In Silico analysis of *Vitis vinifera* Cabernet Sauvignon TOR and its responses to sugar and abscisic acid signaling

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ABSTRACT
Target of rapamycin (TOR) is a very critical protein in plants, which connects with biological process, glycometabolism, life span, and photosynthesis. Here, the evolutionary relationship, conserved motif, gene structure and cis-acting elements of TOR were analyzed. Promoter cis-acting elements analysis indicated various cis-acting elements respond to light, auxin, ABA and multiple signal pathway. Transcriptome sequencing and the co-expression network of VvTOR, sugar and abscisic acid (ABA) related genes from *Vitis vinifera* L. Cabernet Sauvignon berries indicated that VvTOR might participate in sugar and ABA signaling. The expression of VvTOR in grape suspension cells analyzed by quantitative real-time PCR showed that VvTOR responded to ABA and glucose treatment. These results predicted the potential functions of VvTOR in glucose metabolism and ABA signal pathway.

Keywords: target of rapamycin, in silico analysis, grape, sugar, abscisic acid

Introduction

TOR (target of rapamycin) is a large protein (~280 kDa) that belongs to an atypical serine-threonine protein kinase (PK), closely relates to the phosphatidylinositol 3-kinase-related protein kinase (PIKK) family and shares 40% - 60% identity in their primary sequence conserved from yeasts to plants and humans (Loewith & Hall 2011; Robaglia et al. 2012; Aramburu et al. 2014; Maegawa et al. 2015; Xiong & Sheen 2015; Dobrenel et al. 2016). TOR signaling network is a central metabolic network in all eukaryotes, which coordinates cell growth and development in response to all kinds of signals, including light, auxin, glucose, amino acid (Wullschleger et al. 2006; Xiong et al. 2013; Xiong & Sheen 2015; Dobrenel et al. 2016; Inaba & Nagy 2018). Recently, more and more research about TOR protein has been done. So far, every eukaryote genome has been examined containing the TOR, including yeast, plant, animal, algae, and slime mold etc. Comparing with yeast genome which has two different TOR genes, most plants, animals and human genomes have only one TOR gene, except that two TOR genes were found in *Glycine max*, *Populus trichocarpa* and *Brassica rapa* (González & Hall 2017; Shi et al. 2018; Jamsheer et al. 2019). In mammals, there is only one copy of TOR gene, but it forms two TOR complexes, called mTORC1
(mammalian TOR complex 1) and mTORC2 (mammalian TOR complex 2), which are formed by different elements and functionally specified proteins (Van Leene et al. 2019). In plants, TOR exists as only TORC1, while the TORC2 is absent, which is a key evolutionary difference between plants and mammals (Xiong & Sheen 2015; Dobrenel et al. 2016; Van Leene et al. 2019). However, it is possible that there are other undiscovered special TOR complexes in plants, or the plants TORC1 possesses partial function of mammalian TORC2 which can replace the whole function of mammalian TORC2 (Jamsheer et al. 2019).

Grapevine (Vitis vinifera L.) is considered to be one of the major fruit crops in the world. The yield of grapes is very abundant, and the economic value is tremendous. Grape can be used not only for wine but also for fresh fruit, dried fruit, and for grape juice. The quality of grapes depends on the accumulation of sugar to a large degree, including glucose, fructose, sucrose and so on. Sugar not only supports energy for plant growth, but also a critical signaling molecule. TOR and abscisic acid (ABA) are pivotal protein and hormone about sugar metabolism in plants (Ciereszko 2018; Rodriguez et al. 2019). ABA increases carbon allocation in different organs of grapevine plants by inducing accumulation of non-structural carbohydrates in leaves, enhancement of phloem area and expression of sugar transporters (Murcia et al. 2016). The glucose-TOR crosstalk controls many genes that are uniquely required for plant growth, defense or communication to promote fitness, adaptation and survival (Xiong et al. 2013). In a summary, TOR has critical influence on the sugar and ABA signal pathway, and the in silico analysis is able to provide references and theoretical basis for the further study of TOR.

In this study, in silico analysis about phylogenetic tree construction, gene structure analysis, conserved motif analysis and cis-acting elements prediction of TOR were performed to further understand the potential functions of TOR. At the same time, the study selected post-flowering 30-day, 70-day and 90-day development stages of grapevine berries Cabernet Sauvignon and extracted RNA for transcripomte sequencing. The co-expression network of Vitis vinifera TOR (VvTOR), sugar and ABA related genes reveals that VvTOR has a close relationship and works together with a variety of sugar and ABA metabolic genes. Furthermore, the expression of VvTOR, sugar and ABA related genes from the co-expression network were analyzed by transcriptomic analysis, which reveals that the early stage of grapevine berries development has a big difference with the middle and later stage. According to quantitative real-time PCR analysis, we detected the expression levels of VvTOR in grape suspension cells with sugar and ABA treatments to explore the roles of VvTOR in sugar metabolism and ABA signal pathway. Based on the above analyses of VvTOR gene, the study also played analysis for VvTOR by online analysis software, including basic physicochemical properties, hydropathicity and hydrophobicity, transmembrane structure, protein secondary and tertiary structure prediction. In conclusion, the study told us that TOR was a highly conserved protein and TOR promoter sequence contained multiple cis-acting elements. ABA and sugar signals could affect the expression of VvTOR, which implied that VvTOR could participate in sugar metabolism and ABA signal pathway. We expect this work could provide some references for improving the sugar content by the VvTOR gene in grapevine berries and offer some viewpoints for exploring the mechanism of the VvTOR metabolism network.

**Materials and methods**

**Phylogenetic tree construction**

Twenty-one gene and protein sequences of TOR from plants were downloaded from the NCBI (https://www.ncbi.nlm.nih.gov/) databases (Tab. S1). The statistical method of Neighbor-Joining was applied to construct the TOR phylogenetic tree by Mega 7.0. The evolutionary distances were computed using the Maximum Composite Likelihood (Saitou & Nei 1987; Huang et al. 2020).

**Conserved motif and gene structure analysis**

The motif analysis of twenty-one protein sequences of TOR was performed in the MEME (http://meme-suite.org/tools/meme). The conserved motifs were screened and visualized with TBtools (Toolbox for Biologists) v0.664435 (Chen et al. 2020). Gene structure analysis of twenty-one TOR genes was combined with a phylogenetic relationship. The genome information was from NCBI.

**Cis-acting elements prediction**

To further understand the potential functions of TOR, online analysis of 2,000 bp promoter sequence in the coding-sequence (CDS) was performed by PlantCare website (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) for cis-acting prediction (Lescot et al. 2002; Huang et al. 2020). The information about the sequence was downloaded from NCBI.

**Transcriptome Sequencing**

The grapevine berries Cabernet Sauvignon at 30, 70 and 90 days after bloom were collected from Beijing Lainberg International Winery (116.218778, 39.790114) and 90 days after bloom were collected from Beijing Lainberg International Winery (116.218778, 39.790114) and transcriptome sequencing of these berries was performed. Total RNA of grapevine berries was extracted using the RNAprep Pure Plant kit (TIANGEN, Beijing, China) and checked for a RIN number to inspect RNA integrity by an Agilent Bioanalyzer 2100 (Agilent technologies, Santa Clara, CA, US). Qualified cDNA library was constructed and applied for following sequencing (Illumina Hiseq 2000). The original
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image file obtained was subjected to base recognition and error filtering. The sequenced fragments called “Reads” were obtained for analysis. In order to eliminate the influence of gene lengths and sequencing discrepancies on gene expression, the reads were converted into FPKM (Fragments per Kilobase of exon model per Million mapped Reads) for standardization of gene expression (Mortazavi et al. 2008). The number of fragments for each gene was counted by HTSeq and applied normalized by TMM (trimmed mean of M values) method, FPKM value of each gene was calculated using perl script (Robinson & Oshlack 2010; Anders et al. 2015).

**Co-expression network analysis of VvTOR and sugar and ABA related genes**

The co-expression networks of VvTOR and sugar (Glucose, Fructose, Sucrose, Glucan, Starch, Xylan) and ABA related genes were constructed according to the HRR (highest reciprocal rank) method, respectively. Sugar and ABA related genes (Tabs. S2, S3) were selected by the information of the gene description in transcriptome sequencing results and NCBI. Correlation between two genes was calculated by pearson correlation coefficient (r), using R Development Core Team (2012) version 3.5.3 parameters for HRR30 gene correlation coefficient calculation and threshold screening followed by visual analysis using Cytoscape software (Smoot et al. 2011; Yong et al. 2018).

**Analysis of VvTOR Gene and Protein**

The ProtParam tool (https://web.expasy.org/protparam/) was applied for analyzing the physicochemical properties of proteins. ProtScale (https://web.expasy.org/protscale/) was applied to hydropathicity and hydrophobicity analysis of proteins. TMHMM Server v.2.0 (http://www.cbs.dtu.dk/services/TMHMM/) was used for protein transmembrane structure analysis. SOPMA was applied for protein secondary structure prediction, following the link (https://npsa-prabi.ibcp.fr/cgi-bin/npsa_automat.pl?page=npsa_sopma.html). PredictProtein (https://www.predictprotein.org/) had the same function with SOPMA. However, the PredictProtein function was more comprehensive. Swiss-Model Workspace (https://swissmodel.expasy.org/) was used for tertiary structure analysis of proteins. SignalP-5.0 software (http://www.cbs.dtu.dk/services/SignalP/) could predict if there was signal peptide.

**Grape suspension cells preparation and treatments by sugar and ABA**

The calli of grape cultivar Cabernet Sauvignon was cultured at 20 days interval on Gamborg B5 medium containing 0.2 mg L⁻¹ 6-furfurylamo-purine (KT) and 0.1 mg L⁻¹ 1-naphthaleneacetic acid (NAA) at 25 °C in 16 h of light and 8 h of darkness. Appropriate amount of grape suspension cell was cultured in B5 liquid medium at the condition of 25 °C, 110 r/min and cultured at 7 days interval. Four days after cultured, glucose (Gl, 100 mM), sucrose (Suc, 100 mM), fructose (Fru, 100 mM), ABA (10 μM), Glu (100 mM) + ABA (10 μM), Suc (100 mM) + ABA (10 μM), Fru (100 mM) + ABA (10 μM) was added into suspension cell and light culture for 12 h. Mannitol (100mM) treatment was applied as a control to exclude the effects of osmotic stress (Song et al. 2010). After 12 h treatment, cell was vacuum filtered, frozen in liquid nitrogen immediately and stored at -80 °C for subsequent quantitative real-time PCR analysis.

**Quantitative real-time PCR analysis**

To evaluate the expression levels of VvTOR in the suspension cells with different sugar and ABA treatments, quantitative real-time PCR (qRT-PCR) analysis was performed. Total RNA was extracted from grape suspension cells with ABA and sugars treatment using a RNAprep Pure Plant kit (CWBIO, Jiangsu, China). First-stranded cDNA was synthesized from 4 μg RNA using a HiFiScript cDNA Synthesis kit (YEASEN, Shanghai, China) and applied as a template for qRT-PCR. Dissociation curves of qRT-PCR reaction were analyzed for the specificity of primers. qRT-PCR analysis was run on CFX96 Real Time PCR System (Bio-rad, America) using UltraSYBR mixture (CWBIO, Jiangsu, China). The housekeeping gene VvACTIN (XM_002282480) with nearly constant expression level under all experimental conditions was applied as an internal control (Wang et al. 2017). The relative expression of VvTOR under different sugar and ABA treatments was measured according to the method of 2⁻ΔΔCt (Livak & Schmittgen 2001).

**Statistical analysis**

Each experiment was replicated three times, and the mean ± standard deviation (SD) was reported. Statistical differences between means were evaluated by SPSS 20.0 software. Univariate analysis of variance (ANOVA) and Duncan’s test were applied to establish the significance at P < 0.05.

**Results**

**Phylogenetic tree, conserved motif and gene structure analysis**

Phylogenetic tree of TOR was shown in Fig. 1A. It could be observed that *Vitis vinifera* has close ties of consanguinity with *Theobroma cacao*, *Herrania umbralatica*, *Ricinus communis* and *Jatropha curcas*. The amino acid conserved domain analysis of TOR protein sequences was carried out in the online MEME program. All TOR contained conserved motifs 1 to 30 and the motifs are in the same order, except that
Raphanus sativus starts with one more motif 20 in the 5’ of the sequence (Fig. 1B). This is corresponding to the viewpoint that TOR is highly conserved. The gene structure map showed the number of intron-exons in all members of TOR genes (Fig. 2A). Almost all TOR genes have 57 exons and there is a big difference in the full length of TOR genes. VvTOR gene is very long in the gene structure map. In close ties of consanguinity, TOR genes are about the same length, like Raphanus sativus, Brassica oleracea, Capsella rubella and Arabidopsis thaliana.

Cis-acting elements prediction

Analysis of cis-acting elements in the TOR promoters provided the basic for the understanding of potential regulation mechanism of TOR. Promoter element analysis illustrates that TOR contains multiple light responsive regulatory elements (GT1-motif, 3-AF1 binding site, AE-box, AAAC-motif and so on). Some cis-acting elements were associated with auxin (TGA-element, AuxRR-core), gibberellin (TATC-box, p-box, GARE-motif), abscisic acid (ABRE), methyl jasmonate (TGACG-motif, CGTCA-motif), salicylic acid (TCA-element, SARE). Some cis-acting elements respond to low-temperature (LTR), defense and stress (TC-rich repeats) and phytochrome down-regulation expression, while some cis-elements were involved in anaerobic induction (ARE), meristem expression (CAT-box), differentiation of the palisade mesophyll cells (HD-zip 1), MYB binding site (MRE, MBS, MBSI), MYBHv1 binding site (CCTTA-box), anoxic specific inducibility (GC-motif), circadian control (circadian) and the like (Fig. 2B). The TOR promoter contained a large number of hormone and stress response elements, indicating that TOR may plays critical role in hormone signal transduction and environment stress. Almost all TOR promoters can respond to light and anaerobic induction. VvTOR promoter responded to light, auxin, abscisic acid responsive, anaerobic induction and MYB binding site, which implies that VvTOR plays a role in these signal pathways.

Co-expression network and expression analysis of VvTOR and sugar and ABA related genes

The relationship between VvTOR and sugar and ABA related genes was explored by co-expression network analysis, respectively. VvTOR is co-expressed with 40 sugar related genes, including 13 glucose, five sucrose, nine xyloglucan, 10 glucan, one starch, two hexoses related genes (Fig. 3A). VvTOR is co-expressed with 28 ABA related genes (Fig. 3B). The co-expression network analysis indicates that VvTOR has a close relationship with sugar metabolism and ABA signal pathway. In order to understand the role of VvTOR, sugar and ABA related genes in the growth and development of grape berries of three stages (DAB30, DAB70, DAB90) were selected for RNA-Seq analysis. The expression levels of VvTOR, sugar and ABA related genes which were in the co-expression network were performed by FPKM. In contrast, VvTOR expression levels are high in DAB70 and DAB90 (Fig. 4). ABA related genes expression levels were higher in DAB30, which is the opposite with VvTOR. The majority of sugar related genes have higher expression levels in DAB70, like sucrose, glucon, xyloglucan, hexose related genes. At the same time, xyloglucan related genes expression levels were high in DAB30 and sucrose related genes expression levels were high in DAB90 too. Glucose related genes had expression in all three stages of grape. The columns of the heat map were clustered and the result showed that the expression levels of VvTOR, sugar and ABA related genes in DAB70 and DAB90 were more similar compared to that in DAB30.

Expression of VvTOR with sugar and ABA treatments

In order to explore the roles of VvTOR in the sugar metabolism and ABA signal pathway, grape suspension cells were treated with different sugar and ABA, the expression levels of VvTOR in suspension cells with sugar and ABA (mannitol, Glu, Suc, Fru, ABA, Glu + ABA, Suc + ABA, Fru +...
ABA) were detected by quantitative real-time PCR. VvTOR responded to different sugar and ABA treatments (Fig. 5), while the expression of VvTOR in suspension cells with signal sugar and ABA treatment differed from that with sugar and ABA complex treatments. VvTOR was able to respond to Glu and ABA signals. The expression of VvTOR with Glu treatment was lower than mannitol treatments and ABA treatment could improve the expression of VvTOR. The relative expression of VvTOR was strongly induced by ABA+Glu. Compared with mannitol treatments, VvTOR was down-regulated by Suc or Fru and up-regulated by Suc + ABA or Fru + ABA, even though the difference is not significant.

**Figure 2.** Gene structure analyses and the cis-acting elements prediction of TOR. (A) Gene structure analyses of TOR. The gray line indicated intron, while the green and yellow boxes indicate UTR and CDS, respectively. TEL1 superfamily and HEAT_EZ superfamily are conserved domains of TOR. (B) The cis-acting elements prediction of TOR.
Analysis of VvTOR Gene and Protein

The number of amino acids of VvTOR are 2,469. Molecular weight is 277,335,89 Da. Theoretical protein isoelectric point (pI) is 6.40. High amino acids composition is Leu 13.0 %, Ala 10.0 %, Arg 7.4 %. A protein whose instability index is smaller than 40 is predicted as stable, a value above 40 predicts that the protein may be unstable (Wilkins et al. 1999). VvTOR protein instability index is 43.51, which classifies the protein as unstable. Grand average of hydropathicity (GRAVY) is -0.099. The score is less than 0, which represents hydrophobicity. The score is greater than 0, which represents hydrophobicity (Wilkins et al. 1999). Hydropathy and hydrophobicity analysis of VvTOR protein shows that the 127th amino acid is the highest, with a score of 2.733 and hydrophobicity is the strongest. The 548th is the lowest, with a score of -2.989 and the hydrophobicity is the strongest. In total, the numbers and scores of hydropathicity are greater than hydrophobicity (Fig. S1). Above all, we predict that VvTOR protein is hydrophilic, which it accords with GRAVY. TMHMM Server v.2.0 used for protein transmembrane structure analysis shows that all amino acids are outside. VvTOR is an outer membrane protein without transmembrane structure (Fig. S2). SignalP-5.0 is used for predicting if there are signal peptides in the VvTOR protein. Signal Peptide (Sec/SP) likelihood is 0.0005. The closer of the signal peptide probability is to 1, the higher the probability that the protein has a signal peptide; hence, we can draw the predicted result that there is no signal protein (Fig. S3). The result corresponds to the study of hydrophilic and hydrophobic analysis and transmembrane structure analysis of proteins (Figs. S1, S2). SOPMA for protein secondary structure prediction shows that alpha helix, random coil, extended strand and beta turn are 63.57 %, 27.44 %, 5.51 % and 3.48 %, respectively (Combet et al. 2000) (Fig. S4). Alpha helix is the main in the secondary structure of VvTOR protein. Swiss-Model Workspace builds the tertiary structure model of VvTOR protein automatically (Fig. 6).

Discussion

TOR is a critical conserved protein to sense and integrate cellular status information from numerous stimuli, including hormone signals, nutrient and energy availability, and stress information. Although TOR protein is high conserved (Fig. 1A), the full-length of TOR genes varies greatly (Fig. 2A). The full-length of TOR genes are similar in the majority closely related species (Figs. 1A, 2A). In our experiments, VvTOR was co-expressed with 40 sugar genes and 28 ABA related genes, which implies that VvTOR may play a critical role in ABA and sugar metabolism (Fig. 3). VvTOR can respond to Glu and ABA signals, which is according to other researchers (Xiong & Sheen 2012; Fu et al. 2020) (Fig. 5). Recently, scientists found that glucose can activate TOR protein further to promote the development of root hair in Arabidopsis (Xiong & Sheen 2012; Van Leene et al. 2019). TOR has a cis-acting element about meristem (Fig. 2B). Sugar signals can be translated by protein kinase complex, which regulates energy metabolism. VvTOR promoter sequence had the ABA responsive elements (Fig. 2B). Down-regulated TOR signaling by chemical inhibitor AZD-8055 also activates genes involved in stress hormone (e.g., ethylene, jasmonic acid (JA), and ABA) signaling pathways (Fu et al. 2020). Some studies suggest that two important mediators of ABA signaling, YAK1 and ABI4, as the key downstream regulator of TOR signaling to control root growth, meristem activation and seed germination (Kim et al. 2016; Barrada et al. 2019; Fu et al. 2020). Researchers found that TOR has a negative effect on JA signaling pathway and there is a cross-talk between TOR and JA (Song et al. 2017). TOR signaling has a significant influence on JA biosynthesis and the associated signal transduction pathways in cotton and Arabidopsis (Song et al. 2017). TOR and SnRK2s work together to regulate the dynamic balance of growth and defense and stress (Jamsheer et al. 2019). These results are corresponding to TOR cis-acting elements prediction that TOR includes multiple hormone and defense and stress related cis-acting elements (Fig. 2B). TOR has cis-acting elements about circadian and some researchers found that metabolite-mediated TOR signaling regulates the

Figure 3. The Co-expression network diagram of VvTOR with sugar and ABA related genes. (A) The Co-expression network diagram of VvTOR with sugar related genes. (B) The Co-expression network diagram of VvTOR with ABA related genes.
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Figure 4. Expression of VvTOR, sugar and ABA related genes in three growth and development stage of grape berries. Numerical values in small squares of different colors were FPKM values, representing genes expression data.
circadian clock in *Arabidopsis* and identify TOR kinase as an essential energy sensor to coordinate circadian clock and plant growth (Zhang *et al*. 2019). As for the cis-acting element of meristem expression related (Fig. 2B), the glucose-TOR-E2Fa/b signal network promotes root growth by improving cell division activity in the root meristem (Xiong *et al*. 2013). Some researchers found that cold treatment compromises enhanced anthocyanin accumulation in the inducible tor-es mutant under normal temperature, which indicated that TOR may be a negative regulator in cold conditions (Wang *et al*. 2017). This is corresponding to the low temperature responsive cis-acting element (Fig. 2B).

Transcriptome sequencing reveals that the early stage of grapevine berries development has a big difference with the middle and later stage (Fig. 4). VvTOR expressive levels are high in DAB70 and DAB90, which indicates that VvTOR participated in color change and maturity period of grape (Fig. 4). ABA related genes expression levels are higher in DAB30, which suggests more ABA related genes take part in the growth period of grape berries (Fig. 4). The majority of sugar related genes have higher expression levels in DAB70, like sucrose, glucan, xyloglucan, hexose related genes, indicating that these sugar genes may participate in color change of grape (Fig. 4). Sucrose may participate in the maturity period of grapevine because the sucrose related genes have higher expression levels in DAB90 too (Fig. 4). Similarly, xyloglucan related genes expression levels are high in DAB30, indicating that they take part in the growth period of grape berries too (Fig. 4). Glucose related genes had expression in all three stages of grape berries, which may imply that glucose related genes are full participation in the growth and development of grapevine berries (Fig. 4).

VvTOR did not show the presence of signal peptides, suggesting that it was not a secreted protein (Fig. S3). This finding was consistent with its location and function as outer membrane protein without transmembrane structure (Fig. S2). VvTOR protein has an amount of alpha helix, which is corresponding to the result of protein secondary structure prediction (Fig. 6) (Guex *et al*. 2009; Bertoni *et al*. 2017; Bienert *et al*. 2017; Waterhouse *et al*. 2018). As a critical protein in the life of grapevine, VvTOR protein still has many functions which need to be found and researched, including regulating life-span and responding to light,

**Figure 5.** Expression of VvTOR in grape suspension cell under mannitol, glucose (Glu), sucrose (Suc), fructose (Fru), abscisic acid (ABA), Glu + ABA, Suc + ABA, Fru + ABA treatments. Data are mean ± SD of three biological replicates. The same letters on the bar are not significantly different by Duncan’s test (p > 0.05).

**Figure 6.** Prediction of the tertiary structure of VvTOR Protein. The quality of the model is indicated by colors from orange (poor quality) to blue (high quality).
auxin and nutrition etc. (Ren et al. 2012; Li et al. 2017; Schepetilnikov et al. 2017). Lately, 63 novel TOR-regulated proteins that have been previously linked to TOR signaling network were discovered (Van Leene et al. 2019). Therefore, an in-depth study needs to be done to reveal the important functions of VvTOR.

Together, the in silico analysis presented the evolutionary relationship, gene structure, cis-acting elements of TOR. The transcriptomic analysis showed the relationship of VvTOR, sugar and ABA related genes in the different periods of grapevine berries. Meanwhile, the expression of VvTOR in grape suspension cells based on different kinds of sugar and ABA indicated that VvTOR had responses to sugar and ABA. These results imply that the potential functions of VvTOR in the growth and development of grapevine berries, sugar metabolism and ABA signal pathway. VvTOR is hydrophilic and outer membrane protein without transmembrane structure. Consequently, we expect that these in silico analysis are valuable for improving grapevine berries sugar content by regulating VvTOR gene and able to offer some viewpoints for exploring the mechanism of VvTOR metabolism network.

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References


