Comments to Author

Review of the paper "**Effect of SlSAHH2 on metabolites between over-expressed and wild-type tomato fruits**"

The paper investigated the effect of SlSAHH2 on metabolites between over-expressed tomato and wild-type tomatoes. The paper reported that 733 metabolites were obtained which were divided into 12 categories. The contents of serine, tryptophan, kaempferol, hesperidin and other ripening related substances in overexpressed tomato were higher than those in wild type tomato. According to the results, genetically modified tomatoes may ripen more easily than wild-type tomatoes. The results showed that SlSAHH2 had a certain effect on tomato fruit ripening, which provided a certain reference value for the subsequent research on SlSAHH2.

The paper is relatively well written, except for some parts (see below) that require clarification. Since I did not receive the manuscript in a line numbering format, I couldn't generate a separate report. Most of my comments/inquiries can be fixed and the authors should have answers to them. Ultimately, the paper meets the requirements of scientific publications in terms of format and objective, so I recommend publishing after some minor amendments

Effect of *SlSAHH2* on metabolites between over-expressed and wild-type tomato fruits

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Abstract

Background: Tomato (*Solanum Lycopersicum*) is an annual or perennial herb that occupies an important position in daily agricultural production. Tomato is one of the essential fruits and vegetables for humans, and its ripening process is regulated by a lot of genes. *SAHH*(S-Adenosyl-l-Homocysteine hydrolase, AdoHcyase, EC 3.3.1.1) is widespread in organisms and plays an important role in regulating biological methylation reactions. Previous studies have revealed that transgenic tomato over-expressed of *SlSAHH*2 ripened earlier as compared with wild-type, while the differences in metabolite and potential metabolomic mechanisms in fruits are not clear.
Objective: To investigate the effect of *SlSAHH*2 on metabolites between over-expressed tomato and wild-type tomato.
Methods: In this study, *SlSAHH2* over-expressed tomato fruits (OE-5# and OE-6#) and wild-type tomato fruits (WT) at breaker stage (Br) were selected for non-targeted metabolome analysis.
Results: All the metabolites identified by mass spectrometry were identified with the KEGG and HMDB database, and 733 metabolites were obtained. According to the superclass results through comparison in HMDB database, metabolites were divided into 12 categories. The differences between the two methods were analyzed by PLS-DA. According to VIP > 1 and P < 0.05, 103 different metabolites have been found between OE-5# and WT; and 63 different metabolites have been found between OE-6# and WT. Through KEGG pathway enrichment analysis of significantly different metabolites, 30 and 32 metabolic pathways were noted in OE-5# vs WT groups and OE-6# vs WT

groups, respectively.
Discussion: The metabolomic mechanism of *SlSAHH2* promoting tomato fruit ripening has been further elucidated.
Keywords: Tomato, Metabolite, *SlSAHH*, Fruit ripening

1 Introduction

Tomato (*Solanum Lycopersicum* L.) is an annual or perennial herb that occupies an important position in daily agricultural production (Liu et al. 2021). And also one of the most important vegetable crops in China (Su et al. 2021; Chen et al. 2009). Tomato fruits are rich in nutrients such as lycopene, beta carotene, vitamin C, and reduced glutathione (Huang et al. 2006a; Li et al. 2020). It also has some medicinal value where, tomato extracts have anticancer effects and reduces neoangiogenesis in breast tumors (Ma et al. 2009). Tomato is a common model plant for studying fruit ripening(Giovannoni 2004; Inaba 2007; Klee 2004). During fruit development, there are large changes in the levels of primary metabolites, including carbohydrates and acids, while at the onset of ripening, flavonoids and carotenoids begin to accumulate (Muir et al. 2001). Some studies on the interaction of plant hormones (ET and GA) during tomato ripening showed that GA treatment inhibited the metabolite changes during ripening, while ET treatment promoted the metabolite changes(Park & Malka 2022). Knockdown of NAC transcription factors member *SlNAC9* affected the metabolism of carotenoids and the ripening of tomato fruits (Feng et al. 2023). The glycine-rich RNA-binding protein RZ1A-Like (RZ1AL) also influenced tomato fruit ripening by participating in the regulation of carotene biosynthesis and metabolism (Li et al. 2022).

S-adenosyl-L-homocysteine hydrolase *SAHH* (S-Adenosyl-l-Homocysteine hydrolase, AdoHcyase, EC 3.3.1.1) is present in a variety of cells and is closely related to DNA methylation, catalyzing the reversible hydrolysis of S-adenosine homocysteine (SAH) into adenosine (Ado) and homocysteine (Hcy)(Turner et al. 2000). In plants, SAHH can regulate plant growth and development and pathogen defense. For example, after interfering with *SAHH* gene expression in tobacco and *Arabidopsis thaliana*, plants were dwarfed, leaves were wrinkled and dark green, lateral buds were increased, and aging was delayed (Li et al. 2008; Mull et al. 2006; Rocha et al. 2005; Tanaka et al. 1997). Inhibition of the expression of *SAHH* coding gene *PvSAHH1* in switchgrass resulted in decreased SAH content and lignin accumulation, and increased enzymatic hydrolysis efficiency of cell wall polysaccharides (Bai et al. 2018). In terms of pathogen defense, the expression of *SAHH* was significantly upregulated in Phytophthora infestation areas of potato leaves(Arasimowicz-Jelonek et al. 2013). Inhibiting the expression of *SAHH* in tobacco showed less viral replication and stronger viral resistance (Masuta et al. 1995). In tomatoes, simultaneous suppression of the expression of three *SAHH* coding genes by VIGS (viral-induced gene silencing) resulted in severe stunting of plants, drought tolerance and Pseudomonas syringae resistance (Pst DC3000) (Li et al. 2015). In addition, recent studies have shown that tomato *SAHH* also plays a role in fruit ripening. Microarray sequencing results showed that the expression of *SSN-U314915* (corresponding to *SlSAHH2*) was significantly higher at the fruit breaker stage than at the mature green stage and sharply decreased with 1-MCP treatment (Yan et al. 2013). Overexpression of *SlSAHH2* in tomatoes promotes ethylene synthesis and accelerates fruit ripening(Yang et al. 2017a).

Although transgenic tomato overexpressed *SAHH2* ripened earlier, the differences in metabolic components during ripening are not clear. Plant metabolomics is the qualitative and

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quantitative analysis of all metabolites in plant samples using a variety of highly sensitive instruments and common metabolomics techniques e.g., LC-MS, GC-MS, etc(Chang & Wang 2015). For example, some researchers used HSSPME-GC-MS, UHPLC-Q-Exactive HF/MS and HPLC techniques to study the changes of compounds in black tea during the wilting process(Fang et al. 2023). Metabolomics is divided into untargeted metabolomics, targeted metabolomics and broadly targeted metabolomics, in which untargeted metabolomics is a comprehensive, unbiased analysis of metabolites in the organism. Untargeted metabolism refers to metabolomics aimed at integrating biological life processes with end-product outcomes to provide a more complete understanding of the overall mechanism (Guo et al. 2017). As more information becomes available for testing substances, nontargeted metabolomics has a wider coverage (Zhang & Chen 2021). In this study, *SAHH*2 over-expressed (*SAHH*2-OE) tomato at breaker stage (Br) (Br is often used as a sign of ripening, Chen 2020) was chosen for untargeted metabolome analysis (LC-MS) to further investigate the mechanism of the effect of *SlSAHH2* on tomato fruit metabolites.

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2 Materials & Methods

2.1 Plant materials and samples

The seeds of the *SlSAHH2* over-expressed (OE-5# and OE-6#) and wild-type (WT) were placed in a shaker (220 rpm, 25 ℃) until the hypocotyls of the seeds were clearly developed. The seeds were planted in nourishing soil at 25 ℃ (day/night) and watered regularly. Tomato fruit was collected at breaker stage (Br). Fruits of the same size were taken and immediately frozen with liquid nitrogen and refrigerated at -80℃. Five biological replicates were applied in the experiment.

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2.2 Sample collection

Each sample weighed of 50 mg was put in a 2 mL centrifuge tube. A total of 400 μL of extraction solution (methanol: water = 4:1) was added into the tube, and the extraction solution contains 0.02 mg/mL of internal standard (L-2-chlorophenyl alanine). Ground samples with a frozen tissue grinder at -10 °C for 6 min (50 Hz). The temperature-controlled ultrasonic cleaner was then used for freezing ultrasound at 5 °C for 30 min (40 kHz). The samples were put at -20℃ for 30 min. Centrifuge at 4 °C for 15 min (13,000g). The supernatant was taken for LC-MS analysis.

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2.3 LC-MS analysis

UHPLC-Q Exactive system and UHPLC tandem Fourier transform mass spectrometry (Thermo Fisher Scientific) were used for determination. Chromatographic condition: the column is ACQUITY UPLC HSS T3 (100 mm × 2.1 mm i.d., 1.8 μm; Waters Milford, USA）； The mobile phase A was 95% water + 5% acetonitrile (0.1% formic acid); and the mobile phase B was 47.5% acetonitrile + 47.5% isopropanol + 5% water (0.1% formic acid); the flow rate was 0.40 mL/min, the sample size was 2 μL, and the column temperature was 40 ℃. The positive and

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negative ion scanning ionization method was used to collect mass spectrum signals respectively. The scan type was 70-1050. The sheath gas flow rate was 40 arb, the auxiliary gas flow rate was 10 arb, the heating temperature was 400 ℃, the capillary temperature was 320 ℃, the positive spray voltage was 3500 V, and the negative spray voltage was -2800 V. Quality control samples (QC) were prepared by mixing the extracts of all samples in equal volume. The volume of each QC was the same as that of the samples and treated and tested in the same way as the samples which were used for analysis. In the process of instrumental analysis, one QC sample was inserted in every 5-15 analysis sample to investigate the stability of the whole detection process.

2.4 Statistical analysis of data

Through baseline filtering, peak recognition, integration, retention time correction and peak alignment, the raw data were inputted into the metabolomics processing software Progenesis QI (Milford Water, USA), and the resulting data matrix included retention time, mass charge ratio and peak intensity. Then Progenesis QI was used to establish a retrieval library, and the MS and MS/MS mass spectrometry information was matched with the metabolite database. Meanwhile, the mass error of the mass spectrometry was set to be less than 10 ppm, and the metabolite identification was carried out based on the secondary database. The obtained data matrix contained Metabolite, Metab ID, Library ID, KEGG Compound ID, M/Z, Retention time, Mode, Formula, Fragmentation Score, Theoretical Fragmentation Score, Mass Error (ppm), CAS ID, RSD of each metabolite in the sample. Partial Least Squares Discriminant Analysis (PLS-DA) was carried out using unit variance conversion for anionic mode and cationic mode respectively by Par, confidence degree 0.95, conversion times: 200. According to the superclass classification of HMDB (Human Metabolome Database), the total metabolite classification map of tomato fruits was obtained. Functional annotations of metabolic pathways were identified from the KEGG (Kyoto Encyclopedia of Genes and Genomes) to find pathways corresponding to differential metabolites.

3 Results

3.1 The main metabolites in WT and transgenic tomato fruits

The periods of tomato fruit development and ripening include the immature green stage (IMG), mature green stage (MG), breaker color stage (Br), orange color stage (O), red color stage (R) and overripe stage (OR) are shown in Fig. 1A.. After using tomato fruit at the Br stage for LC-MS, 733 metabolites were obtained from primary and secondary mass spectrometry data (File S1). And 547 metabolites were revealed from the HMDB database for a total of 12 categories (File S2). Among them, lipids and lipid-like molecules (41.13%) account for the largest proportion (File S2, Fig 1B).

The total of 15 samples were divided into three groups, corresponding to WT, OE-5# and OE-6#, and five replicated samples were within 95% confidence circle. Thereby, all the samples were valid, and the three substances were mostly separate from each other (Fig. 1C). The results of PLS-DA analysis showed that the main components of metabolism in WT, OE-5# and OE-6#

lines were different.

3.2 Differentially accumulated primary metabolites in WT and transgenic fruits

PLS-DA Analysis showed that all five replicas of WT and OE-5# were uniformly distributed in 95% of the confidence rings with no abnormal samples, so each group of five replicas was a valid sample (Fig. 2A and 2B). The Permutation test of PLS-DA showed that PLS-DA analysis was valid. PLS-DA analysis showed that all five replicas of WT and OE-6# were uniformly distributed in 95% of the confidence rings with no abnormal samples, so each group of five replicas was a valid sample (Fig. 2C and 2D). The Permutation test of PLS-DA revealed the viability of PLS-DA analysis. The distance between the experimental and control group indicates differences in metabolites between the two groups.

Figure 2 PLS-DA analysis of metabolites identified of *SlSAHH2* overexpressed tomato and wild-type tomato. (A) Metabolites identified in OE-5# and WT via PLS-DA analysis. (B) The corresponding Permutation test of PLS-DA: R2= (0, 0.9948), Q2= (0, 0.079). (C) Metabolites identified in OE-6# and WT via PLS-DA analysis. (D) The corresponding Permutation test of PLS-DA: R2= (0, 0.9647), Q2= (0, 0.0617)

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3.3 Map of the volcano for *SlSAHH2* over-expressed tomato and wild-type tomato

It was confirmed that the variation trends of differential metabolites of OE-5# vs WT and OE-6# vs WT were not identical. There were 103 different metabolites in OE-5# and WT, among which 58 red marked metabolites were up-regulated e.g. dehydrotomatine, L-serine, hesperetin and 45 blue marker metabolites were down-regulated (Fig. 3A and File S3). According to the screening conditions of VIP > 1 and $P < 0.05$, 85 out of 103 above mentioned metabolites in OE-5# vs WT group could be classified in the HMDB database (File S4).. Similarly, there were 63 different metabolites in OE-6# and WT, among which 58 red marked metabolites were up-regulated e.g. L-Tryptophan, 1,3-Dicaffeoylquinic acid, Kaempferol, Gallic acid and 5 blue marked metabolites were down-regulated (Fig. 3B and File S5). According to the screening conditions of VIP > 1 and $P < 0.05$, 43 out of the 63 different metabolites between OE-6# and WT, could be classified in the HMDB database (File S6).

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3.4 Bubble diagrams revealed different metabolites in *SlSAHH2* over-expressed and WT fruits

Through KEGG pathway enrichment analysis of significantly different metabolites, 30 metabolic pathways were noted in OE-5# and WT groups (File S7), including 12 significant metabolic pathways ($P < 0.05$), namely glycine, serine, and threonine metabolism, ABC transporters, purine metabolism, aminoacyl-tRNA biosynthesis, pyrimidine metabolism, caffeine metabolism, valine, leucine and isoleucine biosynthesis, tryptophan metabolism, monobactam biosynthesis, glycerol phospholipid metabolism, phenylpropanoid biosynthesis, tyrosine metabolism (Fig. 4A). Among them, glycine, serine and threonine metabolism, ABC transporters, purine metabolism, aminoacyl tRNA biosynthesis, pyrimidine metabolism, caffeine metabolism, valine, leucine and isoleucine biosynthesis, and tryptophan metabolism were included in the most significant metabolic pathways ($P < 0.01$) which had eight pathways (File S7). Through KEGG Pathway enrichment and analysis of significantly different metabolites, 32 metabolic pathways were noted in OE-6# and WT groups (File S8), and there were six significant metabolic pathways ($P<0.05$), namely aminoacyl-tRNA biosynthesis, purine metabolism, pyrimidine metabolism, ABC transporters, beta-Alanine metabolism, Tropane, piperidine and pyridine alkaloid synthesis (Fig. 4B). Among them, aminoacyl tRNA biosynthesis, purine metabolism, pyrimidine metabolism and ABC transporters were included in the most significant metabolic pathways ($P < 0.01$) which had four pathways(File S8).

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4 Discussion

Metabolomics is currently a broad, sensitive and practical approach. The analysis of plant phenotypes by differential metabolites is a good method (Patel et al. 2021). Kissoudis et al. studied the changes of metabolites of GSTs overexpressed tobacco plants under salinity stress. Protective metabolites such as proline and trehalosaccharide of transgenic plants had higher concentrations under salt stress, and the results showed that GmGSTU4 contributed to salt stress tolerance(Kissoudis et al. 2015). In this study, different metabolites in overexpressed SAHH and wild-type tomato were compared to explore the early ripening mechanism of overexpressed tomato. The metabolites of transgenic and wild type were screened by LC-MS non-targeted liquid chromatography metabolomics. According to the comparison of the superclass results of HMDB (Human Metabolome Database), metabolites were divided into 12 classes. PLS-DA and other methods were used to analyze the differences between transgenic and wild type tomato. The upregulated metabolites between OE-5# and WT were mainly dehydrotomatine and L-serine. Interestingly, dehydrotomatine and L-serine were proved to play a role in tomato fruit development or ripening. In detail, dehydrotomatine accumulates in tomato leaves, flowers and ripe green fruits, and has a defense effect against bacteria, fungi and insects (Lee et al. 2019). Serine was increased during fruit ripening due to glycolysis (Lee et al. 2010). The upregulated metabolites between OE-6# and WT were mainly L-Tryptophan, 1,3-Dicaffeoylquinic acid, Kaempferol, Gallic acid. Their role has been reported in several species. For example, tryptophan and glycerol levels increased in ripened ginsing berries (GB) and (Park et al. 2019). During grape berry development, veraison and kaempferol revealed the highest content at ripening phase (Fang et al. 2013). During the ripening of mulberries, gallic acid increased from pink to red

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(Saracoglu 2018).

According to the cysteine and methionine metabolism pathway, S-adenosyl homocysteine (SAH) is hydrolyzed by adenosyl homocysteinase (SAHH) to generate homocysteine (Hcy), so the Hcy in *SAHH2* over-expressed tomato is inferred up-regulated. Generally, L-serine and Hcy can produce L-cystathionine through cystathionine β-synthase(Xu et al. 2017). The abundance of L-serine in OE-5# and OE-6# was up-regulated compared with the wild type (Fig 5), so it was speculated that L-cystathione was also up-regulated. At the same time, under the action of γ-lyase, L-cystathionine can produce L-Homocysteine, so it is speculated that the abundance of L-homocysteine in transgenic fruit may be up-regulated compared with the wild type. Similarly, as L-methionine could be produced from L-homocysteine through methionine synthase, it is speculated that L-methionine in transgenic fruits may increases(Shen 2019). Combined with the trans-methylation pathway of methionine, S-adenosyl-L-methionine could be produced from L-methionine through methionine adenosine transferase. S-adenosyl-L-methionine can accelerate production of 1-aminocyclopropane-1-carboxylic acid and S-methyl-5'-thioadenosine through S-adenosine-l-methionine methionine adenosine lyase(Avila et al. 2004; Tang et al. 2008).

The content of terminal chemical compound (Hesperetin, Kaempfrerol, S-Methyl-5-thioadenosine) were all higher in transgenic tomato fruits than that in WT fruits (Fig 5). ACC is the precursor of ethylene synthesis, the up-regulation of ACC can reflect the increase of ethylene in fruit (Mou et al. 2020; Xie et al. 2009). At the same time, ethylene promotes flavonoid synthesis (Ni et al. 2020). Combined with the increase content of S-adenosyl-L-methionine we just inferred and the reported high ethylene level in transgenic fruit (Yang et al. 2017b), the metabolic mechanism of *SlSAHH2* promoting fruit ripening can be further clarified.

5 Conclusions

In this study, LC-MS non-targeted liquid chromatography metabolomics was used to screen metabolites of transgenic and wild type. PLS-DA was used to analyze whether there was any difference. The results showed a difference between 5OE, 6OE and WT. Then, corresponding screening was conducted and metabolites were labeled through databases (Kyoto Encyclopedia of Genes and Genomes and Human Metabolome database) to obtain the total metabolite classification map of tomato. The contents of serine, tryptophan, kaempferol, hesperidin and other ripening related substances in overexpressed tomato were higher than those in wild type tomato. According to the results, genetically modified tomatoes may ripen more easily than wild-type tomatoes. The results showed that *SlSAHH2* had a certain effect on tomato fruit ripening, which provided a certain reference value for the subsequent research on *SlSAHH2*.

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DECLARATION OF COMPETING INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this article

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.