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SWATH-MS quantitative proteomic investigation of nitrogen starvation in Arabidopsis reveals new aspects of plant nitrogen stress responses

Fu-Yuan Zhu[a,b,1], Mo-Xian Chen[c,1], Wai-Lung Chan[b], Feng Yang[b], Yuan Tian[c], Tao Song[c], Li-Juan Xie[d], Ying Zhou[d], Shi Xiao[d], Jianhua Zhang[c,e,*], Clive Lo[b,**]

[a] Co-Innovation Center for Sustainable Forestry in Southern China, College of Biology and the Environment, Nanjing Forestry University, Nanjing, China
[b] School of Biological Sciences, The University of Hong Kong, Pokfulam Road, Hong Kong, China
[c] Shenzhen Research Institute, The Chinese University of Hong Kong, Shenzhen, China
[d] State Key Laboratory of Biocontrol and Guangdong Provincial Key Laboratory of Plant Resources, School of Life Sciences, Sun Yat-sen University, Guangzhou, China
[e] Department of Biology, Hong Kong Baptist University, and State Key Laboratory of Agrobiotechnology, The Chinese University of Hong Kong, Shatin, Hong Kong, China

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ABSTRACT

Nitrogen is an essential macronutrient for plant growth and crop productivity. The aim of this work was to further investigate the molecular events during plant adaptation to nitrogen stress. Here, we present a SWATH-MS (Sequential window acquisition of all theoretical mass spectra)-based quantitative approach to detect proteome changes in Arabidopsis seedlings following nitrogen starvation. In total, 736 proteins of diverse functions were determined to show significant abundance changes between nitrogen-supplied and nitrogen-starved Arabidopsis seedlings. Functional categorization revealed the involvement of nitrogen stress-responsive proteins in biological processes including amino acid and protein metabolism, photosynthesis, lipid metabolism and glucosinolate metabolism. Subsequent phospholipid profiling of Arabidopsis seedlings showed changes in phospholipid composition that may enhance membrane fluidity as a response to nitrogen starvation. Moreover, an Arabidopsis *grf6* T-DNA insertion mutant was found to have a nitrogen stress-sensitive phenotype. GRF6 is a 14-3-3 protein with elevated abundance upon nitrogen starvation and it may function as a positive regulator during nitrogen stress adaptation.

1. Significance

Low nitrogen use efficiency in crop plants leads to economically inefficient cropping systems while excessive nitrogen fertilization continuously causes environment problems. Here, we describe the proteome changes in Arabidopsis seedlings following nitrogen starvation, highlighting several metabolic processes. A potential positive regulatory role of a 14-3-3 protein (GRF6) was also revealed. Our work provides new insights into plant nitrogen stress responses at the proteome level, potentially useful for dissecting the molecular components of nitrogen responses in plants for adapting to low nitrogen availability.

2. Introduction

Nitrogen nutrition is an important factor essential for plant growth and productivity [1], influencing many biological processes in plants such as flowering, senescence and photosynthesis [2]. Nitrogen sources can be available from organic and inorganic compounds in the soil. Amino acids are the main organic sources whereas nitrate and ammonium are the predominant inorganic sources [3, 4]. Crop growth is often limited by low bio-availability of nitrogen in the soil which needs to be supplemented with extra nitrogen fertilizers during the farming season. However, due to the low nitrogen use efficiency of crop plants, excessive applications of nitrogen fertilizer result in deleterious environmental problems including eutrophication and soil pollution [5, 6]. Better understanding of nitrogen nutrition and stress responses in plants is needed for developing strategies to enhance nitrogen use efficiency, hence reducing excessive fertilizer usages.

Nitrogen use efficiency primarily depends on nitrogen uptake efficiency, which is mediated by plasma membrane-localized influx

* Corresponding author at: Department of Biology, Hong Kong Baptist University, and State Key Laboratory of Agrobiotechnology, The Chinese University of Hong Kong, Shatin, Hong Kong, China.
** Corresponding author at: School of Biological Sciences, The University of Hong Kong, Pokfulam Road, Hong Kong, China
E-mail addresses: jzhang@hkbu.edu.hk (J. Zhang), clivelo@hku.hk (C. Lo).
[1] These authors contributed equally to this work.

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transporters [7, 8]. There are different mechanisms in plants for the uptake of ammonium and nitrate which are the main nitrogen sources in soils. For ammonium uptake, six ammonium transporters belonging to the AMT/MEP/Rh (AMT) superfamily in Arabidopsis have been demonstrated to have high affinity for ammonium [9, 10]. Five of the six Arabidopsis *AtAMT* genes showed root expression which was up-regulated during low nitrogen availability [9]. Under such condition, about 90% of the high affinity ammonium uptake is contributed by AtAMT1;1, AtAMT1;2 and AtAMT1;3 addictively [9, 11]. AtAMT1.1, AtAMT1.3 and AtAMT1.5 are responsible for absorbing ammonium directly from the soil, whereas AtAMT1.2 transports apoplastic ammonium into the cell [9, 11]. For nitrate uptake, the NITRATE TRANSPORTER 1/PEPTIDE TRANSPORTER family (NPF) and NITRATE TRANSPORTER 2 (NRT2) family have been identified in Arabidopsis [12]. NRT2 members are high-affinity nitrate transporters accounting for 95% of the uptake capacity, whereas the NPF members showed a low affinity for nitrate [13], suggesting that NRT2 transporters are critical for efficient nitrate uptake to sustain growth under low nitrogen availability. Furthermore, nitrate flux studies revealed that AtNRT2.1, AtNRT2.2, AtNRT2.4 and AtNRT2.5 are the predominant contributors for nitrate transport. According to their spatial expression patterns during nitrogen deficiency, AtNRT2.4 and AtNRT2.5 are involved in nitrate uptake from soil while AtNRT2.1 plays a role in apoplastic nitrate absorption [14]. Interestingly, AtNRT1.1 has been demonstrated with the dual roles of affinity transport and nitrate-sensing [15].

Transcriptomic and proteomics analyses of plants during nitrogen deficiency have been conducted to provide insights on the regulatory mechanisms of nitrogen utilization and stress responses [16, 17]. So far, knowledge on the regulatory mechanisms of plant nitrogen response at protein levels remains limited due to the small number of identified proteins from these studies. In the present work, changes in the proteome profile in Arabidopsis seedlings under nitrogen deprivation were examined using the SWATH-MS (sequential windowed acquisition of all theoretical mass spectra) approach which provides quantification of protein abundances and large-scale identification of nitrogen stress-responsive proteins. A total of 1676 proteins were quantitatively identified in Arabidopsis seedlings, 736 of which were found to be differentially expressed upon nitrogen starvation (cut-off: z-value ≥ 2.0 or $\leq$-2.0 at $P < 0.05$). The nitrogen stress-responsive proteins have been categorized into different biological processes including amino acid biosynthesis, protein translation, lipid metabolism and glucosinolate metabolism. Phospholipid profiling revealed composition changes in membrane lipid which may enhance fluidity upon nitrogen deficiency. Finally, mutant analysis revealed a potential positive regulatory role for a 14-3-3 protein (GRF6) with increased abundances in nitrogen-starved Arabidopsis seedlings.

3. Results and discussion

To ensure a meaningful nitrogen deficiency treatment for proteomic analyses, several physiological parameters after nitrogen deprivation were first determined. Seven-day-old Arabidopsis seedlings were cultured on 2 mL of either MS or MS medium without nitrogen sources (MS-N) for 48 h. As shown in Fig. 1, nitrogen starvation caused reduction of ammonium, nitrate, protein, and chlorophyll contents in the seedlings. Subsequently, a SWATH-MS-based proteomics investigation was performed to investigate the effects of nitrogen starvation on proteome changes in Arabidopsis seedlings. After combining the data from four biological replicates, a total of 1676 unique proteins were quantified by MarkerView (information of the identified peptides and proteins is shown in Table S1). Proteins with a z-value of above 2.0 or below -2.0 ($P < 0.05$) were considered as nitrogen stress-responsive in this study. As such, a total of 736 proteins were found to show significant abundance changes upon nitrogen starvation: 312 increased and 424 decreased (Table S2).

To examine the potential coordinately regulated proteins contributing to nitrogen-starvation responses, functional classification of the nitrogen stress-responsive proteins was performed using the MapMANBIN system (http://ppdb.tc.cornell.edu/dbsearch/searchacc.aspx) (Table S2). Proteins with significantly increased or decreased abundance changes were assigned to 27 functional categories. Accordingly, proteins belonging to the categories “Protein metabolism”, “Amino acid metabolism” and “Photosynthesis” constitute the largest groups of nitrogen stress-responsive proteins, suggesting that these processes were substantially affected during the nitrogen deficiency. For proteins with increased abundances, “Protein metabolism”, “Signaling, and “Transport” constituted the top 3 functional categories (Fig. 2A). For proteins with decreased abundances, “Protein metabolism”, “Photosynthesis”, and “Amino acid metabolism” constituted the top 3 functional categories (Fig. 2B). These findings suggested that different biological processes were activated or repressed in Arabidopsis seedlings to cope with nitrogen starvation.

3.1. Impact on amino acid biosynthesis and protein translation

KEGG classification of the nitrogen stress-responsive proteins provides a quick overview on the metabolic pathways that are most influenced in the nitrogen-starved Arabidopsis seedlings. We identified 44 enzymes that could be mapped to different amino acid biosynthesis pathways, most of them showing reduced protein abundances (Fig. 3). It is well known that the amino acids glutamine, asparagine and aspartate are important nitrogen carriers in plants generated by the assimilation of inorganic nitrogen [3]. Therefore, nitrogen deprivation would block the incorporation of nitrogen into amino acids, leading to an overall down-regulation of amino acid biosynthesis. A similar phenomenon was observed in barley and wheat during nitrogen starvation [18, 19]. Meanwhile, several proteins associated with amino acid degradation showed increased abundances upon nitrogen starvation (Table S2), presumably representing a feedback response to generate endogenous nitrogen sources.

Limitation of nitrogen supply is also expected to impact protein synthesis. Consistently, 93 proteins involved in translation were found to have reduced abundances, including a large number of ribosomal proteins in cytosol and plastids as well as several initiation and elongation factors (Table S3). This would indicate an overall down-regulation of protein synthesis, consistent with the lower protein content in the nitrogen-starved Arabidopsis seedlings (Fig. 1A). On the contrary, 19 proteins participating in translation showed elevated abundances upon nitrogen starvation (Table S3). Presumably they are involved in the synthesis of proteins specific for nitrogen stress responses. Interestingly, two of the translation initiation factors, eIF4A (At3G19760) and eIF4E (At4G18040), are associated with abiotic and biotic stresses, respectively [20, 21]. *eIF4A* expression is induced following cold or heat treatment and gene disruption resulted in increased sensitivities to both cold and heat stresses [20, 21]. On the other hand, eIF4E and its isoform eIF(iso)4E are indispensable for potyvirus infection and gene disruption led to resistance [22, 23]. In fact, several translation initiation factors were considered as potential targets for genetic manipulation to improve plant performance and adaptation [20, 21]. Hence, the roles of translation initiators like eIF4A and eIF4E in nitrogen starvation responses may be further elucidated for potential applications.

3.2. Impact on photosynthesis: light reactions and carbon dioxide assimilation

Nitrogen deficiency inhibits photosynthesis through reduction of chlorophyll content and suppression of ribulose bisphosphate carboxylase/oxygenase (Rubisco) enzymatic activities [24, 25]. In this study, a total of 29 photosynthesis-related proteins were detected with altered abundances: 22 decreased and 7 increased (Table S2). Those proteins with reduced abundances are essential components of photosynthetic