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Proteomic landscape presents cues for vegetative to reproductive transition in mango

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Abstract

Proteome-based characterization of vegetative and flower bud formation was utilized to identify and differentiate protein species with significant variable abundance during floral transition in mango cv. Dashehari using 2DE and corroborating the identified protein spots using gene expression analysis. Total soluble proteins were phenol-extracted from the vegetative and floral flush of mango cv. Dashehari and separated on 2D gels at pH 4-7. The protein spots with variable intensity were identified through SameSpots software. The protein sequences of differentially accumulated spots were identified based on PI and MW using *Citrus sinensis* proteome isoelectric focusing database. Furthermore, these protein sequences were used to conduct (tBLASTn) against *Mangifera indica* to predict the protein. Real time gene expression was done to corroborate identified proteins. Total 301 spots were detected, out of which 16 were identified as differentially expressed based on *P* value ($P \leq 0.05$) and a 2-fold change. These 16 protein spots were identified on the basis of *in silico* comparative mapping protein against genome of mango and citrus, a close relative. They were classified into eight categories *viz.*, transcriptional regulation, phenylpropanoid pathway and cell wall /cytoskeleton metabolism-related proteins, hormone signalling, flowering time, signal-transduction, transport and protein synthesis associated to flowering. Five genes coding for shortlisted proteins were used for validation of results using gene expression analysis. *SAM* (S adenosyl methionine synthase) was found to up-regulated in floral flush, involved in the biosynthesis of polyamines has an association with flowering and stress responses. Furthermore, *ARF* (Auxin Response Factor) and serine/threonine kinase gene members were also found to play a critical role in determining the floral development process in mango, consistent with results obtained through 2DE. Protein species putatively involved in phenylpropanoid pathway were also identified, showing the process of mango flowering from a new perspective beyond the conventional view. This study of flowering-related proteomics provides an overview of the biological pathways and regulatory mechanisms associated with the developmental physiology of flowering.

Key words: 2-DE, flowering, gene expression analysis, *in silico* comparative proteomics, mango, proteomics.

Introduction

Mango is one of the most important fruit globally and is a commercially relevant crop. It is a terminal-bearing plant with scanty knowledge of the variables influencing the transition from vegetative to reproductive mode. In general, mangoes go through a juvenile stage after germination that lasts for several years and during which flowering does not occur and interactions with the environment, flowering in the previous year, etc., are known to be responsible for phenomena like alternate bearing, thus making it difficult for scientists to interpret the research data.

In accordance with a number of academic publications, scientists are currently using traditional genetic and molecular tools for the discovery, identification, and cloning of floral-specific genes, specifically the cloning of floral homeotic genes (Sung *et al.*, 2000; Weigel and Nilsson, 1995). With the rapid advancement of proteome technology, the study of plant floral induction mechanisms has been greatly accelerated (Kofler *et al.*, 2022; Munoz-Fambuena *et al.*, 2013). There is abundant literature on regulatory mechanisms of flowering in *Arabidopsis*, but there is scarcity regarding molecular regulatory mechanisms and different expression levels of proteins, sugars and signalling molecules during flower bud differentiation in mango. Understanding the systemic mechanism of floral induction in mangoes is complex. Factors like endogenous hormones, genetic makeup, soluble carbohydrates, stress conditions as well as the age of the shoots are crucial for successful flowering (Cho *et al.*, 2017). In this context, effort has been made to identify important proteins associated with floral transition in mango as there is a gap in knowledge regarding the role of regulatory pathways culminating in floral transition under subtropical conditions. The cultivar 'Dashehari' that dominates in Indian Gangetic plains exhibits the typical alternate flowering behaviour, clearly defined on and off years for flowering and fruiting. These characteristics make this cultivar a suitable model for studying the process of mango shoot maturation and flowering. The comparative analyses may aid in a better understanding of the genetic and molecular regulatory mechanisms of flowering critical for phase transition (V-R).

Materials and methods

Plant material and sampling time: The studies were conducted at the experimental farm of Central Institute for Subtropical Horticulture, Lucknow (latitude 26.90" N., longitude 80.76" E.) on a 15-year-old plants. The cultivar used was an alternate bearer, "Dashehari", planted at 10 m spacing, with stabilized yield and

maintained under uniform cultural practices. Two types of shoots (20 in no.) were tagged and their phenotypic fate was observed till the bud burst stage: the mature shoots, called flowering bearing shoots/floral flush and the new flush that continues to grow as vegetative flush (Fig. 1). The fully mature middle leaves of the shoots were selected and it was done from both types of the shoots in January 2021. During the experimental period, maximum (20°C) and minimum (6.8°C) temperatures and relative humidity (88.3-69.3) were recorded. The experiment was carried out with three biological replicates and each replication represented a single tree. Leaf samples were collected, rapidly frozen in liquid nitrogen, and stored at −80°C for further research.

Total protein extraction: Total proteins were extracted from the vegetative and floral flush using the modified phenol method (Carpentier *et al.*, 2005). The protein concentration was calculated using the Bradford protein method using bovine serum albumin as the reference standard (Bradford, 1976).

Two-dimensional (2DE) polyacrylamide gel electrophoresis: Two-dimensional gel electrophoresis was used to separate isolated crude protein in two dimensions using isoelectric focusing (IEF) gel in the first dimension and SDS PAGE in the second dimension as earlier standardized in our lab (Laxmi *et al.*, 2022).

Image and data analysis: The gel was stained with Coomassie blue, digitized using Epson Expression 11000XL Scanner (Fig. 1), and analyzed using the SameSpots software (TotalLab Ltd, UK). The spots were detected, matched, and normalized with default parameters. Differentially expressed protein spots among the vegetative and floral flush samples were ascertained using normalized spots and compared with the reference gel. The fold difference and p-value were calculated using one-way ANOVA. The threshold value for fold change was set at 2.0 for up and down-regulation at $P \leq 0.05$. The protein spots were selected based on spot intensity variations among vegetative and floral flush. Further, these protein spots were identified based on PI and MW characteristics, using *Citrus sinensis* proteome isoelectric focusing database (http://isoelectricpointdb.org), as such database of *Mangifera* proteome is not available in the public domain. The protein sequences were further used to conduct protein-translated nucleotide (tBLASTn) against *Mangifera indica* (Taxid: 29780).

Real-Time Quantitative PCR Validation: RNA isolation and Real-Time were performed in floral and vegetative tissue as reported previously by Bajpai *et al.* (2018). Gene-specific primer sets described were designed using the transcriptomic data sets of vegetative and floral tissue of mango cv. Dashehari submitted to the National Centre for Biotechnology information (NCBI) (Accession numbers SRR11261955, SRR11261956). The housekeeping gene MiActin was taken as endogenous reference gene to normalize the data (Luo *et al.*, 2013). The fold change in expression levels was calculated by ΔΔCT value as reported previously Livak and Schmittgen (2001). All primers used for qPCR are listed in (Table 1)

Fold Change = 2^(-ΔΔCt)

Results and discussion

2DE reveals differential accumulation of flowering responsive proteins in mango: The 2 D gels of mango cv Dashehari (leaves

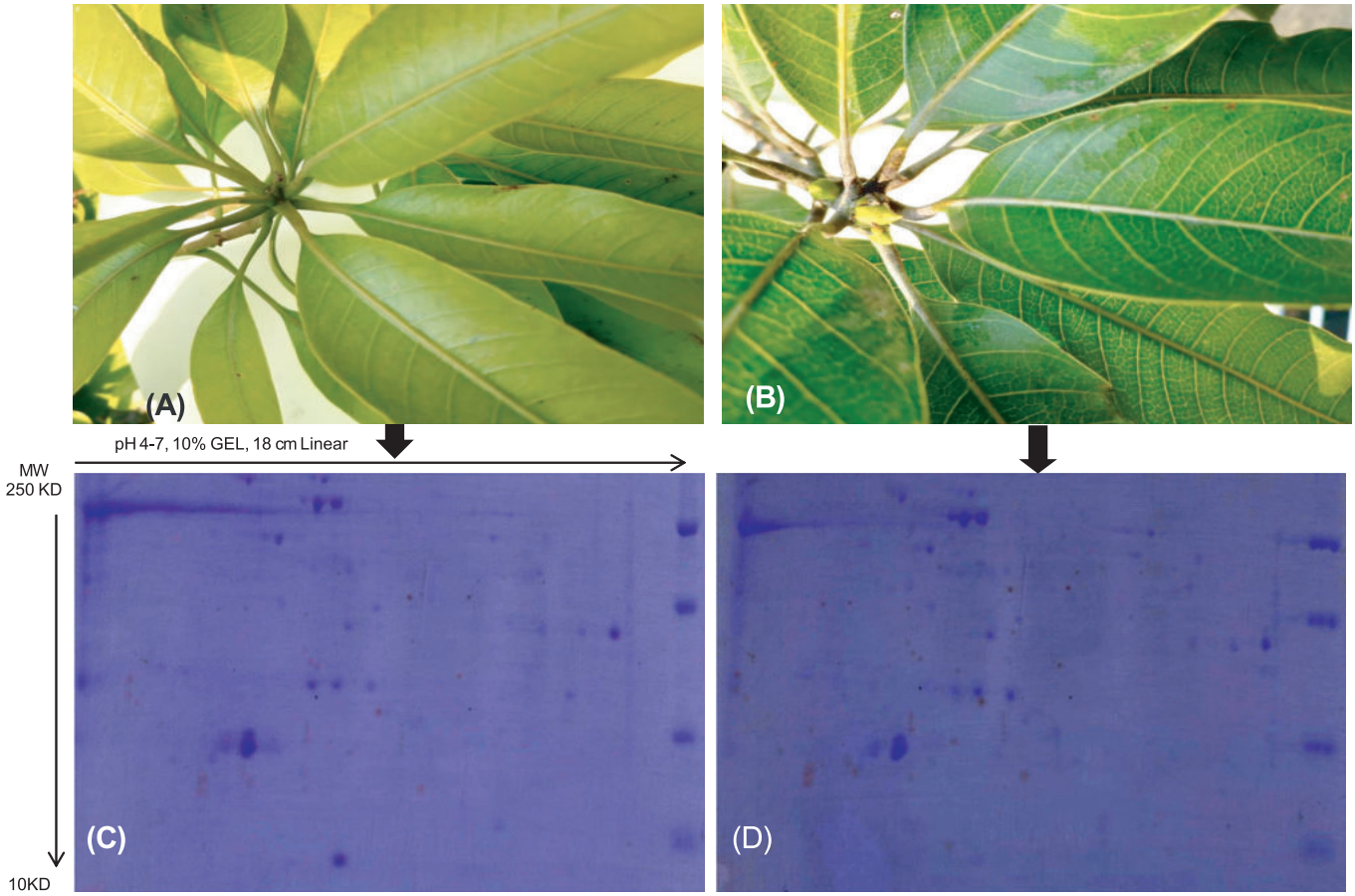

Fig. 1. Type of shoot used for protein isolation and 2DE (A) Vegetative flush, (B) Floral flush, (C) Displaying protein spots in vegetative flush, and (D) in floral flush.

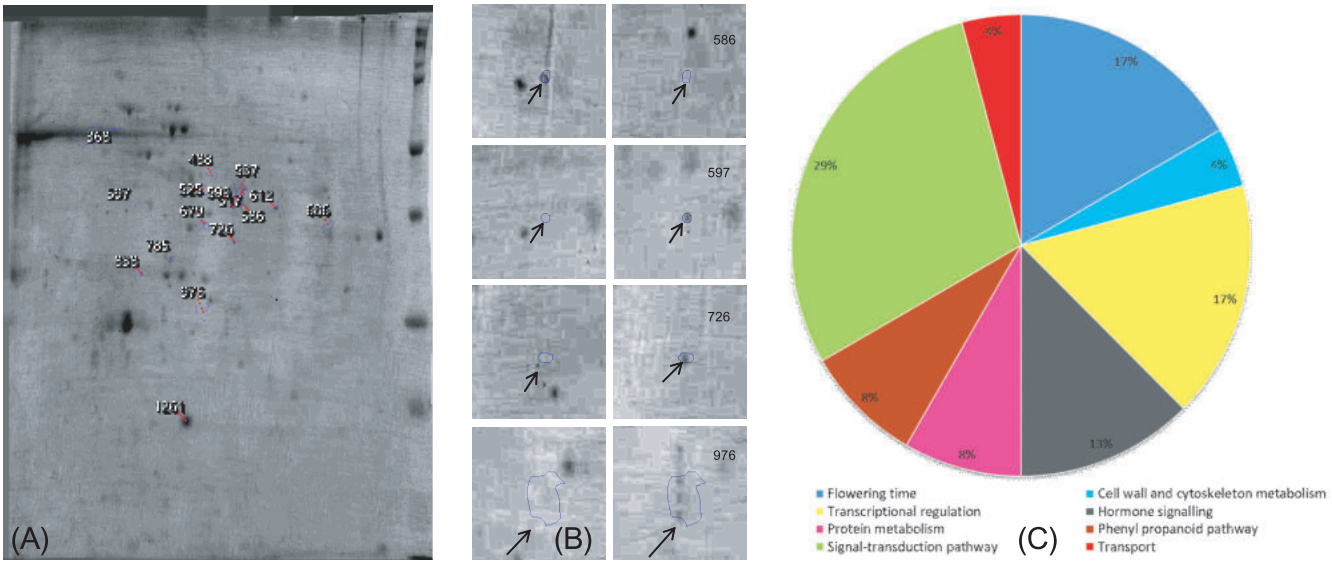

Fig. 2. Total soluble proteins resolved in 2D-gel. (A) Differentially expressed spots identified by Samespots software. (B) Close up view of selected protein spots, in vegetative (left) and floral flush (right) (#586:CO5; #597: Trihelix; #726: AGL26 and #976: ARF) (C) Distribution in different fu MYB53 and COMT)CO5, GA20ox, BCA1, (Identified Key proteins Real time gene expression analysis database iefproteome protein identification using Comparitive software Samespotsprotein spots using Identification of differentially expressed Total protein isolation and 2DE Genetic Background GA3 IAA conditionsgrowth Optimal factorsgenetic Lack of Pathway regulated Vegetative -Up and GTP) AP3, NAC, PPR, KEA3, F3H, SAM, TrihelixAGL 26, (Identified Key proteins Pathway regulated Flowering -UpGA3 IAA Hormonal status Environmental factors Flowering Vegetative growth Mango Shoot Apical Meristem nctional categories based on comparative proteomics.

from vegetative and floral shoot) displayed 301 protein spots cumulatively. The proteins showed a broad distribution in the pI (4.0 to 7.0) and mass (10 to 250 kDa), respectively. Based on spot intensity variations among vegetative and floral flush samples were quantified by SameSpots (version 5.1.012) software, where in 56 (18.60%) protein spots had 2-fold change (up or down) between vegetative and floral flush, and only 16 spots (28.57%) that showed significant differences based on $P \leq 0.05$ and a 2-fold change were used for further analysis (Fig. 2). Among them, ten were up-regulated, and six down-regulated. Identification of proteins based on *in silico* comparative synteny and annotations were selected by maximum query coverage and total score (Table 2). Some spot number codes for more than one protein, and we selected the proteins based on their role in flowering as well as those with the best score by (tBLASTn). The close-up view of representative differentially expressed proteins is shown in Fig. 2.

Classification of protein spots into different functional groups: These proteins were categorized into different functional groups such as transcriptional regulation, phenylpropanoid pathway and cell wall /cytoskeleton metabolism-related proteins, hormone signalling, flowering time, signal-transduction, transport and protein synthesis, and the percentage contribution pattern is illustrated in pie chart (Fig. 2). Spot #488 representing NAC transcription factor was identified which is known to regulate gene expression and flowering time by associating with the histone demethylase JMJ14 (Ning *et al.*, 2015). Another spot, #679, representing oxoglutarate-dependent dioxygenase proteins, was identified. 2-oxoglutarate/Fe (II)-dependent dioxygenases were related to early flowering in alfalfa (Ma *et al.*, 2021). Both of these proteins are found to be differentially up-regulated in the floral flush of mango cv. Dashehari. Protein spot #726 represents the Agamous-like MADS-box protein. *AGL* is the crucial gene regulating floral transition and differentiation (Yadav *et al.*, 2020). Furthermore, spot #586 was assigned as constans5, which encodes a member of the constans-like (*COL*) family. Earlier, overexpression of *ATCOL5* or *ATCOL9* has been indicated to promote or repress flowering, respectively (Cheng and Wang, 2005; Hassidim *et al.*, 2009). The protein spot #525 represents the Apetala3 that controls petal identity and floral meristem patterning in *Nigella damascene* L. (Gonçalves *et al.*, 2013) and determines the flowering fate of saffron (Wafai *et al.*, 2018). Protein spot #597 denotes the trihelix transcription factor which is essential for plant morphological developmental processes and regulating flowering time (Song *et al.*, 2021). Likewise, protein spot #612 represents transcription factor MYB53, which is reported to regulate vegetative growth and is down-regulated by *SPL13* during reproductive development in *Medicago*

Table 1. The list of primers used for qRT-PCR

Spot no	Protein name	Forward primer	Reverse primer
586	*Constans 5* (*CO5*)	5'-AGCCGTTCTTCGACTAGCG-3'	5'-GTTGTTGACACGCGCCACTG-3'
726	S-adenosylmethionine-dependent methyltransferase (*SAM*)	5'-CATGGGGACGCTGGGCTTAC-3'	5'-TCCACCTTGGTGGGGTCCTT-3'
597	Trihelix transcription factor (*Trihelix*)	5'-CACAGAATTGCCGCCAACAA-3'	5'-ACCAGTCTTCCAATGCGACC-3'
726	Agamous-like *MADS*-box protein (*AGL26*)	5'-TTCTTTCGCTCTCTCGGCTT-3'	5'-TGTGGCAGAGACACTCCATTC-3'
976	Auxin response factor-like protein (*ARF*)	5'-AACCACCCGCTGGATTTTGA-3'	5'-AAGCCTGTGTAAGTGGTGGG-3'
537	Serine/threonine kinase gene (*STK*)	5'-TCAGCTGCAGTTGGCCTTGG-3'	5'-GGCTGATCCGGACCTCAACG-3'
Reference gene	Actin	5'GAGAGTTTTGATGTCCCTGCCATG-3'	5'-CAACGTCGCATTTCATGATGGAGT-3'

Table 2. Annotation results of differential spots based on synteny using *in silico* comparative proteomics

Spot No.	Differential expression	MW KD/ PI	Matched Protein	Best score (%)	Accession	Pathway
488	Up	43/5.6	NAC domain-containing protein 71-like (NAC 71-like)	87.50	XR_003066340.1	Flowering time
679	Up	37/5.5	Probable 2-oxoglutarate-dependent dioxygenase (AOP2)	100	XM_006494416.3	
586	Down	40/5.7	Constans 5 (CO5)	73.10	MG677826.1	
726	Up	35/5.7	Agamous-like MADS-box protein (AGL26)	100	XM_025096038.1	
517	Up	42/5.9	Beta-tubulin gene (TUB2)	52.78	MK639361.1	Cell wall and cytoskeleton metabolism
525	Up	42/5.5	AP-3 complex (AP-3)	100	XM_006472177.3	Transcription-al regulation
597	Up	40/4.4	Trihelix transcription factor (Trihelix)	80	XM_006475645.3	
686	Down	36/6.5	ABC transporter I family member 6, (ABCA1)	99.69	XM_006485454.3	
612	Down	39/6.1	Transcription factor MYB53	87.75	XM_024187525.1	
679	Up	37/5.5	F-box proteins (FBPs)	58.08	XM_006451907.2	
686	Down	36/6.5	Gibberellin 20-oxidase-like protein (GA20ox)	93.67	XM_006490166.2	Hormone signalling
976	Up	28/5.5	Auxin response factor-like protein (ARF)	90.3	AY255705.1	
838	Up	32/5.0	Pentatricopeptide repeat-containing protein (PPR)	99.66	XM_006420934.2	Protein metabolism
1261	Down	19/5.2	F-box/kelch-repeat protein (FBPs)	31.69	XM_031107395.1	
586	Down	40/5.7	Caffeic acid O-methyl Transferase COMT)	79.82	FJ645928.1	Phenyl- propanoid pathway
976	Up	28/5.5	Flavanone 3-hydroxylase 2 (F3H)	32.37	KF956031.1	
726	Up	35/5.7	S-adenosylmethionine-dependent methyltransferase (SAM)	97.29	XM_024183454.1	Signal-transduction pathway
1261	Down	19/5.2	Receptor-like protein kinase (RLK)	82.56	XM_025094398.1	
597	Up	40/4.4	GTP-binding protein (GTP)	84.1	XM_006466354.3	
599	Down	39/5.7	NBS-LRR disease resistance protein (NBS-LRR)	42.35	HM446508.1	
537	Up	42/5.8	Serine/threonine kinase gene (STK)	73.7	AY693371.1	
785	Down	33/5.2	Protein NDL1	100	XM_006432969.2	
838	Up	32/5.0	Pentatricopeptide repeat-containing protein (PPR)	99.66	XM_006420934.2	

sativa L. (Gao *et al.*, 2018). These MYB proteins participate in morphogenesis, control of specialized metabolism, circadian clock control, response to phosphate starvation, and flower and fruit development (Feller *et al.*, 2011). One spot, #368, was identified that represented K^+ efflux antiporter 3. K^+ transporters were also shown to be essential for flower development via maintaining K^+ homeostasis in *Arabidopsis* and rice (Isayenkov *et al.*, 2020).

Spot no. #517 represents the up-regulated Beta-tubulin (TUB2) gene, which plays a fundamental role in plant morphogenesis but also was related to flower development in flax (Gavazzi *et al.*, 2017). Further, spot 686 represents the ABC transporter I family member 6, a class of ATP binding cassette proteins also known as ABCG, which transport a variety of substrates necessary for plant growth and reproduction (Zhao *et al.*, 2016). Two Spots #586 and #976, were related to proteins of COMT and F3H, respectively. The protein spot 976 represents Flavanone 3-hydroxylase 2, which is implicated in floral induction in saffron (Tu *et al.*, 2016). Likewise, spot 586 denoted the Caffeic acid O-methyl transferase (*COMT)*, a key enzyme in lignin biosynthesis, whose expression delayed flowering in rice (Li *et al.*, 2009) and altered flowering time in alfalfa (Sun *et al.*, 2019). Two spots, #679 and #1261, represented F-box protein and F-box/kelch-repeat protein, respectively. F-box proteins are involved in regulating flowering, auxin signal transduction, floral organ formation and leaf senescence (Kim *et al.*, 2022). The latter spot was down-regulated, indicating that F-box/kelch-repeat proteins act as a repressor during floral transition stages in mango. Spot #838, which codes for pentatricopeptide repeat-containing protein, was up-regulated, which was reported to be related to early flowering (Saha *et al.*, 2007), organelle biogenesis, and plant development (Emami and Kempken, 2019).

Spot #976 and #686 represented Auxin response factor-like and Gibberellin 20-oxidase-like proteins, respectively. Gibberellin 20 oxidase (GA20ox) is a key catalytic enzyme in the biosynthesis of GA, the concentration of which increases at the meristem immediately before floral induction and promotes flowering by up-regulating *SOC1* expression (Zhou *et al.*, 2019). Furthermore,

another study showed a declining trend in gibberellic acid in floral flush leaves compared to the vegetative flushes in different mangoes, which explains the inhibitory potential of gibberellins on mango flowering (Bajpai *et al.*, 2021). Spot #726 corresponds to the S-adenosyl methionine-dependent methyltransferase (SAM) protein, which has been demonstrated to have a role in flowering by several studies (Ding *et al.*, 2015). Our protein spot results agree with the hormonal theory of flowering, which states that higher auxin levels with a dip in temperature and a decline in gibberellin levels promote inductive flowering in subtropical conditions in mango.

Functional categorization of protein spots from 2D gels placed seven spots (726, 1261, 597, 599, 537, 785, 838) were categorized under the signal transduction group, which are important for the perception of environmental signals and transmission to cellular machinery for activating different growth and development responses in plants. Spot 726, 597, 537 and 838 were up-regulated, while the remaining three spots (1261, 599 and 785) were down-regulated. Likewise, the protein identified by spot #597 represents an up-regulated GTP-binding protein that plays important roles in plant development, cell signalling, activation of DNA-binding protein of vernalization 1 (RTV1), resulting in early flowering in *Arabidopsis* (Heo *et al.*, 2012). Spot # 537 denotes Serine/threonine-protein kinase was up-regulated, which has a role in stress-induced signalling, flower development and regulating flowering time by modulating the photoperiod pathway in *Arabidopsis* (Xie *et al.*, 2022). Spot 599 and 785 display down-regulation and represent NBS-LRR disease resistance protein and

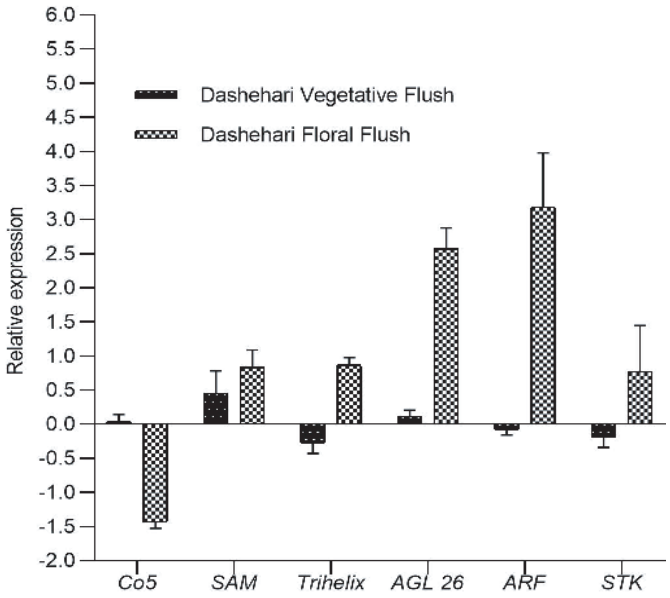

Fig. 3. Validation of selected spots through qRT-PCR.

Protein NDL1, respectively. Pathogenesis-related proteins, *e.g.* receptor-like proteins and toll-interleukin-like receptor (TIR)-nucleotide-binding site (NBS)-LRR class proteins and members of the signalling receptor kinase family LRR proteins are related to early flowering in alfalfa (Ma *et al.*, 2021). The role of NDL1 in the context with flowering is still not clear. Besides this, spot #1261, which codes for the receptor-like protein kinase gene, regulates cell morphogenesis, flowering time, and seed production (Gachomo *et al.*, 2014).

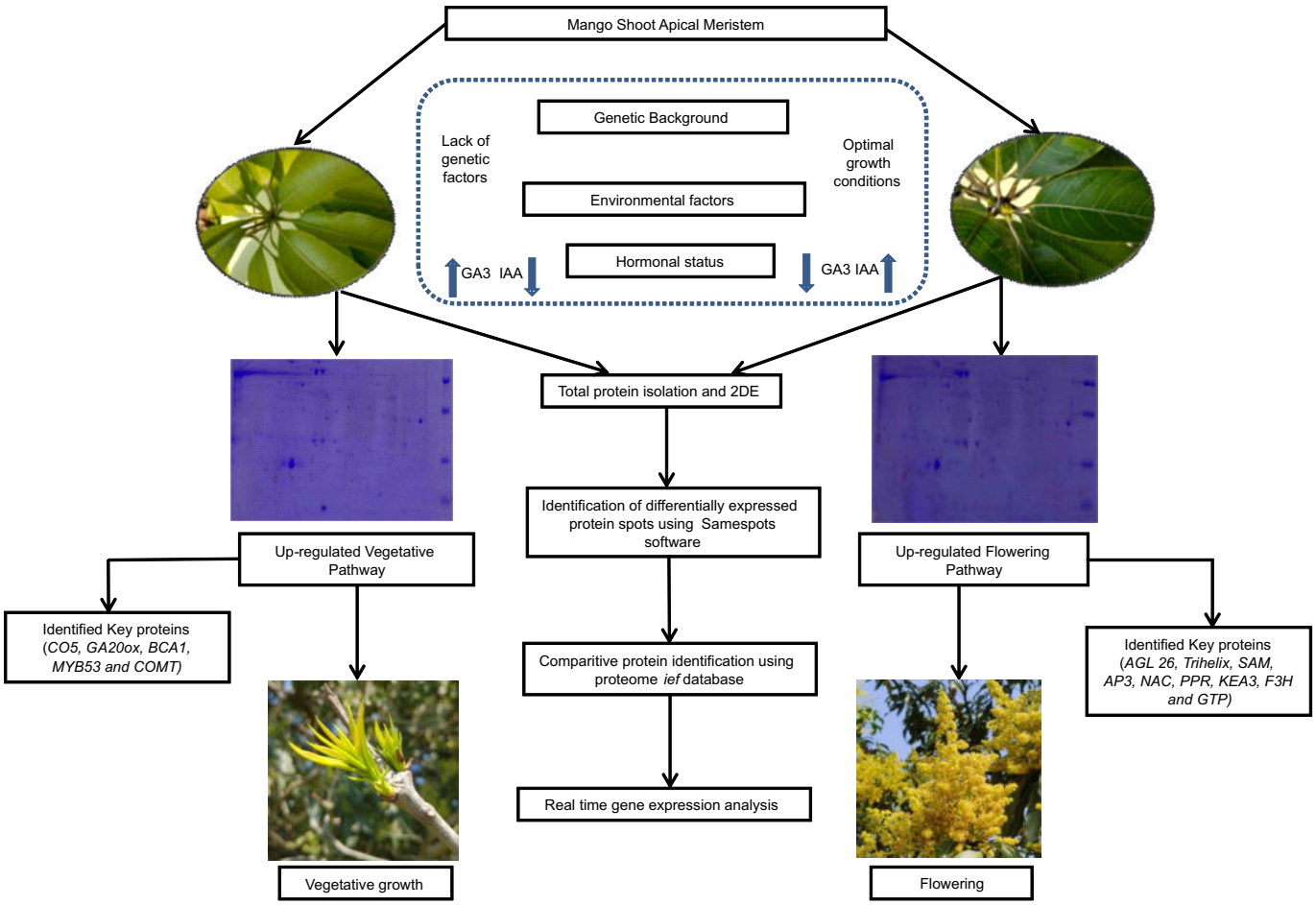

Fig. 4. Proteomics based model presents cues for vegetative to reproductive transition in mango

Validation by RT-PCR: In order to verify our 2DE results, six genes (*CO5, SAM, Trihelix, AGL, ARF* and *STK*) coding for earlier mentioned proteins were selected for the RT-PCR analysis. These genes showed an apparent change between the vegetative and floral flush at the transcriptional level and were majorly up-regulated in floral flush (Fig. 3). The expression profiles of all of these genes at mRNA levels were consistent with those at the protein levels (Table 2). Since we know that *CO5* is an activator of flowering, yet here it is repressed post-transcriptionally in floral flush and up-regulated in vegetative, contributing to see a new perspective to photoperiod sensitive flowering in mango except the conventional view. qRT-PCR showed that *SAM* gene expression was comparatively high in floral flush as compared to vegetative. *SAM* plays a central role in many cellular biochemistry reactions, including flowering and fruit development (Ding *et al.*, 2015). Besides trihelix, a transcriptional activator was found to be up-regulated in floral flush with a log fold change of (0.87). Several studies have demonstrated that a plant-specific trihelix transcription factor, one of a family previously known only as regulators of light-controlled genes, causes early flowering (Song *et al.*, 2021; Yang *et al.*, 2020). We further found an up-regulation of *AGL26* gene in the floral flush (2.57) and its down-regulation in the vegetative flush, which indicates towards the key role of *AGL* in regulating flowering initiation and development in mango. In a study, it was found that knocking down of *AGL* gene, leads to delayed flowering, whereas this gene's constitutive expression caused rapid flowering in *Arabidopsis* (Zhou *et al.*, 2021).

AGL26, integrates multiple flowering pathway signals, and its expression levels eventually determine the exact flowering time (Van and Molenaar, 2017). Furthermore, the *ARF* (Auxin Response Factor) gene was also found to be up-regulated in floral flush (log fold change of 3.18), as compared with vegetative (-0.08). Auxin Response Factors (*ARFs*) is reported to modulate the expression of target genes by binding to auxin response elements (*AuxREs*) and influence the transcriptional activation of down-stream target genes, playing role in flower development (Kumar *et al.*, 2011). The expression trend of serine/threonine kinase during the floral development was found to be up-regulated in floral flush. Previous studies have also demonstrated that serine/threonine kinase gene members may regulate circadian rhythms and vacuolar H+-ATPase (Xie *et al.*, 2022).

There is a scarcity of data on the protein expression profile during flower development in mango. This is the first proteomic profiling study in mango cv. Dashehari during the period of floral bud initiation and renders the possible development of suitable biomarkers for biennial bearing in mango. We therefore performed proteome analysis of *Mangifera indica* cultivar Dashehari vegetative and floral flush wherein putative proteins playing an important role in mango biennial rhythm were identified through a proteomic-based approach and validated through real-time gene expression analysis (Fig. 4). Out of the observed differentially expressed proteins spots from the 2DE data, the transcript abundance of six genes (*CO5, SAM, Trihelix, AGL26, ARF* and *STK*), the key genes regulating floral transition and differentiation, were validated through qRT-PCR. The results of the study provide an overview of the important biological processes occurring during flowering.

In summary, the study unveils distinct protein accumulation patterns between vegetative and floral shoots of mango cv. Dashehari using 2D gel electrophoresis. Among the identified 301 protein spots, 56 exhibited significant 2-fold changes, with 16 spots selected for further analysis. These differentially expressed proteins were categorized into functional groups, revealing their roles in transcriptional regulation, signaling pathways, hormone responses, and metabolic processes related to flowering. Notably, proteins such as NAC transcription factors, dioxygenases, MADS-box proteins, and transporters demonstrated significant changes. Validation through RT-PCR confirmed differential expression of selected genes. This proteomic approach enhances our understanding of mango flowering dynamics and lays groundwork for potential biomarkers for biennial bearing and floral regulation.

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