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ORIGINAL PAPER

Proteomic Analysis of Shoot Tips from Two Alfalfa Cultivars with Different Florescence

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Abstract

Flowering is an indispensable biological process for the complete life cycle of angiosperms, crucial to the regeneration of plants and the continuation of species. In this work, a proteomic approach was applied to investigate differences in protein expression in two alfalfa cultivars with different flowering periods. Shoot tips (containing bud) were collected simultaneously at the bud stage from early flowering (FF) and late flowering (MF) cultivar alfalfa. In total, 442 differentially accumulated proteins were identified, including 230 down-regulated and 212 up-regulated proteins. The identified proteins were mainly involved in metabolism, biosynthetic processes, the immune system, and responses to stimulus and translation and programmed cell death (PCD). The expression profiles demonstrated that the ubiquitin protease pathway and inositol phospholipid signaling pathway are involved in flower development regulation. Furthermore, the transcript-expression patterns of the coding proteins were consistent with the proteomic results of the increased synthesis of amino acids associated with floral organ development and involvement of Sec14p-like phosphatidylinositol transfer family protein and RAB GTPase-like protein A5D, in accordance with early pollen development. The current study is devoted to exploration of protein expression profiles during alfalfa flower development, which would be conducive to illuminate the underlying molecular mechanisms during the alfalfa flowering process. These results may provide further insights into the potential strategies for artificially controlling flowering time in alfalfa.

Keywords Alfalfa · Flowering · Proteomics · Differentially accumulated protein · Function

Hao Sun and Ruicai Long contributed equally to this work.

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Introduction

Under the dual effects of internal physiological and biochemical processes and external environment, angiosperms transform from vegetative growth to reproductive growth. During this process, the floral meristem differentiates from the stem apical meristem and develops into flower formation. Flower development is a complex biological process. It plays an important role in the sexual reproduction of angiosperms, and is a key node in the life cycle of plants. Flowering time is a key event controlling the biological yield of the aboveground part of angiosperms in the process of vegetative growth, and seed yield in the process of reproductive growth, and there are synergistic and antagonistic effects between them. Previous studies have identified a large number of genes related to flowering (Krizek and Fletcher 2005; Lawton-Rauh et al. 2000; Reeves and Coupland 2000; Smyth et al. 1990), but the molecular mechanism of these gene interactions and regulatory networks still need to be revealed. Previous studies have focused on development of floral organs, such as pollen and pollen tubes; however, very little research has focused on gene regulation in flowering time (de Graaf 2005; Koornneef et al. 1991; Wang et al. 2007; Zhang et al. 2009).

Alfalfa (*Medicago sativa* L.) is a perennial legume plant with high nutritional value, which is a typical cross-pollinated plant with self-incompatibility that is widely cultivated all over the world. Previous studies have reported that the yield of the first crop of alfalfa depends on flowering time, which indicates that genes that regulate flowering time play crucial roles in plant development and biomass formation. Although late flowering can increase the first crop yield of alfalfa, this does not mean that late flowering will increase the annual yield of alfalfa. On the contrary, early flowering will not only advance the first crop of alfalfa, so as to increase the stubble times of annual alfalfa harvest as well as decide on their remaining fall harvest options and the possible impact on winter survival and total yield (Stout 1986; Barnhart 2009), but also avoid the influence of the rainy season on alfalfa harvest in temperate continental monsoon climates (Gupta et al. 2010), which is of great significance to alfalfa hay harvest.

Flowering is a complex biological process controlled by environmental conditions and internal development. The physiological and biochemical changes caused by expression of specific genes induce the development of flower organs and accelerate the process of flowering. Furthermore, genes that affect flowering time are divided into four main regulatory pathways: long-day photoperiod, gibberellin (GA), autonomy, and vernalization (Boss et al. 2004; Jack 2004; Mouradov et al. 2002; Simpson and Dean 2002; Simpson et al. 1999). Moreover, proteins encoded by genes involved in the long-day pathway participate mainly in light perception and circadian rhythms (Hayama and Coupland 2003; Mouradov et al. 2002; Reeves and Coupland 2000), and, ultimately, lead to the activation of CONSTANS (CO). Previous studies have reported that CO plays a crucial role in regulating flowering time (Koornneef et al. 1991; Simon et al. 1996). In addition, GA promotes flowering under short days by regulating the expression of the floral integrator, while overwintering stimulates flowering by regulating expression of response genes in the vernalization pathway. Seemingly unlikely, genes on the autonomous pathway function to control flowering in a photoperiod-independent manner. Actually, signals coming from these regulatory pathways are integrated by regulating the expression of response genes. For instance, as a suppressor of CO, expression of *SOC1 MADS-BOX* integrates vernalization, autonomous and gibberellin signals for flowering in Arabidopsis (Moon et al. 2003). Previous study has reported that *GIGANTEA* (*GI*) integrates cellular signals from light sensory transduction and the circadian clock, and activates *CO*, and *Arabidopsis* showed delayed leaf senescence and delayed flowering in a *GI* deletion mutant (Thiruvengadam et al. 2015). Although the molecular mechanisms underlying flowering in model plants have been elucidated, there are still only a few studies on gene regulation of flowering time in alfalfa. Overexpressing of microRNA156 (miR156) ultimately led to delayed flowering time in transgenic alfalfa (*Medicago sativa* L), resulting from *SPL13* being targeted for cleavage by miR156, which proves that *SPL13* contributes to regulating the transition from vegetative growth to reproductive growth (Gao et al. 2018). Overexpression of *MsLFY* specifically caused early flowering, particularly in long-day conditions, which indicated that *MsLFY* plays roles in promoting flowering time (Zhang et al. 2013). In addition, a CCCH-type zinc finger protein gene, *MsZFN*, and a *FRIGIDA*-like gene, *MsFRI-L*, have been identified in our laboratory; expression of *MsZFN* and *MsFRI-L* delay flowering time in transgenic *Arabidopsis thaliana* (Chao et al. 2013, 2014). The classical genetic approach is not enough to elucidate molecular regulation mechanisms of flower development, and proteomics has been developed as a complementary approach to the study of protein functional identification. The impressive set of proteomics tools and approaches to studying differentially expressed protein will help to bring insights into mechanisms responsible for differences in flower development. Proteomic analysis of pollen coat proteins is helpful to understand the interaction between pollen and stigma during flower development (Gong et al. 2015). In addition, in floral developmental proteomics, most of the identified differentially accumulated proteins are involved in signal transduction and programmed cell death, which provides a further understanding of protein expression at different stages during pollen development (Chen et al. 2016). ASK1, encoded by *Arabidopsis SKP1-LIKE1* (ASK1), together with Cullin and F-box proteins constitutes the SCF protein complex (Yang et al. 1999; Samach et al. 2010; Takahashi et al. 2004; Han et al. 2010). For instance, analysis of the proteome changes in *ask1* mutant

versus wild type *Arabidopsis* showed that the expression level of differentially accumulated proteins was not consistent with RNA level, which indicated that ASK1-E3s had a significant effect on protein degradation during flower development (Lu et al. 2016). Furthermore, previous genetic studies have pointed out the essential role of ASK1 in floral organ development, and there are defects in male meiosis in *ask1* mutants (Zhao et al. 2001, 2003, 2006, 2015).

Proteomics offers an effective approach to discover the proteins and pathways that are crucial for flower development and the mechanism of fertilization at a deeper level. Such findings may be conducive to a deeper understanding of the molecular mechanism of the flower development of alfalfa. The current study conducted proteomic analysis on two alfalfa cultivars with different flowering times. The aim of this study was to determine differentially expressed genes related to flower development in alfalfa.

Materials and Methods

Plant Material

Alfalfa FF (early flowering cultivar) is derived from wild germplasm resources collected in the field, and alfalfa MF (late flowering cultivar) is derived from superior individual of Zhongmu NO.1. Two cultivars of alfalfa propagated by cuttings were grown at the experimental base of the Chinese Academy of Agricultural Sciences. Shoot tips containing flower buds of alfalfa FF and MF were collected from the branches at the same physiological stage at the same time. Meanwhile, although a small fraction of the branches were at the early flowering stage in the early flowering cultivar, the flower buds collected for sampling remained at the bud stage, and pollen have not yet been mature. All the samples were collected, frozen immediately in liquid nitrogen and stored at −80 °C for protein and RNA extraction. All experiments were conducted with three biological replicates.

Protein Isolation and Purification

Samples were ground into powder in liquid nitrogen. The powder was then added to lysis buffer containing 7 M urea, 2 M thiourea, 4% CHAPS, 50 ml protease inhibitor cocktail (Roche, product code:04693116001, Sigma-Aldrich, St. Louis, MO) at a ratio of 1:10 (w/v). The mixture was vortexed vigorously. The sample was then subjected to ultrasonication with 22% amplitude for 60 s, 0.2 s on /2 s off. The extract was centrifuged at 15,000 *g* for 1 h at 10°C after extracting for 30 min at room temperature. After centrifugation, the supernatant was collected into a new microcentrifuge tube containing 4× volume of precooling 10% trichloroacetic acid (TCA)/acetone, and the mixture was placed at −20 °C overnight to precipitate proteins. The samples were subjected to centrifugation at 13,000 *g* for 10 min at 4°C. At the end of centrifugation, the supernatant was discarded. The precipitate was washed with acetone and centrifuged, and then the precipitate was repeatedly washed with acetone two to three times until the sample was colorless. Protein concentration was measured using a Bradford assay kit (Bio-Rad, Hercules, CA), and bovine serum albumin (BSA) was used as a reference protein.

Labeling of Peptides with iTRAQ Reagents

Proteins digestion was based on the filter-aided sample preparation (FASP) workflow as follows (Wisniewski et al. 2009). A final concentration of 25 mM DTT was added to 200 μg of each protein sample in a volume of 1.5 mL at 60 °C for 1 h. A final concentration of 50 mM iodoacetamide was added to the latter solution at room temperature for 10 min. The reduced alkylation protein solution was added to a 10 K ultrafiltration tube, centrifuged for 20 min at 12,000 revolutions, and the solution at the bottom of the collecting tube was discarded; 100 μl of the dissolution buffer provided in the iTRAQ kit was added, followed by centrifugation at 12,000 rpm. Again, solution at the bottom of the collecting tube was discarded. These protein samples were digested overnight with trypsin (Promega, Madison, WI) at 37°C. Peptides from the six samples were labelled with isobaric tags from the iTRAQ Reagent-8plex Multiplex Kit (AB Sciex, Framingham, MA) according to the manufacturer's recommended procedure (Abdallah et al. 2012). After a 1-h incubation at room temperature, the iTRAQ® reagents were centrifuged to the bottom of the tube. After adding 150 μl isopropyl alcohol to the iTRAQ® reagents, and vortex oscillation, the mixtures were centrifuged to the bottom of the tube; 50 μl sample (100 μg enzymolytic product) was then transferred to a fresh centrifuge tube. iTRAQ reagent was added to the sample, which was brought to room temperature, centrifuged to the bottom of the tube, and the reaction allowed to proceed at room temperature for 2 h. After that, 100 μl water was added to stop the reaction. In order to test the efficiency and quantitative accuracy of the markers, 1 μl from each of the four groups of samples was taken, and peptides were identified by MALDI TOF/TOF (AB SCIEX 4800 Plus) after desalting with Ziptip, to confirm that iTRAQ derivatization was successful. Labeled samples were mixed by vortex oscillation, then centrifuged to the bottom of the tube. Ultimately, equal amounts of the different samples were pooled and concentrated by evaporation using a SpeedVac (Heto, Saskatoon, SK, Canada). After mixing, the labeled samples were dissolved in 100 μl flowing phase A and centrifuged for 20 min at 14,000 *g*; 100 μl prepared sample was taken for sample loading at a flow rate of 0.7 ml/min. The components obtained from the reverse phase separation with high pH were redissolved in 20 μl 2% methanol and 0.1% formic acid, then centrifuged for 10 min at

12,000 rpm, and the sample drained. The volume of the upper sample is 10 μl, and the upper sample is taken by the clip method. The loading pump speed was 350 nl/min, for 15 min. Separation velocity was 300 nl/min.

Liquid Chromatography, Mass Spectrometry and Database Searching

Tandem mass spectra were extracted by ProteoWizard version 3.0. Charge state deconvolution and deisotoping were not performed. All MS/MS samples were analyzed using Mascot (Matrix Science, London, UK; version 2.6.0). The mascot was set up to search the *Medicago truncatula* database (version 4.0, 57,693 entries) assuming the digestion enzyme trypsin. The mascot was searched with a fragment ion mass tolerance of 0.020 Da and a parent ion tolerance of 10.0 PPM. Carbamidomethyl of cysteine and iTRAQ 8plex of lysine and the N-terminus were specified in Mascot as fixed modifications. Oxidation of methionine, acetyl of the N-terminus and iTRAQ 8plex of tyrosine were specified in Mascot as variable modifications.

Quantitative Data Analysis

Scaffold Q+ (version Scaffold_4.6.2, Proteome Software Inc., Portland, OR) was used to quantitate label based quantitation (iTRAQ, TMT, SILAC, etc.) peptide and protein identifications. Peptide identification were accepted if they could be established at >81.0% probability to achieve a false discovery rate (FDR) of <1.0% by the Scaffold Local FDR algorithm. Protein recognition is acceptable if an FDR of <10.0% is established with a probability of >67.0% and contains at least one identified peptide. The probability of a protein is assigned by the protein prophet algorithm (Nesvizhskii et al. 2003). Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony. As described in the previous study, normalization is iterated over intensity (Oberg et al. 2008). Means are used for differential analysis. Spectra data were log-transformed, pruned of those matched to multiple proteins and those missing a reference value, and weighted by an adaptive intensity weighting algorithm.

qRT-PCR Analysis

SV total RNA extraction system (Beijing, China) was used to extract the total RNA of the sample according to the instructions in the kit, . The first strand of cDNA is synthesized using Prime Script™ RT reagent kit (Takara, Beijing, China). qRT-PCR was conducted on an ABI prism7300 detection system (Applied Biosystems) using Takara TB Green™ Premix Ex Taq™ II (Tli RNaseH Plus) kit (Takara, Beijing, China). In this study, the β-actin gene was used as the internal reference gene. The relative quantification ($2^{-\Delta\Delta CT}$) of gene expression was evaluated using the comparative cycle threshold method (Bustin et al. 2009). Three independent biological replications were completed, and all primer sequences were listed in Supplementary Table 1.

Bioinformatic Analysis of Proteins

Functional category analysis is made of BLAST2GO software (http://www.geneontology.org) (Conesa and Götz 2008). Using KEGG database (http://www.genome.jp/kegg/pathway.html) to obtain the current on the biochemical pathways and other types of molecular interaction of knowledge (Kanehisa 2000). After the expression abundance is standardized, hierarchical cluster analysis is implemented in R (version3.2.2). GO and KEGG enrichment analyses were performed to determine which different cumulative proteins exceeded functional subcategories and metabolic pathways. In addition, the relative quantity of expressed genes was all analyzed statistically using one-way analysis of variance (ANOVA) by SPSS 21.0. Treatment means were separated using Duncan's multiple range test taking $p < 0.05$ as significant.

Results

Phenotypic Identification of FF and MF in Flowering Periods

The average number of days from reviving to the beginning of flowering in 2015 and 2016 were 52.33 and 51.67, respectively, in FF, with corresponding values of 65 and 65 in MF. Thus, flowering days began almost 2 weeks earlier in FF than in MF (Fig. 1).

Functional Categories of Differentially Accumulated Proteins

Ultimately, we obtained 14,315 unique peptides from 3784 identified proteins from the shoot tip. A fold change ratio > 1.20 or < 0.83 ($P < 0.05$) was used to identify differentially accumulated proteins between FF and MF (Supplementary Tables 2 and 3). A total of 442 differentially accumulated proteins were identified, including 230 down-regulated and 212 up-regulated proteins, respectively (Supplementary Table 4). The differentially accumulated proteins between FF and MF were also analyzed by hierarchical clustering (Fig. 2).

In order to understand the functional category, the differently expressed proteins were divided into three groups: biological process(BP), cellular component (CC) and molecular function (Fig. 3, Supplementary Table 5). The major functional categories in the BP were metabolic processes, biosynthetic

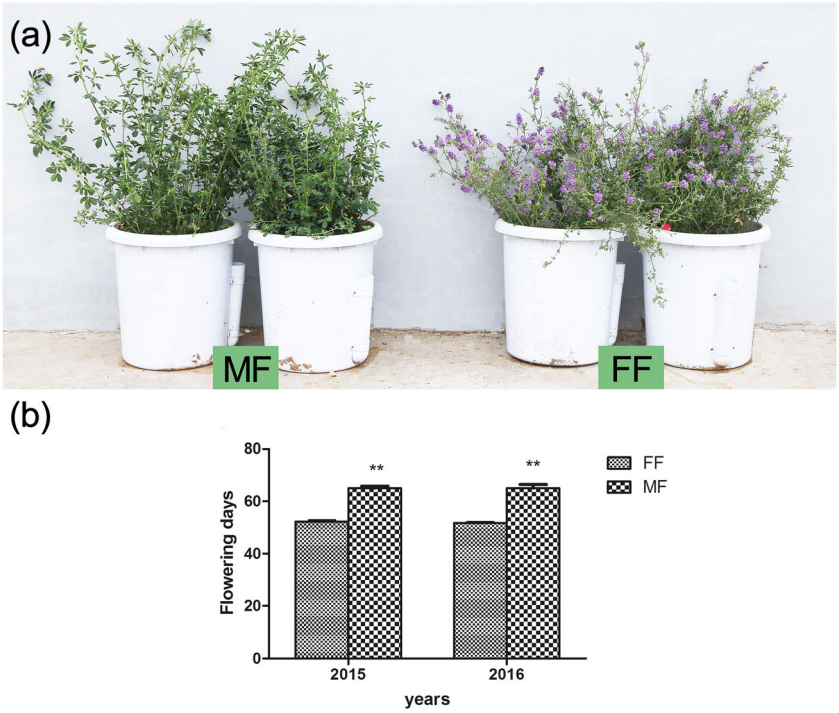

Fig. 1a,b Phenotypic identification of two cultivars in flowering days in 2015 and 2016. **a** Phenotype of two alfalfa cultivars. **b** Flowering days of two alfalfa cultivar in 2015 and 2016, calculated according to time from revival to the start of flowering. *MF* Late flowering cultivar, *FF* early flowering cultivar

process, and response to stimulus and translation. For CC, cell, cell part, cytoplasm, intracellular organelle and chloroplast were the most abundant groups, whereas oxidoreductase activity and catalytic activity accounted for the most abundant groups in terms of molecular function. Gene ontology (GO) enrichment was carried out to elucidate the biological functions of differentially accumulated proteins in developing alfalfa flowers.

Furthermore, Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis was performed to gain insight into the biochemical pathways of identified proteins. A total of 442 proteins were assigned to 83 pathways (Fig. 4, Supplementary Table 6). Among these pathways, the biosynthesis of amino acids, carbon metabolism, glutathione metabolism and phenylpropanoid biosynthesis were significantly enriched according to functional enrichment analysis. Selenoamino acid metabolism was significantly enriched according to functional enrichment analysis. According to the KEGG pathway bubble map, identified proteins ($P < 0.01$) mapped as mainly 34 subtypes.

Transcriptional Expression Analysis of Selected Genes by qRT-PCR

To confirm the proteomic results, qRT-PCR was used to examine 13 randomly selected proteins at the mRNA level using specific primers (Supplementary Table 1). Compared with MF, 11 genes (XP_013444163.1, XP_003596974.1, XP_003597698.1, XP_013447578.1, XP_003611427.2, XP_003594859.2, XP_003627310.1, XP_003607605.1, XP_003624587.1, XP_013451381.1 and XP_013462553.1) were up-regulated and Sec14p-like phosphatidylinositol transfer family protein (XP_013463044.1) and pollen protein Ole E I-like protein (XP_013470510.1) were down-regulated in FF (Supplementary Table 7). The expression profiles of 11 genes (all selected proteins except SAM and AACT) at mRNA levels were consistent with those at protein levels. Nine genes (all selected proteins except SAM and AACT) had expression tendencies at mRNA level that were consistent with protein levels, whereas the mRNA and protein expression levels of SAM domain protein and anthocyanin 5-aromatic acyltransferase exhibited the opposite tendency (Fig. 5).

Discussion

Metabolism-Related Proteins Expressed During Alfalfa Flower Development

In this study, most of the differentially accumulated proteins related to primary metabolism, including carbohydrate metabolism and amino acid metabolism, were identified to be responsible for the nutrient supply during flower development. Compared to the vegetative growth stage, methionine, arginine and seleno amino acid provided basic nutrient metabolism for the reproductive growth stage during flower development (Azevedo et al. 2006). SAMS, which is involved in the biosynthesis of S-adenosyl methionine through cysteine, methionine, and seleno amino acid metabolism (Fig. 6a), is a precursor of polyamines and ethylene biosynthesis (Woodson et al. 1992).

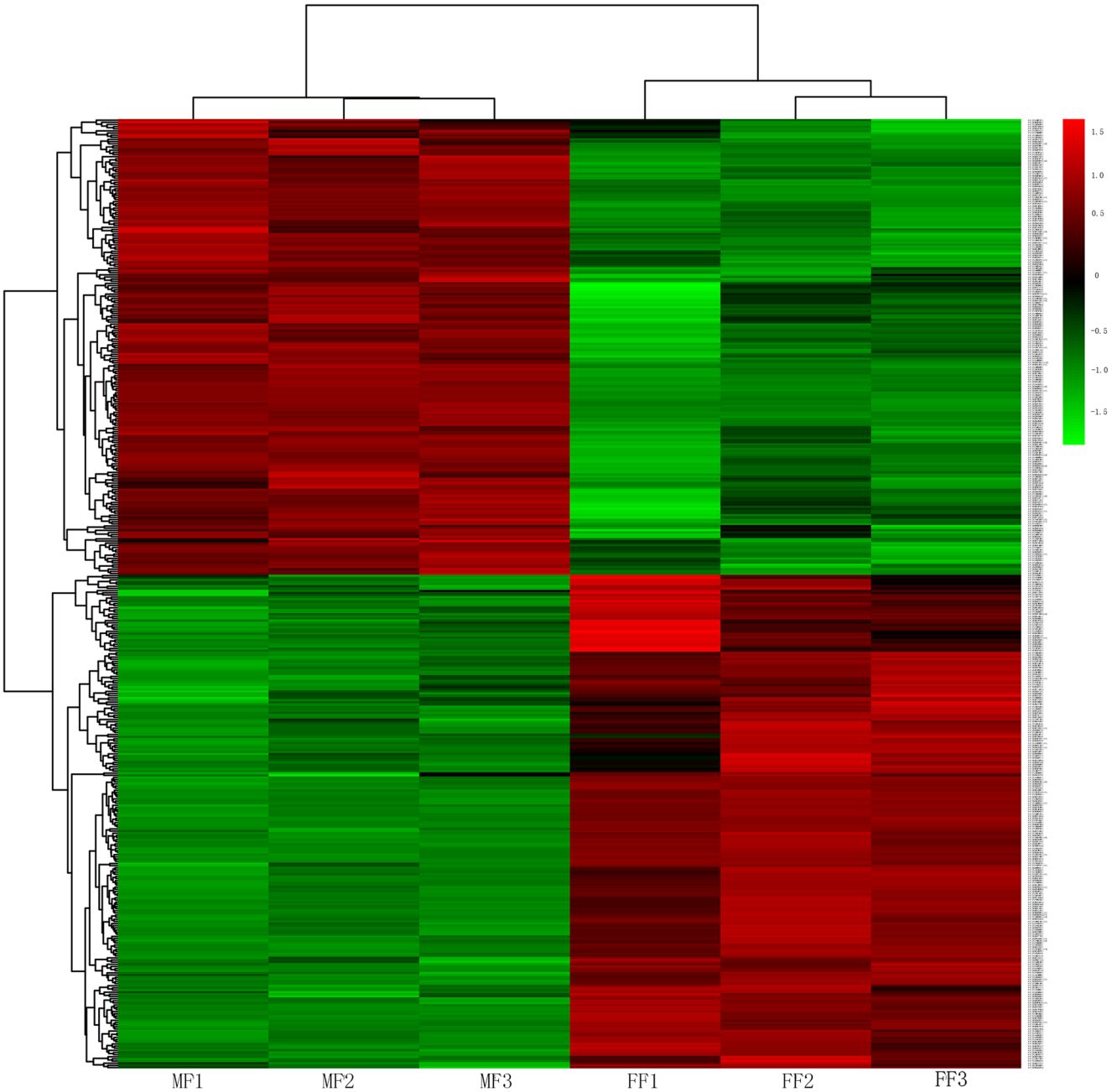

Fig. 2 Hierarchical cluster analysis of differentially accumulated proteins during alfalfa flower development. Dataset clustering was implemented in R (version 3.2.2) after normalization of the expression abundance values. Each colored cell represents the average spot quantity, according to the *color scale* on the right. *FF* Shoot tips (early flowering cultivar), *MF* shoot tips (late flowering cultivar)

Ethylene, as an endogenous plant hormone, regulates a lot of life activities in plants, especially in regulating and inducing flower bud differentiation, while polyamines (PAs), including putrescine, spermidine, spermine and cadaverine, are small molecule compounds in plants, and play an indispensable role in pollen maturation and pollen tube elongation (Aribaud and Martin 1994). In addition, previous studies have reported that polyamines (PAs), one of the determinants of pollen lifespan, are responsible for regulating the plant flowering process (Aloisi et al. 2016) (Fig. 6a). In the current study, SAM domain protein (XP_013444163.1), cobalamin-independent methionine synthase (XP_013451552.1), O-acetylserine (thiol) lyase (XP_003591039.2), aspartate aminotransferase (XP_003607605.1), and 1-aminocyclopropane-1-carboxylate (ACC) (XP_013458217.1) participating in cysteine and methionine metabolism were down-regulated (Fig. 6b), while S-adenosyl-L-methionine-dependent methyltransferase (XP_003595865.1) was up-

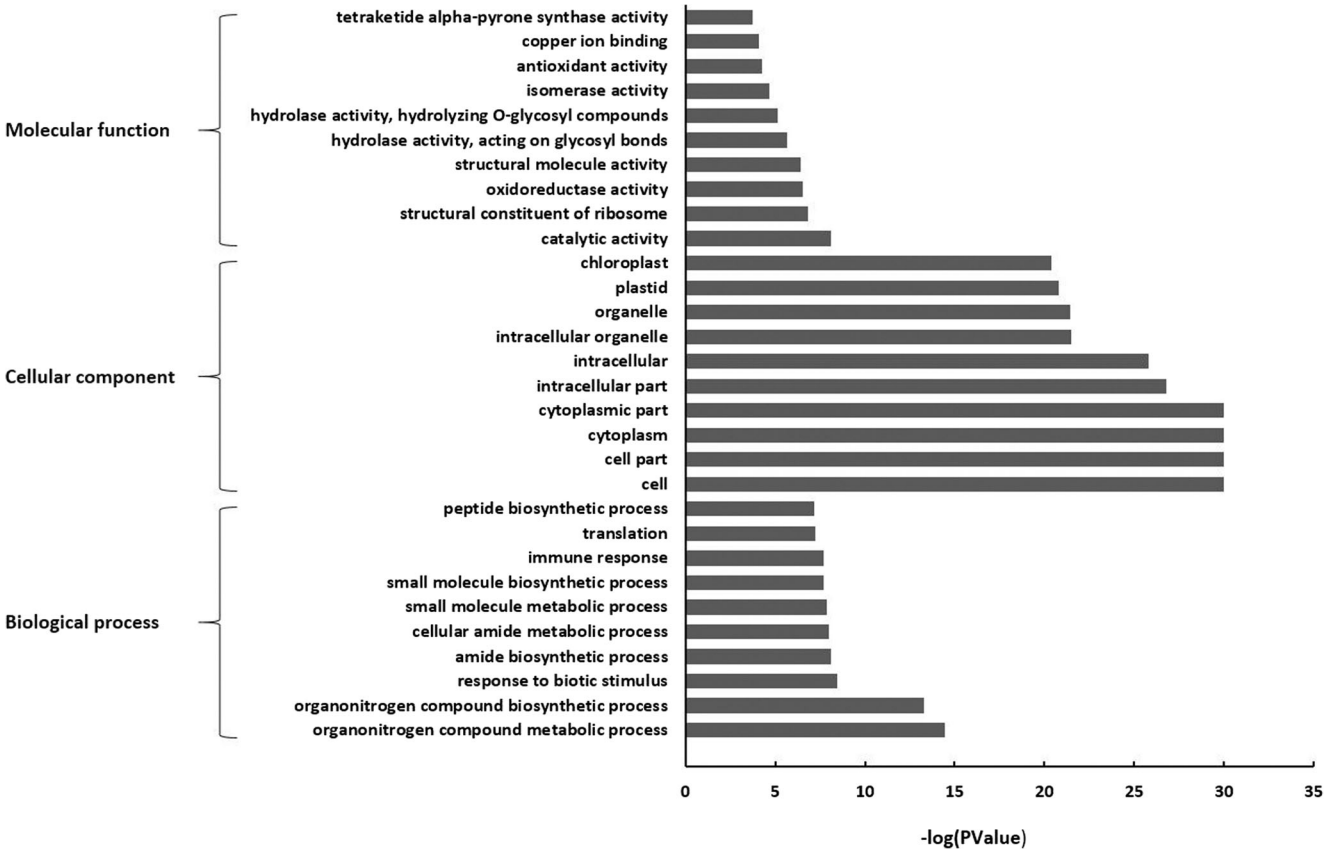

Fig. 3 Gene ontology (GO) classification of the identified proteins during alfalfa flower development. Results are summarized under three main GO categories: biological process, cellular component, and molecular function

regulated. This illustrated that, compared with FF, the changes will result in less polyamines and ethylene in MF, which is due mainly to the earlier flowering time in FF.

In addition, during flower development, the biosynthesis of anthocyanin plays an indispensable role in pollen development, especially for alfalfa, which is cross-pollinated. For self-inbreeding alfalfa, pigmentation, which is conducive to attracting insects and transmitting pollen, plays a vital role in the coloration of flower organs. In the current study, dihydroflavonol 4-reductase (DFR) (XP_003593539.1 (+1))—an essential enzyme participating in anthocyanin biosynthesis through catalyzing dihydrokaempfero to produce anthocyanins (Johnson et al. 2010)—was significantly down-regulated in MF, which resulted from flowering later in MF, consistent with the discussion above. In the current study, compared with FF, chalcone synthase family proteins (XP_013456566.1) were significantly up-regulated in MF, while the chalcone-flavanone isomerase family protein (XP_003592761.1) and chalcone and stilbene synthase family proteins (XP_013453346.1 (+1)) and flavonoid glucosyltransferase (XP_003610163.1) were significantly down-regulated. Furthermore, four differentially accumulated proteins involved in flavonoid biosynthesis were significantly enriched (Fig. 6a). The biosynthesis of phenylpropyl is initiated by the catalysis of chalcone synthase (Heller and Forkmann 1988), and flavonoids are eventually produced through this metabolic pathway (Coe et al. 1981). Studies have reported that, due to the destruction of pollen fertility and flavonoid production, chalcone synthase deletion mutants are infertile. Therefore, flavonoids play a very important role in pollen fertility (Mo et al. 1992) (Fig. 6b).

Furthermore, previous research has reported that genes that affect flowering time are divided into four main regulatory pathways, including long-day photoperiod, gibberellin (GA), autonomy, and vernalization (Boss et al. 2004; Jack 2004; Mouradov et al. 2002; Simpson and Dean 2002). As one of the metabolic pathways in the autonomous pathway, RNA processing has been shown to regulate flowering time in *A. thaliana*. The *A. thaliana* gene *AT PRP39-1*, participating in RNA processing, regulates the flowering time of plants by mediating the contents of FLC, FT and SOC1 in plants (Wang et al. 2007) (Fig. 6b). In the current study, TPR-like protein was significantly down-regulated in MF, which is consistent with the results mentioned above. Meanwhile, two TPR superfamily proteins were significantly up-regulated in MF with a later flowering time, which is inconsistent with previous research (Wang et al. 2007).

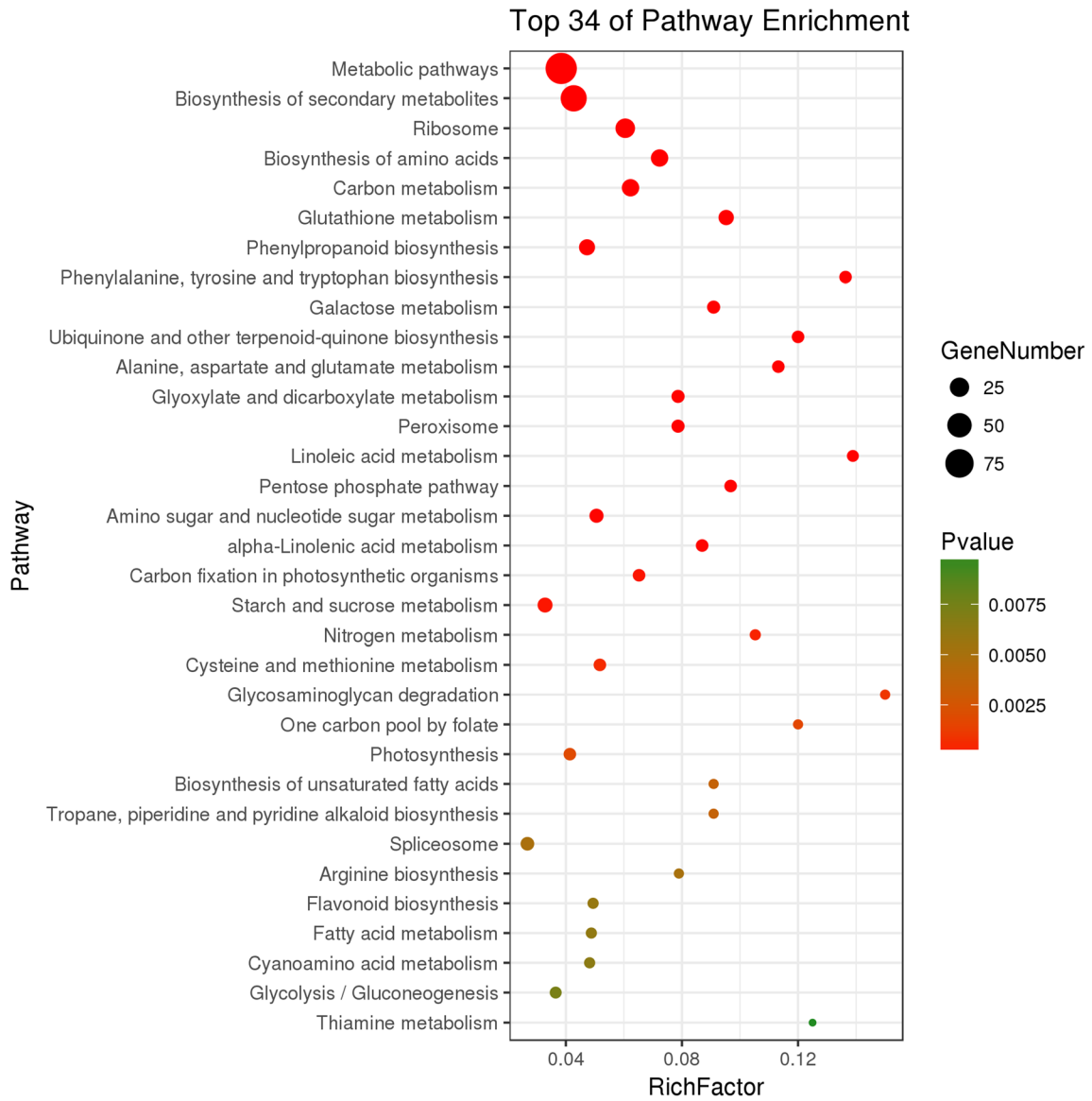

Fig. 4 Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis of differentially accumulated proteins during alfalfa flower development. *Point size* indicates the numbers of annotated differential proteins, and *color depth* indicates the *P* value (*P* value ≤0.01) of enrichment

Inositol Phospholipid Signaling Components Regulate Pollen Development and Pollen Tube Growth

Previous studies have shown that various components of the inositol phospholipid signaling system participate in vacuolar changes during pollen development and vesicle transport during pollen tube growth, including PI (3) P (phosphatidylinositol 3-phosphate), PI3K, PI (4, 5) P2, PIP5K, PLC/IP3/Ca2+, PA (phosphatidic acid), PLD (phospholipase D), IPK (inositol polyphosphate kinase) and IPP (inositol polyphosphate phosphatase) (Munnik and Vermeer 2010). The phospholipase A2 [PLA (2)], including 3 PLA [(2)-β, −γ, and -δ] mainly expressed in the pollen development stage and participated in pollen development and germination as well as in pollen tube growth (Kim et al. 2011). Furthermore, Sec14p-like phosphatidylinositol transfer family proteins as one member of the inositol phospholipid signaling system, participate in vacuolar changes during pollen development and vesicle transport during pollen tube growth (Vincent et al. 2005) (Fig. 6a). In the current study, Sec14p-like phosphatidylinositol transfer family proteins was significantly down-regulated in MF, in accordance with early pollen development in FF.

Nevertheless, Rab GTPases, involved in regulation of membrane trafficking, have an effect on vesicular transport, coordinating the balance between the subcellular components and the plasma membrane in the cell and effectively

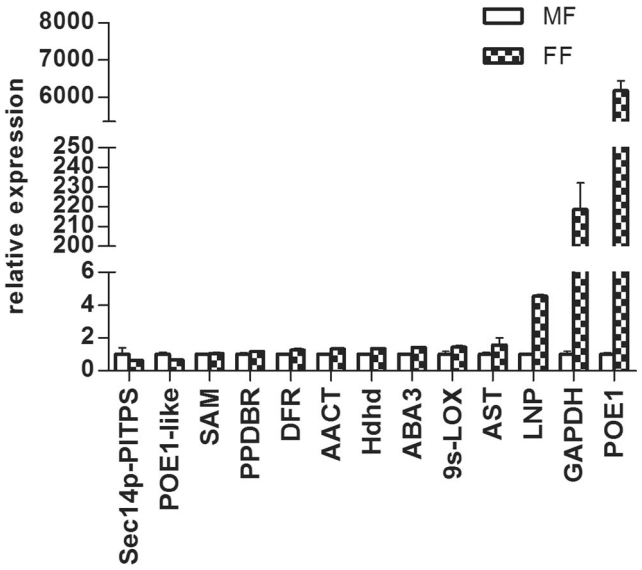

Fig. 5 Quantitative real time PCR analysis of mRNA transcription of the selected expressed proteins during alfalfa flower development. *MF* Shoot tips (containing buds) collected from early flowering cultivar, *FF* shoot tips (containing buds) collected from late flowering cultivar. The average expression of each gene was calculated relatively to the reference gene β-actin. This experiment was conducted three biological replicates. The relative expression value of each gene was normalized to an endogenous control and calculated using the $2^{-\triangle\triangle CT}$ method

controlling the polar growth process (de Graaf 2005) (Fig. 6a). In the current study, RAB GTPase-like protein A5D was significantly down-regulated in MF, in accordance with early pollen development in FF.

Involvement of Jasmonates in Regulation of Male Fertility

The development of floral organs is the result of the balance between external environment and internal environment. Studies in recent years have shown that plant hormones play an important role in the process of plant development. Among them, jasmonic acid (JA) and its derivatives (MeJA) play an indispensable role in the differentiation and development of *Arabidopsis* flower organs, especially in the production of viable pollen. Exogenous application of methyl jasmonate can promote early flowering of plants. Adverse factors promote the activation of phospholipase in the plasma membrane, and further catalyze the degradation of membrane lipids. The released linolenic acid (LA) is the starting material for JA synthesis in plants. Lipoxygenase (lipoxygenase, LOX) catalysis then produces 13-hydrogen peroxide LA (13-hydroperoxylinolenic acid), and then, in turn, after allene oxide synthase (allene oxide synthase, AOS), allene oxide cyclase (allene oxide cyclase, AOC) produce 12-diene acid oxygen generation in plants (12-oxophytodienoic acid, 12-OPDA). As a result, D10 double bond saturation and continuous three-step b-oxidation were generated by 12-opda reductase (OPR) catalysis, and JA was generated (Creelman and Mullet 1997). In the current study, seed linoleate 9S-lipoxygenase, lipoxygenase, allene oxide cyclase (AOC) and linoleate 13S-lipoxygenase were significantly changed and down-regulated compared to FF, which indicated that the biosynthesis of methyl jasmonate was activated, and the improvement of the content of methyl jasmonate provided a guarantee for pollen fertility along with the opening of flowers (Fig. 6b).

Involvement of SCF Complex in Regulation of Gametophytic Incompatibility

The ubiquitin protease pathway is an important pathway for the degradation of redundant proteins in cells, which helps the degradation of redundant proteins in cells and ensures the normal process of complex physiological and biochemical processes in cells. The SCF protein complex is a part of the E3 ubiquitin ligase family, which is composed of SKP1, cullin and f-box protein, and is responsible for regulating the ubiquitination of different substances in plants and degradation by 26S proteasome. As an important component of the SCF protein complex, SKP1, plays an important role in connecting cullin and f-box proteins and is the key to forming the SCF protein complex. Previous studies have pointed out that SKP1 and f-box jointly regulate the formation of flower primordia (Zhao et al. 2001). In the *ask* mutant, vegetative growth of *A. thaliana* was significantly inhibited, and the growth of plants and flower organs was affected. Even in *ask* and *ufo* double mutants, *Arabidopsis* flower organ defects were more serious (Zhao et al. 2015). This suggests that SCF-E3 ubiquitination plays an important role in flower development, SCF is related to the regulation of cell cycle progress and transcriptional control (Hershko et al. 2000; Gagne et al. 2002; Koepp et al. 1999; Schulman et al. 2000). Previously, down-regulated SKP1 expression during pollen grain development may affect the protein ubiquitin degradation pathway during wheat pollen grain development, leading to male infertility (Wang et al. 2015) (Fig. 6c). Furthermore, previous genetic studies have pointed out that ASK1 play an essential effect on floral organ development, and there are defects in male meiosis in ask1 mutant (Zhao et al. 2001, 2003, 2006, 2015). In the current study, the SKP1 family dimerization domain protein (XP_013443867.1), and SCF ubiquitin ligase and SKP1 component (XP_003612227.1), which play an essential role in pollen development, were significantly changed in abundance. In addition, COP9 signaling (CSN) is a multiprotein complex composed of eight subunits, and previous studies have shown that CSN plays a key role in maintaining the activity of SCF and other cullin-based ubiquitin enzymes (Schwechheimer 2004) (Fig. 6c). In the current study, COP9 signalosome complex subunit 1, a component of the COP9 signalosome complex, was successfully down-regulated in MF, which may result from later flowering phenotype.

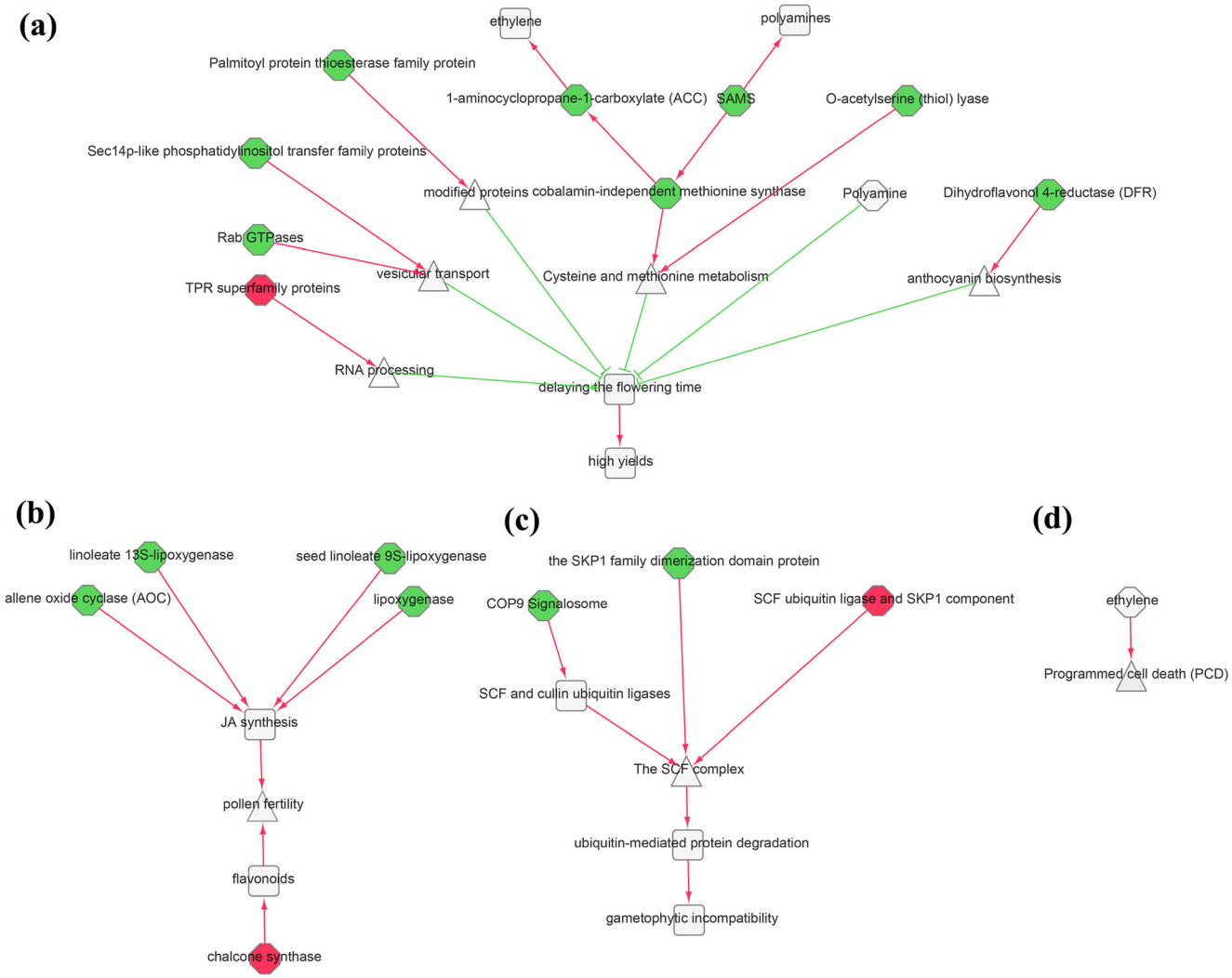

Fig. 6a–d Protein–protein interactions (PPI) in differentially accumulated proteins during alfalfa flower development. **a** Metabolic pathway regulation in flowering time. **b** Metabolic pathway regulation in pollen fertility. **c** Metabolic pathway regulation in gametophytic incompatibility. **d** Metabolic pathway regulation in programmed cell death. Differentially accumulated proteins: *red* up-regulation of protein expression, *green* down-regulation of protein expression. The network model was mapped by directed acyclic graph (DAG) in Omicshare (http://www.omicshare.com)

Programmed Cell Death Regulates Both Flower Pollination and Senescence in Alfalfa

Programmed cell death (PCD) is a common phenomenon during the development of organisms. It refers to the suicide protection measures initiated by gene regulation when cells encounter the stimulation of internal and external environmental factors. The development of flower organs is a critical period for the transition from vegetative growth to reproductive growth, and it also indicates that the plant is about to complete its life cycle. Thus, flower development is accompanied by the beginning of PCD of the plant. Ethylene, as an important plant hormone, plays an important role in PCD. Therefore, the content of ethylene and the expression and activity of key enzymes in ethylene synthesis are important indicators of PCD. Moreover, previous research reported that 1-aminocyclopropane-1-carboxylate (ACC) synthase, which participates in ethylene biosynthesis, has an effect on pollen viability in flower development period (Pan et al. 2005; Zhang et al. 1999) (Fig. 6d). In the current study, ACC (XP_003601593.1) participating in ethylene biosynthesis was significantly down-regulated in MF, indicating that there was an active PCD in FF, resulting from the earlier flowering phenotype. In the current study, the palmitoyl protein thioesterase family protein was lower by half in MF than that in FF. Previous studies have reported that palmitate transferase 10 regulates palmitoylation of modified proteins during the flowering transition, the mutant showed a later flowering time than the wild type, and pollen of homozygous mutant grows slowly in vivo and has poor conductivity, which may be one reason why flowers bloom later in MF (Rahman et al. 2011) (Fig. 6a).

Conclusions

In angiosperms, flowering is the key point of transition from vegetative growth to reproductive growth. It has been reported that by prolonging the vegetative growth stage, delaying the flowering process can promote the accumulation of biomass. Even higher seed yields can be achieved because of greater nutrient reserves (Komeda 2004; Simpson and Dean 2002). For vegetative crops, early flowering can lead to earlier vegetative growth cessation, and more nutrients are transferred to reproductive growth, thus reducing the biomass of vegetative crops (Rahman et al. 2011; Kraus et al. 2011; Hayward and Mcadam 2010). Therefore, delaying the flowering time of alfalfa may be an important way to obtain high yields of alfalfa.

To sum up, in order to obtain high yield and high-quality alfalfa, it is imperative to study the molecular regulation mechanism of alfalfa flower development. This proteomic study aimed to classify the functions of differentially accumulated proteins and to provide ideas for molecular regulatory networks for flower development.

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