Pak. J. Bot., 56(6): 2279-2285, 2024. DOI: http://dx.doi.org/10.30848/PJB2024-6(23)

A COMPARATIVE PROTEOMIC STUDY OF SHOOT APICAL MERISTEM IN PERPETUAL-FLOWERING AND SEASONAL-FLOWERING STRAWBERRY

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Abstract

Strawberry can be categorized into seasonal-flowering(SF) and perpetual-flowering(PF) depending on their flowering habit. To understand the molecular mechanisms underlying the two flowering pattern, a comparative proteomic analysis of the shoot apical meristem(SAM) was performed between the PF cultivar 'Monterey' and the SF cultivar 'Benihoppe'. 1088 differentially expressed proteins (DEPs) between the two cultivars were identified, of which 536 were up-regulated and 552 down-regulated in 'Monterey'. Gene Ontology analysis revealed DEPs were enriched in biological processes related to flavonoid metabolism, pigment metabolism, response to stimuli, circadian rhythm, developmental growth involved in morphogenesis, reproductive structure development and etc.. KEGG analysis indicated that DEPs were involved in several metabolic pathways, including flavonoid biosynthesis and amino acid metabolism. Protein-protein interaction analysis showed flowering related DEPs SPL3, TFL1, ELF4, HDA6, COI1 formed two interacting network. Our findings provide proteomic data for understanding the molecular basis of SAM development during the transition from vegetative to reproductive growth in strawberry.

Key words: Strawberry, Perpetual-flowering, Seasonal-flowering, SAM, Proteomic.

Introduction

The strawberry (*Fragaria x ananassa* Duch.) is a perennial herbaceous plant of the *Rosaceae* family. Strawberries can be categorized into two distinct groups based on their flowering habit, the seasonal-flowering (SF) strawberries which cease flowering in summer, and the perpetual-flowering (PF) strawberries which exhibit year-round blooming (Heide & Sønsteby, 2007; Sønsteby & Heide, 2008; Gaston *et al*., 2013). For example, the 'Benihoppe' strawberry cultivar, developed in Japan, is characterized as a seasonal-flowering variety. In the Hangzhou region, it typically blooms from November through to May of the following year, with flowering ceasing during the summer months due to elevated temperatures. The 'Monterey' strawberry cultivar, hailing from California, is perpetual-flowering. Due to its great tolerance to high temperatures, the cultivar maintain the ability to flower in summer.The study of the flowering mechanism of strawberry is important for the improvement of strawberry yield and quality.

Currently, some progress has been made in strawberry flowering research on flowering gene targeting, environmental and physiological induction mechanisms. Perrotte *et al*., identified the locus FaPFRU, which is tightly linked to the trait PF, by using a selective mapping strategy and recombinant analysis (Perrotte *et al*., 2016a; Perrotte *et al*., 2016b). Lei *et al*., (2020) identified the role of WRKY71 as a flowering promoter through a transcriptional regulatory cascade in wild strawberry. Dong *et al*., (2021) found that auxin-induced AUXIN RESPONSE FACTOR4 activates APETALA1 and FRUITFULL to promote flowering in woodland strawberry. Another study investigated the dormancy mechanisms and its relationship with flowering and stolon formation in day-neutral strawberry cultivars by controlling environmental conditions (Rivero *et al*., 2021). Yang *et al*., (2024). explored the effects of light intensity and quality on flowering formation in strawberry, and found that the expression of the flowering activator FaFT1 was stimulated and that the expression of the flowering repressor FaTFL1 was suppressed after the application of blue light under long day light. Plants generate lateral tissues such as leaves, stolons and flowers through SAM at the growing tips. When flowering is initiated, SAM undergoes a developmental transition from vegetative to reproductive state (Ha *et al*., 2010; Freytes *et al*., 2021).

Previous studies have paid little attention to the changes in the molecular level of SAM during flowering. Proteins are key executor of biological activity. To further understand strawberry flowering mechanisms, we investigated the protein levels in the SAMs of PF and SF strawberry by using a comparative proteomics stragety.

Materials and Methods

Plant materials: The strawberry cultivar 'Benihoppe' and 'Monterey' were planted at Hangzhou Academy of Agricultural Sciences, Zhejiang, China. SAM tissues were collected from both cultivars in August 2022, and stored at -80°C after liquid nitrogen freezing. 10 biological replicates were taken from each group of samples.

Flower bud differentiation analysis: The plant branches were removed, the stem tip was cut off with a scalpel, and excess tissue was carefully removed under a dissecting microscope to expose the growing point. The microscopic view was captured by a microscope digital camera, and the stage of differentiation was determined by referring to Li's study (Li *et al*., 2020).

Total protein extraction and pretreatment: The samples were grounded into powder under liquid nitrogen, and total protein extraction was performed according to the method

in the previous study (Qiu *et al*., 2020a). Briefly, 0.2g of powder was mixed with 800μL of solution (0.7 mol·L^{-1} sucrose, 0.1 mol·L^{-1} KCl, 0.5 mol·L^{-1} Tris, 2% 2-Mercaptoethanol, and 50 mmol·L^{-1} EDTA, pH 8.0), and then added with 800μL of Tris saturated phenol (pH 8.0) to extract proteins. After centrifugation of the above mixture, the phenol layer was pipetted, and precipitated overnight at -20℃ with 0.1 mol·L^{-1} ammonium acetate in methanol. The precipitate was washed by methanol for 3 times and lyophilized at -70℃~-20℃ into protein powder. Proteins were solubilized with 8 mol·L^{-1} urea and quantified by the Bradford method and digested into peptides using FASP (Filter-Aided Sample Preparation) method (Wisniewski *et al*., 2009). The resulting peptides were desalted and lyophilized with Pierce C18 Tips (Thermofisher, USA), and reconstituted in a 0.1% formic acid aqueous solution for further quantitative analysis.

Quantitative proteomic analysis: Quantitative proteomic analysis was performed on a liquid chromatography tandem Orbitrap mass spectrometry system. Peptides were separated by liquid chromatography on Easy nLC1000 platform (Thermo Fisher Scientific, USA), equipped with a homemade reversed-phase column. Referring to our previous study (Qiu *et al*., 2020b), a 120 min gradient program was used to elute the peptides and DIA (data independent acquisition) mode was used in mass spectrometry for data acquisition. Mobile phase A is 0.1% formic acid aqueous solution, and phase B is 0.1% formic acid acetonitrile solution. The gradient program was set as follows: 0~3min, 4~7% B;3~103min, 7~18% B; 103~113min, 18~35% B; 113~117min, 35~75% B; 117~120min, 75% B, at a flow rate of 350 nl·min^{-1}. Mass spectrometry scan was performed on Q Exactive system (Thermo Fisher Scientific, USA), with a range of 350 to 1,350 m/z in positive ion mode and 40 isolation windows with each window at 25m/z in DIA mode. The MS1 resolution was set to 70,000 at m/z 200, and MS2 resolution was 17,500 at m/z 200. The spray voltage was 2.0 KV and collision energy was set to 27%.

Data analysis: The LC-MS raw data was processed with software of Spectronaut 13 (Biognosys AG, Switzerland) and sequence database of *Fragaria x ananassa* Camarosa Genome v2.0, for protein identification and quantification (Edger *et al*., 2019; Liu *et al*., 2021). The quantification matrix was analyzed for data filtering, NA filling, and hypothesis testing. Proteins with adj. $P<0.05$ and $|\text{Fold change}|\geq 1.5$ were considered as differentially expressed proteins. The differentially expressed proteins were functionally annotated through Pannzer (Toronen & Holm, 2022), and analyzed by TBtools (Chen *et al*., 2020) for functional enrichment in gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways (Ogata *et al*., 1999; Ashburner *et al*., 2000), respectively. Interaction prediction of differentially expressed proteins was performed by STRING (Szklarczyk *et al*., 2021) and network mapping was performed by Cytoscape (Shannon *et al*., 2003).

Results

Stereoscopic observation of SAM: The microscopic results showed that, the SAM of 'Monterey' became flat with small, rounded protuberances, indicating it was in early hypertrophic period, the initial stage of flower bud differentiation (Fig. 1a). In contrast, the SAM of 'Benihoppe' was enveloped by a young leaf with a distinctly triangularly tapered young leaf primordium, indicating that it would develop into a leaf (Fig.1b). This suggests that the SAM of 'Monterey' continues to develop flowers during the summer, whereas 'Bennihope'stops flower bud differentiation.

Quantitative proteomic analysis: A total of 70524 peptides, of which 18096 were protein group specific, corresponding to 10695 proteins were identified from LC-MS raw data by searching against the reference database of *Fragaria x ananassa* Camarosa Genome v2.0. After data filtering and missing value filling, 7062 proteins were retained for quantification. A 1.5-fold-change cut off with p-value<0.05 was used to identify Differentially expressed proteins (DEPs) between 2 cultivars. As a result, 1088 DEPs were found in the 'Monterey'/'Benihoppe' comparison, of which 536 proteins were up-regulated, and 552 proteins were down-regulated (Fig. 2).

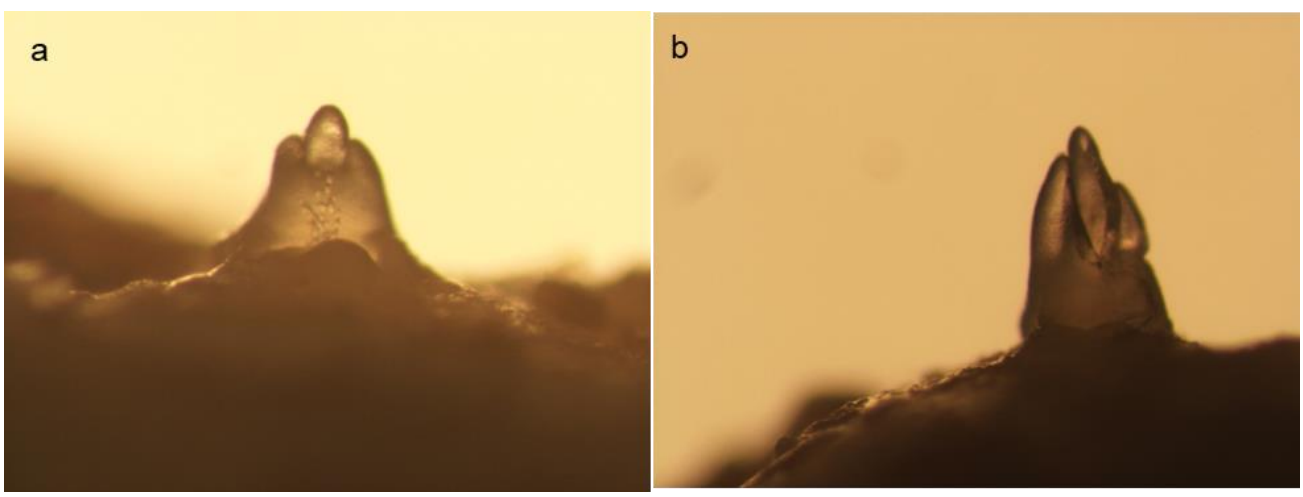

Fig. 1. Stereoscopic view of SAM of 'Monterey'(a) and 'Benihoppe'(b).

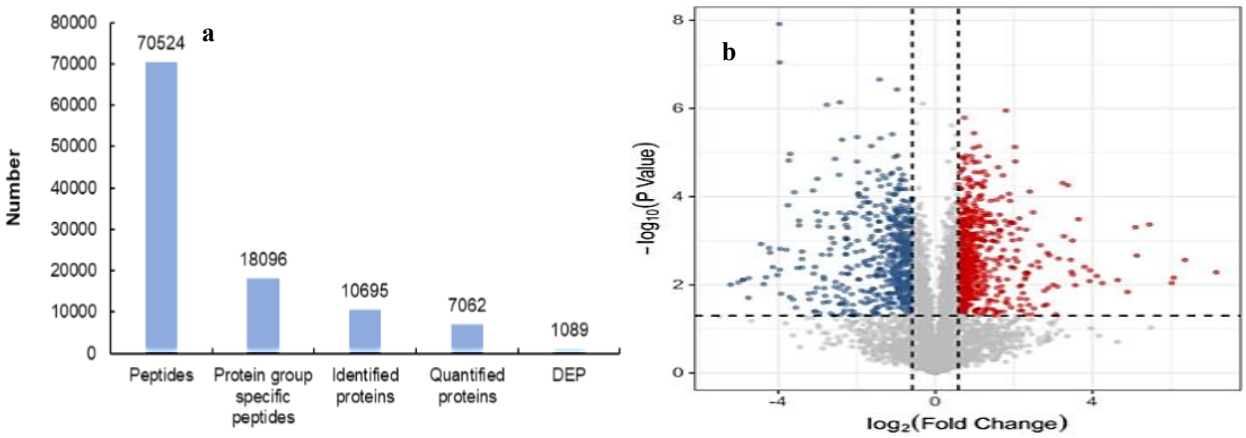

Fig. 2. Overview of proteomic data(a) and volcano of DEPs(b).

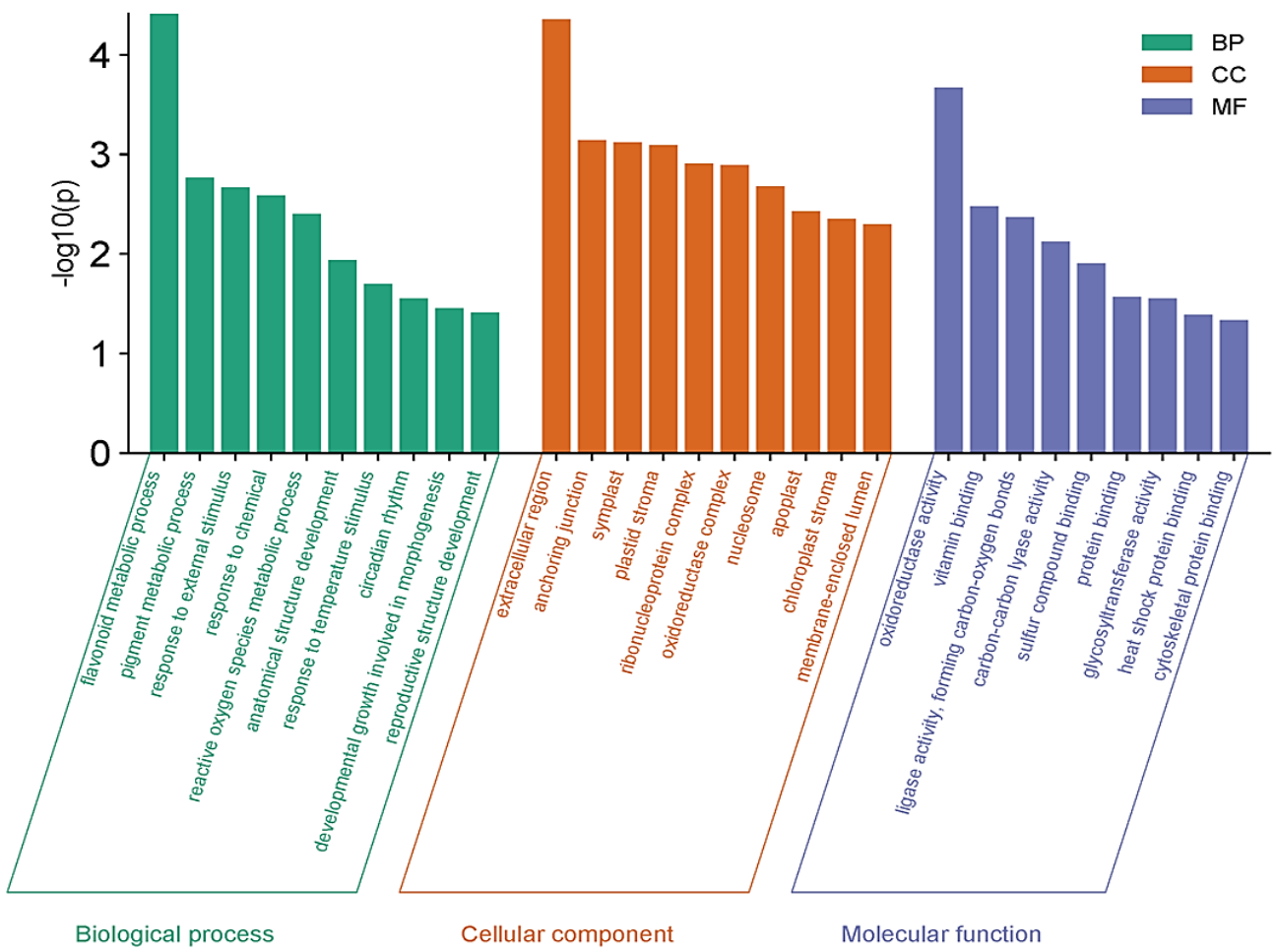

Fig. 3. GO enrichment analysis of DEPs between 'Monterey' and 'Benihoppe'.

GO annotation and enrichment analysis of DEPs: The Gene Ontology (GO) is a commonly used databases for studying the function of gene products, which is defined based on 3 dimensions: biological process, molecular function and cellular component. The DEPs (1088) between 'Monterey' and 'Benihoppe' were enriched to 43 GO terms. The top 10 most significant biological process enriched was flavonoid metabolic process, pigment metabolic process, response to external stimulus, response to chemical, reactive oxygen species metabolic process, anatomical structure development, response to temperature stimulus, circadian rhythm, developmental growth involved in morphogenesis and reproductive structure development. As for cellular component, extracellular region, anchoring junction, symplast, plastid stroma, ribonucleoprotein complex, oxidoreductase complex, nucleosome, apoplast, chloroplast stroma and membrane-enclosed lumen were the main site. The molecular functions performed by DEP were oxidoreductase activity, vitamin binding, ligase activity, forming carbon-oxygen bonds, carbon-carbon lyase activity, sulfur compound binding, protein binding,

glycosyltransferase activity, heat shock protein binding and cytoskeletal protein binding (Fig. 3).

Furthermore, through the 'reproductive structure development' term from the GO enrichment results, we selected twelve DEPs related to flowering, listed in Table 1. These proteins are involved in flower development, photoperiodism, meristem specification and inflorescence morphogenesis. Five of them were up-regulated in 'Monterey', while the other seven were down-regulated. Based on the results of protein sequence blasts performed at uniprot, FxaC_21g45850.t1 is a homolog of Terminal flower 1(TFL), which is involved in the negative regulation of floral development and was significantly down-regulated in 'Monterey'. FxaC_22g20081.t1 and FxaC_27g39840.t1 are homologs of Protein EARLY FLOWERING 4(ELF4), involved in flowering induced by photoperiod, and were significantly up-regulated in 'Monterey'.

KEGG analysis of DEPs: The biological pathway involved in DEPs were obtained by KEGG enrichment analysis. At the threshold of Adjusted P-value<0.05, DEPs were enriched to 10 metabolic pathways: biosynthesis of other secondary metabolites, amino acid metabolism, limonene and pinene degradation, carbohydrate metabolism, arginine and proline metabolism, beta-Alanine metabolism, pantothenate and CoA biosynthesis, amino sugar and nucleotide sugar metabolism, flavonoid biosynthesis, and metabolism of cofactors and vitamins (Fig. 4).

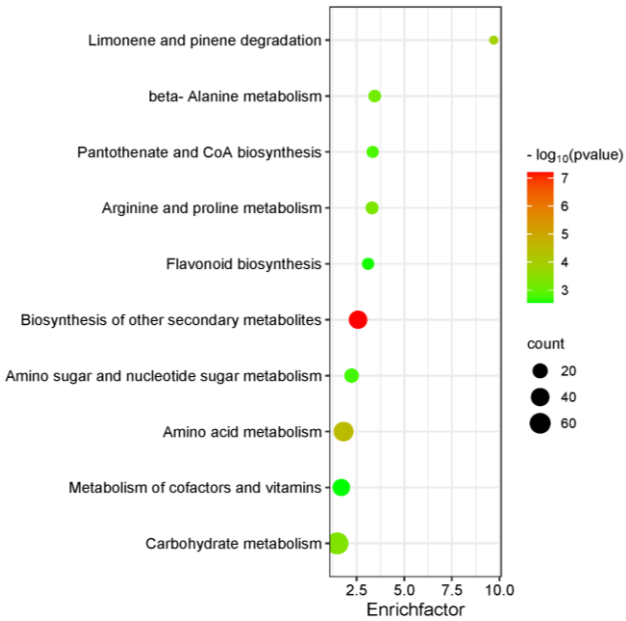

Fig. 4. KEGG pathway enrichment analysis of DEPs between 'Monterey' and 'Benihoppe'.

Table 1. Twelve differentially expression protein associated with flowering.

Protein	Uniprot blast results	GO annotation	log2 Fold change	p-value
FxaC_10g10040.t1	SPL3 (Squamosa promoter-binding-like protein 3)	GO:0009908 flower development	20.48	0.0034
FxaC_10g31450.t1	ABH1 (Nuclear cap-binding protein subunit 1)	GO:0048574 long-day photoperiodism, flowering	-17.35	0.0007
FxaC_13g07120.t1	CUL3A (Cullin-3A)	GO:0009911 positive regulation of flower development	20.69	1E-06
FxaC_17g08471.t1	HDA6 (Histone deacetylase 6)	GO:0010228 vegetative to reproductive phase transition of meristem	-19.03	0.0002
FxaC_18g02530.t1	ACC1 (Acetyl-CoA carboxylase 1)	GO:0010072 primary shoot apical meristem specification	-2.75	0.0002
FxaC_20g00250.t1	OVA6 (Proline--tRNA ligase, chloroplastic/mitochondrial)	GO:0048481 plant ovule development	19.32	0.0002
FxaC_21g44780.t1	SPY (UDP-N-acetylglucosamine--peptide N-acetylglucosaminyltransferase)	GO:0009908 flower development	-3.42	0.0011
FxaC_21g45850.t1	TFL1(Terminal flower 1)	GO:0009910 negative regulation of flower development	-20.71	0.0002
FxaC_22g20081.t1	ELF4 (Protein EARLY FLOWERING 4)	GO:0048573 photoperiodism, flowering	18.28	0.0035
FxaC_23g53111.t1	Auxin transport protein BIG	GO:0048281 inflorescence morphogenesis	-17.57	0.0031
FxaC_26g13890.t1	COI1 (Coronatine-insensitive protein 1)	GO:0009909 regulation of flower development	-19.39	0.0002
FxaC_27g39840.t1	ELF4 (Protein EARLY FLOWERING 4)	GO:0048573 photoperiodism, flowering	20.44	3E-09

Protein-protein interaction (PPI) analysis: The PPI network of DEPs was analyzed by String. 960 of 1088 DEPs were matched in the String database, and 1744 interactions were predicted at a confidence level of 0.7. To focus on the flowering-associated network, we selected flowering-associated DEPs for sub-network analysis. Ten flowering-associated DEPs interacted with 53 other DEPs, and two direct interaction networks were generated. SPL3(FxaC_10g10040.t1) directly interact with TFL1(FxaC_21g45850.t1), which interact with ribonucleoproteins such as RPL28, RPL29, RPL27, etc. ELF4(FxaC_22g20081.t1) interact with HDA6(FxaC_17g08471.t1), which interact with COI1(FxaC_26g13890.t1), AFP homolog2 and MBD10.

Discussion

Precise regulation of plant flowering is important for plant reproduction and crop yield. There are six main pathways for the molecular regulation of flowering time in *Arabidopsis thaliana*: photoperiod, vernalization, autonomous, thermosensory, gibberellin and age pathway, with a number of proteins involved in each pathway (Simpson & Dean, 2002; Blazquez *et al*., 2003; Amasino, 2004; Simpson, 2004; Jeong & Clark, 2005). In this study, we investigated the proteins that play key roles in the process of SAM differentiation of SF strawberry 'Bennihope' and PF strawberry 'Monterey' during summer, by performing quantitative proteomic analysis of growth point tissues.

Through GO analysis, 36 DEPs were enriched to the 'reproductive structure development' term, of which 12 proteins were associated with flowering. Among them, SPL3(FxaC_10g10040.t1) and TFL1(FxaC_21g45850.t1) are known to interact with each other (Fig. 5a). SPL3(SQUAMOSA PROMOTER BINDING PROTEIN-LIKE 3) is a transcription factor, which promotes flowering by binding to the flower meristem identity genes such as *LEAFY*, *FRUITFULL* and *APETALA1* to activate their expression in *Arabidopsis thaliana* (Yamaguchi *et al*., 2009; Pastore *et al*., 2011; Jiang *et al*., 2022). While TFL1(TERMINAL FLOWER 1), a small protein belonging to phosphatidylethanolamine-binding protein family, is an inhibitor of flowering by repressing *LEAFY* and *APETALA1* in *Arabidopsis thaliana* (Bradley *et al*., 1997; Hanano & Goto, 2011). TFL1 inhibits the expression of SPL3, helping to maintain the nutritional properties of meristematic tissues and delay flowering (Cerise *et al*., 2023). Here we found that the level of TFL1 was significantly lower in 'Monterey' undergoing flower bud differentiation in summer, than in 'Benihoppe' which stopped flower bud differentiation, and SPL3 showed the opposite expression pattern, consistent with the study of both in Arabidopsis.

HDA6 is a histone deacetylase that can regulate gene and transposon expression through histone deacetylation (Probst *et al*., 2004). In *Arabidopsis*, HDA6 regulates gene expression by modulating the acetylation levels of flowering repressor *FLC(FLOWERING LOCUS C)* (Michaels & Amasino, 1999; Wu *et al*., 2008; Yu *et al*., 2011). COI1 (CORONATINE INSENSITIVE 1) is a receptor protein for jasmonate (JA) signaling, and COI1-dependent signaling pathway delays the flowering time of *Arabidopsis thaliana* by inhibiting the expression of the florigen gene *FLOWERING LOCUS T (FT)* (Corbesier *et al*., 2007; Yan *et al*., 2009; Zhai *et al*., 2015). ELF4 (EARLY FLOWERING 4) is an essential component of the core oscillator of the *Arabidopsis* circadian clock, involved in photoperiod perception and circadian regulation, and was considered as a negative regulator of flowering (Doyle *et al*., 2002; Zhao *et al*., 2021). According to PPI results, HDA6, COI1 and ELF4 form a small interacting network with SGT1B, MPK4, JMT and etc. Compared to 'Benihoppe', HDA6 (FxaC_17g08471.t1) and COI1(FxaC_26g13890.t1) in 'Monterey' were significantly down-regulated, in concordance with their flowering-promoting role in *Arabidopsis*; whereas ELF4(FxaC_22g20081.t1) was significantly up-regulated, inconsistent with its function as a flowering repressor in *Arabidopsis*.

Flowering is a highly intricate process that is influenced by both environmental and genetic factors. We investigated the compositional differences between PF and SF strawberry SAMs by proteomics, which showed that the two cultivars differed significantly in the expression of several key proteins in the photoperiodic and autonomic flowering pathways. The current study may provide further information for investigating the mechanism of strawberry flower formation.

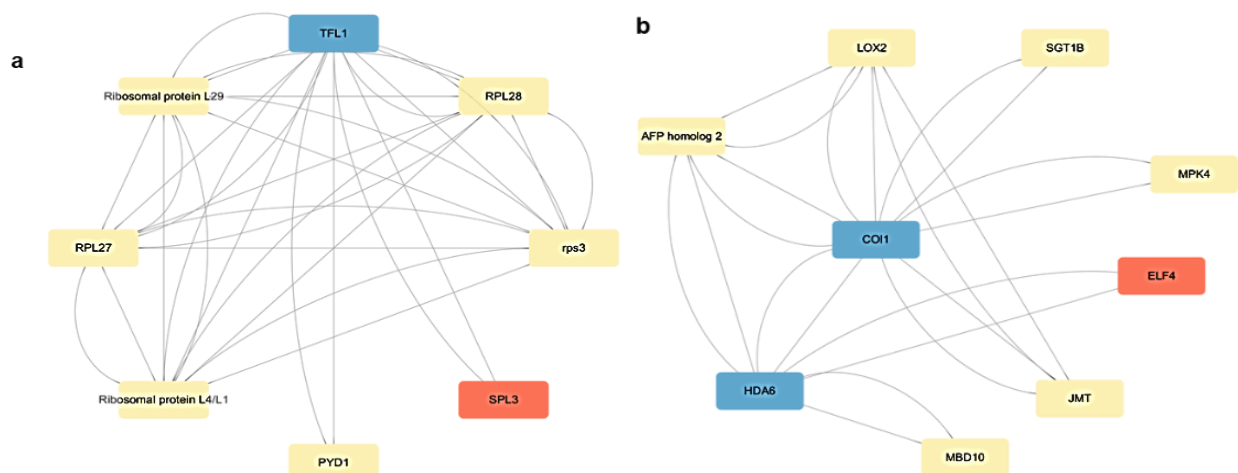

Fig. 5. Protein-protein interaction of flowering-associated DEPs TFL1 and SPL3(a), COI 1, HDA6 and ELF4(b).

Acknowledgements

This research was supported by Hangzhou Science and Technology Development Program (No. 20201203B109 and No. 202205B05).

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(Received for publication 12 August 2023)