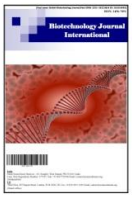

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Prediction of *Solanum lycopersicum* Target of Rapamycin (SlTOR) Protein

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Authors' contributions

This work was carried out in collaboration among all authors. Authors CN, DLN, WR and ZJ designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors DLN and WHR managed the analysis of the study. Authors YL and WXY managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Aims: Tomato (*Solanum lycopersicum*) is an important protected vegetable in China. Its yield and quality receive much concern, however, its growth is often adversely affected by environmental stress, and so improving stress-resistance of tomato has become an urgent issue to be resolved in facility cultivation. Recent studies find that the TOR(Target of Rapamycin) complex acts as a central coordinator of energy, growth, hormones and stress signals, as well as plays a critical role in regulating transcription, protein synthesis, cell size, cell division, and basal metabolism. To study the mechanism of SlTOR in tomato growth and development as well as in stress responses, we did a series of bioinformatics analysis on SlTOR.
Study Design: In order to explore the mechanism of TOR in regulating tomato resistant to adverse conditions, we systematically analyzed the SlTOR gene with bioinformatics methods, and carried out the determination of its tissue-differential expression, aiming at laying down the basis for further experiment research.

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Place and Duration of Study: College of Bioscience and Biotechnology, between March 2018 and April 2019.
Methodology: Bioinformatics analysis was conducted by online programmes. The expression of SlTOR gene in different tissues of tomato was determined by qRT-PCR.
Results: Our results showed that SlTOR was an evolutionarily conserved protein kinase, of which the molecular formula was $C_{12366}H_{19734}N_{3490}O_{3584}S_{104}$, the relative molecular weight was 277978.19Da and the number of amino acid residues was 2470. Besides, it was predicted to be an acidic and unstable protein. SlTOR protein did not contain the signal peptide or transmembrane region, showing that it might be an intracellular protein. And SlTOR was speculated to be targeted to the chloroplast. Moreover, SlTOR had five domains including HEAT, FAT, PIKKc_TOR, FRB and FATC. The KEGG database displayed the only one SlTOR metabolism pathway related to autophagy. The STRING database found that SlTOR probably interacted with SlSnRK1 and SlPP2C. The experimental results of the expression of SlTOR gene in different tissues suggested that in the mature tomato plant, it was expressed the most highly in the root, followed by in the fruit let and in the mature fruit. Our experimental results were roughly consistent with the predicted results.

Keywords: Tomato; TOR; ABA; SnRK protein kinase; stress response.

1. INTRODUCTION

Tomato, known as "vegetal gold", which is one of the most widely cultivated commodities, has not only diverse varieties, but also certain health benefits [1,2,3,4]. However, in practical production, tomato is highly sensitive to stress conditions, such as chilling, drought, high salt and germs, causing significant reduction in production and decline in quality [5,6,7,8]. Therefore, exploring the anti-stress mechanism of tomatoes and active adversity-resistance breeding are of great significance to increase the yield and improve the quality of tomatoes.

Until now, the mechanism of tomato in adversity resistance has been elucidated in depth. For example, plant hormone growth regulators regulate the tomato stress resistance, while calcium signal and small GTPase also play an indispensable role in stress resistance regulations, but, how these signals function through being integrated into a regulatory network is poorly understood [9,10,11,12]. Recent studies have found that the specific target protein of rapamycin (TOR) is an evolutionarily conserved kinase, it can regulate cell cycle, control protein synthesis, guide cell substances/energy metabolism, coordinate energy, growth, hormone, stress signals, and have regulatory functions in response to biotic and abiotic stresses in eukaryotes [13,14, 15,16,17].

The aim of this study was to investigate the mechanism of TOR in regulating tomato resistant to adverse conditions; we systematically analyzed the SlTOR gene using bioinformatics methods and carried out the determination of its tissue-specific expression, intending to lay down the basis for future experiment research.

2. MATERIALS AND METHODS

2.1 Plant Material and Growth Condition

Tomato cultivar "Jinguan 5"(*S. lycopersicum*) was used in all experiments, provided by Liaoning Academy of Agricultural Sciences.

Seeds were surface sterilized to reduce the potential for seeds borne bacterial diseases. The plants were subsequently raised in a greenhouse of which the temperature was 25°C with light/darkness for 16/8 hours a day. When seedlings had developed two true leaves, the roots, stems, cotyledon and true leaves were sampled and frozen in liquid nitrogen and stored at −80°C until used.

When the other group of tomato plants grew to two true leaves, they were planted in the greenhouse. Then the roots, stems, leaves, flowers and fruit lets, swollen fruits and reap fruits were sampled and frozen in liquid nitrogen and stored at −80°C until used. Three replicates were used for each sample.

2.2 Methods

2.2.1 Bioinformatics analysis

Bioinformatics analysis was conducted using online programs in the following table.

Table 1. Tools for bioinformatics analysis

Programme	Name	Website
Physicochemical properties	ProtParam	http://web.expasy.org/protparam/
Signal peptide	SignalP 5.0	http://www.cbs.dtu.dk/services/SignalP/
Transmembrane region	TMHMM	http://www.cbs.dtu.dk/services/TMHMM/
Subcellular localization	Plant-mPLoc	href="http://www.csbio.sjtu.edu.cn/bioinf/plant-multi/
Protein secondary structure	SOPMA	href="http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_sopma.html
Functional domains	NCBI	https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi
Phylogenetic analysis	MEGA7	Software
Protein-protein interaction	STRING	http://string-db.org/
Tissue-differential expression	BAR	http://bar.utoronto.ca/
Pathway analysis	KEGG	https://www.genome.jp/kegg/pathway.html

2.2.2 Differential gene expression analysis

Total RNA of different tissues of tomato was extracted by referring to the instructions of the Plant Total RNA Extraction Kit (Tiangen, Beijing). The RNA integrity was detected by 1.5% agarose gel electrophoresis. Total RNA was reverse-transcribed into cDNA using Quant Reverse Transcriptase Reverse Transcription Kit (Tiangen, Beijing).

SlTOR coding region sequence (CDS) was offered on line by NCBI (http://www.ncbi.nlm.nih.gov/). The primers of Real-time fluorescent quantitative PCR were designed by Primer Premier 5.0, and the primer sequences were shown in Table 2. After reverse transcription, standard cDNA was diluted three times and made a homogenized qRT-PCR reaction mixture using Fluorescence Quantification Kit (Tiangen, Beijing). The reaction system was as follows:

2×SuperReal PreMix Plus	4.5 μL
Forward Primer	0.1 μL
Reverse Primer	0.1 μL
cDNA	1 μL
ddH_2O	4.3 μL
Total	10 μL

Using a CFX96 Real Time System (Bio-Rad), qRT-PCR reactions were conducted using a two-step procedure as follows: 40 cycles of 95°C for 15 min, 95°C for 10s, 60°C for 20 s, 72°C for 30s; 95°C for 0.5s, 60°C for 1 min, 50°C for 30s.

Data analysis was carried out by Bio-Rad CFX Manager. The *SlActin* was taken as the internal reference gene, and the relative expression level of gene was calculated by the $2^{-\Delta\Delta CT}$(Livak method).

3. RESULTS

3.1 Basic Properties, Prediction and Analysis for SlTOR Protein Kinase

3.1.1 Prediction and analysis of physicochemical properties of SlTOR

ProtParam analysis of SlTOR sequence revealed that its molecular formula was $C_{12366}H_{19734}N_{3490}O_{3584}S_{104}$ and relative molecular weight was 277978.19Da. It was composed of 2470 amino acid residues, of which 292 were negatively charged and 278 were positively charged. Overall, the protein was acidic, with a possible theoretical isoelectric point of 6.66. The aliphatic index was computed to be 100.13. The instability index was computed to be 44.46 (>40), suggesting that the protein was unstable. The grand average of hydropathicity was found to be -0.113(>-0.5 and <+0.5) which classified the protein as amphiphilic.

Table 2. Primers used for qRT-PCR assay

Name	Primer sequences (5' - 3')
Q-SlTOR	F: 5' - CTGGGCTTTCCGTAACACT - 3' R: 5'- GAACTAACGCAGAACCACTCA - 3'

3.1.2 Prediction and analysis of signal peptide, transmembrane region and subcellular localization

Secreted proteins and membrane proteins are synthesized in the form of precursor peptides, of which *N*-terminal contains a sequence composed of about 15 to 30 amino acid residues as the transmembrane signal which is called signal peptide or signal sequence[18]. The signal peptide and transmembrane region of SITOR were examined by Signal 5.0 Server and TMHMM program online. The results indicated that the protein did not contain the signal peptide ortransmembrane region (Fig. 1). In summary of these two points, SITOR was an intracellular protein (Fig. 2). With the help of Plant-mPLoc program, subcellular localization prediction suggested a chloroplast localization of SITOR, which was consistent with *Arabidopsis thaliana.*

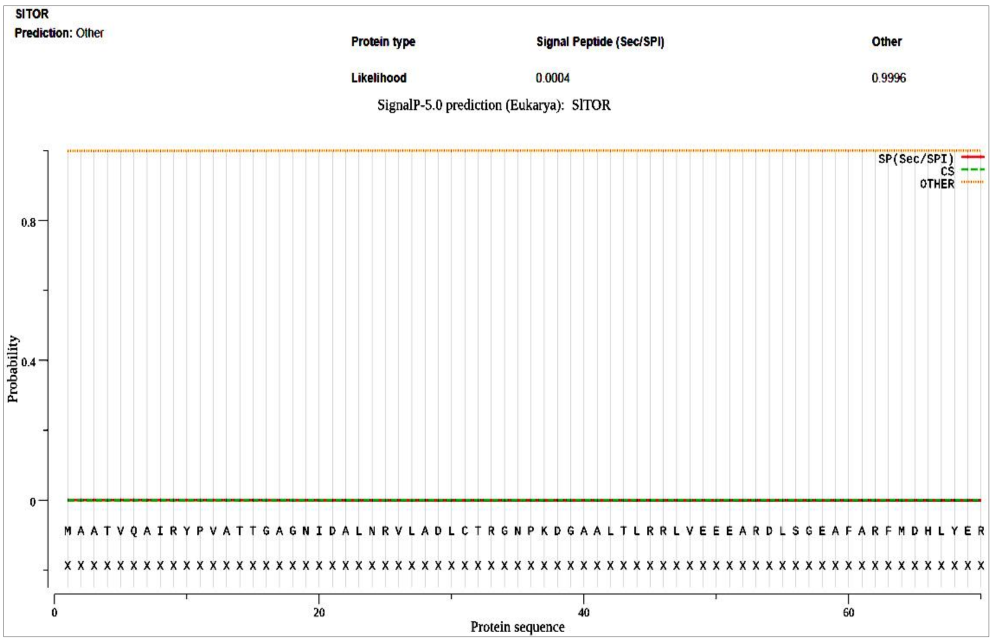

Fig. 1. Prediction for SITOR signal peptide by signal P5.0 database

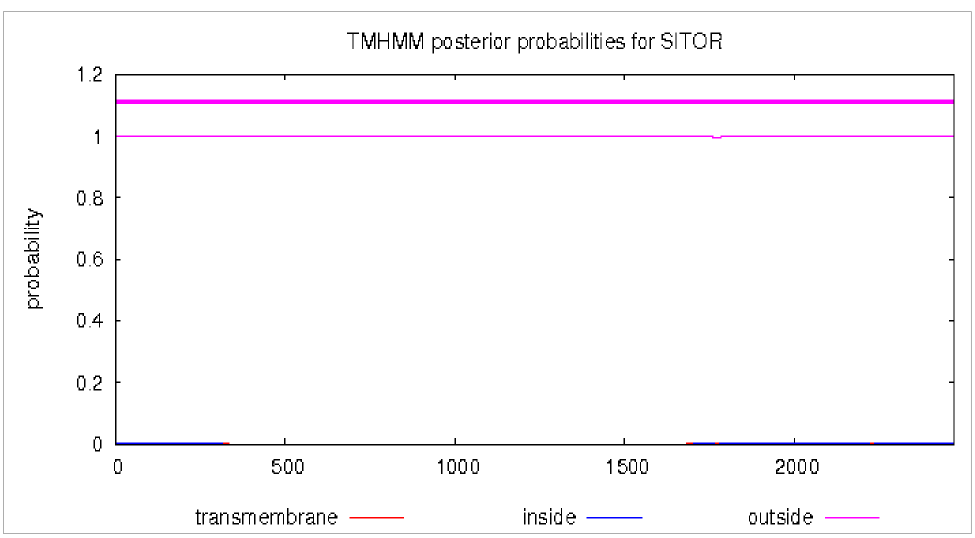

Fig. 2. Prediction for SITOR transmembrane region by TMHMM database

3.1.3 Prediction and analysis of secondary structure for SlTOR protein

Protein secondary structure is the three dimensional form of local segments of proteins. The common secondary structural elements are alpha helix (Hh), extended strand (Ee), Beta turn (Tt) and random coil (Cc) [19]. Secondary structure is mainly maintained by the pattern of hydrogen bonds between the amino (—NH_2) hydrogen and carboxyl (—COOH) oxygen atoms in the peptide backbone. The α-helix is the most extreme and the most stable from sequence, as well as the most prevalent, among types of local structure in proteins. The extended strand is normally formed from repeating structural units composed of two or three short β-strands linked by short loops. β turn is a type of non-regular secondary structure in proteins that causes a change in direction of the polypeptide chain [20], and it generally presents in globular proteins. A random coil is a statistical distribution of shapes for all the polypeptide chains in a population of macromolecules.

The secondary structure of SlTOR was predicted by SWISS-MODEL program indicating that SlTOR contained 1482 α-helixs at 62.16%, 117 extended strands (4.94%), 84 β-turns (3.55%) and others were random coils (28.90%). It could be seen that SlTOR structure was relatively stable.

3.1.4 Conserved domains prediction of SlTOR

Functional domains in the SlTOR protein sequences were predicted by Conserved Domain Search (NCBI), Ensembl Plants and Quick GO, to initially understand the structural basis for SlTOR function. The results were displayed in Fig. 3. Three highly conserved domains, HEAT repeats, FAT and PIKKc_TOR (kinase) were hit specifically. FAT, a domain of PIk related kinase, was an intracellular sensor controlling information transmission. PIKKc_TOR was phosphatidylinositol-3 kinase catalytic domain of TOR protein and found in all PIKKs, which was involved in various biochemical processes, including cell movement, Ras pathway, vesicle transportation and secretion as well as apoptosis. The HEAT repeats had been indicated to mediate protein-protein interactions. In addition, there were 29 non-specific hits. Among them, there were two important functional domains, FRB and FATC. FRB was the FKBP12-Rapamycin binding domain. The rapamycin and FKBP12 could form a complex which specifically inhibited the TORC1 complex, leading to growth arrest. FATlikely played an essential role in redox-dependent structural and cellular stability (Fig. 3).

These predictions have been verified in *S. cerevisiae* TOR1 and TOR2 [21], *H. sapiens* TOR [22] and *A. thaliana* TOR [23]. However, further experiments will be required in tomatoes to confirm these predicted results.

3.2 Multiple Sequence Alignment, Phylogenetic Analysis for SlTOR Protein Sequence

After the TOR sequences were obtained, we generated a phylogenetic tree using MEGA7.0 software through Neighbor-Joining method with 1000 bootstraps (Fig. 4) to investigate the phylogenetic relationships among the nine TOR proteins. The results showed that SlTOR was highly conserved during evolution.

Furthermore, it had the highest homology with TOR protein from *Cucumis sativus* and *Fragaria vesca* because all of them belong to dicotyledon.

3.3 Prediction and Analysis of Metabolism Pathway for SlTOR

The KEGG database displayed the only one SlTOR metabolism pathway related to autophagy (Fig. 5). The process of autophagy could be divided into four stages: Induction, vesicle nucleation, elongation and closure, as well as fusion and digestion. The results indicated that TOR played a vital part in the induction periods.

According to the pathway graph, SlTOR kinase suppresses cell autophagy by forming a complex with RAPTOR and LST8. SlTOR inactivation enhances the autophagy pathway. However, TOR is involved in different metabolic pathways in mammals and yeast, hinting that themetabolic pathways of SlTOR in tomatoes were still not clear, so more experimental evidence will be needed in the future.

3.4 Prediction of Protein Interaction

Using the STRING database to predict the interaction of SlTOR, it was found that SlTOR might interact with SlSnRK1 and SlPP2C. What's more, SlTOR and SlSnRK1 were connected by SlPP2C (Fig. 6).

SnRK1 is activated by PP2C inhibition, which positively regulates cell autophagy, thus assisting plants to respond to stress [24]. Furthermore, SnRK2 can also be dephosorylated and inactivated by PP2C, allowing plants to grow normally in the absence of endogenous abscisic acid (ABA) [25]. Furthermore, previous studies have shown that the antagonistic effect exists between TOR protein kinase and SnRK2 in stress responses [26].

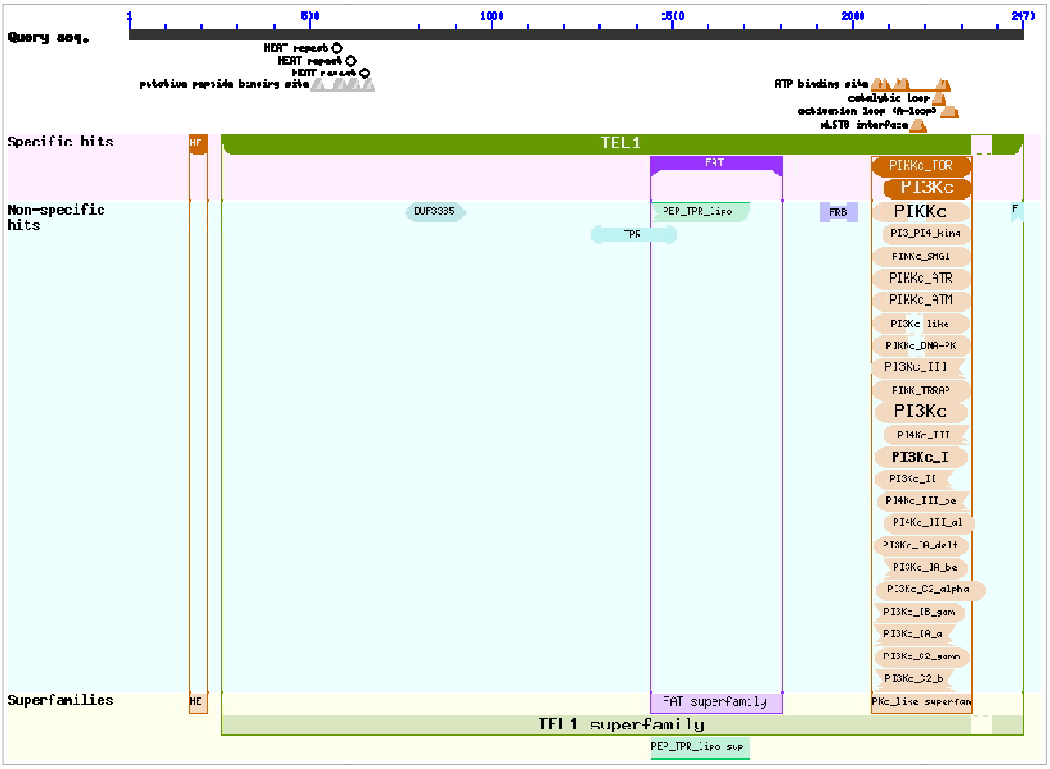

Fig. 3. Conserved domains analysis of SlTOR in tomato

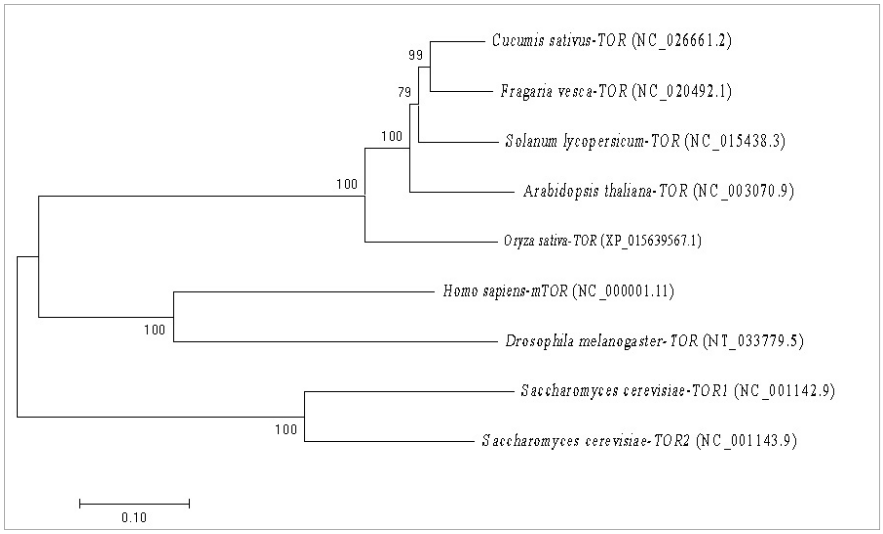

Fig. 4. Phylogenetic tree of *TOR* in different species

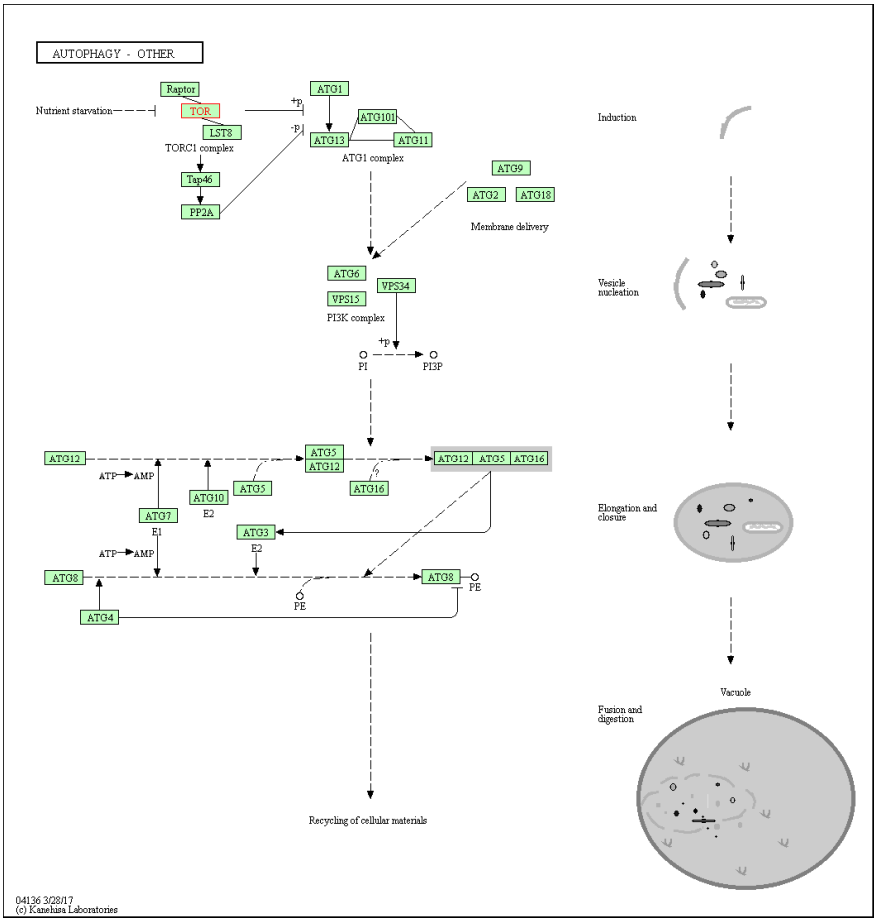

Fig. 5. Pathway of SlTOR protein in tomato

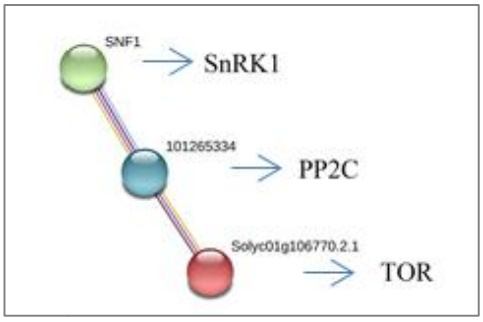

Fig. 6. The prediction of protein interaction among SlTOR, SlSnRK1 and SlPP2C in tomato

3.5 Specific Expression Analysis of SlTOR in Tissues of Tomato

The predicted distributions of SlTOR expression levels in each tissue were shown in Fig. 7. Obviously, the expression levels were variable, and SlTOR was expressed the most highly in roots and mature fruits.

We determined the expression of SlTOR in different tissues of tomato. It was found that the cotyledon had the highest expression of SlTOR in tomato seedlings (Fig. 8A). When it came to mature tomato plants, it was expressed the most highly in roots, followed by in fruitlets and in mature fruits, while at low level in other tissues (Fig. 8B).

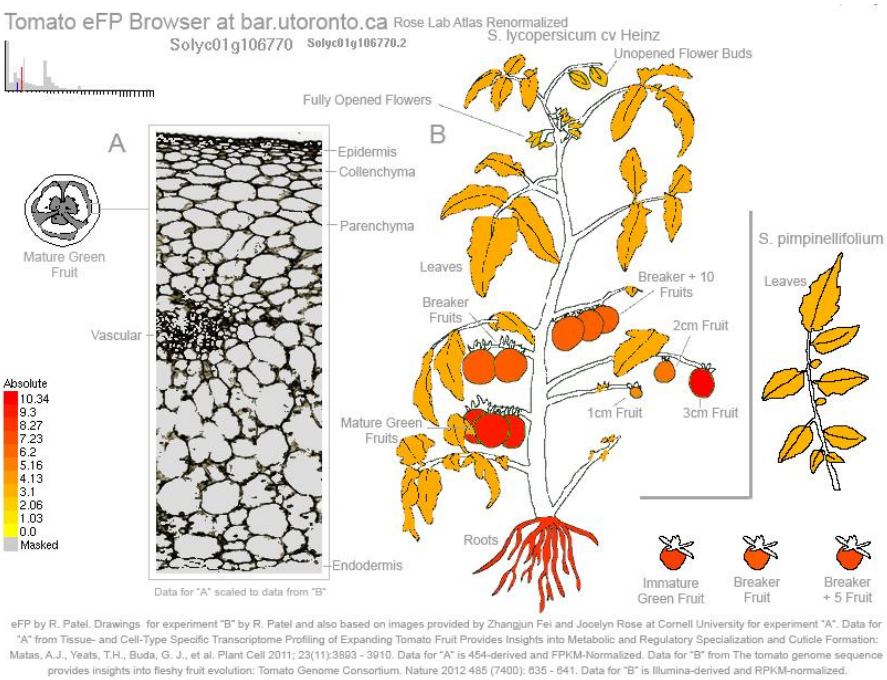

Fig. 7. Predicted tissuespecific expression of SITOR in tomato

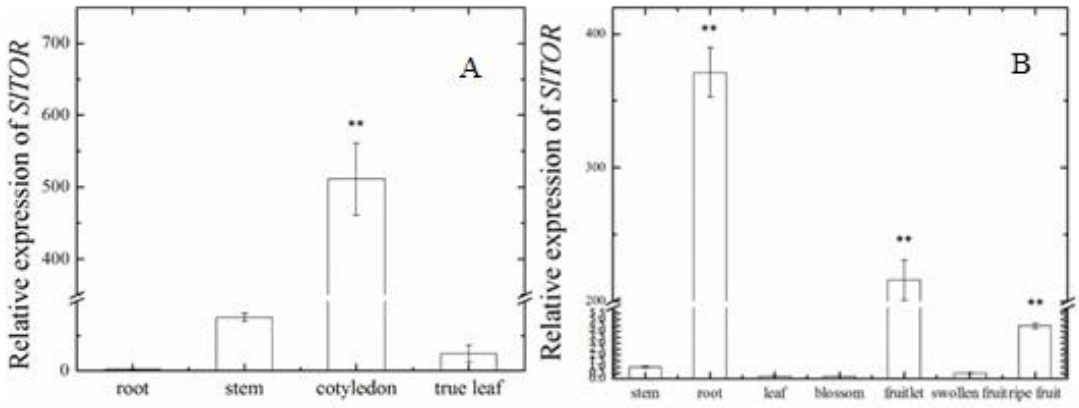

Fig. 8. Tissuespecific expression of SITOR in tomato

4. DISCUSSION

All organisms rely on nutrients to maintain cellular metabolism and energy production. Under stress conditions, it is necessary to adjust according to the existing resources to maintain the steady state of life. TOR is an evolutionarily conserved central regulator that correlates environmental information, such as the quantity and quality of nutrients with development and metabolic processes to maintain cell homeostasis [27,28,29]. As an important kinase to promote growth and metabolism, TOR plays an adjust role in nitrogen or carbon metabolism [30]. It is inactivated under nutrition deficient conditions or in some cell life activities, such as cell division and translation, which consume more energy, thus inducing cell autophagy [31]. There have been some studies on the regulation of TOR in *Arabidopsis* and some other crops [32], but the mechanism in tomato is still unclear.

To study the mechanism of SITOR in tomato growth and development as well as stress responses, we performed a series of bioinformatics analysis on SITOR. Our results

showed that SlTOR was an evolutionarily conserved protein kinase, of which the molecular formula was $C_{12366}H_{19734}N_{3490}O_{3584}S_{104}$, the relative molecular weight was 277978.19Da and the number of amino acid residues was 2470. Besides, it was predicted to be an acidic and unstable protein. SlTOR protein did not contain the signal peptide or transmembrane region, showing that it might be an intracellular protein. And SlTOR was speculated to be targeted to the chloroplast. Moreover, SlTOR had five domains including HEAT, FAT, PIKKc_TOR, FRB and FATC. The STRING database found that SlTOR probably interacted with SlSnRK1 and SlPP2C. The experimental results of the expression of SlTOR in different tissues suggested that in the mature tomato plant, it was expressed the most highly in the root, followed by in the fruit let and in the mature fruit. Our experimental results were roughly consistent with the predicted results. The KEGG database revealed the only one SlTOR metabolism pathway related to cell autophagy which played a crucial role in plant stress responses. Therefore, further research is needed to elucidate the mechanism of SlTOR in autophagy.

In addition, bioinformatics analysis manifested that SlTOR might interact with SlSnRK1 and SlPP2C. Previous studies have shown that ABA signal transduction triggers different pathways based on endogenous ABA content. In the absence of ABA, PYL ABA receptor mostly exits as a dimer or can be phosphorylated by TOR at a conserved serine residue. Under this condition, PYL cannot associate with ABA and PP2C phosphatase effectors, leading to inactivation of SnRK2 kinase, thus plants maintain normal growth [26]. Under stress, ABA accumulates rapidly and binds to PYLs [33]. The ABA-PYL receptor complex subsequently inhibits downstream protein phosphatases PP2C; PP2C inhibition releases SnRK2s, which phosphorylate downstream effectors to trigger a series of defence responses [34]. Besides, SnRK1, a member of the SnRK family, is also a substrate of PP2C. Experiments have shown that ABA can obviously up-regulate the expression of SnRK1.1 and SnRK1.2 in *Arabidopsis* [35]. SnRK1 in ABA-induce cell autophagy activation is activated by PP2C inhibition, thus positively regulating autophagy [36].

SnRK1 and TOR are evolutionarily conserved protein kinases that lie at the heart of sugar sensing and energy management. Furthermore, they play an antagonistic role in the regulation of metabolism and gene expression [37]. The information in this study may be useful to further in-depth research on SlSnRK1-SlPP2C-SlTOR interaction.

Our subsequent research will concentrate on analyzing and verifying SlTOR motifs and functional domains, proving its binding sites and related metabolic pathways by using molecular biology experimental methods. The bioinformatics analysis results of this study have laid a theoretical foundation for further illustrating the mechanism of SlTOR in regulating the balance between growth and development as well as stress in tomato, improving tomato resistance in production.

5. CONCLUSION

Using bioinformatics methods, we had analyzed SlTOR in tomato, showing that SlTOR was an evolutionarily conserved and amphiphilic protein kinase. And it was speculated to be a high-molecular weight and intracellular protein. Besides, SlTOR was in the chloroplast, so it might be closely related to photosynthesis. Moreover, SlTOR had five domains including HEAT, FAT, PIKKc_TOR, FRB and FATC. The STRING database found that SlTOR probably interacted with SlSnRK1 and SlPP2C. The experimental results of the expression of SlTOR in different tissues suggested that in the mature tomato plant, it was expressed the most highly in the root, followed by in the fruitlet and in the mature fruit. Our experimental results were roughly consistent with the predicted results. The KEGG database revealed the only one SlTOR metabolism pathway related to cell autophagy which played a vital role in plant stress responses. Therefore, the subsequent research needs to pay more attention to the mechanism of SlTOR in autophagy.

ACKNOWLEDGEMENT

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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