J Plant Mol Breed (2019)7(1): 56 - 66
DOI: 10.22058/jpmb.2020.114871.1193

RESEARCH ARTICLE

miR395 is involved in response to cold stress and modulation of sulfate and phosphate deficiency in Grape (*Vitis vinifera*)

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ABSTRACT: Low temperature is a major abiotic stress which can significantly affect the grape production. microRNAs play an important role in the control of plant development and response to adverse environmental conditions. Although miRNAs and their targets have been identified in several Vitis species, their participation during cold accumulation remains largely unknown. One such microRNA is miR395, which is conserved and regulates sulfate assimilation and distribution in plants. In this study, the possible role of miR395 in cold stress response was investigated. Identification of target genes, gene ontology and biological system analysis were performed to identify the major networks in which this miRNA is involved. Finally, the effects of gradual chilling and also a shock chilling on the expression of miR395 were investigated. In total, five target genes were identified, which all of them are targeted by miR395s a to m, whereas of the five target genes, only one is identified by miR395n as a target. Three of these genes, including ATP sulfurylase, sat-1 and, LAST3-like are involved in the control of sulfur metabolism and transport. Pathway analysis showed that miR395 was involved in response to cold stress in grape through cellular response to sulfate and phosphate deficiency. Based on RT-PCR results, contrary expression patterns of miR395 under gradual (up-regulated) and shock chilling stress (down-regulated) were observed. The changes of sulfate assimilation process would influence the formation of sulfur-containing antioxidant compounds. These results provide an insight into the regulatory roles of miR395 in response to low-temperature stress in *V. viniferae*.

KEYWORDS: Abiotic stress, Grape, miRNAs, RT-PCR, Oxidative stress

INTRODUCTION

Cold tolerance is a survival strategy of some overwintering plants in frigid winter. Cold-tolerant plants respond and adapt to cold stress through a number of biochemical changes, such as accumulation of compatible metabolites (4, 21), production of antifreeze proteins (57), and increase of heat shock proteins (31). In response to cold stress, plants have established stress-responsive molecular regulation mechanisms at different levels (27). MicroRNAs (miRNAs) are a class of endogenous small non-coding RNAs (20-24 nt in length) which originate from pre-miRNAs with stem-loop structures and have been reported as a new regulator in plant adaptation to environmental stresses (10, 11). miRNAs play an important role in regulating gene expression and various metabolism and biological processes, including growth development, phytohormone signaling, flowering, and sex determination (34, 41). Plant miRNA was first identified in Arabidopsis thaliana by different research groups (37, 40) and currently, miRNAs have been reported in 72 plant species; all their sequences have been

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Received: 25 September 2019 / Last revised: 17 January 2020
Accepted: 28 January 2020

deposited in the miRBase database (24). Numerous researches concentrated on their role in plant response to various stresses including cold, drought, salinity, and nutrient deficiency (7, 29). Increasing evidence has shown that plant miRNAs are extensively involved in biotic and abiotic stresses responses (6, 46).

miR395 is a conserved small RNA that has been found in Arabidopsis, rice, and many other plant species (49). miR395 was amongst the first such miRNAs identified (16) which overexpression of it results in accumulation of sulfate in plant shoots and leaves due to increased translocation from the roots (25). miR395 targets multiple genes from two gene families involved in sulfate uptake and assimilation. miR395 is strongly induced by sulfate deficiency, and is crucial for the regulation of sulfate homeostasis (8, 11). The abiotic stress factors like cold and drought which the organelle experiences lead to creation of an oxidative environment and production of reactive oxygen species (ROS). Sulfur metabolites containing thiol residues with reversible oxidation-reduction potential effectively scavenge ROS in a series of biochemical reactions.

Computational tools of system biology, such as common targets analysis, common regulators analysis, and pathway discovery, have created new opportunities to understand the molecular mechanisms of various stresses, including cold stress. The discovery of common targets and downstream pathways based on enrichment analysis is a reliable way to gain a comprehensive view of the molecular mechanisms that can provide a particular transcriptomic, genomic or proteomic profile. The common goal is to find the target or the result of changing proteins, genes, or variants. Transcription factors and miRNAs are major molecules in the regulation of gene networks and are generally shared between different networks (2).

Grapevine (*Vitis vinifera* L.) is one of the most important and widely cultivated fruit crops in the world and western of Iran; its whole genomic sequence was released in 2007 (20). Low temperature is an important environmental factor that negatively affects grapevine productivity and quality. To address this issue, the genetic mechanisms of cold accumulation in grapevine have been widely studied (47). Recently, our group has investigated the physiological and biochemical mechanisms of cold-tolerance in three grapevine cultivars (43). Also, we examined the effect of gradual and shock chilling stress on abscisic acid, soluble sugars and antioxidant enzymes changes in 'Sultana' grapevine and reported that under gradual chilling stress, grapevine plants show better cold acclimation by lower oxidative stress and higher accumulation of osmo-regulants in compared with shock chilling stressed vines (42). The purpose of this study is to determine the potential function of miR395, its target genes and its possible involvement in biological processes. The results would help illustrate the roles of miR395 during cold stress response in grapevine. Real-time RT-PCR was also used to investigate the expression pattern of miR395 in grapevine under gradual and shock chilling stress.

MATERIALS AND METHODS

Plant material and growth conditions

The cuttings of 'Sultana' grapevine (*V. vinifera* L.) were planted and treated according to Beheshty Rooy et al (2017) (42).

Controlled chilling tests

Potted vines were subjected to chilling stress in a programmable cooling chamber. Chilling was accomplished at two distinct forms including of gradual temperature decrease of 2 °C/h and shock temperature decrease of 5 °C/h from 24 °C to +4 °C and kept at +4 °C for 12 hours (42). The plants incubated at 24 °C were considered as control vines. After chilling exposure, the first three fully expanded leaves were taken for RNA extraction and immediately frozen in liquid N_2.

Identification of miR395 family members in *V. vinifera*

MiR395 family members of *V. vinifera* were taken from miRBase 21 (http://www.mirbase.org/), the main data repository for the storage of miRNA information, for the analysis. The reference *V. vinifera* genome assembly was taken from NCBI, accession: GCF_000003745.3. Identification of miR395 family member location on the grape genome and identification of location of all small non-coding RNAs on chromosome 1 was performed using NCBI Genome Browser. ClustalW was used to generate multiple alignments of miRNAs stem-loop sequences (13) and MEGA7 was used to represent multiple alignments.

Prediction of target genes and their biological importance

Targeted genes for vvi-miR395 family members were identified using the psRNATarget server

(http://plantgrn.noble.org/psRNATarget/) and by considering default parameters (15). To determine the potential function of miR395 and its target genes and their possible involvement in biological processes, the putative functions were assigned to miRNA target genes through BLASTx at NCBI. The assumed function for these genes was determined based on their interpretation in other plant species. Finally, analysis of the biological system was performed using Pathway Studio 11 software to identify the major networks in which selected miRNAs are involved.

RNA extraction and Quantitative Real-Time PCR analysis

The expression of miR395 was analyzed by quantitative real-time PCR. Total RNA was extracted using Trizol reagent (Invitrogen) according to manufacturer's instructions. The quantity and quality of extracted RNAs were evaluated by Nano-Drop, and the integrity was also assessed by electrophoresis on a 1.3 % agarose gel. Stem-loop qPCR technique was used to evaluate the expression of miR395. The sequence of mature miR395 has been obtained from online miRNAs database (mirbase 21). Stem–loop RT–PCR and miRNA gene-specific primers were designed according to Chen et al. 2005 (12). GAPDH (56) was used as reference genes (Table 1). miRNA stem–loop reverse transcription was performed using RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific) according to Varkonyi-Gasic et al., 2007 (53). qRT-PCR reactions were performed using HOT FIREPol® EvaGreen® qPCR Supermix (Solis BioDyne) on a Rotor-Gene Q (5-plex) according to the manufacturer's instructions (33). The $2^{-\Delta\Delta CT}$ method was used to analysis the data (45).

RESULTS AND DISCUSSION

Identification of miR395s and its target genes

Initially, the sequence of all identified miR395s in grape was examined and, it was found that all but one of them located on chromosome 1 (only miR395n located on chromosome 11). The distribution of these miRNAs on chromosome 1 was evaluated. Then, the locations of all short non-coding RNA coding genes on chromosome 1 were determined. The results showed that all of these miRNAs are located in a specific region of the chromosome by relatively short intervals, and this region has the highest density of small non-coding RNAs (Fig 1). Subsequently, alignment of the stem-loop sequences of these miRNAs revealed that the shoot-loop sequence of these miRNAs was very similar and the mature region of all miRNAs are completely identical. The length of stem-loop structure of all miRNAs on chromosome 1 was less than 100 nucleotides, but the length of miR395n located on chromosome 11 was 132 nucleotides. All mature sequence produced by miR395a to m was completely identical (Fig 2).

The target genes were identified for miR395s using psRNATarget and, five target genes were predicted. All of them are targeted by miR395 a to m, whereas of the five target genes only one is identified by miR395n as a target (ATP sulfurylase). The reason is that the miR395n mature sequence is different from other miRNAs in this family. Of these five genes, the target site in two genes was in the 3 UTR region, in two genes was in the CDS region and one gene was in the 5 UTR region (Fig 1). Three of these target genes including ATP sulfurylase, sat-1 and LAST3-like, are involved in the control of sulfur metabolism and transport (5, 35, 50, 51).

Table 1. Primers designed to investigate miR395 expression change using Real-time PCR.

Gene name		Primer sequence (5-3)
miR395a	RT primer	gtcgtatccagtgcagggtccgaggtattcgcactgg atacgacgagttc
	Forward primer	caatgctgaagtgtttgggg
	Reverse primer	gtgcagggtccgaggt
GAPDH	Forward primer	ccacagacttcatcggtgaca
	Reverse primer	ttctcgttgagggctattcca

Cellular response to sulfate deficiency

Among the differentially expressed miRNAs in response to cold in the present study, different members of miR395 including miR395a, miR395e, miR395c, miR395b, miR395d and miR395f are present in the sulfate deficiency cell response network that identified by

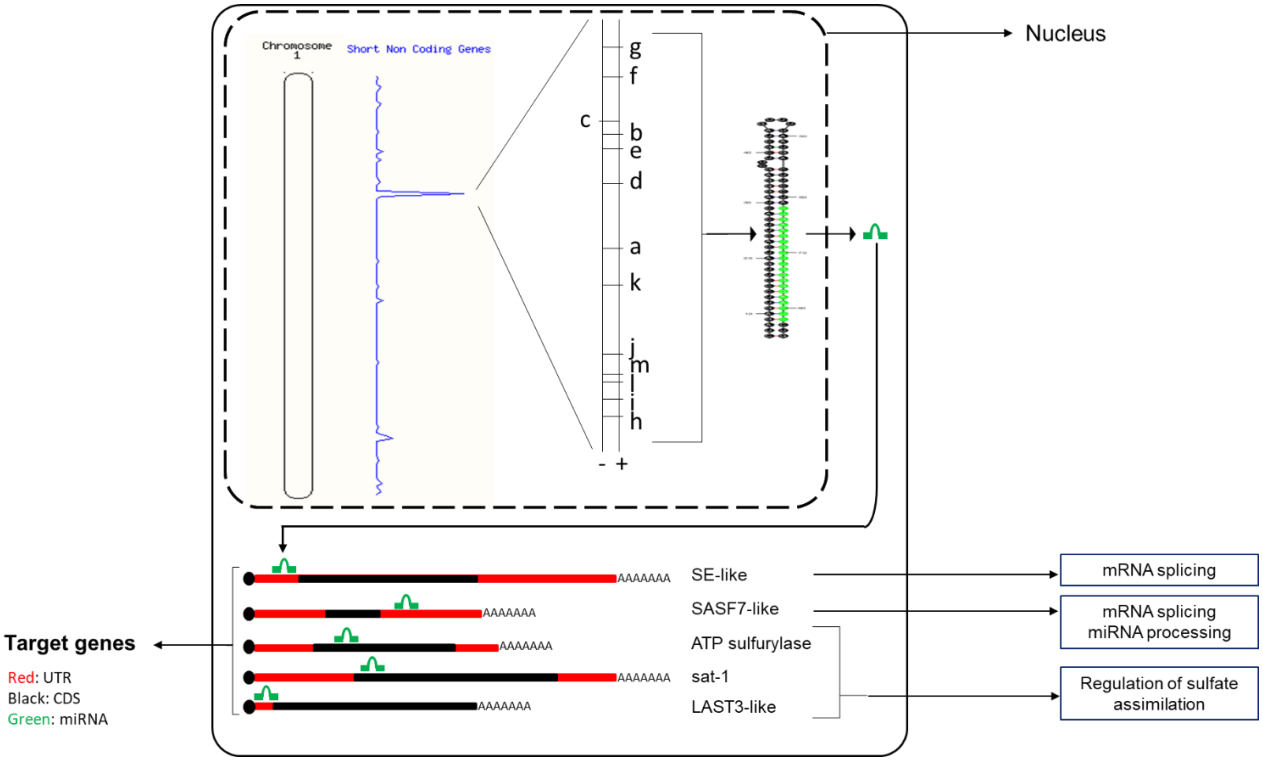

Figure 1: All small non-coding RNAs and miR395 family member location on chromosome 1. Five genes were predicted to be the targets of miR395 family members.

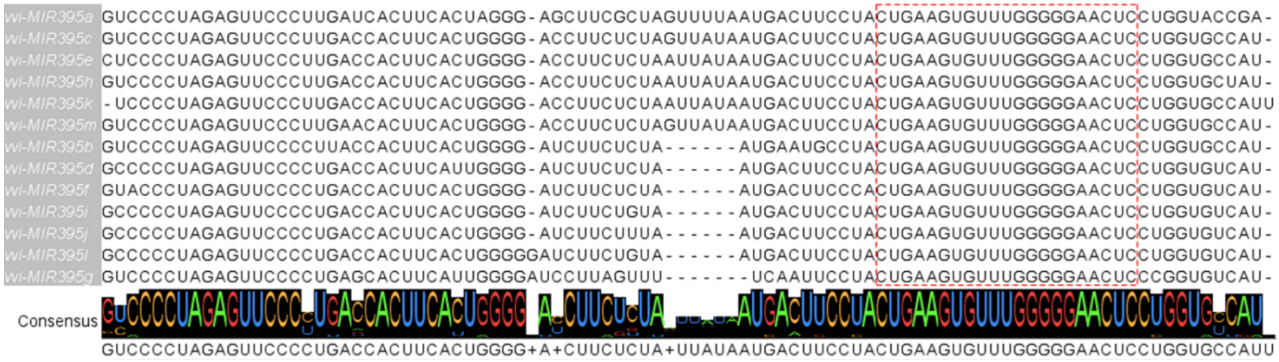

Figure 2: Multiple sequence alignment analysis of miR395 family member stem-loops. The red box represents the mature miRNA producing region.

enrichment analysis using Pathway Studio software (Fig 3). By analyzing the network of common targets, miRNAs, transcription factors, micro-molecules, and other contributing factors to sulfate deficiency are revealed. Sulfur is one of the essential inorganic minerals that is absorbed by plants via the roots from soil in the form of sulfate and reduced to sulfide. Sulfur is an essential element in the structure of cysteine, methionine, glutathione and Fe-S proteins and several other cofactors (38). During sulfate deficiency, uptake and metabolism of sulfur in plants are regulated at different levels and miRNAs are part of this regulatory network. miR395, which is specifically induced in response to sulfate deficiency, targets the low-affinity sulfate transporter (AST68/SULTR 2) and three members of the ATP sulfurylase family (APS1, APS2, APS4) (48). Recent evidence has suggested that an increase in miR395 expression leads to a structural decrease in APS1, APS2, and APS4 under normal conditions as well as in sulfate deficiency. APS1 is up-regulated by sulfate starvation at the transcriptional level but is down-regulated by miR395 (1).

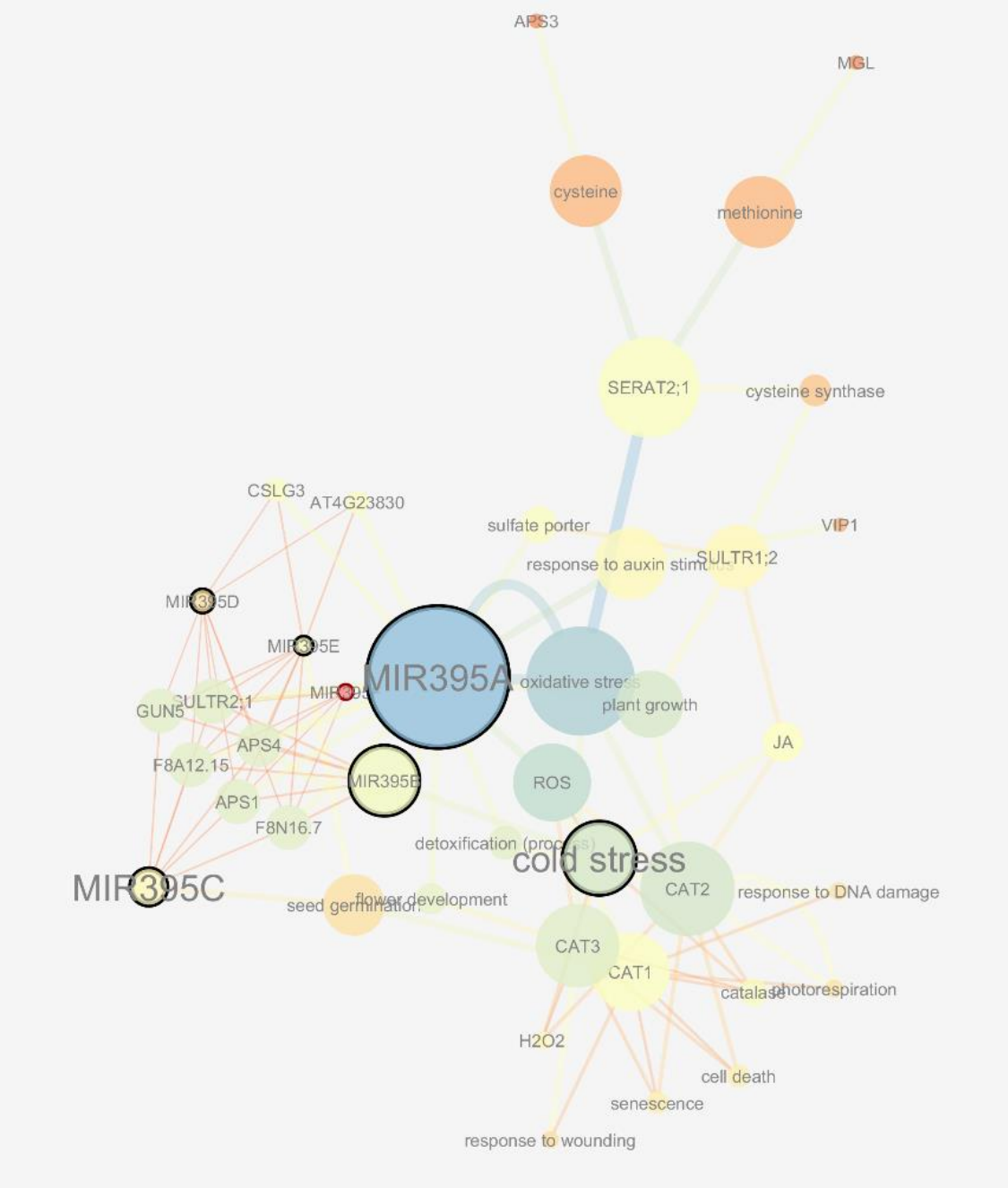

Figure 3: Displays the topological properties of the sulfate deficiency network. Node size (small to large) and color of nodes (yellow: low to blue: high) indicates the betweenness and closeness, respectively

Silencing of SULTR1;1 and SULTR2;1 by T-DNA insertion gene disruption completely disrupts sulfate uptake, which causes plant growth is strongly affected under sulfur deficiency. Liang et al., (2010) showed that under sulfate deficiency, increasing the expression of miR395 limits SULTR2;1 expression and, consequently, restricts sulfate transfer from mature leaves to young leaves (28).

miR395 is strongly induced by sulfate deficiency, and under these conditions, regulates sulfate transfer from old leaves to young leaves as well as from roots to shoots (18). Overexpression of miR395c or miR395e has various effects on Arabidopsis seed germination under different stress conditions (22). There are many published studies that have reported, miR395 has different expression pattern in response to abiotic stress such as cold stress, there is probably a relationship between the role of this miRNA and its target genes in regulating sulfate homeostasis and the response to cold stress. The plant's metabolic processes are affected by temperature, and

plant increases the concentration of sulfate, nitrogen, phosphate, and other essential elements in its cell to maintain its metabolism at low temperatures (44).

By investigation of the algorithm of common regulator, SLIM1, a transcription factor from the EIL family, was identified that induces miR395 induction and maintain sulfate homeostasis under Sulfur deficiency conditions. The expression of SULTR1,1 and SULTR1,2 is controlled by SLIM1. SLIM1 is a central transcription regulator of sulfur responses and metabolism, and that the induction of miR395 by sulfate starvation is dependent on SLIM1 (1). APS1 and APS2 are controlled by the LH5 transcription factor (18). Transcript level of HY5 transcription factor, which is a kind of BZIP transcription factors, was increased in response to cold treatment in wild-type tomatoes grown in high infrared light condition compared to those grown in low infrared light conditions. Growth under high infrared light conditions also increased the level of HY5 transcripts in phyB1B2 mutant leaves under cold stress, but this increase in the phyA and phyAB1B2 mutants was not observed. Exposure to cold, induces HY5 in wild-type and phyB1B2, but mutation in phyA inhibits cold-affected transcription in both high-infrared and low-infrared light conditions (55). On the other hand, studies have shown that HY5 binds the SULTR1,2 promoter and activates it; SULTR1,2 is responsible for sulfate uptake (23). These results indicate the effect of cold on upstream transcription factors of sulfate homeostasis regulator genes. As mentioned earlier, miRNAs and transcription factors are key regulators of gene networks and, when affected by various stimuli such as hormones, stress, and etc., regulate a network of downstream genes. Considering the regulation of miR395 by SLIM1, investigation of SLIM1 stimuli will help to understand the relationship between cold and miR395. On the other hand, investigating the association of HY5 and miR395 also leads to a better understanding of this regulatory mechanism.

Cellular response to phosphate deficiency

In the present study, among differentially expressed miRNAs under cold stress, miR398b, miR398c, miR395b, miR395c, miR395f, miR156g, miR156f, miR166, miR169 and miR399 involved in this network (Fig 4). Meng et al. (2010) have also demonstrated repression of miR169, miR395, and miR398 under phosphate deficiency conditions (32). Inorganic phosphate is one of the essential and often limiting nutrients for plant growth (52). Plants have evolved a diverse array of strategies to adapt phosphate deficiency, including mechanisms to regulate phosphate-deficient genes by small RNAs (14). PHO2 encodes a ubiquitin-conjugating (UBC) E2 enzyme that binds to phosphate transporters and inhibits them. PHO2, in combination with the NLA1 protein, polyubiquitinates the PHT1 phosphate vector, located in the plasma membrane, which is subsequently degraded by the 26 s proteasome (54).

Under phosphate-deficient conditions, the expression on APS4 and SULTR2,1 was increased; and suppression of miR395 under phosphate deficiency conditions may be partially influenced by this procedure. Overexpression of APS4 and SULTR2,1 may increase sulfate uptake and its involvement in sulfolipid biosynthesis to partially compensate phospholipids decreasing during phosphate deficiency (17).

miR395 expression regulate sulfate and phosphate hemostasis in response to cold stress

In the present study, the expression of miR395 was increased after 12h of cold stress, but due to the decrease in expression in the samples exposed to cold shock, it is likely that reducing this miRNA and increasing the expression of its target genes is the plant's initial response to phosphate deficiency, which in turn increase its sulfate uptake and replace it with phosphate-containing compounds as much as possible; as under stress conditions, plants should maintain essential elements such as phosphate to preserve the plant’s critical processes. Reich and Oleksin (2004) have suggested that low temperatures can reduce the rate of biochemical reactions catalyzed by N-rich enzymes and P-rich RNAs in plant tissues. Therefore, plants typically allocate more nutrients to these molecules to compensate for this reduced rate under low temperature conditions (39). As shown in Figure 4, a number of cold responsive-miRNAs work together in a phosphate deficiency response network to optimize plant performance under these adverse conditions.

Phosphate deficiency leads to a gradual increase in proline content in wild-type Arabidopsis as well as transcriptional activation of P5CS1 and proline dehydrogenase 2 genes. Induction of P5CS1 transcription and proline accumulation during phosphate deficiency is reduced by phr1 and phl1 mutations and also is defective in ABA mutants (3).

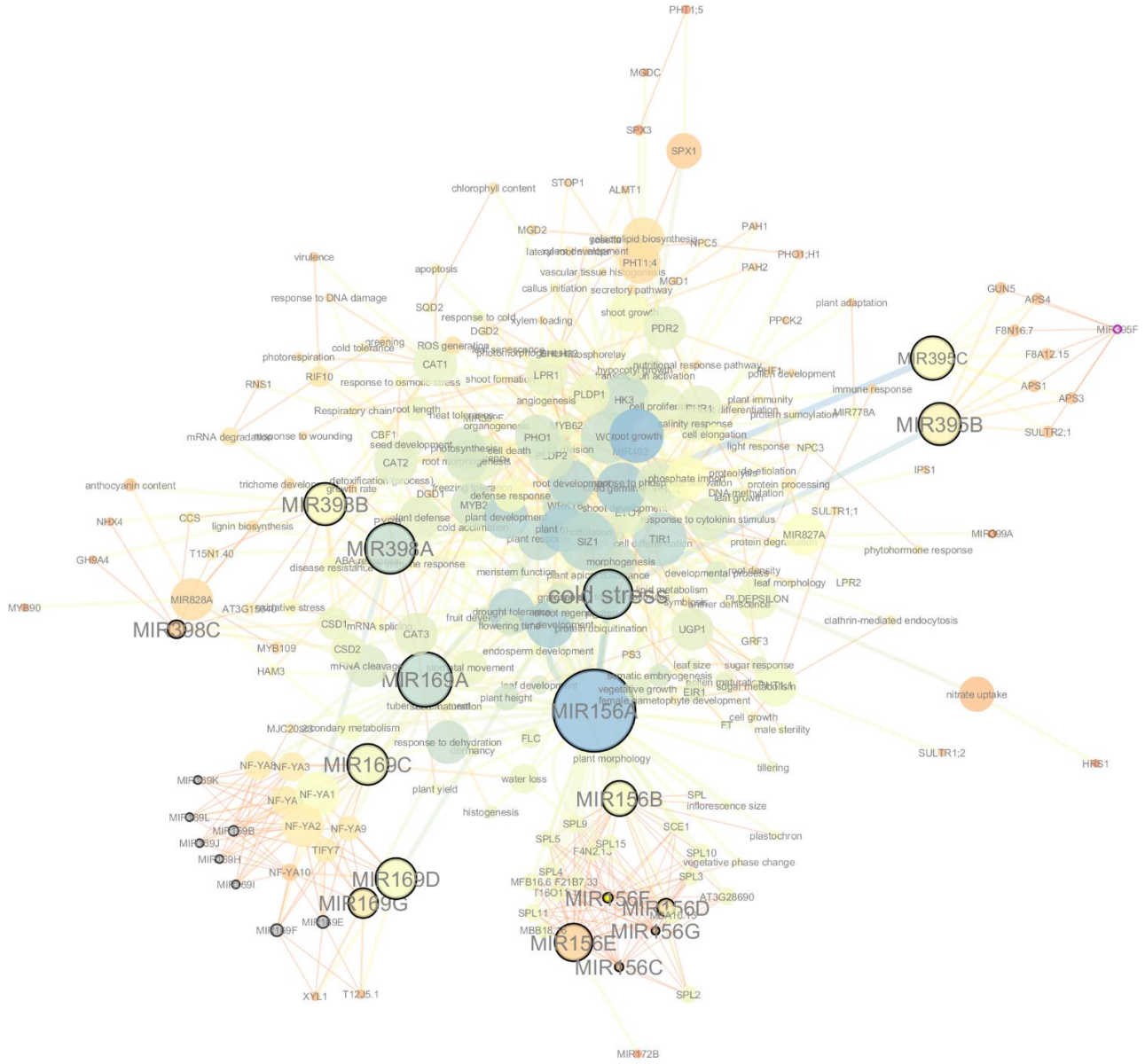

Figure 4. Displays the topological properties of the phosphate deficiency network. Node size (small to large) and color of nodes (yellow: low to blue: high) indicates the betweenness and closeness, respectively.

In the previous study (9), proline content was increased in samples exposed to cold stress, which may be partly affected by phosphate deficiency.

Effects of gradual and shock stress treatment on the expression of miR395

Environmental stresses such as cold, salinity, and drought have been shown to modulate the expression of miRNA in plants (26, 36, 59). In this study, the expression of miR395 was studied against cold stress. This miRNA showed contrary expression pattern under gradual and shock stress condition. Exposure to gradual chilling stress induced the up-regulation of miR395 in Grape shoots; however, shock chilling stress decreased the expression of this miRNA (Fig. 5). The High Throughput Sequencing analysis also showed that expression of this miRNA increased under gradual stress conditions (data not shown). The previous results showed that levels of miR395 were also increased during sulfate deprivation, and arsenate, cadmium and copper stress, all of which imposed oxidative stress (19, 30, 58). The result showed that MIR395 could be induced under the conditions that cause oxidative stress, and the induction of MIR395 might play important roles in oxidative stress response by regulating sulfur metabolism.

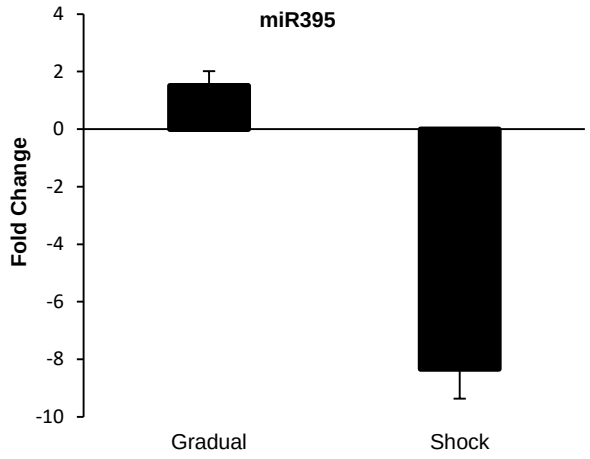

Figure 5: miR395 expression pattern under gradual and shock chilling stress in self-rooted grape cutting with 15-20 leaves.

CONCLUSION

In conclusion, this study found that miR395 was involved in response to cold stress in grape shoots through Cellular response to sulfate and phosphate deficiency. miR395 was induced to regulate sulfate assimilation and translocation, mediating the increase in the sulfur-containing antioxidant compounds during cold exposure. These results would shed new light on the regulatory role of miRNAs in the response to environmental stress like as cold stress in plants.

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بررسی نقش miR395 در پاسخ به تنش سرما و تعدیل کمبود سولفات و فسفات در انگور (*Vitis vinifera*)

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چکیده

درجه حرارت پایین یکی از مهم‌ترین تنش‌های غیر زیستی است که می‌تواند به طور معنی‌داری بر تولید انگور اثرگذار باشد. میکروRNA ها نقش مهمی در کنترل نمو گیاهان و پاسخ آنها به شرایط نامساعد محیطی دارند. اگرچه miRNAها و اهداف آنها در چندین گونه از انگور شناسایی شده‌اند، اما مشارکت آنها در طی تنش سرما تا حد زیادی ناشناخته است. یکی از این میکرو RNAها، miR395 است که یک میکروRNA حفاظت شده است و در گیاهان عالی، اسیمیلاسیون و توزیع سولفات را تنظیم می‌کند. در این مطالعه، نقش احتمالی miR395 در پاسخ به تنش سرما مورد بررسی قرار گرفت. شناسایی ژن‌های هدف، آنتولوژی ژن و آنالیز سیستم بیولوژیکی به منظور شناسایی شبکه‌های اصلی که این miRNA در آنها دخیل است، انجام شد. در نهایت، اثرات تنش سرما به صورت تدریجی و شوک بر بیان miR395 مورد بررسی قرار گرفت. در کل، پنج ژن هدف شناسایی شدند که همه آنها توسط میکروRNAهای ۳۹۵ a تا m هدف قرار گرفته‌اند؛ و تنها یک ژن، توسط miR395n به عنوان یک هدف شناسایی شده است. سه مورد از این ژن‌ها شامل ATP سولفوریلاز، sat-1 و LAST3 در کنترل متابولیسم سولفور و انتقال آن نقش دارند. آنالیز مسیر (Pathway analysis) نشان داد که miR395 در پاسخ به تنش سرما در اندام هوایی انگور از طریق پاسخ سلولی به کمبود سولفات و فسفات نقش دارد. بر اساس نتایج RT-PCR، ما الگوهای بیان متضاد miR395 را تحت تنش سرمایی تدریجی (افزایش بیان) و شوک (کاهش بیان) مشاهده کردیم. تغییر در فرآیند اسیمیلاسیون سولفات، تشکیل ترکیبات آنتی اکسیدانی حاوی سولفور را تحت تاثیر قرار می‌دهد. این نتایج دورنمایی از نقش‌های تنظیمی miR395 را در پاسخ به تنش درجه حرارت پایین در انگور ارائه می‌دهد.

کلمات کلیدی: تنش سرمایی، انگور، miR395، RT-PCR، تنش اکسیداتیو