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Proteomics reveals key proteins participating in growth difference between fall dormant and non-dormant alfalfa in terminal buds

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ABSTRACT

To explore the molecular mechanism of growth differences between fall dormant (FD) and non-FD alfalfa, we conducted iTRAQ-based quantitative proteomics on terminal buds of Maverick (FD) and Cuf101 (non-FD) cultivars, identified differential abundance protein species (DAPS) and verified expression profiling of certain corresponding mRNA by qRT-PCR. A total of 3872 protein species were annotated. Of the 90 DAPS, 56 and 34 were respectively up- and down-accumulated in Maverick, compared to Cuf101. They were grouped into 35 functional categories and enriched in seven pathways. Of which, auxin polar transport was up-regulated, while phenylpropanoid biosynthesis, pyruvate metabolism and transportation, vitamin B1 synthesis process and flavonoid biosynthesis were down-regulated in Maverick, comparing with Cuf101. In Maverick, mRNA abundances of L-asparaginase, chalcone and stilbene synthase family protein, cinnamyl alcohol dehydrogenase-like protein, thiazole biosynthetic enzyme, pyruvate dehydrogenase E1 beta subunit, and aldo/keto reductase family oxidoreductase were significantly lower at FD than at other stages, and lower than in Cuf101. We also observed opposite mRNA profiles of thiazole biosynthetic enzyme, chalcone and stilbene synthase family protein, pyruvate dehydrogenase E1 beta subunit in both cultivars from summer to autumn. Our results suggest that these DAPS could play important roles in growth difference between FD and non-FD alfalfa.

Biological significance: Up to now, as far as we know, currently the proteins related with the growth differences between FD and non-FD alfalfa cultivars in autumn have not yet been identified in terminal buds. This study identified the protein species expressed in alfalfa terminal buds, selected differentially abundant protein species in terminal buds between Maverick (FD) and Cuf101 (non-FD) cultivars in autumn and identified the important protein species participated in the growth differences. This study lays a foundation for further investigation of the molecular mechanism of the growth differences between FD and non-FD alfalfa and the cultivation of advanced alfalfa cultivars.

1. Introduction

Alfalfa (*Medicago sativa* L.) is a perennial, high-quality forage crop cultivated worldwide, and contributes to improve the quality of milk, meat, and eggs. The crop can be harvested multiple times throughout its lifecycle. The vast genetic and phenotypic variations in alfalfa cultivars attribute to its cultivation over large spatial scales with diverse climate/environment conditions. According to the amount of growth reduction during the fall dormancy (FD), alfalfa cultivars are divided into three types: FD (FD class I-III), semi-FD (FD class IV-VI) and non-FD (FD class VII-IX) [1,2]. FD is one of the most important factors influencing adaptation and production performance; it also helps in choosing suitable alfalfa cultivars for specific regions. FD cultivars are more suitable for cold climatic regions, while non-FD cultivars are generally grown in warm and tropical climate regions. During fall seasons, FD cultivars exhibit slow growth rate with small leaves and thin stems, leading to low yields. However, these cultivars exhibit strong cold hardiness with overwintering ability. In contrast, non-FD cultivars grow vigorously, but have poor ability of cold tolerance and overwintering [3,4].

These differences in growth habit between FD and non-FD cultivars are proposed to be determined by their genetic differences with respective adaptation mechanisms to environmental change [5]. To

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understand the underlying biomolecular basis associated with alfalfa genetic diversity, Bogovid Zivkovi estimated the genetic distance among various alfalfa genotypes by use of morphometry, seed storage protein analysis and random amplification of polymorphic DNA analysis [6]. Remarkably, 204 novel polymorphic expressed-sequence tag simple sequence repeat (EST-SSRs) markers were developed from cultivated alfalfa genotypes [7]. These EST-SSRs can be applied to assess alfalfa genetic diversity, and it was shown that alfalfa populations are genetically and phenotypically heterogeneous with high levels of inter- and intra-population genetic variability [8,9]. The genetic basis of FD alfalfa is different from non - dormant alfalfa [7,10]. FD has generally been associated with the reduction of day length and temperature [11,12], and photoperiod was identified as the key environmental limiting factor [5]. However, the molecular basis of these differences among different FD types remains largely unknown.

Although genes associated with alfalfa's FD have not yet been identified, dormancy-related genes have been identified in other perennial plants. It has been verified that CONSTANS, Flower Locus T and dormancy-associated MADS-box participate in *Populus tremula* and peach [*Prunuspersica (L.) Batsch*] dormancy [13,14]. Light, ethylene and abscisic acid (ABA) signal transduction pathways control the dormant bud formation sequentially in poplar [15,16]. ABA and ethylene are involved in bud dormancy of birch trees [17,18]. AINTEGUMENTA, Cyclin-Dependent Kinase A (and B) and Cyclin D3 are down-regulated, whereas Cyclin D1, Cyclin D2 and the expressions of protein homologous genes controlling flowering time are up-regulated during the key stage of dormancy initiation in poplar [16,19]. Sixteen key genes may play important roles in transition process from summer to autumn in *Euphorbia esula* L. [20]. In our earlier studies, 28 candidate microRNAs associated with alfalfa FD were identified via high-throughput small RNA sequencing [21] and 2064 differentially expressed genes in the leaves of FD and non-FD alfalfa were found by RNA sequencing [22]. Therefore, these genes may be involved in alfalfa FD, and the active proteins and genetic regulations need to be thoroughly studied.

Terminal bud is the primary growing point at the top of the stem. As one of the most active growing points, its development and differentiation determine the growth rate of the entire plant. Therefore, we hypothesized that active proteins and genetic regulations in this particular point during the FD period may have essential influence on the dormancy behaviour of alfalfa, demonstrating stark differences in the growth rate and plant height between FD and non-FD cultivars in autumn. However, up-to-date, the proteins relating to the differences between FD and non-FD genotypes in autumn have not yet been identified in terminal buds. Therefore, the objectives of this study were to: (1) identify protein species synthesized in alfalfa terminal buds; (2) quantify differentially abundance protein species (DAPS) in terminal buds between FD and non-FD alfalfa in autumn; and (3) determine key protein species relating to alfalfa FD and growth. The study was performed by comparing the terminal bud proteomes of Maverick and Cuf101 using isobaric tags for relative and absolute quantitation (iTRAQ)-based labelling quantitative proteomics approach and analysing the mRNA profiles of genes coding for a subset of DAPS from the non-FD to FD stage. The present research will provide an insight into the possible molecular mechanisms which explain the difference between different fall dormant alfalfa cultivars in autumn and should be helpful for future alfalfa breeding.

2. Materials and methods

2.1. Plant materials and growth condition

Two alfalfa cultivars, Maverick and Cuf101, were the test material. They are the representative standard FD (FD class I) and non-FD (FD class IX) cultivars, respectively, in the USDA classification system, with distinct growth habit from summer to autumn. Seeds of Maverick and Cuf101 obtained from Beijing Bytway Ecotechnology CO., LTD. were planted by hand on sandy loam soil at the experimental station of Henan Agricultural University (34°19′N, 113°35′E) on 23 September 2013. Both cultivars were randomly grown under identical natural environment conditions, and each cultivar was grown in three test plots. The alfalfa plants were well watered, weeds were manually removed and no fertilizer was applied during growth. The region has a temperate climate, with four distinctive seasons and September being considered part of the autumn season. Ten terminal buds of ten randomly-chosen plants in each test plot were collected as a biological sample replicate. Three replicates of each cultivar were sampled for iTRAQ analysis from three test plots at 8:00–9:00 on 25 September 2014 (the 14th day after cutting).

Changes of DAPS mRNA abundance in terminal buds at consecutive time points derived from three growth cycles of non-FD and FD alfalfa cultivars were analyzed by qRT-PCR. A growth cycle was defined as the period between two consecutive cuttings. The terminal buds were collected from three replicated plots for each cultivar at 8:00–9:00 on the 8, 18 and 28 July, 7 and 18 August, and 1, 12 and 23 September 2015 for detecting mRNA profiles of DAPS. Each time point is relevant within the vegetable growth stage and referred to as C1 for sampling dates in July; C2 for sampling dates in August; and C3 for sampling dates in September.

All collected samples were immediately frozen in liquid nitrogen and stored at − 80 °C until use. At the same time, 30 plants were chosen at random, and plant heights, angles between the stem and the ground, and stem diameters were measured. Photoperiod (daylength) and temperature were recorded. The average growth rate was determined by the re-growth height of the plant divided by the number of days in one growth cycle. The variation and significance of the measured variables were statistically analyzed using SPSS 19.0 (IBM Corp. Released 2010. IBM SPSS Statistics for Windows, Version 19.0. Armonk, NY: IBM Corp.).

2.2. Protein extraction, iTRAQ labelling and liquid chromatography-tandem mass spectrometry (LC–MS/MS) separation

Proteomic analysis was performed on the samples of each cultivar collected on 25 September 2014, and these samples were labelled as FD1 AB1, FD1 AB2, FD1 AB3, FD9 AB1, FD9 AB2 and FD9 AB3. Total proteins were extracted using trichloroacetic acid as per the cold acetone method with some modifications [23]. Two-g sample was cut into small pieces using a pair of scissors, and put in a 1.5-mL EP tube. Then, add 200 μL TPE solution [1 M triethylamonium bicarbonate (TEAB), 1 mM phenylmethanesulfonyl fluoride (PMSF) and 2 mM ethylenediaminetetraacetic acid (EDTA)]. After 5 min, add dithiothreitol (DTT) in the test tube. Cells were lysed by ultrasound in an ice bath and centrifuged. Five-fold of the volume of cold acetone solution and DTT were added in the collected supernatant, followed precipitation at − 20 °C overnight, the supernatant was discarded after centrifuged. The protein pellet was air-dried. Add 200 μL TPE solution, and after 5 min, add DTT in the test tube. Followed an ultrasound in an ice bath and centrifugation, the supernatant was collected into a 1.5-mL EP tube and DTT was added. The tube was incubated in a water bath at 56 °C for 1 h. Subsequently, 55 mM IAM and five-fold of the volume of cold acetone solution was added, followed precipitation at − 20 °C overnight and centrifugation, the supernatant was discarded, the remaining protein pellet was air-dried. The protein pellet was resuspended in 200 μL of 1 M TEAB and disrupted with ultrasound for 5 min in an ice bath. After centrifugation of the protein solution, the supernatant was collected and stored at − 80 °C. Concentration of DTT was 10 mM and centrifugation procedure was at 25,000 × *g* under 4 °C for 20 min in all the protein extraction process. The protein concentration of the supernatant was determined using the Bradford protocol and the quality of protein extraction was detected by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (Fig. S1).

The protein samples (100 μg each) of FD1 AB1, FD1 AB2, FD1 AB3,