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REGULAR ARTICLE

Proteomics combined with BSMV-VIGS methods identified some N deficiency-responsive protein species and ABA role in wheat seedling

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Abstract

Aims Crops often encounter a soil deficiency of nitrogen (N), the most important macronutrient for plants; however, the molecular mechanism of plant responses to N deficiency remains unclear. In this study, proteome-level changes that occur in bread wheat seedlings suffering from N deficiency were investigated to identify some N deficiency-responsive protein species in bread wheat.

Methods We utilized isobaric tagging for relative and absolute quantification (iTRAQ) to measure changes in the proteome patterns of N-deficient wheat seedlings and validated the role of abscisic acid (ABA) using the barley stripe mosaic virus-induced gene-silencing (BSMV-VIGS) method.

Results A total of 1515 N deficiency–responsive protein species were successfully identified in both root and leaf tissues of wheat seedlings suffering from 8-d N deficiency. Of these, abundance of wheat zeaxanthin epoxidase (TaZEP), a key ABA synthesis-related enzyme, was significantly upregulated, and the endogenous ABA contents also markedly increased. After *TaZEP* gene was further silenced using BSMV-VIGS method, BSMV-VIGS-TaZEP infected wheat seedlings showed enhanced sensitivity to N deficiency, suggesting silencing of *TaZEP* gene decreased the tolerance to N deficiency remarkably.

Conclusion Our results identified some N deficiency-responsive protein species and revealed the role of ABA in wheat responses to N deficiency.

Keywords Abscisic acid · BSMV-VIGS · iTRAQ · Nitrogen deficiency · Proteome · *Triticum aestivum* L

Abbreviations

ABA	abscisic acid
BSMV-VIGS	barley stripe mosaic virus-virus induced gene-silencing
CTK	cytokinin
DW	dry weight
ETH	ethylene
FW	fresh weight
IAA	auxin

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iTRAQ	isobaric tagging for relative and absolute quantification
JA	jasmonic acid
MW	molecular weights
pI	isoelectric points
qRT-PCR	quantitative real-time PCR
SA	salicylic acid
ZEP	zeaxanthin epoxidase

Introduction

Nitrogen (N) is one of three most essential macronutrients for plant growth and development. It constitutes approximately 2% of plant dry matter and is also an important constituent of most cellular molecules, including proteins (enzymes), chlorophyll pigments, amino acids, nucleic acids, and phytohormones (Criado et al. 2007; Shi et al. 2010). It plays key roles in many physiological processes, such as protein synthesis, amino acid metabolism, and carbon metabolism (Cai et al. 2012). Plant growth and yield require abundant N, and the element is deficient in most cultivated soils worldwide. Therefore, in many agricultural regions, large amounts of chemical N fertilizers are applied annually to compensate for this deficiency, and the consumption of chemical N fertilizers has increased more than 9-fold worldwide since 1960 (Qin et al. 2019). However, less than 50% of applied nitrogen is effectively absorbed, and the other 50% or more is ineffectively dissipated in the wider environment by leaching into the soil, water, and air, leading to environmental pollution and health hazards (Hakeem et al. 2011; Nazir et al. 2015). Therefore, it is essential to improve the absorption and utilization efficiency of N fertilizer and minimize its input, and implementing these changes requires detailed understanding of the molecular mechanisms of N absorption and utilization.

Plant responses to N deficiency involve morphological, physiological, developmental, and cellular processes with known responses, including the improvement of N absorption and translocation, remobilization of N from source organs to newly growing tissues, and alteration of carbohydrate partitioning (Krapp et al. 2011). These processes are effectively controlled by N deficiency-responsive genes, and thus, identification of N deficiency-responsive genes is a fundamental step to understanding these complex processes (Liang et al. 2013). In addition to identification on the genetic basis of nutrient use efficiency, such as genome-wide association studies, forward genetic tools such as omics are suitable to obtain a comprehensive overview of the changes in response to stress and may help to close the information gap between genotype and phenotype (Marioni et al. 2008). Transcriptomic technology can take into consideration all aspects of nutrient management in plants and is an integrated approach to investigating biotic and abiotic factors, and using this technology combined with other protocols, many N deficiency-responsive genes have been identified in the past few decades (Gelli et al. 2014; Yang et al. 2015; He et al. 2016a; Quan et al. 2016; Wei et al. 2016; Curci et al. 2017; Nawaz et al. 2018). These studies have transcriptionally provided a basis for elucidating the underlying molecular mechanism of physiological changes associated with N deficiency and have improved our understanding of plant responses to N deficiency. Phytohormones regulate many developmental processes and adaptive stress processes and are considered to be the foremost signaling molecules in plants (Lee and Luan 2012), and some studies have shown that the genes involved in the biosynthetic pathway of some plant hormones (e.g., abscisic acid, jasmonic acid, and gibberellin) are significantly regulated (Ristova et al. 2016; Kuang et al. 2017). For example, ABA contents significantly increase under low-N conditions and exogenous ABA application promotes the nitrogen use efficiency in plants (Oka et al. 2012; Han et al. 2017). ABA is synthesised via the carotenoid biosynthetic pathway, zeaxanthin epoxidase (ZEP) enzyme catalyses the conversion of zeaxanthin to alltrans-violaxanthin in a two-step oxidation, and is considered as one of the key enzymes in this biosynthetic pathway because its mutant displays the remarkably decreased levels of ABA (Agrawal et al. 2001; Cuming and Stevenson 2015).

However, many questions need to be further answered because gene expression can also be regulated at the posttranscriptional, translational, and posttranslational levels (Hu et al. 2015). Cellular proteins directly perform most of the catalytic work required by biological processes, and proteomics is an alternative approach to complement transcriptomic analyses because changes at the transcriptional level are not always reflected at the translational level (Findlay et al. 2009; Grobei et al. 2009; Kosová et al. 2011). Some N deficiency-responsive protein species in higher plants have been identified using a proteomic approach in some plant

species, with most of the focus on two model plant species (Arabidopsis and rice) (Møller et al. 2011; Deng et al. 2014; Jin et al. 2015; Qin et al. 2019). To some extent, different plant species have diverse responses to the same abiotic or biotic stresses (Kang et al. 2015), and it is important to identify N deficiency-responsive protein species in important food crops because we are facing challenges to the worldwide food supply. Bread wheat is one of the three most important cereal crops globally and is widely cultivated worldwide. Its production accounts for over 20% of the world's food supply (He et al. 2015). To our knowledge, however, quantitative proteomics studies on N deficiency have not been reported in this species so far because this species is characterized by a very large and rather complex, 17-gigabase-pair (Gb) allohexaploid genome and more than 85% of its genome consists of highly repetitive sequences (Brenchley et al. 2012), although the draft genome sequences of bread wheat and its diploid ancestors have recently been completed (Jia et al. 2013; Ling et al. 2013; IWGSC 2014 and 2018).

Short-term nutrient deficiency is a result of a significant decrease in the level of the corresponding nutrients, whereas long-term nutrient deficiency is characterized by the major alteration of metabolic activities and the presence of a distinct phenotypic symptom (Liang et al. 2013). More nutrient-responsive proteins (genes) have been identified under long-term nutrient deficiency than under short-term deficiency (Ma et al. 2014). Identifying differentially abundant protein species in plants suffering from long-term nutrient deficiency could reveal the in-depth mechanisms of nutrient deficiencies (Liang et al. 2013). When wheat seedlings suffered from 8 d of N deficiency in our previous study, the phenotype, morphological and physiological parameters (e.g., plant height, shoot fresh and dry weights, and nitrate and protein contents) of wheat seedlings were significantly inhibited (Guo et al. 2014). These results suggested that wheat seedlings at this time point suffered from long-term N deficiency. At this time point in the present study, an iTRAQ-based large-scale quantitative proteomics analysis was used to identify a large number of N deficiency-responsive protein species in the root and leaf tissues of wheat seedlings responding to long-term N deficiency. This technique was sensitive enough to delineate 1515 N deficiency-responsive protein species. Moreover, an important role of phytohormone ABA in wheat seedlings responding to long-term N deficiency was verified using both physiological protocols and BSMV-VIGS. This study identifies some N deficiency-responsive protein species in important crops in response to N deficiency and reveals ABA roles.

Methods

Plant growth conditions and N deficiency treatment

A spring bread wheat cultivar Yumai 34, characterized with good quality, and high biotic and abiotic tolerance, and released in 1994, was developed by the Agricultural and Forestry Science Institute of Zhengzhou and its seeds were kindly provided by Dr. Xiangzheng Liao in this institute. Seeds (sterilized in 0.01% $HgCl_2$, washed in distilled water) were grown hydroponically in glass dishes (15 cm diameter) in full-strength Hoagland solution (Hoagland and Arnon 1950; Elberse et al. 2003). These glass dishes were placed in an FPG-300C-30D illumination incubator (Ningbo Laifu Technology Co., Ltd., China) with a 14-h photoperiod, 25/15 °C day/night temperatures, relative humidity of 60/75% (day/night), and light intensity of 250 μmol m^{-2} s^{-1}. Each dish contained approximately 50 seedlings. After two weeks, the wheat seedlings were divided into two groups: one group remained in Hoagland medium in full N conditions (6 mM) as a control, and the other group was transferred to N-free (DN) Hoagland medium for N deficiency. KNO_3 and $CaNO_3$ were replaced by KCl and $CaCl_2$, respectively (Guo et al. 2014). The nutrient solutions were renewed every 2 days, and the pH was adjusted to 5.5~6.0 with HCl or NaOH each time. Root and leaf tissues of the wheat seedlings were collected at 0, 2, 4, 6, and 8 days after incubation in two nutrient media, immediately frozen in liquid nitrogen, and stored at −80 °C for extracting the total proteins.

iTRAQ labeling, LC-MS/MS analysis, and protein identification

For iTRAQ, three biological replicates of protein samples were extracted from root and leaf tissues. Total proteins were extracted using the trichloroacetic acid/acetone method (Li et al. 2013) and then reduced, alkylated, and digested using the filter–aided sample preparation procedure (Wisniewski et al. 2009). The digested peptide mixture was labeled using the iTRAQ reagents 8-plex Kit according to the manufacturer's instructions (AB Sciex, Inc., Foster City, CA, USA).

Extracted protein samples were labelled with 113, 114, 115, 116, 117, 118, 119, and 121 labels in each independent 8-plex iTRAQ reagent, each label represented one sample, and three 8-plex iTRAQ tags containing three biological replicates of each sample were performed. In three 8-plex iTRAQ tags, 113-control root, 114-N-deficient root, 117-control leaf, and 118-N-deficient leaf have been labelled and used in this study, whereas the other four labels (115, 116, 119, and 121) were used in other experiments (Li et al. 2017).

The MS/MS data were analyzed by the cHiPLC Nanoflex microchip system (Dublin, CA, USA) with nanoLC–MS/MS (Nano Ultra 2D Plus, Eksigent) equipped with an AB SCIEX TripleTOF 5600 MS (Toronto, Concord, Canada) (Kang et al. 2015). MS/MS spectra were identified using the MASCOT engine software (Matrix Science, London, UK; version 2.2) embedded in the Proteome Discoverer 1.4 software (Thermo Electron, San Jose, CA). The protein species were filtered using the cRAP database (ftp://ftp.thegpm.org/fasta/cRAP) (Torabi et al. 2009). Moreover, the functional/biological processes were searched by using NCBInr databases (http://blast.ncbi.nlm.nih.gov) (version 10/06/2015, 4,075,097 entries), and then grouped into biological functions using Gene Ontology terms (Wang et al. 2012). For protein identification, the following parameters were used: Viridiplantae as the taxonomy, MS/MS as the ion search type, trypsin as the digestion enzyme, carbamidomethyl (C), iTRAQ8plex (N-term), and iTRAQ8plex (K) as the fixed modfication, oxidation (M) as the variable modification, the matched unique peptides at ≥1, monoisotopic as the mass values, the max missed cleavage at 2.0, the fragment mass tolerance at 0.1 Da, peptide mass tolerance at ±20 ppm, and the peptide false discovery rate (FDR) at ≤0.01 as described in our previous study (Li et al. 2017).

For protein quantification, the MS/MS spectra revealed unique proteins that were identified by extract peak intensities of the iTRAQ labeling tags. Then, they were normalized among samples with the built-in bias correction function of ProteinPilot (AB Sciex, Inc). Using the software's standard procedures, we generated relative protein quantification for comparisons of interest. In one 8-plex iTRAQ reagent, only one replicate of each sample was labelled and ratios of the identified protein species between DN and CK (e.g., replicate 1 of N-deficient root vs replicate 1 of N-sufficient root) were calculated and compared according to the protein abundance. According to methods of Ma et al. (2012), we used the 1.2-fold cutoff for further analysis in order to contain more candidate genes involving in wheat response to N deficiency because there were fewer up- or down-regulated protein species with the abundance change more than 1.5 or 2.0-fold cutoff.

Determination of the contents of selected phytohormones

Root and leaf tissues of wheat seedlings were harvested separately at 8 days after incubation in two nutrient media. Sampled tissues (2.5 g) were homogenized with 3 cm^3 10% trichloroacetic acid, then incubated overnight at 20 °C, and centrifuged at 8000 g for 1 h at 4 °C. Precipitates were dried at 40 °C in vacuum and homogenized with lysate solution (1.35 g of urea, 0.1 g of CHAPS, and 2.5 mm^3 of double-distilled water). The contents of ABA, auxin (IAA), salicylic acid (SA), jasmonic acid (JA), cytokinin (CTK), and ethylene (ETH) were determined using an enzyme–linked immunosorbent assay kit (R&D, USA) according to the manufacturer's instructions.

In vitro transcription of virus-induced genes and plant inoculation

ABA role in response to N deficiency was identified using the BSMV-VIGS method by identifying the function of wheat *TaZEP* gene. Because of asymmetric expression patterns of two or more homoeoalleles in allopolyploid plants (Møller et al. 2011), only one copy (gi|474,244,839) of *TaZEP* was identified in our study. In this study, cDNA sequences of three copies were identified in the genome of bread wheat, and they had high similarities (98.2%) (Supplementary Fig. S1). To prevent functional complementation and allow commonly silencing of three copies (homologues) of *TaZEP* gene in genome of bread wheat, a conserved cDNA fragment (254 bp), which shares high similarities (99.5%) among three homologues (Supplementary Fig. S1), was used to construct silencing vector (BSMV-TaZEP) to possibly result in the simultaneous silencing of three homologues in the wheat genome. In BSMV-TaZEP-inoculated wheat plants, transcription levels of *TaZEP* gene were to identify the total expression profile of all three homologues. PCR primers specific to the family member but shared by the three homologues of the same gene were designed and used for quantitative

real-time PCR (qRT-PCR) (Song et al. 2012; Grün et al. 2018), and primers and the amplified fragment are indicated in Supplementary Fig. S1.

In this study, the BSMV-VIGS method was performed as previously described (Zhang et al. 2016). The α, β, and γ and α-, β-, and γ-ZEP transcripts of the BSMV genome were synthesized using RiboMAX™ Large Scale RNA Production System-T7 (Promega, USA). The RNA-α, RNA-β, and RNA-γ and RNA-α-, −β-, and -γ-ZEP transcripts were separately mixed in a 1:1:1 ratio, added to diethylpyrocarbonate-treated distilled water (1:1), and subsequently added with an abrasive FES buffer (Scofield et al. 2005). BSMV-γ:00 and BSMV-*TaZEP* were composed of α, β, and γ transcripts and RNA-α-, −β-, and -γ-ZEP transcripts, respectively. Two-week-old wheat seedlings were separately inoculated with BSMV-γ:00 and BSMV-*TaZEP*, and 10 mm^3 viral RNA was inoculated on the downmost second leaf of each wheat seedling (Supplementary Fig. S2). Inoculated leaves were sprayed with nuclease-free water and then covered with plastic film to maintain high relative humidity (85.0%) and darkness for 24 h in an illumination incubator. After inoculation, the wheat seedlings were immediately transferred to N-free Hoagland solution for the N deficiency stress treatment. At 8 days after inoculation, wheat seedlings with chlorosis were hypothesized to be BSMV-infected plants (Liu et al. 2016). In this study, 40 (80.0%) and 43 (86.0%), and 38 (76.0%) and 44 (88.0%) of the 50 BSMV-γ:00-infected or BSMV-*TaZEP*-inoculated wheat seedlings showed chlorosis under full-length N supply and nitrogen deficiency conditions, respectively (Supplementary Table S1). At the same time, the inoculated and most adjacent and higher leaves of BSMV-inoculated wheat seedlings were sampled, and qRT-PCR was performed to determine the transcription levels of *TaZEP* gene for further identification of the BSMV-*TaZEP*-inoculated wheat seedlings. The primers are indicated in Supplementary Fig. S1. At 8 days after inoculation, dry weight and ABA contents of the BSMV-*TaZEP*-inoculated wheat seedlings identified above were examined as described. N contents were measured as described by He and his colleagues (He et al. 2016b).

Statistical analysis

In this study, all experiments were repeated in triplicate, and each sampling was analyzed separately. The results were analyzed using SPSS 17.0 statistical software (SPSS Inc., Chicago, IL., USA), and Duncan's multiple range test (DMRT) was used to identify significant ($P < 0.05$) differences among the group means.

Results

A large number of N deficiency-responsive protein species identified using the iTRAQ method in wheat seedlings suffering from N deficiency

To identify the N deficiency-responsive protein species, proteome profiles were generated using the iTRAQ method in the root and leaf tissues of wheat seedlings subjected to N deficiency for 8 days. Three independent biological replicates were used for each sample for iTRAQ labeling, and then the spectra were analyzed using the NCBInr database. A total of 10,001 peptides from 151,654 spectra (Supplementary Table S2), and 7336 protein species were identified (Supplementary Table S3), of which 4571 occurred in all three biological replicates and were included in subsequent analyses (Supplementary Table S4). The mass spectrometry proteomics data for the present study are available via ProteomeXchange with the dataset identifier PXD71498.

In this study, the ratios (≥ 1.20- and ≤ 0.83-fold) in N deficient vs. control plants were considered to be changes in the altered abundance of N deficiency-responsive protein species, and Duncan's multiple-range tests were performed to determine the differences ($P < 0.05$). The protein species identified were further filtered using the cRAP database. Terminology for the identified protein species was based on the method of Schlüter and his colleagues (Schlüter et al. 2009). Based on this method, some protein species were identified with the same names and accession numbers but had different isoelectric points (pIs) or molecular weights (MWs) (Supplementary Table S5). These proteins should be classified into different protein species because one gene could have different protein products and diverse cellular tasks (Kosová et al. 2018). These could be due to nucleotide polymorphisms, proteolytic cleavage, alternative splicing, post-translational modifications, etc. (Schlüter et al. 2009). For example, two phenylalanine ammonia-lyase enzymes (gi|357,150,254) were identified in root proteomic data, but they should be treated as two different protein species because they had different pIs and MWs.

Based on the above criteria, in the present study, 1515 N deficiency-responsive protein species were identified in both root and leaf tissues of wheat seedlings suffering from 8-d N deficiency. Of these protein species, 866 (427 ≥ 1.20- and 439 ≤ 0.83-fold) and 649 (348 ≥ 1.20- and 301 ≤ 0.83-fold) were from the root and leaf tissues, respectively (Table 1, Supplementary Table S5). Our findings indicated that most of the N deficiency-responsive protein species were expressed at low abundance, and the abundance of most N deficiency-responsive protein species in the root (720/866, 83.1%) and leaf (543/649, 83.7%) tissues of N-deficient wheat seedlings was altered by 1.20 ~ 1.49- or 0.67 ~ 0.83-fold, respectively (Fig. 1).

Functional classification of N deficiency-responsive protein species and differences in the root and leaf tissues

According to the biological functions listed on the UniProt and Gene Ontology website (Li et al. 2014), the 1515 N deficiency-responsive protein species identified were classified into ten and eleven functional categories in the root and leaf tissues, respectively (Fig. 2; Supplementary Table S5). The functional categories in the root tissue were involved in hormone synthesis (2.5%), signal transduction (5.0%), nucleotide metabolism (5.3%), lipid and phosphate metabolism (1.7%), protein metabolism (22.1%), carbohydrate metabolism (12.6%), stress and defense (14.1%), transportation (9.6%), other metabolisms (3.3%), and unknown function (23.8%) (Fig. 2a). Analogous functional categories in the leaf tissue of wheat seedlings were related to hormone synthesis (2.0%), signal transduction (4.6%), nucleotide metabolism (5.7%), lipid and phosphate metabolism (2.2%), protein metabolism (18.2%), carbohydrate metabolism (9.9%), photosynthesis (5.7%), stress and defense (14.2%), transportation (11.4%), other metabolisms (2.6%), and unknown function (23.6%) (Fig. 2b). Predicted, hypothetical, uncharacterized, and unnamed N deficiency-responsive protein species were classified into the unknown function category. A large proportion (23.8% and 23.6% in root and leaf tissues, respectively) of unknown function protein species were not annotated or identified. A high proportion of unknown function protein species were not annotated (Fig. 2; Supplementary Table S5).

The amount and percentage of the carbohydrate metabolism-related protein species identified in root tissue (109/866, 12.6%) were greater than those in leaf tissue (64/649, 9.9%). The amount and percentage (66/109, 60.6%) of downregulated carbohydrate metabolism-related protein species in root tissue were greater than those (36/64, 56.2%) in leaf tissue. Some (155/1515, 10.2%) of the identified N deficiency-responsive proteins were found in both the root and leaf tissues of N-deficient wheat seedlings (Table 1, Supplementary Table S6). However, most (1360/1515, 89.8%) of the protein species identified appeared specifically between the root and leaf tissues.

Table 1 Fold changes and tissue-specificity of the identified N deficiency-responsive protein species in wheat plants

Tissue	Fold changes			Common or specific expression	
	Total	≥1.20	≤0.83	Common	Specific
Root	866	427	439	155	711
Leaf	649	348	301	155	394

Two week-old wheat seedlings were transferred to N-deficient full-length Hoagland medium for 8 d, an iTRAQ proteomics was performed in the root and leaf tissues of N-deficient wheat seedlings, and the N deficiency-responsive protein species were identified in these two tissues

Some phytohormones could be involved in N deficiency in wheat seedlings

Our study identified a few protein species related to the synthesis of important plant hormones, such as ABA, jasmonic acid (JA), ethylene (ETH), auxin (IAA), salicylic acid (SA), and cytokinin (CTK). In the root tissue, 2, 4, 9, 2 and 5 protein species were involved in the synthesis of ABA, SA, JA, CTK, and ETH, respectively. In the leaf tissue, 3, 3, 1, 3, 2 and 1 were separately related to the synthesis of ABA, JA, IAA, SA, CTK, and ETH (Supplementary Table S5). However, there were some differences in the changes in the contents of these plant hormones between the root and leaf tissues (Fig. 3).

Phytohormone contents (IAA, ABA, SA, JA, CTK and ETH) were measured next in both root and leaf tissues of wheat seedlings at 8 days after N deficiency. Our data indicated that ABA, JA, and ETH contents markedly increased by 37.3% and 43.6%, 17.5% and 23.6%, and 17.2% and 14.0% in both root and leaf tissues, respectively (Fig. 3a-c). However, the contents

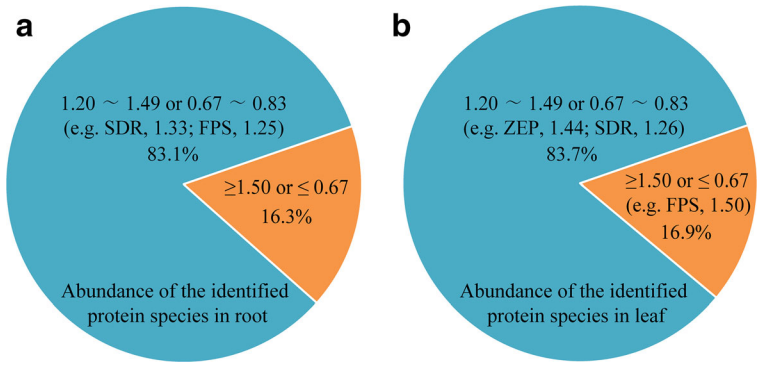

Fig. 1 Proportions of the identified protein species with decreased (blue) and increased (yellow) abundances in root (**a**) and leaf (**b**) tissues of N-deficient wheat seedlings with respect to control plants. Two weeks-old wheat seedlings were transferred to N-deficient full-length Hoagland medium for 8 d. At this timepoint, some growth and physiological parameters (plant height, root length, and root or leaf dry weights) showed significant changes between N-deficient wheat seedlings and N-sufficient wheat seedlings, and thus, iTRAQ proteomics was used to measure the global protein expression patterns in the root (**a**) and leaf (**b**) tissues of N-deficient wheat seedlings. Venn diagrams show the proportions of the identified protein species with low (blue) and high (yellow) abundances. Only protein species commonly identified in three biological replicates for each sample were considered. Names and abundance of the identified representative protein species related to ABA biosynthesis (e.g. zeaxanthin epoxidase, ZEP; short-chain dehydrogenase, SDR; farnesyl pyrophosphate synthase, FPS) are indicated in brackets

of IAA, CTK, and SA significantly decreased by 60.5% and 58.0%, 69.4% and 70.0%, and 46.8% and 37.2% in both root and leaf tissues, respectively (Fig. 3d-e). At 8 d after N deficiency, the contents of ABA in both root and

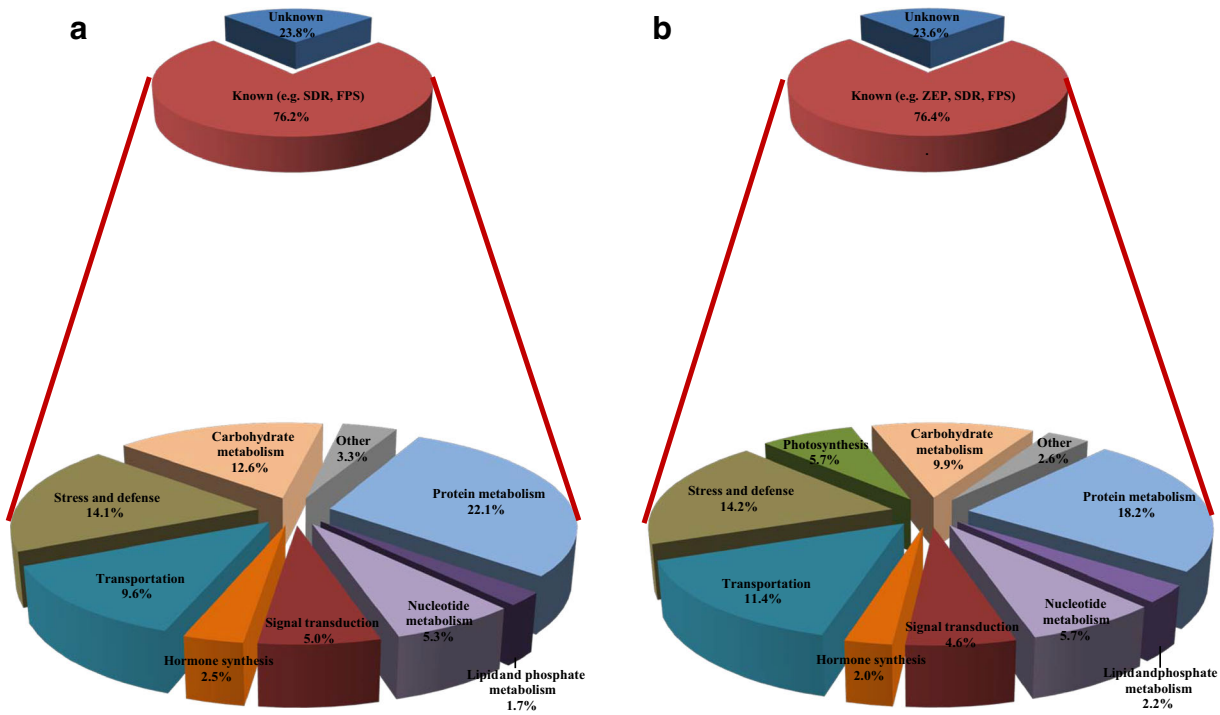

Fig. 2 Functional categorization of N-responsive protein species in root and leaf tissues of bread wheat seedlings suffering from N deficiency for 8 days. Two weeks-old wheat seedlings were transferred to N-deficient full-length Hoagland medium for 8 days, and an iTRAQ proteomics was performed in the root and leaf tissues of N-deficient wheat seedlings. The N deficiency-responsive protein species identified in root (**a**) and leaf (**b**) tissues were functionally categorized according to the biological functions listed on the UniProt and Gene Ontology website (Li et al. 2014). Names of the identified and representative protein species related to ABA synthesis (ZEP, SDR, and FPS) are indicated in brackets, and ZEP, SDR, and FPS have been annotated in Fig. 1

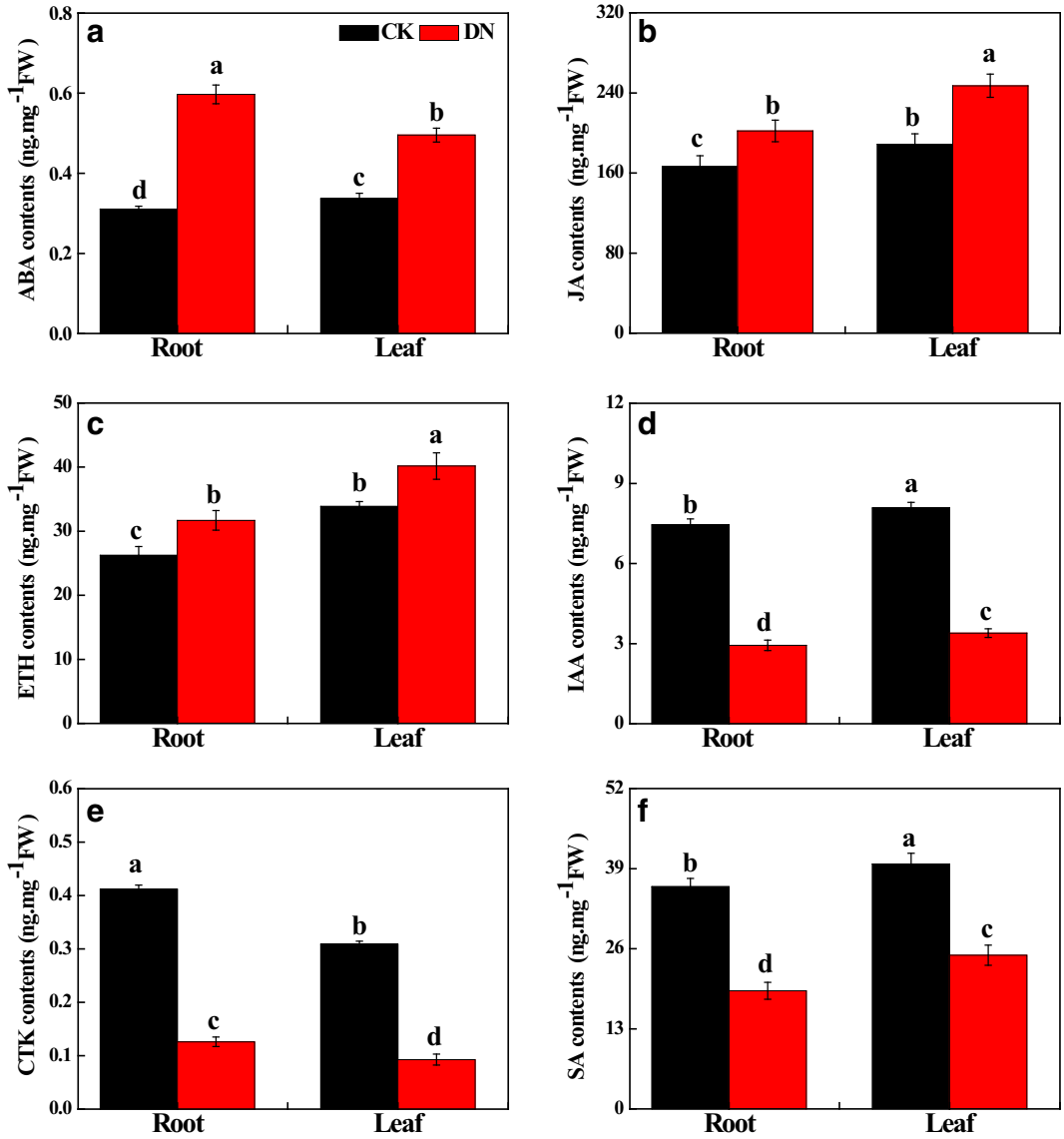

Fig. 3 Contents of the phytohormones in N-deficient wheat seedlings. Two weeks-old wheat seedlings were transferred to full N conditions (6 mM, CK) or N-free Hoagland solution for the N-deficient (DN) full-length Hoagland medium for 8 d, and contents of phytohormones ABA (**a**), JA (**b**), ETH (**c**), IAA (**d**), CTK (**e**), SA (**f**) both in root and leaf tissues of N-sufficient and -deficient wheat seedlings were measured. Each value is mean ± standard deviation (SD) of three biological replicates, and different letters indicate significant differences ($P < 0.05$) relative to control by using two-way ANOVA followed by Duncan's multiple range test

leaf tissues were increased by 4.50- and 5.20-fold, respectively, far higher than those of other phytohormones (Fig. 3a-c).

Roles of ABA hormone in N-deficient wheat seedlings

A conserved fragment (254 bp) of *TaZEP* gene was used to construct a BSMV-derived *TaZEP* silencing vector, which was inoculated into the leaves of wheat seedlings. BSMV-γ:00-inoculated wheat plants were used as control. Our results indicated that BSMV-VIGS-induced chlorosis appeared in the middle of seedling leaf tissue at 8 days after inoculation (Fig. 4), and the efficiency of the BSMV-VIGS was over 76% (Supplementary Table S1). Total transcription levels of three *TaZEP* homologues and contents of ABA in the leaf tissue of BSMV-*TaZEP*-inoculated wheat seedlings were markedly reduced by 56.7% and 61.6%, respectively (Fig. 5),

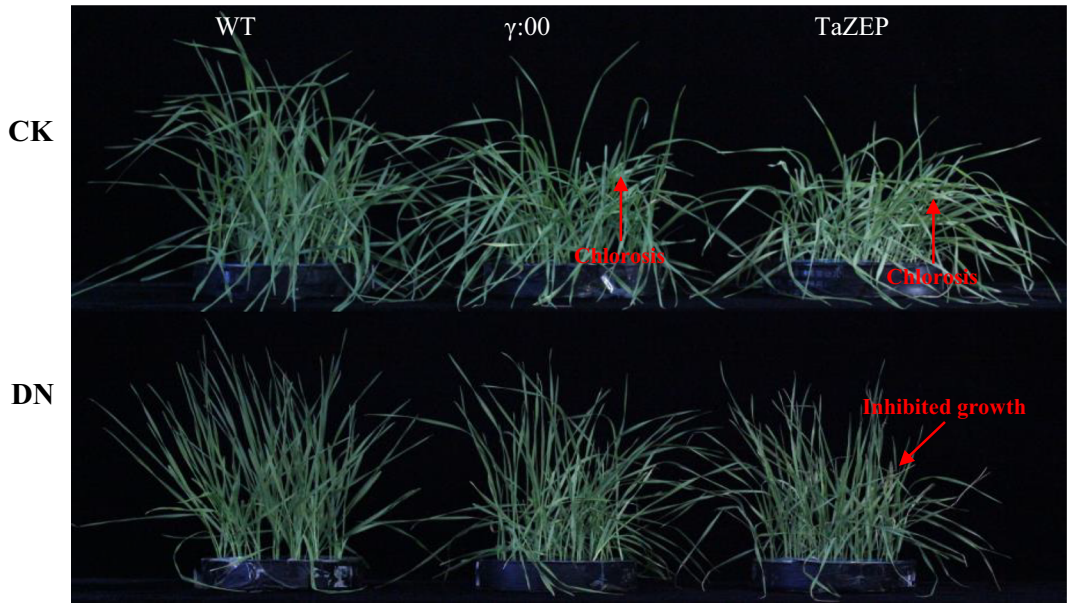

Fig. 4 Phenotypes of the BSMV-TaZEP vector-inoculated wheat seedlings at 8 days after nitrogen deficiency. Two-week-old wheat seedlings were sprayed with nuclease-free water (WT), or separately inoculated with 10 mm^3 BSMV-γ:00 (γ:00) and BSMV-TaZEP (TaZEP) viral RNA on the downmost second leaf of the wheat seedlings. After inoculation, wheat seedlings were immediately transferred to full N conditions (6 mM, CK) or N-free Hoagland solution for the N deficiency (DN) for 8 days to observe their phenotypes. The efficiency of the BSMV-VIGS was over 76%, and the number is indicated in Supplementary Fig. S1

suggesting that *TaZEP* was successfully silenced and ABA synthesis was greatly inhibited in wheat seedlings. Our data indicated that, compared to BSMV-γ:00-inoculated wheat seedlings, the growth of the BSMV-TaZEP-inoculated wheat seedlings was substantially inhibited (Fig. 4) and the total dry weights and N contents of the BSMV-*TaZEP*-silenced wheat seedlings were significantly reduced (Fig. 5). These results suggested that silencing the gene encoding TaZEP enzyme resulted in a decrease in the tolerance to N deficiency in wheat seedlings, suggesting a role of ABA in response to N deficiency.

Discussion

A large number of identified N deficiency-responsive protein species helped further reveal the adaptive mechanism of N deficiency in both root and leaf tissues of bread wheat

In this study, we identified 866 and 649 N deficiency-responsive protein species in the root and leaf tissues, respectively (Supplementary Table S5). Numbers of the identified N deficiency-responsive protein species in this study were far more than those (≤ 65) in the above previous studies (Møller et al. 2011; Deng et al. 2014; Meise et al. 2017). The differences in the number of N deficiency-responsive protein species identified from our study and those of previous studies could be attributed to proteomic technologies, experimental designs, the divergences of genome sequences between such distantly related species, and the different statistical cut-off and fold-change thresholds. Thus, our work provided more molecular information on important crops responses to N deficiency. In the present study, however, a large proportion (359/1515, 23.7%) of unknown function protein species were not annotated (Fig. 2, Supplementary Table S5), possibly because genome of bread wheat is huge and complex, and this has resulted in slower research progress compared with homologous species (rice, maize, etc) of relatively simple genomes (Brenchley et al. 2012).

There were some differences in the categories of N deficiency-responsive protein species identified between the root and leaf tissues of N-deficient wheat seedlings (Supplementary Table S6), suggesting that they may have similar biological functions in the response to N deficiency in the two tissues. However, most (1360/1515, 89.8%) of the protein species

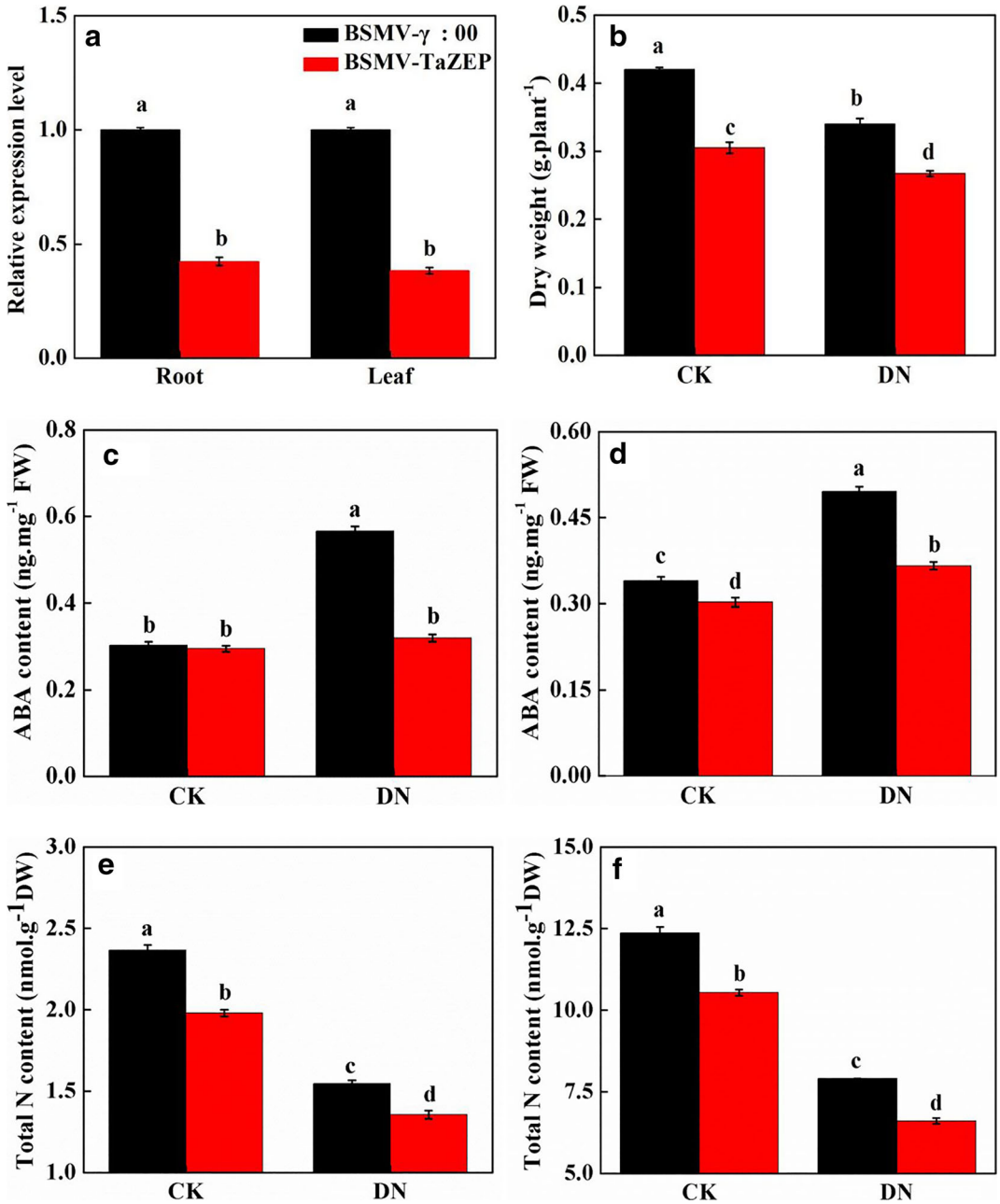

Fig. 5 Transcription levels of *TaZEP* gene, and some physiological parameters on BSMV-*TaZEP* vector-inoculated wheat seedlings suffering from nitrogen deficiency. Two-week-old wheat seedlings were separately inoculated with 10 mm^3 BSMV-γ:00 (γ:00) and BSMV-TaZEP (TaZEP) viral RNA on the downmost second leaf of the wheat seedlings. After inoculation, the wheat seedlings were immediately transferred to full N conditions (6 mM, CK) or N-free Hoagland solution for the N deficiency (DN) for 8 days to measure the transcription levels of *TaZEP* gene (**a**), dry weight (**b**), ABA contents in both root (**c**) and leaf (**d**) tissues, total nitrogen contents in both root (**e**) and leaf (**f**) tissues. The transcription levels of *TaZEP* gene were to identify the total expression profile of all three homologues. BSMV-γ:00 vector-inoculated wheat seedlings were used as positive control. Each value is mean ± standard deviation (SD) of three biological replicates, and different letters indicate significant differences ($P < 0.05$) relative to control by using two-way ANOVA followed by Duncan's multiple range test

identified appeared specifically between the root and leaf tissues (Supplementary Table S6), implying that root and leaf tissues have differential N deficiency-responsive mechanisms. The differences in the protein

species identified between the root and leaf tissues might be related to their different functions, growth environments, and sensitivities to long-term N deficiency. The different proteome profiles for root and leaf tissues further suggest that long-term N deficiency may have profound and varying effects on growth in the root and leaf tissues of wheat seedlings.

Comparison of proteome profiles in this study with transcriptome profiles in previous studies in plant responses to N deficiency

Our leaf proteome profile of N-deficient bread wheat seedlings was compared with the barley leaf transcriptome profile of N-deficient barley seedlings (Wei et al. 2016), because the plant materials (bread wheat and barley) used in these two studies were close relatives. Moreover, there were many similarities between the two studies, such as similar sampling tissues (leaves) and similar environment conditions (N-deficient solutions). We found that the 176 (176/528, 33.3%) function-known leaf protein species in our study were commonly identified in the leaf transcriptome analysis of N-deficient barley seedlings (Wei et al. 2016). These common N deficiency-responsive protein species (genes) were involved in carbohydrate metabolism, protein metabolism, stress and defense, and photosynthesis (Supplementary Table S7). However, most (352/528, 66.7%) of the N deficiency-responsive protein species in our results were not identified in the leaf transcriptome profiles of N-deficient barley seedlings (Supplementary Table S7) (Wei et al. 2016), further suggesting that these protein levels rarely correlated with mRNA expression levels because of posttranscriptional, translational, posttranslational modifications, as well as differential mRNA and protein degradation rates (Torabi et al. 2009; Vogel and Marcotte 2012; Hu et al. 2013). Therefore, our proteome identified some N deficiency-responsive protein species and enhanced our further understanding of plant responses to N deficiency.

Roles of phytohormone ABA in the N-deficient wheat seedlings

Most stress and defense-, signal transduction-, photosynthesis-, carbohydrate metabolism-, protein metabolism-, nucleotide metabolism-, and other metabolism-related genes have been identified, and their functions have also been discussed in previous N-transcriptomic and proteomics results (Gelli et al. 2014; Yang et al. 2015; He et al. 2016a; Quan et al. 2016; Wei et al. 2016; Curci et al. 2017; Nawaz et al. 2018). Thus, the protein species involved in the functional categories above and identified in this study were not discussed further. However, phytohormones are very important elements of signaling pathways in plants, and they regulate most developmental and physiological processes (Lee and Luan 2012; Chao et al. 2016). In addition, data on the protein species that regulate phytohormones are scarce (Møller et al. 2011; Deng et al. 2014; Meise et al. 2017), possibly because of the low abundance of hormone-related enzymes.

In the present study, a large number (35) of protein species involved in synthesis of phytohormones ABA, JA, ETH, IAA, CTK and SA were first identified in both root and leaf tissues of wheat seedlings (Supplementary Table S5), and their contents were greatly changed in these two tissues (Fig. 3). These implied that they could be involved in the response to N deficiency in wheat seedlings. However, there were some differences in the changes in the contents of these plant hormones between the root and leaf tissues (Fig. 3), partly accounting for their differential roles or the caused differences in growth parameters (e.g., root and shoot fresh and dry weights) in these two tissues of N-deficient wheat seedlings (Zhang et al. 2019). At 8 d after N deficiency, the contents of ABA in both root and leaf tissues were far higher than those of other phytohormones (Fig. 3a-c), similar to the altered abundance of the hormone synthesis-related protein species identified (Supplementary Table S5). Thus, we speculated that ABA could play an important role in wheat seedlings in response to N deficiency.

Bread wheat (*Triticum aestivum* L.) has a very large, 17-gigabase-pair (Gb), allohexaploid genome (2n = 6x = 42, AABBDD; 2n is the number of chromosomes in each somatic cell, and 6x is the basic chromosome); therefore, each wheat gene potentially exists as a trio of A, B, and D homoeologs (Feldman et al. 2012). This species may be the last cereal to be genetically transformed because the efficiency of the two main transformation methods (particle bombardment and cocultivation with *Agrobacterium tumefaciens*) in this species is very low due to cultivar specificity, multiple-copy insertions, time consumption, low transformation efficiency, etc. (Wu et al. 2003; Zhao et al. 2006). BSMV-VIGS mainly exploits the BSMV-mediated antiviral defense

mechanism of plants to explore the function of endogenous genes based on homology-dependent gene silencing because it is not required for plant transformation, facilitates rapid generation of gene knockdown phenotypes, offers a fast and rapid transient assay for silencing of gene expression, and has been developed as more successful genetics approach to assess gene functions than simple constitutive transgene in polyploid plant species (Cao et al. 2011; Tufan et al. 2011). Although results from BSMV-VIGS are not comparable to results from stable transformation experiments (e.g., RNAi or CRISPR/Cas9), BSMV-VIGS findings are useful for increasing our understanding of gene function and may lead to the development of a strategy that is more successful than simple constitutive transgene overexpression (Borisjuk et al. 2019).

ZEP is one key enzyme of ABA synthesis in higher plants (Cuming and Stevenson 2015), and its abundance was greatly regulated in leaf tissue of wheat seedlings suffering from N deficiency (Supplementary Table S5). To verify the above speculation, in this study, the function of ABA in wheat seedlings response to N deficiency was determined by using BSMV-VIGS-mediated ZEP silence (Supplementary Fig. S1). After 8 d of N deficiency, BSMV-TaZEP-inoculated wheat seedlings displayed the inhibited growth (Fig. 4), and the dry weights, ABA and total nitrogen contents in both root and leaf tissues of BSMV-VIGS-TaZEP silenced wheat seedlings were markedly reduced (Fig. 5). These results suggested that silencing the gene encoding TaZEP enzyme resulted in a decrease in the tolerance to N deficiency in wheat seedlings, suggesting a role of ABA in response to N deficiency.

Based on our experimental data, we proposed a putative ABA-dependent schematic model in higher plants under N deficiency (Fig. 6). In this pathway, plant cells could perceive external N deficiency in cell membrane, and N deficiency signaling is subsequently transduced by Ca^{2+} sensors, kinases, or other signaling components, which then induce translational changes in many functional protein species related to phytohormones (e.g., ABA). ABA and other phytohormones then regulate the downstream transcriptional, translational, and posttranslational responses, and, finally, plants exert many morphological and physiological adaptive changes that assist survival under N-deficiency stress.

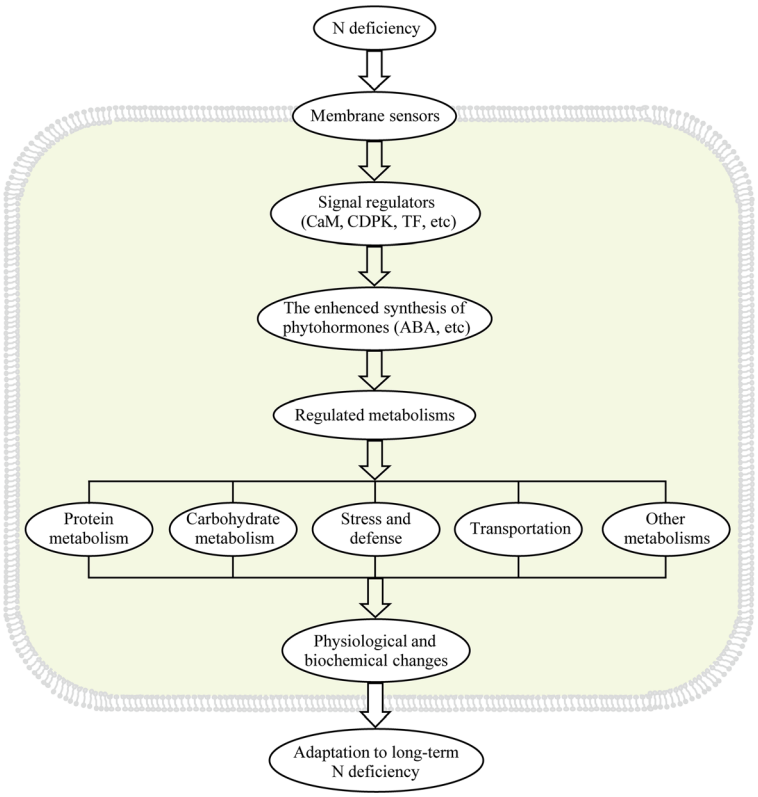

Fig. 6 A schematic model of the molecular responses underlying adaptation to N deficiency in higher plants. ABA, abscisic acid; CaM, calmodulin; CDPK, Ca^{2+}-dependent protein kinase; TF, transcription factor

Conclusions

Based on large-scale proteomics, in this study, we identified 1515 N deficiency-responsive protein species with significantly altered abundance in wheat seedlings, and these species were functionally related to diverse biological processes. Of the identified protein species, the abundance of an important ABA synthesis-related enzyme (TaZEP) was significantly enhanced, and ABA contents were also increased, suggesting the possible role of ABA in response to N deficiency. Silencing of *TaZEP* gene markedly decreased the tolerance of wheat seedlings to N deficiency. These results suggest a role for ABA in wheat response to N deficiency. Extensive further investigations are merited to integrate ABA and other signaling regulatory components into a comprehensive signaling network, which would greatly increase our understanding of plant responses to N-deficiency stress.

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