE and flower development genes were inhibited in 5660M

Strikingly, FVE (spot 92) was downregulated in 5660M at the T-S and DR-S compared with that in 5660 (Table 1 and Fig. 3B). This protein plays an important role in the transition from vegetative to reproductive phases in *Arabidopsis* [16]. It has been reported that at least seven genes (*FLOWERING LOCUS D*, *FVE*, *FCA*, *FPA*, *FY*, *FLK*, and *LUMINIDEPENDENS*) are part of the autonomous pathway [17–19]. The finding that FVE was downregulated caused us to consider if the expression of the other six genes also changed in the apices of 5660M at the T-S and DR-S. We assayed the transcription of the aforementioned seven genes in 5660M and 5660, all of which were more highly expressed in 5660 than in 5660M (Fig. 4A), which suggested that the developmental spike inhibition phenotype in 5660M may be relevant to the downregulation of these genes. These findings were consistent with the observation that 5660M apices, for the most part, cannot complete the transition from the vegetative to the reproductive phase. We also assayed the transcription of the downstream genes regulated by the autonomous pathway, namely short vegetative phase (*SVP*), vernalization2 (*VRN2*, analogous to flowering locus C (*FLC*)), suppression of overexpression of constans 1 (*SOC1*), flowering locus T (*FT*), *FD*, agamous-like 24 (*AGL24*), leafy (*LFY*), and vernalization1 (*VRN1*, *APETALA1* (*API*) in *Arabidopsis*), which have been reported to be regulators of flowering during the floral transition in *Arabidopsis* [19–21]. Six of the genes, *SOC1*, *FT*, *FD*, *AGL24*, *LFY*, and *VRN1*, which are positive regulators of the floral transition, were all downregulated, whereas *SVP*, which negatively regulates *SOC1* and *FT* [20], was upregulated in 5660M. *VRN2*, another negative regulator [21], was not detected in 5660M or 5660 at both developmental stages after winter. The expression patterns of these genes involved in flowering were similar to those in the autonomous pathway. Most of the flowering genes were upregulated from the T-S

A



B

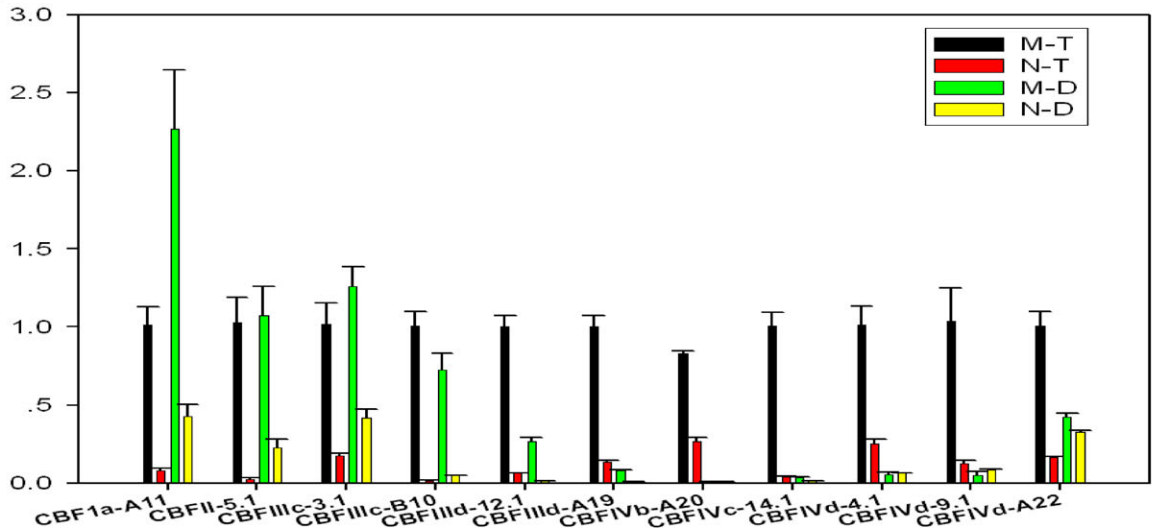


Figure 4. Quantitative RT-PCR expression analysis of flowering genes and *TaCBF* genes in 5660M and 5660 at T-S and DR-S. (A) The transcriptional expression pattern of 24 spike development genes in wheat at T-S and DR-S. These regulators of flowering are involved in the photoperiod, vernalization, ambient temperature, and autonomous pathways or are downstream regulators and integrators that promote flowering [19]. The repressors of the floral transition are indicated in orange; the activators are indicated in blue; arrows indicate the activators of downstream genes, and bars indicate the repressor of downstream genes. (B) The transcription level of *TaCBF* genes.

to DR-S. The transcription of the flowering genes was closely correlated with the spike development inhibition phenotype in M-D-D, M-I-D, and normal development of shoot apices in N-D.

3.7 C-repeat binding factors (CBFs) regulated *COR* genes are all upregulated in 5660M

Aforementioned several detected *COR* proteins that might be transcriptionally activated through the CBF-mediated pathway were all intensely upregulated in 5660M. Therefore, we used QRT-PCR to characterize the expression patterns of genes encoding CBFs, which are involved in transcriptional regulation of LT acclimation and considered as key regulators of cold-responsive (*COR*) genes [22]. Of the 15 known wheat CBFs (*TaCBF1a-A11*, *TaCBF11-5.1*, *TaCBF11a-6.1*, *TaCBF11c-3.1*, *TaCBF11c-B10*, *TaCBF11d-12.1*, *TaCBF11d-A15*, *TaCBF11d-A19*, *TaCBF11a-A2*, *TaCBF11b-A20*, *TaCBF11c-14.1*, *TaCBF11d-4.1*, *TaCBF11d-9.1*, *TaCBF11d-A22*) [23], 11 were specifically expressed in the 5660 and 5660M shoot apices at the T-S and DR-S. All of them were upregulated in 5660M, especially at the T-S (Fig. 4B). They had the same expression pattern to *COR* proteins and were downregulated, as the temperature increased from the T-S to DR-S.

4 Discussion

4.1 Cold stress affected the number of spikes in 5660M

In the present study, we systemically characterized gene transcription and protein expression in the mutant wheat 5660M line that has a spike inhibition phenotype. Strikingly, many proteins strongly responded to cold stress, particularly the cold-regulated *WCOR18*, *WCOR615*, and *WCOR719*, which were more highly expressed in 5660M than in 5660 at the T-S and DR-S. *WCOR719* can be rapidly and strongly upregulated by LT and also help preserve the structural integrity of plant cells during freezing stress [24]. *WCOR18* is a member of the phosphatidyl ethanolamine binding protein superfamily, whose members regulate protein phosphorylation and inhibit the serine proteases chymotrypsin and elastase [25–28]. *COR* genes contain a C-repeat/dehydration response element (CRT/DRE) in their promoters, and CBFs, which are CRT/DRE-binding transcription factors, are key regulators of the cold-response pathway [22, 29]. We further analyzed the transcription expression of CBFs and found all of them were upregulated in 5660M. These results indicated that 5660M might be more sensitive to cold stress. By the morphological and anatomical analysis, we found the number of spikes in 5660M that planted in spring was significantly increased in comparison to that planted in autumn and 60.5%

of the 5660M shoot apices did not survive through the winter. The number of spikes and spike development inhibition phenotype in 5660M was distinctly impacted and correlated to the low temperature, especially the freezing temperature. Finally, the shoot apices of the 5660M were vulnerable to the freezing temperature commonly experienced by wheat, and they did not develop into the spikes due to sensitivity to low temperature.

Secondary signals such as ROS can induce Ca^{2+} signaling to influence cold signaling [30–33]. However, high-level ROS can lead to oxidative stress. Our results showed that most of the above-mentioned antioxidases involved in the antioxidant defense against oxidation stress were upregulated in 5660M, especially the sample of M-D-D. By analyzing the spike development inhibition phenotype and expression of key enzymes involved in the antioxidant defense system, we found that the ROS level correlated directly with the slow-growth phenotype (M-I-D) and death phenotype (M-D-D) at the DR-S. Thus, the spike development inhibition phenotype might be a result of both cold stress and oxidative stress. Although the *COR* proteins and the key enzymes that involved in ROS detoxification were all upregulated in 5660M, *COR* proteins were gradually downregulated from the T-S to DR-S while the key enzymes were expressed at constant high levels, especially the sample of M-D-D in 5660M. The results suggested that the 5660M might be triggered by the oxidative stress that was the result of intensive response to cold stress.

4.2 Spike development of 5660M cannot complete the floral transition from the vegetative to reproductive state

In *Arabidopsis*, the gene network controlling the floral transition has been well characterized, and regulation of the flowering pathway has also elucidated in *B. distachyon*, which serves as a model for temperate grasses [19, 34]. In the present study, we identified *FVE* (spot 92) as having altered expression; this protein is involved in regulating the floral transition via the autonomous pathway [19, 35]. *FVE* expression in the M-D-D samples was the lowest measured (Fig. 3B). In M-I-D samples, its mean expression level was the same as that for N-T. For the 5660 at DR-S, *FVE* expression was maximal when the shoot apex began the floral transition. *FVE* expression patterns in 5660M significantly correlated with the floral transition and the spike development inhibition phenotype. Seven genes involved in the autonomous pathway have been found in *Arabidopsis*, and homologous genes have been characterized in rice and *B. distachyon* [34]. Owing to the limitations of 2DE, we did not identify other regulators of the autonomous pathway that might have been expressed in small amounts. Consequently, we investigated the transcription of the autonomous pathway genes and those downstream flowering genes that encode regulators and integrators of flowering. Most of these genes were downregulated (Fig. 8), which

suggested that flowering-time pathways were blocked or delayed in 5660M. By the phenotype analysis, it was shown that the 5660M shoot apices did not complete the floral transition from the vegetative to reproductive state and finally did not develop into spikes. Our molecular and morphological data have all shown that the spike development of 5660M was inhibited and finally did not complete the floral transition.

Although the fertile tiller inhibition gene has not been cloned and the molecular mechanism of the 5660M mutation still remains unknown, the 5660M mutation material can be taken for further studying the spike development mechanism. In the present study, the proteomics and flowering genes transcription data were original and significant to study the spike development of the common wheat, although the altered expression of these proteins and genes in 5660M maybe a consequence, rather than a cause. These flowering genes and proteins, especially the protein of FVE were closely related to spike development inhibition phenotype. FVE, which is a component of the histone deacetylase complex involved in transcription repression, regulates flowering time, and the cold response [35–37]. FVE is also involved in the thermosensory pathway that mediates ambient temperature signaling to repress *SVP* and, as discussed above, is involved in the autonomous pathway to repress *FLC* to regulate flowering and acts as a negative regulator of the CBF/DREB pathway during cold acclimation by binding to and thereby silencing COR chromatin. Overexpression of cold-inducible CBFs caused late flowering and there is a feedback loop between cold response and flowering-time regulation in *Arabidopsis* [20, 38]. Our results suggest that the CBFs genes and COR proteins were all intensely upregulated and FVE and spike development genes was repressed in 5660M. By the phenotype analysis, low temperature affected the number of spikes and spike development of 5660M. FVE might play an important role in regulating cold response and flowering time as that in *Arabidopsis*. Are there the feedback loop and the link between the cold response and spike development of the wheat? Further work is needed to study this problem.

In the present study, these DEPs and flowering genes related to spike development of the wheat might be important for understanding the mechanism of spike development inhibition and further study on spikes development of wheat. Our results provided the theoretical basis for studying spike development, and further analysis of wheat yield formation.

We thank Professor Hong-Xia Wang and Wei-Hua Li from the Military Medical Science Academy of the People's Liberation Army for help with the nano LC-HDMS MS/MS study.

This work was supported by the National High Technology Research and Development Program of China (Grant numbers 2011AA100102; 2006AA10Z174).

The authors have declared no conflict of interest.

5 References

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