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IN SILICO IDENTIFICATION OF PUTATIVE miRNAs TARGETING GME TO INCREASE ASCORBIC ACID IN TOMATO

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Abstract

The primary natural sources of ascorbic acid or vitamin C are fruits and vegetables, with tomatoes being one of the most readily available and accessible examples. The biosynthesis of ascorbic acid through the L-galactose pathway plays a key role in regulating vitamin C production in tomatoes. microRNAs (miRNAs) are among the various regulators that interact with mRNA involved in this process. Investigating how miRNA regulates mRNA could provide crucial information for developing genetic engineering techniques to boost vitamin C production. The main purpose of this research is to identify potential miRNA regulators of GME using in silico approach. The research findings demonstrated that ascorbic acid biosynthesis in tomato involves 19 gene IDs. It also discovered that GME interacts with multiple miRNAs in tomatoes, specifically sly-miR-159b, sly-miR-159a, sly-miR-167, sly-miR-319c, sly-miR-319b and sly-miR-319a. Among these miRNAs, sly-miR-159a displays higher expression, which allows it to exert a suppressive effect on GME activity (negative regulator), leading to a decrease in vitamin C production. Decreasing the expression of this miRNA provides a potential molecular approach for enhancing the nutritional quality of tomato cultivars in response to the rising demand for functional foods.

Keywords: *Ascorbic Acid; GME; miRNA in Tomatoes; Plant miRNA*

Abstrak

Sumber alami utama asam askorbat atau vitamin C berasal dari buah dan sayuran, dengan tomat menjadi salah satu contoh yang paling mudah didapat dan terjangkau. Biosintesis asam askorbat melalui jalur L-galaktosa memiliki peran penting dalam mengatur produksi vitamin C pada tomat. mikroRNA (miRNA) merupakan salah satu dari berbagai pengatur yang berinteraksi dengan mRNA yang terlibat dalam proses ini. Penelitian mengenai bagaimana miRNA mengatur mRNA dapat memberikan informasi penting untuk pengembangan teknik rekayasa genetik dalam meningkatkan produksi vitamin C. Tujuan utama dari penelitian ini adalah untuk mengidentifikasi kandidat miRNA potensial yang berperan sebagai regulator GME dengan pendekatan in silico. Hasil penelitian menunjukkan bahwa biosintesis asam askorbat pada tomat melibatkan 19 ID gen. Penelitian ini juga menemukan bahwa GME berinteraksi dengan beberapa miRNA pada tomat, yaitu sly-miR-159b, sly-miR-159a, sly-miR-167, sly-miR-319c, sly-miR-319b, dan sly-miR-319a. Di antara miRNA tersebut, sly-miR-159a menunjukkan tingkat ekspresi yang lebih tinggi sehingga mampu memberikan efek supresi terhadap aktivitas GME (sebagai regulator negatif), yang berdampak pada penurunan produksi vitamin C. Penurunan ekspresi miRNA ini menjadi salah satu pendekatan molekuler potensial dalam meningkatkan kualitas nutrisi kultivar tomat sebagai respons terhadap meningkatnya permintaan pangan fungsional.

Kata Kunci: *Asam Askorbat; GME; miRNA pada Tomat; miRNA Tanaman*

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INTRODUCTION

In silico experiments are studies that use databases, software/applications or other computing systems as a method of data collection and analysis. Utilising this method enables efficient data collection, reducing the risks associated with laboratory experiments and minimising the need for large sample sizes. This approach enables researchers to gather valuable genetic, proteomic, and metabolomic data that can enhance the quality of agricultural output, ultimately leading to more sustainable farming practices including improving the quality of plant nutrition (Gomez-Casati et al., 2018).

Ascorbic acid, commonly referred to as vitamin C, is a well-established essential micronutrient that functions as a cofactor for enzymes participating in diverse cellular metabolic processes. Its role in maintaining cellular homeostasis and supporting enzymatic activity underscores its biological significance. Adequate levels of vitamin C intake have been associated with positive health outcomes, acting as a non-enzymatic antioxidant that supports immune function and enhances skin health by facilitating depigmentation, boosting collagen synthesis, and mitigating aging effects (See et al., 2024).

Vitamin C is available through natural sources and can also be artificially synthesized. Numerous horticultural crops are rich in vitamin C such as potato with 1-14.8 mg/100 g (Tatarowska et al., 2023), spinach 51.9 mg/100 g, kale 135 mg/100 g, and purslane 152 mg/100 g (Nemzer et al., 2021). Tomatoes (*Solanum lycopersicum*), among all horticulture species, is widely regarded as a popular fruit that can be cultivated with relative ease, requiring minimal effort to grow, and is capable of producing a substantial yield within a period of 65 to 85 days (Li et al., 2018). Ripe tomatoes contain approximately 193.82 mg of vitamin C per 100 grams of dry weight and this level can be further increased through specific treatments (Motlhalamme et al., 2025). The concentration can also increase by modifying various molecular components involved in ascorbic acid biosynthesis pathway, such as protein and enzyme-forming genes and their regulators.

GDP-mannose 3,5-epimerase is an enzyme encoded by the GME with an important role in ascorbic acid biosynthesis. GME represents a critical control point in the L-galactose pathway of ascorbic acid biosynthesis, as it catalyzes the first committed step toward vitamin C production. Its central role in directing metabolic flux toward ascorbate underscores its importance as a strategic target for metabolic engineering aimed at enhancing vitamin C content in plants. The GME encoding this enzyme in tomatoes consists of 376 amino acids and is located on chromosome 9. This gene is recognized for its role in the production of ascorbic acid in a variety of plant species including cabbage, rice, *Arabidopsis thaliana*, and tomatoes (Beerens et al., 2022).

Like other genes, before it becomes a functional protein, it must be turned into mRNA first. This mRNA is certainly regulated by non-coding RNAs including microRNAs (miRNAs). miRNAs are small non-coding RNA molecules, typically ranging from 21 to 25 nucleotides in length. In the regulation of gene expression, miRNAs function by targeting genes during the post-transcriptional stage, either through the cleavage of mRNA or the inhibition of gene translation (Dong et al., 2022). Therefore, compared to other methods, the use of miRNAs in plant engineering offers distinct advantages by functioning through natural regulatory mechanisms to modulate gene expression without cleaving target DNA. This approach preserves genomic integrity, as it does not induce permanent alterations to the genome, thereby presenting a safer strategy for plant genetic modification.

Bioinformatics has emerged as a robust approach for forecasting potential miRNA-mRNA interactions. Earlier investigations effectively utilized computational biology techniques to pinpoint three miRNAs that bind to Tomato Leaf Curl Virus (ToLCV) in tomato plants, with subsequent experimental confirmation (Tousi et al., 2017). Among the available tools, psRNAtarget has proven particularly valuable, elucidating miRNA profiles associated with drought resistance in maize root systems (Tang et al., 2022). Furthermore, examining expression profiles of both miRNAs and their target genes enables a more comprehensive understanding of probable binding affinities and associated biological processes. Such analyses are supported by bioinformatics resources, including curated plant gene expression datasets (Sullivan et al., 2019) and specialized miRNA databases (Guo et al., 2020).

Previous studies have explored genetic engineering to enhance ascorbic acid levels in plants. Koukounaras et al. (2022) demonstrated that GGP1 overexpression under a fruit-specific promoter (PG) tripled ascorbic acid content in ripe tomatoes, influencing both nutrition and fruit development. Fenech et al. (2021) identified GDP-L-galactose phosphorylase (GGP) as a key regulator of ascorbic acid metabolism. Liu et al. (2024) found that the CsGME gene in cucumber modulates leaf size and ascorbic acid biosynthesis, with mutations reducing both. Zhu et al. (2022) reported that miR4415 inhibits L-ascorbate oxidase, elevating ascorbic acid in *Ammopiptanthus nanus*. However, post-transcriptional regulation of GME by miRNAs, particularly in tomatoes, remains underexplored. This study aims to identify ascorbic acid biosynthesis genes in the L-galactose pathway and predict potential miRNA regulators of GME in tomatoes using in silico approaches.

METHODS

Data analysis in this study used open resources (all websites are 2025 version) to ensure data accessibility and reproducibility. This study commenced with the identification of predicted genes involved in tomatoes ascorbic acid biosynthesis, utilizing data from the Pathway Tools website which is available at https://solcyc.solgenomics.net/. The genes within the metabolic pathway were determined based on their corresponding gene and protein names displayed on the website. In addition, the Bio-Analytic Resource for Plant Biology (absolute mode) which is available at https://bar.utoronto.ca/ (Waese et al., 2017) was utilized to examine their distribution in tomatoes. Furthermore, the nucleotide sequences corresponding to GME1 and GME2 were accessed through the GenBank (GenBank Overview) on the NCBI platform (https://www.ncbi.nlm.nih.gov/). The nucleotide sequence was then submitted to psRNATarget (Dai et al., 2018) through the 'Submit target candidates' option (penalty for G:U pair: 0.5, expectation cutoff: 5, missmatches allowed: 2, translation inhibition range: 10-11 NT.) to predict miRNAs that could target GME (https://www.zhaolab.org/psRNATarget). Following this, RNAhybrid (Krüger & Rehmsmeier, 2006) was employed to predict the Minimum Free Energy (MFE) of interactions between miRNAs and their targets (https://bibiserv.cebitec.uni-bielefeld.de/rnahybrid), with the requirement that the value be negative. In the final step, miRNA expression was determined using the Plant microRNA Encyclopedia (pmiRen), where the miRNA name was entered into the search field at https://www.pmiren.com (Guo et al., 2020).

RESULTS AND DISCUSSION

Results

1. Genes involved in ascorbic acid pathway based on Pathway

   The Pathway Tools revealed 19 registered gene identities involved in ascorbic acid biosynthesis. However, some of these gene identifiers correspond to the same protein/enzyme. In total, there are 11 distinct proteins/enzymes participating in the L-galactose pathway of ascorbic acid synthesis in tomatoes (Table 1).

Table 1. Gene IDs Involved in Ascorbic Acid Pathway based on Pathway Tools

Gene ID	Protein/Enzyme Name
Solyc06g060100.2	Mannose-6-phosphate isomerase
Solyc02g063220.2	
Solyc02G086090.2	
Solyc08g076560.1	Phosphomannomutase
Solyc08g008670.2	
Solyc08g076550.1	
Solyc05g026490.2	Phosphoglucomutase (alpha-D-glucose-1,6-bisphosphate-dependent)
Solyc05g046340.1	Phosphomannomutase
Solyc05g048760.2	
Solyc04g005030.2	Phosphoglucomutase (alpha-D-glucose-1,6-bisphosphate-dependent)

Gene ID	Protein/Enzyme Name
Solyc09g011220.2 Solyc06g051270.2 Solyc03g096730.2	GDP-D-mannose pyrophosphorylase
Solyc03g113790.2	ADP-glucose pyrophosphorylase family protein
Solyc09g082990.2	GDP-D-mannose3,5 -epimerase 2
Solyc01g097340.2	GDP-D-mannose 3,5-epimerase 1
Solyc02g091510.2 Solyc06g073320.2	GDP-D-glucose phosphorylase
Solyc10g079470.2	L-galactono 1,4 lactone dehydrogenase

2. GME1 profile expression on tomato
The expression of GME1 was not restricted to fruits alone but was also observed in various other tomato plant organs. The expression levels of GME1 ranged from 13.59 to 157.3 RPKM (Figure 1).

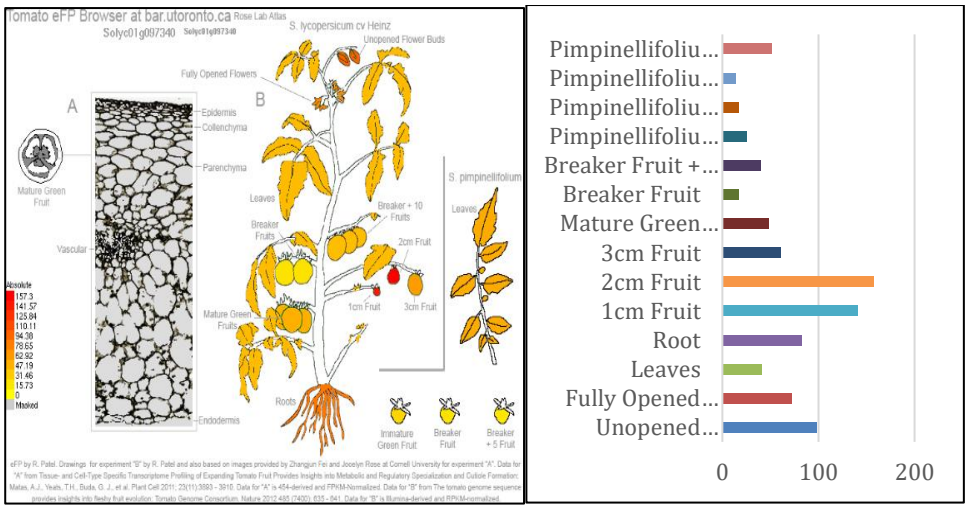

Figure 1. GME1 Profile Expression on Tomato (Data are Shown as RPKM)

3. GME2 profile expression on tomato
Similar to GME1, the GME2 was also expressed in nearly all tomato plant organs, with expression levels ranging between 17.55 and 211.74 RPKM (Figure 2).

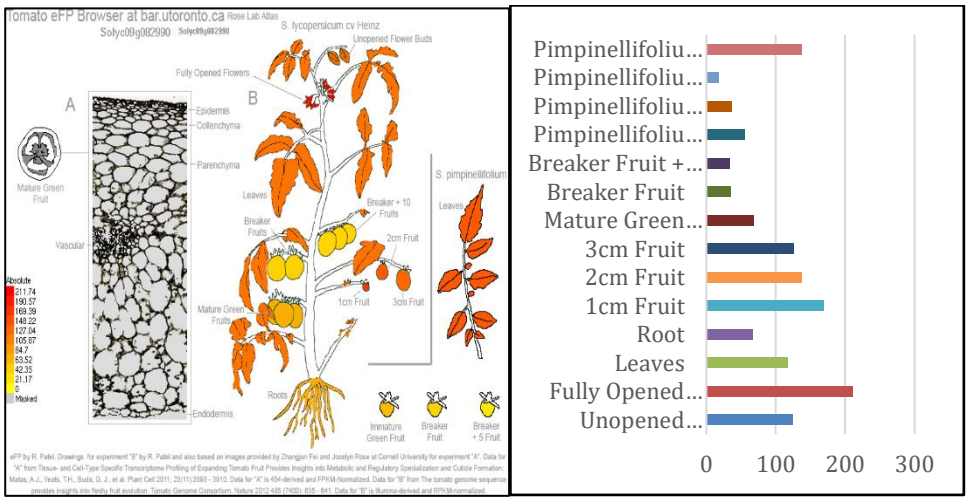

Figure 2. GME2 Profile Expression on Tomato (Data are Shown as RPKM)

4. miRNAs that target GME

This study identified six candidate miRNAs potentially targeting GME: sly-miR-159b, sly-miR-159a, sly-miR-167, sly-miR-319c, sly-miR-319b, and sly-miR-319a. These GME-targeting miRNAs exhibited expectation values ranging from 4.0 to 5.0 and minimum free energy (MFE) values between -29.0 and -23.9 kcal/mol (Table 2).

Table 2. Tomatoes miRNAs that Target GME

miRNA	Target	Alignment	Expectation	MFE (kcal/mol)
sly-miR-319a	GME2	miRNA 20 CCUCGAGGGAAGUCAGGUUC 1 :.::::.: ::::::: :: Target 314 UGGGCUUCAUUCAGUCGAAC 333	5.0	-23.9
sly-miR-319b		miRNA 21 UCCCUCGAGGGAAGUCAGGUU 1 :.::::.: ::::::: :: Target 312 UAUGGGCUUCAUUCAGUCGAA 332	5.0	-27.9
sly-miR-319c-3p		miRNA 21 UUCCUCGAGGGAAGUCAGGUU 1 : :.::::.: ::::::: :: Target 312 UAUGGGCUUCAUUCAGUCGAA 332	5.0	-27.3
sly-miR-319a	GME1	miRNA 20 CCUCGAGGGAAGUCAGGUUC 1 :.:.:.: :::::::::: Target 317 UGGGUUUCAUUCAGUCCAAC 336	4.0	-28.2
sly-miR-319b		miRNA 21 UCCCUCGAGGGAAGUCAGGUU 1 :.:.:.: :::::::::: Target 315 CAUGGGUUUCAUUCAGUCCAA 335	4.0	-29.0
sly-miR-319c-3p		miRNA 21 UUCCUCGAGGGAAGUCAGGUU 1 : :.:.:.: :::::::::: Target 315 CAUGGGUUUCAUUCAGUCCAA 335	4.0	-28.1
sly-miR-159		miRNA 21 AUCUCGAGGGAAGUUAGGUUU 1 :.:.:.: ::::.:::::: Target 316 AUGGGUUUCAUUCAGUCCAAC 336	4.5	-26.4
sly-miR-167b-3p		miRNA 21 UAACUUCGACGAUCUACUGGA 1 ::::::: :. :::.::: Target 481 AAAGAAGCUGAUGCAUGGCCU 501	5.0	-26.5

5. Expression of miRNAs that target GME

In tomato roots, leaves, and fruits, miRNAs that target GME showed expression levels ranging from 1 to 3.957 Reads Per Million (Figure 3).

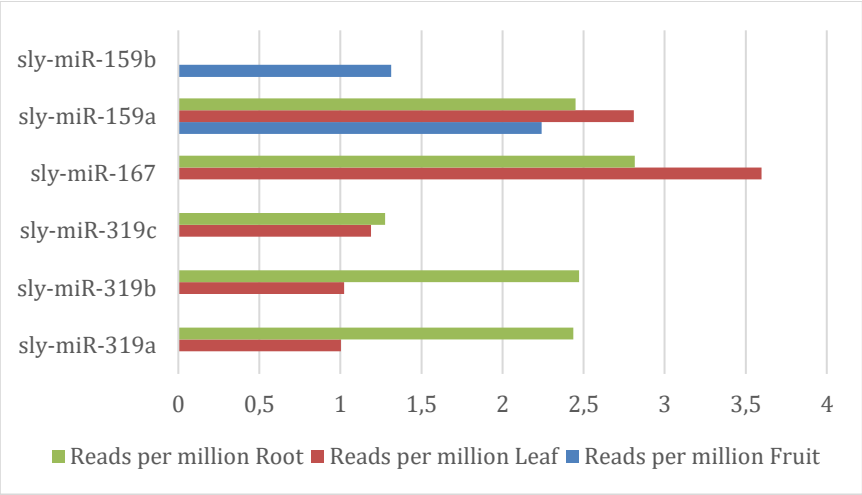

Figure 3. Expression Patterns of miRNAs Targeting GME
(Data are Shown as $\log_{10}(x+1)$, where x Represents the Expression Level)

Discussion

Plants synthesize ascorbic acid primarily through four metabolic routes: the L-galactose pathway (Smirnoff-Wheeler pathway), myo-inositol pathway, L-gulose pathway, and D-galacturonate pathway. The D-mannose/L-galactose pathway, or Smirnoff-Wheeler pathway, is essential for ascorbic acid biosynthesis in plants, producing compounds that are highly advantageous for human health. Although alternative metabolic pathways exist for the production of vitamin C, this pathway is the primary and most significant biosynthetic route in plants, including tomatoes (Li et al., 2019) and rice (Broad et al., 2020). The L-gulose pathway utilizes GDP-D-mannose-3,5-epimerase to produce GDP-L-gulose (rather than GDP-L-galactose as in the L-galactose pathway), which is subsequently converted to vitamin C via GULO (homologous to the animal pathway), while the L-galactose pathway depends on GME and GGP/VTC2. In contrast, both the D-galacturonate and myo-inositol pathways serve as secondary routes that employ non-standard precursors (derived from cell wall pectin and phospholipids, respectively), differing fundamentally from the L-galactose pathway which uses GDP-D-mannose as its primary substrate, and typically contribute less to overall vitamin C production (Zheng et al., 2022).

There are six main web data providers used, namely Pathway Tools, NCBI, Uniprot, BAR, psRNATarget, RNAhybrid and pmiRen. The Pathway Tools diagram are identified by a unique code referred to as gene ID or gene identity (Table 1). The ascorbic acid biosynthetic pathway comprises 19 gene IDs, with certain gene IDs exhibiting identical names. Upon identification of the genes, their nucleotide sequences can be accessed from GenBank (NCBI), notably for GME1 and GME2, which are the primary focus of this investigation. NCBI deliver extensive genomic and protein datasets that include information from all types of living organisms, ranging from viruses and bacteria to plants, animals, and humans. This platform is freely accessible without any registration requirements.

The plant genetic data website, BAR owned by the University of Toronto, provides various genetic information for food crops in plant genetics research such as rice, Arabidopsis thaliana, and tomatoes. GME1 and GME2 expression images were obtained from this website via the electronic Fluorescent Pictograph Browser provided (Waese et al., 2017). In tomatoes, the GME1 and GME2 are expressed in nearly all plant organs, with varying levels of expression, and are not confined to the fruit. Moreover, higher expression of GME has been linked to increased vitamin C accumulation (Li et al., 2022). Based on Figure 1, the highest expression level of the GME1 gene is found in a fruit with diameter of 2 cm, which is 157.3 RPKM (Reads Per Kilobase per Million). The expression levels following the highest were detected in 1 cm diameter fruits (141.5 RPKM) and unopened flower buds (98.65 RPKM). The findings underscore the pivotal role of GME1 in ascorbic acid biosynthesis during fruit development. GME1 expression escalates with fruit diameter, potentially correlating with enhanced vitamin C accumulation in larger fruits.

GME1 and GME2 are isoforms that have different nucleotide sequences and may have similar roles. Interestingly, GME1 was also seen to have high expression in unopened flower bud. In contrast to GME1, the GME2 showed the highest expression in fully opened flower with an expression level of 211.74 RPKM, followed by fruits with diameters of 1 cm (168.86 RPKM), 2 cm (138.29 RPKM) and 3 cm (126.79 RPKM) as shown in Figure 2. The expression of GME2 in tomatoes demonstrated an inverse proportionality to GME1, with smaller fruit diameters associated with elevated GME2 expression. The occurrence of both gene families in non-fruit plant organs is hypothesized to be tied to their involvement in cell wall biosynthesis (Mounet-Gilbert et al., 2016), alongside their critical roles in pollen germination, pollen tube elongation, and male gametophyte transmission and development (Qi et al., 2017).

psRNATarget is an online resource that delivers information on plant miRNAs, including their target genes, the expectation value (E) of miRNA-gene interactions, and their functional mechanisms, specifically cleavage or translation inhibition. The expected value (E) offers insight into the discrepancy between the miRNA sequence and its target. The greater the E value, the greater the mismatch between the miRNA and the target gene, with a recommended range of 3 to 5 (Dai et al., 2018). Since the MFE value is unavailable on this site, this research uses RNAhybrid to provide insights into miRNA-gene interactions based on the MFE value.

According to 110 publications related to psRNAtarget analysis of tomatoes, the miRNAs capable of binding to GME are the sly-miR-319 family (a, b and c), sly-miR-159 and sly-miR-167 (Table 2). These three miRNAs have wide range of expression in tomatoes organs based on pmiREN (Figure 3). The sly-miR-319 family is recognized as one of the most conserved miRNAs in plants. Despite its conservation, the expression of sly-miR-319b is undetectable in tomato fruit, suggesting that there are no natural inhibitory mechanisms preventing GME1 from functioning efficiently in the biosynthesis of ascorbic acid. The interaction between GME2 and this miRNA is most pronounced when the gene binds to sly-miR-319b, with an MFE value of -27.9 kcal/mol. Hence, to increase vitamin C levels in tomatoes, the environmental conditions must be managed to avoid overexpressing sly-miR-319b. The presence of this miRNA in various tomatoes organs may be linked to additional functions, including shaping plant structure (Jian et al., 2022), enhancing fungal resistance (Wu et al., 2020), initiating trichome development, and boosting insect resistance (Fan et al., 2020), as well as activating secondary cell wall biosynthesis (Sun et al., 2017).

The sly-miR-159 family (a and b) is expressed at levels sufficient to potentially inhibit GME1 activity, particularly in the fruit. Sly-miR-159b, in particular, shows higher expression in the fruit than in other organs. The interaction between this miRNA and GME1 suggests a strong affinity because of the secondary structure stability, as evidenced by an MFE value of -26.4 kcal/mol (Chaudhary et al., 2022). Consequently, one strategy to enhance ascorbic acid production in tomatoes could involve reducing the expression level of this miRNA. In contrast, overexpression of this miRNA can lead to sterility in tomatoes male gametes. However, another significant aspect of this miRNA is its involvement in tomatoes morphology through gibberellin modulation (Zhao et al., 2022). In addition to its expression in tomatoes fruit, this miRNA is also present in the leaves and roots. This widespread expression may be associated with its role in drought tolerance (López-Galiano et al., 2019) and its function in facilitating normal plant growth by regulating GAMYB, which is known to induce cell death (Millar et al., 2019).

Another microRNA identified as a potential regulator of GME2 is sly-miR-167b. Data obtained from psRNAtarget analysis indicate that this miRNA is specifically expressed in leaf and root tissues, with no detectable expression observed in fruit tissues. This tissue-specific expression pattern implies that sly-miR-167b is unlikely to play a significant role in the regulation of GME gene expression during fruit development, highlighting its potential function as a regulatory agent primarily in vegetative tissues. Although the role of sly-miR-167b has not been extensively studied, experiments involving its relative, sly-miR-167a, have shown that reducing its expression triggers early ripening of tomato fruits before harvest and slows down their aging process after harvest (Duan et al., 2025). It is well-established that this miRNA family also modulate SlARF8A and SlARF8B, which are essential for the formation of locular and placental tissues in tomatoes, ultimately impacting auxin balance (Hua et al., 2024).

Given that miRNAs exert their regulatory function by binding to complementary target genes, elevated miRNA expression enhances the probability of gene inhibition or cleavage, which could consequently modulate vitamin C biosynthesis in tomatoes. Existing functional studies have confirmed that miRNAs participate in the regulation of ascorbic acid biosynthesis pathway through artificial miRNA-induced silencing other gene in L-galactose pathway (VTC2 that encodes GDP-L-galactose phosphorylase) leads to significant reductions in ascorbate levels (Vidal-Meireles et al., 2017). While our in silico analysis suggests potential miRNA-mediated regulation of GME expression, these computational predictions require experimental validation using molecular docking and functional studies to identify the most biologically relevant miRNA regulators. Most importantly, we strongly recommend experimental validation to confirm the presence of miRNAs in tomato and elucidate its regulatory mechanism in vivo.

CONCLUSIONS

Bioinformatic analysis provides a useful starting point for identifying genes and regulatory elements involved in ascorbic acid biosynthesis, despite its preliminary nature. This study highlights that GME is broadly expressed across tomato organs and is potentially regulated by miRNAs such as sly-miR-159a/b, sly-miR-167, and sly-miR-319a/b/c. Given its central role in ascorbic acid biosynthesis, GME stands out as a key genetic target for enhancing the nutritional quality of tomato through increased vitamin C content. Combining bioinformatics with experimental approaches—such as high-throughput sequencing, qRT-PCR, and transgenic overexpression—can clarify miRNA-mediated regulation of GME and support genetic strategies to enhance ascorbic acid content.

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