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ORIGINAL ARTICLE

ABA and sucrose co-regulate strawberry fruit ripening and show inhibition of glycolysis

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Abstract

Abscisic acid (ABA) and sucrose play an important role in strawberry fruit ripening, but how ABA and sucrose co-regulate this ripening progress remains unclear. The intention of this study was to examine the effect of ABA and sucrose on strawberry fruit ripening and to evaluate the ABA/sucrose interaction mechanism on the strawberry fruit ripening process. Here, we report that there is an acute synergistic effect between ABA and sucrose in accelerating strawberry fruit ripening. The time frame of fruit development and ripening was shortened after the application of ABA, sucrose, and ABA + sucrose, but most of the major quality parameters in treated-ripe fruit, including fruit weight, total soluble solids, anthocyanin, ascorbic acid, the total phenolic content, lightness (L^*), chroma (C^*), and hue angle (h°) values were not affected. Meanwhile, the endogenous ABA and sucrose levels, and the expression of ABA and sucrose signaling genes and ripening-related genes, such as *NCED1*, *NCED2*, *SnRK2.2*, *SuSy*, *MYB5*, *CEL1,* and *CEL2*, was all significantly enhanced by ABA or sucrose treatment alone, but in particular, by the ABA + sucrose treatment. Therefore, improving the ripening regulation efficiency is one synergetic action of ABA/sucrose. Another synergetic action of ABA/sucrose shows that a short inhibition of glycolysis occurs during accelerated strawberry ripening. ABA and sucrose can induce higher accumulation of H_2O_2, leading to a transient decrease in glycolysis. Conversely, lower endogenous H_2O_2 levels caused by reduced glutathione (GSH) treatment resulted in a transient increase in glycolysis while delaying strawberry fruit ripening. Collectively, this study demonstrates that the ABA/sucrose interaction affects the ripening regulation efficiency and shows inhibition of glycolysis.

Keywords Abscisic acid · Sucrose · Hydrogen peroxide · Glycolysis · Strawberry · Ripening

Introduction

The regulation of fruit ripening is an important research area in fleshy fruit production. Strawberry is an ideal model plant for the study of non-climacteric fruit ripening (Cherian et al. 2014; Li et al. 2011). In recent years, substantial progress has been made towards clarifying the regulatory mechanism of strawberry ripening. It has been demonstrated that abscisic acid (ABA) (Ayub et al. 2016; Chai et al. 2011), auxin (Liu et al. 2011; Mezzetti et al. 2004; Narayanan et al. 1981), jasmonate (JA) (Concha et al. 2013; Han et al. 2019; Mukkun and Singh 2009), brassinosteroids (BRs) (Chai et al. 2013), ethylene (ETH) (Merchante et al. 2013; Sun et al. 2013; Trainotti et al. 2005), gibberellic acid (GA) (Martínez et al. 1994), and sucrose (Jia et al. 2013), each play different roles in the regulation of strawberry ripening. Jia et al. (2016) constructed a model to provide a preliminary overview of the relationships between ABA, indole acetic

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acid (IAA), JA, and sucrose in the regulation of strawberry ripening. In this model, IAA plays an important role in the early stages of strawberry fruit development, and is responsible for inducing cell division and expansion. In the middle and later stages of fruit development, ABA, sucrose, and JA are the main molecules involved in regulating gene expression. ABA and sucrose can regulate one other and both can induce the JA signaling pathway(Jia et al. 2016). In particular, perception and transduction of the ABA signal by FaPYR1–FaPP2C–FaSnRK2 are essential in the regulation of strawberry ripening (Chai et al. 2011; Han et al. 2015; Li et al. 2011). Recently, Han et al. (2019) reported that methyl jasmonate (MeJA) promoted strawberry fruit ripening with the induction of anthocyanin, sucrose, cell wall, JA, and ABA metabolism-related gene expression. Wu et al. (2019) reported that strigolactones were involved in fruit development of the woodland strawberry (*Fragaria vesca*). Therefore, strawberry fruit ripening is likely to be the result of the concerted actions of multiple signaling pathways. However, the synergistic mechanism among these factors associated with strawberry fruit ripening remains elusive.

Previous reports have showed that there are complex interactions between ABA and sugar. Some of ABA-biosynthetic mutants display sugar-response phenotypes, and many *Arabidopsis* sugar-signaling mutants isolated turned out to be allelic to *ABA-biosynthetic* (*aba*) or *ABA-insensitive* (*abi*) mutants (Arenas-Huertero et al. 2000; Rolland et al. 2006). Furthermore, ABA and sucrose have synergistic effects on diverse biological processes. For example, ABA and sucrose targets include the ADP-glucose pyrophosphorylase large subunit (*ApL3*) genes in *Arabidopsis* (Rook et al. 2001) and rice (Akihiro et al. 2005) that are involved in starch biosynthesis; the ABA-stress-ripening (*ASR*) gene in grape (Cakir et al. 2003) that is involved in ABA and sucrose signaling; and the *VvMybA1* gene in grape that is responsible for anthocyanin biosynthesis (Gambetta et al. 2010).

Sucrose and starch are the main substrates of glycolysis in plants. The fundamental function of glycolysis are to provide energy, thus connecting carbohydrate metabolism with growth and development (Plaxton 1996). Glycolysis and sugar levels need to be finely coordinated to provide flexibility to the demands of plant development and to environmental stress acclimation (Koch et al. 2000; Rontein et al. 2002). In spite of a growing number of studies have reported that ABA and sucrose are involved in many important biological processes, the interactions between ABA and sucrose, and the role played by glycolysis in these interactions in the regulation of strawberry fruit ripening is lacking. Given the central role of sucrose and ABA in the regulation of strawberry fruit ripening, the characterization of possible mechanisms of ABA/sucrose interaction in promoting strawberry fruit ripening is essential. In this study, strawberry fruit at the de-greening stage (DG, 18 days after anthesis) was, therefore, treated with ABA, sucrose, or both in a greenhouse, and then analyzed step-by-step through a series of experiments to obtain both ripening-related physiological and RNA sequencing (RNA-seq) data. We provide new insights into the links between ABA/sucrose interaction and glycolysis in the regulation of strawberry ripening.

Results

Effects of exogenous ABA and sucrose on strawberry fruit ripening

The effects of ABA and sucrose on strawberry fruit ripening were first evaluated in a greenhouse. Exogenous ABA,

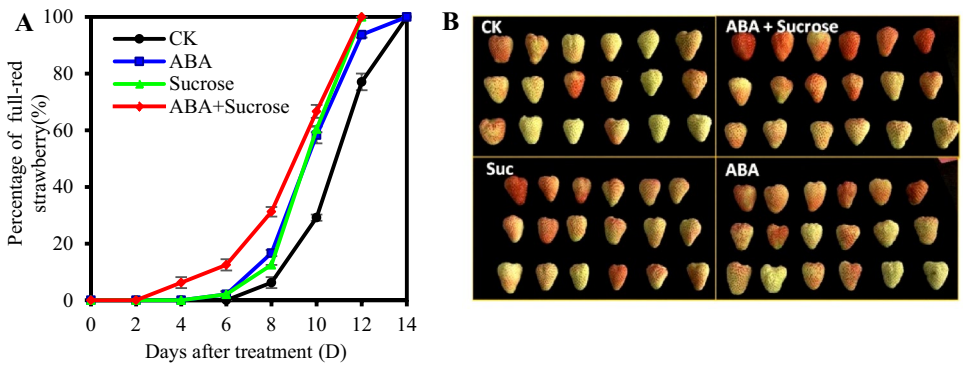


Fig. 1 Influence of exogenous abscisic acid (ABA) and sucrose on fruit coloring of strawberry. **a** Percentages of full-red strawberries after ABA, sucrose and ABA + sucrose treatments in the first sampling group. **b** Fruit coloring of each treatment on the 8th day in the second sampling group. All fruits used for analyses were randomly harvested from the field. Strawberries were treated with exogenous concentrations of 95 µM ABA, 100 mM sucrose and 95 µM ABA + 100 mM Sucrose at the de-greening stage. Percentages of full-red strawberries were calculated for each observation time by counting the number of full-red strawberry fruit. Data were shown as mean ± SD of three replicates (color figure online)

sucrose, or ABA + sucrose were applied to the de-greening (DG) fruit. Application of ABA, sucrose, or ABA + sucrose obviously promoted strawberry fruit coloring, with the combined ABA + sucrose treatment having the faster effect (Fig. 1). Fruit treated with ABA + sucrose all reached the full-red color (FRC) stage on the 12th day after treatment (DAT), and this was at least 2 days earlier than the ABA treatment and control. The on,ly significant differences in coloring between the ABA and sucrose applications during fruit development and ripening were on the 8th, 12th and 14th DAT (Fig. 1a).

To ensure that the effect of ABA and sucrose was not limited to promoting development, the major fruit physiological parameters of full-red strawberries were measured. In general, application of ABA, sucrose, or ABA + sucrose had no significant effect on fruit weight, total soluble solids (TSS), anthocyanin accumulation, ascorbic acid content, the total phenolic content (TPC), lightness (L^*), chroma (C^*), and hue angle ($h°$) values when compared with the control (Table 1). However, the titratable acidity (TA) of fruit treated with ABA or sucrose and the total flavonoid content (TFC) of fruit treated with sucrose or ABA + sucrose were significantly reduced in comparison with the control (Table 1). Collectively, these results indicate that while ABA and sucrose promote strawberry ripening and reduce the time frame of fruit development and ripening, they have no or little effect on the final fruit quality of full-red strawberries including anthocyanin accumulation.

Changes in ABA and sucrose levels in the receptacles of treated fruit

To further explain the differences between treatments in accelerated ripening, we determined the levels of endogenous ABA and sucrose in treated fruit. Application of exogenous sucrose or ABA + sucrose markedly increased endogenous ABA and sucrose concentrations beyond the standard development and ripening-related increases observed for these compounds (Fig. 2). However, ABA application did not have such obvious effects. By comparing the three treatments, the ABA + sucrose treatment had a much greater effect than the ABA or sucrose treatments alone on the accumulation of endogenous ABA and sucrose in the fruit (Fig. 2). Combined with the previous statistical data regarding the effect of exogenous ABA and sucrose on fruit coloring in greenhouses (Fig. 1), these results suggest that the endogenous ABA and sucrose content is closely related to shorten the period length of strawberry fruit ripening.

Table 1 Effect of ABA, sucrose, and ABA + sucrose on fruit quality

Treatment	Weight (g)	TSS (°Brix)	TA (%)	Anthocyanin (mg/100 g)	AsA (mg/100 g)	TPC (mg/100 g)	TFC (mg/100 g)	L^*	C^*	$h°$
Control	14.42 a	12.65 a	1.08 a	3.25 a	94.42 a	39.23 a	15.20 a	38.94 a	50.18 a	32.08 a
Sucrose 100 mM	17.04 a	12.32 a	0.88 b	2.96 a	94.47 a	35.25 a	12.60 b	40.31 a	52.31 a	34.04 a
ABA 95 µM	16.19 a	13.09 a	0.95 b	3.18 a	100.01 a	39.49 a	14.46 ab	39.49 a	50.68 a	33.26 a
ABA + sucrose	14.87 a	12.62 a	1.13 a	3.42 a	95.03 a	38.18 a	11.71 b	38.50 a	51.52 a	32.18 a

Full-red strawberries once appeared after ABA, sucrose and ABA + sucrose treatment were collected for fruit physiochemical analysis. Values are means of three samples (each sample includes eighteen fruits). Overall significant difference ($P \leq 0.05$) is represented by different lower case letters as determined by Duncan's multiple range test. Lowercase letters indicate significant differences between treatments in each parameter. Anthocyanin, TPC, and TFC were measured as mg of pelargonidin 3-glucoside, gallic acid, and quercetin equivalents per 100 g FW

TSS total soluble solids, *TA* titratable acidity, *AsA* ascorbic acid, *TPC* total phenolic content, *TFC* total flavonoid content

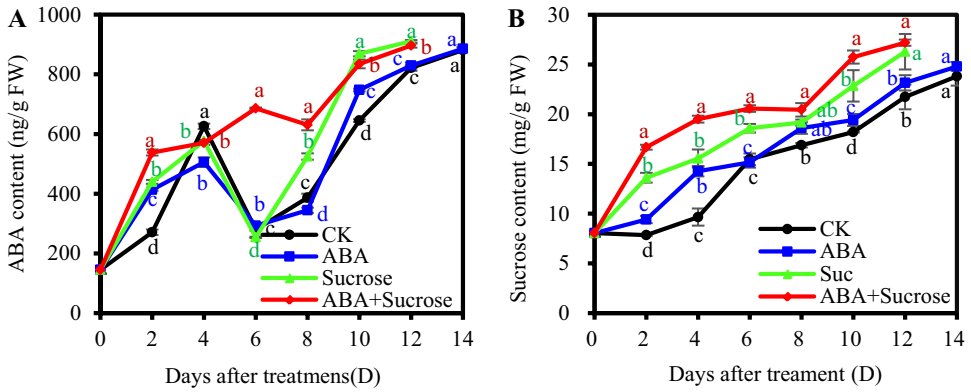


Fig. 2 ABA **a** and sucrose **b** contents in the receptacles of 95 μM ABA, 100 mM sucrose and 95 μM ABA + 100 mM sucrose-treated fruits during the development and ripening of strawberry. Values are mean ± SD of three biological replicates. Overall significant difference ($P \leq 0.05$) is represented by different lower case letters as determined by Duncan's multiple range test. Lowercase letters indicate significant differences between treatments on the same day after treatments

Effects of exogenous ABA and sucrose on ripening-related gene transcripts levels

To elucidate the molecular mechanism of exogenous ABA and sucrose in the regulation of strawberry fruit ripening, several ABA-responsive, sucrose-responsive, ripening-related, and glycolysis-related genes were examined, including the ABA biosynthesis gene (*NCED1*, *NCED2*), ABA-signaling regulators (*ABI4*, *SnRK2.2*), sucrose phosphate synthase (*SPS1*), sucrose synthase (*SuSy*), pigment and cell-wall metabolism-related genes (*MYB5*, *Cellulase 1*, *CEL1,* and *Cellulase 2*, *CEL2*), and fructose–bisphosphate aldolase (*ALDO*). Compared with their respective controls, the ABA biosynthesis gene (*NCED1*, *NCED2*), ABA signaling gene (*SnRK2.2*), and pigment and cell-wall metabolism-related genes (*MYB5*, *Cellulase 1*, *CEL1* and *Cellulase 2*, *CEL2*) were all up-regulated by ABA or sucrose treatment alone, but in particular, by the ABA + sucrose treatment on the 4th and 8th DAT (Fig. 3a–f), whereas ABI4 was not (Fig. 3g). The *SPS1* was down-regulated by ABA, sucrose, and ABA + sucrose treatment at the 4th DAT, but up-regulated by the ABA + sucrose treatment on the 8th DAT (Fig. 3h). Meanwhile, the expression of *SuSy* was up-regulated in ABA + sucrose treatment on the 4th DAT, 8th DAT, and 12th DAT, and down-regulated by ABA and sucrose treatment alone on the 8th DAT and 12th DAT (Fig. 3i). Except the fruit treated with sucrose on the 4th DAT and 12th DAT, the expression of *ALDO* was up-regulated by ABA, sucrose, and ABA + sucrose treatment (Fig. 3j). Taken together, ABA + sucrose treatment influenced some ABA biosynthesis genes, sucrose biosynthesis genes, anthocyanin, and cell-wall metabolism-related genes to regulate strawberry fruit ripening. The higher expression of the above genes in ABA + sucrose treatment suggested that ABA and sucrose accelerate fruit ripening by interaction.

Analyses of differentially expressed genes (DEGs)

RNA-seq was performed to further understand the molecular mechanism of ABA and sucrose in the regulation of fruit development and ripening. The clean raw data have been submitted to SRA database of NCBI (Accession ID: PRJNA565646; Link: https://www.ncbi.nlm.nih.gov/sra/PRJNA565646). Samples were taken from fruit treated with ABA, sucrose, or ABA + sucrose on the treatment day and on the 8th DAT. Comparing the ABA, sucrose, and ABA + sucrose libraries with the control library on the 8th DAT (i.e., CK8 versus ABA8, CK8 versus Suc8, and CK8 versus AS8), we identified 2575 DEGs (FDR < 0.05, $|\log_2 FC| \geq 1$) (Fig. 4a). In the comparison between CK8 and ABA8, a total of 394 genes were identified as being significantly differentially expressed, with 299 up-regulated and 95 down-regulated genes in the ABA8 fruit (Fig. 4a, Tables S1, S2). In the Suc8 fruit, there were 993 DEGs, including 558 up-regulated and 435 down-regulated genes (Fig. 4a, Tables S3, S4). In the AS8 fruit, there were 1188 DEGs with 616 up-regulated and 572 down-regulated genes (Fig. 4a, Tables S5, S6).

As shown in the Venn diagram (Fig. 4b), there were 163 DEGs that were common to the ABA, sucrose, and ABA + sucrose groups, and there were overlaps of 58 genes between CK8 versus ABA8 and CK8 versus AS8, 395 genes between CK8 versus AS8 and CK8 versus Suc8, and 61 genes between CK8 versus Suc8 and CK8 versus ABA8 (Fig. 4b). Furthermore, there were 141, 374 and 572 DEGs which were specific to the ABA, sucrose, and ABA + sucrose treatments, respectively (Fig. 4c). These results show that strawberry fruit exhibits distinct gene expression patterns in response to ABA, sucrose, and ABA + sucrose treatments. Moreover, the expression pattern in response to the ABA + sucrose treatment was not just an additive algorithm

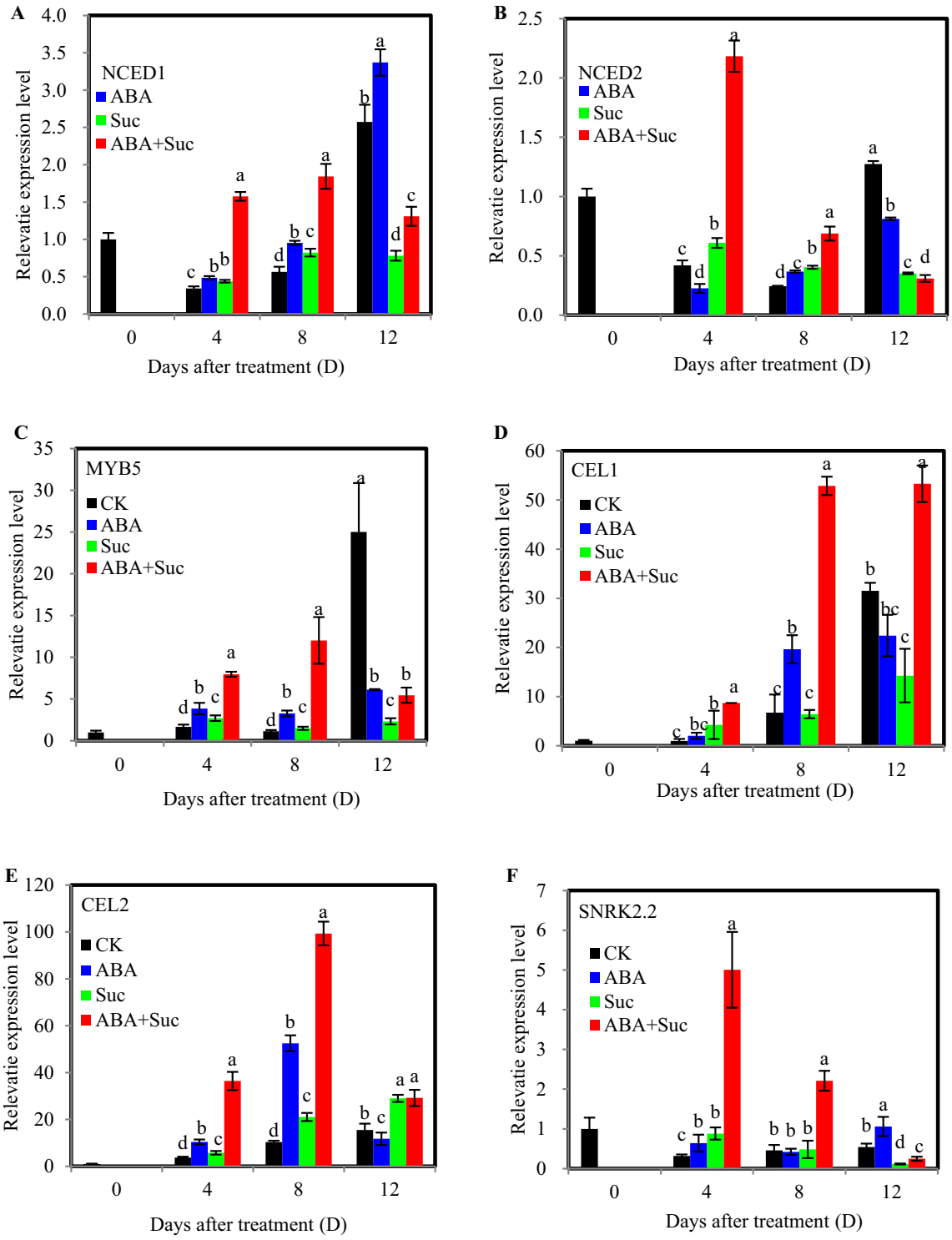


Fig. 3 Influence of exogenous ABA, sucrose and ABA + sucrose on the major ABA, sucrose, anthocyanins, glycolysis and cell wall metabolism genes. 9-cis-epoxycarotenoid dioxygenase (*NCED*), cellulase (*CEL*), sucrose non-fermenting 1-related protein kinase 2.2 (*SnRK2.2*), abscisic acid insensitive 4 (*ABI4*), sucrose phosphate synthase (*SPS1*), sucrose synthase (*SuSy*) and fructose-bisphosphate aldolase (*ALDO*). Values are mean ± SD of three biological replicates. Overall significant difference ($P \leq 0.05$) is represented by different lower case letters as determined by Duncan's multiple range test. Lowercase letters indicate significant differences between treatments on the same day after treatments

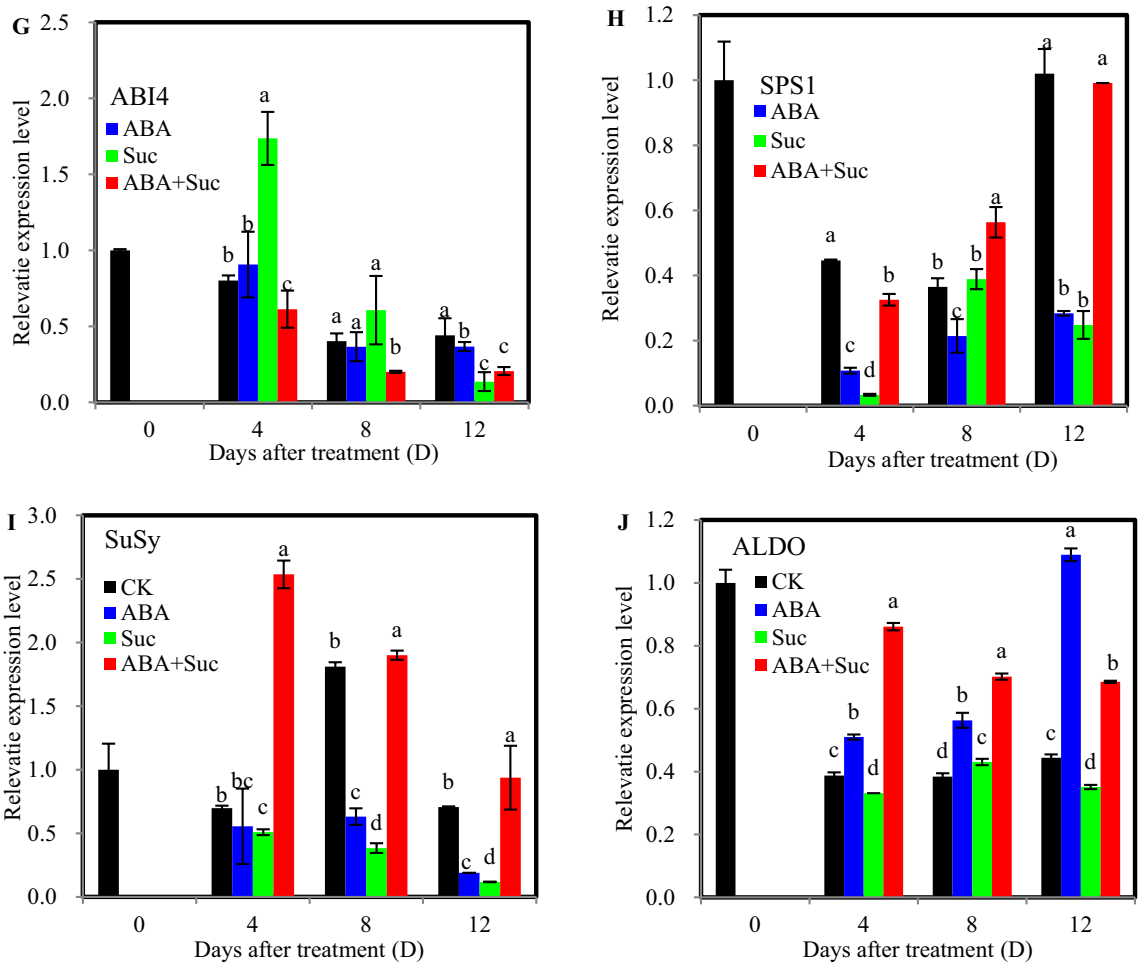


Fig. 3 (continued)

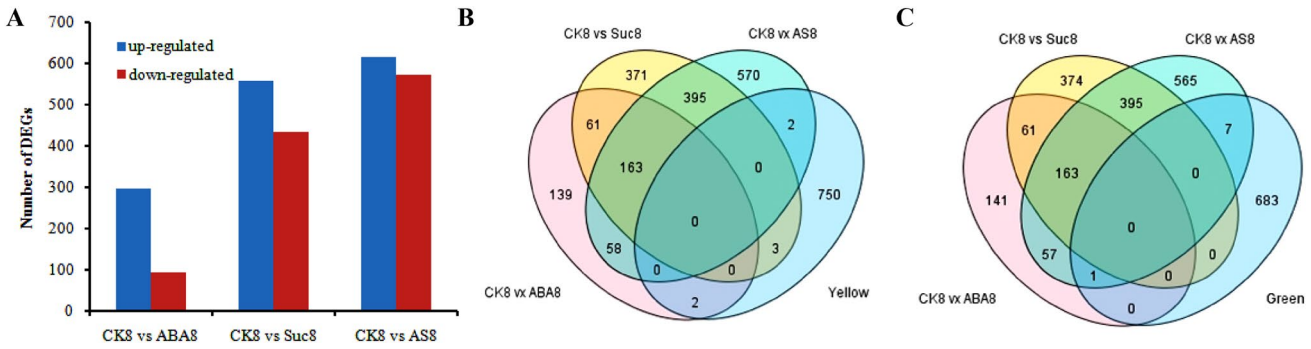


Fig. 4 Number of difference expression genes (DEGs) on the 8th day of ABA, sucrose, or ABA + sucrose (AS) treatment. **a** Number of up- and down-regulated DEGs in each comparison group. **b** Venn diagram showing the number of DEGs between three comparison groups and the yellow module. **c** Venn diagram showing the number of DEGs between three comparison groups and the green module

of the individual responses to the ABA and sucrose treatments, but included genes specifically induced by the combined ABA + sucrose treatment.

Co-expression network analysis using WGCNA

To discover associated co-expressed genes responding to ABA, sucrose, and ABA + sucrose treatments during the

ripening of strawberry fruit, weighted gene co-expression network analysis (WGCNA) was applied to the RNA-seq data. WGCNA is a systems biology method for understanding the correlation patterns and networks among genes (Langfelder and Horvath 2008). We constructed the co-expression network based on pairwise correlations between all genes across five sampled fruits and the major fruit ripening-associated parameters (ABA, ascorbic acid, sucrose, flavones, total acids, carotenoids, and anthocyanin). Eleven distinct modules (clusters) of highly correlated genes were obtained that are identified by different colors (Fig. 5a, b). Each tree branch in the dendrogram constitutes a module and each leaf in the branch represents one gene (Fig. 5a). The module eigengene can be considered the module's representative gene expression profile that was summarized using the first principal component of all modules' genes.

Among the 11 modules, the yellow module identifies 757 ABA-specific genes, and the green module identifies 691 AS-specific genes respect to threshold value (Fig. 5b). Meanwhile, correlation analysis showed that only those genes shown in yellow and green modules are significant correlated with the ABA8 and AS8 treatment, respectively (Fig. 5b). Interestingly, there was no significant correlation between sucrose treatment and gene expression in any modules, although sucrose treatment accelerates strawberry ripening (Fig. 5b). In addition, the Venn diagram showed that there were only two ABA-specific DEGs in the yellow module (Fig. 4b): these were homologs of genes encoding a DNAJ homolog subfamily C member 21-like (*DNAJ-like*) protein and histidine triad nucleotide-binding protein 3 (*HINT3*). There were seven AS-specific DEGs in the green module (Fig. 4c) that were homologs of nucleobase cation symporter-1 (*NCS1*), pyruvate kinase (*PK*), high-temperature protein (*HtpG*), glucose-6-phosphate isomerase (*GPI*), glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), fructose-bisphosphate aldolase (*ALDO*), and small subunit ribosomal protein S11e (*RP-S11e*), respectively. The expression patterns of these nine DEGs are presented as a heatmap (Fig. 5c). Interestingly, *PK*, *GPI*, *GAPDH,* and *ALDO* are all involved in glycolysis. In comparison with the CK8 control library, *PK*, *GPI*, *GAPDH,,* and *ALDO* were all significantly down-regulated, especially in the AS8-treated fruits (Fig. 5c). These results suggest that the above nine genes might be very important in regulating strawberry fruit ripening. ABA combined with sucrose can synergistically influence the expression of *NCS1*, *PK*, *molecular chaperone HtpG*, *GPI*, *GAPDH*, *ALDO,* and *RP-S11e*. Given that there was significant down-regulation of important enzymes (*PK*, *GPI*, *GAPDH,* and *ALDO*) in the glycolysis pathway (Fig. 5c) and additional DEGs identified in an enriched pathway branch of the ABA + sucrose-treated fruit that were related to glycolysis (Table 2), suggesting that inhibition of glycolysis occurs during accelerated strawberry ripening.

Verification of glycolysis inhibition

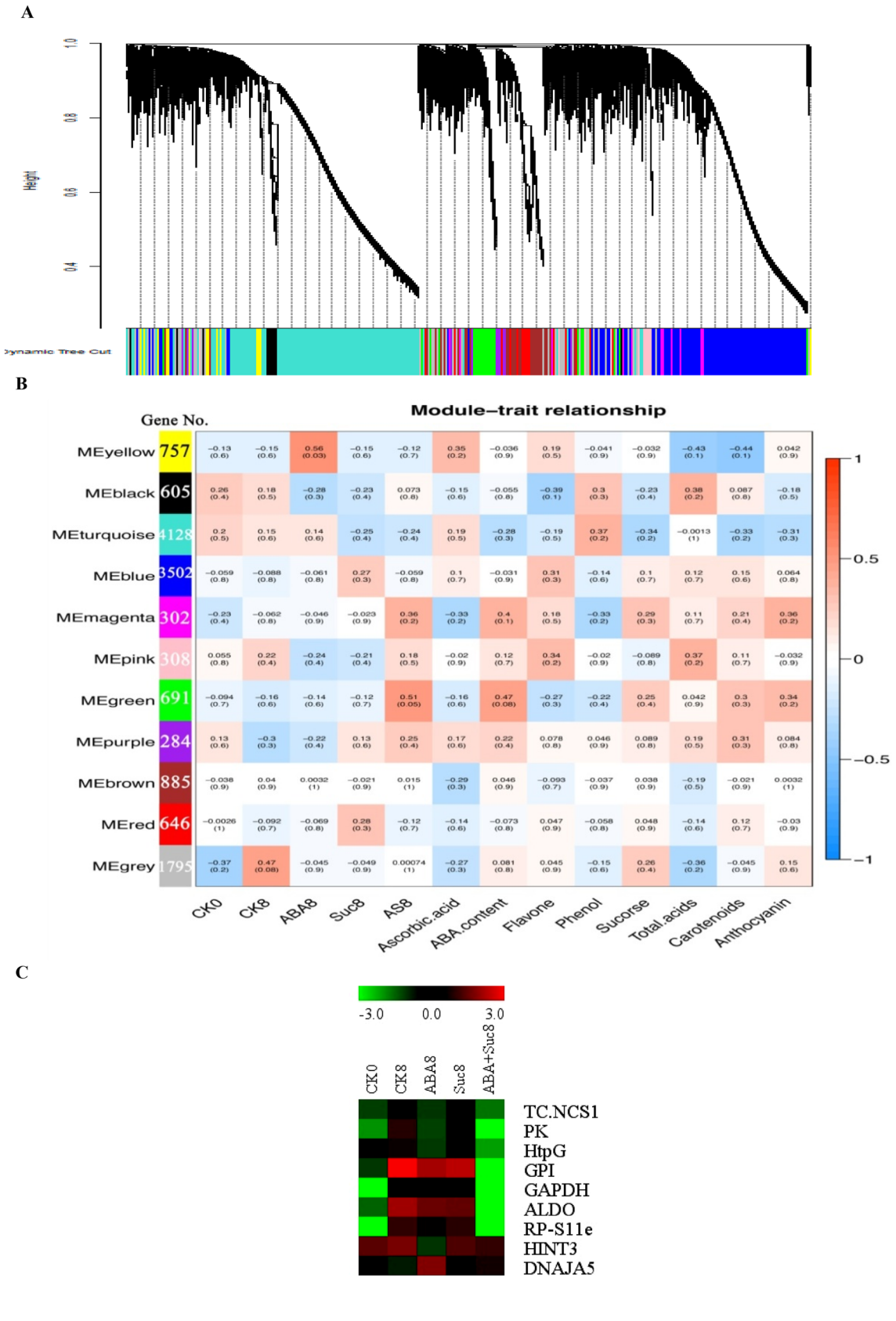
Pyruvate kinase is one of the rate-limiting enzymes in glycolysis. To further investigate whether inhibition of glycolysis was taking place at the expression level, we analyzed *FaPK* expression and the pyruvate content. Application of exogenous ABA, sucrose, and ABA + sucrose significantly increased *FaPK* expression levels on the 4th DAT compared to CK, but *FaPK* was down-regulated on the 8th DAT, especially in the ABA + sucrose treatment (Fig. 6a). Meanwhile, all treatments induced a significant decrease in pyruvate content on the 8th DAT (Fig. 6b). Furthermore, in vitro application of the glycolysis inhibitors 3-bromopyruvate (3-BrPA) and iodoacetamide (IAM), as well as ABA + sucrose, all led to faster fruit coloring than in the controls (Fig. 6c). These results show that inhibition of glycolysis promotes fruit coloring and that a short inhibition of glycolysis occurs during the development and ripening of strawberry fruit after application of ABA, sucrose, and ABA + sucrose.

H_2O_2 is involved in accelerating strawberry fruit ripening

Fruit ripening is closely related to senescence with these two overlapping and different processes occurring during the development and ripening of fruit (Brady 1987). Accelerating ripening is associated with more rapid senescence. Therefore, we wondered whether H_2O_2 is involved in accelerating strawberry fruit ripening. To investigate this hypothesis, we injected 100 mM GSH or a water control into the de-greening stage fruit, and then observed the subsequent fruit coloring. The percentage of full-red strawberries in the control was 31% and 85% on the 6th day and the 8th day after injection, respectively, while the percentages in the GSH-injected group were 4% and 60%, respectively (Fig. 7a). Therefore, GSH treatment obviously delays strawberry fruit coloring (Fig. 7a, b).

Next, we measured the H_2O_2 content in GSH-treated fruit, as well as in the ABA-, sucrose-, and ABA + sucrose-treated fruit. Higher levels of H_2O_2 on the 4th and 12th DAT were detected in the ABA-, sucrose-, and ABA + sucrose-treated fruits; the levels were particularly high in the ABA + sucrose-treated fruit (Fig. 7c). Significantly lower H_2O_2 concentrations on the 4th DAT were detected in GSH-treated fruit (Fig. 7d). These data support the hypothesis that H_2O_2 is involved in the modulation of fruit ripening.

The effects of GSH treatment on fruit coloring were the opposite to that of the ABA + sucrose treatment, so we wondered whether the *FaPK* expression levels and pyruvate content in GSH-treated fruit also had the opposite


A
Height
1.0
0.8
0.6
0.4
Dynamic Tree Cut
B
Module–trait relationship
Gene No.
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MEblack 605
MEturquoise 4128
MEblue 3502
MEmagenta 302
MEpink 308
MEgreen 691
MEpurple 284
MEbrown 885
MEred 646
MEgrey 1795
CK0
CK8
ABA8
Suc8
AS8
Ascorbic.acid
ABA.content
Flavone
Phenol
Sucorse
Total.acids
Carotenoids
Anthocyanin
1
0.5
0
−0.5
−1
C
-3.0
0.0
3.0
CK0
CK8
ABA8
Suc8
ABA+Suc8
TC.NCS1
PK
HtpG
GPI
GAPDH
ALDO
RP-S11e
HINT3
DNAJA5

◂**Fig. 5** Weighted gene co-expression network analysis (WGCNA) of genes in fruit. **a** Dendrogram produced by hierarchical clustering showing co-expressing modules identified by the WGCNA. Colors represent different modules. **b** Module eigengene network showing correlations between genes across five sampled fruits and the major ripening trait. Each row responds to a given module. The number of genes is indicated in the left modules. Eleven distinct modules of highly correlated genes were obtained that are identified by different colors. Each column responds to a specific treatment's fruit and the major ripening-related parameters. The correlation coefficient and the *P* value are both indicated in the cells and represent, respectively, the correlation degree, and the significance of correlation coefficient. A difference of $P \leq 0.05$ was significant. **c** Heatmap of ABA-specific difference expression genes (DEGs) in the yellow module and AS-specific DEGs in the green module generated using hierarchical clustering. Nucleobase Cation Symporter-1 (*NCS1*); Pyruvate Kinase (*PK*); High-temperature Protein (*HtpG*); Glyceraldehyde 3-Phosphate Dehydrogenase (*GAPDH*); Fructose-Bisphosphate Aldolase (*ALDO*); Small Subunit Ribosomal Protein S11e (*RP-S11e*); Histidine Triad Nucleotide-Binding Protein 3 (*HINT3*); DNAJ homolog subfamily C member 21-like (*DNAJ-like*); glucose-6-phosphate isomerase (*GPI*); *Fa*, *Fragaria ananassa*. The expression variance of each gene is indicated by colors ranging from low (green) to high (red) (color figure online)

trend to that detected in ABA + sucrose-treated fruit. We found that the GSH treatment significantly down-regulated *FaPK* expression and increased the pyruvate content until the 4th DAT (Fig. 7e, f). These results confirm that there are transient opposing patterns of *FaPK* expression between the GSH- and ABA + sucrose-treated fruit (Figs. 6a, 7e).

Discussion

The interaction between ABA and sucrose is central to the regulation of strawberry fruit ripening

In recent years, it has been shown that ABA and sucrose play a key role in strawberry fruit ripening (Ayub et al. 2016; Chai et al. 2011; Jia et al. 2011, 2013). Although it has also been shown that ABA and sucrose undergo extensive cross-talk (Carrari et al. 2004; Finkelsteina and Gibson 2002), more detailed information regarding the ABA/sucrose interaction in strawberry fruit ripening is not available. A better understanding of this ABA/sucrose interaction will greatly enhance our knowledge of the mechanisms at biochemical or molecular level that regulate strawberry fruit development and ripening. This study provides new information about how strawberry fruit ripening is altered by exogenous ABA and sucrose.

ABA and sucrose co-regulate strawberry fruit ripening (Jia et al. 2013, 2016). To demonstrate the role of the ABA/sucrose interaction in the regulation of strawberry fruit development and ripening, we first performed field experiments in a greenhouse. As shown in Fig. 1a, ABA, sucrose, and ABA + sucrose treatments dramatically accelerated fruit ripening, with the ABA + sucrose treatments having the greatest effect, similar to reported by Jia et al. (2017). To further explain the different degrees of accelerated fruit ripening observed among treatments, the endogenous ABA and sucrose content of treated fruit were measured after treatments. Interestingly, we observed that changes in endogenous ABA and sucrose content were closely correlated with the transition phase of fruit development, that is, the levels of ABA and sucrose in treated fruit nearly always remained higher than in the control, especially with the ABA + sucrose treatment (Fig. 2). Meanwhile, the expression levels of ABA biosynthesis genes (*FaNCED1* and *FaNCED2*) were up-regulated on the 4th and 8th after ABA or sucrose treatment alone but in particular, the ABA + sucrose treatment (Fig. 3a, b). In addition, the sucrose metabolism-related genes (*FaSPS1* and *FaSuSy*) were also up-regulated after the ABA + sucrose treatment (Fig. 3h, i). Previous reports have shown that ABA is the core signal, and sucrose also acts as a signal to induce ABA accumulation and promote strawberry fruit ripening by ABA-dependent and ABA-independent pathways (Jia et al. 2013). These results provide strong evidence that ABA and sucrose can regulate one other, and ABA and sucrose accelerate fruit ripening by interaction. Meanwhile, the distinct gene expression patterns of DEGs in response to ABA, sucrose, and ABA + sucrose treatments further suggest that the interaction is not the simple additive algorithm of ABA or sucrose treatment alone, but is a synergetic action. The complexity of the multiple, synergistic signals is probably the requirement for an orderly alteration of fruit development and ripening. Similar synergistic effects of ABA/sucrose interactions in promoting the synthesis of anthocyanin and starch and the expression of an ABA-stress-ripening (*ASR*) transcription factor have been previously observed in *Arabidopsis*, maize and strawberry (Huang et al. 2016; Jia et al. 2016; Loreti et al. 2008).

Physiological characteristics of full-red strawberry through ABA, sucrose, and ABA + sucrose application

Strawberry is a good source of flavonoids (Pillet et al. 2015). Due to their potential cytotoxicity and antioxidant abilities, flavonoids functions span from pigmentation, UV protection, and reactive oxygen species (ROS) scavengers, to human health protection (Pourcel et al. 2007). Flavan-3-ols and anthocyanins are the most abundant classes of strawberry fruit flavonoids (Carbone et al. 2009). Flavan-3-ols presumably protect immature berries from feeding by pest insects and animals and from pathogen attack at early growth stages. Anthocyanins impart a visual attractant to animals for berries consumption at full ripening (Almeida et al. 2007). The flavan-3-ols and

Table 2 List of significant DEGs-rich pathways after ABA, sucrose, and ABA + sucrose treatment

Pathway id	Pathway name	Sample number		
		CK8 versus ABA8	CK8 versus Suc8	CK8 versus AS8
Carbohydrate metabolism				
ko00052	Galactose metabolism	14		40
ko00040	Pentose and glucuronate interconversions	17	31	38
ko00650	Butanoate metabolism	8		20
ko00660	C5-Branched dibasic acid metabolism	6		
ko00500	Starch and sucrose metabolism		61	
ko00620	Pyruvate metabolism		42	73
ko00053	Ascorbate and aldarate metabolism		21	
ko00020	Citrate cycle (TCA cycle)			50
ko00630	Glyoxylate and dicarboxylate metabolism			64
Energy metabolism				
ko00195	Photosynthesis	18		44
ko00196	Photosynthesis—antenna proteins			41
ko00190	Oxidative phosphorylation			107
ko00710	Carbon fixation in photosynthetic organisms		63	
Signal transduction				
ko04075	Plant hormone signal transduction	30		
Lipid metabolism				
ko00592	α-Linolenic acid metabolism	19	23	35
ko00591	Linoleic acid metabolism	11	12	
Amino-acid metabolism				
ko00270	Cysteine and methionine metabolism	22	37	73
ko00290	Valine, leucine and isoleucine biosynthesis	10	13	
ko00260	Glycine, serine and threonine metabolism			60
ko00250	Alanine, aspartate and glutamate metabolism		39	
Metabolism of other amino acids				
ko00480	Glutathione metabolism	19		
Biosynthesis of other secondary metabolites				
ko00941	Flavonoid biosynthesis	11	16	
ko00940	Phenylpropanoid biosynthesis	20	16	
ko00950	Isoquinoline alkaloid biosynthesis			20
ko00966	Glucosinolate biosynthesis			5
Metabolism of terpenoids and polyketides				
ko00906	Carotenoid biosynthesis	9		
Metabolism of cofactors and vitamins				
ko00770	Pantothenate and CoA biosynthesis	12	18	
Metabolism of terpenoids and polyketides				
ko00909	Sesquiterpenoid and triterpenoid biosynthesis	7		
Transport and catabolism				
ko04145	Phagosome		36	
Environmental adaptation				
ko04712	Circadian rhythm—plant		18	27

flavonol levels show a decreasing trend during the development of strawberry fruit, and *p*-coumaric acids and anthocyanins levels show opposite developmental changes (Carbone et al. 2009). The developmental regulation of flavonoid metabolism can be regarded as an adaption of strawberry to environment (Carbone et al. 2009). In our

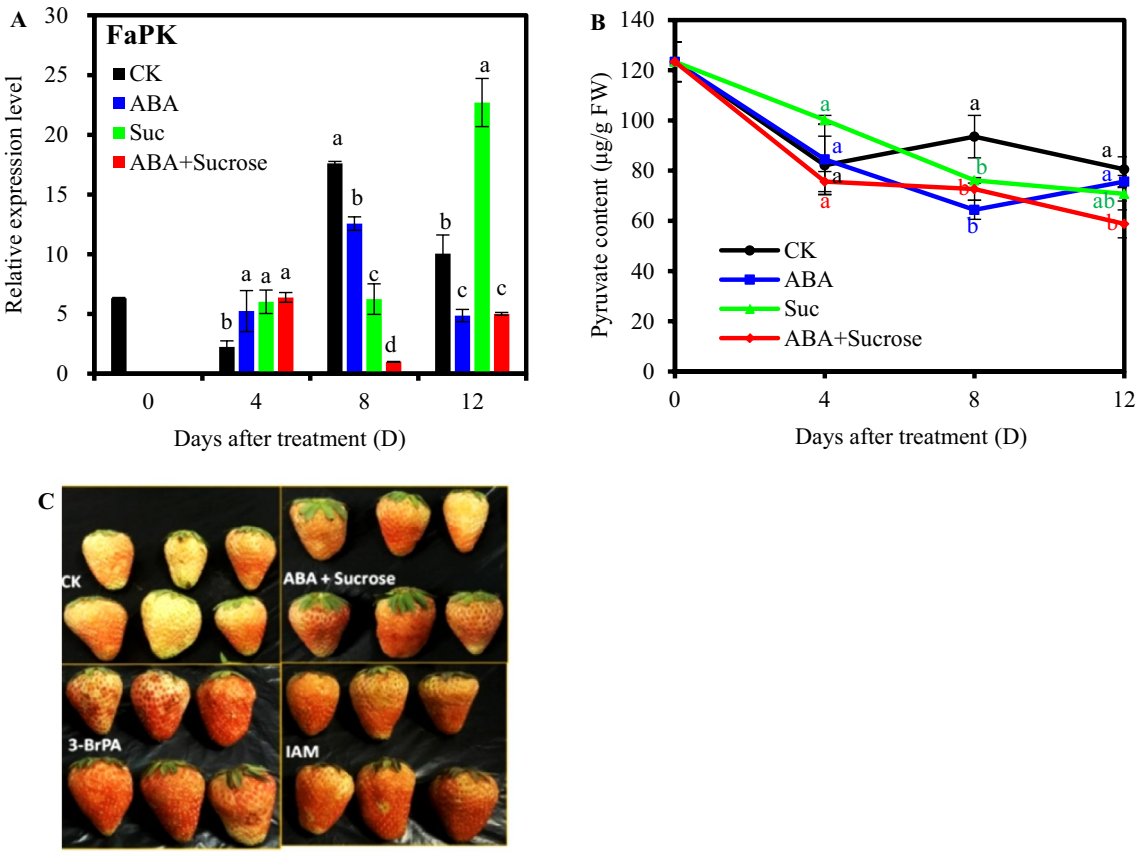

Fig. 6 Effect of exogenous ABA, sucrose and ABA + sucrose treatment on *FaPK* expression levels and pyruvate content, and exogenous ABA + sucrose, 3-bromopyruvate (3-BrPA) and iodoacetamide (IAM) on fruit coloring. **a** Changes in *FaPK* expression levels in ABA, sucrose and ABA + sucrose-treated fruits during the development and ripening of strawberry fruit. **b** Changes in pyruvate content in ABA-, sucrose- and ABA + sucrose-treated fruits. **c** Coloring of fruit treated with exogenous ABA + sucrose, 3-BrPA and IAM on the 2th day after treatment. *PK*: pyruvate kinase. Strawberries were treated with 95 μM ABA, 100 mM sucrose and 95 μM ABA + 100 mM sucrose at the de-greening stage for the measurement of *FaPK* expression levels and pyruvate content. Strawberry were treated with 95 μM ABA + 100 mM sucrose, 5 mM 3-BrPA, and 5 mM IAM at white stage for fruit coloring observation in vitro. Values are mean ± SD of three biological replicates. Overall significant difference ($P \leq 0.05$) is represented by different lower case letters as determined by Duncan's multiple range test. Lowercase letters indicate significant differences between treatments on the same day after treatments (color figure online)

study, sucrose could cause a negative impact on the total flavonoid content, and the synergistic effect with ABA was more detrimental on the TFC accumulation. Remarkably, besides the accelerated ripening, the unique difference between ABA + sucrose and the other treatments is the reduction on TFC (Table 1). ABA + sucrose application could induce a large amount of H_2O_2 production (Fig. 7c), the obvious decrease of TFC in ABA + sucrose-treated fruit may be attributed to flavonoids antioxidant abilities to scavenge H_2O_2.

Anthocyanins are accumulated at high levels at full-red stage and closely related with strawberry fruit quality. Most reports showed that ABA and sucrose application can promote the rapid accumulation of anthocyanin by regulating anthocyanin biosynthesis-related genes (Jia et al. 2013; Jiang and Joyce 2003). Interestingly, in the present study, we observed that ABA, sucrose, and ABA + sucrose treatment did not significantly affect the final levels of anthocyanin and the main color parameter values including L^*, C^*, and h° of full-red strawberries (Table 1). Meanwhile, the fruit weight and TSS were also not affected (Table 1). Of note, the time frame of fruit development and ripening was shortened, but most of the major quality parameters in treated-ripe fruit were not affected (Table 1). One possible explanation for this is that ABA/sucrose interaction improves the efficiency of the regulation of strawberry fruit ripening. The obvious increase in endogenous ABA and sucrose concentrations, enhanced in ABA and sucrose signaling, and up-regulation of ripening-related genes in ABA + sucrose-treated fruit provide for strong evidence for this hypothesis(Jia et al. 2016, 2017).

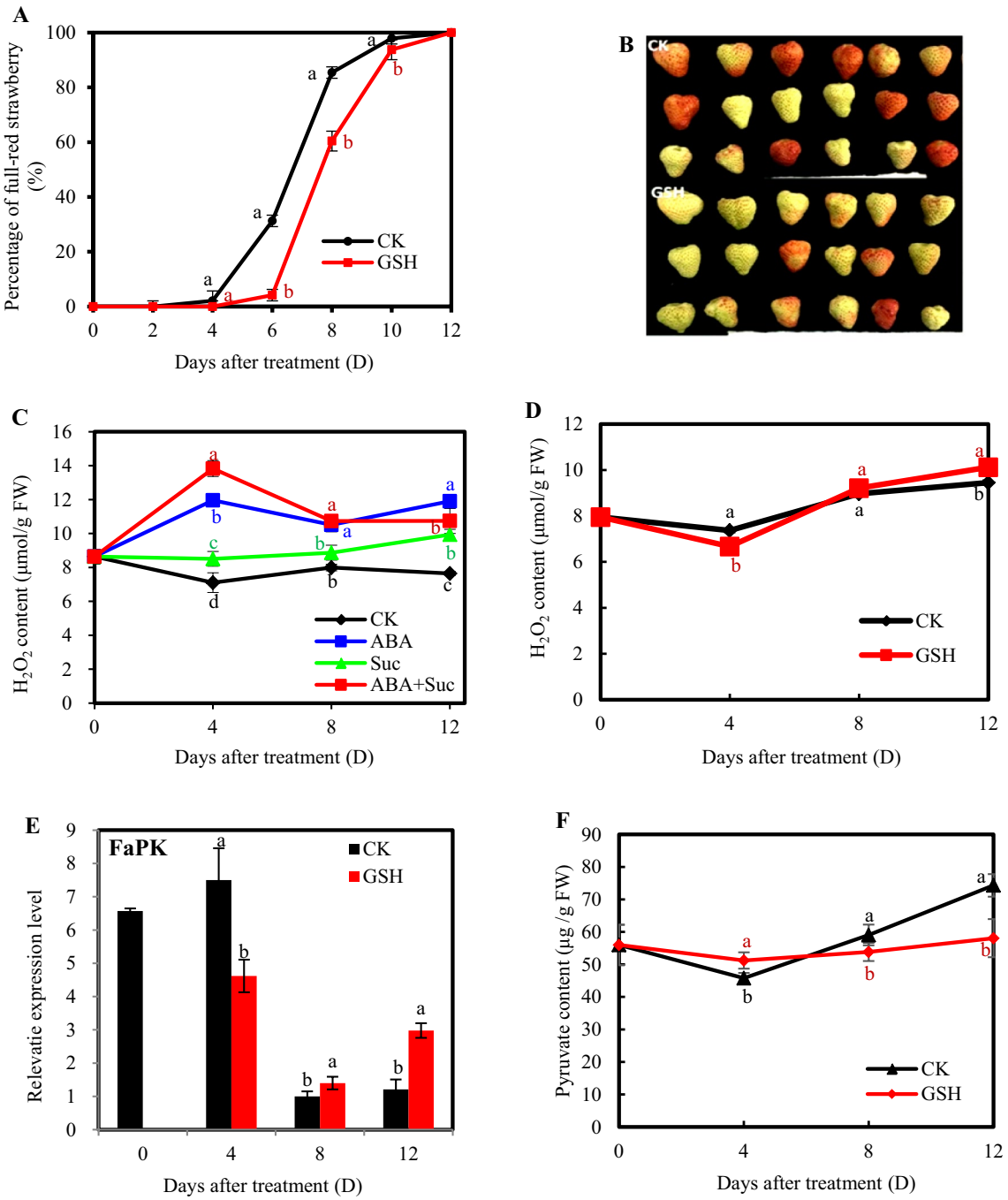

Fig. 7 Influence of exogenous ABA, sucrose, ABA + sucrose and GSH on H_2O_2 content and exogenous GSH on strawberry fruit coloring, *FaPK* expression levels, and pyruvate content. **a** Percentages of full-red strawberry after GSH treatment. 100 mM GSH and distilled water (control) were injected into the de-greening stage fruits. The percentage of full-red strawberries was recorded every 2 days intervals after treatment. **b** Fruit coloring following exogenous GSH treatment on the 4th day after treatment in the second group. These fruit were randomly harvested from the field. **c** Changes in H_2O_2 content in ABA-, sucrose- and ABA + sucrose-treated fruit during development and ripening. Exogenous ABA at 95 μM, sucrose at 100 mM and 95 μM ABA + 100 μM sucrose was applied to fruit at the de-greening stage. **d** Changes in the H_2O_2 content in GSH-treated fruit. **e** Changes in *FaPK* expression levels in GSH-treated fruits during the development and ripening of strawberry fruit. **f** Changes in pyruvate content in GSH-treated fruits. GSH: reduced glutathione; *PK*: pyruvate kinase. Values are mean ± SD of three biological replicates. Overall significant difference ($P \leq 0.05$) is represented by different lower case letters as determined by Duncan's multiple range test. Lowercase letters indicate significant differences between treatments on the same day after treatments (color figure online)

Inhibition of glycolysis is involved in the regulation of strawberry fruit ripening

Glycolysis is well known to be a crucial metabolic pathway that provides energy and precursors for fatty-acid and amino-acid syntheses (Plaxton 1996). Glycolysis converts glucose into pyruvate. In fact, recent studies have provided evidence that glycolysis have additional non-glycolytic functions, such as transcriptional regulation [hexokinase (HK)-2, lactate dehydrogenase (LDH)-A, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and enolase (ENO) 1], stimulation of cell motility [glucose-6-phosphate isomerase (GPI)] and apoptotic regulation (HK and GAPDH), which is attributed to some glycolytic enzymes (Kim and Dang 2005). The existence of multifaceted roles of glycolytic proteins suggests that links between metabolic sensors and transcription are established directly through enzymes that participate in metabolism (Kim and Dang 2005).

Our WGCNA analysis of RNA-seq data and pyruvate determination provided direct evidence that temporary inhibition of glycolysis is occurred. Meanwhile, the application of glycolysis inhibitors, 3-BrPA and IAM, which can inhibit the enzyme activity of glycolysis (Ganapathy-Kanniappan et al. 2009), contributed to strawberry fruit ripening (Fig. 6c). The inhibition of glycolysis would reduce the availability of pyruvate and hence could limit the availability of ATP and the synthesis of substrates required for tricarboxylic acid cycle (TCA) (Gahan et al. 1987). Recently, another study presented indirect evidence that suggested a link between glycolysis and strawberry fruit ripening. The E1 component subunit alpha of the pyruvate dehydrogenase gene (*PDHE1α*) is responsible for the conversion of pyruvate to acetyl-CoA that is then completely oxidized in the next TCA. Down-regulation of *PDHE1α* accelerated strawberry fruit ripening by inhibiting respiration and ATP synthesis (Wang et al. 2017). Therefore, we suggest that temporary inhibition of the glycolytic pathway may be an important aspect of strawberry fruit ripening induced by ABA and sucrose.

H_2O_2 may play a role in connecting ABA/sucrose interaction and glycolysis

H_2O_2 is an important signaling molecule that is involved in many biological processes, including stress response, programmed cell death, seed germination, plant growth, development, and fruit ripening (Gechev and Hille 2005; Kumar et al. 2016). ABA concentration is closely related to H_2O_2 generation (Kwak et al. 2003; Wang and Song 2008). In our study, we found that ABA, sucrose, and ABA + sucrose treatment increased the endogenous H_2O_2 level of strawberry fruit (Fig. 7c). This enhanced H_2O_2 production suggested that oxidative stress was being induced. Normally, plants can sense oxidative stress and then transmit "current status" information to activate protective mechanisms (Foyer and Noctor 2011) with the pentose phosphate pathway (PPP) being the major target under oxidative conditions (Krüger et al. 2011). Recently, it was reported that cytosolic GAPDH in glycolysis is considered to be an information hub linking metabolic alteration and stress signaling(Yang and Zhai 2017). Cytosolic GAPDH from *Arabidopsis* is involved in ABA-mediated H_2O_2 signaling in response to stress (Guo et al. 2012; Yang and Zhai 2017). The enhanced H_2O_2 production may lead to GAPDH inactivation and a rapid increase in G6PDH flux, and then result in rearrangement of central carbon metabolism and redirection of carbon flux to the PPP (Bedhomme et al. 2012; Kuehne et al. 2015; Ralser et al. 2007). Different degrees of down-regulation of *FaGAPDH* were also observed in our experiment after ABA and sucrose treatment (Fig. 5), supporting these findings. Of note, GSH acts as one of the most important antioxidants and scavengers (Kuehne et al. 2015); GSH treatment clearly delays strawberry fruit coloring and reduced H_2O_2 accumulation in our study (Fig. 7d), but also increased glycolysis (Fig. 7e, f). Therefore, the results suggest that H_2O_2 may play a role in connecting ABA/sucrose interaction and glycolysis. In addition, the majority of reports regarding the glycolysis/PPP transition counteracting oxidative stress have been in microorganisms, human tumors, and cancer cells (Krüger et al. 2011; Kruger and Schaewen 2003; Kuehne et al. 2015; Preter et al. 2016) with few reports in plants (Lehmann et al. 2009). Whether the glycolysis/PPP metabolic switch is involved in the regulation of **A**BA and sucrose mediated strawberry fruit ripening deserves further investigation.

In summary, this work uncovered a possible new mechanism for the alteration of ripening by ABA + sucrose. It was demonstrated that there is an acute synergistic effect between ABA and sucrose in accelerating strawberry fruit ripening. One of the aspect of this synergistic effect is that exogenous ABA and sucrose co-regulate endogenous ABA and sucrose levels and the expression of several ripening-related genes; another is the exogenous ABA and sucrose induces differences in the H_2O_2 level, which may affect glycolysis. Finally, this work suggests that ABA/sucrose interaction affects the ripening regulation efficiency and shows inhibition of glycolysis, and H_2O_2 may play a role in connecting ABA/sucrose interaction and glycolysis in regulating strawberry fruit ripening. This study provides new insights into the link between ABA/sucrose interaction and plant primary metabolism in regulation strawberry fruit ripening.

Materials and methods

Plant materials and treatments

Strawberry (*Fragaria*×*ananassa* cv. Benihoppe) plants were grown in a plastic greenhouse under natural culture conditions in Chengdu, China. Fruits were classified in seven developmental stages based on color changes (Jia et al. 2013): small green (SG, 7 days after anthesis), large green (LG, 14 days after anthesis), de-greening stage (DG, 18 days after anthesis), white (WT, 20 days after anthesis); initial red (IR, 23 days after anthesis), partial red (PR, 25 days after anthesis), and full red (FR, 28 days after anthesis) (Supplementary Figure 1). The experiment was carried out in the spring of 2016. Based on our previous experiments, totally about 2000 secondary flowers from at least 500 plants were tagged and the fruits at the de-greening stage (DG, 18 days after anthesis) were sprayed with 95 μM ABA, 100 mM sucrose, or 95 μM ABA + 100 mM Sucrose (ABA and sucrose were mixed in equal volumes) until dripping and with water as control and then divided the treated strawberries into two sampling groups. In one group, the processing of fruit development was recorded at 2-day intervals and the full-red berries once appeared were collected for fruit quality analyses, amounting to 18 berries from each treatment. In another group, 18 berries per treatment were randomly sampled from the beginning of the treatment to the time that all treated fruits reached at the full-red stage with 2-day intervals for the determination of ABA, sugar, and RNA extraction. All the berries for per treatment were sampled individually with three biological replications.

Fruit physiochemical analysis

After fruits were weighed using the electronic balance (Quintix124, Sartorius®, Germany), their color was determined by a chroma meter (CR-400, Konica Minolta, Japan) with color characteristics—L^*, $h°$ and C^* at three different parts around the equatorial region of each fruit. L^* means lightness. $h°$ means hue angle, which was calculated as $h° = \arctan(b^*/a^*)$, and C^* means chroma as $C^* = (a^{*2} + b^{*2})^{1/2}$). a^* indicates bluish-green/red–purple hue component, while b^* means yellow/blue hue component (McGuire 1992). Total soluble solids (TSS) were measured using a pocket refractometer (PAL-1, Atago, Japan), while the titratable acidity (TA) was determined by repeated titrations with 0.1 mol L^{-1} NaOH to a faint pink and the citric acid content was estimated as described previously (Han 1996).

The total anthocyanin determination was conducted using the pH differential method (Cheng and Breen 1991). Briefly, the mixed strawberry fruit (5.0 g) was extracted with 25 mL of cold 1% HCl-ethanol and centrifuged at 8000×*g* for 25 min at 4 °C, and then, the supernatants were used for measuring the total anthocyanin content. Results were shown as milligram of pelargonidin 3-glucoside equivalents per 100 g of fresh weight.

To determine the total phenolic content (TPC) and total flavonoid content (TFC), approximately 5.0 g of mixed strawberry fruit was used with 25 mL of 80% acetone extracting for 1 h at room temperature, followed by centrifugation (10 min, 4500×*g*) at room temperature and the supernatant was collected for the measurement of TPC and TFC according to the method described by Molan et al. (2009) and Chang et al. (2002), respectively. TPC and TFC were exhibited as mg gallic acid and quercetin equivalents per 100 g of fresh weight, respectively.

The ascorbic acid (AsA) measurements were based on the method of Sun (2007). About 5.0 g of mixed strawberry fruit was extracted using 30 mL of 5% (w/v) metaphosphoric acid, followed by centrifugation at 22,000 × *g* for 15 min and quantified at 525 nm for AsA. Results were expressed as mg of AsA per 100 g of fresh weight. All analyses were repeated three times using biological replicates.

Endogenous ABA and sucrose determination

The endogenous ABA and sucrose content were assayed by the ABA and sucrose determination kit (Beijing Chenglin Biotechnology Co., Ltd. and Suzhou Keming Biotechnology Co., Ltd.), respectively. The mixed strawberry fruit (0.5 g) was extracted with 10 mL of 80% methanol and centrifuged at 10,000×*g* for 20 min. The supernatant liquid was used for the ABA and sucrose determination, following the manufacturer's protocol. The experiments were performed as triplicates in three biological replicates.

RNA extraction and library preparation for RNA-seq

Considering that the percentage of full-red strawberry among treatments had a large difference 10 day after treatment (DAT) (Fig. 1), and the potential time scale from gene expression to phenotypic appearance, the fruits of 0 and 8 DAT were selected as timepoint of RNA-seq. The samples were denoted as CK0, CK8, ABA8, Suc8, and AS8 accordingly. RNA extraction of each sample consisting of six randomly selected treated fruits, using the RNeasy Plant Mini Kit (Qiagen, Dusseldorf, Germany) in conjunction with RNase-Free DNase (Qiagen). RNA quality and concentration were assessed using a Nanodrop 1000 spectrophotometer and an Agilent 2100 Bioanalyzer. Subsequently, 15 cDNA libraries for three biological replicates, corresponding to CK0, CK8, ABA8, Suc8, and AS8, respectively, were

constructed and sequenced with the Illumina HiSeq 2000 platform by Novogene (Beijing, China).

Sequencing data analysis and assembly

To ensure the quality of biological information analyses, clean reads were screened by removing the low quality reads (Qphred ≤ 20), including adapter and reads with more than 10% ambiguous bases (Li et al. 2015). Considering the incompleteness of the octaploid strawberry genome, all clean reads were then de novo assembly by Trinity software (version 2.4.0) (Grabherr et al. 2011). Only the longest transcripts were selected as unigenes.

Statistical of difference expression genes (DEGs)

The reads per kb per millon reads (FPKM) method was used to the calculation of gene expression (Shen et al. 2011). DEGs (FDR < 0.05, $|\log_2 FC| \geq 1$) of three comparisons were conducted: CK8 versus ABA8, CK8 versus Suc8, and CK8 versus AS8.

Validation of gene expression using quantitative real-time PCR (qRT-PCR)

qRT-PCR was conducted to confirm the reliability of RNA-seq results. Ten genes, which were, respectively, related to ABA biosynthesis (*NCED1* and *NCED2*), ABA-signaling regulators (*ABI4* and *SnRK2.2*), sucrose metabolism (*SuSy*, *SPS1,* and *ALOD*), pigment, and cell-wall metabolism (*MYB5*, *CEL1,* and *CEL2*) were selected, whose expression levels were listed in Fig. 3. The method of total RNA extraction was as described above. The same RNAs used in the RNA-seq were used in this assay and cDNA were synthesized as described above. The specificity of qPCR primers (Table S7) was confirmed by melting curve analyses. The relative transcription levels were calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001). *FaActin* gene (AB116565) was used as the internal control. The qPCR results were obtained from three technical replicates using biological replicates for all sequenced samples.

Co-expression network construction and relationship analysis

First, we constructed a gene co-expression network from genome-wide gene expression data and clustered thousands of transcripts into 11 co-expression modules. Next, the relationship between each treatment as well as physiological data (i.e., ABA content, sucrose content, and anthocyanin, etc.) and each module was identified combining with physiological data. Co-expression network was constructed using the weighted gene co-expression network analysis (WGCNA, v1.42) software package (Langfelder and Horvath 2008). A total of 13,905 genes with the coefficient of variation (cv) filtering (between 0.7 and 10, and where 20% of samples had expression > 1) were used for the WGCNA signed co-expression network analysis. The goal of CV filtering is to remove genes that are constitutively expressed, unexpressed, or vary only modestly across experimental treatments or conditions. The normalized reads per kilo base of transcript per million (NRPKM) was imported into WGCNA. Gene modules were obtained using the automatic network construction function blockwise modules with default settings except that the power was set to 20, TOMType was signed, min Module Size was set to 30, and merge Cut Height 0. The trait profiles including ABA, anthocyanin, sucrose, flavonoid, total acids, carotenoids, total polyphenols, and ascorbic acid contents were used as respective input files for the detection of relationship among traits and the eigengene of each module. The network was visualized using Cytoscape (v.3.4.0).

Treatment of glycolysis inhibitors and reduced glutathione (GSH)

Fruits ($n = 6$) at the white stage (WT, 20 days after anthesis) were selected and sprayed with 5 mM 3-bromopyruvate (3-BrPA), 5 mM iodoacetamide (IAM), or sucrose + ABA (a mixture of 95 μM ABA and 100 mM sucrose) in vitro. Then, these fruits were immersed into MS solutions with only their peduncle in controlled conditions (25 °C, 90% relative humidity, 8/16 h day/night). The treatment of water was used as control ($n = 6$). Two days after treatment, the phenotypes of these fruits were investigated. The experiment was repeated three times.

GSH treatment was carried out in the spring of 2017. The fruits at the de-greening stage (DG, 18 days after anthesis) were injected with 0.5 mL 100 mM GSH solution until dripping and with water as control, and then divided the treated strawberries into two sampling groups. In one group, the percentage of full-red strawberries was calculated every 2 days. In another group, 18 fruits of each treatment were randomly harvested at a 4-days interval for the determination of H_2O_2 and pyruvate level during the development and ripening of strawberry.

Determination of pyruvate and hydrogen peroxide (H_2O_2)

For the measurement of pyruvate, the method proposed by Anthon and Barrett (2003) was used with some modifications. The mixed strawberry fruit (0.5 g) was extracted with 10 mL of 8% (w/v) trichloroacetic acid for 30 min and centrifuged at 8000×*g* for 10 min. The 100 μL of supernatant was mixed with 200 μL of 8% (w/v) trichloroacetic acid

and 100 µL of 0.1% (w/v) 2, 4-two nitrophenyl hydrazine in 2 M HCl. The mixture was placed into a 37 °C water bath for 10 min, and then, 500 µL of 1.5 M NaOH was added. Pyruvate contents were determined by Microplate Spectrophotometer (Multiskan GO, Thermo, USA).

According to the method described by Cao and Jiang (2007) for the measurement of H_2O_2, the mixed strawberry fruit (0.5 g) was immersed in 25 mL of 80% acetone for 1 h, followed by centrifuging at 12,000×*g* for 20 min, and then the 1 mL of supernatant was mixed with 0.2 mL of 13 M ammonia and 0.1 mL of 5% (w/v) titanium sulphate. The mixture was centrifuged at 6000×*g* and 4 °C for 10 min, and then, the pellets were dissolved in 3 mL of 10% (v/v) H_2SO_4 and centrifuged at 5000×*g* for 10 min. The supernatant was quantified at 410 nm for H_2O_2. Results were expressed as µmol of per 1 g of fresh weight. All samples for per treatment were measured as triplicates in three independent biological replicates.

Statistical analysis

The data were analyzed by one way ANOVA test using SPSS software (Version 20; IBM, USA), and were expressed as mean ± SD. *P* value of ≤ 0.05 was considered a statistically significant difference (Duncan's multiple range test).

Author contributions YL and HT designed the experiments and wrote the manuscript. YL performed most of the experiments and analyzed the results together with Cong Ge. All authors discussed the results and commented on the manuscript.

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Compliance with ethical standards

Conflict of interest All the authors declare no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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