Horticulture, Environment, and Biotechnology (2022) 63:557–569
https://doi.org/10.1007/s13580-022-00419-5

RESEARCH REPORT

STAY-GREEN (*SGR*) genes in tomato (*Solanum lycopersicum*): genome-wide identification, and expression analyses reveal their involvements in ripening and salinity stress responses

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Received: 11 August 2021 / Revised: 6 January 2022 / Accepted: 7 January 2022 / Published online: 18 July 2022

Abstract

Chlorophyll (Chl) is present in many plant organs and plays vital roles during growth and development. Degradation of Chl causes the loss of green colour that typically occurs during senescence, and fruit ripening. The present study addresses genome-wide identification and bioinformatics analyses of tomato (*Solanum lycopersicum*), *SlSGR1* (Solyc08g080090.2.1) *SlSGR2* (Solyc12g056480.1.1) and *SlSGR-like* (Solyc04g063240.2.1) genes. Multiple sequence alignment indicated that the three tomato SGR proteins have conserved domains. Motif, sequence, and protein structure analysis showed that SlSGR-like differentially evolved from SlSGR proteins. Co-expression analyses were performed for each *SlSGR* using transcriptomic data of two fruit ripening stages [mature green (MG) and ripe fruit (R)] of Ailsa Craig (AC) tomato cultivar. According to the co-expression network analyses, SlSGRs participate in sulphur homeostasis, fatty acid biosynthesis, and biological processes of plant development during ripening stages of tomato. Six common genes were identified in the merged co-expression network. Also, 38 transcription factor families (TFFs) were searched in the co-expression network. Only 13 transcription factors, belonging to seven TFFs, were found to be involved in regulation of these two genes. Of these TFFs, *GRAS* and *GeBP* had five and three members for *SlSGR-like* and *SlSGR1* in the network. The expression profiles of *SlSGR1/2* and *SlSGR-like* in different tissues and different fruit ripening stages showed that *SlSGR1/2* are highly expressed in ripening fruits, whereas *SlSGR-like* is more involved in leaf maturation. Moreover, expression of *SlSGR1/2* and *SlSGR-like* in the leaves of 'Ciko' and 'Black' tomato varieties under 200mM salt stress indicated that significant expression changes occurred in *SlSGR2* suggesting that *SlSGR* genes may be involved in signalling responses to abiotic stress. The findings provide new insight into the functions of these genes in growth and salinity stress.

Keywords Bioinformatics · Co-expression · Fruit ripening · Salt stress · Tomato

Communicated by Sung-Chur Sim.

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1 Introduction

Many immature fleshy fruits and young leaves undergo dramatic changes during maturation and ripening stages in terms of chemical composition and ultrastructure (Abdelrahman et al. 2017). Chlorophyll (Chl), including *Chla* and *Chlb* molecules, plays vital roles in absorption, transmission, and transformation of light energy in the process of photosynthesis in green plants (Hörtensteiner 2009). Environmental stress-induced leaf senescence is also characterized by degradation of *Chls*, proteins, nucleic acids and lipids, as well as lower metabolic activities (Abdelrahman et al. 2017). Fruit ripening and leaf senescence involve the degradation of chlorophylls, and enhancement of carotenoids and anthocyanins (Matile et al. 1999). The breakdown of

chl-protein complexes, leading to chlorophyl degradation, is a prerequisite of the de-greening process. It also helps meet the nutritional needs of fast-growing vegetative tissues and reproductive organs (Lim et al. 2007). The '*Stay Green*' trait is one of the most physiologically and agronomically important traits allowing plants maintain photosynthetic activity and improve stress tolerance.

The biochemical pathway of Chl breakdown, called the PAO pathway, has been characterized by the functions of Chl catabolic enzymes (CCEs) (Kuai et al. 2018). Before the Chl degradation pathway begins, chl *b* is converted to chl *a* through a two-step reaction (Kusaba et al. 2007; Meguro et al. 2011). Chl *a* is then converted into a primary fluorescent Chl catabolite (pFCC) by several CCEs in four successive steps (Jiao et al. 2020). The genes (*SGR, RCCR, PAO, PPH, NYC1* and *NOL*), involved in these processes, which encode the enzymes involved in leaf senescence and fruit ripening, have been identified (Shimoda et al. 2016; Schelbert et al. 2009; Pružinská et al. 2007). In the first four successive steps of the conversion, magnesium (Mg) in Chl *a* is removed by a Mg-dechelatase named STAY-GREEN (SGR) in *Arabidopsis*, and Chl *a* is converted into pheophytin a (Phein a) (Shimoda et al. 2016). The functional mechanism and regulatory networks of SGRs and SGR homologs showed that SGR removes Mg not only from free chlorophyll but also from chlorophyll-protein complexes (Shimoda et al. 2016).

The stay-green phenotype, induced by the *sgr* mutation was initially identified in pea by Mendel (1901). After that, the SGRs and SGR homologs were identified in multiple species, including rice (Rong et al. 2013), *Arabidopsis* (Sakuraba et al. 2012, 2014b), tomato (Barry et al. 2008; Luo et al. 2013), pepper (Barry et al. 2008) and alfalfa (Zhou et al. 2011). In recent years, Chl breakdown in response to several abiotic/biotic stresses has become a hot topic in the scientific community due to the relationship between "*STAY GREEN*" and beneficial agricultural characteristics such as tolerance to abiotic/biotic stresses as well as improved yield production. In this regard, while *gr1/nye1-1* and *sgrl* mutants displayed a stay-green phenotype under abiotic stress conditions, including high salinity, drought and heat, the overexpression of *SGR1* and *SGR-like* results in early leaf yellowing (Sakuraba et al. 2014a). On the other hand, *Arabidopsis* plants overexpressing *SGR2*, exhibited a '*Stay Green*' phenotype (Sakuraba et al. 2014b), suggesting that whilst *SGR1* and *SGR-like* positively regulate Chl degradation, *SGR2* negatively regulates the process during senescence. Tomato fruit ripening leads to fruit colouration and is dependent on the accumulation of carotenoids accompanied by the degradation of Chl (Liu et al. 2015). Silencing of *SlSGR1* inhibited chlorophyll degradation, exhibited a stay-green phenotype and extended the shelf-life of tomato (Hu et al. 2011; Luo et al. 2013a). In a recent study, lycopene levels were increased in CRISPR/Cas9- multiplex genome edited tomato, with edited *SlSGR1* and four other genes (Li et al. 2018). The overexpression of the *SGR-like* gene results in earlier leaf yellowing than wild-type (WT) lines under ABA treatment (Yang et al. 2020). Therefore, these results suggest that *SGR* genes play a pivotal regulatory role in Chl degradation and colour formation during fruit ripening. Although the functions of *SGR/SGR-like* genes during Chl degradation and the interactions between *SGR/SGR-like* genes and other CCEs have been demonstrated in various organisms, little is known about how *SGR/SGR-like* genes interact and which transcription factors are involved in their regulation of fruit ripening, and how *SGR/SGR-like* genes are expressed under both fruit ripening and salt stress conditions in tomato. For this reason, we performed comprehensive bioinformatics analyses of *SlSGR* genes in tomato. By conducting co-expression network analyses, we investigated how *SlSGRs* are interacted with each other and on which transcription factor families (TFFs) are more involved in their regulations under fruit ripening conditions. We also tried to identify gene expression profiles of *SlSGR* genes in different tomato organs and ripening stages. Lastly, we investigated the expression of *SlSGR* genes under salt stress using two different tomato varieties, 'Ciko' and 'Black'.

2 Materials and methods

2.1 Identification and annotation of *SGR* genes in *S. lycopersicum*

To identify *SlSGR* genes in tomato, we obtained all the SGR protein sequences as query sequences from the TAIR (https://www.arabidopsis.org/), SGN (https://solgenomics.net/) and PMRD (http://bioinformatics.cau.edu.cn/PMRD/) databases. The proteins, encoded by the putative *SlSGR* genes, were analysed for domain analysis with the Pfam database (http://pfam.xfam.org/).

2.2 Sequence analysis of *SlSGR* genes

The physico-chemical characteristics of *SlSGR* genes, including protein length, isoelectric point (pI), and molecular weight (MW) were predicted by ExPASy ProtParam (https://web.expasy.org/protparam/) (Gasteiger et al. 2003). Generic phosphorylation sites in SlSGRs were searched online using NetPhos − 3.1 (https://services.healthtech.dtu.dk/ service.php?NetPhos-3.1) (Blom et al. 1999). Amino acid sequences of SlSGR proteins from different plant species were aligned using BioEdit (Hall et al. 2011). CELLO

(http://cello.life. nctu.edu.tw/) was used to predict the subcellular localizations (Yu et al. 2006).

2.3 Phylogenetic evolution and gene structure analyses

The phylogenetic tree was constructed using MEGA-X software with the maximum likelihood estimation (MLE) method and was analysed with 1000 bootstrap replications (Kumar et al. 2018). Predicted functional annotation of SlSGR proteins was performed on the PANNZER server (http://ekhidna2.biocenter.helsinki.fi/sanspanz/) (Törönen et al. 2018). The exon-intron organizations were constructed according to the Gene Structure Display Server (http://gsds.gao-lab.org/) (Guo 2007). To search conserved motifs in SlSGR protein sequences, Multiple Expectation Maximization for Motif Elicitation (https://meme-suite.org/meme/tools/meme) was used (Bailey et al. 2009).

2.4 Promoter and miRNA analyses

To search for cis-acting elements in the promoter sequences of *SlSGR* genes, genomic DNA sequences in the promoter region (1500 bp) were downloaded from Phytozome and the promoter regions were scanned using the PlantPAN 3.0 server (http://plantpan.itps.ncku.edu.tw/) (Chow et al. 2019). The potential target miRNAs of *SlSGRs* were predicted by psRNA target server (http://plantgrn.noble.org/psRNATarget/) based on default parameters (Dai and Zhao 2011).

2.5 Co-expression gene network analyses

The co-expression networks were created using a sub-dataset of the study conducted by Silva et al. (2021). The full raw data and its count matrix are available with the GSE148217 number on Gene Expression Omnibus (GEO), NCBI (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE148217). Three *SlSGR* genes *SlSGR1* (Solyc08g080090), *SlSGR2* (Solyc12g056480.1.1) and *SlSGR-like* (Solyc04g063240) were searched in the count matrix file and only two *SlSGR* genes (*SlSGR1* and *SlSGR-like*) were found. The correlation analyses were conducted with about 19, 000 genes after filtration of the sub-dataset and using the read counts of mature green (MG) and ripe fruit (R) stages of tomato cultivar Ailsa Craig (AC), selected from the count matrix of the study. The details of correlation analysis were described by (Contreras-López et al. 2018). Then the most highly correlated genes with each *SlSGR* were filtered from the correlation result file at a > 0.9 correlation coefficient (r) level using Pandas package of Python programming language (Mckinney 2010). Then, each *SlSGR* gene file was merged into a single "result file" and the members of 38 transcription factor families, obtained from iTAK TF database (http://itak.feilab.net/cgi-bin/itak/index.cgi) (Zheng et al. 2016), were searched and labeled within the result file. The data on the file was visualized on Cytoscape 3.8.2 (Shannon et al. 2003).

2.6 Plant materials and salt stress treatments

Laboratory experiments were conducted at Burdur Mehmet Akif Ersoy University, Turkey; between June-September in 2020. Flower, young/mature leaf, and pericarp at four developmental stages including IMG (immature green), MG (mature green), BR (Breaker) and Ripe (R) were collected from 'red cherry' tomato cultivar to determine *SlSGR* gene expression profiles at fruit ripening stages.

Two tomato (*Solanum lycopersicum* L.) hybrids 'Ciko F1' and 'Black' were used for salt treatment. Plants were grown with Hoagland solution for two months and salt treatment was applied gradually (50 mM, 100 mM, 150 mM and finally 200 mM NaCl) over 3-day intervals to avoid osmotic shock (Akbudak and Filiz 2019). After 24 h of the final treatment of 200 mM NaCl, plant leaves were picked for RNA isolation. For each stage, three biological replicates were prepared. All the samples were immediately frozen in liquid nitrogen and were transferred to -80 °C until RNA isolation.

2.7 RNA isolation and gene expression analysis by RT-qPCR

Total RNA was extracted from harvested plant tissues (flower, young / mature leaf, fruit pericarps and salinity treated plants) using PureLink™ Plant RNA Reagent (Thermo Fisher Scientific) in accordance with the manufacturer's instructions. Total RNA was dissolved in 50 µL of elution buffer, the concentration and quality of RNA were determined by NanoDrop (Epoch Microplate, Biotek). Total RNA was reverse transcribed into cDNA by the RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific). The mean of tomato *Actin* (Diretto et al. 2020) and *Elongation Factor 1-α* (Pokalsky et al. 1989) genes were used as the internal control for normalization of the expression of the target genes. The RT-qPCR reaction was performed in a 20 µl reaction mixture using the CFX96™ Real-Time System (Bio-Rad). The thermal program for PCR was set using the following conditions: 95 °C for 5 min, 35 cycles of 5 s at 95 °C, 30 s at 60 °C, and 30 s at 72 °C. The relative expression level of *SlSGR* genes from three biological and two technical replicates was calculated by the $2^{-\Delta Ct}$ method (Livak and Schmittgen 2001). Primers used in this experiment are listed in (Supplementary Table S1). Changes

Table 1 Sequence features of SGR proteins in tomato, *Arabidopsis*, and rice

Phytozome ID	Species	Exon no	Protein length (aa)	*Domain family	Mol. wt. (kDa)	*pI*	SL	**NetPhos
AT4G22920.1	*Arabidopsis*	4	268	PF12638	300.52	8.83	C	34
AT4G11910.1	*Arabidopsis*	4	271	PF12638	307.52	8.88	C	27
AT1G44000.1	*Arabidopsis*	4	260	PF12638	298.92	8.83	C	24
SlSGR1	tomato	4	272	PF12638	305.33	8.85	C	24
SlSGR2	tomato	4	247	PF12638	279.00	8.56	N	29
SlSGR-like	tomato	5	242	PF12638	274.83	6.66	C	33
Os04g59610.2	rice	4	334	PF12638	281.81	9.32	Ext	23
Os09g36200.1	rice	3	274	PF12638	308.79	8.42	M	8

* PF12638: Staygreen protein, C: chloroplast, N: nucleus, **NetPhos 3.1 server predicts serine, threonine or tyrosine phosphorylation sites in eukaryotic proteins

in relative expression levels of the gene were checked for statistical significance in accordance with the one-way ANOVA and the means and standard deviation of the replications were compared by the least significant difference (LSD) test at the 5% level.

3 Results and discussion

3.1 Identification of the *SGR* genes

Members of the *SlSGR* family were downloaded from the *Solanum lycopersicum* genome (https://solgenomics.net/) and SGR domains (PF12638) were identified using the Pfam database (http://pfam.xfam.org/) profile of the SGR domain. The protein properties of SlSGRs including ID number, exon number, protein length, theoretical isoelectric point (*pI*), molecular weight, domain family, subcellular localization and phosphorylation sites are presented in Table 1. The protein lengths of SlSGRs ranged from 242 to 334 amino acids (aa) with an average length of 271 residues. Molecular weight varied from 27.4 kDa to 30.8 kDa, and the *pI* values varied from 6.66 to 9.32, with an average of 8.5, suggesting that most SlSGR proteins were weakly basic. The subcellular localization predictions for SlSGRs indicated their localizations to be in the chloroplast, nucleus, extracellular matrix, and mitochondria. The number of predicted phosphorylation sites of SlSGR proteins ranged from 8 to 33 with an average of 25. The highest prediction values for phosphorylation sites are chloroplast, nucleus, EM and mitochondria, suggesting dynamic regulation of SlSGR proteins in cell metabolism.

3.2 Conserved motif and phylogenetic analyses

To better understand the SlSGR proteins, the identity matrix of the SlSGR protein sequences of tomato, *Arabidopsis* and rice were calculated, and ranged between 0.699 and 0.281 (Supplementary Table S2). The highest identity was about 70% between AtSGR1 and AtSGR2, followed by about 62% between AtSGR1 and SlSGR1, and about 61% between SlSGR1 and SlSGR2. The lowest sequence identity was determined between AtSGR1 and OsSGR1. Consequently, the motif analysis showed that there were sequence variations among SGR proteins.

To identify conserved domains of SGR proteins, amino acid sequences of eight proteins from *Arabidopsis*, tomato, and rice were aligned with BioEdit. The results showed that SGR domains had three different conserved sites, including chloroplast signal peptides, SGR domains and variable C-terminal regions (Fig. 1). SGR proteins have four Cys-rich motifs (C-X3-C-X-C2-F-P-X5-P) at the C-terminus, but SGR-like proteins, whose unstable region is 12 to 38 amino acids long, lack this motif (Pilkington et al. 2012). This result is consistent with that of Bade et al. (2016) suggesting that SlSGR-like proteins do not have the CRM domain, which plays unique roles in the conformational change and Mg-dechelating activity (Xie et al. 2019).

To explore the evolutionary relationship of the SlSGR family, SGR protein sequences of *S. lycopersicum*, *A. thaliana*, and *O. sativa* were used to generate the phylogenetic tree (Fig. 2). A total of eight SGRs were clearly divided into two groups, called SGR and SGR-like with well-supported bootstrap values (1000 replicates). The phylogenetic tree showed that SlSGR-like belonged to the SGR-like subfamily.

3.3 Gene structure and protein motif composition analyses of *SGR* genes

Protein motifs are conserved amino acid sequences, which are functionally important portions of proteins. To identify the structural diversification of SGR proteins, four conserved motifs were identified by MEME server and annotated with SMART and Pfam database (Fig. 3a). The lengths of these conserved motifs varied from 29 to 50 amino acids. All SGR

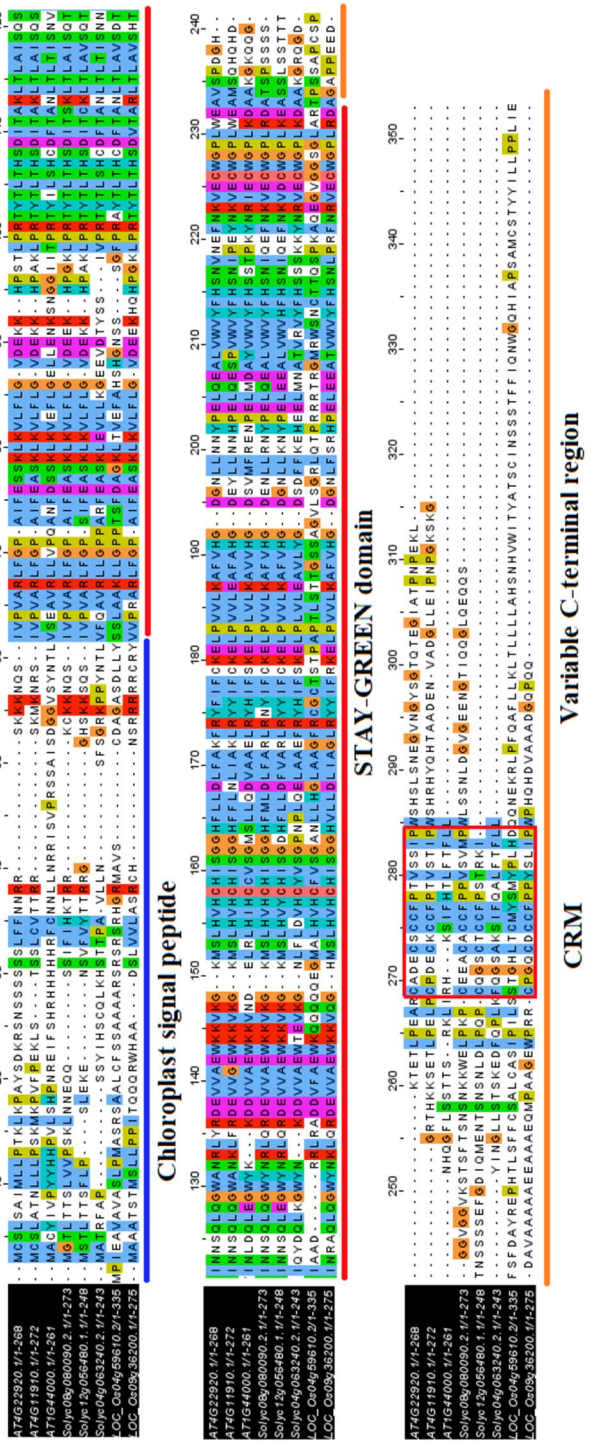

Fig. 1 Multiple alignments of SGR proteins in tomato, *Arabidopsis*, and rice using BioEdit program. Black shading indicates the identical regions of SGR proteins. Blue, red, and orange lines indicate the chloroplast signal peptide, the STAY-GREEN protein domain structure (PF12638), and variable C-terminal region, respectively. CRM is shown in the red frame

proteins had similar motifs (Fig. 3b). Of these, motif 1 was found in all the SGRs, except for LOC_Os04g59610.2. This motif similarity indicates that *SGR* genes may share similar functions (Fig. 3c.).

In order to compare the diversity of gene structure, the exon-intron organization of *SGR* genes was analysed (Fig. 3d). The number of introns in *SGR*s varied from two to four. Although *AtSGRs* contain two introns, *SlSGR1* contains three introns, *SlSGR2* consists of three introns with no UTRs and *SlSGR-like* is comprised of four introns. Overall, *SGR* genes show a complex gene structure with varying intron positions and lengths.

3.4 Gene ontology enrichment analysis

In terms of GO categories, we predicted the functions of all SlSGR proteins, including biological process, molecular function, and cellular component using PANNZER server (Törönen et al. 2018). A total of three biological processes, three molecular functions, and four cellular components were identified (Fig. 4). Chloroplast organization (GO:0009658), chlorophyll metabolic processes (GO:0090056) and tetrapyrrole catabolic processes (GO:1,901,404) were identified in the biological process category indicating that *SlSGR* genes mostly function in chlorophyll metabolism. Protein binding (GO:0005515), tRNA 2'-phosphotransferase activity (GO:0000215), and ribosyltransferase activity (GO:0003950) were predicted for the molecular function category. Therefore, *SlSGR* genes may be involved in tRNA metabolism. Plastid thylakoid membrane (GO:0055035), integral component of membrane (GO:0016021), plastid membrane (GO: 0042170) and extracellular region (GO: 0005576) were identified under the cellular component category. In conclusion, these SGR proteins may have a wide range of roles in chlorophyll metabolism.

3.5 Prediction of miRNA targets

MicroRNAs (miRNAs) are small noncoding single-stranded RNA molecules that play important regulatory roles by targeting mRNAs for cleavage or translational repression (Li et al. 2017). To identify potential miRNA target sites within the putative *SlSGR* genes, the mRNA sequences were analysed with the psRNATarget server. By querying the miRNA database, we identified two miRNAs, sly-miR9469-3p and sly-miR156e-5p, targeting *SlSGR-like* and *SlSGR2* genes, respectively, for translation and cleavage (Supplementary Table S3). Consequently, transcriptional regulation of *SlSGR*s may be controlled by miRNAs.

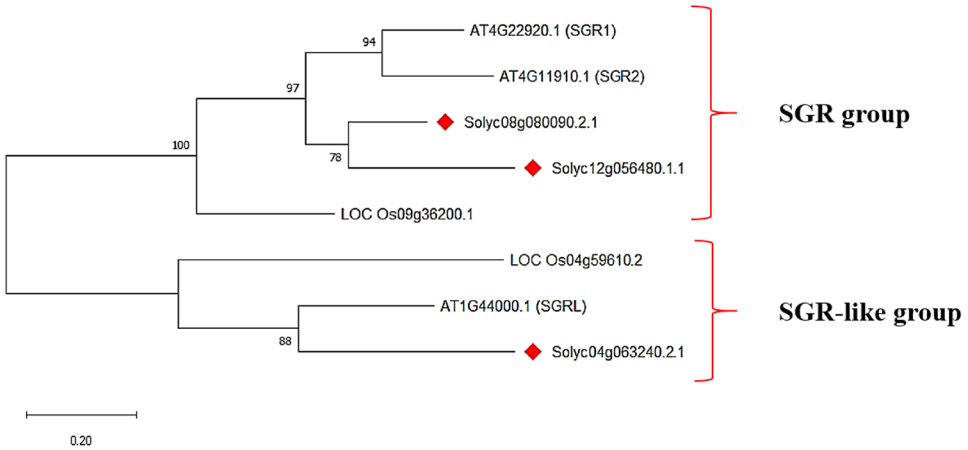

Fig. 2 Phylogenetic tree of *SGR* genes in *S. lycopersicum*, *Arabidopsis* and *O. sativa*. The rootless phylogenetic trees of *S. lycopersicum*, *Arabidopsis* and *O. sativa* SGR protein sequences

3.6 *Cis*-regulatory elements analysis

cis-regulatory elements are special motifs involved in gene transcriptional regulation during plant development and in the response to stress (Le et al. 2012). To explore the transcriptional regulation and potential functions of *SlSGRs*, the putative cis-regulatory elements in the 200 bp upstream region of the initiation codon were analysed using the PlantCARE database and, eight cis-regulatory elements were identified (Fig. 5). AT-hook transcription factors, are found specifically in land plants, and are involved in stress responses and regulation of growth and development (Favero et al. 2020). MYB TFs are characterized by a highly conserved DNA-binding domain and regulate plant-specific processes like hormone responses, differentiation, biotic and abiotic stresses, and phenylpropanoid metabolism (Segarra et al. 2009). WRKY TFs regulate many different plant processes such as biotic/abiotic stresses, seed development, and senescence (Zou et al. 2004). Additionally, different functions related to *cis*-elements were found in the promoter region of *SlSGRs*, such as *GATA* (phytohormone), *DOF* (light, seed maturation and germination), *bHLH* (regulation of fruit dehiscence, stress responses), *bZIP* (photomorphogenesis and energy homeostasis), and homeodomain (responses to environmental stresses) (Perotti et al. 2017). Overall, the *cis*-regulatory elements found in the *SlSGRs* indicate that *SlSGR* genes may participate in responses to hormones and stress signals.

3.7 Co-expression network analyses of *SlSGR* genes

Co-expression network analysis can reveal modules of co-expressed genes, which share similar biological functions in large transcriptomic datasets (Rao and Dixon 2019). In the present study two co-expression networks were set up for *SlSGR1* and *SlSGR-like* genes. Later, these two networks were amalgamed to identify co-expressed common genes. Each co-expression network was constructed using transcriptomic data of two fruit ripening stages, mature green (MG) and ripe fruit (R) stages, of the AC tomato cultivar. After correlation analyses, 3875 and 2287 genes clustered in the networks of *SlSGR1* and *SlSGR-like*, respectively, at $r > 0.9$. The top ten identified neighbours of *SlSGR1* in the co-expression network functioned in autophagocytosis, calcium signalling, carbon fixation and sucrose metabolism, cell wall matrix polysaccharide biosynthesis, arginine metabolism, cysteine oxidation and proteins phosphorylation (Table 2). The top ten neighbour genes in the network of *SlSGR-like* were found to take part in magnesium (Mg) metabolism, DNA methylation, oxidoreductase activity, anthocyanin biosynthesis, acyl lipid metabolism, extracellular signaling and ubiquitin-mediated proteolysis (Table 3). The association of *SlSGR* genes with very broad metabolic pathways supports the importance of the gene family in cell metabolism.

In addition, six common genes were the identified when two co-expression networks were merged. The functional annotations of these genes are shown in Table 4. Of these genes, thioredoxin proteins play vital roles in catalysing the thiol-disulfide interchange and regulation of the redox environment of the cell (Gelhaye et al. 2005). Enoyl-acyl carrier protein (ACP) reductases (ENRs) are involved in fatty acid biosynthesis, catalyzing the fatty acid elongation cycle (Massengo-Tiassé and Cronan 2009). Cupin family protein family like germin and germin-like proteins (GLPs) are involved in plant development and defence (Wang et al. 2014; Hu et al. 2014) stated that Pollen Ole e 1 domain-containing proteins act as developmental regulators as well

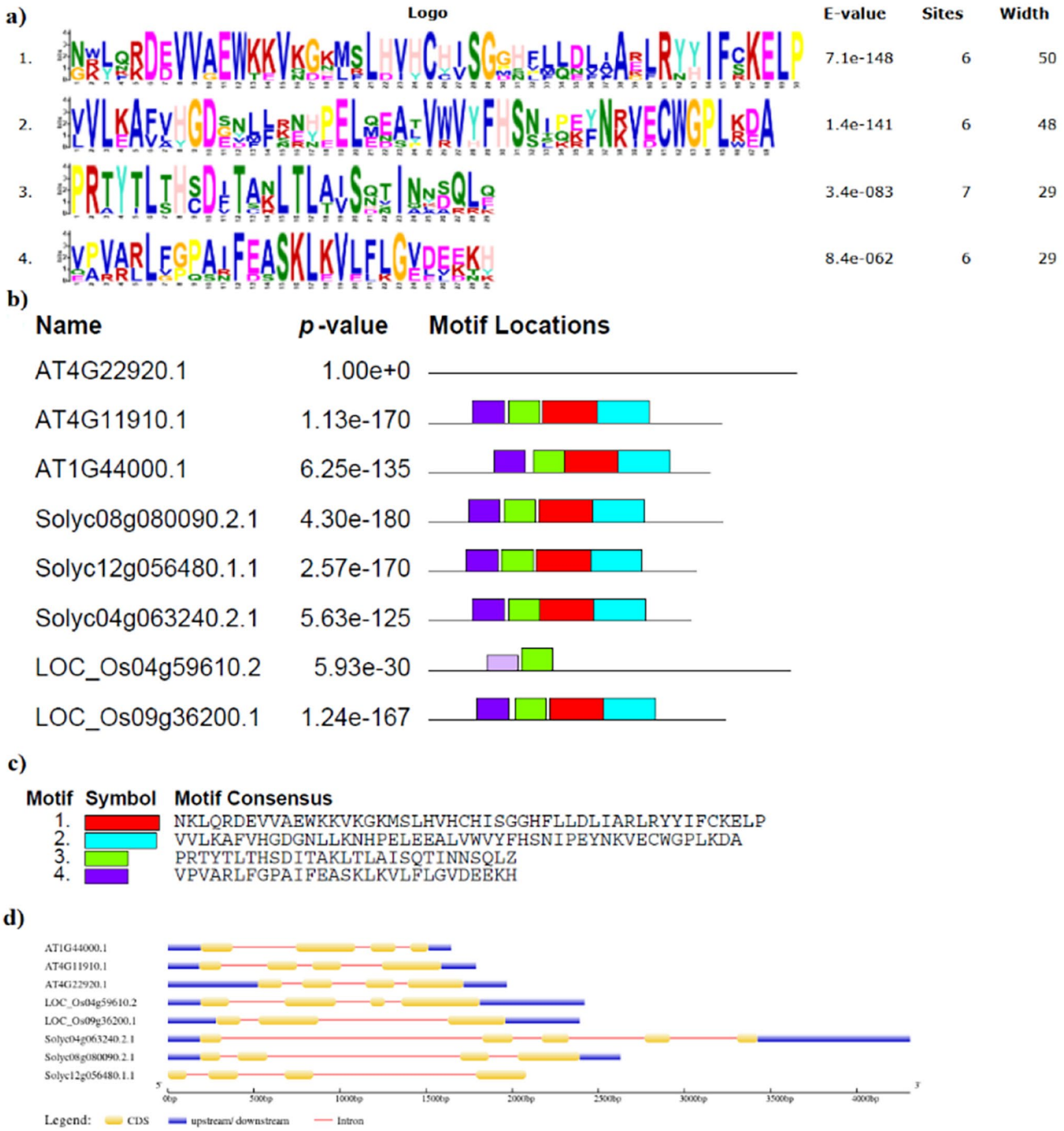

Fig. 3 Motif composition and gene structure of the *SGR* genes of tomato. **(a)** Motif patterns of the tomato SGR proteins generated using MEME. **(b)** Motif locations of SGR proteins of *A. thaliana*, *S. lycopersicum* and *O. sativa*. **(c)** Consensus motif symbol of SGR proteins. **(d)** Gene structure of SGR genes generated using Gene Structure Display Server of *A. thaliana*, *S. lycopersicum* and *O. sativa*. Upstream/downstream regions are represented by a blue box, coding regions (CDS) are shown as yellow round-corner rectangles, and introns are shown as red lines

as major allergens in many plant tissues. The co-expression network of the *SlSGR* genes showed that they may be involved in sulphur homeostasis and fatty acid biosynthesis, during fruit ripening of tomato.

3.8 TF genes in the co-expression network of *SlSGR* genes

Metabolic networks and their regulations are vital for fitness of an organism. In addition, transcriptional control of

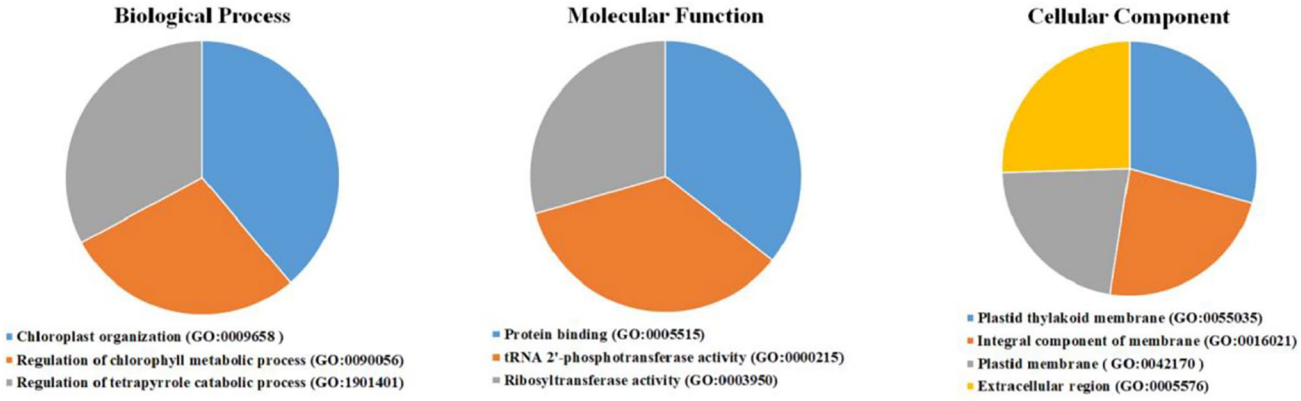

Fig. 4 Functional annotation of SlSGR proteins in terms of biological process, molecular function, and cellular components

enzyme-encoding genes plays essential roles in response to environmental and developmental stimuli (Gaudinier et al. 2015). In this study, transcriptional networks of *SlSGR* genes were analysed using gene co-expression networks. A total of 13 TF genes, directly connected to *SlSGR* genes, were identified. Of these TFs, three TFs were found in the *SlSGR1* network, and 10 TFs were identified for the *SlSGR-like* network (Table 5; Fig. 6B). Two of the three identified TFs in the *SlSGR1* network belonged to the *GRAS* TF family. In addition, three *GRAS* TF members were identified as first neighbours of *SlSGR-like*. The *GRAS* family is a plant-specific TF family involved in regulation of plant growth and development. The *GRAS* family is divided into eight subfamilies, *SHR*, *SCR*, *SCL3*, *DELLA*, *HAM*, *LS*, *LISCL* and *PAT1* (Hirsch and Oldroyd 2009). DELLA proteins, which negatively regulate senescence, increase the expression of downstream senescence-related *SGR* genes (Zhang et al. 2018). In addition, members of the *GeBP, ERF, MYB* and *bHLH* TFs were also found in the co-expression networks of two *SlSGR* genes. It was reported that an ethylene response factor, *CitERF13*, trans-activated the *CitSGR* promoter during citrus fruit de-greening (Yin et al. 2016). In conclusion, *SGR* genes may be either in the same pathway or have molecular interactions with *GRAS* TF family in cellular metabolism. In addition, *SGR* genes are co-expressed with different TFs involved in cellular metabolism.

3.9 Expression patterns of *SlSGR* genes in different tissues and under salt stress

The expression profiles of *SlSGR* genes in seven different tissues/organs and under 200 mM salinity treatment (two

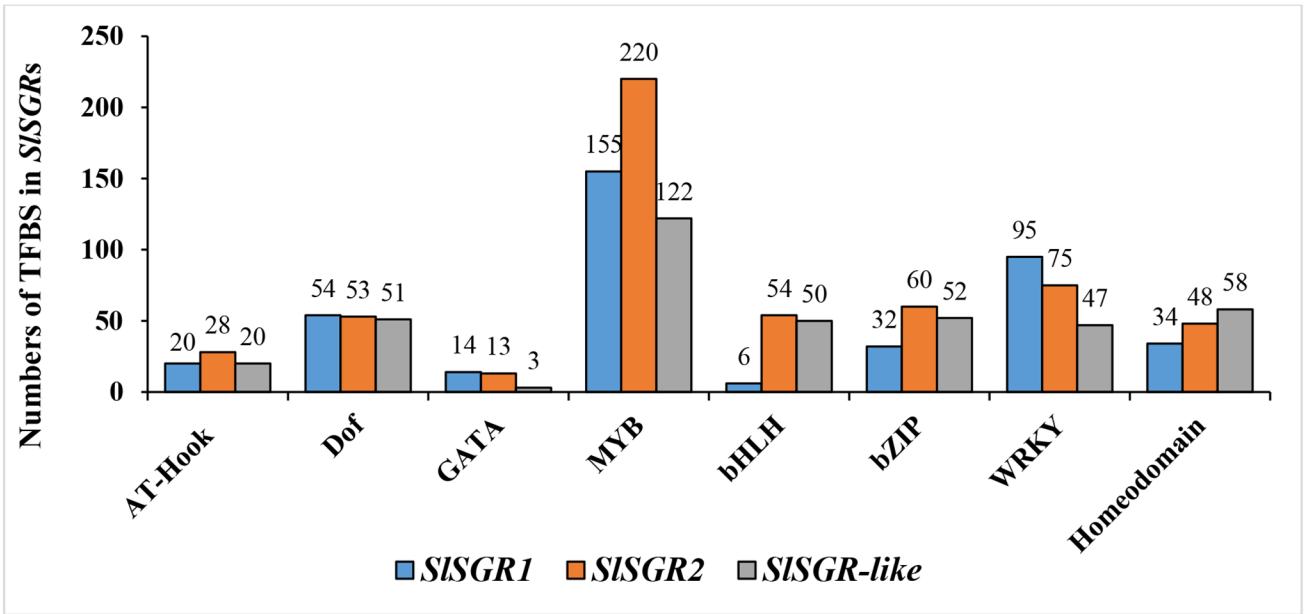

Fig. 5 Predicted transcription factor binding sites (TFBSs) in promotor sequences of *SlSGR* genes

Table 2 Top ten neighbours of *SlSGR1* in co-expression network at r > 0.9

Gene ID	*Arabidopsis* homologue	Putative function
Solyc09g047840.3.1	AT3G07525.1	Autophagocytosis-associated family protein
Solyc08g075970.3.1	AT1G32120.1	Transmembrane protein 45B
Solyc01g111040.4.1	AT4G38810.2	Calcium-binding EF-hand family protein
Solyc12g009270.1.1	AT5G20740.1	Plant invertase/pectin methylesterase inhibitor
Solyc02g091790.3.1	AT3G49660.1	WD40 repeat-like superfamily protein
Solyc10g083570.3.1	AT2G36460.1	Aldolase superfamily protein
Solyc10g007200.3.1	AT1G53290.1	Galactosyltransferase family protein
Solyc05g012270.3.1	AT4G24830.1	Arginosuccinate synthase family
Solyc01g100320.3.1	AT2G47470.1	Thioredoxin family protein
Solyc03g006500.3.1	AT3G09010.1	Protein tyrosine kinase

Table 3 Top ten neighbours of *SlSGR-like* in the co-expression network at r > 0.9

Gene ID	*Arabidopsis* homologue	Putative function
Solyc04g064720.3.1	AT5G17520.1	Maltose excess protein 1-like
Solyc10g077040.2.1	AT3G56940.1	Magnesium-protoporphyrin
Solyc10g008020.3.1	AT1G23360.1	Methyltransferase
Solyc02g068710.3.1	AT1G31170.2	Sulfiredoxin
Solyc07g062030.3.1	AT1G53520.1	Chalcone-flavanone isomerase
Solyc09g005320.3.1	AT5G01450.1	RING/U-box superfamily protein
Solyc07g040740.3.1	AT2G19450.1	Membrane-bound O-acyl-transferase family
Solyc08g080570.4.1	AT1G12780.1	UDP-D-glucose
Solyc11g066320.2.1	AT2G28110.1	Exostosin family protein
Solyc03g120680.3.1	AT1G79870.1	D-isomer specific 2-hydroxyacid dehydrogenase

Table 4 Details of the six common genes in the co-expression networks of *SlSGR1* and *SlSGR-like*

Gene ID	*Arabidopsis* homologue	Putative function
Solyc04g071560.4.1	AT1G76760.1	Thioredoxin
Solyc06g071910.3.1	AT1G24360.1	Enoyl-(Acyl carrier protein) reductase
Solyc06g068080.4.1	AT1G15370.1	ZETA-COAT PROTEIN
Solyc11g073200.2.1	AT1G07750.1	Cupin
Solyc04g054810.3.1	AT4G08685.1	Pollen proteins Ole
Solyc08g067830.3.1	AT1G34350.1	Transmembrane protein 18

Table 5 Transcription factor (TF), regulator and repressor genes directly connected with *SlSGR* genes (*SlSGR1* and *SlSGR-like*) in the co-expression networks

SlSGR gene	Co-expressed gene	*Arabidopsis* homologue	Putative function
SlSGR1	Solyc09g018460.1.1	AT4G08250.1	GRAS family TF
SlSGR1	Solyc02g085600.1.1	AT4G36710.1	GRAS family TF
SlSGR1	Solyc11g066460.1.1	-	Unknown Protein
SlSGR-like	Solyc02g087970.1.1	AT3G28917.1	Mini zinc finger 2
SlSGR-like	Solyc11g006670.1.1	AT5G04820.1	Transcriptional repressor (OFP13)
SlSGR-like	Solyc02g085340.1.1	AT5G66770.1	GRAS family TF
SlSGR-like	Solyc04g078420.1.1	AT2G23290.1	MYB70 TF
SlSGR-like	Solyc01g097890.2.1	AT3G04930.1	Transcriptional regulator
SlSGR-like	Solyc05g051330.1.1	AT5G28040.1	Transcriptional regulator
SlSGR-like	Solyc11g013150.1.1	AT4G08250.1	GRAS family TF
SlSGR-like	Solyc02g070880.1.1	AT1G29950.1	bHLH144 TF
SlSGR-like	Solyc03g025170.1.1	AT5G52510.1	GRAS family TF
SlSGR-like	Solyc09g066360.1.1	AT3G23240.1	ERF1 TF

tomato cultivars) were analysed using RT-qPCR (Fig. 7). Each gene showed a range of expression patterns in different tissues/organs. *SlSGR1/2* and *SlSGR-like* were moderately expressed in flower tissues. However, while *SlSGR1* expression was almost undetectable in leaves, the expression of *SlSGR-like* was the highest in photosynthetically active leaves. The expression *SlSGR1/2* increased steadily during fruit ripening and peaked in ripe fruits (R stage). In contrast, *SlSGR-like* expression was almost undetectable in ripening fruit.

In contrast to its expression in vegetative and reproductive tissues/organs *SlSGR2* seemed to be more active than the others under salinity stress in tomato. While the expression patterns of *SlSGR1* and *SlSGR-like* did not change under salinity in the two different tomato cultivars, *SlSGR2* exhibited a drastic increase under salinity stress compared to the controls in both cultivars. These results indicated that expression of *SlSGR1* and *SlSGR2* increases during fruit ripening and CHL degradation, and *SlSGR2* is involved in salinity stress.

It was previously suggested that the SGR subfamily proteins play a critical role in the initiation of Chl degradation and senescence in various plant species (Barry et al. 2008; Zhou et al. 2011; Jiao et al. 2020). Upregulation of

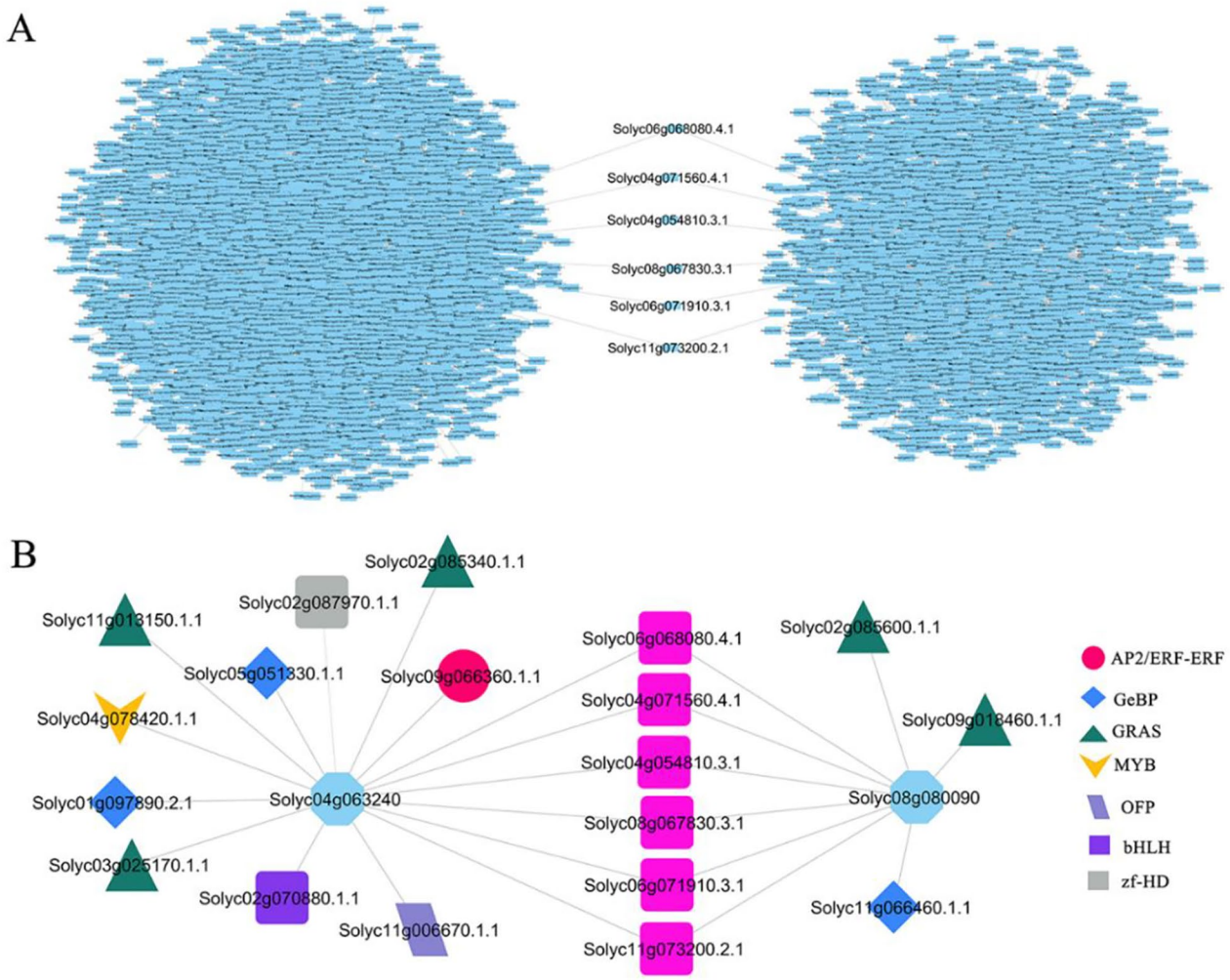

Fig. 6 The merged co-expression networks of *SlSGR1* and *SlSGR-like* (A) and the identified members of transcription factor families in each network (B). Six genes (shown in magenta) in each network are identified as common genes between the networks. The networks were constructed based on correlation analyses and genes in the networks were selected at the r > 0.9 level

Chl degradation and downregulation of Chl biosynthesis result in de-greening. The upregulation of *SlSGR1/2* during fruit ripening and of *SlSGR-like* during leaf senescence coincides with Chl breakdown (Park et al. 2007). Previous comparative investigations of the expression patterns of *SlSGR1* in tomato have shown that unlike *SlSGR-like*, *SlSGR1* was strongly expressed in ripening fruits (Hu et al. 2011). To the best of our knowledge, there are no studies about on the role of *SlSGR2* in ripening tomato fruit. In this study, the expression of *SlSGR1* and *SlSGR2* began to increase in MG fruits and reached a very high level in ripe (R) fruits. This expression pattern indicated that *SlSGR1/2* play a vital role in Chl degradation in ripening fruits. Similar to this finding, the repression of *SlSGR1* in transgenic tomato fruits elevated *SlPSY1* mRNA accumulation, which encodes a key carotenoid synthesis enzyme, at the early stages of fruit ripening, resulting in increased lycopene and β-carotene levels in red ripe fruits (Luo et al. 2013). Moreover, RNAi silenced *SlSGR1* fruits had a longer shelf-life with the decreased expression of *ethylene response* (*ETR) 1, 3, 4* and *5* genes and delayed activity of *PG2A* and *pectinesterase* (*PE*) enzymes up to seven days post-breaker stage. However, more research is needed to clarify the exact role of *SlSGR2* in tomato ripening. It was also previously shown that *SlSGR1* mRNA was present mainly in senescent leaves, but almost undetectable in young and mature leaves (Hu et al. 2011). This result was well-supported in this study as *SlSGR1/2* expression levels were very low in young and mature leaves. In contrast to *SlSGR1/2*, *SlSGR-like* was hardly detected in tissues with little or no Chl, such as the mature fruits at BR and R stages. Overall, these results imply that *SlSGR1/2* and *SlSGR-like* may function at different stages, and they have opposite expression patterns in tomato leaves and ripening fruit.

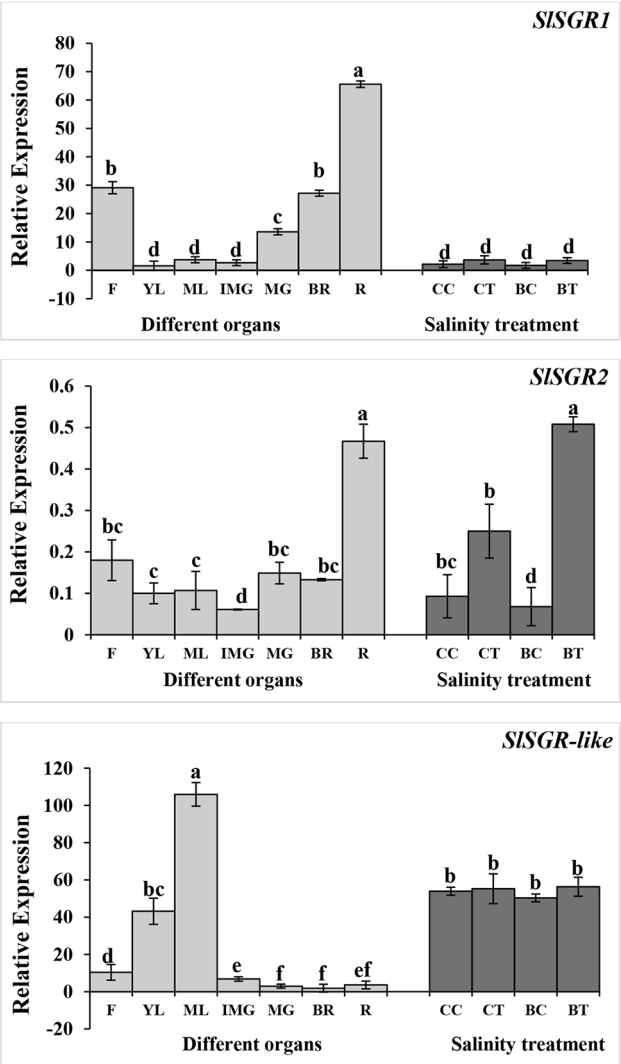

Fig. 7 The expression profiles of *SlSGR1/2* and *SlSGR-like* in different tomato organs and salinity treated tomato cultivars. Light grey bars: Flower (F), young leaf (YL), mature leaf (ML), immature green (IMG), mature green (MG), breaker fruit (BR), ripe fruit (R). Dark grey bars: 'Ciko' variety control (CC), 'Ciko' variety 200 mM salinity treatment (CT), 'Black' variety control (BC), 'Black' variety 200 mM salinity treatment. Mean values and SE ± were obtained from three biological and two technical replicates. The bars with different letters indicate significant differences, $p < 0.05$

To gain more insight about *SlSGR* genes in response to salinity stress, gene expression levels were analysed in two tomato varieties using RT-qPCR. Interestingly, while the expression levels of *SlSGR1* and *SlSGR-like* did not significantly change under salinity stress, *SlSGR2* expression was upregulated 5-fold in both varieties. Similar to the finding of the present study, previous studies reported that the *SlSGR* genes are regulated differently under other stress conditions. For example, the expression level of *SlSGR1* was enhanced under water-deficit and flooding (Hu et al. 2011). Five days of heat treatment in tomato upregulated the transcript abundance of *SlSGR1* and *SlSGR2* up to 11.79- and 18.82-fold for the genes, respectively, relative to no heat treatment (0 h) (Jahan et al. 2021). Moreover, the expression of *SlSGR-like* was induced by darkness, PEG, and ABA, and downregulated by ethylene and H_2O_2 (Yang et al. 2020). While the expression of *SlSGR* genes needs to be further studied under different stress treatments, *SlSGR2* might have a role in the breakdown of chlorophyll under various abiotic stresses.

Genome-wide identification, gene structure and protein sequences analyses, and expression profile analyses of *SlSGR* genes in different tissues and under salt stress were performed in the present study. The findings showed that *SlSGR2* is not only involved in fruit ripening and Chl degradation but participates in salt response mechanisms in tomato. The upregulation of *SlSGR2* under salt stress and of *SlSGR1* in fruit ripening may be related to their roles in stress-signalling and senescence pathways, respectively. Additionally, the roles of *SlSGR* genes in fruit ripening may be regulated by several TFs, among which GRAS family members may have more specific roles.

Acknowledgements We would like to thank Graham B. Seymour and Barbara Blanco-Ulate for providing detailed information about RNA-Seq data used for co-expression analysis.

Author Contribution SU, AK and EF conceived the study; SU and AK conducted the lab experiments, AK, EF, FK generated bioinformatic analysis; All authors wrote the manuscript, read, edited, and approved the manuscript.

Funding This research did not receive any specific grant from funding agencies in the public, commercial or not-for-profit sectors.

Declarations

Conflict of interest The authors declare no conflict of interest.

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