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IN SILICO ANALYSIS OF BES1 TRANSCRIPTION FACTORS IN *CITRUS SENESCENCE*

Fereshteh Izadi*, Hamid Najafi Zarrini, Nadali Babaeian Jelodar

Department of Plant Breeding, Sari Agricultural Sciences and Natural Resources University (SANRU), sari, Iran

ABSTRACT: Brassinosteroids (BRs) are a class of plant steroid hormones governing a wide range of physiological and developmental processes in plant kingdoms. They are essential for plant normal development and reacting to several of environmental fluctuations. Brassinosteroid transcription Factors (BRTFs) receiving BRs signals regulate target genes in several of BR responses. In this regard BES1 is a transcription factor involved in BR signaling pathway. There is couple of contradict evidences of the role of BRs in fruit senescence. Thereby in order to investigating the role of BES1 as a novel plant-specific transcription factor in Citrus senescence process, we used BES1 as hub genes for identification of the downstream targets by reconstructing a gene regulatory network (GRN) based on a reverse engineering approach. we then annotated the target genes and as a result our in silico analysis showed that these transcription factors regulate the expression of genes which highly connected with biological process such as oxidative stress, redox and hormone signaling and nutrient transportation all overrepresented in energy depletion, programmed cell death and finally leading to Citrus senescence.

KEYWORDS: Brassinosteroids, Gene Regulatory Network, Citrus Senescence, BES1 Transcription Factor

***Corresponding Author: Dr. Fereshteh Izadi** Ph.D.

Department of Plant Breeding, Sari Agricultural Sciences and Natural Resources University (SANRU), sari, Iran

*Email Address**:** izadi1991@yahoo.com

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1.INTRODUCTION

Citrus genus is one of the most important crop fruits with extensive economic footsteps and containing essential nutrients such as Vitamins, minerals and fibers influencing human heath that respect to ethylene metabolism and transduction classified into non-climacteric fruit [1, 2]. Fruits ripening and senescence due to various internal and external biological interactions remained as an inevitable process that exploring underlying mechanisms has concerned a great dealt of researches in plant sciences [3]. Experimental evidences have shown that different transcription factor families such as NAC, WRKY, C2H2-type zinc finger, AP2/EREBP, MYB [4] and Phytohormones including abscisic acid (ABA), auxin, salicylic acid, ethylene, BRs and Jasmonic acid [5] leading to irreversible changes in fruit features and finally senescence phenomenon [6,7]. In this regard researches mainly focused on climacteric fruits and ethylene signal transduction [8] demonstrating the fruit senescence as an oxidative process. Therefore elucidating senescence in non-climacteric fruits could extract prominent information for delaying or at least controlling this process [3]. Citrus postharvest senescence in spite of climacteric fruits which relays on ethylene signaling, is a step wise process [8, 9]. Indeed exploring the complex regulation of this phenomenon in citrus might discover some characteristics of aging in non-climacteric fruits. several evidences illustrated the external application of BRs delays the citrus senescence [10, 11], because of the decrease in ethylene metabolism and respiration [12] also external treatment with this Phytohormone lead to an increase in the fruit firmness and shelf life of sweet cherry fruit during postharvest [13]. However other contradict results showed that ethylene, ABA, Jasmonic acid, salicylic acid, auxin, and BRs are inducers/promoters of senescence [14]. Furthermore pre-harvest treatments of BRs accelerated fruit ripening while this advancement depended on dosage, time and mode of external application [15]. Consistently [16] also showed that high concentrations application of epi-brassinolide (EBL) in wheat (*Triticum aestivum* L.) leaf segments, promoted senescence. BRs are the sixth groups of plant-specific steroid hormones with pleotropic effects on developmental process such as senescence and abscission. Brassinosteroid transcription Factors (BRTFs) receiving BRs signals regulate target genes in several of BR responses. In this context BES1 (BRI1-EMS-SUPPRESSOR 1) and/or BRASSINAZOLERESISTANT1 (BZR1) are a family of plant specific transcription factors that regulate the expression of BRs responding genes in frame of transcriptional network including thousands of genes [1]. However the role of BES1 as a plant novel transcription factor in citrus senescence is not fully understood and this *in silico* analysis showed that these transcription factors correlate with promotion of citrus postharvest senescence.

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2. MATERIAL AND METHODS

Used data sets and preprocessing

We downloaded GEO Series GSE63706 from NCBI Gene Expression Omnibus (GEO) database [18]. In this experiment four citrus varieties were grouped in rind and flesh tissues and sampled every 10 days during 50 days after harvest (0, 10, 20, 30, 40 and 50 DAH). Raw CEL files were normalized with Robust Multiarray Averaging (*RMA*) [19]. Using limma R package, gene expression were considered to be biologically significant between time points, verities and tissues if expression level changes was above the defined threshold (absolute Log2FC $\geqq$ 2 and with p-value < 0.01) where 18575 probsets passed the filter (Supplementary file).

Transcriptional network inferred by ARACNE

Using ARACNE (Algorithm for the Reconstruction of Accurate Cellular Networks) embedded in geworkbench (http://wiki.c2b2.columbia.edu/workbench/index.php/ARACNe) we reconstructed a GRN by setting mutual information (MI) to 0.5, Adaptive Partitioning and DPI tolerance to 0. We used two Citrus BRTF genes Cit.13168.1.S1_at and Cit.30289.1.S1_at which encode Brassinosteroid signaling positive regulator-related proteins as hub genes.

Annotation analysis

Ultimately GO analysis was done on the 582 BES1's predicted Targets. To this end 582 Citrus probsets were mapped into their Arabidopsis homologous gene identifier (AGI) by Plexdb tool (http://www.plexdb.org/modules/MPT/mpt_Input.php) and converted IDs then feed in PANTHER database (http://pantherdb.org/) with default parameters.

Results and discussion

BRs regulatory interactions

We firstly took BRTFs from NICCE database (http://citrus.adelaide.edu.au/nicce/home.aspx) and performed blastn against Citrus affymetrix chip where we found six BES1 genes Cit.28950.1.S1_at, Cit.15563.1.S1_at, Cit.24829.1.S1_at, Cit.29783.1.S1_s_at, Cit.13168.1.S1_at and Cit.30289.1.S1_at of which only Cit.13168.1.S1_at and Cit.30289.1.S1_at probsets were differentially expressed therefore used as hub genes in inferring GRN by ARACNE algorithm. Figure 1 is a schematic representation of inferred GRN using two hub genes. ARACNE [20] an information-theoretic approach has been successfully applied for reconstructing GRNs [21]. In this approach first a pair-wise MI matrix is been calculated between all possible pairs of genes afterward the resulted matrix manipulated for identifying regulatory interactions between nodes [22]. As a result 582 genes showed regulatory interactions used for annotation analysis (Fig. 1). In order to verify that mentioned 582 genes are the potential targets of BES1 transcription factors, we performed promoter analysis. To do

so we intersected between our identified targets and a list of BES1 differentially regulated genes obtained by supplementary data of [23] where we found 15 common genes. Finally 1000 bp from up-stream of 15 overlapping genes was scanned by JASPAR CORE Plantae (http://jaspar.genereg.net/cgi-bin/jaspar_db.pl?rm=browse&db=core&tax_group=plants) for investigating BZR transcription factor models MA0549.1 and MA0550 (Fig. 2). Detailed results of promoter analysis summarized in Table 1. We observed that the upstream of target genes contained motifs matched with BZR1 compared to BZR2. The higher scores for BZR1 model confirmed that 582 identified genes are potential targets of BES1.

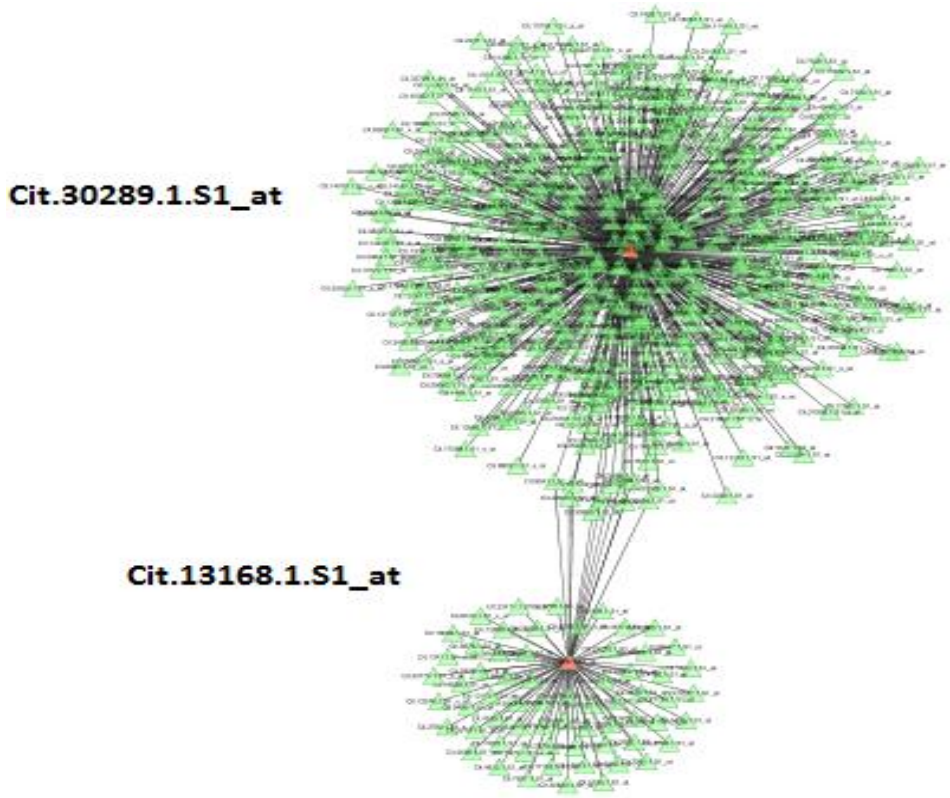

Fig. 1 Gene regulatory network of BES1's target genes. Red triangles represent BES1 and green ones BES1's target.

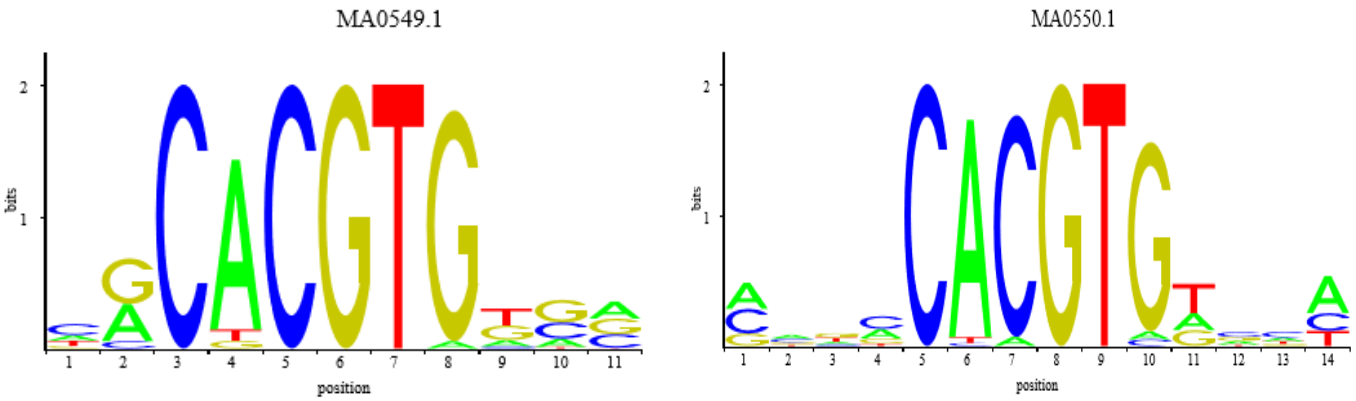

Fig. 2 sequence logos of BZR1 and BZR2 scanned in promoter of 15 Overlapping genes between BES1's Targets and BRs responding Differentially Expressed Genes Identified By (23).

Table. 1 Promoter Analysis of 15 Overlapping Genes between BES1's Targets and BRs responding Differentially Expressed Genes Identified By (23).

Model ID	Model name	Score	Relative score	Start	End	Strand	predicted site sequence
MA0550.1	BZR1	6.139	0.81	399	412	1	AACGTACGTGTCTT
MA0549.1	BZR2	5.715	0.804	401	411	1	CGTACGTGTCT
MA0550.1	BZR1	7.640	0.837	728	741	-1	GATATACGTGGCCC
MA0550.1	BZR1	8.230	0.848	728	741	1	GGGCCACGTATATC
MA0549.1	BZR2	7.393	0.839	729	739	-1	TATACGTGGCC
MA0550.1	BZR1	8.865	0.859	757	770	-1	GGACCACGTAAGCA
MA0549.1	BZR2	6.037	0.811	758	768	-1	ACCACGTAAGC
MA0550.1	BZR1	7.666	0.838	832	845	1	CCAACACGTCACGT
MA0549.1	BZR2	6.669	0.824	834	844	1	AACACGTCACG
MA0550.1	BZR1	8.111	0.846	837	850	1	ACGTCACGTCGCTA
MA0550.1	BZR1	7.235	0.83	984	997	-1	GTTACACGTTACAA
MA0549.1	BZR2	6.668	0.824	985	995	-1	TACACGTTACA

Functional classification of BES1's target genes

GO analysis (Fig. 3) classified 582 identified target genes so that most of them enriched in these terms: catalytic activity (GO: 0003824), metabolic process (GO: 0008152), cell part (GO: 0044464), oxidoreductaes (PC00176) and ubiquitin proteasome pathway (P00060). Significant Enriched GO terms showed several interesting results such as catalytic activity, oxidoreductase activity and proteasomes. In agreement with these results, [24] demonstrated the role of proteasomes in citrus senescence. Reactive Oxygen Species (ROS) are kind of messengers that are produced in response to biotic and abiotic stresses and play an important role in senescence signal transduction [25]. In apple ROS signaling induced some changes in the expression of mitochondrial proteins such as superoxide dismutase and oxidoreductase [3]. The oxidoreductase activity in BES1's target genes suggests possible role of this transcription factor in citrus senescence via ROS signaling. However [24] declared the small role of ROS in citrus senescence. Considerable catalytic and hydrolase activities might cause to release of smaller molecules like sugars from degradation of complex macromolecules such as organic acids toward senescence. The resulted molecules also small sugars coming from fatty acid metabolic process in organelles will be used in glycolysis and tricarboxylic acid cycle pathways for producing energy and demanded precursor substances. Sugars are important signal molecules mediating in senescence [26] and [27] showed the role of hexoses as a signaling molecule during plant development. Consistently with previous findings BES1's target genes included probsets involving in sugar signaling, dismutases and catalases, ubiquitin proteasome and transporters. Furthermore high binding activity in molecular function might be due to nucleotide and-or ATP binding activities in regulating cell cycle progression or sugar-binding proteins which play a role in signal transduction.

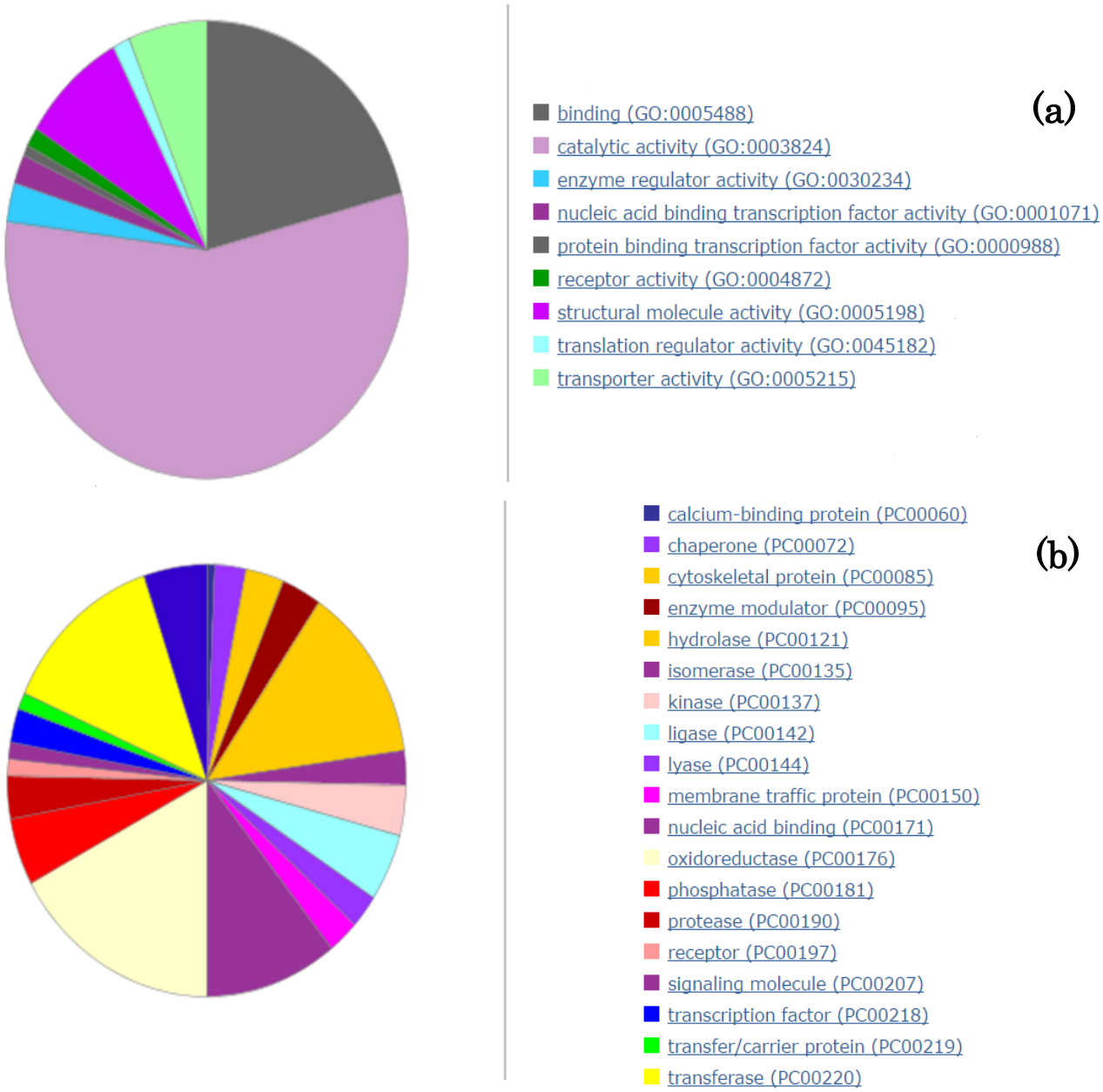

Fig. 3 Pie chart of functional classification of BES1's Targets. (a) Molecular function and (b) protein classes of BES1's predicted target genes.

Expression pattern of BES1's targets

Figure 4 illustrates hierarchical clustering of target genes. Evidently different number of genes grouped in different clusters depend on varieties, tissues and time points but we generally focused on genes obtained by transcriptional regulation neglecting the sampling levels. We observed distinct patterns of up or down-regulation in genes toward senescence (Fig 4). Interestingly genes related to transporters were up-regulated also we observed a down-regulation of genes involving in protein turn over and signal transduction toward aging. Our Results showed that BES1 regulate a number of biological and cellular process including generation of precursor metabolites required for several chemical reactions such as metabolism of tricarboxylic acid cycle compounds, amino acids and lipids also some process like cell wall modification, energy production, respiration, organelles electron transport, Post translational modification and cofactor metabolic process. BRs directly activate a number of genes related to cellular transportation contributing in solute uptake also cell wall modification enzymes like Pectinesterases [17]. A couple of studies demonstrated that BRs enhance the plant ability against stresses such as high and low temperature, pathogens [28] and oxidative stress that existing the stress related genes (heat, cold, salt and PR proteins) and GST among BES1's targets is in accordance with these findings. Also [1] declared that senescence in non-climacteric fruits is because of response to water, nutrient and temperature stresses that gradually induces the genes such as stress responsive genes and genes that encode proteins mediating in energy production and providing substances for maintaining fruit activities. In this regard genes involved in energy production such as DEAD/DEAH box helicase, ribonuclease T2 family protein and Adenine nucleotide alpha hydrolase like protein family targeted by BES1 might be relevant. Among identified predicted genes there were about 17 probsets annotated as transcription factors like bZIP, zinc finger, MYB, bHLH, BZR and WRKY from which WRKY, zinc finger and MYB characterized to be related to senescence [29,30] and MYB known as a key regulator of hormone signaling [31], and anthocyanin biosynthesis in citrus [32]. We also found genes related to plant hormones like auxin, ethylene, BRs, gibberellin and ABA that all except for gibberellin are believed to be senescence inducers/promoters. Briefly in BES1's target list, genes mediating in cell wall modification like Pectinesterases and Polygalactoronase, ABC, sulfate and amino acid transporters, lignin biosynthesis, ethylene metabolism and signaling, mitochondrial electron transport, stress related genes, sucrose and Threhalose metabolism, Thioredoxin, sugar and calcium signaling suggests a higher metabolism in response to environmental stresses specifically water deficiency that subsequently fallowed nutrient depletion, texture modification and finally senescence. We also identified a considerable number of genes related to secondary metabolites in particular flavonoids that might be due to the exhaustive

use of nutrients after over transportation and consumption. Another interesting aspect of target list was participating several genes involving in lipid metabolism. Higher rate of nutrient consumption due to abiotic stresses will force fruits to supply energy via pathways such as fatty acid metabolism in peroxisomes mediating by the groups of cytochrome P450 and lipases that likely will yield the formation of signal molecules such as Jasmonic acid [33] and hydrogen peroxide as by product that in turn will be converted to water and O_2 by catalases. The mentioned signal molecules produced by fatty acid metabolism participate in citrus senescence [23] and their role may be implied by accumulation of cytosolic calcium and MAPK activation [23]. There is couple of evidences in relationship of cytosolic calcium and senescence [34] also recently loss of function of a MAPK cascade in *Arabidopsis* delayed the senescence [35]. In agreement, up-regulation in genes encoding cytochrome P450, lipases, phospholipid hydroperoxide glutathione peroxidase, catalases, dismutases, MAPKs and several genes involving in lipid metabolism and calcium signaling in target list may be biologically relevant with a possible relationship between BRs and pathways happening during fatty acid metabolism that produce signal molecule and a cascade toward senescence. However MapMan annotation of BES1's targets did not show genes related to Jasmonic acid (Supplementary file). Gradual postharvest aging in climacteric and non-climacteric fruits induced by environmental stresses is mostly controlled by ethylene and ABA where ethylene causes ABA

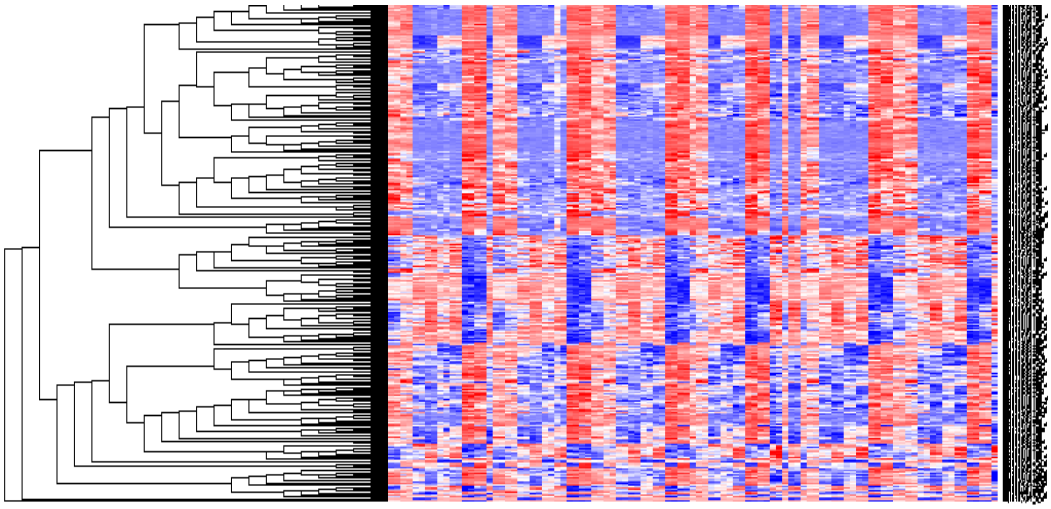

Fig. 4 Cluster analysis of BES1's target genes. There are distinct expression pattern toward senescence each pattern contains different number of genes grouped in different clusters depend on varieties, tissues and time points.

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Accumulation and subsequently fruit ripening [36, 37, and 5]. Previous researches showed that external application of *epi*brassinolide (*e*BR) induced senescence in mung bean plants [38]. Also Arabidopsis BRs mutant showed delaying in leaf senescence [39] where [1] found several similarities between leaf and fruit senescence. [40] Showed that tomato pericarp treatment with BRs accelerated the senescence by decreasing organic acids and increasing carbohydrates. Taken together this finding is consistent with BES1's target list containing genes that suggested to be BR-regulated and aging promoter. Considering the negligible amount of produced ethylene in Citrus as a non-climacteric fruits, external use of BRs delays senescence via cross-talking by ethylene. In this context acceleration in senescence process by BRs could be due to the hyperpolarization of membranes, stimulate ATPase activity, and alter the orientation of cortical microtubules [38]. Furthermore endogenous BRs supposed to be aging promoter via participating in oxidative stress, redox and hormone signaling and nutrient transportation. Inferring regulatory interactions from omics data is a prominent strategy for extracting meaningful information of several natural process such as senescence. BES1 targets identified by inferring a GRN included genes which in orchestrate with other senescence related genes involved in changing fruit texture and firmness, promoting nutrient transportation and consumption during the postharvest period in response to environmental stimuli and exhaustive use of energy. These genes can be targets for future researches based on desired goals.

ABBREVIATIONS

ABA abscisic acid AGI Arabidopsis gene identifier BES1 BRI1-EMS-SUPPRESSOR1 BRs brasinosteroids BRTF brasinosteroid transcriptional factor DAH day after harvest GRN gene regulatory network GTS glutathione S transferase MI mutual information NICCE Network inference for Citrus Co-Expression ROS reactive oxygen species

COMPETING INTERESTS

The authors declared that they have no competing interests.

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