

Title A comprehensive metabolic map reveals major quality regulations in red-flesh kiwifruit (*Actinidia chinensis*)

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1 A comprehensive metabolic map reveals major quality regulations in red flesh
2 kiwifruit

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20 Summary

- 21 • Kiwifruit (*Actinidia chinensis*) is one of the popular fruits worldwide, and its
 22 quality is mainly determined by key metabolites (sugars, flavonoids and vitamins).
 23 Previous works on kiwifruit are mostly done via a single omics approach or
 24 involve only limited metabolites. Consequently, the dynamic metabolomes during
 25 kiwifruit development and ripening and the underlying regulatory mechanisms are
 26 poorly understood.
- 27 • In this study, using high-resolution metabolomic and transcriptomic analyses, we
 28 investigated kiwifruit metabolic landscapes at 11 different developmental and
 29 ripening stages and revealed a parallel classification of 515 metabolites and their
 30 co-expressed genes into 10 distinct metabolic vs gene modules (MM vs GM).
 31 Through integrative bioinformatics coupled with functional genomic assays, we
 32 constructed a global map and uncovered essential transcriptomic and
 33 transcriptional regulatory networks for all major metabolic changes that occurred
 34 throughout the kiwifruit growth cycle.
- 35 • Apart from known MM vs GM for metabolites such as soluble sugars, we
 36 identified novel transcription factors that regulate the accumulation of
 37 procyanidins, vitamin C and other important metabolites.
- 38 • Our findings thus shed light on the kiwifruit metabolic regulatory network and
 39 provide a valuable resource for the designed improvement of kiwifruit quality.

40 **Key words:** kiwifruit, multi-omics, metabolic regulatory network, transcription factor,
 41 procyanidins, vitamin C

42

43 **Introduction**

44 The flavor and nutritional value of commercial fruit are usually determined by the key
 45 metabolites. For example, sucrose, glucose, fructose and many soluble sugars
 46 contribute to determine fruit taste (Kader, 2008). On the other hand, different bioactive
 47 compounds such as flavonoids and vitamins with antioxidant activities play a vital role
 48 in maintaining human health (Pignocchi *et al.*, 2003; Rao & Rao, 2007; Mandl *et al.*,
 49 2009; Skibola & Smith, 2000; Chen & Chen, 2013; Fiedor & Burda, 2014; Young *et al.*,
 50 2015; Pullar *et al.*, 2017; Rodriguez-Concepcion *et al.*, 2018). Thus, improvement
 51 in fruit quality not only makes fruits appealing to consumers, but also brings health
 52 benefits to humans. However, engineering metabolite biosynthesis to enhance quality
 53 is not an easy task and it has become a major challenge in the fruit breeding and biotech
 54 industry.

55 A prerequisite for manipulating traits to improve fruit flavor and nutritional quality
 56 requires a fine dissection of metabolic pathways (MPs) and their underlying metabolic
 57 regulatory networks (MRNs). This can be achieved by multi-omics technologies such
 58 as high-resolution metabolomic and global transcriptomic profiling along with efficient
 59 bioinformatic mining and functional genomic assays. Indeed, omics analyses have led
 60 to the reveal of numerous MPs and MRN genes that are involved in the production of
 61 flavor- and/or nutrition-associated metabolites in the model fruit crop tomato. For
 62 instance, the dynamics of both primary and secondary metabolisms during tomato
 63 development and fruit ripening and corresponding MRNs were uncovered through
 64 integrative analyses of metabolomes and transcriptomes (Carrari *et al.*, 2006). Another
 65 example shows that transcriptome, genetic diversity, and metabolite profiling unveil
 66 specific MP and MRN for carotenoid accumulation, and in particularly *SIERF6* as a
 67 key regulator that controls this process in tomato fruit (Lee *et al.*, 2012). Recently, the
 68 change of flavor and other metabolites and relevant regulatory mechanisms during
 69 tomato domestication have also been revealed by interrogation of the combined genome,
 70 metabolome, and transcriptome data (Tieman *et al.*, 2017; Gao *et al.*, 2019; Zhu *et al.*,
 71 2019). More recently, a MicroTom tomato metabolic regulatory network (MMN)

throughout the fruit growth cycle was generated by integrative analysis of high-resolution spatio-temporal metabolome and transcriptome (Li *et al.*, 2020b). From the MMN, two novel transcription factors (TFs) were identified to regulate the steroidal glycoalkaloid and flavonoid metabolism (Li *et al.*, 2020b). These studies demonstrate that integrating multi-omics analyses can be a highly efficient means to dissect MPs and MMN, as well as to identify key TFs that regulate metabolisms responsible for fruit flavor and nutrition in tomato. However, MPs and underlying MRN-mediated mechanisms in many other fruits including kiwifruit remain largely unclear.

Kiwifruit (*Actinidia chinensis*) is known as ‘the king of fruits’, rich in metabolites including flavor-associated compounds, vitamins, flavonoids and various bioactive chemicals (Bulley *et al.*, 2009; Zuo *et al.*, 2012; Park *et al.*, 2013; Hu *et al.*, 2016; Richardson *et al.*, 2018; Ampomah-Dwamena *et al.*, 2019). During the last decades, given the importance of metabolites to the quality of kiwifruit, MPs or MRNs for flavor- and nutrition-related compounds have been widely investigated (Zhang *et al.*, 2020; Wang *et al.*, 2022a). For example, anthocyanin as an important fruit quality indicator of the red-fleshed kiwifruit cultivar, its biosynthesis and regulators have been extensively studied. TFs such as AcMYB10, AcMYB110, AcMYB123, AcbHLH42, and AcMYB27 have been shown to control anthocyanin metabolism (Fraser *et al.*, 2013; Li *et al.*, 2017; Wang *et al.*, 2019b; Liu *et al.*, 2021; Nardozza *et al.*, 2020). Moreover, numerous studies reported that Vitamin C (L-ascorbic acid, AsA) contents vary in different kiwifruit cultivars, and several structural and TF genes such as AcERF92, AceMYBS1-like and AceGBF3 are involved in AsA metabolism (Nishiyama *et al.*, 2004; Tavarini *et al.*, 2008; Bulley *et al.*, 2009; Chen *et al.*, 2021; Liu *et al.*, 2022). However, these studies were mostly performed on fruits at a particular developmental and ripening stage using either a metabolomic or transcriptomic approach, and cannot reveal links between the landscape of metabolic changes and the underlying MRNs in kiwifruit throughout the fruit growth cycle.

In this study, we utilized a multi-omics means and generated metabolomes and transcriptomes for kiwifruit pericarp tissues at 11 fruit developmental and ripening

101 stages (FDRSs). Through integrative bioinformatics analyses and functional genomics
102 assays, we established a comprehensive kiwifruit metabolic regulatory network
103 (KMRN) and revealed novel “MM vs GM” for transcriptomic and transcriptional
104 regulation of biosynthesis of major metabolites. The potential application of such
105 KMRN to manipulate and improve kiwifruit quality is also discussed.

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Materials and Methods

Plant materials

Kiwifruits (*Actinidia chinensis* cv. Hongyang) were grown in a research orchard in Shifang, Sichuan Province, China. Fruits at different developmental and ripening stages were collected at 10, 25, 40, 55, 70, 85, 100, 115, 130, 145, and 170 days post anthesis (DPA). Pericarps (without skin and seeds) sampled from ten fruits of uniform sizes were pooled into one biological replicate. Fruit tissues were then cut into small pieces, rapidly frozen in liquid nitrogen and stored in -80°C freezers. Three biological replicates were used for subsequent metabolome and transcriptome profiling.

Metabolome and transcriptome profiling

Metabolome profiles were generated by using a widely targeted metabolomics method (Zhu *et al.*, 2018; Li *et al.*, 2020; Zhang *et al.*, 2022). In brief, kiwifruit pericarp tissues were lyophilized using a vacuum freeze-dryer (Scientz-100F) and ground into fine powers using a mixer mill (MM 400, Retsch) with a zirconia bead at 30 Hz for 1.5 min. 100 mg tissue powders were extracted with 1.0 mL 70% aqueous methanol at 4°C overnight and followed by centrifugation at 10,000 g for 10 min. Supernatants were then collected, filtered through a membrane (SCAA-104, 0.22 mm pore size; ANPEL, Shanghai, China, <https://www.anpel.com.cn>), and analyzed by LC-MS/MS. Quantification of metabolites was carried out using scheduled multiple reaction monitoring methods (Dong *et al.*, 2013; Zhu *et al.*, 2018; Li *et al.*, 2020; Yuan *et al.*, 2022).

Transcriptome profiling was performed as described previously (Pertea *et al.*, 2016). Briefly, raw reads from NovaSeq 6000 platform were subjected to adaptor trimming, low quality and short reads removal using Fastp 0.22.0 (Chen *et al.*, 2018). HISAT2 v2.2.1 (Kim *et al.*, 2019) was used to align clean reads against the kiwifruit reference genome v3 (https://www.ncbi.nlm.nih.gov/assembly/GCA_009663005.1) with default parameters (Wu *et al.*, 2019). Alignment files were sorted by Samtools 1.13 (Li *et al.*, 2009). For each alignment file, we re-assembled a transcriptome using StringTie 2.1.6

(Pertea *et al.*, 2015) based on the latest reference annotation from the kiwifruit reference genome v3. To qualify as a valid reconstructed transcript, we applied a threshold of 10 and 5 reads as minimum base-pair coverage (-c) and junction coverage (-j), respectively. The resulting assemblies were merged using StringTie's merge function to create the merged assembly which was subsequently fed into TransDecoder 5.5.0 (Haas *et al.*, 2013) to generate the final reference assembly with novel protein-coding genes. With the guidance of the final reference assembly, we reused StringTie to quantify the transcript level of each protein-coding gene.

PCA analysis and clustering of metabolomes and transcriptomes

PCA analysis and Hierarchical clustering were performed using procomp function and ComplexHeatmap (Gu *et al.*, 2016) in R software, respectively to compare the protein-coding gene profiles and metabolite profiles among different developmental and ripening stages of kiwifruit. The transformed and normalized gene and metabolite accumulation values with Z-scores were used for PCA analysis and hierarchical clustering.

Metabolite and gene modules (MM and GM) clustering and analysis

The metabolite profile containing the CAS number and the co-expressed gene profile after $\log_2$ transformation was fed to the Mfuzz (Kumar & Futschik, 2007) separately to obtain MMs and GMs. The heatmaps and line graphs of MMs and GMs were visualized through ComplexHeatmap (Gu *et al.*, 2016) and ggplot2 (Wickham, 2011) respectively. The KEGG analysis of the GMs was carried out using clusterProfiler (Wu *et al.*, 2021), and p-values ≤ 0.05 was considered significant enrichment. The SCMG was calculated as follows:

$$\mathbf{M} = \{x_1, x_2, x_3, \dots, x_m\}, \mathbf{G} = \{y_1, y_2, y_3, \dots, y_g\}$$

$$SCMG(\mathbf{M}, \mathbf{G}) = \frac{\sum_{i=1}^m \sum_{j=1}^g cor(x_i, y_j)}{m * g}$$

$\mathbf{M}$ represents the accumulation value matrix of m metabolites in a MM, and $\mathbf{G}$ represents the expression value matrix of g genes in a GM. $cor(x_i, y_j)$ represents the

correlation coefficient between the accumulation values of a metabolite and the expression values of a gene calculated using the Pearson correlation coefficient algorithm.

Construction of kiwifruit metabolic regulatory network (KMRN)

Metabolic regulatory networks (MRNs) were performed as described previously (Wang *et al.*, 2022a). In brief, the position frequency matrices (PFMs) of transcription factors were downloaded from PlantRegMap (Tian *et al.*, 2019), which were used to predict cis-acting element information in the promoter region (1500 bp before ATG) of structural genes under the condition of p-values $\leq 1e^{-5}$ by FIMO (Grant *et al.*, 2011). KMRNs were generated by combining the PCC ($PCC \geq 0.6$) between TFs and structural genes, the availability of cis-element binding sites in the promoter regions of structural genes and the PCC ($PCC \geq 0.6$) between TFs and metabolites. KMRNs were visualized using Cytoscape 3.7.1 (Kohl *et al.*, 2011).

Phylogenetic analysis

In MEGA X software (Kumar *et al.*, 2018), the clustalw algorithm was used to align protein sequences to generate alignment results, which were used to construct the molecular phylogenetic tree applying the maximum likelihood model with bootstrap value 1000.

Transient dual luciferase reporter assays in tobacco protoplasts

Full-length coding sequences of TF genes of interest were cloned into pGreen II 62-SK vector to generate the effector gene expression constructs as earlier described (Wang *et al.*, 2022a), while the promoter sequences of target genes were cloned into the pGreen II 0800-LUC double-reporter vector. Both reporter and effector plasmids were then transferred into tobacco leaf protoplasts by polyethylene glycol 6000-mediated transfection. The empty pGreen II 62-SK vector was used as the negative control (CK). Results are presented as the ratio of LUC to REN activity from three biological replicates. Primers used for vector construction are listed in Table S2.

189 **Transient gene overexpression and virus-induced gene silencing in kiwifruit and** 190 **tobacco leaves.**

191 To generate gene overexpression constructs *pBI121-AcMYB123-2*, *pBI121-AcERF192*,
 192 *pBI121-AcAO1*, and *pBI121-AcAPX2*, the coding sequence of each TF gene was
 193 amplified with primers (Table S2) and recombined into the binary pBI121 vector,
 194 respectively. To generate virus-induced gene silencing (VIGS) constructs *pTRV2-*
 195 *AcMYB123-2*, *pTRV2-AcERF192* and *pTRV2-AcAO1*, the partial coding sequence of
 196 each TF gene was amplified with specific primers (Table S2) and cloned into the
 197 Tobacco rattle virus-based pTRV2/pTRV1 vector system. Each of these constructs was
 198 transformed into *Agrobacterium tumefaciens* GV3101, separately. TF gene sequences
 199 in all constructs were confirmed by Sanger sequencing.

200 To transiently overexpress or silence TF genes in kiwifruits, 30-fruits attached on vines
 201 at 120 DPA were injected at a depth of approx. 0.5 cm inside pericarp tissues with 0.1
 202 ml agrobacterium (1.0 OD₆₀₀) harboring each of the individual overexpression or VIGS
 203 constructs. Empty pBI121 or pTRV2/pTRV1 vector was used as negative controls. At
 204 7 days post-agroinfiltration (i.e., 127 DPA), these agroinfiltrated fruits were harvested
 205 and their outer pericarps were collected, rapidly frozen in liquid nitrogen, and stored at
 206 -80°C for further molecular analyses.

207 For transient structure gene overexpression in tobacco, two young leaves of *Nicotiana*
 208 *benthamiana* plants at the six-leaf stage were infiltrated with 0.1 ml agrobacterium (1.0
 209 OD₆₀₀) harboring each of the individual overexpression. An empty pBI121 vector was
 210 used as the negative control. Subsequently, tobacco plants were maintained at 20 °C in
 211 dark for 48 hours, and at 25 °C under light for 24 hours. Agroinfiltrated leaves were
 212 then harvested and stored at -80 °C until use.

213 **Electrophoretic mobility shift assay (EMSA)**

214 EMSA was performed as previously described in our laboratory (Wang *et al.*, 2022a).
 215 Full-length coding sequences of AcMYB4 and AcERF192 were cloned into pGEX-4T-
 216 1 vector and both TFs were expressed in BM Rosetta (DE3) Competent Cells after

induction with 0.5 mM of IPTG (isopropyl- β -D-1-thiogalactopyranoside) for 5 hours at 30 °C. EMSA was performed using an Electrophoretic Mobility Shift Assay kit (Thermo Fisher, 20148) according to the manufacturer's instructions. Specific probes for promoters containing the putative cis-elements, i.e., the binding sites of corresponding target TFs, were labeled with biotin using a DNA 3' End Biotinylation Kit (Thermo Fisher, 89818) and annealed to form double-stranded oligonucleotides. Gel shifting was detected using a Lightshift Chemiluminescent EMSA kit (Thermo Fisher, 20158). Cold probes and mutated probes (200-fold concentration of unlabeled oligonucleotides) were utilized in the competition experiments.

RNA extraction and RT-qPCR analysis

Total RNA from kiwifruit tissues was isolated using a Plant RNA Purification Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Treatment to prevent genomic DNA contamination and first-strand complementary DNA generation was carried out following the previously described protocols (Deng *et al.*, 2018). Quantitative reverse-transcription PCR (qRT-PCR) was performed using 2 $\times$ SYBR Green qPCR Mix (Vazyme, Q431-02) on a Bio-Rad CFX384 Real-Time System, following the manufacturer's instructions. With *AcActin1* as an internal control (Wang *et al.*, 2019b), the relative transcript level of each gene was calculated using the Δ Ct method. Primers used in qRT-PCRs are listed in Table S2.

Determination of proanthocyanidins and AsA

Kiwifruit pericarp tissues from three independent biological/pooled samples were lyophilized and ground into fine powders. 0.1 g freeze-dried powders were extracted with 1mL 80% methanol/water (v/v) containing 0.1% formic acid. The mixture was vortexed for 30 sec, sonicated at 4°C for 20 min, vortexed again for 30 sec, centrifuged (14,000g, 10 min, 4°C), and filtered through a 0.2-mm polytetrafluoroethylene membrane filter. Procyanidin B and Procyanidin C were measured by MS using the SCIEX Triple Quad 5500 LC-MS/MS System with a UPLC column connected online to a photodiode array detector (Shimadzu).

Reduced AsA and total AsA contents were measured as previously described (Liu *et al.*, 2016). In brief, 0.5g freeze-dried sample powders were extracted with 10 mL 1% oxalic acid at 4 °C for 12 hours and centrifuged (14,000g, 10 min, 4 °C). To determine the total AsA level, 1 mL of the supernatant was transferred to a 2-ml Eppendorf tube containing 10 uL of 5 mmol dithiothreitol (DTT) and incubated at 4 °C for 1 hour to convert the oxidized ascorbate into the reduced form. Supernatants were collected and filtered before the LC-MS analysis was performed using the SCIEX Triple Quad 5500 LC-MS/MS System. To assay reduced AsA, DTT was replaced by the same volume of potassium phosphate buffer (pH 7.4), while the rest of the procedure was the same as for the total AsA assay. All samples were analyzed in three independent biological replicates.

Statistics

For comparison of individual treatments with relevant controls, paired two-tailed Student's *t*-tests in R software were performed, and a p-value ≤ 0.05 was considered statistically significant.

Results

Construction of kiwifruit metabolic regulatory network (KMRN)

To reveal metabolic dynamics and relevant underlying mechanisms in kiwifruit (*A. chinensis* cv. Hongyang), we collected kiwifruit pericarp tissues at 11 FDRSs, ranging from 10 days post anthesis (DPA) to 170 DPA (Fig. 1a). These samples can be also divided into three groups I, II and III based on phenotypic changes in the color of kiwifruit pericarps during FDRSs (Fig. 1a). Group I fruit at 10 DPA had purple pericarps, whilst pericarps in early Group II fruits at 25, 40, 55, 70 and 85 DPA developed dark-green color, and changed to light-green in later Group II fruits at 100, 115 and 130 DPA. However, the pericarp color of fully ripe Group III fruits at 145 and 170 DPA became yellow (Fig. 1a).

We then carried out LC-tandem MS (LC-MS/MS)-based metabolic profiling on these pericarp tissues (Li *et al.*, 2020b). In total, 693 metabolites were identified and annotated in kiwifruits at one or more FDRSs (Dataset S1). We also generated transcriptomes for all the same fruit samples. After removing the adapter, ambiguous and low-quality reads, approx. 431-Gb clean data were obtained (Dataset S2). Uniquely mapped mRNA reads were used to calculate gene expression levels in transcripts per million (TPM) (Li *et al.*, 2010; Wagner *et al.*, 2012). In total, 47,433 protein-coding genes (maximum TPM>0) were identified to be expressed at least once at one or more stages of the 11 different FDRSs (Dataset S3).

Furthermore, hierarchical cluster analysis (HCA) demonstrates that metabolomes or transcriptomes at each FDRS were highly correlated among the three biological replicates, and that the 11 FDRS samples formed three distinct clusters (Fig. 1b, c). Such clusters were further evidenced by the principal component analysis (PCA, Fig. 1d, e). Intriguingly, the metabolic and/or transcriptomic clustering was consistent with the pericarp color-based phenotypic grouping of kiwifruits throughout the 11 FDRSs (Fig. 1a). These results imply that the accumulation of metabolites is parallel to and

288 associated with global gene expression in response to the progress of kiwifruit
289 development and ripening.

290 **Establishment of ‘metabolite verse gene’ modules during kiwifruit development** 291 **and ripening**

292 To gain insight into metabolic profiles during kiwifruit development and ripening, we
293 explored our KMRN and identified 515 metabolites with Chemical Abstracts Service
294 Registry Number (CAS Number). These metabolites include 36 alkaloids, 74 amino
295 acids and derivatives, 60 flavonoids, 64 lipids, 17 lignans and coumarins, 38
296 nucleotides and derivatives, 57 organic acids, 71 phenolic acids, 13 tannins, 48
297 saccharides and alcohols, 14 terpenoids, 12 vitamins and 11 other unclassified
298 compounds (Table S1). Ten metabolic modules (MMs) were generated based on the
299 accumulation patterns of metabolites using the fuzzy c-means clustering algorithm (Fig.
300 2a; Dataset S4). Metabolic module I (MM-I) contains metabolites that preferentially
301 accumulated at 10 DPA, while metabolites in MM-VI were mainly produced at 25 DPA
302 (Fig. 2a; Fig. S1). MM-II represents metabolites that gradually decreased throughout
303 the entire FDFS. In contrast, metabolites in MM-IX progressively increased from 10 to
304 170 DPA (Fig 2a; Fig. S1). We also noticed various distributions of metabolites in
305 different MMs. For example, amino acids and derivatives were highly clustered in MM-
306 I, MM-VI, and MM-VIII, flavonoids in MM-I, whereas saccharides and alcohols in
307 MM-IX (Fig 2c; Fig. S1).

308 To reveal the genetic basis of metabolite accumulation at different FDFSs, 33371 genes
309 (maximum TPM ≥ 1) from our KMRN (Dataset S5) were used to perform the co-
310 expression analyses with metabolites (Serin *et al.*, 2016; Zhu *et al.*, 2018; Li *et al.*,
311 2020b). We calculated the Pearson Correlation Coefficient (PCC) between $\log_2$
312 transformed gene expression values and metabolite accumulation values. In total,
313 24325 genes were identified that were significantly correlated with at least one
314 metabolite (PCC ≥ 0.8). Like clustering metabolites, the 24325 genes were also grouped
315 into ten gene modules (GMs) based on their expression patterns (Dataset S6).

Interestingly, both MMs and GMs showed a high consistency (Fig. 2a, b; Fig. S1). This finding is further supported by the substantial similarity coefficients between the metabolite and gene modules (SCMG) (Dataset S7).

We then performed Kyoto Encyclopedia Genes and Genomes (KEGG) analysis (Kanehisa *et al.*, 2016) on the ten GMs, and found that MPs enriched in GMs are directly correlated with the biosynthesis of the metabolites in the corresponding MMs (Dataset S8). For instance, the enriched MPs in GM-I include ribosomes, DNA replication, pyrimidine metabolism and flavonoid biosynthesis (Fig. 2d; Dataset S8), which are responsible for the production of nucleotides and derivatives, flavonoids and tannins, all of which were the consistently clustered into MM-I (Fig. 2c; Table S1). The enriched MPs for linoleic acid and alpha-linoleic acid metabolism in GM-V (Fig. 2e; Dataset S8) are involved in the accumulation of alpha-linolenic acid and gamma-linolenic acid in MM-V (Dataset S4). Moreover, saccharides and alcohols are mainly clustered into MM-IX (Fig. 2c; Table S1), and relevant MPs such as glycolysis/gluconeogenesis, citrate cycle, pentose phosphate pathway, carbon/pyruvate/starch/sucrose/galactose metabolism are enriched in GM-IX (Fig. 2f; Dataset S8). In summary, our data indicate that the dynamics of major metabolisms are largely paralleled with the expression of genes that control each of the respective metabolic pathways during kiwifruit development and ripening.

Key regulatory factors in soluble sugar accumulation

Sweetness is one of the most important quality traits of kiwifruit and is favored by consumers (Harker *et al.*, 2009). Soluble sugars including D-sucrose, D-fructose, D-glucose, myo-inositol and other saccharides are the main determinant of kiwifruit sweetness (Nardozza *et al.*, 2013). Accumulation of soluble sugars mainly involves the starch/sugar metabolism pathway in kiwifruit (Nardozza *et al.*, 2013; Wang *et al.*, 2022a). Targeting this pathway and mining the KMRN, we detected 5 metabolites i.e., D-sucrose, D-glucose, D-glucose-6P, D-fructose, and D-fructose-6P (Fig. 3a). Among them, the three main soluble sugars D-sucrose, D-fructose and D-glucose were all

344 clustered in MM-IX, whilst D-fructose-6P and D-sucrose-6P were clustered into MM-
 345 I and MM-X, respectively. Expression of genes encoding multiple α -amylase/ β -
 346 amylase (AMY/BAM) (6/15), and sucrose phosphate synthase (SPS) (3/4) reached high
 347 levels at 145 DPA and 170 DPA, and these genes were clustered into GM-IX. In
 348 contrast, only low expression of hexokinase (HK) (6/7) and fructokinase (FK) (3/4)
 349 genes occurred in kiwifruit at 145 DPA and 170 DPA (Fig. **3b**; Dataset **S9**). Moreover,
 350 two *BAMs* *AcBAM3.1* and *AcBAM3.5* shown in MM-IX have been demonstrated to
 351 encode key enzymes that are involved in soluble sugar accumulation in kiwifruit
 352 (Zhang *et al.*, 2018; Sun *et al.*, 2021). These results suggest that the accumulation of
 353 soluble sugars at the later FDRSs in kiwifruit is mainly due to starch degradation,
 354 consistent with previous studies (Wegrzyn & MacRae, 1995; Nardoza *et al.*, 2013).

355 To further identify key TFs associated with soluble sugar accumulation, we combined
 356 cis-acting elements with co-expression modules (Fig. **3c**) as previously described
 357 (Wang *et al.*, 2022a). Using this approach, we constructed a soluble sugar MRN by
 358 establishing a close correlation between metabolites in MM-IX and genes in GM-IX
 359 (Fig. **S2**; Dataset **S9**). Analyzing the TOP 10 TF families in our KMRN, we found that
 360 NAC, C2H2, AP2/ERF, MYB and bHLH are the most likely TF families (52.54%) that
 361 may regulate the accumulation of soluble sugars in kiwifruits (Fig. **S2**). Considering
 362 accumulation of soluble sugars is one of the most important characteristics of fruit
 363 ripening (Burdon, 2015), we focused on the ERF-type TFs since they are known to be
 364 involved in ethylene-mediated ripening processes. Interestingly, *AcERF182*, which has
 365 recently been shown to regulate soluble sugar accumulation by activating the
 366 expression of *AcBAM3.5* (Wang *et al.*, 2022a), was found in our soluble sugar MRN
 367 (Fig. **3d**). These results demonstrate that the KMRN described in this study can be used
 368 to validate previously known metabolic regulatory pathways.

369 **Key regulatory factors in anthocyanidin accumulation.**

370 The most important agricultural trait for red-flesh kiwifruit is the high content of
 371 anthocyanins and proanthocyanidins (PAs) (Dawes & Keene, 1999; Montefiori *et al.*,

2005; Drummond, 2013; Hellstrom *et al.*, 2009). Using KMRN, we identified 13 metabolites including naringenin, dihydrokaempferol, dihydroquercetin, catechin, epicatechin, kuromanin (cyanidin-3-glucoside) and 6 procyanidins that are associated with anthocyanidin/procyanidin biosynthesis (Fig. 4a) (Dixon *et al.*, 2005). These metabolites were mostly clustered into MM-I/II except kuromanin (anthocyanin) was clustered into MM-IX (Fig. 4b). Consistent with metabolite accumulation patterns, most anthocyanin / procyanidin biosynthesis genes such as *chalcone synthase (CHS)*, *flavanone 3-hydroxylase (F3H)*, *Flavonoid 3'-hydroxylase (F3'H)*, *dihydroflavonol reductase (DFR)*, *anthocyanidin reductase (ANR)* and *leucoanthocyanidin reductase (LAR)* (Borevitz *et al.*, 2000; Dixon *et al.*, 2005; Jeong *et al.*, 2006) were clustered into GM-I/II (21/23) (Fig. 4b). These results suggest that during the development of kiwifruit, PAs are formed in the early stage, while anthocyanins are formed in the middle and later stages, which is in line with previous studies (Fig. S3) (Wang *et al.*, 2019b).

Previous studies have shown that AcMYB123 and AcMYB10/AcMYB75/AcMYB110 are able to regulate anthocyanin biosynthesis genes *AcF3'H1*, *AcANS*, *AcF3GT1* and *AcGST1* to modulate anthocyanin formation in red-fleshed kiwifruit (Liu *et al.*, 2010; Li *et al.*, 2017; Liu *et al.*, 2019; Wang *et al.*, 2019b; Wang *et al.*, 2022b). Our transcriptomic data for anthocyanin biosynthesis genes and related transcription factors (excluding 10 DPA) further showed that *AcF3'H1*, *AcF3GT1*, *AcMYB10/AcMYB75/AcMYB110*, *AcMYB123* clustered on the same clade, especially *AcF3GT1* (PCC = 0.82) and *F3'H1* (PCC = 0.67) was strongly co-expressed with *AcMYB10/AcMYB75/AcMYB110* (Fig. S3). In summary, we can use the KMRN to accurately verify previously known metabolic regulatory networks throughout the kiwifruit growth cycle.

Novel TFs in regulation of proanthocyanidin metabolism

Unlike studies in anthocyanin metabolism, MM and GM related to PA metabolism in kiwifruit remain largely unknown. To address this question, we constructed a

400 procyanidin MRN by establishing a tight correlation between the metabolites in MM-
 401 I/II and the genes in GM-I/II (Fig. **S4**; Dataset **S10**) by using the same strategy as afore-
 402 descried for soluble sugars (Fig. **3c**). Detailed analyses of this network further unveiled
 403 that bHLH, MYB, MYB-related and AP2/ERF in the top 4 putative TF families may
 404 participate in the regulation of the PA metabolism in kiwifruit (Fig. **S4**).

405 TFs in the bHLH, MYB and MYB-related families are known to regulate PA
 406 metabolism in *Arabidopsis*, rice and other plant species (Baudry *et al.*, 2004; Furukawa
 407 *et al.*, 2007; Dubos *et al.*, 2008). MYB, bHLH and WD proteins can form the MBW
 408 complex to modulate the expression of PA metabolic genes, and MYB is particularly
 409 responsible for binding to target gene promoters to activate or repress their expression
 410 (Baudry *et al.*, 2004). Based on these reports, the MYB-associated subnetwork was
 411 chosen and screened within the procyanidin MRN (Fig. **4c**). Phylogenetic analysis
 412 revealed candidate genes *AcMYB90*, *AcMYB111* and *AcMYB123-2* in the MYB family
 413 (Fig. **4d**), homologous to *AtTT2* and *VvMYBPA2* which are involved in the regulation
 414 of PA accumulation in *Arabidopsis* and grape, respectively (Baudry *et al.*, 2004; Terrier
 415 *et al.*, 2009). These genes are predicted to have similar functionality in kiwifruit.

416 We then carried out functional genomics analyses to testify if our prediction would be
 417 valid. In plants, prior to the last step in procyanidin biosynthesis, catechin and
 418 epicatechin are converted from leucocyanidin and cyanidin, respectively. Conversion
 419 of leucocyanidin→catechin and cyanidin→epicatechin is catalyzed by either LAR or
 420 ANR (Fig. **4a**). Therefore, to verify the role of *AcMYB90*, *AcMYB111* and
 421 *AcMYB123-2* in the regulation of procyanidin metabolism, we performed dual
 422 luciferase reporter assays in tobacco protoplasts. Promoters of six *LAR* and *ANR* genes
 423 were isolated from the kiwifruit genome and cloned into the LUC-reporter gene
 424 expression vectors *proAcANR1::LUC*, *proAcANR2::LUC*, *proAcANR3::LUC*,
 425 *proAcLAR2::LUC*, *proAcLAR3::LUC* and *proAcLAR4::LUC*. We also generated
 426 *35S::AcMYB90*, *35S::AcMYB111* and *35S::AcMYB123-2* to express the 3 TFs as
 427 effectors. After co-transfection of protoplasts, we found that *AcMYB111* was unable
 428 to activate promoters of any *ANR* or *LAR* genes, whereas *AcMYB90* and *AcMYB123-2*

enhanced the activities of *AcLAR3* or *AcLAR2* promoter, respectively (Fig. 4e; Fig. S5). Since the impact of *AcMYB123-2* on the *AcLAR2* promoter was stronger than that of *AcMYB90* on the *AcLAR3* promoter, we then focused on *AcMYB123-2* for further functional studies. We performed EMSA and uncovered that *AcMYB123-2* could directly interact with the *AcLAR2* promoter dependent on the Myb-p binding motif “CCWACC” (Fig. 4f). Moreover, we analyzed how overexpression or silencing of *AcMYB123-2* would affect procyanidin accumulation in kiwifruit. For this purpose, we produced *Agrobacterium tumefaciens* GV3101 harboring 35S::*AcMYB123-2* for *AcMYB123-2* overexpression or a virus-induced gene silencing (VIGS) vector *pTRV2::AcMYB123-2* for *AcMYB123-2* knockdown. Fruits of ‘Hongyang’ at 120 DPA were then agroinjected and collected at 7 days post-agroinjection. RT-qPCR analysis showed that the transcript level of *AcLAR2* was significantly up-regulated in kiwifruits in which *AcMYB123-2* was transiently overexpressed. In contrast, *AcLAR2* was down-regulated in *AcMYB123-2* VIGS kiwifruits. Consequently, procyanidin B/C accumulated much higher in *AcMYB123-2*-overexpressing but reduced in *AcMYB123-2*-silenced kiwifruits when compared to controls (Fig. 4g). Thus, our *in vitro* and *in vivo* functional assays demonstrate that *AcMYB123-2* is involved in the control of procyanidin metabolism through direct transcriptional regulation of the expression of key structure gene *AcLAR2* in kiwifruit.

In addition to MYB, AP2/ERF is another TF in the top 4 putative TF families that are predicted to regulate PA metabolism from our KMRN analysis (Fig. S4). To validate whether AP2/ERF TFs can indeed regulate PA metabolism in kiwifruit, we extracted the AP2/ERF-associated subnetwork (Fig. 5a) from the procyanidin MRN (Fig. S4). Among AP2/ERF members, *AcERF192* was selected for investigation based on (1) the close correlation between *AcERF192* expression with the accumulation pattern of procyanidin B2 (Dataset S10), and (2) its homology with *AtRAP2.5* and *AtERF8* (Fig. 5b) which are involved in the regulation of anthocyanins under the light in Arabidopsis (Koyama & Sato, 2018). Like *AcMYB123-2*, *AcERF192* activated the *AcANRI* promoter (Fig. 5c), and bound directly to the GCC-box in the *AcANRI* promoter (Fig.

5d). Moreover, overexpression and VIGS experiments demonstrate that AcERF192 regulated *AcANRI* transcription that subsequently influenced the biosynthesis of procyanidin B and procyanidin C in kiwifruit (Fig. 5e).

Taken together, we uncovered procyanidin MM vs GM and MRNs, and the roles of two key TFs AcMYB123-2 and AcERF192 in transcriptional regulation of PA metabolism in kiwifruit.

Involvement of recycling pathway in L-Ascorbate metabolism in kiwifruit

Using the same bioinformatics and functional genomics strategies described above, we attempted to reveal the metabolic regulatory mechanism for AsA, an essential nutrition quality characteristic of kiwifruit. To achieve this, we analyzed two most important AsA biosynthetic pathways, the D-mannose/L-Galactose (Wheeler *et al.*, 1998) and the galacturonate pathway in higher plants (Agius *et al.*, 2003). Both pathways involve a final step catalyzed by L-galactonolactone dehydrogenase (GLDH) to produce AsA (Fig. 6a). In our KMRN, we detected four metabolites D-fructose-6P, D-glucose-6P, D-galacturonate, and L-ascorbate in the AsA biosynthetic pathway (Fig. 6a). However, we noticed that accumulation patterns of L-ascorbate and other three metabolites differed at different developmental and ripening stages in kiwifruit (Fig. 6b; Dataset S11).

L-ascorbate was clustered into MM-VI. It accumulated to the maximum level at 25 DPA, and then gradually declined during subsequent kiwifruit development and ripening (Fig. 6b; Dataset S11). To our surprise, the accumulation of L-ascorbate and expression of AsA biosynthetic genes were found to have contrasting patterns at 10 DPA. Indeed, most of AsA biosynthetic genes including two key structural genes *AcGME2* and *AcGGP3* were clustered into GM-II rather than GM-VI (Fig. 6b). Such contradictory MM vs GM profiles prompted us to analyze the AsA recycling pathway (Fig. 6a). This pathway contains L-ascorbate peroxidase (APX) and L-ascorbate oxidase (AO) that oxidize L-ascorbate into L-mono/dehydroascorbate (Wilkinson *et al.*, 2002; Pignocchi *et al.*, 2003). Interestingly, L-dehydroascorbate displayed the highest

accumulation, consistent with the peak expression of *AcAOI* and *AcAPX2* at 10 DPA (Fig. 6b). In particular, the transcript level of *AcAOI* was about 10-fold higher at 10- than 25-DPA (Fig. S6). These analyses suggest that the degradation of L-ascorbate mediated by key structural genes in the recycling pathway might account for its low accumulation at 10 DPA.

To validate this hypothesis, we agroinfiltrated *35S::AcAOI* or *35S::AcAPX2* into tobacco leaves. LC-MS analysis showed that *AcAOI* or *AcAPX2* overexpression significantly lowered contents of reduced L-Ascorbate and total AsAs (L-ascorbate and L-dehydroascorbate) in agroinfiltrated leaf tissues compared with controls at 3 days post-agroinjection (Fig. S6). Furthermore, overexpression or *pTRV2::AcAOI*-mediated VIGS of *AcAOI* in kiwifruits at 120 DPA caused a reduction or increase in contents of reduced AsA and total AsA, respectively (Fig. 6c). Taken together, these results indicate that *in planta* accumulation of L-Ascorbate is mediated by the AsA recycling pathway.

A novel TF regulates AsA recycling pathway

To further understand how AsA recycling pathway is modulated at the transcriptional level, we screened putative TFs in our KMRN and generated a regulatory network targeting *AcAOI* and *AcAPX2* in GM-II (Fig. 6d; Dataset S11). Interestingly, AcMYB123-2 and AcERF192 presented in the procyanidin MRN (Fig. 4c; Fig. 5a) were also found in the *AcAOI* and *AcAPX2* regulatory network (Fig. 6d; Dataset S11). To examine whether the two TFs are involved in AsA metabolism through directly regulating *AcAOI* transcription in kiwifruit, we tested how AcMYB123-2 and AcERF192 affect the *AcAOI* promoter activity in the dual luciferase reporter assays. We discovered that only AcMYB123-2 could activate *AcAOI* promoter (Fig. 6e). Using the same experimental setting-ups for the study of PA metabolic regulation (Fig. 4g), we found that contents of reduced AsA and total AsA were significantly decreased in the *AcMYB123-2*-overexpressing but increased in *AcMYB123-2*-silenced kiwifruits when compared to controls. Besides, the transcript level of *AcAOI* was also higher in

514 *AcMYB123*-2-overexpressing but lower in *AcMYB123*-2-silenced kiwifruits (Fig. **6f**).
515 Moreover, EMSA further indicated that AcMYB123-2 bound the Myb-p binding site
516 (CCWACC) in the *AcAOI* promoter (Fig. **6g**). Taken together, these results
517 demonstrate that TF AcMYB123-2 regulates AsA recycling pathway by directly
518 binding to the promoter region of *AcAOI* to affect its transcription in kiwifruit.
519

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520 Discussion

521 Dissecting metabolic pathways of key compounds that contribute to fruit flavor and
 522 nutrition and revealing their underlying transcriptomic and transcriptional regulatory
 523 networks are two important steps toward fruit quality improvement through molecular
 524 breeding (Klee & Tieman, 2018). To date, although many compounds related to fruit
 525 quality have been identified via advanced metabolite detection technologies, the
 526 metabolic pathways of these compounds and their underlying regulatory network
 527 remain largely unclear. Here, through integrative analyses of metabolic and
 528 transcriptomic data across 11 kiwifruit developmental and ripening stages, we revealed
 529 the dynamic landscapes of the metabolic vs gene modular changes and constructed a
 530 high-resolution kiwifruit metabolic regulatory network (KMRN). Using this KMRN
 531 coupled with *in vitro*, *in vivo* and *in planta* gene functional assays, we not only validated
 532 MMs vs GMs in known metabolic pathways but also identified novel regulators and
 533 illustrated the underlying regulatory mechanisms.

534 Metabolomic and transcriptomic conjoint analysis has become a powerful means to
 535 generate MRNs in different plants (Li *et al.*, 2020b; Wang *et al.*, 2022a; Yang *et al.*,
 536 2022). Using this strategy, we previously established the primary KMRN and revealed
 537 regulatory mechanisms involving key TFs to modulate the metabolism of flavor
 538 (soluble sugars, organic acids, and important volatiles) during the fruit developmental
 539 and ripening process in kiwifruit (Wang *et al.*, 2022a). In the current study, we further
 540 dissected the secondary KMRN and identified novel TFs that regulate the accumulation
 541 of important metabolites including PAs and vitamin C. Through the two studies, we
 542 constructed both primary (flavor) and secondary (nutrition) MRNs in kiwifruit and
 543 provided rich resources for designed molecular breeding to enhance and improve flavor
 544 and nutrition in kiwifruit (Fig. 7).

545 Both anthocyanins and PAs are products of the flavonoid metabolic pathway. For
 546 anthocyanins, previous studies have shown that anthocyanin (kuromanin) starts to
 547 accumulate at 85 DPA and gradually increases at later developmental stages of kiwifruit

(Wang *et al.*, 2019b), which is consistent with most of our metabolic data (except for 10 DPA) (Fig. 7; Fig. S3). Meanwhile, multiple genes including *AdMYB10/75/110*, *AcMYB123*, and *AcF3'H1* and *F3GT1* (Li *et al.*, 2017; Liu *et al.*, 2018; Wang *et al.*, 2019b; Wang *et al.*, 2022b) that regulate anthocyanin accumulation in kiwifruit have been identified (Fig. 7). However, how PAs change in the development of kiwifruit and the regulatory mechanisms behind them are rarely studied. In *Arabidopsis*, PAs accumulate in the seed coat (Debeaujon *et al.*, 2003; Xie *et al.*, 2003; Routaboul *et al.*, 2006). This differs from PA biosynthesis in fleshy fruit crops such as persimmon, strawberry and grape. PA accumulation occurs in fleshy fruits usually at the early developmental stage (Deluc *et al.*, 2008; Akagi *et al.*, 2009; Terrier *et al.*, 2009; Schaart *et al.*, 2013; Wang *et al.*, 2019a). Consistently, we found that PAs accumulate preferentially at 10 DPA and genes such as *LARs* and *ANRs* encoding key enzymes responsible for the PA biosynthetic pathway were highly expressed at the same developmental stage in kiwifruits (Fig 4b; Fig. 7). These works suggest that a conserved accumulation pattern of PAs exists in different fruit crops. Since PAs play an important role in protecting plants against herbivores and diseases (Dixon *et al.*, 2005), the early accumulation of PAs may be also related to such a defensive role during initial kiwifruit development.

R2R3-type MYB TFs have been shown to act as key regulators in the regulation of PA biosynthesis in different plants (Nesi *et al.*, 2001; Baudry *et al.*, 2004; Bogs *et al.*, 2007; Lepiniec *et al.*, 2006; Akagi *et al.*, 2009; Schaart *et al.*, 2013; Wang *et al.*, 2019b; Zhou *et al.*, 2019; Peng *et al.*, 2020;). We showed a novel R2R3-type *AcMYB123-2*, homologous to *AtTT2* and *VvMYBPA2* plays an important role in PA metabolism in kiwifruit by directly activating the transcription of *AcLAR2*, a key gene for PA biosynthesis. These results suggest a conserved function of R2R3-type MYB in different plants. Interestingly, in addition to *AcMYB123-2*, we also identified an ERF-type TF *AcERF192* that acts as a key regulator in PA metabolism (Fig. 7). Although the role of ERF in anthocyanin accumulation has been reported in various plants (Yao *et al.*, 2017; An *et al.*, 2018; Koyama & Sato, 2018; Ni *et al.*, 2019), the involvement

of ERF in the regulation of PA biosynthesis in fruit crops remains largely unknown (Li *et al.*, 2020a). In this regard, our study has extended our understanding of the regulation of PA and flavonoid metabolism in plants.

The D-mannose/L-galactose pathway has been regarded as the predominant pathway of AsA biosynthesis in high plants (Wheeler *et al.*, 1998; Agius *et al.*, 2003; Wolucka & Van Montagu, 2003; Lorence *et al.*, 2004; Chen *et al.*, 2021). However, in this study, we showed that the recycling pathway mediated by AO and APX also plays an important role in the accumulation of L-ascorbate during kiwifruit development and ripening. This is supported by the fact that high *AO* and *APX* transcriptions are associated with the low content of L-ascorbate in fruits at 10 DPA, but a sharp decrease in *AO* and *APX* mRNA accounts for the high accumulation of L-Ascorbate in fruits at 25 DPA (Fig. 6b; Fig. 7). Consistent with our finding, the level of *AO* expression is negatively correlated with the accumulation of L-ascorbate in tomato (Abdelgawad *et al.*, 2019; Zhang *et al.*, 2011). Moreover, although homologous *AO* genes have been identified in different plants, regulation of *AOs* at the transcriptional level remains largely known. Unlike previous studies that focused on the L-ascorbate biosynthesis pathway (Chen *et al.*, 2021; Liu *et al.*, 2022), we established a regulatory network for *AcAOI* and revealed *AcAOI* transcription is controlled by AcMYB123-2 through its directly binding to the *AcAOI* promoter (Fig. 7). Thus, our study has provided new insight into the metabolic regulation of L-ascorbate during kiwifruit development. Intriguingly, AcMYB123-2 acts as a key regulator in both PA and AsA metabolisms during kiwifruit development, suggesting that this TF can simultaneously modulate two metabolic pathways. We envisage that *AcMYB123-2* can be a useful genetic resource for the biotech improvement of multiple traits contributing to kiwifruit quality. Indeed, several recent reports have shown that manipulation of a single TF can concurrently affect nutrition- and/or flavor-related metabolites from different biosynthetic pathways in various fruit crops (Ampomah-Dwamena *et al.*, 2019; Jian *et al.*, 2019; Wu *et al.*, 2020; Shi *et al.*, 2021).

A considerable number of metabolites were identified in our current studies. However, it is worthwhile noting partial metabolites such as carotenoids and ester, were not quantified due to the detection limit of the broadly target LC-MS/MS used here. Therefore, a higher-resolution metabolome analysis may be required to reveal accumulation patterns for novel compounds and to construct more comprehensive metabolic regulatory pathways in the future. Nevertheless, the metabolomes, and transcriptomes along with the co-expression analysis of metabolomes and transcriptomes described in this work provide a rich, accurate, and robust resource for a wide-ranging study of KMRN throughout the entire growth cycle of kiwifruit fruit.

In summary, our study produces high-resolution metabolomes and transcriptomes at 11 fruit developmental and ripening stages in kiwifruit. The KMRN brings about a comprehensive overview of the major metabolic changes that occur during kiwifruit development and ripening, and the transcriptomic and transcriptional regulation mechanisms that underpin these changes. Mining this KMRN coupled with functional genomics analysis has revealed important insights into the control of major kiwifruit metabolites, guiding future work on the designed manipulation of fruit quality. Our KMRN and the experimental strategies for gene functional analyses would be useful in the discovery and verification of other MM vs GM regulation in kiwifruit and stipulates a blueprint for similar works for other fruit crops.

625 **Data availability**

626 RNA sequencing data sets have been submitted to the Genome Sequence Archive in
 627 National Genomics Data Center, Beijing Institute of Genomics, Chinese Academy of
 628 Sciences (<http://bigd.big.ac.cn>). The accession number is CRA006020 and is publicly
 629 accessible.

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638 **Author contributions**

639 M.Liu., Y.Z. and P.S. designed the experiments; Z.Z., Y.W., Y.C., K.L., H.D., J.Z.,
 640 X.Z., J.W. conducted the experiments; Z.L., Y.X., K.D., M.Li. analyze the data; M.Liu.
 641 , P.S. Z.Z and Y.Z. wrote the paper with the input of all the authors. Y.H. helped to
 642 revise and improve the manuscript. All authors have approved this manuscript.

643 **Conflict of interest**

644 All authors declare no competing interests.

645

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990

991 **Supporting Information**

992 **Table S1 Class categories and metabolic modules of 515 metabolites with CAS**
 993 **number.**

994 **Table S2 Primer list in this study.**

995 **Fig. S1 Ten metabolite or gene modules generated using a clustering algorithm**
 996 **based on the dynamic changes of metabolites or genes.**

997 **Fig. S2 Soluble sugar MRN in kiwifruit.**

998 **Fig. S3 anthocyanidin MRN in kiwifruit.**

999 **Fig. S4 Procyanidin MRN in kiwifruit.**

1000 **Fig. S5 Functional analysis of TFs AcMYB90 (a) and AcMYB111 (b) in Dual**
 1001 **luciferase reporter assays.**

1002 **Fig. S6 AcAO1 and AcAPX2 regulate L-Ascorbate accumulation in Nicotiana**
 1003 **benthamiana.**

1004 **Dataset S1. Primary and secondary metabolites in kiwifruits at 10, 25, 40, 55, 70,**
 1005 **85, 100, 115, 130, 145 and 170 DPA.**

1006 **Dataset S2. Summary of transcriptome data in kiwifruits at 10, 25, 40, 55, 70, 85,**
 1007 **100, 115, 130, 145 and 170 DPA.**

1008 **Dataset S3. Genes expression level in kiwifruits at 10, 25, 40, 55, 70, 85, 100, 115,**
 1009 **130, 145, and 170 DPA.**

1010 **Dataset S4. Cluster results of primary and secondary metabolites in kiwifruits at**
 1011 **10, 25, 40, 55, 70, 85, 100, 115, 130, 145, and 170 DPA.**

1012 **Dataset S5. Protein-coding genes expression in development at 10, 25, 40, 55, 70,**
 1013 **85, 100, 115, 130, 145, and 170 DPA.**

1014 **Dataset S6. Cluster results of co-expression genes in kiwifruit at 10, 25, 40, 55, 70,**
 1015 **85, 100, 115, 130, 145, and 170 DPA.**

1016 **Dataset S7. Similarity coefficient between ten metabolite modules and ten gene**
1017 **modules.**

1018 **Dataset S8. Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis of ten**
1019 **gene modules (GM-I – GM-X).**

1020 **Dataset S9. Construction of soluble sugar MRN in kiwifruit.**

1021 **Dataset S10. Construction of procyanidin MRN in kiwifruit.**

1022 **Dataset S11. Construction of AsA MRN in kiwifruit.**

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1024 Figure legends

1025 **Fig. 1 Summary of metabolome and transcriptome data sets.** (a) Phenotypic
 1026 changes in kiwifruits (*Actinidia chinensis* cv Hongyang) at 11 different fruit
 1027 developmental and ripening stages (FDRSs). Intact and sectioned kiwifruits were
 1028 photographed at 10, 25, 40, 55, 70, 85, 100, 115, 130, 145, and 170 days post anthesis
 1029 (DPA). Bar = 1 cm. (b, c) Cluster analysis for metabolomes (b) and transcriptomes (c).
 1030 The color scales 0-1 represent Pearson correlation coefficient (PCC). (d, e) Principal
 1031 component analysis (PCA) for metabolomes (d) and transcriptomes (e). Kiwifruits at
 1032 the 11 FDRSs can be categorized into three major Groups I, II, and III based on purple,
 1033 green or yellow phenotypes in pericarps (a), metabolomes (b, d), and transcriptomes
 1034 (c, e).

1035 **Fig. 2 Construction of kiwifruit metabolite regulatory network (KMRN) -**
 1036 **Association of dynamic metabolisms of major metabolites with gene expression**
 1037 **patterns at different kiwifruit developmental and ripening stages.** (a, b) Metabolite
 1038 modules (MMs) vs gene modules (GMs). Accumulation patterns of metabolites in ten
 1039 MMs (MM-I – MM-X; a), and the expression patterns of 24325 co-expressed genes in
 1040 ten GMs (GM-I – GM-X; b) are shown. Z-scores of data sets were standardized to -4
 1041 to 4. (c) Statistics of the class categories of metabolites in ten MMs. (d-f) Examples of
 1042 KEGG analyses. KEGG analysis of co-expressed genes in GM-I (d), GM-V (e), and
 1043 GM-IX (f) are shown. The color marks #F8766D, #619CFF, and #00BAF8 represent
 1044 various enrichments in metabolic pathways that correspond to the metabolites in MM-
 1045 I, MM-V, and MM-IX, respectively.

1046 **Fig. 3 Mining KMRN reveals key regulatory factors in soluble sugar accumulation.**
 1047 (a) Soluble sugar metabolism pathways. Metabolites detected in KMRN are color
 1048 (#FFA500) coded. AMY, α -amylase; BAM, β -amylase; SPS, Sucrose phosphate
 1049 synthase; AGPase, ADP glucose pyrophosphorylase; INV, Sucrose invertase; SUS,
 1050 Sucrose synthase; HK, Hexokinase; FK, Fructokinase. (b) Heatmap of metabolites and
 1051 genes in soluble sugar metabolism pathways. Z-scores of data were standardized to -3

to 3. **(c)** Schematic representation of MRN construction. TF: transcription factors; SG: structural genes; MET: metabolites. Promoter region: cis-acting elements of structural genes' promoter sequences. **(d)** The AP2/ERF-associated subnetwork within the soluble sugar MRN. Regulatory relationships have been reported are shown by color-coded (#FFA500) dotted lines.

Fig. 4 TF AcMYB123-2 modulates procyanidin accumulation by transcriptional regulation of *AcLAR2* in kiwifruit. **(a)** Procyanidin-associated metabolism pathways.

Metabolites detected in KMRN are indicated by color-coded (#AE323F) words. CHS, Chalcone synthase; CHI, Chalcone isomerase; FLS, Flavonol synthase; F3H, Flavanone 3-hydroxylase; F3'5'H, Flavonoid 3'-5'-hydroxylase; F3'H, Flavonoid 3'-hydroxylase; DFR, Dihydroflavonol reductase; LAR, Leucocyanidin reductase; ANS, Anthocyanin synthase; ANR, Anthocyanin reductase; F3GT, Flavonoid 3-O-glucosyltransferase. **(b)** Heatmap of metabolites and genes in procyanidin-associated metabolism pathways. Data were z-score standardized to -3 to 3. **(c)** MYB-associated subnetwork in procyanidin MRN. **(d)** Four classes of MYB transcription factors in procyanidin MRN. Anthocyanidin- (#FF0000), procyanidin- (#AE323F), anthocyanidin repressor-associated (#C3E49F) procyanidin MRNs, and others based on the phylogenetic tree are shown. **(e)** Impact of TF AcMYB123-2 on activity of its potential target gene promoters. Data are represented as Mean $\pm$ SD (n = 3). Asterisks indicate statistical significance by Student's *t*-tests ($P < 0.05$). **(f)** AcMYB123-2 binding to the Myb-p motif in *AcLAR2* promoter. Wild-type (WT) probe containing the Myb-p element was biotin-labeled (Biotin-P). Competition for AcMYB123-2 binding was performed with cold or Myb-p mutated probes (Cold-P or Mutant-P). The symbols – and + represent absence or presence of the probes and GST-tagged AcMYB123-2 protein. **(g)** *In planta* gene functional assays. Relative mRNA levels of *AcMYB123-2* (top) and *AcLAR2* (middle), and the relative contents of procyanidin B and procyanidin C (bottom) in *AcMYB123-2* overexpressing or silenced kiwifruits are shown. Data are represented as Mean $\pm$ SD (n = 3). Asterisks indicate statistical significance by Student's *t*-tests ($P < 0.05$).

1081 **Fig. 5 TF AcERF192 modulates procyanidin accumulation by transcriptional**
 1082 **regulation of *AcANR1* in kiwifruit.** (a) AP2/ERF-associated subnetwork in
 1083 procyanidin MRN. (b) Phylogenetic tree of Arabidopsis and kiwifruit AP2/ERF TFs.
 1084 (c) Dual luciferase reporter assays of AcERF192 function. Data are represented as
 1085 Mean $\pm$ SD (n = 3). Asterisks indicate statistical significance by Student's *t*-tests ($P <$
 1086 0.05). (d) AcERF192 binding to the DRE/CRT element in the *AcANR1* promoter.
 1087 Biotin-labeled wild-type (WT) probe (Biotin-P), cold- (Cold-P) and the DRE/CRT
 1088 element-mutated probes (Mutant-P) were used in EMSA. The symbols – and +
 1089 represent absence or presence of the probes and GST-tagged AcERF192 protein. (e)
 1090 Relative mRNA levels of *AcERF192* (top) and *AcANR1* (middle), and the relative
 1091 contents of procyanidins B and procyanidins C (bottom) in *AcERF192* overexpressing
 1092 or silenced kiwifruits are shown. Data in (c) and (e) are represented as Mean $\pm$ SD (n =
 1093 3). Asterisks indicate statistical significance by Student's *t*-tests ($P <$ 0.05).

1094 **Fig. 6 TF AcMYB123-2 modulates vitamin C (L-Ascorbate, AsA) degradation by**
 1095 **regulation of *AcAO1* in kiwifruit.** (a) AsA metabolism pathways. The color-coded
 1096 (#508548) words represent the metabolites detected in KMRN. D-mannose/L-
 1097 Galactose and Galacturonate pathways are the two most important vitamin biosynthesis
 1098 pathways in plants. Recycling pathway is the AsA recycling pathway. PGI, Glucose-6-
 1099 phosphate isomerase; PMI, Mannose-6-phosphate isomerase; PMM,
 1100 Phosphomannanase; GMP, Mannose-1-phosphoguanilate transferase; GME, GDP-D-
 1101 Mannose 3',5'-epimerase; GGP, GDP-L-galactose phosphorylase; GPP, L-galactose 1-
 1102 phosphate phosphatase; GDH, L-galactose dehydrogenase; GLDH, L-galactose-1,4-
 1103 lactone dehydrogenase; GALUR, D-galacturonate reductase; MDHAR,
 1104 Monodehydroascorbate reductase; DHAR, L-Hydroascorbate reductase; APX, L-
 1105 Ascorbate peroxidase; AO, L-Ascorbate oxidase. (b) Heatmap of metabolites and genes
 1106 in AsA pathways. Data were z-score standardized to -3 to 3. The color #508548- or
 1107 #9398FF-coded line corresponds to genes in AsA biosynthesis or recycling pathway,
 1108 respectively. (c) Relative mRNA levels of *AcAO1* (top), and contents of reduced AsA
 1109 (L-Ascorbate) and total AsA (L-Ascorbate + L-Dehydroascorbate) (bottom) in *AcAO1*-

overexpressing or silenced kiwifruits are shown. FW, flesh weight. **(d)** *AcAO1* and *AcAPX2* in AsA MRN. **(e)** Activation of *AcAO1* promoter by *AcMYB123-2* in dual luciferase reporter assays. Data are represented as Mean $\pm$ SD (n = 3). Asterisks indicate statistical significance by Student's *t*-tests ($P < 0.05$). **(f)** Relative mRNA levels of *AcAO1* (top), and contents of reduced AsA and total AsA (bottom) in *AcMYB123-2* overexpressing and silenced kiwifruits. FW, flesh weight. **(g)** *AcMYB123-2* binding to the Myb-p motif in the promoter of *AcAO1*. Biotin-labeled wild-type (WT) probe (Biotin-P), cold- (Cold-P) and the Myb-p element-mutated probes (Mutant-P) were used in EMSA. The symbols – and + represent absence or presence of the probes and GST-tagged *AcMYB123-2* protein. Data in **(c)**, **(e)** and **(f)** are represented as Mean $\pm$ SD (n = 3). Asterisks indicate statistical significance by Student's *t*-tests ($P < 0.05$).

Fig. 7 Schematic diagram represents the dynamics of major metabolites changes and regulatory mechanisms that underlie them during the quality formation of kiwifruit. DPA, days post anthesis. The content of metabolites at different developmental stages in kiwifruit is normalized to 0-1, and the ovals represent transcription factors and the rectangles represent structural genes in the transcriptional regulation patterns.

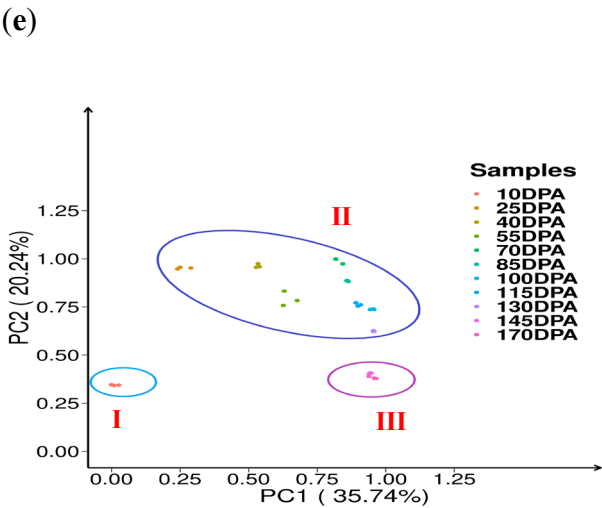
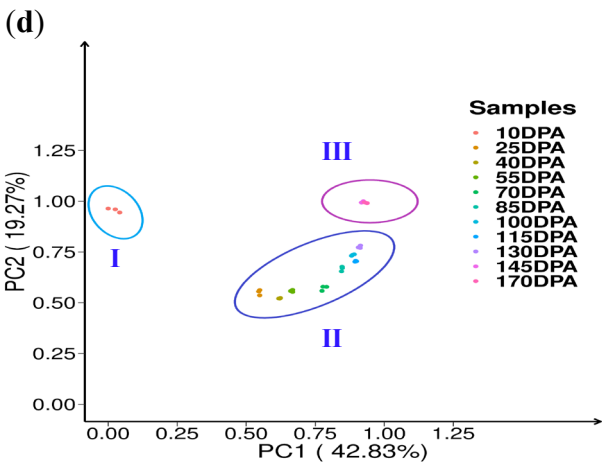
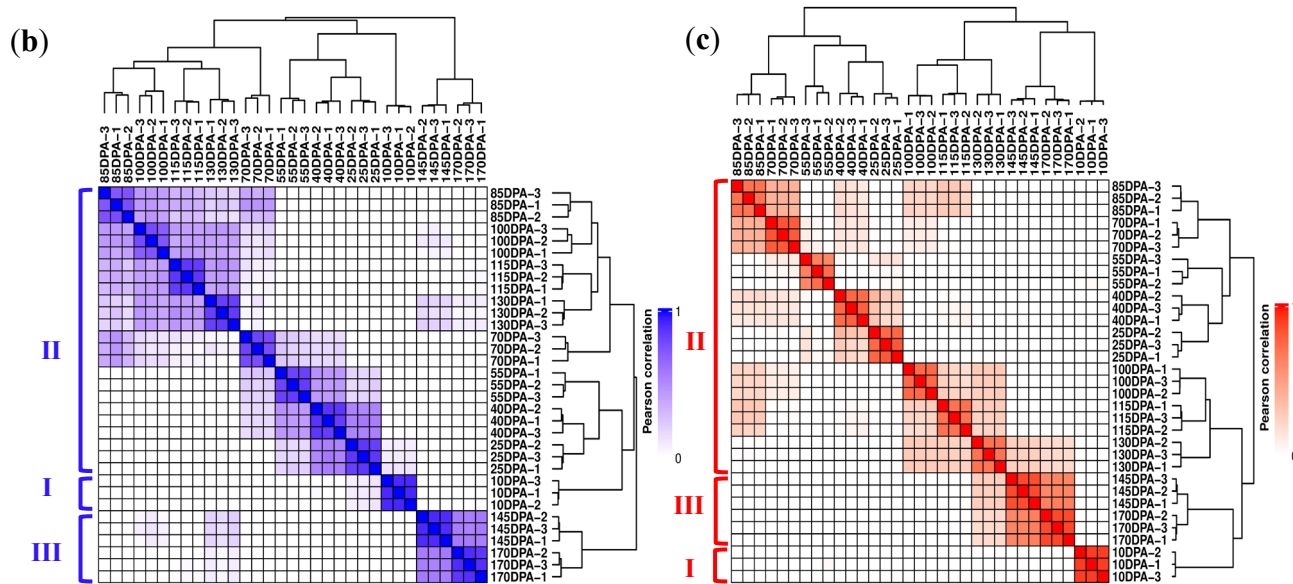
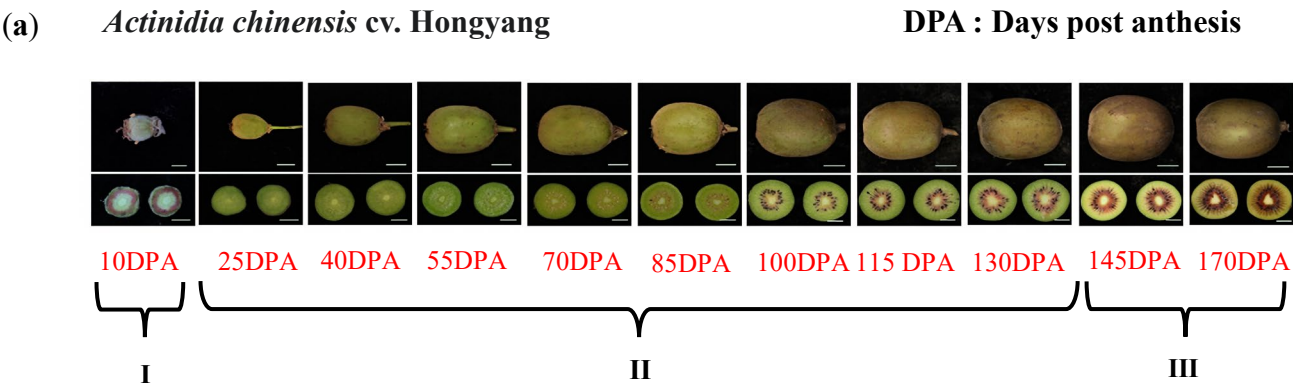


Fig. 1 Summary of metabolome and transcriptome data sets. (a) Phenotypic changes in kiwifruits (*Actinidia chinensis* cv Hongyang) at 11 different fruit developmental and ripening stages (FDRSs). Intact and sectioned kiwifruits were photographed at 10, 25, 40, 55, 70, 85, 100, 115, 130, 145, and 170 days post anthesis (DPA). Bar = 1 cm. (b, c) Cluster analysis for metabolomes (b) and transcriptomes (c). The color scales 0-1 represent Pearson correlation coefficient (PCC). (d, e) Principal component analysis (PCA) for metabolomes (d) and transcriptomes (e). Kiwifruits at the 11 FDRSs can be categorized into three major Groups I, II, and III based on purple, green or yellow phenotypes in pericarps (a), metabolomes (b, d), and transcriptomes (c, e).

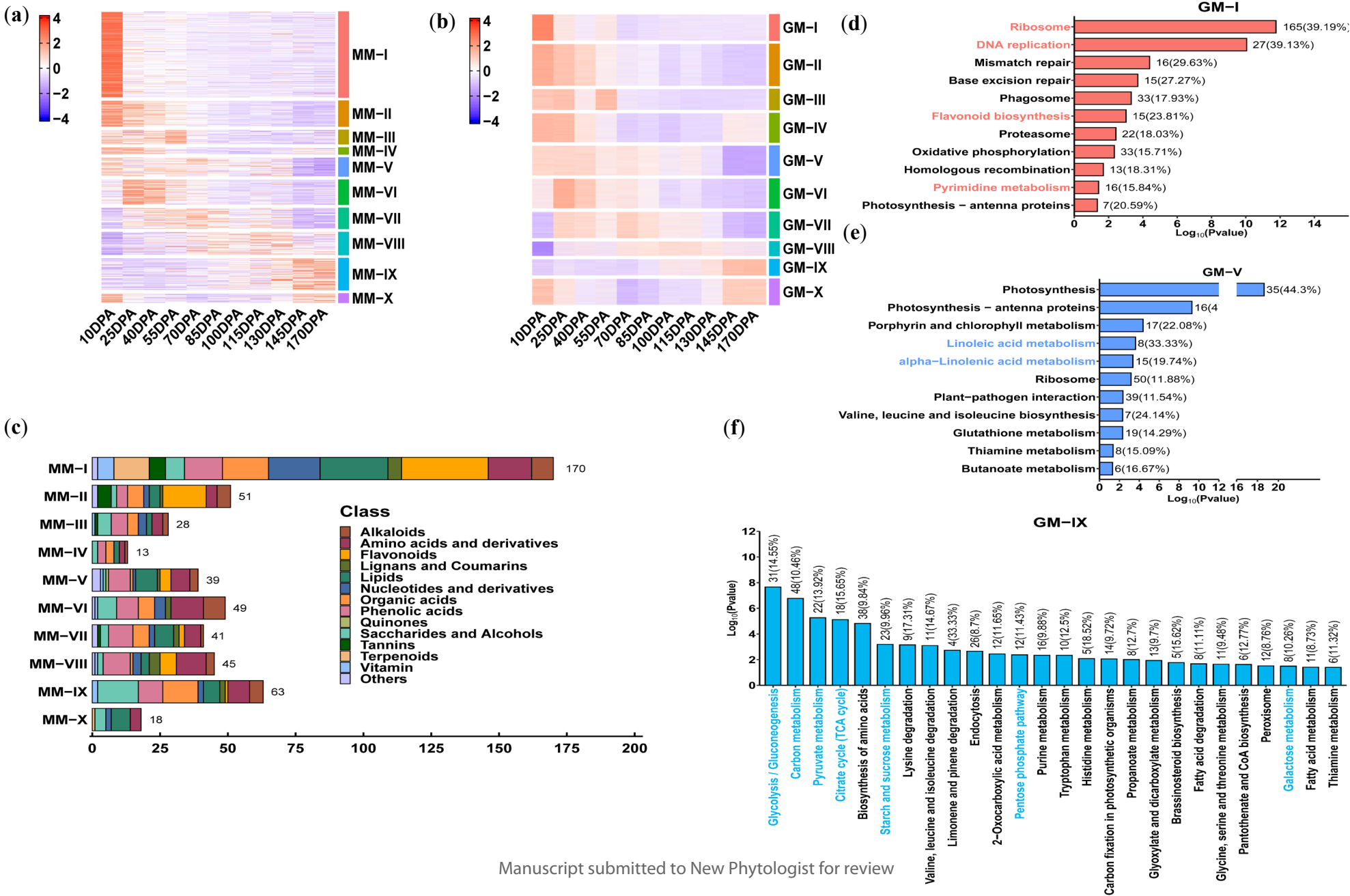
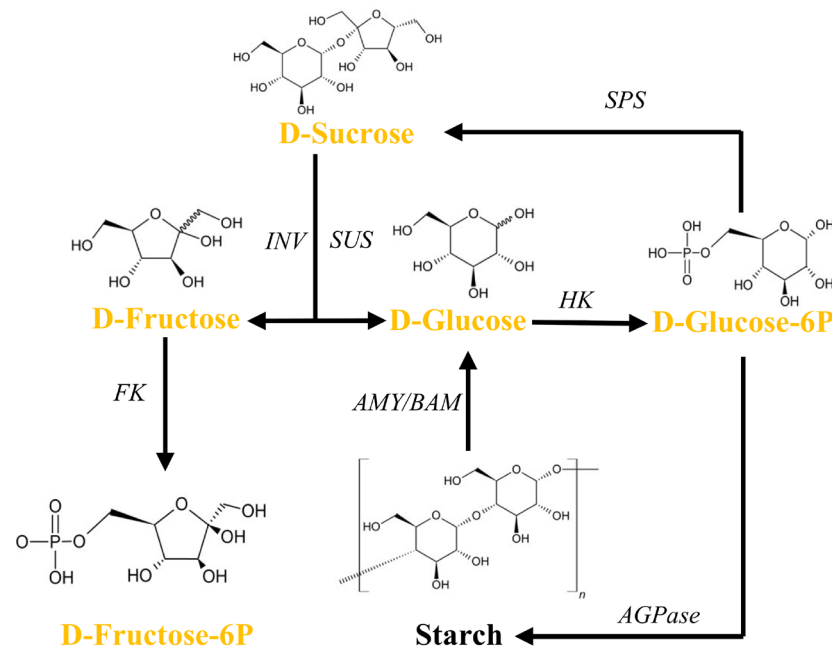
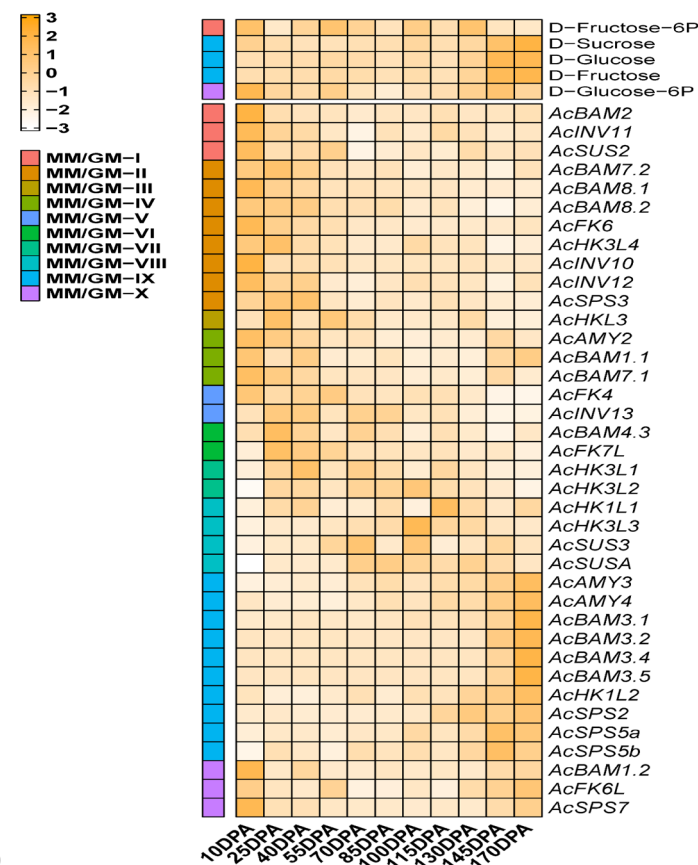


Fig. 2 Construction of kiwifruit metabolite regulatory network (KMRN) - Association of dynamic metabolisms of major metabolites with gene expression patterns at different kiwifruit developmental and ripening stages. (a, b) Metabolite modules (MMs) vs gene modules (GMs). Accumulation patterns of metabolites in ten MMs (MM-I – MM-X; **a**), and the expression patterns of 24325 co-expressed genes in ten GMs (GM-I – GM-X; **b**) are shown. Z-scores of data sets were standardized to -4 to 4. (c) Statistics of the class categories of metabolites in ten MMs. (d-f) Examples of KEGG analyses. KEGG analysis of co-expressed genes in GM-I (**d**), GM-V (**e**), and GM-IX (**f**) are shown. The color marks #F8766D, #619CFF, and #00BAF8 represent various enrichments in metabolic pathways that correspond to the metabolites in MM-I, MM-V, and MM-IX, respectively.

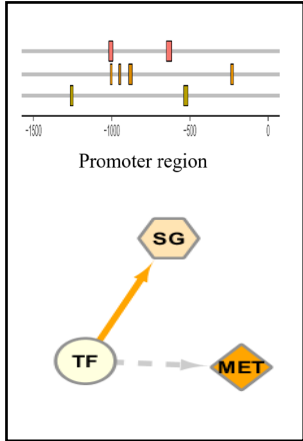
(a)



(b)



(c)



(d)

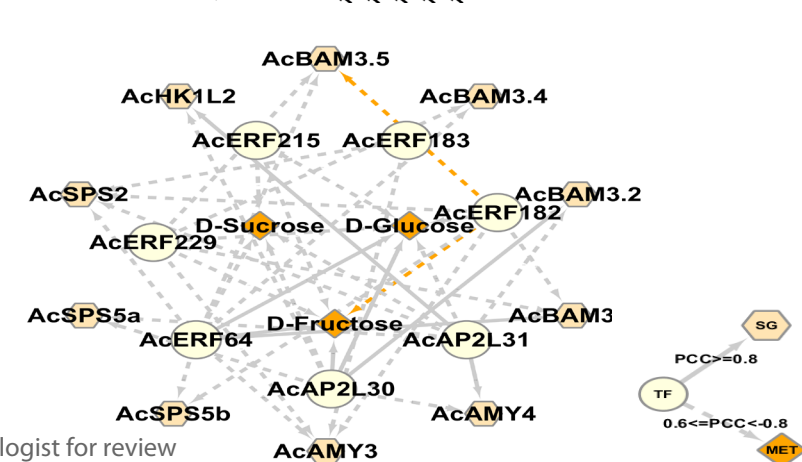


Fig. 3 Mining KMRN reveals key regulatory factors in soluble sugar accumulation. (a) Soluble sugar metabolism pathways. Metabolites detected in KMRN are color (#FFA500) coded. AMY, α -amylase; BAM, β -amylase; SPS, Sucrose phosphate synthase; AGPase, ADP glucose pyrophosphorylase; INV, Sucrose invertase; SUS, Sucrose synthase; HK, Hexokinase; FK, Fructokinase. **(b)** Heatmap of metabolites and genes in soluble sugar metabolism pathways. Z-scores of data were standardized to -3 to 3. **(c)** Schematic representation of MRN construction. TF: transcription factors; SG: structural genes; MET: metabolites. Promoter region: cis-acting elements of structural genes' promoter sequences. **(d)** The AP2/ERF-associated subnetwork within the soluble sugar MRN. Regulatory relationships have been reported are shown by color-coded (#FFA500) dotted lines.

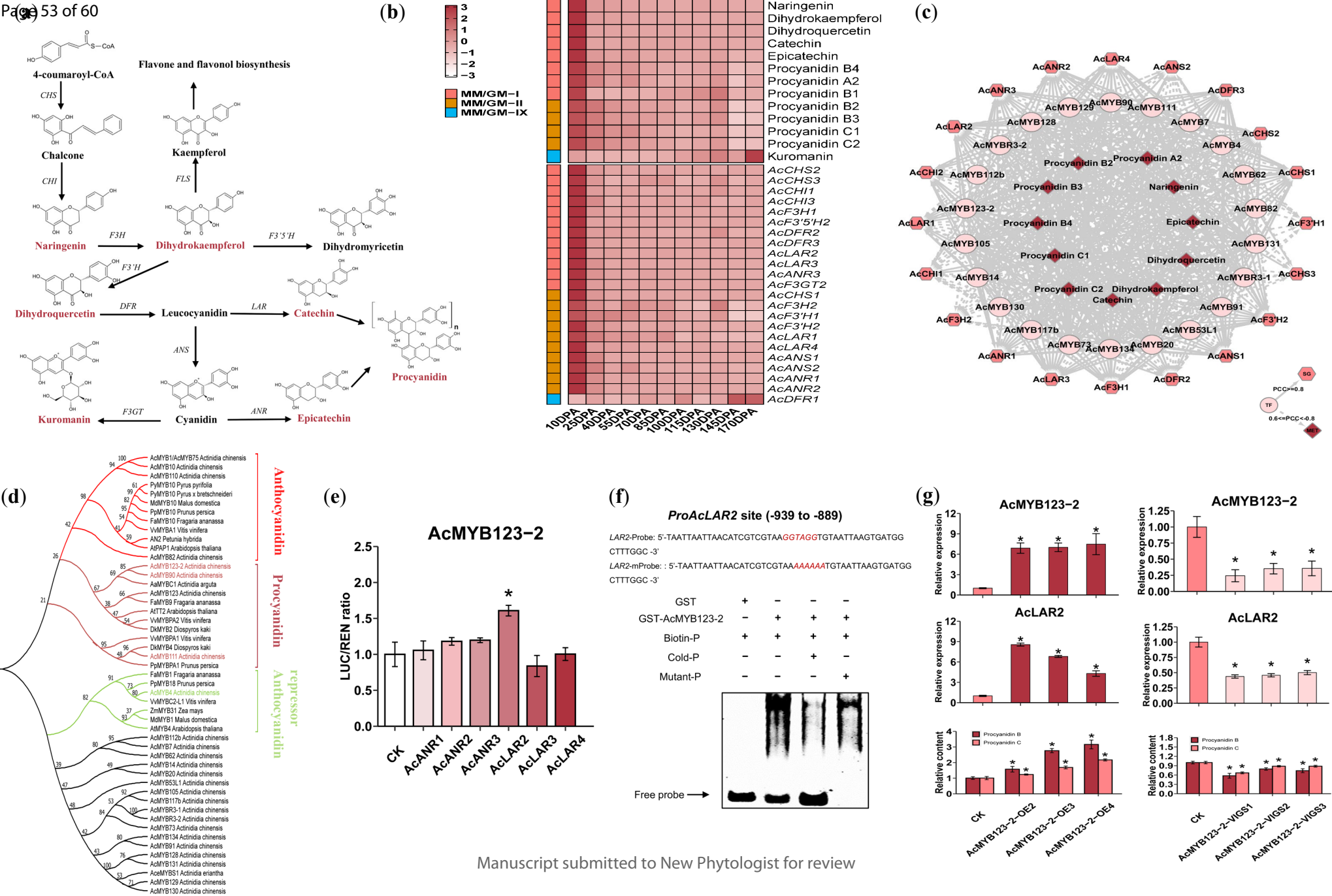


Fig. 4 TF AcMYB123-2 modulates procyanidin accumulation by transcriptional regulation of *AcLAR2* in kiwifruit. (a) Procyanidin-associated metabolism pathways. Metabolites detected in KMRN are indicated by color-coded (#AE323F) words. CHS, Chalcone synthase; CHI, Chalcone isomerase; FLS, Flavonol synthase; F3H, Flavanone 3-hydroxylase; F3'5'H, Flavonoid 3'-5' -hydroxylase; F3'H, Flavonoid 3' -hydroxylase; DFR, Dihydroflavonol reductase; LAR, Leucocyanidin reductase; ANS, Anthocyanin synthase; ANR, Anthocyanin reductase; F3GT, Flavonoid 3-O-glucosyltransferase. (b) Heatmap of metabolites and genes in procyanidin-associated metabolism pathways. Data were z-score standardized to -3 to 3. (c) MYB-associated subnetwork in procyanidin MRN. (d) Four classes of MYB transcription factors in procyanidin MRN. Anthocyanidin- (#FF0000), procyanidin- (#AE323F), anthocyanidin repressor-associated (#C3E49F) procyanidin MRNs, and others based on the phylogenetic tree are shown. (e) Impact of TF AcMYB123-2 on activity of its potential target gene promoters. Data are represented as Mean $\pm$ SD (n = 3). Asterisks indicate statistical significance by Student's *t*-tests ($P < 0.05$). (f) AcMYB123-2 binding to the Myb-p motif in *AcLAR2* promoter. Wild-type (WT) probe containing the Myb-p element was biotin-labeled (Biotin-P). Competition for AcMYB123-2 binding was performed with cold or Myb-p mutated probes (Cold-P or Mutant-P). The symbols – and + represent absence or presence of the probes and GST-tagged AcMYB123-2 protein. (g) *In planta* gene functional assays. Relative mRNA levels of *AcMYB123-2* (top) and *AcLAR2* (middle), and the relative contents of procyanidin B and procyanidin C (bottom) in *AcMYB123-2* overexpressing or silenced kiwifruits are shown. Data are represented as Mean $\pm$ SD (n = 3). Asterisks indicate statistical significance by Student's *t*-tests ($P < 0.05$).

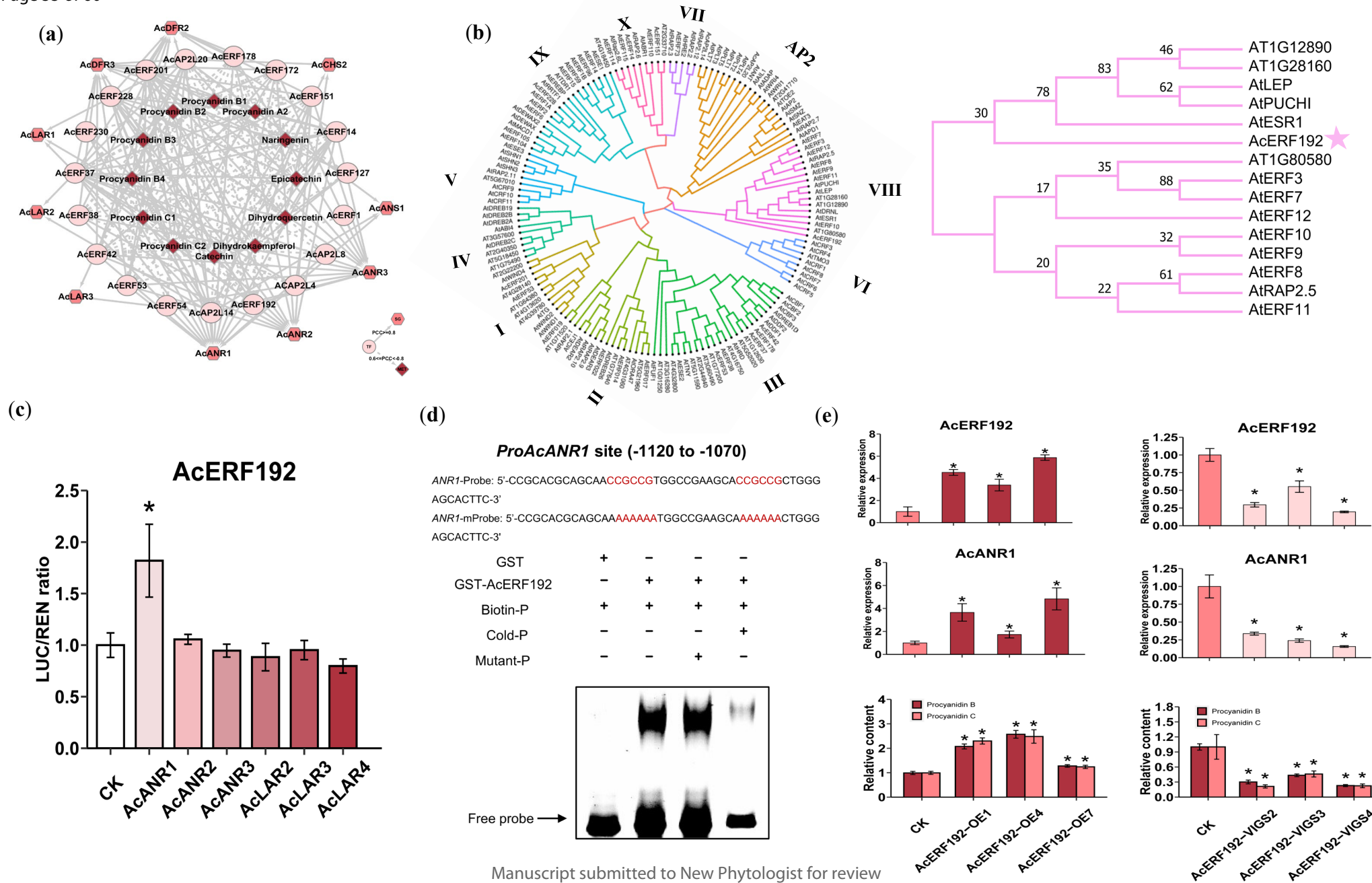


Fig. 5 TF AcERF192 modulates procyanidin accumulation by transcriptional regulation of *AcANR1* in kiwifruit. (a) AP2/ERF-associated subnetwork in procyanidin MRN. (b) Phylogenetic tree of Arabidopsis and kiwifruit AP2/ERF TFs. (c) Dual luciferase reporter assays of AcERF192 function. Data are represented as Mean $\pm$ SD (n = 3). Asterisks indicate statistical significance by Student's *t*-tests ($P < 0.05$). (d) AcERF192 binding to the DRE/CRT element in the *AcANR1* promoter. Biotin-labeled wild-type (WT) probe (Biotin-P), cold- (Cold-P) and the DRE/CRT element-mutated probes (Mutant-P) were used in EMSA. The symbols – and + represent absence or presence of the probes and GST-tagged AcERF192 protein. (e) Relative mRNA levels of *AcERF192* (top) and *AcANR1* (middle), and the relative contents of procyanidins B and procyanidins C (bottom) in *AcERF192* overexpressing or silenced kiwifruits are shown. Data in (c) and (e) are represented as Mean $\pm$ SD (n = 3). Asterisks indicate statistical significance by Student's *t*-tests ($P < 0.05$).

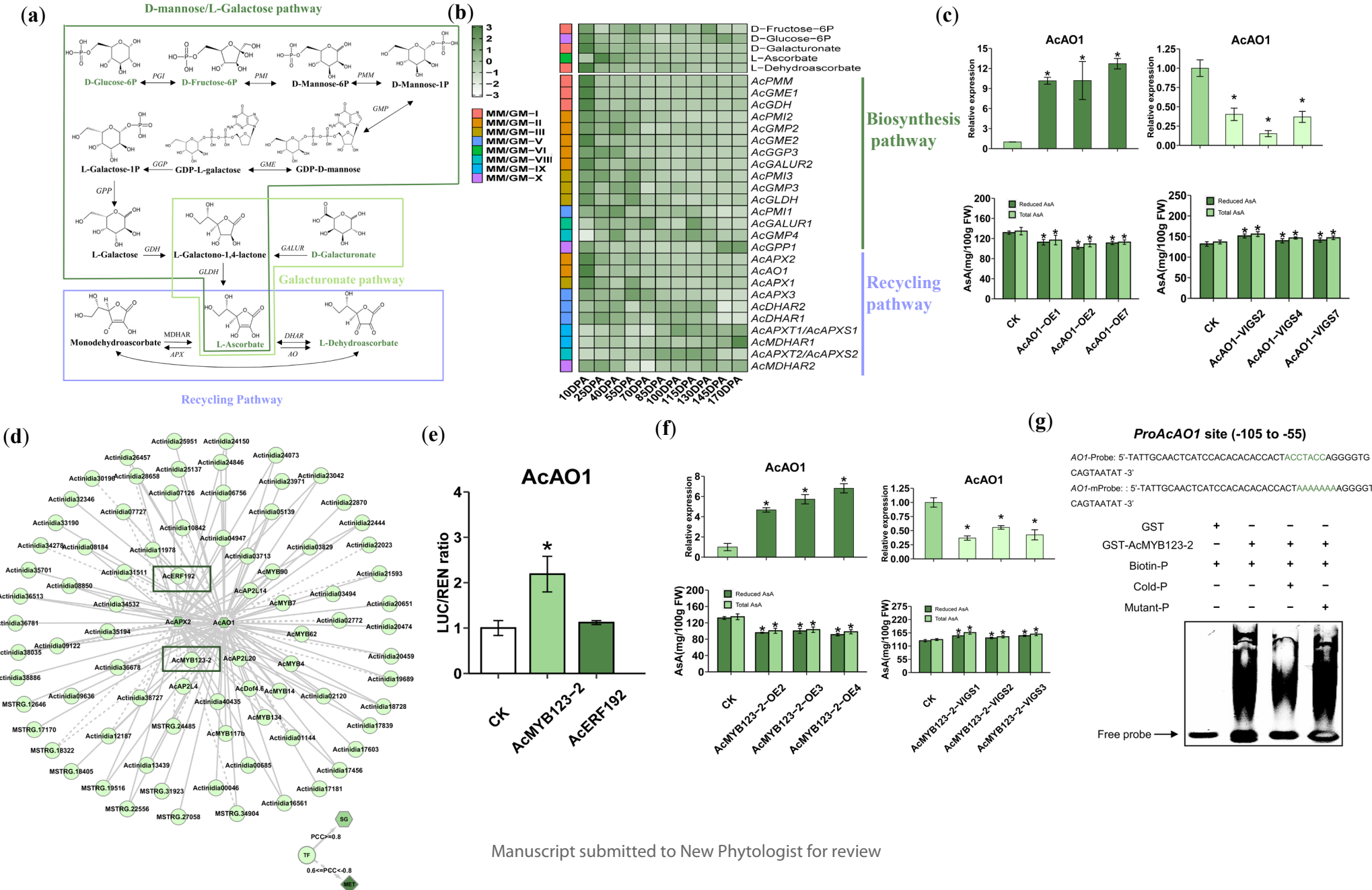


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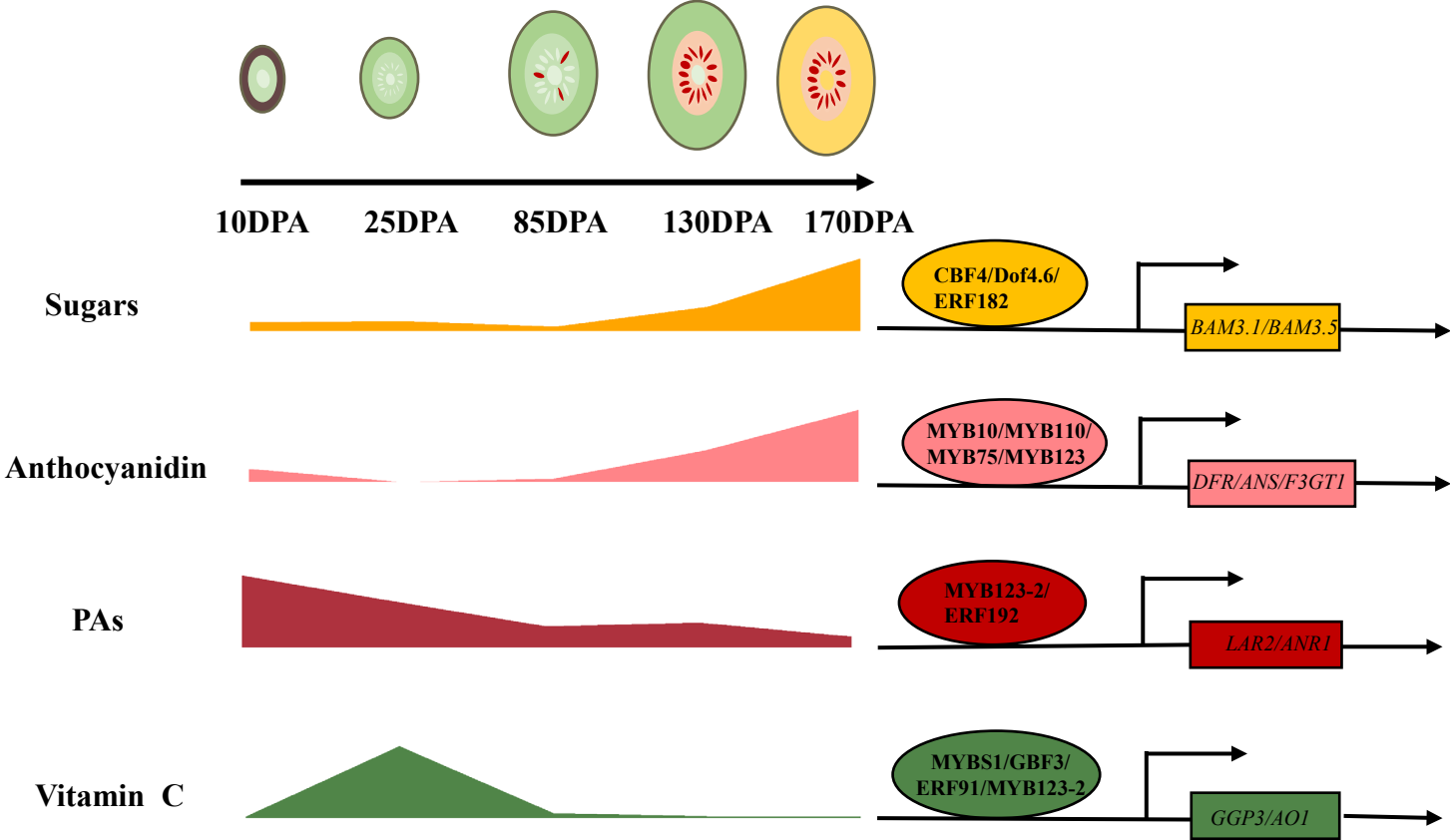


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