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Mechano-stimulated modifications in the chloroplast antioxidant system and proteome changes are associated with cold response in wheat

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Abstract

Background: Mechanical wounding can cause morphological and developmental changes in plants, which may affect the responses to abiotic stresses. However, the mechano-stimulation triggered regulation network remains elusive. Here, the mechano-stimulation was applied at two different times during the growth period of wheat before exposing the plants to cold stress (5.6 °C lower temperature than the ambient temperature, viz., 5.0 °C) at the jointing stage.

Results: Results showed that mechano-stimulation at the Zadoks growth stage 26 activated the antioxidant system, and substantially, maintained the homeostasis of reactive oxygen species. In turn, the stimulation improved the electron transport and photosynthetic rate of wheat plants exposed to cold stress at the jointing stage. Proteomic and transcriptional analyses revealed that the oxidative stress defense, ATP synthesis, and photosynthesis-related proteins and genes were similarly modulated by mechano-stimulation and the cold stress.

Conclusions: It was concluded that mechano-stimulated modifications of the chloroplast antioxidant system and proteome changes are related to cold tolerance in wheat. The findings might provide deeper insights into roles of reactive oxygen species in mechano-stimulated cold tolerance of photosynthetic apparatus, and be helpful to explore novel approaches to mitigate the impacts of low temperature occurring at critical developmental stages.

Keywords: Mechano-stimulation, Cold, Reactive oxygen species, Chloroplast, Wheat

Background

Chilling temperature significantly affects the early growth of winter wheat plants causing considerable reduction of grain yield and is one of the major factors limiting growth and productivity of crops [1]. Cold induced photosynthesis inhibition results in a complex array of reactive oxygen species (ROS) generation, especially in chloroplasts [2]. Over-accumulation of ROS may cause rigidification and leakage of the cell membrane, and destabilization of protein complexes [1]. Recent proteomic studies have revealed differential expression of proteins in wheat exposed

to cold stress [3, 4]. Among the down-regulated proteins due to cold stress, some key enzymes involved in Krebs cycle (isocitrate dehydrogenase, malate dehydrogenase) have been identified, together with many photosynthesis-related proteins (e.g. oxygen-evolving complex proteins, ATP synthase subunits, ferredoxin NADPH oxidoreductase, and some Calvin cycle enzymes) [3]. Proteomic analysis of spring freezing stress responsive proteins in leaves revealed an increased accumulation of stress defense proteins, including LEA-related COR protein, Cu/Zn superoxide dismutase, and ascorbate peroxidases, which may play crucial roles in enhancing tolerance to spring freeze stress in bread wheat [4]. In addition, proteomic analysis of wheat in response to prolonged cold stress showed reinforcement in expressions of enzymes involving in

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ascorbate recycling (dehydroascorbate reductase, ascorbate peroxidase) and involving in tetrapyrrole re-synthesis (glutamate semialdehyde aminomutase) [3].

Mechanical wounding can be caused by surrounding environmental factors, such as wind, rainstorms, and herbivores, and it has broad impacts on plants, including changes in morphogenetic characteristics, membrane potential [5], ROS, hormone signaling and gene expression [6]. Several alterations induced by mechanical wounding can allow plants to resist and acclimate to environmental stresses [6]. As previously observed in maize, bean, and rice, denser but smaller stomata in mechanically stimulated leaves could help plants to control transpirational water loss, thereby avoiding drought stress [7]. Mechano-stimulation was reported to increase cold tolerance in beans, tomato, and maize through maintenance of higher Photosystem II (PSII) efficiency and accumulation of higher levels of soluble sugars [8]. It was also suggested that similar defense mechanisms are operated in cold acclimation and mechano-stimulation, resulting in similar morphological and developmental changes [8]. Recently, analysis of transcript profiles indicated various defense response genes were induced by mechano-stimulation, and were related to cold stress response, including general stress response (GSR), rapid stress response (RSR), and rapid wound response (RWR) [9]. In addition, it has been proposed that mechanical disruption of the cell wall may induce stress signaling [10]. Cold stress is perceived by the plant through detection of changes in membrane fluidity and protein conformation. Secondary messengers such as Ca^{2+} and ROS are implicated in the initial signaling cascades in response to cold stress [1].

Many studies reported changes in ROS levels following mechano-stimulation [11, 12]. For instance, mechano-stimulation induced a significant increase in ROS levels in *Mesembryanthemum crystallinum* leaves [12]. Furthermore, proteomic studies have shown that plants transiently produce superoxide and H_2O_2 , which might play critical roles in signal transduction during early wound response [13]. Mechano-stimulation induced increased expression of cytosolic H_2O_2 -detoxifying enzyme, ascorbate peroxidase 2 (APX2) [14]. This increase in APX2 was independent of other mechanical wounding signals such as jasmonic acid (JA) or abscisic acid (ABA) [15]. It has also been suggested that NADPH-dependent H_2O_2 signals contribute to the activation of specific mechano-stimulated signals which are not activated by the JA or ABA [15]. The cellular steady-state level of ROS is tightly regulated by a complex network involving Ca^{2+} , protein phosphorylation, and ROS-scavenging/producing enzymes during wound response [15]. In addition, mechanical wounding has been found to induce a burst of superoxide and apoplastic

peroxidase with both oxidative and peroxidative activities [15, 16].

In this study, mechano-stimulation was applied to two contrasting winter wheat cultivars that differed in cold tolerance at different growth stages in order to investigate the effects of mechano-stimulation on the performance of the chloroplastic antioxidant system and changes of the proteome under late spring low temperature stress. The results obtained in this study may provide deeper insights into the roles of mechano-stimulated modifications within chloroplast antioxidant systems and proteome in cold tolerance in wheat. This information will be helpful for exploring novel approaches to mitigate the impacts of low temperatures which occur during critical developmental stages in wheat plants.

Methods

Plant materials

This experiment was carried out at Lianyungang Experimental Station of Nanjing Agricultural University (119° 32'E, 34°30'N) during the wheat growing season in 2011–2012. The soil is a clay, contains 11.4 g kg^{-1} organic matter, 1.1 g kg^{-1} total N, 79.8 mg kg^{-1} available N, 32.4 mg kg^{-1} Olsen-P, and 132.4 mg kg^{-1} available K. Before sowing, 120 kg N ha^{-1} , 60 kg P_2O_5 ha^{-1} and 120 kg K_2O ha^{-1} were applied as basal fertilizer and a further 120 kg N ha^{-1} was used as a topdressing after jointing to avoid the potential impacts on stress treatments. Two winter wheat cultivars differing in cold tolerance but having close genetic backgrounds (Jimai 17 displays similar morphology and is related with Yannong 19 in pedigree), Yannong19 (YN19, cold tolerant) and Lianmai6 (LM6, cold susceptible, parents: YN19// Jimai 17/Zheng9023) were used in this experiment. The sowing date was 14 October 2011, with a seedling density of 160 m^{-2} and a row space of 0.25 m. The jointing date was confirmed through spike development checked with a Dino-Lite digital microscope (AM411 Version 1.4.1; Vidy Precision Equipment Co. Ltd, Wuxi, China).

Mechano-stimulation and cold treatments

To investigate the effects of mechano-stimulation applied at different stages on seedling performance under cold stress, four treatments were imposed: P₁L, the early priming of mechano-stimulation for plants was applied at the Zadoks growth stage 26 (25 March 2012) and then subjected to a 4-day cold event at the Zadoks growth 31 (jointing stage, 8–12 April 2012); P₂L, the later mechano-stimulation for plants was carried out 6 days before the cold event (2 April 2012); CL, the cold stress at jointing without early mechano-stimulation; CC, the normal temperature control. Mechano-stimulation was carried out using a cylinder roller with weight of 150 kg and diameter of 40 cm. The roller was rolled over

the wheat plants with a pressure of $7000 \text{ N} \cdot \text{m}^{-2}$ at 9:00–9:30 am, which resulted in less than 20 % of the leaf area being damaged at jointing. A 4-day cold stress was applied using four temperature control systems operated in the open top chamber condition. Air was cooled by a compressor, and then the cooled air was driven by an air blower to the field through ducting [16]. During cold treatment, plots were surrounded by 180-cm-high plastic film. All tubes were removed just after cooling treatment to avoid shading. Six temperature and humidity sensors were used to record the real-time data in each plot. The mean temperature in the cold treatment was $5.60 \text{ }^\circ\text{C}$ lower than the normal temperature control. The mean temperature at night was $-1.14 \text{ }^\circ\text{C}$, and the lowest temperature recorded during the cold treatment was $-4.97 \text{ }^\circ\text{C}$ (detailed temperature data are shown in Additional file 1: Figure S1). The experiment had a split-plot design with temperature treatment as the main plot and wheat cultivar as the subplot, with three replicates for each treatment. The size of each plot was $3 \text{ m} \times 4 \text{ m}$.

Chl a fluorescence transient

The fast chlorophyll a fluorescence induction curve was measured using a Plant Efficiency Analyzer (Pocket-PEA; Hansatech, Norfolk, UK) [17]. Before measuring, plants were dark adapted for 0.5 h. The collected data were processed by the program PEA Plus 1.04, and Biolyzer 3.0 software (Bioenergetics Lab., Geneva, Switzerland, http://www.fluoromatics.com/biolyzer_software-1.php) was used to calculate the fast chlorophyll a fluorescence induction (OJIP) test parameters.

Chloroplast extraction and enzyme activity analysis

Chloroplasts were isolated and purified from the latest fully expanded leaves following our previous method with a few modifications [16]. Leaf samples (6 g) were ground in 30 ml of extraction buffer (0.45 M sucrose, 15 mM 3-(*N*-morpholino) propanesulfonic acid (MOPS), 1.5 mM ethylene glycol tetraacetic acid (EGTA), 0.6 % polyvinylpyrrolidone (PVP), 0.2 % bovine serum albumin (BSA), 0.2 mM phenylmethylsulfonyl fluoride (PMSF) and 10 mM dithiothreitol (DTT)). The homogenate was filtered through eight layers of gauze, and the filtrate was then centrifuged at $2\,000 \times g$ for 5 min. The sediment was resuspended with sorbitol resuspension medium (SRM, 0.33 M sorbitol in 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)), and then layered on the top of a 7-ml layered system (35 %, 80 % Percoll) for step gradients. The chloroplasts were collected and washed with 2 ml SRM followed by centrifugation at $1100 \times g$ for 10 min. Finally, the intact chloroplasts were maintained in 2 ml SRM at $-4 \text{ }^\circ\text{C}$.

Following the methods of Zheng et al. [18], H_2O_2 concentration was measured by monitoring the absorbance of titanium peroxide complex at 410 nm, and the release rate of O_2^- was determined at an absorbance at 530 nm. APX (EC 1.11.1.11) activity was determined by monitoring the decrease in absorbance at 290 nm, the activity of SOD (EC 1.15.1.1) was measured by monitoring the inhibition of photochemical reduction of nitroblue tetrazolium (NBT), and GPX (EC 1.11.1.7) activity was calculated by monitoring the increase in absorbance at 470 nm due to the oxidation of guaiacol. GR (EC 1.6.4.2) activity was determined by the oxidation of NADPH at 340 nm, and CAT (EC 1.11.1.6) activity was measured following the method of Tan et al. [19]. DHAR (EC 1.8.5.1) was assayed by monitoring changes in absorbance at 265 nm after the addition of ascorbate oxidase as described by Miyake and Asada [20]. The activities of Ca^{2+} -ATPase and Mg^{2+} -ATPase in the chloroplasts suspension were measured following the method of Zheng et al. [18].

Rubisco activity

Leaf samples (0.2 g) were ground in 40 ml of extraction buffer (50 mM Tris-HCl, 1 mM EDTA, 1 mM MgCl_2 , 10 % PVP and 10 mM β -mercaptoethanol), and then centrifuged at $15\,000 \times g$ for 15 min. The supernatant was gently collected to measure Rubisco activity. The activity of Rubisco (EC 4.1.1.39) before (initial activity) and after (total activity) active site carbamylation was assayed using a spectrophotometric procedure coupled to NADH oxidation [21]. Rubisco activation was estimated as the percentage ratio of initial to total activities for each sample.

Protein extraction and 2-DE procedure

The extraction of protein in the latest fully expanded leave for 2 DE was performed following the trichloroacetic acid (TCA) acetone precipitation method described by Ding et al. [22].

Immobiline DryStrip gels (117 cm length: Bio-Rad) were used for first dimension isoelectrofocusing (IEF) at pH 4 to 7. Rehydration and focus were performed using PROTEAN IEF apparatus (Bio-Rad) at $50 \mu\text{A}$ per strip at $20 \text{ }^\circ\text{C}$, using the following programme: 12 h of rehydration at 50 V in rehydration buffer (7 M urea, 2 M thio-urea, 4 % (w/v) CHAPS, 0.5 % (v/v) IPG buffer, 10 mM DTT, and 0.1 % bromophenol blue), 1 h at 500 V, 1 h at 1 000 V, 2 h at 8 000 V, and 85 000 V · hours at 8 000 V. After dimension isoelectrofocusing, strips were equilibrated for 15 min in SDS equilibration buffer solution (6 M urea, 37.5 mM Tris-HCl (pH 6.8), 20 % (v/v) glycerol, 2 % (w/v) SDS, and 1 % (w/v) DTT), followed by equilibration with a buffer containing 135 mM iodoacetamide for 15 min. After equilibration, proteins were

distributed in the second dimension (SDS-PAGE) using 10 % polyacrylamide gels (250 × 200 × 1 mm), and the gels were stained with silver nitrate solution.

Image analysis, protein identification, and functional annotation

The gels were scanned using a VersaDoc4000 image system (Bio-Rad) and the images were analysed with PDQUEST 8.0 software (Bio-Rad, USA). There were three biological replicates per treatment with at least three gels for each biological replicate. Only spots with a variation rate of ± 0.5 in the three replicates were considered for further analysis. Stained protein spots were excised manually from the gels, in-gel digested with trypsin, and analysed using a MALDI-TOF/TOF mass spectrometer (ABI 4800). The MASCOT database search engine (<http://matrixscience.com>) was used to search for peptide mass lists from the obtained spectra against the NCBI database. The mass error tolerance was set to 80 ppm, and the score threshold was above or equal to 110.

RNA extraction and qRT-PCR for gene expression analysis

RNA was extracted from wheat leaves using Trizol according to the manufacturer's instructions. The gene-specific primers were constructed using the Primer 3 programme, on the basis of wheat gene sequences in the GenBank (<http://www.ncbi.nlm.nih.gov/>) [23]. The following primers were used for amplification: *Cu/Zn SOD*, 5'-CGCTCAGAGCCTCCTCTTT-3' and 5'-CTCCTGGGGTGGAGACAAT-3'; *Fe SOD*, 5'-GAAGCTTGAGGTGGCACA-3' and 5'-TAAGCATGCTCCCAC AAGTC-3'; *CAT*, 5'-CCATGAGATCAAGGCCATCT-3' and 5'-ATCTTACATGCTCGGCTTGG-3'; *tAPX*, 5'-G CAGCTGCTGAAGGAGAAGT-3' and 5'-CACTGGGGCCACTCACTAAT-3'; β -*actin*, 5'-GCTCGACTCTGGTGATGGTG-3' and 5'-AGCAAGGTCCAAACGAAGGA-3'. The qPCR analysis was performed using the TaKaRa® SYBR Premix Ex Taq™ II on an ABI PRISM 7300 Sequence Detection System (ABI, Foster, CA, USA). The PCR conditions consisted of denaturation at 95 °C for 3 min, followed by 40 cycles of denaturation at 95 °C for 15 s, annealing at 54 °C for 20 s, and extension at 72 °C for 18 s. To minimize sample variations, β -*actin* was used as the reference gene. Each extraction and qRT-PCR was replicated three times. The quantification of mRNA levels was based on the relative quantification method ($2^{-\Delta\Delta C_t}$) [24].

Statistical analysis

All data were subjected to the two-way ANOVA using the SigmaSATA (Systat Software Inc., CA, USA). The Duncan's multiple range test was used to check the significance of difference between treatments. In 2-DE

analysis, the difference of expression level at the given protein spots between treatments and the control (CC) for each cultivar was calculated and converted to a color scale by PageMan software (<http://mapman.mpimp-golm.mpg.de/pageman/>).

Results

Chl a fluorescence transient

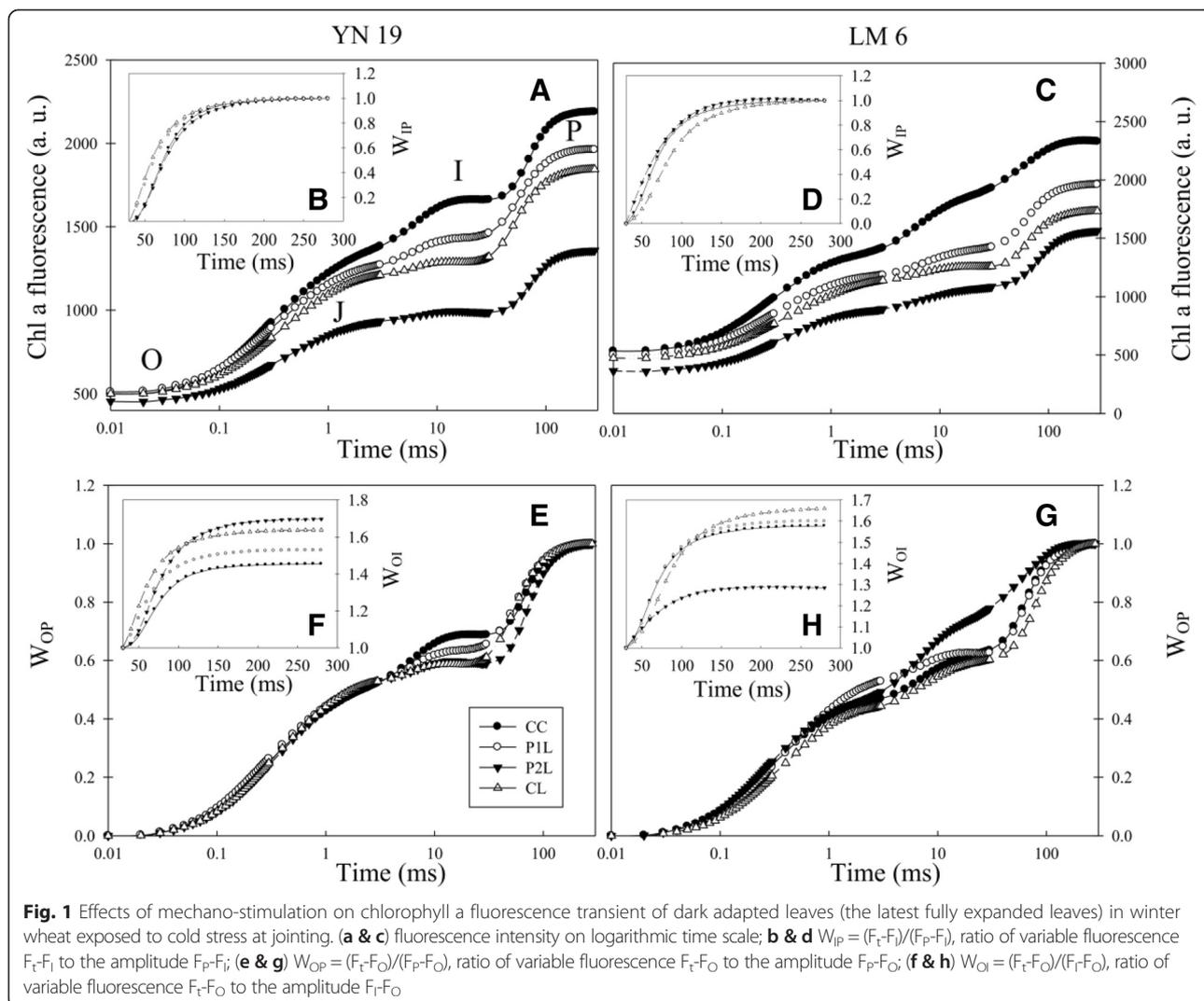
The increase in leaf fluorescence transients observed in CC treatment showed a typical OJIP shape in YN19 and LM6 (Fig. 1a, c). However, P₁L (early mechano-stimulation + cold stress), P₂L (later mechano-stimulation + cold stress) and CL (non-mechano-stimulation + cold stress) showed repressed fluorescence transients in these two cultivars, particularly at step I (30 ms) and P. The main changes of fluorescence data were normalized between step I (30 ms) and P (300 ms) and presented as relative variable fluorescence W_{IP} (Fig. 1b, d). Obvious changes in W_{IP} during the fast rise period were observed under P₂L and CL in YN19, while under P₁L, P₂L, and CL in LM6, compared with CC. W_{OP} (Fig. 1e, g) and W_{OI} (Fig. 1f, h) showed relatively variable fluorescence from O to step P (300 ms) and from O to I (30 ms). A significant decrease in W_{OP} at step I was found in P₁L, P₂L, and CL in YN19, while W_{OP} was increased remarkably by P₂L at step I in LM6. Significant changes in W_{OI} were found among P₁L, P₂L, and CL treatments in YN19, which were related to the reductions between PSI and reduced NADP⁺. However, with the exception of the P₂L treatment, W_{OI} was only slightly affected in LM6.

Rubisco activities and activation

Initial and total Rubisco activities and Rubisco activation in the latest fully expanded leaves were significantly decreased with CL, compared with CC in YN19 and LM6 (Fig. 2, $P < 0.001$). Both traits were slightly and marginally significantly increased by P₁L ($P = 0.077$), whereas they were depressed by P₂L compared with CL ($P < 0.001$). Rubisco activation in P₁L was relatively higher than in CL, but was still lower than in CC for both cultivars ($P < 0.001$). In addition, no difference in Rubisco activation was found between P₂L and CL.

ROS production, activities of antioxidant enzymes, and expressions of their encoding genes in chloroplasts

In YN19, CL increased the concentration of H₂O₂ in chloroplasts in the latest fully expanded leaves by 63 % as compared with CC, whereas P₂L showed a 34 % increment compared with CL (Fig. 3a). However, no difference was observed between CC and P₁L. A similar pattern was observed in LM6. The highest rate of O₂⁻ release was found in P₂L, followed by CL and P₁L, whereas that in CC was lowest in both cultivars (Fig. 3b, $P < 0.001$). SOD activity in chloroplasts was increased



by 16 % and 25 % with P₁L in YN19 and LM6, respectively. Compared with CC, P₁L increased while P₂L and CL significantly decreased chloroplastic SOD activity in the two cultivars (Fig. 3c). In addition, in both cultivars, expression of *Cu/Zn SOD* was up-regulated by P₁L compared with CL (Fig. 4a), whereas an up-regulation of *Fe SOD* due to P₁L was only observed in YN19 (Fig. 4b). For both cultivars, CAT activity was lower in CL than in CC, whereas it was higher in P₁L than in CL (Fig. 3d). In LM6, P₂L decreased CAT activity by 20 % compared with CL, whereas no significant difference was found in YN19 ($P = 0.112$). An increase in the expression of *CAT* was found in P₁L and P₂L compared with CL in YN19; however, the difference was not statistically significant (Fig. 4c). In both cultivars, the combination of mechano-stimulation and low temperature (P₁L and P₂L) and CL enhanced APX activity compared with CC. In particular APX activity in P₂L was significantly higher than in CL (Fig. 3e). Further, the same trend was found in

thylakoid-bound APX (tAPX) expressions in YN19, whereas in LM6, a significant up-regulation of *tAPX* was only observed in P₁L (Fig. 4d). In both cultivars, GPX activity was enhanced with P₁L, but depressed with P₂L and CL (no significant difference between P₂L and CL) (Fig. 3f). In both cultivars, P₁L and CL resulted in a significant increase in GPX activity, compared with CC ($P < 0.001$), whereas P₂L slightly increased GPX activity (Fig. 3g, $P = 0.105$). Low temperature significantly enhanced DHAR activity in both cultivars ($P < 0.001$); however, P₁L and P₂L had opposite effects on DHAR activity in the two cultivars—namely, P₂L decreased DHAR activity compared to CL in YN19, but increased activity in LM6; P₁L increased DHAR activity in LM6, whereas no difference between P₁L and CL was found in YN19 (Fig. 3h). Thus, P₁L and P₂L showed opposite patterns in the concentration of H₂O₂ in chloroplasts, O₂⁻ release rate and most of the antioxidant enzyme activities, but the APX activity showed a similar trend in P₁L and P₂L.

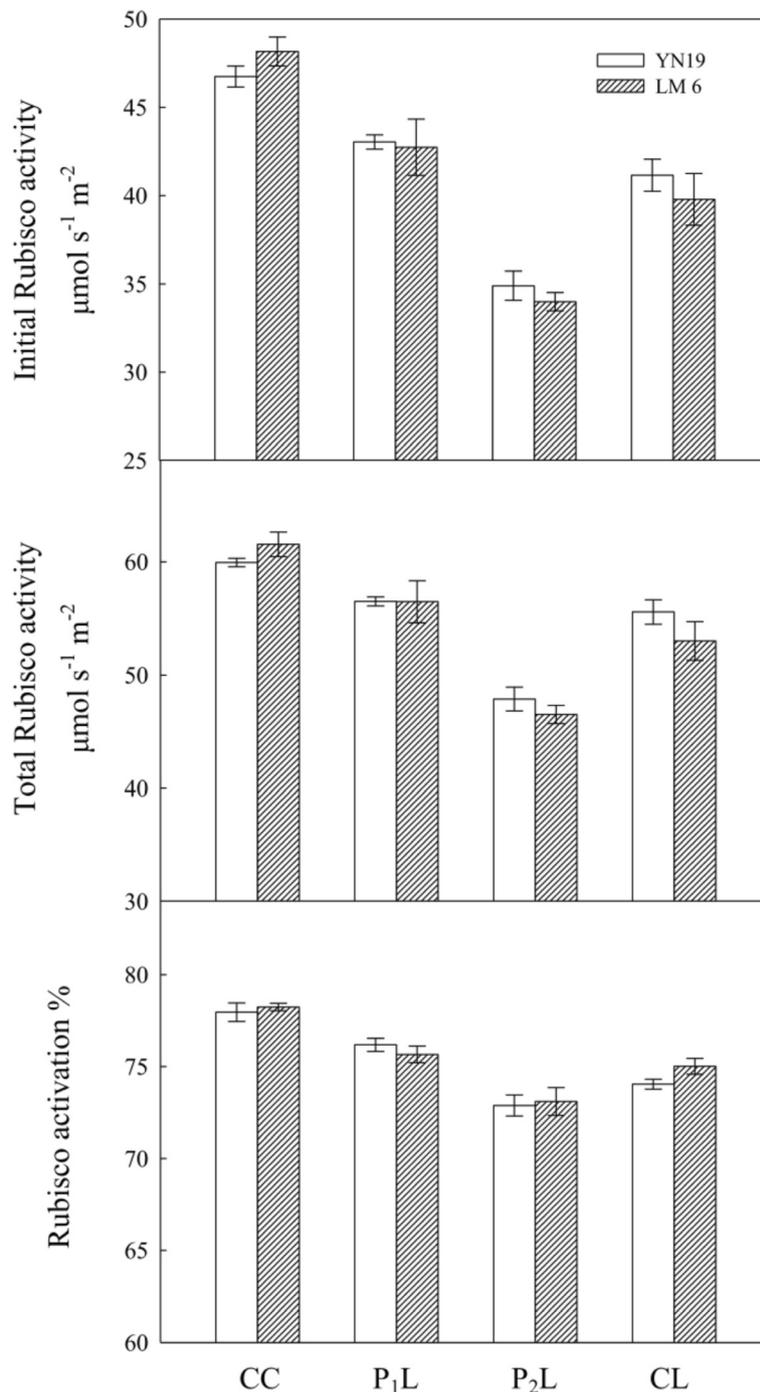


Fig. 2 Effects of mechano-stimulation on initial and total Rubisco activities and activation in the latest fully expanded leaves in winter wheat exposed to cold stress at jointing

ATPase activities in chloroplasts

In YN19, the Activities of both Mg²⁺-ATPase and Ca²⁺-ATPase were significantly decreased by CL as compared with CC (Fig. 5, $P < 0.001$). However, both ATPase activities were increased by P₁L, whereas they decreased by P₂L. The activities of Mg²⁺-ATPase and

Ca²⁺-ATPase in LM6 in response to different treatments were similar to those in YN19.

Proteomics

The reference 2-DE gel of proteins in wheat leaves affected by combination of mechano-stimulation and cold

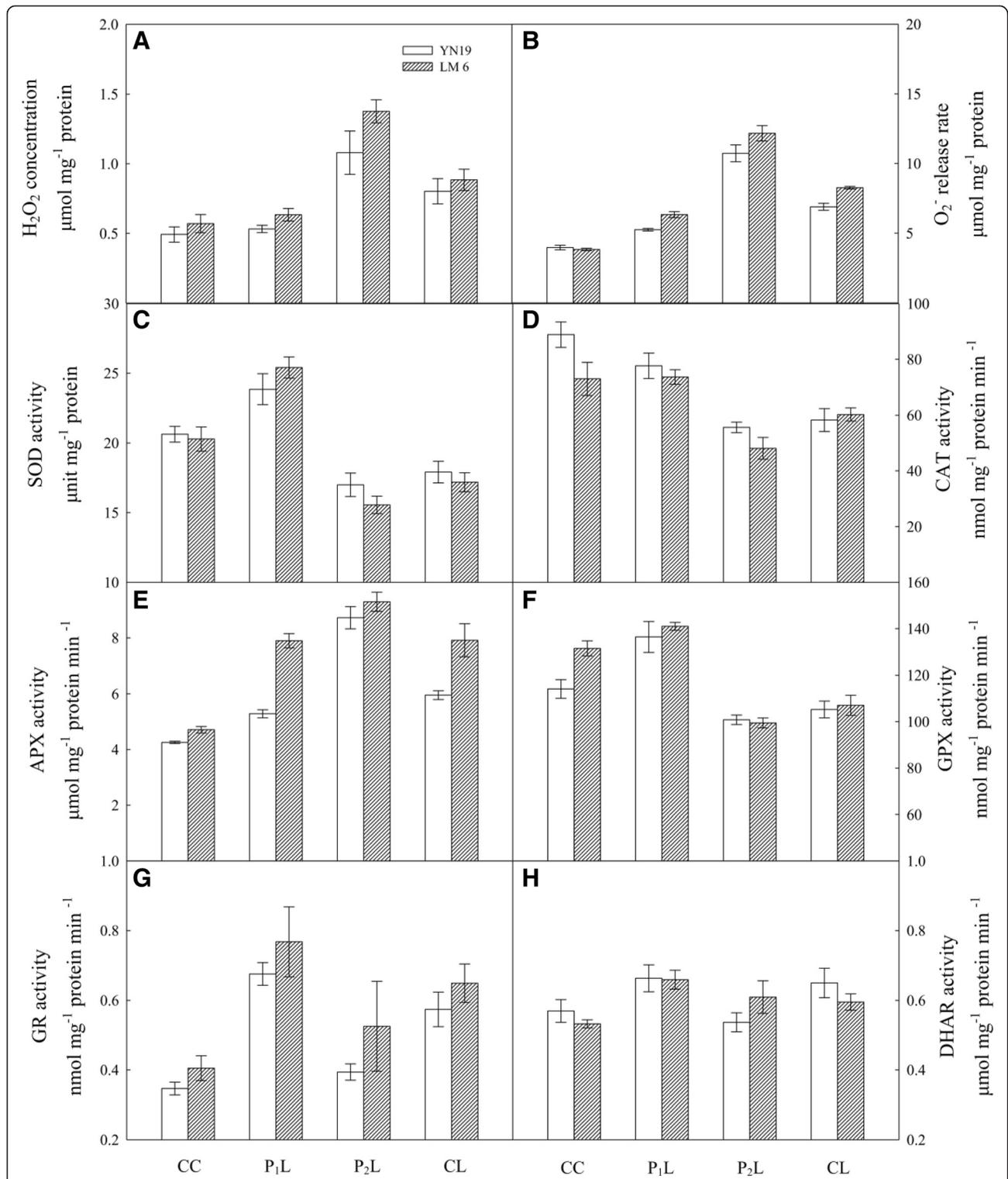
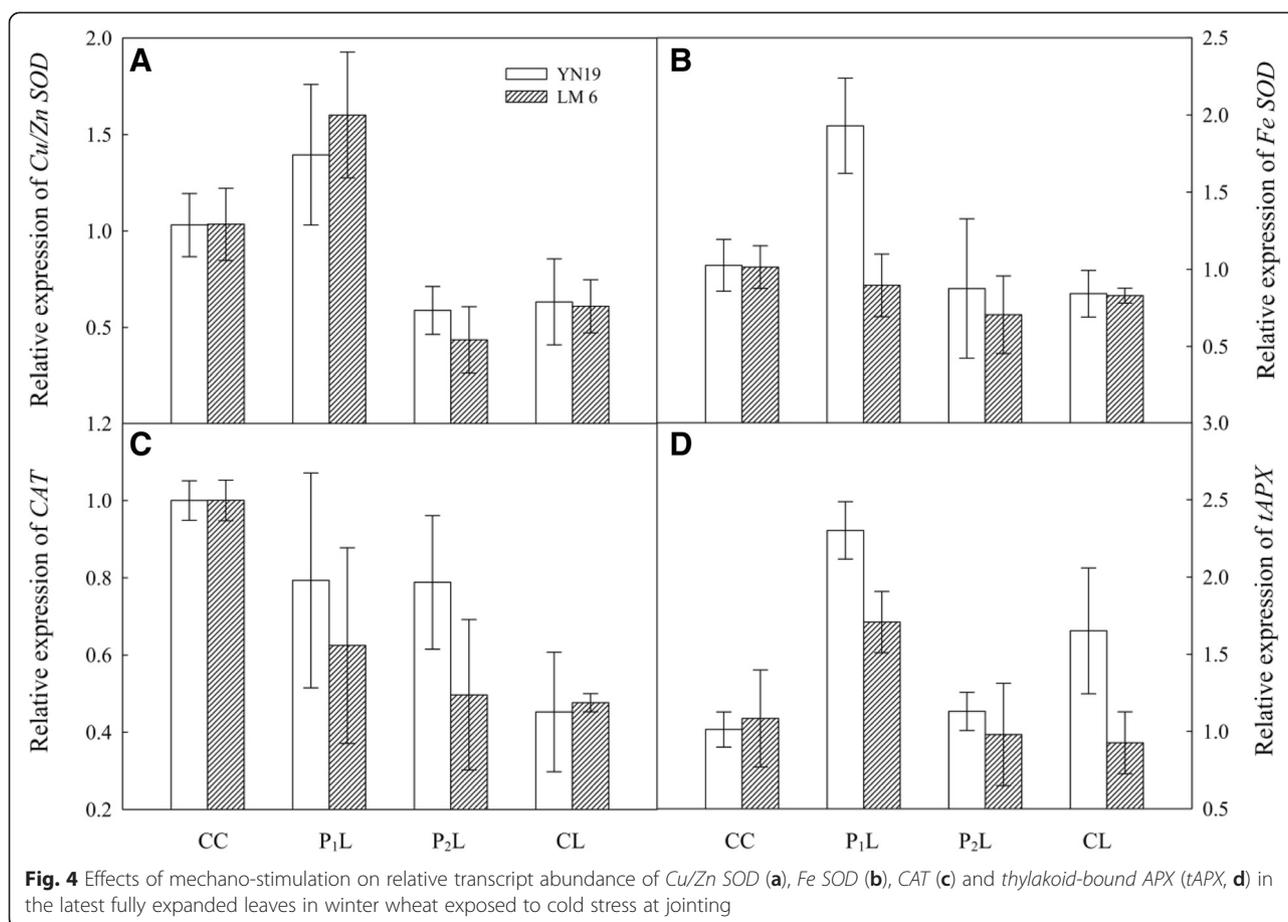


Fig. 3 Effects of mechano-stimulation on reactive oxygen species and antioxidant enzyme system in chloroplasts in the latest fully expanded leaves in winter wheat exposed to cold stress at jointing. **a** H₂O₂, hydrogen peroxide; **b** O₂⁻, superoxide anion radical; **c** SOD, superoxide dismutase; **d** CAT, catalase; **e** APX, ascorbate peroxidase; **f** GPX, glutathione peroxidase; **g** GR, glutathione reductase; **h** DHAR, monodehydroascorbate reductase



stress is shown in Fig. 6. More than 600 protein spots were detected in each gel. To demonstrate the proteomic response of the photosynthetic apparatus to mechano-stimulation and cold stress, variation in the expression of 12 protein spots related to photosynthesis, energy production, stress defense in chloroplasts is specifically shown in Fig. 7. The differentially expressed protein spots were identified by mass spectrometry (MS, Table 1). In the cluster related to photosynthesis, five protein spots, including enzymes involved in the Calvin cycle and Rubisco protein subunit—ferredoxin-NADP(H) oxidoreductase (spot 10), ribulose-1, 5-bisphosphate carboxylase activase (spot 11) and the Rubisco large subunit-binding protein subunit alpha (6)—were up-regulated by P₁L in both cultivars; the exception being spot 10, which was missing in P₁L in YN19. CL induced up-regulation in chloroplastic glutathione reductase (spot 5) and ascorbate peroxidase (spot 7) in both cultivars, whereas the expression of catalase-1 (spot 8) was down-regulated by CL. These proteins were, however, all up-regulated by P₁L in both cultivars, except for catalase-1 in YN19. In addition, in both cultivars, the expression of ATP synthase β subunit (spot 9) was depressed by CL compared with CC, but was increased by P₁L compared with CL. Interestingly, the

chloroplastic fructose-bisphosphate aldolase (spot 2) was up-regulated by CL in the two cultivars, whereas under P₁L, it was decreased in YN19 but increased in LM6. Proteomic analyses revealed that the oxidative stress defense, ATP synthesis, and photosynthesis-related proteins were similarly modulated by the mechano-stimulation and the cold stress.

Discussion

It is well known that ROS production is a universal response to mechanical wounding in various plants [11]. The defense system can also be activated to alleviate ROS-induced oxidative stress and repair the damaged tissues [25]. Furthermore, many of the genes encoding enzymes involved in ROS metabolism are regulated by mechanical wounding [26]. Here, the leaf chloroplastic H₂O₂ concentration in P₁L plants was very close to that in CC plants. In contrast, P₂L plants have a significantly higher H₂O₂ concentration than CC plants (Fig. 3). This difference is related to the efficient ROS scavenging capacity of the antioxidant enzyme systems, particularly the water-water cycle in chloroplasts, which mainly includes SOD and APX [27]. The scavenging capacity of SOD and CAT activated by the mechano-stimulation in P₁L

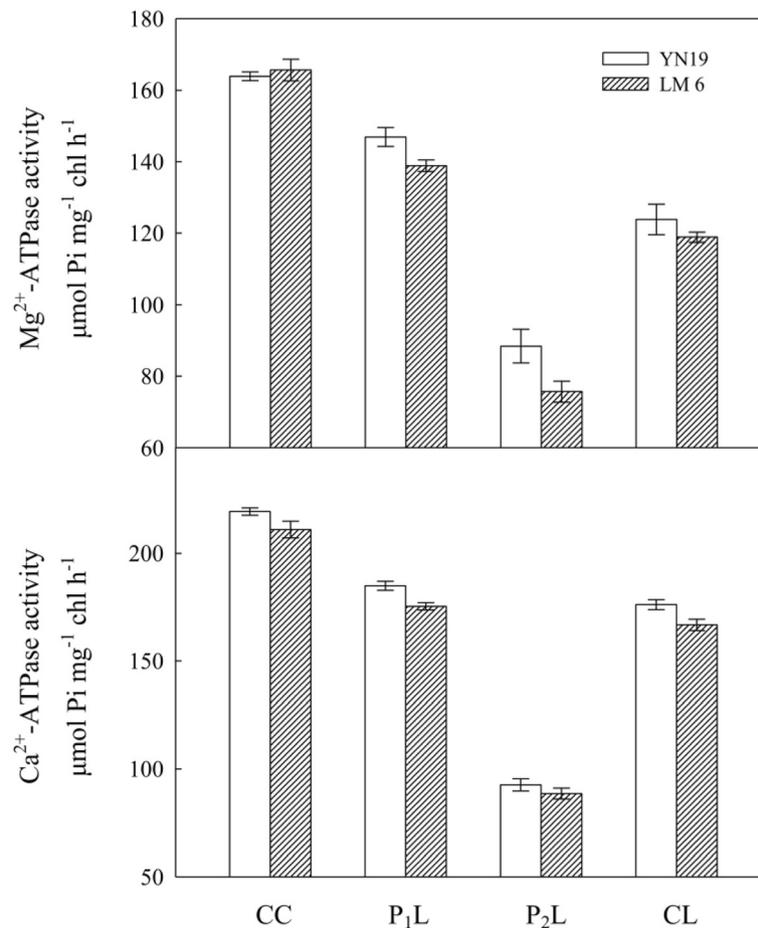
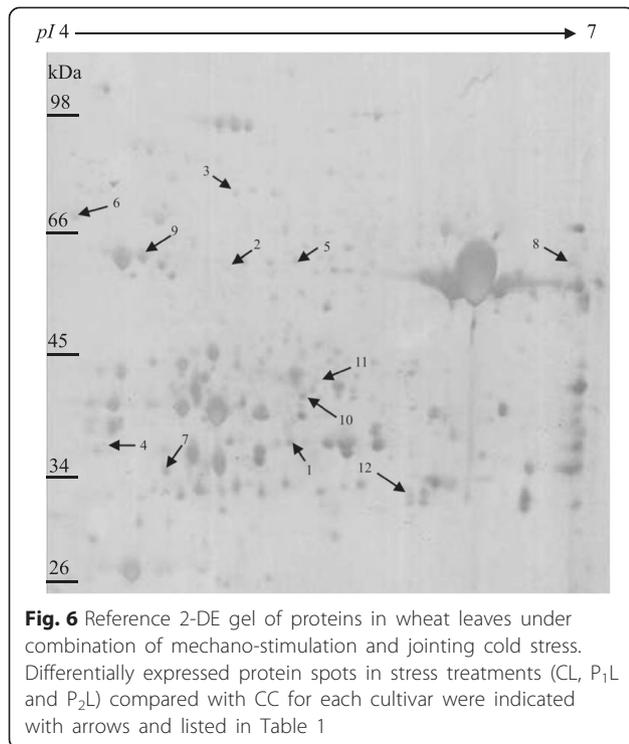


Fig. 5 Effects of mechano-stimulation on activities of Mg²⁺-ATPase and Ca²⁺-ATPase in chloroplasts in the latest fully expanded leaves in winter wheat exposed to cold stress at jointing

had a significant inhibitory effect on the oxidative burst under low temperature stress. Further analysis revealed that the enhanced activities of SOD and CAT could be largely explained by the up-regulated expression of *Cu/Zn SOD*, *Fe SOD*, and *CAT* in P₁L (Fig. 7). Wound-induced activation of H₂O₂-detoxifying enzymes has previously been demonstrated using proteomic tools [26]. Our proteome analysis showed that the expression levels of ascorbate peroxidase and catalase-1 were paralleled by the activities of APX and CAT, respectively, under different treatments (Fig. 7). However, in YN19, the activity of APX in P₁L was decreased compared to CL, whereas no significant difference was found in LM6. The qPCR analysis also showed that the *tAPX* expression was only slightly affected by P₁L in YN19, but it was increased by P₁L in LM6. APX activity under the combination of mechano-stimulation and cold stress was only partly consistent with that previously reported. It has been reported that expression of ascorbate peroxidase 2 (APX2) is involved in modulation of cellular H₂O₂ levels in response to wounding [15, 26, 27]. The

increase in APX activity in P₂L and no increase in P₁L suggested that APX may not play vitally important roles in the mechano-induced cold tolerance in wheat.

Increasing evidence supports the multi-signaling functions of H₂O₂ in response to abiotic stresses in higher plants [11]. Here, under low temperature, for both texted cultivars the H₂O₂ concentration in P₁L was very close to the normal level in CC. However, the release rate of O₂⁻ in P₁L was significantly higher than in CC. It was suggested that activated antioxidative enzymes, such as SOD and CAT induced by mechano-stimulation, modify the H₂O₂ concentration to an appropriate level as a signal molecule, which prevents H₂O₂-induced damage to plant tissues [11]. In addition, modified GPX and GR activities have also been shown to be related to the down-regulation of H₂O₂ levels in chloroplasts [28]. Here, the increased activities of GPX and GR did favour the relatively low level of H₂O₂ in P₁L (Fig. 3). Although the concentrations of AsA and GSH are only in the millimolar range in plant tissues, the AsA-GSH cycle plays a very important role in neutralizing H₂O₂ released by



disproportionation of O₂⁻ [28]. As key members in the AsA-GSH cycle, the altered expression of glutathione reductase (GR)- and dehydroascorbate reductase (DHAR)-related proteins were found via proteome analysis in the present study; chloroplastic GR was increased in P₁L in both cultivars, whereas DHAR was enhanced in P₁L only in LM6 compared to CL (Fig. 7). The changes in expression of these enzymes are in accordance with their activities in chloroplasts. Thus, we suggest that

the AsA-GSH cycle is involved in mechano-stimulated cold tolerance in winter wheat.

Hardening with a previous abiotic stress endows plant with higher tolerance to recurring stresses [29]. For example, pre-anthesis heat hardening (or pre-treatment) can partially protect wheat plants from photosynthetic inhibition and oxidative damage under post-anthesis high-temperature stress, which is attributed to the modified expressions of photosynthesis-responsive and antioxidant enzyme-related genes [30]. Furthermore, many studies have shown that the mechanism underlying hardening includes the accumulation of soluble sugars, reduction of photosynthetic apparatus [30], scavenging of reactive oxygen species (ROS) [30], accumulation of osmoprotective proteins (dehydrins) [31], and other compatible solutes such as proline and betafina [31]. It is well known that cold acclimation reduces frost damage, and that this phenomenon involves a mechanism similar to that of drought acclimation [32]. Mechano-stimulation may induce many types of cold response proteins and genes [8]. In addition to the antioxidant system activated by mechano-stimulation, shown in the present study, many types of proteins related to photosynthesis, energy production, and C metabolism were modified by mechano-stimulation (Fig. 7). With respect to photosynthetic C assimilation, ribulose-1, 5-biophosphate carboxylase activase and its isoform 1 had a higher level of expression in P₁L, but a relatively low level in CL, in the two tested cultivars. Ribulose-1, 5-biophosphate carboxylase activase and Rubisco large subunit-binding protein have been shown to play a critical role in the activation of Rubisco [33]. Carbonic anhydrase enhances the CO₂ concentration in chloroplasts, which improves the carboxylation rate of

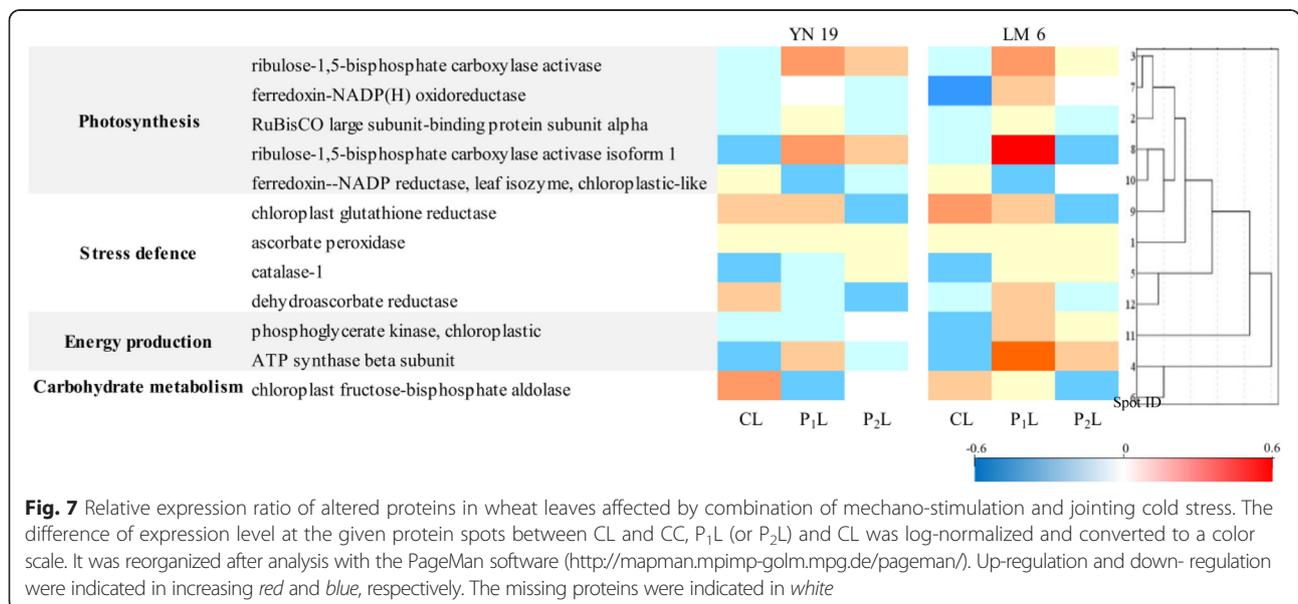


Table 1 Identification of differentially expressed proteins in wheat leaves affected by combination of mechano-stimulation and jointing cold stress through MALDI-TOF/TOF

Spot ID	Protein name	GI accession no.	Theor. Mr (kDa)/pI	Score	SC (%)	Taxonomy
1	ferredoxin-NADP reductase, leaf isozyme, chloroplastic-like	357110920	40.81/6.72	103	5	<i>Brachypodium distachyon</i>
2	chloroplast fructose-bisphosphate aldolase	223018643	42.22/5.94	800	30	<i>Triticum aestivum</i>
3	Phosphoglycerate kinase, chloroplastic	129915	49.98/6.58	323	10	<i>Triticum aestivum</i>
4	ribulose-1,5-bisphosphate carboxylase activase isoform 1	167096	47.37/8.62	549	45	<i>Hordeum vulgare subsp</i>
5	chloroplast glutathione reductase	148250114	50.87/6.17	309	10	<i>Dasypyrum villosum</i>
6	RuBisCO large subunit-binding protein subunit alpha, chloroplastic precursor	134102	57.66/4.83	841	26	<i>Triticum aestivum</i>
7	ascorbate peroxidase	15808779	27.96/5.10	471	27	<i>Hordeum vulgare subsp. Vulgare</i>
8	catalase-1	2493543	57.00/6.52	512	13	<i>Triticum aestivum</i>
9	ATP synthase beta subunit	110915710	53.02/5.17	326	17	<i>Vulpia microstachys</i>
10	ferredoxin-NADP(H) oxidoreductase	20302473	40.49/6.92	739	34	<i>Triticum aestivum</i>
11	ribulose-1,5-bisphosphate carboxylase activase	37783283	22.49/4.98	475	34	<i>Triticum aestivum</i>
12	dehydroascorbate reductase	28192421	23.46/5.88	118	22	<i>Triticum aestivum</i>

Spot ID are named according to Fig. 6. GI refers to accession number. NCBI refers to database accession number. Mr/pI refers to molecular weight and isoelectric point of identified protein. Score refers to Mascot protein score. SC refers to Sequence Coverage

Rubisco enzyme [21]. Our proteomic data also showed a higher abundance of Rubisco large subunit-binding protein subunit α in P₁L than that in CL in both cultivars. This implies increases in the Rubisco activation state and carboxylation rate induced in the early mechano-stimulated plants under low temperature stress (Fig. 2). The unaltered expression of ferredoxin-NADP(H) oxidoreductase and down-regulation of ferredoxin-NADP reductase, leaf isozyme, and chloroplastic-like protein in P₁L observed in the present study, resulted in an increased level of NADPH-dependent H₂O₂ as compared with CL. It has been suggested that increased NADPH-dependent H₂O₂ is required for the activation of systemic wound responses [11]. The mechano-stimulation induced H₂O₂ production is also involved in plant defence responses against invading pathogens [34]. It was reported that ROS can control Ca²⁺-permeable channel activity to regulate the intracellular Ca²⁺ level [35]. The changes of ROS and Ca²⁺ following mechano-stimuli were implicated in the induction of defense genes in response to fungal pathogens [34, 36].

Photosynthetic electron transport generates energy (ATP) and reducing power (NADPH) to support carbon reduction and photorespiratory carbon oxidation in the dark reaction in photosynthesis and plays a key role in the maintenance of optimum photosynthetic rate and ensuring effective energy flow for growth [37]. To further investigate the interactive effects of mechano-stimulation and cold stress on the process of photosynthetic electron transport, transient fluorescence kinetics were analysed (Fig. 1). The I-P phase of the transient fluorescence kinetics revealed changes in the

electron flux from PQH₂ to the final electron acceptor and the size of the final electron acceptor pool of PS I [38]. The present study showed no significant effects of the combination of mechano-stimulation and cold stress on the electron flux from PQH₂ to the final electron acceptor. However, the O-I part of the kinetics was affected by cold stress and mechano-stimulation, which reveals changes in the process involving exciton capture to PQ reduction [38]. In addition, the rise in fluorescence transient from O to P was faster in P₂L in LM6, which indicates that the re-oxidation of Q_A was inhibited by the combination of cold and later mechano-stimulation [39]. However, it was not markedly affected by cold stress alone. The ATPases in chloroplasts, Mg²⁺-ATPase and Ca²⁺-ATPase, play a key role in ATP formation [18]. In this study, the activities of these two functional enzymes in chloroplasts were enhanced by mechano-stimulation in response to cold stress, which might favor the ATP formation. Consistently, the proteome data showed that, in both cultivars tested, there was a higher abundance of ATP synthase β subunit in P₁L.

Conclusions

Early mechano-stimulation at the stage of at Zadoks growth stage 26 activated the antioxidant system and hence maintained the balance of reactive oxygen species, improved the electron transport and photosynthesis rate under cold stress applied at the jointing stage, whereas mechano-stimulation applied 6 days before the cold event induced an opposite effect, except for APX activity and ATPase activities in chloroplasts. Proteomic and transcriptional analysis revealed that the oxidative stress

defense, ATP synthesis, and photosynthesis-related proteins and genes are up-regulated by the mechano-stimulation, which were involved in the responses of wheat plants to the cold stress.

Additional file

Additional file 1: Figure S1. Temperature difference between low temperature treatment and the normal temperature control during the cold stress treatment at jointing. (DOCX 1175 kb)

Abbreviations

ABA: Abscisic acid; APX: Ascorbate peroxidase; AsA: Ascorbate; ATP: Adenosine triphosphate; BSA: Bovine serum albumin; CAT: Catalase; CC: the normal temperature control; CL: The cold stress at jointing without early mechano-stimulation; DHAR: Dehydroascorbate reductase; DTT: Dithiothreitol; EGTA: Ethylene glycol tetraacetic acid; GPX: Glutathione peroxidase; GR: Glutathione reductase; GSR: General stress response; HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; H₂O₂: Hydrogen peroxide; IEF: Isoelectrofocusing; JA: Jasmonic acid; LEA-related COR protein: Late embryogenesis abundant protein-related cold-responsive protein; MOPS: 3-(N-morpholino) propanesulfonic acid; MS: Mass spectrometry; NADPH: Nicotinamide adenine dinucleotide phosphate; NBT: Nitroblue tetrazolium; O₂: Singlet oxygen; P₁L: The combined treatment of early priming of mechano-stimulation at the Zadoks growth stage 26 and a 4-day cold event at the jointing stage; P₂L: The combined treatment of the later mechano-stimulation 6 days before the cold event and a 4-day cold event at the jointing stage; PMSF: Phenylmethylsulphonyl fluoride; PSII: Photosystem II; PVP: Polyvinylpyrrolidone; ROS: Reactive oxygen species; RSR: Rapid stress response; RWR: Rapid wound response; SDS: Sodium dodecyl sulfate; SOD: Superoxide dismutase; SRM: Sorbitol resuspension medium; TCA: Trichloroacetic acid.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

D.J., Q.Z. and J.C. conceived the idea and led the study design. X.L., C.H. and J.Z. carried out the experiment, performed analyses and wrote the paper. F.L., T.D. and W.C. assisted with study design, data analysis, and writing. All authors contributed to the editing of the manuscript. All authors have read and approved the final version of the manuscript.

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