Scientific Research Publishing

American Journal of Plant Sciences, 2018, 9, 1037-1050
http://www.scirp.org/journal/ajps
ISSN Online: 2158-2750
ISSN Print: 2158-2742

In Search of Regulators of *LeSPL-CNR* by South-Western Blotting and Yeast One-Hybrid Library Screening System

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How to cite this paper: Wang, X.H., Ye, B.S., Wang, Y., Zhou, T. and Lai, T.F. (2018) In Search of Regulators of *LeSPL-CNR* by South-Western Blotting and Yeast One-Hybrid Library Screening System. *American Journal of Plant Sciences*, **9**, 1037-1050.
https://doi.org/10.4236/ajps.2018.95080

Received: March 15, 2018
Accepted: April 17, 2018
Published: April 20, 2018

Abstract

LeSPL-CNR is a crucial transcription factor for fruit ripening of *Solanum lycopersicum*. The *cnr* (*colorless non-ripening*) epimutation resulted from hypermethylation in a 286 bp region of *LeSPL-CNR* promoter inhibits normal fruit ripening. In present study, potential regulators of *LeSPL-CNR*, which could bind to the specific 286 bp region, were screened via south-western blotting and yeast one-hybrid (Y1H) library screening system. Results indicated that a total of 13 and 19 candidate proteins were acquired respectively, and both ribulose-1,5-bisphosphate carboxylase/oxygenase and 40S ribosomal protein were identified by two methods. These would provide some information for revealing roles of DNA methylation and the regulatory mechanism for *LeSPL-CNR*.

Keywords

Solanum lycopersicum, *LeSPL-CNR*, 286 bp Region, South-Western Blotting, Yeast One-Hybrid

1. Introduction

Fruit ripening is a genetically regulated process involving a series of coordinated biochemical, physical and organoleptic changes in color, flavor, texture, aroma, and nutritional status, which is controlled by endogenous hormonal, genetic regulators and environmental stimuli [1] [2] [3]. To understand biological mechanisms of fruit ripening, tomato (*Solanum lycopersicum*) has been studied

most extensively, in view of biological advantages of this model system such as well-known structural genomics, rich transcriptome and proteome databases, amenability to genetic transformation, and short life cycle [4] [5]. *Solanum lycopersicum* cv. Ailsa Craig (AC) with a medium size has been cultivated for around 60 years. It can be grown indoors or out and produces a regular looking fruit which is grown on heavy trusses. In addition, AC has good disease resistance which makes it a nice reliable variety. Therefore, it usually serves as a background for derived-mutants or transgenic lines.

Considerable progress has been made in elucidating the biochemical and molecular basis of fruit ripening that has established the important role of ethylene during fruit ripening in tomato [6] [7] [8] [9] [10]. Fruit-specific transcriptional control of ripening as well as crosstalk among the already characterized components in tomato also has received considerable attention [11] [12]. For example, *LeMADS-RIN*, *LeSPL-CNR*, *SlFUL*1/*SlFUL*2, *LeHB*-1, *SlTAGL*1, *SlMADS*1, *SlAP*2*a*, *SlNAC*4 and *SlZFP*2 all play crucial roles in tomato fruit ripening by both ethylene-dependent and independent pathways [13]-[21].

Recent studies have suggested that epigenetic changes play pivotal roles in plant development and fruit ripening progress [22] [23]. Liu *et al.* [24] showed a direct cause and effect relationship between active DNA demethylation (mainly mediated by tomato DEMETER-like DNA demethylases) and fruit ripening. Zhong *et al.* [25] found that the epigenome of tomato was dynamic, and the global methylation level gradually declined during wild type fruit development and ripening but remained high in *cnr* (*colourless non-ripening*) and *rin* (*ripening inhibitor*) mutants. As a dominant pleiotropic mutation, the *cnr* epimutation results from a naturally occurring hypermethylation in a 286 bp region of *LeSPL-CNR* promoter. The *cnr* mutant greatly inhibits ethylene production and softening, and possesses a non-ripening phenotype with a yellow skin and a colorless mealy pericarp (**Figure 1(a)** and **Figure 1(b)**) [13] [26]. The distinct phenotype is not reversed by exposure to exogenous ethylene. In *cnr* mutant, the biosynthesis of ripening-related pericarp carotenoid is abolished due to the reduced ability to synthesize the carotenoid precursor geranylgeranyl diphosphate (GGPP) [27]. Meanwhile, the solubilisation of homogalacturonan-rich pectic polysaccharides and the proportion of carbohydrate are less than those in wild type. Those lead to reduced cell-to-cell adhesion which reflects changes in the structure of *cnr* cell walls [28]. *LeSPL-CNR* is mapped in the euchromatin region on the long arm of tomato chromosome 2 and encodes a transcription factor of the Squamosa Promoter Binding-like Protein. Evidences indicated that a sophisticated regulatory mechanism existed at the upstream of *LeSPL-CNR.* Besides the binding site of *LeMADS-RIN* in the promoter of *LeSPL-CNR* [18], the 3'-UTR also possessed a potential *miRNA*156*/7* target. *Slymi*R157 could regulate the expression of *LeSPL-CNR* in a likely dose-dependent manner through miRNA-induced mRNA degradation and translation repression [29]. Meanwhile, infection of tomato plants with cucumber mosaic virus (CMV) carrying

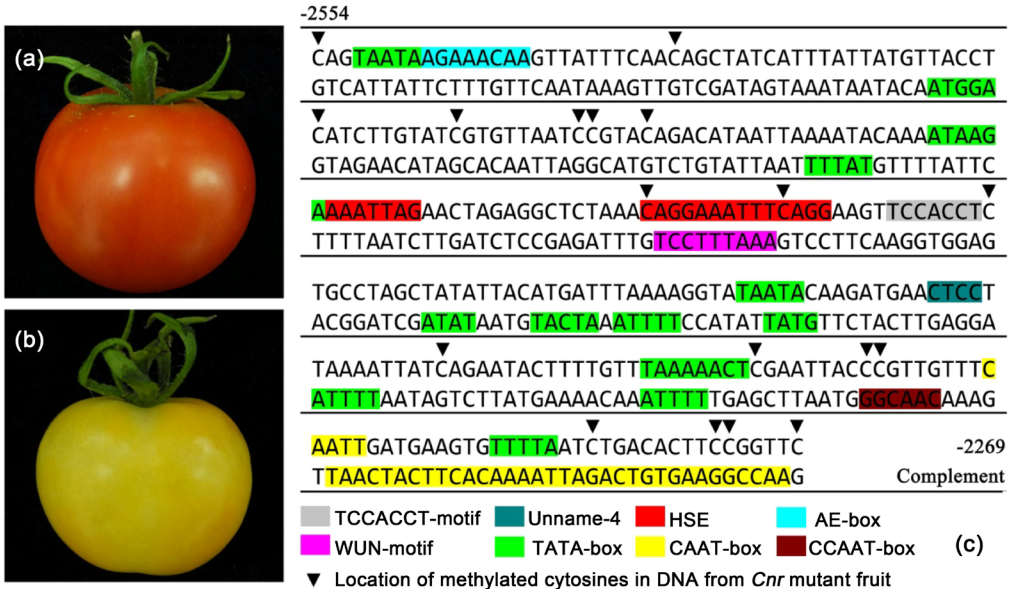

Figure 1. Ripening phenotypes of wild-type AC (a) and *cnr* mutant fruits (b) with structural and methylation site analysis of a 286 bp region in *LeSPL-CNR* promoter (c).

the *LeSPL-CNR* promoter could induce epigenetic changes and result in inhibition of fruit ripening and mottled phenotype [30].

In this context, we surmised that the 286 bp region of *LeSPL-CNR* promoter contained some valuable sites. The hypermethylation of this region could prevent the binding of some potential regulators, and these regulators might play a critical role in complicated transcriptional cascade or epigenetic controls for fruit ripening. Thus, a screening via south-western blotting and yeast one-hybrid library screening system was carried on to find these potential regulators in present study. Results would provide some information to reveal roles of DNA methylation in plant genomes and elucidate the regulating mechanism for *LeSPL-CNR.*

2. Materials and Methods

2.1. Plant Materials and Cis-Acting Regulatory Element Analysis

Solanum lycopersicum cv. Ailsa Craig (AC) and the *cnr* mutant were grown in glasshouses at 25˚C and 80% humidity with a photoperiod of 16 h day/8 h night. Tomato flowers were tagged at anthesis and fruit development and ripening stages were recorded as days post anthesis (DPA). *In silico* analysis of the 286 bp region of *LeSPL-CNR* promoter were performed in PlantCARE which was a database of plant cis-acting regulatory elements, enhancers and repressors. A motif search algorithm called Motif Sampler and a probabilistic approach based on Gibbs Sampling were used [31]. The PlantCARE relational database was available at http://bioinformatics.psb.ugent.be/webtools/plantcare/html/. Regulatory elements are represented by positional matrices, consensus sequences and individual sites on particular promoter sequences.

2.2. Two-Dimensional Electrophoresis and South-Western Blotting

Total genomic DNA of AC fruits at breaker stage was extracted using a DNeasy Plant Mini Kit (Qiagen, Germany), following the manufacturer's instruction. The 286 bp region was amplified from genome DNA with specific primers (P1F and P1R in Table 1) and used as a probe in sequent experiment. PCR reactions were conducted in a total volume of 20 μL containing 1 μL DNA template, 0.5 μL forward primer, 0.5 μL reverse primer, 1 μL dNTP, 0.1 μL PrimeSTAR HS DNA polymerase, 4 μL 5 × PrimeSTAR Buffer (Mg^{2+} plus) and 12.9 μL deionized water. PCR conditions for amplification were 94˚C for 3 min followed by 25 cycles of 94˚C for 20 s, 53˚C for 30 s and 72˚C for 40 s. Then, a PCR DIG Probe Synthesis Kit (Roche, USA) was used to acquire high labeled and sensitive [digoxigenin(DIG-11)]-dUTP-labelling probe. After purification by High Pure PCR Product Purification Kit (Roche, USA), a series of dilutions of DIG-labeled probe and control DNA were applied to determine the working concentration of labeled probe according to the product manual.

The extraction and quantitation of total cellular proteins of AC fruits at breaker stage used methods described by [32]. About 100 μg of proteins were loaded on a rehydrate immobilized pH gradient gel strip (7 cm, pH 3 - 10) (Bio-Rad, USA) and two-dimensional electrophoresis (2-DE) was carried out according to previous reports [33]. Coomassie Brilliant Blue R-250 was used to stain the proteins in a duplicate gel to monitor the equaling of samples. The proteins in one of polyacrylamide gels were transferred to PVDF membrane (0.45 μm, Millipore, USA) by a Trans-Blot Turbo (Bio-Rad, USA). After washing, renaturation, and incubation with DIG-labeled probe, immunological detection using anti-digoxigenin-AP conjugate (Roche, USA) and NBT/BCIP solution (Promega, USA) was performed according to product manual. The distribution of visible black spots representing the potential regulatory factors of *LeSPL-CNR* on the PVDF membrane was photographed. The total experiment was performed three times independently, and the spots detected at least twice at same position were considered as candidates. Then, the corresponding proteins at same positions on duplicated 2-DE gel were manually excised and sent to Sangon Biotech (Shanghai) Co., Ltd for identification by mass spectrometry. The in-gel protein digestion and analysis of matrix assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF-MS) were performed using a service from Sangon Biotech (Shanghai) Co., Ltd. MS data acquired on an AB SCIEX TOF/TOF 5800 system (Applied Biosystems, USA) were used to search against NCBInr protein databases (http://www.ncbi.nlm.nih.gov) with Mascot MS/MS Ions Search program on the Matrix Science public web site (http://www.matrixscience.com). Search parameters were set as proteolytic enzyme, trypsin; max missed cleavages, 1; fixed modifications, carbamidomethyl (C); variable modifications, oxidation (M); peptide mass tolerance, 1.2 Da; and fragment mass tolerance, 0.6 Da. Only significant hits as defined by Mascot

probability analysis were considered.

2.3. Yeast One-Hybrid (Y1H) Screening

A Matchmaker Gold Yeast One-Hybrid Library Screening System (Clontech, USA) was used as described by the manufacturer. Briefly, the 286 bp fragment was amplified using a pair of primers (P2F and P2R in Table 1), sequenced, and cloned into the pAbAi vector in digestion sites of restriction enzymes *Hin*dIII/*Sal*I. After linearizing by *Bst*BI, the pBait-AbAi plasmid was transformed to Y1HGold strain following the protocol of the Yeastmaker Yeast Transformation System 2 (Clontech, USA) to generate bait yeast strain. Then, the working concentration of Aureobasidin A (AbA) used to suppress basal expression of our bait construct in the absence of prey was determined on SD/-Ura media with different concentrations of AbA.

Total RNA was extracted from AC fruits at breaker stage using an RNeay Plant Mini Kit (Qiagen, Germany). The first-strand SMART cDNA synthesis, amplifying SMART (Switching Mechanism at 5' end of RNA Transcript) cDNA by long distance PCR, and purifying the ds cDNA with a CHROMA SPIN + TE-400 column (Clontech, USA) were performed following protocol of Clontech's SMART technology. The purified SMART ds cDNA and pGADT7-Rec AD Cloning Vector (*Sma*I-linearized) were co-transformed into bait yeast strain using the Yeastmaker Yeast Transformation System 2. The aliquot suspensions of the library transformation reaction were dispersed evenly on the 150 mm plates with SD/-Leu/ABA^{100}. After incubation for 3 - 5 days, the positive clones were isolated and used to extract prey plasmids responsible for activation of reporters by a TIANprep Yeast Plasmid DNA Kit (Tiangen, China). At last, cDNA inserts from pGADT7-Rec vectors were identified by PCR amplification with T7 sequencing primer (T7 SP) and 3'AD sequencing primer (3'AD SP) (Table 1) according to the protocol of A Matchmaker Gold Yeast One-Hybrid Library Screening System, and sequencing in Shanghai Sunny Biotechnology Co., Ltd. Sequencing results were analyzed on https://blast.ncbi.nlm.nih.gov/Blast.cgi. Search parameters were set as Database: Nucleotide collection (nr/nt); Organism: *Solanum lycopersicum*; Exclude: Uncultured/environmental sample sequences; Limit to: No; Entrez Query: No.

Table 1. Primers used in this study.

Name	Sequence	Annealing temperature (˚C)
P1F	CAGTAATAAGAAACAAGTTATTTCAAC	53
P1R	GAACCGGAAGTGTCAGATTAAAACAC	
P2F	GCTACCAAGCTTCAGTAATAAGAAACAAGTTATTTC	55
P2R	GAGTGCGTCGACGAACCGGAAGTGTCAGATTAAAAC	
T7 SP	TAATACGACTCACTATAGGGC	55
3'AD SP	AGATGGTGCACGATGCACAG	

3. Results

3.1. Cis-Acting Regulatory Element Analysis in Specific 286 bp Region

In this study, potential *cis*-acting regulatory elements of the specific 286 bp region were analyzed online in PlantCARE database. Total eight kinds of motifs (including AE-box, CAAT-box, CCAAT-box, HSE, TATA-box, TCCACCT-motif, WUN-motif and unname-4 motif) were forecasted in both forward and reverse complement sequences (Figure 1(c)). These motifs were composed of 135 nucleotides which accounted for about 47% of the 286 bp region. Unexpectedly, most of the methylated cytosine residues in the 286 bp region did not showed a correlation with these motifs. These results indicated that the 286 bp region possessed structural basis for potential regulators binding.

3.2. Screening Potential Regulators by South-Western Blotting

The DIG system is a simple, effective system for nonradioactive labeling and detection of nucleic acids. In this study, the polymerase chain reaction was performed for incorporation of DIG-11-dUTP into the specific 286 bp probe (Figure 2(a)). The labeling efficiency was determined by comparison to the DIG-labeled control DNA. And 1 ng/μL of DIG-labeled DNA probe was used as

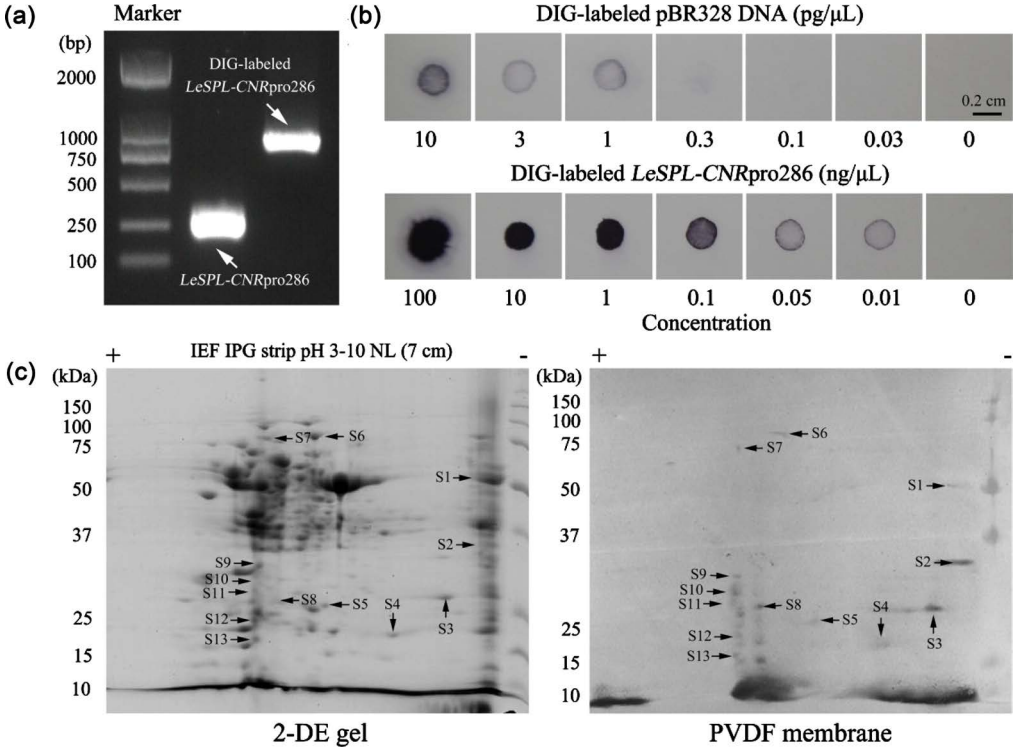

Figure 2. Digoxigenin-labeled *LeSPL-CNR*pro286 probes (a), determination of labeling efficiency (b), and *LeSPL-CNR* regulatory factor screening by two-dimensional electrophoresis (2-DE) combined with south-western blotting analysis (c). Spots indicated by black arrow in 2-DE gel and PVDF membrane were potential regulatory factors which could bind with DIG-labeled *LeSPL-CNR*pro286 probes. The spots were numbered and identified by MS/MS, corresponding to those in Table 2.

working concentration in the subsequent hybridization detection (**Figure 2(b)**). Proteins extracted from tomato fruits at breaker stage were separated using 2-DE. More than 300 protein spots were detected in the gel after ignoring very faint spots and spots with undefined shapes by Image Master 2D Elite software. After south-western blotting detection, a total of 20 spots on the PVDF membrane were considered as positive signals (**Figure 2(c)**), and the corresponding proteins were identified by mass spectrometry and were matched in NCBInr database. Among them, 13 proteins were matched to proteins of *Solanum lycopersicum* with known or predicted functions (**Table 2**) including elongation factor 1-alpha, basic 30 kDa endochitinase precursor, osmotin-like protein precursor, ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit, etc.

3.3. Screening Potential Regulators by Yeast One-Hybrid Library Screening System

Yeast one-hybrid system is a powerful technique to rapidly identify heterologous proteins that can interact with a specific regulatory DNA sequence of interest (the bait sequence) and uses a single fusion protein in which the activation domain (AD) is linked directly to the binding domain. The binding domain in this case also may be constituted by a library. In this study, our target-reporter

Table 2. Proteins information acquired through south-western blotting combined with MALDI-TOF-MS analysis.

Number	Name	NCBI accession[a]	Theo. Mass/PI[b]	Species	Mascot Score/Threshold[c]	SC (%)[d]	NP[e]
S1	Elongation factor 1-alpha	NP_001234035	49613/9.24	*Solanum lycopersicum*	345/45	12	4/4
S2	Basic 30 kDa endochitinase precursor	NP_001234403	33209/8058	*Solanum lycopersicum*	356/43	16	3/3
S3	Osmotin-like protein precursor	NP_001234714.1	28159/8.15	*Solanum lycopersicum*	151/44	10	2/2
S4	Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit	AAA19771.1	53398/6.55	*Solanum lycopersicum*	69/45	2	1/1
S5	Predicted triosephosphate isomerase	XP_004236746.1	27251/5.73	*Solanum lycopersicum*	390/44	20	4/4
S6	Predicted 5-methyltetrahydropteroyltriglutamate homocysteine methyltransferase	XP_004249374	84898/5.93	*Solanum lycopersicum*	1200/44	16	9/9
S7	Predicted transketolase	XP_004248560.1	80341/5.94	*Solanum lycopersicum*	677/43	12	6/6
S8	Cytosolic ascorbate peroxidase 1	NP_001234782.1	27733/5.61	*Solanum lycopersicum*	622/44	34	6/6
S9	Oxygen-evolving enhancer protein 1	NP_001296294.1	25377/5.89	*Solanum lycopersicum*	250/44	11	2/2
S10	40S ribosomal protein S8	XP_004242427.1	24847/10.39	*Solanum lycopersicum*	196/44	12	2/2
S11	Predicted triosephosphate isomerase	XP_012084383.1	33880/6.13	*Solanum lycopersicum*	225/45	9	2/2
S12	Predicted ribulose-phosphate 3-epimerase, cytoplasmic isoform-like	XP_004238785.1	23944/5.29	*Solanum lycopersicum*	99/43	6	1/1
S13	Predicted: adenine phosphoribosyltransferase 4	XP_004238096.2	19993/5.17	*Solanum lycopersicum*	389/45	20	4/4

[a]NCBI Accession: the accession number of non-redundant protein database; [b]Theo. Mass/PI: theoretical molecular mass and isoelectric point based on amino acid sequence of the identified protein; [c]Mascot Score/Threshold: Mascot scores are statistically significant ($P < 0.05$); [d]SC: Amino acid sequence coverage for the identified proteins; [e]NP: The total number of matched peptides/the number of matched peptides whose mascot scores are statistically significant ($P < 0.05$).

construct (pBait-AbAi) contained one copy of the specific 286 bp sequence inserted in the upstream of *AbA*^{r} reporter gene in pAbAi (Figure 3(a)). After detection of basal expression of the bait reporter in the absence of prey, 100 ng/mL was consider as minimal inhibitory concentration of AbA and used in the subsequent library screening. The cDNA library of AC fruit at breaker stage were constructed by SMART technology (Figure 3(b) and Figure 3(c)) and the number of screened clones was about 0.3 million by counting the number of colonies on SD/-Leu. Meanwhile, far fewer (about 80) colonies, which contained targeted prey plasmid as well as the positive interaction of the potential regulatory factors with the specific 286 bp region, were acquired on SD/-Leu/AbA^{100}. After confirming reporter phenotype by restreaking onto fresh selective media (Figure 3(d)), yeast colony PCR, rescue and isolation of the library plasmid responsible for activation of reporters, a total of 28 genuine positive colonies were

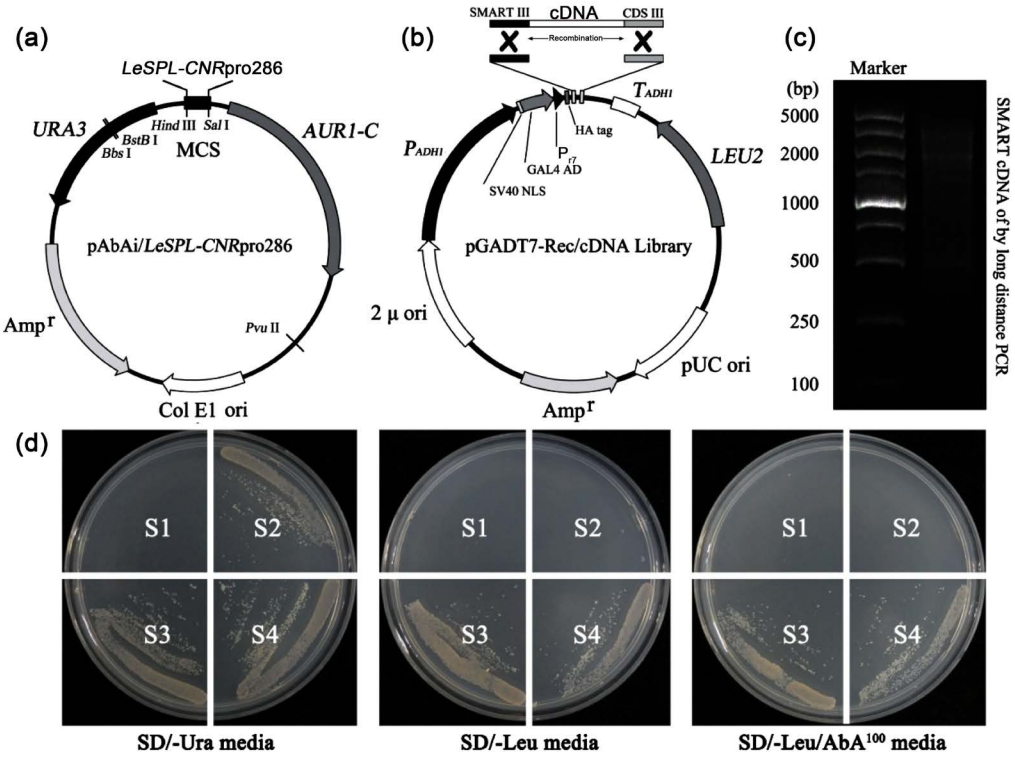

Figure 3. *LeSPL-CNR* regulatory factors screening by yeast one-hybrid library screening system. A 286 bp region of the *LeSPL-CNR* promoter was cloned into the pAbAi reporter vector which was then integrated into the Y1HGold genome to create a bait-specific reporter strain (a). Activation of the AbA resistance gene (AbA^r) occurred if a potential regulatory factor could bind to the bait sequence. A cDNA library from tomato fruit at breaker stage was created by SMART cDNA synthesis technology (c) and the cDNA pool with flanking end sequences were homologous to the prey vector, pGADT7-Rec (b). When the cDNA and linear prey vector were transformed into the reporter strain together, the yeast recombined the cDNA and the vector. Transformed cells were plated on SD/-Ura, SD/-Leu and SD/-Leu/AbA^{100} media to determine if pAbAi report vector or pGADT7-Rec existed in the cells, and select positive colonies which had activated the AbA^r reporter (d). S1: Y1HGold wild type strain, as a negative control; S2: Y1HGold [pAbAi/*LeSPL-CNRpro*286] strain; S3: Y1HGold [pGADT7-Rec/p53, pAbAi/p53] as a positive control; S4: Y1HGold [pGADT7-Rec/BP1, pAbAi/*LeSPL-CNRpro*286].

distinguished from false positive colonies. Finally, a total of 19 prey inserts were identified by sequencing and the detailed information of these candidates was shown in Table 3.

4. Discussion

Gene expression regulatory networks are comprised of *cis*- and *trans*-acting factors, and differences in gene expression are attributable to genetic variation. Epigenetic change can also be considered as an important factor in understanding phenotypic change. *LeSPL-CNR* of *Solanum lycopersicum* is an excellent research subject which possesses important biological functions and is controlled by both epigenetic and classical genetics approaches. In this study, a 286 bp contiguous region at 2.4 kb upstream from the *LeSPL-CNR* coding sequence was used as a bait to search potential regulatory factors by south-western blotting and yeast one-hybrid library screening system. A total of 32 candidate proteins were acquired, and they distributed in nucleus, membrane, mitochondrial, cytoplasm and chloroplast. Except three uncharacterized candidates (BP12, BP15 and BP19), other candidates have known or predicted functions in an extensive range including structural protein, enzyme, and cell signaling, etc. Among them,

Table 3. Information of sequences received from yeast one-hybrid library screening system.

Number	NCBI accession	Species	Description
BP1	NM_001247170	*Solanum lycopersicum*	Alcohol dehydrogenase
BP2	XM_004247745.2	*Solanum lycopersicum*	Predicted: putative G3BP-like protein-like
BP3	XM_004246323.2	*Solanum lycopersicum*	Predicted: phosphatidylinositol: ceramide inositolphosphotransferase 2
BP4	XM_004247148.2	*Solanum lycopersicum*	Predicted: GDP-mannose transporter GONST4
BP5	XM_004242375.2	*Solanum lycopersicum*	Predicted: peptidyl-prolyl cis-trans isomerase FKBP62-like
BP6	XM_004241364.2	*Solanum lycopersicum*	Predicted: chloroplast stem-loop binding protein of 41 kDa b
BP7	NM_001308004.1	*Solanum lycopersicum*	Predicted: mitochondrial CBS domain-containing protein CBSX3
BP8	XM_004249296.2	*Solanum lycopersicum*	Predicted: calmodulin-7
BP9	XM_004229193.2	*Solanum lycopersicum*	Predicted: 40S ribosomal protein S21-2-like
BP10	XM_004229308.2	*Solanum lycopersicum*	Predicted: histone H3.2
BP11	XM_004245431.2	*Solanum lycopersicum*	Predicted: ABC transporter F family member 3
BP12	XM_004250280.2	*Solanum lycopersicum*	Uncharacterized LOC101249421
BP13	XM_004250067.2	*Solanum lycopersicum*	Predicted: histone H3.3
BP14	NM_001308944.1	*Solanum lycopersicum*	Chloroplastic ribulose bisphosphate carboxylase small chain 2A
BP15	AK324254.1	*Solanum lycopersicum*	LEFL1074DA11, HTC in leaf
BP16	XM_004230295	*Solanum lycopersicum*	Predicted: shaggy-related protein kinase NtK-1
BP17	XM_004232376.2	*Solanum lycopersicum*	Predicted: mitochondrial-like Gamma carbonic anhydrase 1,
BP18	XM_004251215.2	*Solanum lycopersicum*	Predicted: ATP synthase subunit d, mitochondrial-like
BP19	AK319554.1	*Solanum lycopersicum*	LEFL1043BF01, HTC in leaf

BP10 and BP13 were predicted as histone H3.2 and H3.3, S4 and BP14 belonged to Ribulose-1,5-bisphosphate carboxylase/oxygenase, and S10 and BP9 belonged to 40S ribosomal protein. Same results acquired from same or different approaches indicated that screening methods in this study were feasible and available.

It is known that histone H3 is one of five main histone proteins involved in the structure of chromatin in eukaryotic cell. Featuring a main globular domain and a long N-terminal tail, H3 is involved with the structure of the nucleosomes on the beads on a string structure. Its sequence variants and variable modification stages are thought to play a role in the dynamic and long term regulation of genes [34]. Variant H3.2 (BP10) closely with H3.1 (only differing in a Cys-Ser substitution at amino acid position) is coupled to DNA synthesis during DNA replication and possibly DNA repair, whereas histone variant H3.3 (BP13) is necessary to mediated DNA-synthesis-dependent and -independent nucleosome assembly [35]. Therefore, the interaction between 286 bp fragment and Histone H3 is reasonable.

Calmodulin (BP8), an abbreviation for calcium-modulated protein, is a multifunctional intermediate calcium-binding messenger protein expressed in eukaryotic cells. It is a small highly conserved protein that is 148 amino acids long (16.7 kDa). This protein has two approximately symmetrical globular domains each containing a pair of EF-hand motif separated by a flexible linker region for a total of four Ca^{2+} binding sites. Once bound to Ca^{2+}, calmodulin acts as part of a signal transduction pathway by modifying its interactions with various target proteins such as kinases or phosphatases [36]. Calmodulin can undergo post-translation modifications, such as phosphorylation, acetylation, methylation and proteolytic cleavage, each of which has potential to modulate its actions [37]. For absence of clues about connection between calmodulin and the 286 bp fragment, a possibility is that calmodulin might form a complex with other protein or proteins to fulfil the regulatory responsibilities. This mode can refer the report of Shima *et al.* [38] that tomato RIN forms a complex with FUL1 and FUL2, and this complex can bind to a typical target DNA motif for MADS-box proteins.

Petidyl-prolyl *cis-trans* isomerase (BP5), as another candidate, is an enzyme that interconverts the *cis* and *trans* isomers of peptide bonds with the amino acid proline (http://www.uniprot.org/uniprot/P62937). Proline has an unusually conformationally restrained peptide bond duo to its cyclic structure with its side chain bonded to its secondary amine nitrogen. This structure can overcome the energetic preference for the *trans* peptide bond conformation and stabilize the *cis* form so that both isomers are populated under biologically relevant conditions. The process of *cis-trans* isomerization can be the rate-limiting step in the process of protein folding, and petidyl-prolyl *cis-trans* isomerase therefore functions as protein folding chaperones [39]. It probably makes petidyl-prolyl *cis-trans* isomerase participate in transcription regulation of *LeSPL-CNR* in an indirect way.

Up to now, there is insufficient evidence confirming the positive correlations

between other candidate proteins not mentioned and transcription regulation of *LeSPL-CNR*. For example, 5-methyltetrahydropteroyltriglutamate homocysteine methyltransferase (S6) belongs to the family of transferases which catalyzes the transfer of a methyl group from 5-methyltetrahydrofolate to homocysteine resulting in methionine formation (http://enzyme.expasy.org/EC/2.1.1.14); Transketolase (S7) is a ubiquitous enzyme that catalyzes the reversible transfer of a two-carbon ketol group from fructose-6-phosphate or sedoheptulose-7-phosphate to glyceraldehyde-3-phosphate to yield xylulose-5-phosphate and erythrose-4-phosphate or ribose-5-phosphate [40]. These two proteins involve in methionine metabolism and Calvin cycle, respectively. Therefore, further exploration about function of the 286 bp region via multiple approaches from different aspects is necessary. Furthermore, it has a potential of obtaining more valuable information and excluding the false positive results via optimizing experiment conditions.

5. Conclusion

In this study, potential regulators of *LeSPL-CNR*, which could bind to the specific 286 bp region of *LeSPL-CNR* promoter, were screened via south-western blotting and yeast one-hybrid library screening system. A total of 13 and 19 candidate proteins were acquired respectively. Although current knowledge could not reasonably explain the molecular mechanism of interactions between acquired candidates and 286 bp fragment, results still provided some interesting information for further exploration in transcriptional cascade or epigenetic controls for fruit ripening.

Acknowledgements

This work was supported by the Zhejiang Provincial Natural Science Foundation of China (LY18C150009) and the National Natural Science Foundation of China (31501810, 31401926).

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