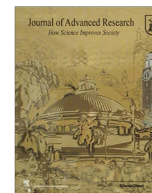
Journal of Advanced Research 42 (2022) 177–188

Contents lists available at ScienceDirect

Journal of Advanced Research

journal homepage: www.elsevier.com/locate/jare



Original Article

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

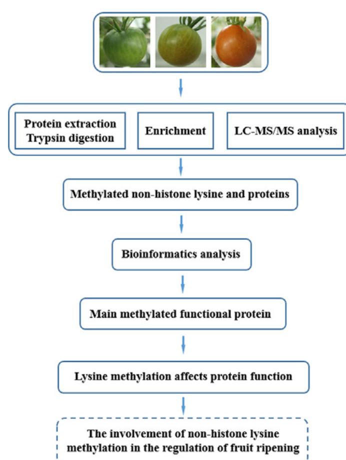
Lu Xiao [a,d,e], Hanzhi Liang [c], Guoxiang Jiang [a], Xiaochun Ding [a], Xuncheng Liu [a,b], Jian Sun [g], Yueming Jiang [a,b], Lili Song [f,*], Xuewu Duan [a,b,g,*]

[a] Guangdong Provincial Key Laboratory of Applied Botany, South China Botanical Garden, Chinese Academy of Sciences, Guangzhou 510650, China
[b] Center of Economic Botany, Core Botanical Gardens, Chinese Academy of Sciences, Guangzhou 510650, China
[c] University of Chinese Academy of Sciences, Beijing 100049, China
[d] Institute of Quality Standard and Monitoring Technology for Agro-products of Guangdong Academy of Agricultural Sciences, Guangzhou 510640, China
[e] Key Laboratory of Testing and Evaluation for Agro-product Safety and Quality, Ministry of Agriculture and Rural Affairs, Guangzhou 510640, China
[f] State Key Laboratory of Subtropical Silviculture, Zhejiang A&F University, Lin'an 311300, Zhejiang Province, China
[g] Agro-food Science and Technology Research Institute, Guangxi Academy of Agricultural Sciences, Nanning 530007, China

## 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.

## GRAPHICAL ABSTRACT




## ARTICLE INFO

*Article history:*
Received 12 December 2021
Revised 20 February 2022
Accepted 22 February 2022
Available online 24 February 2022

## 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.

Peer review under responsibility of Cairo University.

* Corresponding authors at: Guangdong Provincial Key Laboratory of Applied Botany, South China Botanical Garden, Chinese Academy of Sciences, Guangzhou 510650, China (X. Duan).

*E-mail addresses:* lilisong@zafu.edu.cn (L. Song), xwduan@scbg.ac.cn (X. Duan).

https://doi.org/10.1016/j.jare.2022.02.013

2090-1232/© 2022 The Authors. Published by Elsevier B.V. on behalf of Cairo University.
This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

*Keywords:*
Non-histone
Lysine
Methylome
Post-translational modification
Tomato
Ripening

*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_xxxExxxxxx and xxxxxxExxx_K_xxxxxxxxxx. 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.
© 2022 The Authors. Published by Elsevier B.V. on behalf of Cairo University. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

## 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 [1]. 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-κB [16], AR [17], RB [18], ERα [19], E2F1 [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.

Tomato is an important model plant for fruit development and ripening research. Here, we report a proteomics study of lysine methylation on non-histones in tomato. The methylated substrates and lysine methylation sites during tomato ripening were identified by high-resolution mass spectrometry. This is the first report of non-histone methyl proteome profiling of horticultural crops. Our findings provide information regarding lysine methylation on non-histones, which will have important implications for further elucidating the underlying mechanism of plant growth and development, especially fruit ripening, from the perspective of non-histone lysine methylation modification.

## 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 C 18 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 | Polygalacturonase-2 | Trimethyl | chloroplast |
| A0A3Q7EQS7 | 172, R; 182, R | Pectin acetylesterase | Methyl | extracellular |
| P10967 | 12, K | 1-aminocyclopropane-1-carboxylate oxidase homolog | Trimethyl | nucleus |
| A4ZYQ6 | 279, K | 1-aminocyclopropane-1-carboxylate oxidase | Trimethyl | cytoplasm |
| A0A3Q7IBL6 | 124, K | Squamosa promoter-binding-like protein | Methyl | nucleus |
| Q9XEX8 | 122, K | Remorin 1 | Methyl | nucleus |
| A0A3Q7G3L5 | 55, K; 56, K | ribosome biogenesis protein BOP1 homolog | Methyl | nucleus |
| A0A3Q7GBC1 | 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 |
| A0A3Q7GAU5 | 475, K | PREDICTED: ALG-2 interacting protein X | Methyl, Dimethyl, Trimethyl | chloroplast |
| | 484, R | | Methyl | chloroplast |
| P27161 | 116, K | Calmodulin | Trimethyl | nucleus |
| A0A3Q7H3D2 | 32, R | Non-specific serine/threonine protein kinase | Methyl | plasma membrane |
| A0A3Q7GIA3 | 987, K | serine/threonine-protein kinase 4 homolog B isoform X2 | Trimethyl | nucleus |
| A0A3Q7EPA9 | 629, K | PREDICTED: probable serine/threonine-protein kinase DDB_G0282963 isoform X1 | Methyl | chloroplast |
| A0A3Q7EPH8 | 162, K;269, K | PREDICTED: probable serine/threonine-protein kinase kinX isoform X1 | Methyl | cytoplasm |
| A0A3Q7H3D2 | 32, R | serine/threonine protein kinase | Methyl | plasma membrane |
| A0A3Q7EPH8 | 162, K; 269, K | PREDICTED: serine/threonine-protein kinase kinX isoform X1 | Methyl | cytoplasm |
| A0A3Q7HY85 | 233, R | PREDICTED: disease resistance protein TAO1-like | Methyl | cytoplasm |
| K4AUK7 | 23, R; 26,R | PREDICTED: F-box/LRR-repeat protein At3g48880-like | Methyl | mitochondria |
| ***Methylated proteins related to energy metabolism*** | | | | |
| A0A3Q7GAX9 | 385, K | Fructose-bisphosphate aldolase | Methyl | chloroplast |
| | 386, K | | Methyl, Dimethyl | |
| | 391, K | | Methyl, Dimethyl, Trimethyl | |
| A0A3Q7HGJ9 | 813, K | Phosphoglycerate kinase | Trimethyl | chloroplast |
| A0A3Q7HG29 | 832, K | Multifunctional fusion protein | Methyl, Dimethyl, Trimethyl | chloroplast |
| | 835, K | | Methyl | chloroplast |
| A0A3Q7IN81 | 126, K | PREDICTED: enolase-like | Trimethyl | cytoplasm |
| A0A3Q7EYM6 | 374, K | Fructose-bisphosphate aldolase | Methyl | chloroplast |
| | 385, K | | Methyl, Trimethyl | |
| | 391, K | | Methyl, Dimethyl, Trimethyl | |
| A0A3Q7ETK4 | 387, K;393, K | Fructose-bisphosphate aldolase | Methyl, Dimethyl, Trimethyl | chloroplast |
| A0A3Q7F980 | 377, K | Fructose-bisphosphate aldolase | Methyl | chloroplast |
| | 378, K | | Methyl, Dimethyl | |
| | 383, K | | Methyl, Dimethyl, Trimethyl | |
| A0A3Q7FAC9 | 502, K | ATP synthase subunit beta | Methyl | mitochondria |
| A0A3Q7GD18 | 508, K | ATP synthase subunit beta | Dimethyl | mitochondria |
| ***Methylated proteins related to stimulus and stress response*** | | | | |
| A0A3Q7EYX0 | 267, K | ubiquitin receptor RAD23d isoform X1 | Dimethyl, Trimethyl | cytoplasm |
| 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 | Trimethyl | cytoplasm |
| A0A3Q7HV43 | 524, K | hsp70-Hsp90 organizing protein 2 | Dimethyl | peroxisome |
| A0A3Q7F5P8 | 119, K | late embryogenesis abundant protein 76-like | Methyl | chloroplast |
| A0A3Q7F9P0 | 148, K | 22.7 kDa class IV heat shock protein-like | Trimethyl | vacuolar membrane |
| A0A3Q7HJ62 | 185, K | uncharacterized LOC107830885 | Trimethyl | cytoplasm |
| A0A3Q7HK83 | 206, K | Glutathione peroxidase | Trimethyl | chloroplast |
| A0A3Q7F690 | 284, K | cotton fiber (DUF761) | Trimethyl | plasma membrane |
| A0A3Q7FN48 | 286, K | protein BOBBER 1 | Methyl, Dimethyl | nucleus |
| A0A3Q7FG90 | 322, K | protein SGT1 homolog | Methyl | cytoplasm |
| A0A3Q7IP07 | 249, K; 252, K; 262, K; 254, R; 257, R | PREDICTED: trihelix transcription factor GT-2-like | Methyl | nucleus |
| A0A3Q7JBS2 | 228, K | PREDICTED: glutathione S-transferase T1 | Methyl, Dimethyl | mitochondria |
| | 230, R | | Methyl | |
| A0A3Q7FRW2 | 54, K; 57, K | PREDICTED: protein PXR1 | Methyl | nucleus |
| A0A3Q7H3N2 | 662, K | PREDICTED: autophagy-related protein 11 | Methyl, Dimethyl | nucleus |
| | 663, R | | Methyl | |
| A0A494G999 | 28, R | PREDICTED: cellulose synthase-like protein G1 | Methyl, Dimethyl | nucleus |
| | 29, R | | Methyl | |
| A0A3Q7IZ90 | 132, K | PREDICTED: transcription factor TGA2.3-like isoform X1 | Methyl | nucleus |
| A0A3Q7HWZ9 | 141, K | PREDICTED: GDSL esterase/lipase At5g03820-like | Methyl | extracellular |
| A0A3Q7G067 | 259, K | PREDICTED: putative methyltransferase DDB_G0268948 | Trimethyl | chloroplast |

(*continued on next page*)

**Table 1** (*continued*)

| UniProt no. | Methylation position, amino acid | Description | Type | Subcellular localization |
|---|---|---|---|---|
| ***Methylated proteins related to oxidation–reduction process*** | | | | |
| A0A3Q7EPI9 | 482, K | lycopene beta/epsilon cyclase | Trimethyl | chloroplast |
| A0A3Q7F0Q5 | 807, K | PREDICTED: kinesin-like protein KIN-14E | Trimethyl | chloroplast |
| A0A3Q7G6F7 | 682, K;687, K | PREDICTED: cytochrome P450 81F3-like | Methyl | chloroplast |
| A0A3Q7HE49 | 91, K | Peptide-methionine (R)-S-oxide reductase | Methyl | chloroplast |
| A0A3Q7HK83 | 206, K | Glutathione peroxidase | Trimethyl | chloroplast |
| K4C5K7 | 262, K | Arogenate dehydrogenase | Methyl | chloroplast |
| P00060 | 80, K | Cytochrome *c* | Dimethyl,Trimethyl | mitochondria |
| | 94, K | | Trimethyl | |
| A0A3Q7F9X5 | 81, K; 82, K;95, K;96, K;100, R | PREDICTED: cytochrome *c* | Methyl | mitochondria |
| P27065 | 14, K | Ribulose bisphosphate carboxylase large chain | Dimethyl, Trimethyl | cytoplasm |
| | 18, K | | Dimethyl, Trimethyl | |
| | 21, K | | Dimethyl | |
| P28032 | 338, K | Alcohol dehydrogenase 2 | Trimethyl | cytoplasm |
| P54153 | 144, K | Peptide methionine sulfoxide reductase (Fragment) | Trimethyl | mitochondria |
| Q2MI87 | 93, K | Cytochrome *f* | Trimethyl | mitochondria |
| Q2MIA0 | 38, K | Photosystem I P700 chlorophyll *a* apoprotein A1 | Trimethyl | plasma membrane |
| Q5NE18 | 352, K | Formate dehydrogenase, mitochondrial | Trimethyl | mitochondria |
| Q8H6B5 | 406, K | Putative dehydrogenase | Methyl | cytoplasm |
| A0A3Q7IET1 | 19, K; 21, K | PREDICTED: thioredoxin-like protein Clot | Methyl | cytoplasm |
| A0A3Q7IAC1 | 733, R;735, R | bystin-like protein | Methyl | cytoplasm |
| K4B3H5 | 161, R | NADH dehydrogenase [ubiquinone] iron-sulfur protein 8 | Methyl | chloroplast |
| K4C5K7 | 260, R | Arogenate dehydrogenase | Methyl | chloroplast |
| A9LRT7 | 188, K | Isopentenyl diphosphate isomerase | Methyl | chloroplast |
| ***Methylated proteins related to carbohydrate biosynthetic*** | | | | |
| P27065 | 14, K | Ribulose bisphosphate carboxylase large chain | Dimethyl, Trimethyl | cytoplasm |
| | 18, K | | Dimethyl, Trimethyl | |
| | 21, K | | Dimethyl | |
| A0A3Q7F5U3 | 882, K | PREDICTED: callose synthase 11-like | Trimethyl | plasma membrane |
| A0A494G999 | 28, R | PREDICTED: cellulose synthase-like protein G1 | Methyl, Dimethyl | nucleus |
| | 29, R | | Methyl | |
| A0A3Q7EQS7 | 172, R; 182, R | Pectin acetylesterase | Methyl | extracellular |
| A0A3Q7FVH3 | 623, K | PREDICTED: alpha-xylosidase 1 | Trimethyl | cytoskeleton |
| A0A3Q7ETK4 | 387, K; 393, K | Fructose-bisphosphate aldolase | Methyl, Dimethyl, Trimethyl | chloroplast |
| A0A3Q7EYM6 | 374, K | Fructose-bisphosphate aldolase | Methyl | chloroplast |
| | 385, K | | Methyl, Trimethyl | |
| | 391, K | | Methyl, Dimethyl, Trimethyl | |
| A0A3Q7F980 | 377, K | Fructose-bisphosphate aldolase | Methyl | chloroplast |
| | 378, K | | Methyl, Dimethyl | |
| | 383, K | | Methyl, Dimethyl, Trimethyl | |
| K4D422 | 36, K | UDP-glycosyltransferase 73C4 | Trimethyl | cytoplasm |
| | 64, K | | Methyl | |
| A0A3Q7H9X4 | 391, K | Glycosyltransferase | Methyl | chloroplast |
| A0A3Q7IMY2 | 546, K | PREDICTED: UDP-glycosyltransferase 73C5-like | Trimethyl | nucleus |

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 $H_2O$ for two times. The bound peptides were then eluted from the beats 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 methylation 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. 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 predicated 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 annotation terms, 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* (*Solyc03g078400*) 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 $2^{-\Delta\Delta CT}$. 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 spectrogram 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 methylome 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, xxxxxxExxx_K_xxxExxxxxx and xxxxxxExxx_K_xxxxxxxxxx, 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

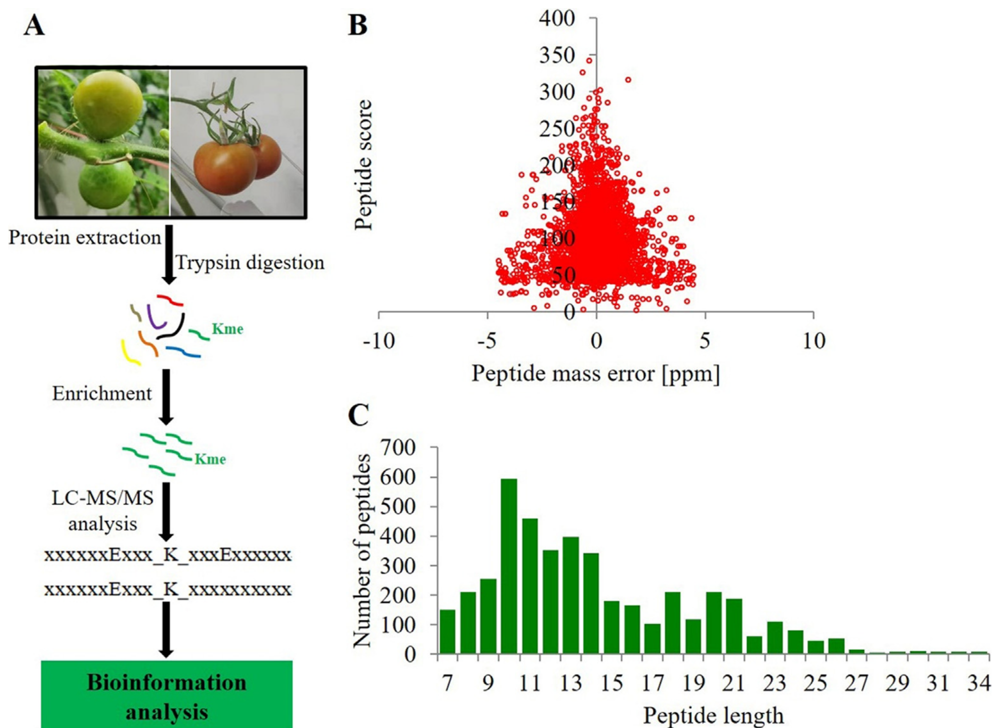



**Fig. 1.** Global identification of lysine methylation sites in tomato. A. Workflow for the lysine methylome analysis. B. Mass error distribution of the identified lysine methylated peptides. C. Length distribution of the lysine methylated peptides.

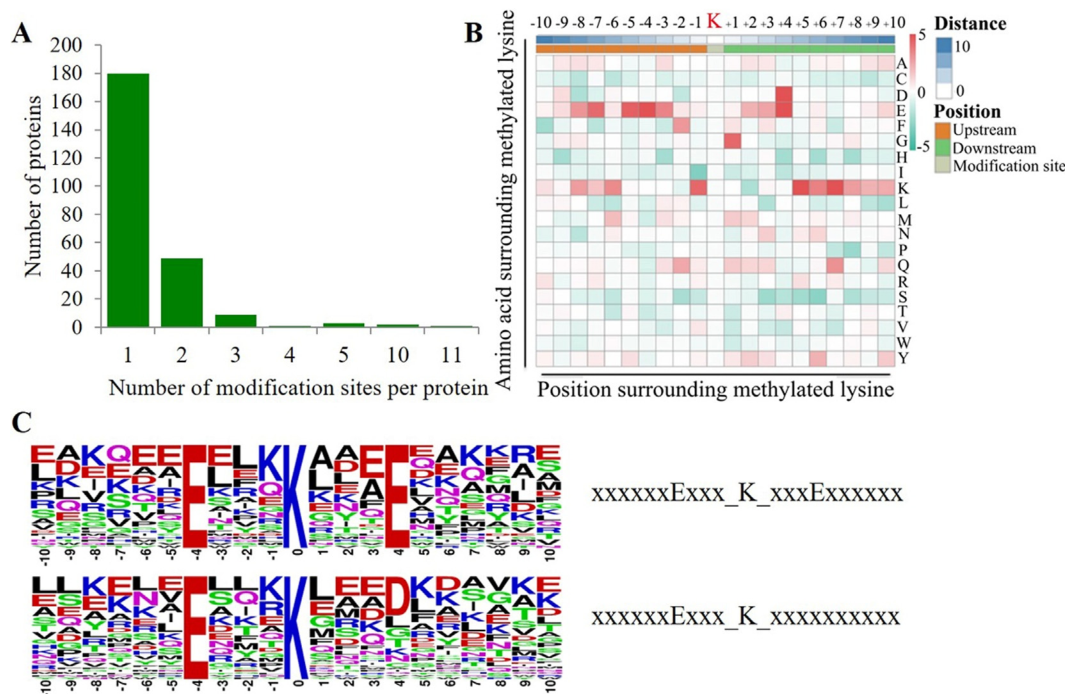



**Fig. 2.** Motif analysis of all identified Kme sites. A. Number of the identified lysine methylation sites per protein. B. Heat map of amino acid compositions of methylation site motifs. The central K refers to a methylated lysine. C. Enriched methylated motif logos. Size of each letter represents the frequency of the amino acid residues in that position.

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%).

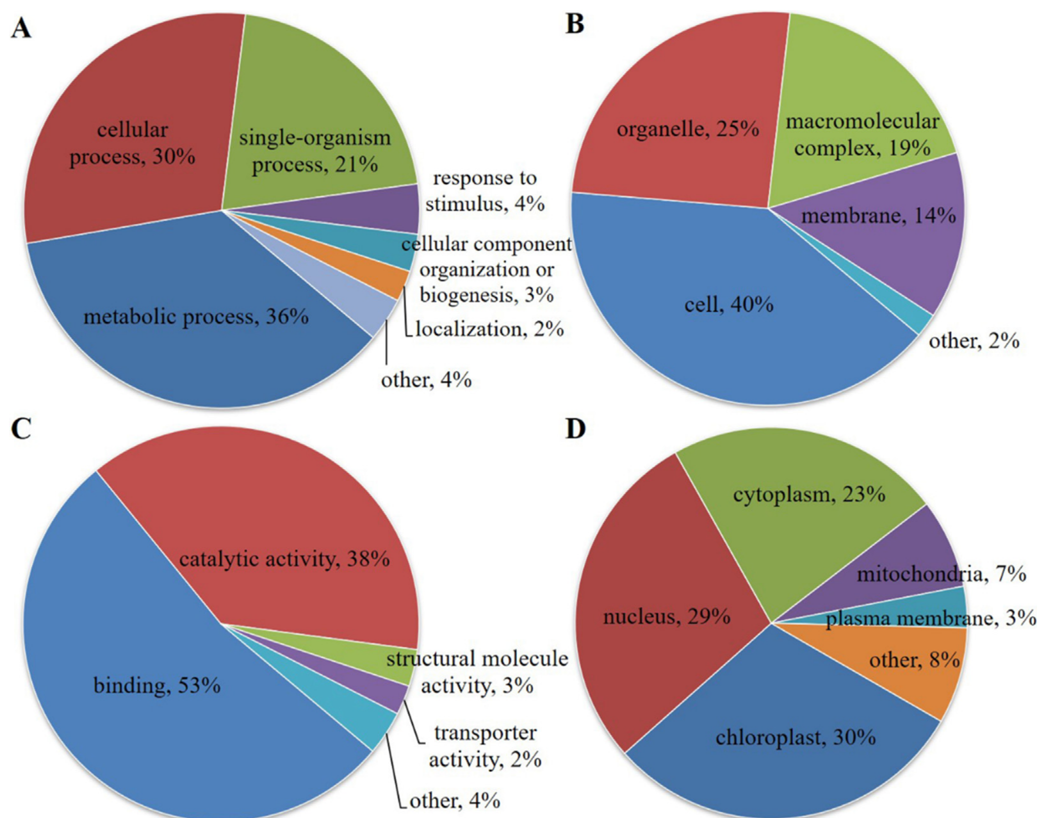



**Fig. 3.** Functional distribution and subcellular location of lysine-methylated proteins in tomato. A. Classification based on biological process. B. Classification based on cellular component. C. Classification based on molecular function. D. Subcellular location prediction.

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 *L*11, 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]. Polygalacturonases (PGs) play important roles in pectin degradation and fruit softening [34–36]. Pectin acetylesterase (PAE) is another enzyme that may be involved in cell wall degradation. PAE cleaves the acetylester bond 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 acetylesterase, was reported

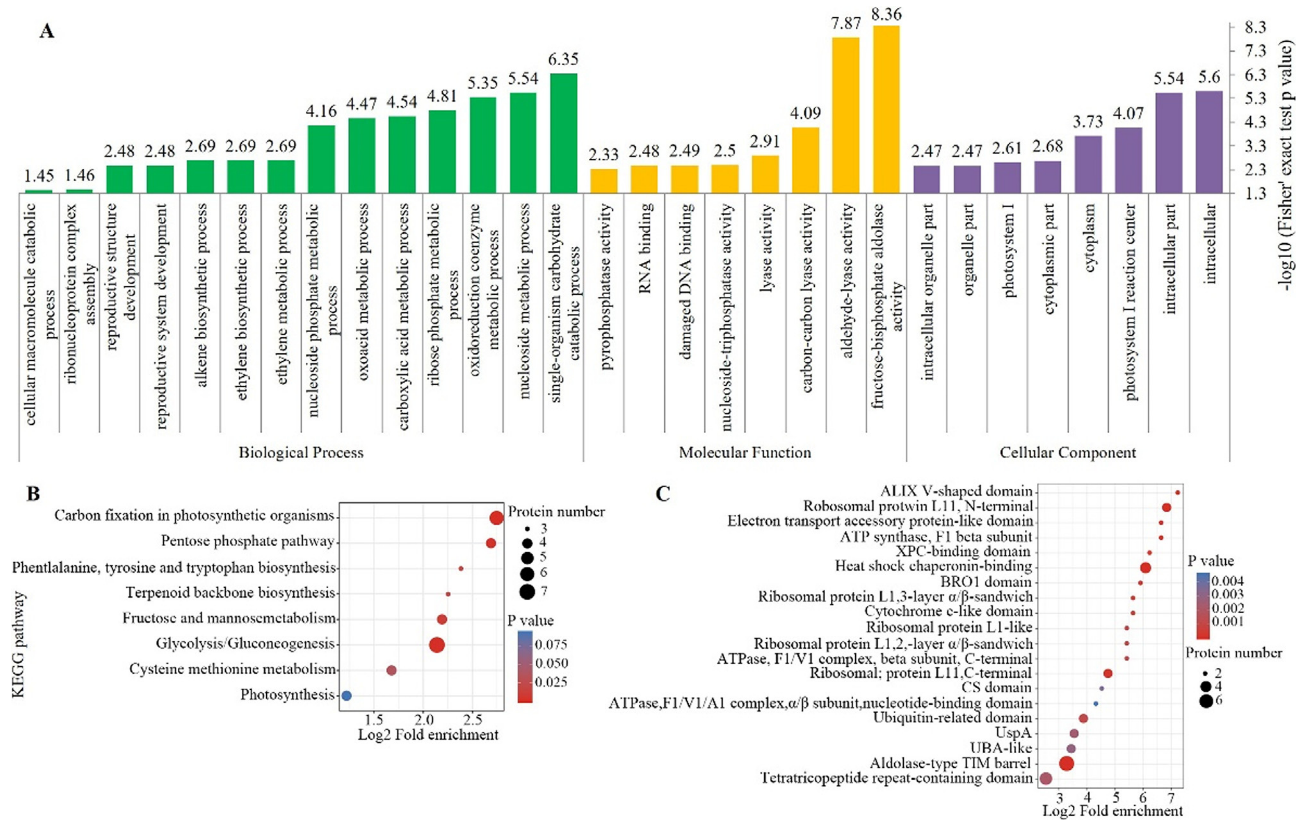



**Fig. 4.** Enrichment analysis of lysine-methylated proteins in tomato. A. Enrichment based on GO annotation. B. Enrichment based on KEGG pathways. C. Enrichment based on protein domain.

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 PAE 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 (Msrs) 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 $H_2O_2$ and hydroperoxides and plays important roles in plant development and response to stresses [48]. Methionine sulfoxide reductases (Msrs), 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 (Trxs) 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 DlGpx 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 SlCaM2 is the major calmodulin during ripening and plays a double role to regulate fruit ripening [52]. Recently, Tang et al. reported that SlCML37, a calmodulin-like protein, enhances tomato fruit chilling stress tolerance by interacting with proteasome maturation factor SlUMP1 [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.

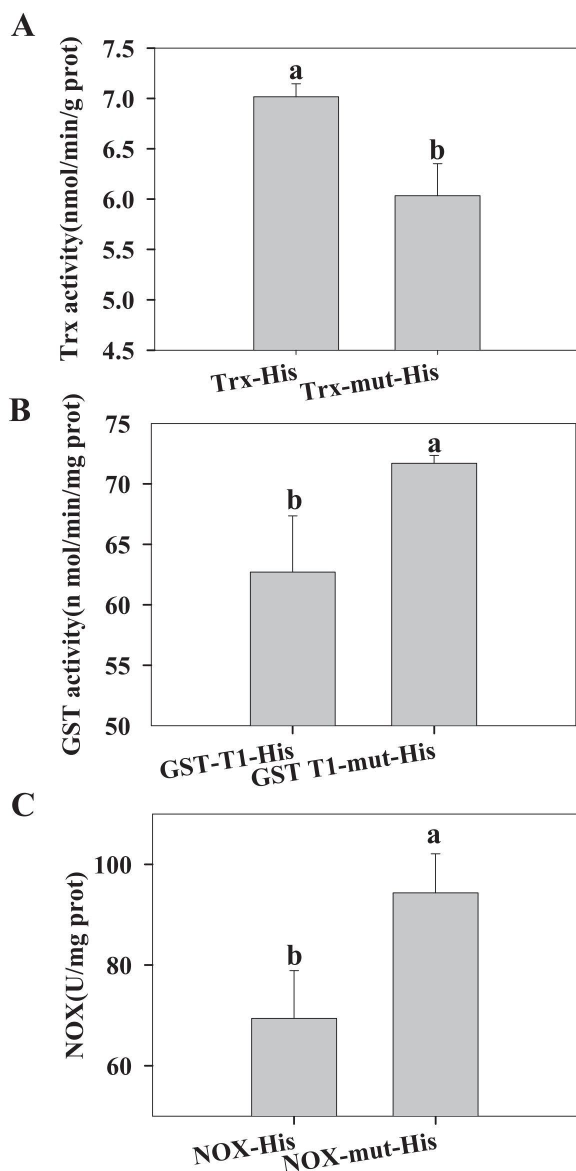



**Fig. 5.** Analysis of enzyme activity of recombinant proteins His-Trx, GST T1, NOX, and their mutations. Mutation of lysine or arginine to alanine leads to failed methylation. Trx: K19A and K21A; NOX: R161A; GST: K228A and R230A. The values with different letters are significantly different ($p < 0.05$).

***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 play 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 mannans [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 SlTGA2.2 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

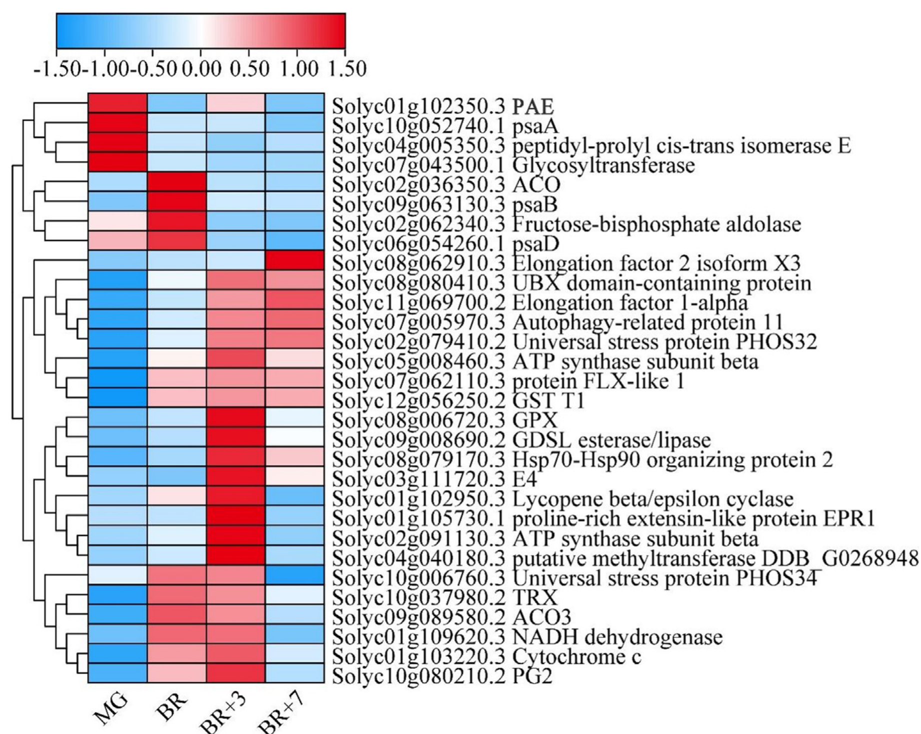



**Fig. 6.** RT-qPCR analysis of some methylated proteins during the ripening of tomato. These selected proteins are mainly associated with fruit ripening and senescence, stress and defence responses, signal transduction, the oxidation–reduction process, energy metabolism, carbohydrate biosynthesis, and other physiological processes. 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 sulfhydryl 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 *NOR* gene is upregulated during tomato fruit ripening and the transcriptional activity of NOR transcription factor is decreased due to sulfoxidation. Shan et al. [72] reported that transcriptional activity of NAC2 transcription factor is supressed 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 post-translational 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:** . **Yueming Jiang:** . **Lili Song:** . **Xuewu Duan:** 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.

## Acknowledgments

This work was supported by National Natural Science Foundation of China (Nos. 31830070, 31871856 and 32102452), Joint Foundation of Basic and Applied Basic Research of Guangdong Province (No. 2020A1515110100), Guangxi Natural Science Foundation (No. 2021GXNSFGA196001), China Postdoctoral Science Foundation (No. 2020M682954).

## Appendix A. Supplementary data

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

## References

[1] Jiang G, Zeng J, Li Z, Song Y, Yan H, He J, et al. Redox regulation of the NOR transcription factor is involved in the regulation of fruit ripening in tomato. Plant Physiol 2020;183(2):671–85. doi: https://doi.org/10.1104/pp.20.00070.
[2] Wang Q, Wang K, Ye ML. Strategies for large-scale analysis of non-histone protein methylation by LC-MS/MS. Analyst 2017;142:3536–48.
[3] Murn J, Shi Y. The winding path of protein methylation research: milestones and new frontiers. Nat Rev Mol Cell Biol 2017;18(8):517–27.
[4] Carlson SM, Gozani Or. Nonhistone lysine methylation in the regulation of cancer pathways. CSH Perspect Med 2016;6(11):a026435. doi: https://doi.org/10.1101/cshperspect.a026435.
[5] Barski A, Cuddapah S, Cui K, Roh T-Y, Schones DE, Wang ZB, et al. High-resolution profiling of histone methylations in the human genome. Cell 2007;129:823–37.
[6] Paik WK, Paik DC, Kim S. Historical review: the field of protein methylation. Trends Biochem Sci 2007;32(3):146–52.
[7] Levy D, Kuo AJ, Chang Y, Schaefer U, Kitson C, Cheung P, et al. Lysine methylation of the NF-κB subunit RelA by SETD6 couples activity of the histone methyltransferase GLP at chromatin to tonic repression of NF-κB signaling. Nat Immunol 2010;12:29–36.
[8] Malodobra-Mazur M, Dziewulska A, Kozinski K, Dobrzyn P, Kolczynska K, Janikiewicz J, et al. Stearoyl-CoA desaturase regulates inflammatory gene expression by changing DNA methylation level in 3T3 adipocytes. Int J Biochem Cell Biol 2014;55:40–50.
[9] Huang J, Dorsey J, Chuikov S, Zhang X, Jenuwein T, Reinberg D, et al. G9a and Glp methylate lysine 373 in the tumor suppressor p53. J Biol Chem 2010;285(13):9636–41.
[10] Jansson M, Durant ST, Cho E-C, Sheahan S, Edelmann M, Kessler B, et al. Arginine methylation regulates the p53 response. Nat Cell Biol 2008;10:1431–9.
[11] Huang J, Perez-Burgos L, Placek BJ, Sengupta R, Richter M, Dorsey JA, et al. Repression of p53 activity by Smyd2-mediated methylation. Nature 2006;444:629–32.
[12] Huang J, Sengupta R, Espejo AB, Lee MG, Dorsey JA, Richter M, et al. p53 is regulated by the lysine demethylase LSD1. Nature 2007;449:105–8.
[13] Ciocca DR, Calderwood SK. Heat shock proteins in cancer: diagnostic, prognostic, predictive, and treatment implications. Cell Stress Chaperones. 2005;10:86–103.
[14] Cho HS, Shimazu T, Toyokawa G, Daigo Y, Maehara Y, Hayami S, et al. Enhanced HSP70lysine methylation promotes proliferation of cancer cells through activation of Aurora kinase B. Nat Commun 2012;3:1072.
[15] Hamamoto R, Saloura V, Nakamura Y. Critical roles of non-histone protein lysine methylation in human tumorigenesis. Nat Rev Cancer 2015;15:110–24.
[16] Lu T, Stark GR. NF-κB: Regulation by methylation. Cancer Res 2015;75:3692–5.
[17] Gaughan L, Stockley J, McCracken SRC, Treumann A, Armstrong K, et al. Regulation of the androgen receptor by SET9-mediated methylation. Nucleic Acids Res 2010;39(4):1266–79.
[18] Munro S, Khaire N, Inche A, Carr S, La Thangue NB. Lysine methylation regulates the pRb tumour suppressor protein. Oncogene 2010;29:2357–67.
[19] Li K-X, Sun Q, Wei L-L, Du G-H, Huang X, Wang J-K. ERα gene promoter methylation in cognitive function and quality of life of patients with Alzheimer disease. J Geriatr Psychiatry Neurol 2019;32(4):221–8.
[20] Xie Qi, Bai Y, Wu J, Sun Yu, Wang Y, Zhang Ye, et al. Methylation-mediated regulation of E2F1 in DNA damage-induced cell death. J Recept Signal Transduction 2011;31(2):139–46.
[21] He A, Shen XH, Ma Q, Cao JJ, Von Gise A, Zhou PZ, et al. PRC2 directly methylates GATA4 and represses its transcriptional activity. Genes Dev 2012;26:37–42.
[22] Kim E, Kim M, Woo D-H, Shin Y, Shin J, Chang N, et al. Phosphorylation of EZH2 activates STAT3 signaling via STAT3 methylation and promotes tumorigenicity of glioblastoma stem-like cells. Cancer Cell 2013;23(6):839–52. doi: https://doi.org/10.1016/j.ccr.2013.04.008.
[23] Dhami GK, Liu HD, Galka M, Voss C, Wei R, Muranko K, et al. Dynamic methylation of numb by Set8 regulates its binding to p53 and Apoptosis. Mol Cell 2013;50:565–76.
[24] Wu ZR, Connolly J, Biggar KK. Beyond histones – the expanding roles of protein lysine methylation. FEBS J 2017;284:2732–44.
[25] Xu XY, Cao XW, Yang J, Chen LH, Liu T, Jin Q. Proteome-wide identification of lysine propionylation in the conidial and mycelial stages of *Trichophyton rubrum*. Front Microbiol 2019;10:1–10.
[26] Rice JC, Allis CD. Histone methylation versus histone acetylation: new insights into epigenetic regulation. Curr Opin Cell Biol 2001;13:263–73.
[27] Martin C, Zhang Yi. The diverse functions of histone lysine methylation. Nat Rev Mol Cell Biol 2005;6(11):838–49.
[28] Rea S, Eisenhaber F, O'Carroll D, Strahl BD, Jenuwein T. Regulation of chromatin structure by site-specific histone H3 methyltransferases. Nature 2000;406:593–9.
[29] Sneeringer CJ, Scott MP, Kuntz KW, Knutson SK, Pollock RM, Richon VM, et al. Coordinated activities of wild-type plus mutant EZH2 drive tumor-associated hypertrimethylation of lysine 27 on histone H3 (H3K27) in human B-cell lymphomas. P Natl Acad Sci USA 2010;107(49):20980–5.
[30] López-Gómez R, Cabrera-Ponce JL, Saucedo-Arias LJ, Carreto-Montoya L, Villanueva-Arce R, Díaz-Perez JC, et al. Ripening in papaya fruit is altered by ACC oxidase cosuppression. Transgenic Res 2009;18:89–97.
[31] Jiang YM, Fu JR. Ethylene regulation of fruit ripening: Molecular aspects. Plant Growth Regul 2000;30:193–200.
[32] Alexander L, Grierson D. Ethylene biosynthesis and action in tomato: a model for climacteric fruit ripening. J Exp Bot 2002;53:2039–55. doi: https://doi.org/10.1093/jxb/erf072.
[33] Xiao L, Li T, Jiang G, Jiang Y, Duan X. Cell wall proteome analysis of banana fruit softening using iTRAQ technology. J Proteomics 2019;209:103506. doi: https://doi.org/10.1016/j.jprot.2019.103506.

[34] García-Gago JA, Posé S, Muñoz-Blanco J, Quesada MA, Mercado JA. The polygalacturonase *FaPG1* gene plays a key role in strawberry fruit softening. Plant Signal Behav 2009;4:766–8.
[35] Chun JP, Huber DJ. Reduced levels of beta-subunit protein influence tomato fruit firmness, cell-wall ultrastructure, and PG2-mediated pectin hydrolysis in excised pericarp tissue. J Plant Physiol 2000;157:153–60. doi: https://doi.org/10.1016/s0176-1617(00)80185-6.
[36] Paniagua C, Ric-Varas P, García-Gago JA, López-Casado G, Blanco-Portales R, Muñoz-Blanco J, et al. Elucidating the role of polygalacturonase genes in strawberry fruit softening. J Exp Bot 2020;71(22):7103–17. doi: https://doi.org/10.1093/jxb/eraa398.
[37] Philippe F, Pelloux J, Rayon C. Plant pectin acetylesterase structure and function: new insights from bioinformatic analysis. BMC Genomics 2017;18(1). doi: https://doi.org/10.1186/s12864-017-3833-0.
[38] Wu B, Shen F, Chen CJ, Liu L, Wang X, Zheng WY, et al. Natural variations in a pectin acetylesterase gene, *MdPAE10* , contribute to prolonged apple fruit shelf life. Plant Genome 2021;14(1). doi: https://doi.org/10.1002/tpg2.v14.110.1002/tpg2.20084.
[39] Cai JH, Qin GZ, Chen T, Tian SP. The mode of action of remorin1 in regulating fruit ripening at transcriptional and post-transcriptional levels. New Phytol 2018;219:1406–20.
[40] Manning K, Tör M, Poole M, Hong Y, Thompson AJ, King GJ, et al. A naturally occurring epigenetic mutation in a gene encoding an SBP-box transcription factor inhibits tomato fruit ripening. Nat Genet 2006;38:948–52.
[41] Choudhury FK, Rivero RM, Blumwald E, Mittler R. Reactive oxygen species, abiotic stress and stress combination. Plant J 2017;90:856–67.
[42] Pitzschke A, Hirt H. Mitogen-activated protein kinases and reactive oxygen species signaling in plants. Plant Physiol 2006;141:351–6.
[43] Rey P, Tarrago L. Physiological roles of plant methionine sulfoxide reductases in redox homeostasis and signaling. Antioxidants (Basel) 2018;7:114. doi: https://doi.org/10.3390/antiox7090114.
[44] MØller IM, Jensen PE, Hansson A. Oxidative modifications to cellular components in plants. Annu. Rev. Plant Biol 2007;58:459–81.
[45] Jiang G, Xiao L, Yan H, Zhang D, Wu F, Liu X, et al. Redox regulation of methionine in calmodulin affects the activity levels of senescence-related transcription factors in litchi. BBA-Gen. Subjects. 2017;1861(5):1140–51. doi: https://doi.org/10.1016/j.bbagen.2017.02.004.
[46] Tian S, Qin G, Li B. Reactive oxygen species involved in regulating fruit senescence and fungal pathogenicity. Plant Mol Biol 2013;82(6):593–602. doi: https://doi.org/10.1007/s11103-013-0035-2.
[47] Wu F, Jiang G, Yan H, Xiao L, Liang H, Zhang D, et al. Redox regulation of glutathione peroxidase by thioredoxin in longan fruit in relation to senescence and quality deterioration. Food Chem 2021;345:128664. doi: https://doi.org/10.1016/j.foodchem.2020.128664.
[48] Kim I, Ji S, Jeong Y. Antioxidant activity of *Suaeda japonica* makino sprout extracts. J Korean Soc Food Sci Nutr 2019;48(1):40–8.
[49] Demidchik V, Maathuis F, Voitsekhovskaja O. Unravelling the plant signalling machinery: an update on the cellular and genetic basis of plant signal transduction. Funct Plant Biol 2018;45:1–8. doi: https://doi.org/10.1071/fp17085.
[50] Li L, Xing Y, Chang D, Fang S, Cui B, Li Q, et al. CaM/BAG5/Hsc70 signaling complex dynamically regulates leaf senescence. Sci Rep 2016;6(1). doi: https://doi.org/10.1038/srep31889.
[51] Nie H, Zhao C, Wu G, Wu Y, Chen Y, Tang D. SR1, a calmodulin-binding transcription factor, modulates plant defense and ethylene-induced senescence by directly regulating *NDR1* and *EIN3*. Plant Physiol 2012;158:1847–59. doi: https://doi.org/10.1104/pp.111.192310.
[52] Yang T, Peng H, Bauchan GR. Functional analysis of tomato calmodulin gene family during fruit development and ripening. Hortic Res 2014;1(1). doi: https://doi.org/10.1038/hortres.2014.57.
[53] Tang M, Xu C, Cao H, Shi Y, Chen J, Chai Y, et al. Tomato calmodulin-like protein SlCML37 is a calcium (Ca2+) sensor that interacts with proteasome maturation factor SlUMP1 and plays a role in tomato fruit chilling stress tolerance. J Plant Physiol 2021;258-259:153373. doi: https://doi.org/10.1016/j.jplph.2021.153373.
[54] Park G, Servin JA, Turner GE, Altamirano L, Colot HV, Collopy P, et al. Global analysis of serine-threonine protein kinase genes in *Neurospora crassa*. Eukaryot Cell 2011;10:1553–64.
[55] Li C, Chang P-P, Ghebremariam KM, Qin L, Liang Y. Overexpression of tomato *SpMPK3* gene in Arabidopsis enhances the osmotic tolerance. Biochem Biophys Res Commun 2014;443(2):357–62. doi: https://doi.org/10.1016/j.bbrc.2013.11.061.
[56] Mercier V, Laporte MH, Destaing O, Blot B, Blouin CM, Pernet-Gallay K, et al. ALG-2 interacting protein-X (Alix) is essential for clathrin-independent endocytosis and signaling. Sci Rep 2016;6:1–15.
[57] García-León M, Cuyas L, El-Moneim DA, Rodriguez L, Belda-Palazón B, Sanchez-Quant E, et al. Arabidopsis ALIX regulates stomatal aperture and turnover of abscisic acid receptors. Plant Cell 2019;31(10):2411–29. doi: https://doi.org/10.1105/tpc.19.00399.
[58] Somerville C. Cellulose synthesis in higher plants. Annu Rev Cell Dev Biol 2006;22(1):53–78.
[59] Richmond TA, Somerville CR. Integrative approaches to determining Csl function. Plant Mol Biol 2001;47:131–43.
[60] Zhu JH, Lee B-H, Dellinger M, Cui XP, Zhang CQ, Wu S, et al. A cellulose synthase-like protein is required for osmotic stress tolerance in Arabidopsis. Plant J 2010;63:128–40.
[61] Zhao H, Li Z, Wang Y, Wang J, Xiao M, Liu H, et al. 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. Plant Biotechnol J 2021. doi: https://doi.org/10.1111/pbi.13729.
[62] Li P, Li Z, Xie G, Zhang J. Trihelix transcription factor ZmThx20 iIs required for kernel development in maize. Int J Mol Sci 2021;22(22):12137. doi: https://doi.org/10.3390/ijms222212137.
[63] Magwanga RO, Kirungu JN, Lu P, Yang X, Dong Q, Cai X, et al. Genome wide identification of the trihelix transcription factors and overexpression of Gh_A05G2067 (GT-2), a novel gene contributing to increased drought and salt stresses tolerance in cotton. Physiol Plant 2019;167(3):447–64. doi: https://doi.org/10.1111/ppl.12920.
[64] Völz R, Kim S-K, Mi J, Mariappan KG, Guo X, Bigeard J, et al. The Trihelix transcription factor GT2-like 1 (GTL1) promotes salicylic acid metabolism, and regulates bacterial-triggered immunity. PLoS Genet 2018;14(10):e1007708. doi: https://doi.org/10.1371/journal.pgen.1007708.
[65] Gatz C. From pioneers to team players: TGA transcription factors provide a molecular link between different stress pathways. Mol Plant Microbe Interact 2013;26(2):151–9.
[66] Shimizu K, Suzuki H, Uemura T, Nozawa A, Desaki Y, Hoshino R, et al. Immune gene activation by NPR and TGA transcriptional regulators in the model monocot *Brachypodium distachyon*. Plant J 2022. doi: https://doi.org/10.1111/tpj.15681.
[67] Qi P, Huang M, Hu X, Zhang Y, Wang Y, Li P, et al. A *Ralstonia solanacearum* effector targets TGA transcription factors to subvert salicylic acid signaling. Plant Cell 2022. doi: https://doi.org/10.1093/plcell/koac015.
[68] Lemaire-Chamley M, Koutouan C, Jorly J, Assali J, Yoshida T, Nogueira M, et al. A chimeric TGA repressor slows down fruit maturation and ripening in tomato. Plant Cell Physiol 2022;63(1):120–34. doi: https://doi.org/10.1093/pcp/pcab150.
[69] Karam JA, Parikh RY, Nayak D, Rosenkranz D, Gangaraju VK. Co-chaperone Hsp70/Hsp90-organizing protein (Hop) is required for transposon silencing and Piwi-interacting RNA (piRNA) biogenesis. J Biol Chem 2017;292(15):6039–46.
[70] Hatton PJ, Dixon D, Cole DJ, Edwards R. Glutathione transferase activities and herbicide selectivity in Maize and associated weed species. Pestic Sci 2015;46:267–75.
[71] Fromm S, Senkler J, Eubel H, Peterhnsel C, Braun H-P. Life without complex I: Proteome analyses of an Arabidopsis mutant lacking the mitochondrial NADH dehydrogenase complex. J Exp Bot 2016;67:3079–93.
[72] Shan W, Kuang J-f, Wei W, Fan Z-q, Deng W, Li Z-g, et al. MaXB3 modulates MaNAC2, MaACS1, and MaACO1 stability to repress ethylene biosynthesis during banana fruit ripening. Plant Physiol 2020;184:1153–71.