Original Article

Proteome-wide identification of non-histone lysine methylation in tomato during fruit ripening

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highlights

• A total of 241 sites of lysine methylation in 176 proteins were identified in tomato during fruit ripening.
• The methylated proteins were mainly related to fruit ripening, redox process, signalling, stress responses, and energy metabolism.
• Mimicking demethylation led to decreased TRX activity but increased GST T1 and NOX activities.

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abstract

Introduction: Histone and non-histone methylations are important post-translational modifications in plants. Histone methylation plays a crucial role in regulating chromatin structure and gene expression. However, the involvement of non-histone methylation in plant biological processes remains largely unknown.

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Introduction

Post-translational modifications (PTMs) are important mechanisms that modulate protein functions in organisms. PTMs regulate protein functions by affecting stability, localization, interactions with other biomacromolecules. There are multiple PTMs in organisms, including methylation, acetylation, phosphorylation, ubiquitination, SUMOylation and S-nitrosylation. Of these modifications, protein methylation plays important regulatory roles in gene expression, protein activity and stability, and signal transduction (2). (See Table 1.)

Protein methylation mainly occurs on lysine or arginine residues. Lysine is a frequent modification target of all amino acids, which can provide docking sites for the binding of effector proteins or inhibit alternative PTMs at the same lysine residue. Lysine can be methylated to different degrees, including mono-, di-, or trimethylation, which reflects its functional diversity and regulatory complexity compared to other PTMs (3). Protein methylation mainly includes histone and non-histone methylation. Most studies have focused on histone lysine methylation to understand its regulatory roles in chromatin biology and gene regulation (4). Methylation is associated with not only gene activation but also gene silencing, compared with acetylation that is associated with gene activation only. Genes associated with histone H3K9 or H3K27 methylation are usually repressed, whereas those marked by histone H3K4 or H3K36 methylation are generally activated (5).

Expanding the function of methylation beyond histones, lysine methylation on non-histones also plays important regulatory roles in multiple biological processes (6-8). p53, an important tumour suppressor, is the first non-histone protein to be found susceptible to methylation. Methylation enhances or suppresses its activity depending on the methylation site and degree (9-12). Recently, more non-histones have been found to be modified by methylation. Heat shock proteins (HSPs) are non-histone proteins and play important roles in cancer (13). The increased methylation level of K561me2 in HSP70 accompanies the development of tumours (14,15). Other non-histone proteins susceptible to methylation include some transcription factors, such as NF-kB (16), AR (17), RB (18), ERα (19), EZF1 (20), GAGA (21), STAT (22), and Numb (23) and their activities can be regulated by methylation. Protein methylation regulates the occurrence and development of diseases by affecting protein activity, or subcellular location, or interaction with other proteins, or crosstalk with other modifications (15,24). However, studies on the function of non-histone methylation in plants are rarely reported, and the study results presented to date need to be clarified.

Methods: The methylated substrates and methylation sites during tomato fruit ripening were identified by LC-MS/MS. Bioinformatics of lysine methylated proteins was conducted to analyze the possible role of methylated proteins. The effects of methylation modification on protein functions were preliminarily investigated by site-directed mutation simulation.

Results: A total of 241 lysine methylation (mono-, di- and trimethylation) sites in 176 proteins were identified with two conserved methylation motifs: xxxxxxExxx_K_xxxxExxxxxxxx and xxxxxxExxx_K_xxxxExxxxxxxx. These methylated proteins were mainly related to fruit ripening and senescence, oxidation reduction process, signal transduction, stimulus and stress responses, and energy metabolism. Three representative proteins, thioredoxin (Trx), glutathione S-transferase T1 (GST T1), and NADH dehydrogenase (NOX), were selected to investigate the effect of methylation modifications on protein activity. Mimicking demethylation led to decreased Trx activity but increased GST T1 and NOX activities. In addition, RT-qPCR exhibited that the expression of many genes that encode proteins subjected to methylation was upregulated during fruit ripening.

Conclusion: Our study suggests that tomato fruit ripening undergo non-histone lysine methylation, which may participate in the regulation of fruit ripening. It is the first report of methyl proteome profiling of non-histone lysine in horticultural crops.

Materials and methods

Sample collection and protein extraction

Wild-type tomato (Solanum lycopersicum, Mill. cv Ailsa Craig) was grown in a greenhouse at 20–24 °C under a 16 h: 8 h, light: dark photoperiod. Fruits at mature green (MG), break (BR) and red ripening (RR) stages were obtained and mixed. The mixture was ground into cell powder with liquid nitrogen, and the total proteins were extracted with the Plant Total Protein Extraction Kit (BANGFEI BIOSCIENCE, Beijing, China).

Trypsin digestion and HPLC fractionation

The extracted proteins were reduced with dithiothreitol (5 mM) at 56 °C for 30 min, followed by alkylation with iodoacetamide (11 mM) at 25 °C for 15 min under dark condition. The alkylated proteins were then digested with trypsin at a mass ratio of 1:50 (trypsin: protein) for 12 h, followed by 1:100 for 4 h. Finally, the peptide solution was desalted with a Sep-Pak SPE column and lyophilized.

High pH reverse-phase HPLC with a C18 column (5 μm particles, 10 mm i.d., 250 mm in length) was used to fractionate the tryptic peptides, which was separated into 60 fractions with an ammonium phosphate (pH 9.0) acetonitrile linear gradient from 8% to 32% within 60 min. The separated peptides were then combined into four fractions and dried by vacuum.

Affinity enrichment of lysine-methylated peptides

The tryptic peptides were dissolved in 50 mM Tris-HCl buffer (pH 8.0) containing 1 mM EDTA, 100 mM NaCl and 0.5% NP-40, and incubated with mono-, double-, and tri-methylated resin...
Table 1
Methylated proteins related to many important biological processes.

| UniProt no. | Methylation position, amino acid | Description | Type | Subcellular localization |
|-------------|---------------------------------|-------------|------|--------------------------|
| **Methylated proteins related to fruit ripening and senescence** | | | | |
| P05117      | 210, K                           | POLYGAUCTURONASE-2 | Trimethyl | chloroplast |
| A0A3Q7EQS7  | 172, R; 182, R                   | Pectin acetyl esterase | Methyl | extracellular |
| F10957      | 12, K                            | 1-aminoacyclopropene-1-carboxylate oxidase homolog | Trimethyl | nucleus |
| A2QVQ6      | 279, K                           | 1-aminoacyclopropene-1-carboxylate oxidase | Trimethyl | cytoplasm |
| A0A3Q7IBL6  | 124, K                           | SQUAMOSA PROMOTER-BINDING-LIKE PROTEIN | Methyl | nucleus |
| Q9XEX8      | 122, K                           | REMORIN 1 | Methyl | nucleus |
| A0A3Q7CIL3  | 55, K; 56, K                     | Ribosome biogenesis protein BOP1 homolog | Methyl | nucleus |
| A0A3Q7GEC1  | 38, K                            | PREDICTED: mitotic spindle checkpoint protein BUBR1 | Trimethyl | nucleus |
| **Methylated proteins related to signaling** | | | | |
| A0A3Q7FXM4  | 479, K                           | PREDICTED: ALG-2 interacting protein X-like | Methyl, Dimethyl | chloroplast |
| 488, R      |                                  | Methyl | chloroplast |
| A0A3Q7GUAU5 | 475, K                           | PREDICTED: ALG-2 interacting protein X | Methyl, Dimethyl, Trimethyl | chloroplast |
| 464, R      |                                  | Methyl | chloroplast |
| P27161      | 116, K                           | Calmodulin | Trimethyl | nucleus |
| A0A3Q7HID2  | 32, R                            | Non-specific serine/threonine protein kinase | Methyl | plasma |
| A0A3Q7GIA3  | 987, K                           | Serine/threonine-protein kinase 4 homolog B isoform X2 | Trimethyl | nucleus |
| A0A3Q7EPF9  | 629, K                           | PREDICTED: probable serine/threonine-protein kinase | Methyl | chloroplast |
| A0A3Q7HHC0  | 111, K                           | Universal stress protein PHOS34 | Methyl, Dimethyl | chloroplast |
| A0A3Q7IBN5  | 110, K                           | Universal stress protein PHOS34 | Methyl, Dimethyl | chloroplast |
| K4B9G5      | 101, K                           | Universal stress protein PHOS32 | Methyl | cytoplasm |
| K4C741      | 4, K                             | UBC34 | Methyl | cytoplasm |
| A0A3Q7ETK4  | 387, K; 393, K                   | Fruuctose-bisphosphate aldolase | Methyl, Trimethyl | chloroplast |
| A0A3Q7F980  | 377, K                           | Fruuctose-bisphosphate aldolase | Methyl, Dimethyl | chloroplast |
| 378, K      |                                  | Methyl | chloroplast |
| 383, K      |                                  | Methyl | chloroplast |
| A0A3Q7FAC9  | 502, K                           | ATP synthase subunit beta | Methyl | mitochondria |
| A0A3Q7GDB8  | 508, K                           | ATP synthase subunit beta | Methyl | mitochondria |
| **Methylated proteins related to energy metabolism** | | | | |
| A0A3Q7GAX9  | 385, K                           | Fruuctose-bisphosphate aldolase | Methyl | chloroplast |
| 386, K      |                                  | Methyl | Dimethyl |
| 391, K      |                                  | Methyl | Dimethyl, Trimethyl |
| A0A3Q7HC9   | 813, K                           | Phosphoglycerate kinase | Trimethyl | chloroplast |
| A0A3Q7HC9   | 832, K                           | Multifunctional fusion protein | Methyl, Dimethyl, Trimethyl | chloroplast |
| 835, K      |                                  | Methyl | chloroplast |
| A0A3Q7IN81  | 126, K                           | PREDICTED: enolase-like | Trimethyl | cytoplasm |
| A0A3Q7EYF6  | 374, K                           | Fruuctose-bisphosphate aldolase | Methyl | chloroplast |
| 385, K      |                                  | Methyl | Trimethyl |
| 391, K      |                                  | Methyl | Dimethyl, Trimethyl |
| A0A3Q7ETK4  | 387, K; 393, K                   | Fruuctose-bisphosphate aldolase | Methyl, Dimethyl, Trimethyl | chloroplast |
| A0A3Q7F980  | 377, K                           | Fruuctose-bisphosphate aldolase | Methyl, Dimethyl | chloroplast |
| 378, K      |                                  | Methyl | chloroplast |
| 383, K      |                                  | Methyl | chloroplast |
| A0A3Q7FA9   | 502, K                           | ATP synthase subunit beta | Methyl | mitochondria |
| A0A3Q7GDB8  | 508, K                           | ATP synthase subunit beta | Methyl | mitochondria |
| **Methylated proteins related to stimulus and stress response** | | | | |
| A0A3Q7EYX0  | 267, K                           | Ubiquitin receptor RAD23d isoform X1 | Dimethyl, Trimethyl | cytoplasm |
| A0A3Q7HCO1  | 111, K                           | Universal stress protein PHOS34 | Methyl, Dimethyl | chloroplast |
| A0A3Q7BN5   | 110, K                           | Universal stress protein PHOS34 | Methyl, Dimethyl | chloroplast |
| K4B9G5      | 101, K                           | Universal stress protein PHOS32 | Methyl | cytoplasm |
| K4C741      | 4, K                             | UBC34 | Methyl | cytoplasm |
| A0A3Q7HV4   | 524, K                           | Hsp70-Hsp90 organizing protein 2 | Dimethyl | peroxisome |
| A0A3Q7FSP8  | 119, K                           | Late embryogenesis abundant protein 76-like | Methyl | chloroplast |
| A0A3Q7FSP9  | 148, K                           | 22.7 kDa class IV heat shock protein-like | Trimethyl | vacuolar membrane |
| A0A3Q7HJ62  | 185, K                           | Uncharacterized LOC107830885 | Trimethyl | cytoplasm |
| A0A3Q7HKS3  | 206, K                           | Glutathione peroxidase | Trimethyl | chloroplast |
| A0A3Q7F690  | 284, K                           | Cotton fiber (DUF761) | Trimethyl | plasma membrane |
| A0A3Q7FN4   | 286, K                           | Protein BOBBER 1 | Methyl | mitochondria |
| A0A3Q7FC0   | 322, K                           | Protein SGT1 homolog | Methyl | cytoplasm |
| A0A3Q7FP0   | 249, K; 252, R; 262, K; 254, R; 257, R | PREDICTED: trihelix transcription factor GT-2-like | Methyl | nucleus |
| A0A3Q7BS2   | 228, K                           | Glutathione S-transferase T1 | Methyl, Dimethyl | mitochondria |
| A0A3Q7FWR2  | 54, K; 57, K                     | PREDICTED: protein PXR1 | Methyl | nucleus |
| A0A3Q7HUN2  | 662, K                           | Autophagy-related protein 11 | Methyl, Dimethyl | nucleus |
| 663, R      |                                  | Methyl | nucleus |
| A0A49G5999  | 28, R                            | PREDICTED: cellulose synthase-like protein G1 | Methyl, Dimethyl | nucleus |
| 29, R       |                                  | Methyl | nucleus |
| A0A3Q7IG90  | 132, K                           | GDSL esterase/lipase TGA2.3-like isoform X1 | Methyl | extracellular |
| A0A3Q7HW29  | 141, K                           | GDSL esterase/lipase At5g53820-like | Methyl | extracellular |
| A0A3Q7G667  | 259, K                           | PREDICTED: putative methyltransferase DDB_G0268948 | Trimethyl | chloroplast |

(continued on next page)
antibody beads at 4 °C to enrich the methylated peptides. After 12 h of incubation, the beads were washed with the above-mentioned buffer for four times and with H2O for two times. The bound peptides were then eluted from the beads with 0.1% trifluoroacetic acid. The eluted peptides were desalted using C18 ZipTips (Millipore, Germany).

**LC-MS/MS analysis**

The tryptic peptides dissolved in 2% acetonitrile in aqueous 0.1% formic acid (solvent A) were subjected to separation using a C18 reversed-phase column (15-cm length, 75 μm i.d.) on an EASY-nLC 100 UPLC system. The gradient elution was performed with 5% to 20% solvent B (0.1% formic acid in 90% acetonitrile) from 0 to 20 min, 20% to 35% solvent B from 20 to 32 min, 35% to 80% solvent B from 32 to 36 min, and 80% solvent B for the last 3 min. The flow rate was 500 nl/min. The eluted peptides were subjected to nanospray ionization and then MS/MS analysis in a Q Exactive Plus (Thermo) as previously described [25]. The electrospray voltage applied was set as 2.0 kV with a scan range from 100 to 1600 m/z. The MS/MS parameters were conducted as previously described [25].

**Database search and motif analysis**

The resulting MS/MS data were processed using MaxQuant (v.1.5.2.8, http://www.maxquant.org/) and searched against the Solanum lycopersicum data in UniProt database (https://www.uniprot.org/). The mass tolerance allowed for precursor ions in the first search and the main research were 20 ppm and 5 ppm, respectively. The mass tolerance allowed for fragment ions was 0.02 Da. The oxidation on Met and carbamidomethyl on Cys were specified as variable modification and fixed modification, respectively. FDR was set at < 1%. The minimum score of the methylated peptides was set at > 40. MoMo software (v.5.0.2, http://meme-suite.org/tools/momo) was used to analyze the amino acid compositions from −10 to + 10 around the methylated lysine. The minimum number of occurrences was set to 20.
Bioinformatics analysis

UniProt-GOA database (http://www.ebi.ac.uk/GOA/) was used to perform the GO annotation (v.5.14–53.0, http://www.ebi.ac.uk/interpro/) of the methylated proteome. Perl module (v.1.31, https://metacpan.org/pod/Text::NSP::Measures::2D::Fisher) was used for functional enrichment analysis. The enrichment of the methylated proteins against all proteins in the species database was analyzed by two-tailed Fisher’s exact test. The p value<0.05 was considered significant. KEGG database was used to annotate the methylated protein pathways. KAAS software (v.2.0, http://www.genome.jp/kaas-bin/kaas_main) was firstly used to annotate the protein KEGG database description (http://www.genome.jp/kegg/). KEGG mapper (v.2.5, http://www.kegg.jp/kegg/mapper.html) was used to map the methylated protein annotation result to the KEGG pathway. The subcellular localization of methylated proteins was predicted using Wolf PSORT software (http://wolfpsort.seq.cbrc.jp/) and CELLO (v.2.5, http://cello.life.nctu.edu.tw/). Protein domain annotation was performed using the software InterProScan (v.5.14–53.0, http://www.ebi.ac.uk/interpro/) based on protein sequence algorithm and the corresponding InterPro domain database. For enrichment or depletion (right-tailed test) of specific amino acid residues, the p values were analyzed by Fisher’s exact test and the p values<0.05 were considered significant.

Site-directed mutagenesis, purification of recombinant proteins and activity analysis

The site-directed mutagenesis in GST-T1, Trx and NOX were performed as described previously [1]. We mutated the lysine and arginine sites, which were prone to be methylated, to alanine to simulate locking to methylation modification. The mutations were as follows: GST-T1-mut (K228A, R230A), NOX-mut (R161A), and Trx-mut (K19, 21A). The encoding sequences of GST-T1, Trx, NOX and their mutants were subcloned into the pET-28a vector. The recombinant proteins were expressed in E.coli BL21 and purified with Ni-NTA affinity chromatography. The protein activities were determined using the assay kits of GST-T1, Trx and NOX (Comin Biotechnology Co., Ltd., Suzhou, China), respectively.

Real-time quantitative PCR (RT-qPCR) analysis

Total RNA from tomato pericarp was extracted and reverse-transcribed to synthesize the first-strand cDNA. RT-qPCR was carried out as described previously [1]. SLActin (Solanum lycopersicum) was used as the internal control. The results of RT-qPCR were normalized using the Ct value corresponding to SLActin. The gene relative expression levels were calculated by the formula 2−DDCT. The primers were shown in Supplementary Table S1.

Statistical analysis

The data are expressed as the means ± SD. Differences among different treatment groups were compared using SPSS v.7.5 (SPSS Inc., Chicago, IL, USA).

Results and discussion

Proteome-wide analysis of lysine methylation sites and proteins in tomato

Lysine methylation is an important PTM that plays important roles in transcription regulation, DNA replication, repair and recombination. Methylation of lysine residues on histone proteins is vital to chromatin structure and function [26-28] Dysregulation of histone methylation results in reprogramming of gene expression networks and many diverse disease states [27-29]. However, non-histone protein substrates of methylation modification remain largely unknown. Here, we performed a proteome-wide analysis of non-histone lysine methylation in tomato during fruit ripening (Fig. 1A).

The proteins from a mixed samples at different ripening stages, including mature green (MG), break (BR) and red ripening (RR) stages, were extracted and analyzed (Supplementary Figure S1 and Table S2). A total of 2,448 methylated peptides were identified by spectrumogram analysis (Supplementary Table S3). As shown in Fig. 1B, the majority of the mass errors were near to zero, showing good accuracy.

The methylated peptides ranged in length from 7 to 34 amino acid residues, most of which were from 7 to 21 amino acid residues (Fig. 1C). A total of 292 methylation sites, including 241 lysine sites and 51 arginine sites, were identified in 201 proteins, including three methylated histones (Supplementary Table S4). The sequences of ‘predicted’ proteins with low proportion of protein annotation in UniPort were blasted in NCBI database (plant) to increase the proportion of protein annotation (Supplementary Table S5). In this method, the peptides were enriched twice to obtain more methylation sites, and some arginine methylation sites were identified (nonspecific binding). Subsequent bioinformatics analysis was carried out for the identified lysine methylation sites and proteins. The MS data have been deposited in proteome Xcharge (Project accession: PXD023985). As the first lysine methylation map of horticultural crops, our results provide valuable resources for further studies on fruit development and ripening in relation to non-histone lysine methylation modification.

Motif characteristics of methylated peptides in tomato

Fig. 2A shows the distribution of lysine methylation site per protein. The number of lysine methylation sites in each protein ranged from 1 to 28 and 74.2% of methylated proteins had only one lysine methylation site. To further explore the methylated site attributes in Solanum lycopersicum, the −10 to + 10 amino acid residues around the methylated lysine were analyzed. Some strong bias were found in the amino acid residues with specific methylation site motifs. As shown in Fig. 2B and C, glutamic acid (E) was enriched at the −4 or + 4 positions, and glutamic acid was also occurred at −3, −5, −7, −8, +2, and + 3 positions. In addition, lysine (K) and glycine (G) were enriched at the −1 and + 1 positions, respectively, and lysine (K) also occurred at the + 5, +6, +7, +8, +9, and + 10 positions. Aspartic acid (D) occurred at the + 4 position. These amino acid residues are hydrophilic, indicating that the hydrophilicity may be vital for methylation. Currently, only the many conserved domains in methylated proteins have been reported, such as the SET (suppressor of variegation, enhancer of zeste and trithorax) domain. The consensus motifs for methylated non-histones were not identified. In the present study, two conserved Kme motifs, xxxxxxxxxK_xxxExxxxxx and xxxxxxxxxK_xxxxxxxx, were enriched (Fig. 2C), and the enrichment statistics from MoMo software are shown in Supplementary Table S6.

Functional annotation of methylated proteins in tomato

To explore the possible roles of the methylated proteins in fruit ripening, all the identified methylated proteins were subjected to GO annotation according to their biological process, cellular component, molecular function and subcellular location (Fig. 3). For the biological process, 36%, 30% and 21% of the methylated proteins were related to metabolic process, cellular process and single-organism process, respectively (Fig. 3A). Regarding the cellular
components, 40% of cells, 25% of organelles, 19% of macromolecular complexes, and 14% of membranes were included (Fig. 3B).

For molecular function, binding activity and catalytic activity accounted for 53% and 38% of all the methylated proteins, respectively, indicating that the methylated proteins were involved in enzyme metabolism in tomato (Fig. 3C). As shown in Fig. 3D, the identified methylated proteins were predicted to be mainly localized to cytoplasm (30%), nucleus (29%), and cytoplasm (23%), and a few proteins were in mitochondria (7%) or plasma membrane (3%).
Furthermore, we performed a GO enrichment analysis of proteins with lysine methylation (Fig. 4). Among the 201 identified methylated proteins, 196 methylated proteins were annotated in terms of their biological processes and 166 in terms of their molecular functions. In the GO enrichment analysis, the proteins preferentially methylated included those in intracellular parts (11.4%), photosystem I reaction centres (6.68%), cytoplasmic parts (6.41%) and intracellular organelle parts (4.94%). In addition, the molecular functions of the methylated proteins were related to fructose-bisphosphate aldolase activity (8.36%), aldehyde-lyase activity (7.87%), carbon–carbon lyase activity (4.09%), lyase activity (2.91%), and nucleoside-triphosphatase activity (2.5%). According to biological process, the methylated proteins were mainly enriched in single-organism carbohydrate catabolic process (6.35%), nucleoside metabolic process (5.54%), oxidoreduction coenzyme metabolic process (5.35%), ribose phosphate metabolic process (4.81%), carboxylic acid metabolic process (4.54%), oxoacid metabolic process (4.47%), nucleoside phosphate metabolic process (4.16%), and ethylene metabolic and biosynthetic process (5.38%) (Fig. 4A). The KEGG pathway category enrichment analysis indicated that the methylated proteins were related to carbon fixation in photosynthetic organisms, pentose phosphate pathway, fructose and mannose metabolism, and cysteine and methionine metabolism (Fig. 4B).

Analysis of protein functional domains can provide some information on the particular functions of target proteins. Using the PFAM database, we performed a functional domain enrichment analysis of the methylated proteins. As shown in Fig. 4C, the most enriched proteins were the aldolase-type TIM barrel, heat shock chaperonin-binding, ribosomal protein L11, N- and C-terminal, ubiquitin-associated domain, electron transport accessory protein-like domain, ATP synthase F1 bate subunit, and cytochrome c-like domain, indicating that the methylated proteins might be involved in glycometabolism, responses to stress, protein synthesis, energy metabolism, and redox process.

Methylated proteins related to important biological processes

In our study, the methylated proteins were mainly involved in fruit ripening and senescence, signal transduction, stimulus and stress response, oxidation–reduction process, energy metabolism and carbohydrate biosynthesis.

**Fruit ripening and senescence.** Fruit ripening is an active process driven by both endogenous and exogenous factors. The process is accompanied by dramatic changes in ethylene biosynthesis, colour, texture, flavour, aroma and nutrition [30]. In the present study, several known proteins involved in ethylene biosynthesis, cell wall degradation and carotenoids biosynthesis were identified to be methylated in lysine sites during fruit ripening. At the onset of ripening, the ethylene production of climacteric fruits increase sharply [31]. 1-Aminocyclopropane-1-carboxylic acid oxidase (ACO) is one of the key enzymes responsible for ethylene synthesis, which plays a crucial role in fruit ripening [32]. During fruit ripening, cell wall synthesis gives way to degradation, involving in numerous proteins and enzymes [33]. Polygalacturronases (PGs) play important roles in pectin degradation and fruit softening [34–36]. Pectin acetyltransferase (PAE) is another enzyme that may be involved in cell wall degradation. PAE cleaves the acetyl group from acetylated galacturonic acids in pectin. Unexpectedly, the biochemical properties and physiological function of PAE are unclear [37]. Until recently, MdPAE10, a candidate gene from apple encoding pectin acetyltransferase, was reported
to negatively affect fruit shelf life [38]. Fruit ripening was accompanied by the synthesis and accumulation of carotenoids. Lycopene beta/epsilon cyclase acts downstream of carotenoids biosynthesis, which is responsible for carotene production. Our results showed that two ACO, one PG, one PAA and one lycopene beta/epsilon cyclase underwent lysine methylation in tomato during fruit ripening. In addition, two proteins that possibly participate in the regulation of tomato fruit ripening, including remorin1 and SBP-box protein, also were methylated in lysine sites. Remorin1 is a positive regulator of tomato fruit ripening that can interact with ethylene biosynthesis proteins ACO1 and ACS2 [39]. SBP-box protein is critical for tomato fruit ripening as a transcription factor [40]. Despite the established significance of ACO, PG, lycopene beta/epsilon cyclase, remorin1 and SBP-box protein in tomato fruit ripening, the post-translational regulatory mechanisms of these proteins are unclear. Our results suggest that these ripening-related functional proteins and regulators are subjected to lysine methylation during ripening, which will affect their function and be involved in the regulation of fruit ripening. However, whether lysine methylation positively or negatively regulate tomato fruit ripening and the underlying mechanism need to be further verified by molecular and genetic approach.

**Oxidation-reduction process.** Plants accumulate excessive reactive oxygen species (ROS) during senescence or under stress conditions, which cause oxidative damage of macromolecules, such proteins, nucleic acid, lipids and polysaccharides, leading to structural and functional loss or, potentially, cell death [41–44]. Recent studies have shown that fruit senescence is related to ROS accumulation and oxidative damage to proteins [45–47]. To prevent oxidative damage, plants have evolved antioxidant or scavenging mechanisms, as well as macromolecule repair systems. In this study, many proteins related to ROS scavenging and oxidized-protein repair, including one glutathione peroxidase (GPX), two methionine sulfoxide reductases (Msr) and one thioredoxin (Trx), were subjected to lysine methylation during tomato fruit. Glutathione peroxidase (GPX) is a thiol-based antioxidant enzyme that catalyzes the reduction of H2O2 and hydroperoxides and plays important roles in plant development and response to stresses [48]. Methionine sulfoxide reductases (Msr), as important oxidized protein repair-related enzymes, can reduce methionine sulfoxide to methionine under senescence condition or in response to stresses. Jiang et al. reported that the redox modifications of methionine in CaM by MsrA1/B1 are implicated in the regulation of senescence in litchi fruit [45]. Recently, Jiang et al. proposed that Msr-mediated redox modification of NOR, a master transcription factor regulating tomato fruit ripening, regulates the expression of ripening-related genes, thereby influencing tomato fruit ripening [1]. Thioredoxins (Trx) are another types of proteins that modify the redox state of target proteins through thiol-disulfide exchange. More recently, Wu et al. reported that Trx-mediated redox regulation of DLPx is involved in senescence or quality deterioration of harvested longan fruit [47]. These identified redox-related proteins susceptible to lysine methylation indirectly regulate fruit ripening or senescence by scavenging ROS and reducing oxidized methionine and cysteine in functional proteins. However, the PTMs of these protein remain unclear. It is suggested that the lysine methylation in Gpx, Msr and Trx is an important PTM of these protein and influence their roles in indirectly regulating fruit ripening and senescence.

**Signalling.** Plant signalling pathways convert external and internal signals into physiological response via a network of signalling proteins such as receptors, transporters, kinases [49]. Signal transduction plays a central role in the regulation of biological processes in plants. In the present study, several signalling-related
proteins, including calmodulin, serine/threonine protein kinases, were also found to be methylated in lysine sites during tomato fruit ripening. Calmodulin is a ubiquitous calcium-binding protein in signal transduction pathways in all eukaryotic cells and plays important roles in plant growth and development and in response to stresses [50,51]. Tomato contains six calmodulin isoforms, of which SiCaM2 is the major calmodulin during ripening and plays a double role to regulate fruit ripening [52]. Recently, Tang et al. reported that SICML37, a calmodulin-like protein, enhances tomato fruit chilling stress tolerance by interacting with proteasome maturation factor SIUMP1 [53]. Serine/threonine (S/T) protein kinases are crucial components of diverse signalling pathways in eukaryotes. Phosphorylation function of protein kinases leads to the activation of signal-transduction pathways and function in a great number of biological processes [54]. In tomato, SpMPK3, a serine/threonine protein kinase, is involved in resistance to abiotic stress [55]. Alix (ALG-2 interacting protein X) is implicated in membrane deformation and fission both in endosomes and at the plasma membrane. Alix is required for rapid endocytosis and downstream signaling [56]. In Arabidopsis, ALIX regulates stomatal aperture and turnover by controlling the accumulation of abscisic acid specific PYR/PYL/RCAR receptors [57]. Our results showed that lysine methylation occurred in one calmodulin, five serine/threonine protein kinase and two Alix during tomato fruit ripening. These proteins have not been reported to be directly involved in the regulation of fruit ripening, especially the ethylene and auxin-related signalling pathway. It is suggested that the lysine methylation in these proteins possibly indirectly regulate tomato ripening.

**Stimulus and stress responses.** During growth and development, stimulus and stress response often occur in plants. Stress responsive proteins play important roles in adaptation to various stimuli and stresses in plants. Surprisingly, a number of stimulus and stress-related proteins were identified to undergo lysine methylation during tomato fruit ripening, including cell wall synthesis-related proteins, transcription factors, heat shock proteins. The biosynthesis of cell wall polysaccharides plays important roles in plant growth, development and stress responses. Cellulose synthase-like (CSL) proteins, which belong to the glycosyltransferase family, are implicated in the biosynthesis of cellulose, xylans, xyloglucans, galactans and mannan [58,59]. Zhu et al. reported that the CSL proteins AtCSLD5 is required for osmotic stress tolerance in Arabidopsis, which is related to the regulation of ROS [60]. Similarly, the cellulose synthase-like protein OsCSLD4 plays an important role in the response of rice to salt stress by mediating abscisic acid biosynthesis to regulate osmotic stress tolerance [61]. Transcriptional regulation plays a vital role in plant growth and development and involves the participation of numerous transcription factors. In the present study, two transcription factors, GT-2-like and TGA2.3-like isoform X1, were identified to be methylated in lysine sites during tomato fruit ripening. GT-2 factors are the members of trihelix transcription factors that regulate plant growth and development [62] and more importantly, respond to different abiotic [63] and biotic stresses [64]. TGA family is an important group of plant bZIP transcription factors that regulate the resistance or development of floral organ [65]. Of the TGA family, TGA2 subgroup are mainly involved in disease resistance or immune response [66,67]. Recently, Lemaire-Chamley et al. reported that SITGA2.2 is a transcriptional repressor, which slows down fruit maturation and ripening in tomato [68]. Heat shock proteins (HSPs), as molecular chaperones, enhance organism resistance to stress by facilitating refolding and preventing denaturation and aggregation of proteins.

In particular, Hsp90 and Hsp70 are the most abundant classes of chaperones and function under conditions of cellular stress and/or elevated temperature [69]. The methylation of Hsp70 was reported in the development of tumours, while the methylation of HSP in plants has not been reported [14]. SGT1 is an indispensable resistance-related protein that has been shown to be a cofactor for HSP90 and HSP70. In addition to CSL, GT-2-like protein, TGA2.3-like isoform X1, HSP and SGT1, other stimuli and stress-related proteins, including the ubiquitin receptors RAD23d, UBC34, PXR1, glutathione peroxidase and glutathione S-transferase T1, were also found to be methylated in lysine sites during tomato fruit ripening. These proteins have been reported to mainly play roles in response to stresses. The roles of lysine methylation of these proteins in fruit ripening are worthy of further study.

In our study, many methylated proteins were also found to participate in other biological processes during tomato ripening, such as energy metabolism (fructose-bisphosphate aldolase, phosphoglycerate kinase and ATP synthase subunit beta), carbohydrate biosynthesis (ribulose bisphosphate carboxylase, cellulose

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**Fig. 5.** Analysis of enzyme activity of recombinant proteins His-Trx, GST T1, NOX, and their mutations. Mutation of lysine or arginine to alanine leads to failed methylation. Trx: K19A and R21A; NOX: R161A; GST: K228A and R230A. The values with different letters are significantly different (p < 0.05).
synthase-like protein, pectin acetylesterase, alpha-xylosidase 1 and fructose-bisphosphate aldolase). These results indicates that lysine methylation is a common post-translational modification of proteins in tomato fruit, which is involved in the regulation of diverse biological processes in tomato during fruit growth and development.

Effects of the methylation modification on protein activity

In this study, we selected three identified methylated proteins (Trx, GST T1 and NOX) (Supplementary Figure S3) in tomato during fruit ripening to investigate the effect of methylation on protein function through site-directed mutation-simulated demethylation. Thioredoxins (Trxs) function mainly by reducing disulfide bonds in the sulphydryl groups of proteins and play important roles in senescence or stress responses in plants [56]. The Glutathione S-transferases (GSTs) are a group of multi-gene cellular detoxification enzymes that catalyze the conjugation of glutathione to endogenous and exogenous electrophilic compounds [70]. NADH dehydrogenase (NOX) is a multi-subunit respiratory chain reduced nicotinamide adenine dinucleotide (NADH):ubiquinone oxidoreductase. NOX is located in the inner mitochondrial membrane and a key enzyme in oxidative phosphorylation [71]. We purified the recombinant GST T1-His, Trx-His, NOX-His and their mutant ronproteins (Supplementary Figure S2) and compared the activities of the wild type and the mutant proteins. As shown in Fig. 5A, when the methylated sites (K19 and K21) were mutated to alanine to simulate demethylation, the thioredoxin (Trx) activity decreased significantly, suggesting that methylation modification may have a positive effect on Trx. Different from Trx, simulated demethylation resulted in the increased activity of GST T1 (Fig. 5B), which shows that methylation negatively regulates GST T1 activity. Similarly, NADH dehydrogenase activity also was increased when subjected to demethylation, suggesting that methylation may decrease NADH dehydrogenase function (Fig. 5C). These results suggest that the regulation of protein function by methylation is complex and may promote or inhibit protein functions.

Expression profiles of many genes encoding proteins subjected to methylation during fruit ripening

To understand the importance of these methylated proteins in fruit ripening, we selected 30 methylated non-histones to analyse the expression of their genes during fruit ripening. These proteins were mainly related to fruit ripening and senescence (ACO, ACO3, PAE, and PG-2), energy metabolism (fructose-bisphosphate aldolase and ATP synthase subunit beta), oxidation–reduction process (lycopene beta/epsilon cyclase, cytochrome c, E4, GPX, NADH dehydrogenase, Trx, psaA, psaD, psaB, and bystin-like protein), carbohydrate biosynthetic (pectin acetylesterase, glycosyltransferase, UDP-glycosyltransferase 73C4, and fructose-bisphosphate aldolase), response to stimulus and stress (GPX, Hsp70-Hsp90 organizing protein 2, universal stress protein PHOS32 and PHOS34, autophagy-related protein 11, GDSL esterase/lipase, and putative methyltransferase DDB_G0268948) and other biological processes. As shown in Fig. 6, the transcript levels of most of these genes were upregulated at different fruit ripening stages, especially in break stage, which indicates that these genes may be involved in tomato fruit ripening. Moreover, these ripening-related genes also were subjected to lysine methylation during fruit ripening. Therefore, it is suggested that numerous ripening-related genes are involved in the regulation of tomato fruit ripening which also are regulated via lysine methylation in their encoding proteins. Interestingly, among the analysed genes, the expression of Trx and NOX were upregulated during tomato fruit ripening (Fig. 6), but methylation might result in a decrease in their activity (Fig. 5). Similar results also have been reported in other post-translational modification.
Jiang et al. [1] found that the expression of NLR gene is upregulated during tomato fruit ripening and the transcriptional activity of NLR transcription factor is decreased due to sulfation. Shan et al. [72] reported that transcriptional activity of NAC2 transcription factor is suppressed due to ubiquitination, but the expression of NAC2 is upregulated during banana fruit ripening. These results suggest that the function of ripening-related genes is regulated at multilayer levels, such as transcriptional regulation and posttranslational modification. However, whether the lysine methylation positively or negatively regulates fruit ripening and the underlying mechanisms remain to be clarified.

Conclusion
In this study, a proteome-wide identification of non-histone lysine methylation in tomato during fruit ripening was performed. A total of 241 sites of lysine methylation (mono-, di-, or tri-methylation) in 176 proteins were identified, which are related to fruit ripening and senescence, signal transduction, oxidation reduction processes, stimuli and stress, energy metabolism. Furthermore, simulated non-histone methylation promoted or suppressed the activities of three selected proteins, including Trx, GST T1, and NOX. It is suggested that lysine methylation is involved in the regulation of tomato fruit ripening. This is the first proteome of non-histone lysine methylation in fruit, which will provide cues and directions for studying the mechanism and ripening from the perspective of non-histone methylation modification.

CRediT authorship contribution statement
Lu Xiao: Conceptualization, Data curation, Formal analysis, Methodology, Software, Writing – original draft. Hanzhi Liang: Methodology. Guoxiang Jiang: Methodology. Xiaochun Ding: Methodology. Xuncheng Liu: Methodology. Jian Sun: Methodology. Yueming Jiang: Conceptualization, Data curation, Formal analysis, Funding acquisition, Project administration, Supervision, Writing – review & editing.

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 paper.

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Appendix A. Supplementary data
Supplementary data to this article can be found online at https://doi.org/10.1016/j.jare.2022.02.013.

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