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Article

# Set of miRNAs Involved in Sulfur Uptake and the Assimilation Pathway of Indian Mustard (*B. juncea*) in Response to Sulfur Treatments

Sadia Iqrar, Kudsiya Ashrafi, Shazia Khan, Monica Saifi, Nazima Nasrullah, and Malik Zainul Abdin*

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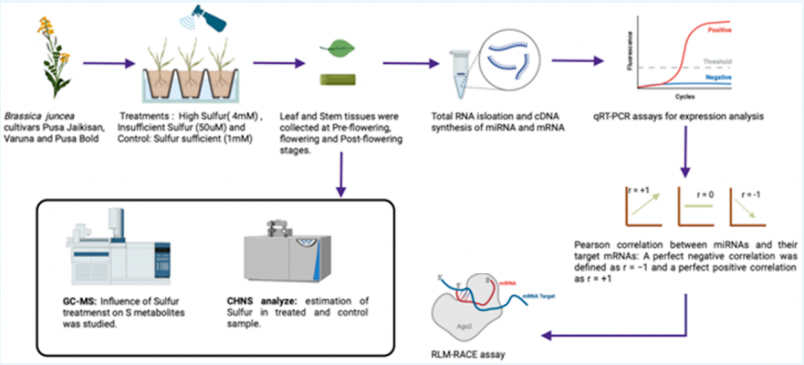


**ABSTRACT:** MicroRNAs (miRNAs) play an important role in the regulation of gene expression. They play a regulatory role in various nutrient assimilatory pathways of plants; however, their role in the regulation of sulfur uptake and assimilatory pathways in mustard cultivars under high/low sulfur conditions is not elucidated. Sulfur is essential for plant growth and development, and its deficiency can cause a decline in oil seed content and thus lower the economic yield in *Brassica juncea*. In this study, different miRNAs involved in the regulation of sulfur uptake and assimilation pathways in *B. juncea* were identified using a psRNA target analyzer and miRanda database tools. The predicted miRNAs that belong to 10 highly conserved families were validated using stem-loop RT-PCR. The *B. juncea* cultivars Pusa Jaikisan, Pusa Bold, and Varuna were kept in sulfur-excessive (high) and -deficient (insufficient) conditions, and expression studies of miRNAs and their target mRNAs were carried out using qRT-PCR. The correlation between the expression pattern of miRNAs and their target genes showed their potential role in sulfur uptake and assimilation. Analysis with 5′ RACE revealed the authentic target of miRNAs. The influence of S treatments on metabolites and sulfur content was also studied using GC-MS and a CHNS analyzer. Our study showed the potential role of miRNAs in the regulation of sulfur uptake and assimilation and put forward the implications of these molecules to enhance the sulfur content of *B. juncea*.

## INTRODUCTION

Sulfur (S) is an essential element needed for plant growth and development. It is an essential component for synthesizing S-amino acids, cysteine and methionine, which are the building blocks of various proteins.[1] Not only it is necessary for plants and microbes, but also S-containing amino acids (cysteine and methionine) are essential in animals and humans.[2] Further, some of the S-containing secondary metabolites, for example, glucosinolates, are a valuable source of anticancer phytochemicals and, therefore, act a valuable component of therapeutic molecules.[3]

Plants mainly uptake sulfur from the soil in the form of sulfate, and within the plants, sulfate either remains in the roots or is transported via the xylem to the leaves, the main sites of sulfate reduction and assimilation.[1,4] The sulfate distribution processes within the plant are controlled by sulfate transporters (SULTRs).[5,6] Uptake and distribution of sulfate within the plant are demand-driven processes, and not only sulfate itself but also cysteine and/or glutathione (GSH) are supposed to serve as signals in this regulation.[6,7] The response includes increased expression of several sulfate transporters and enzymes involved in sulfate assimilation such as ATP sulfurylase (ATPS) and adenosine 5-phosphosulfate (APS) and reductase (APR).[5,8]

The demand for S changes as per the different developmental stages and may vary from species to species.[5] Mustard is an

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Table 1. Potential miRNA−Target Pair Derived from the miRanda Database Tool above the Threshold Value

| miRNAs | target binding position | score | MFE (kcal/mol) | % complementarity | target genes | inhibition |
|---|---|---|---|---|---|---|
| 395a | 809−831 | 153 | −20.47 | 75 | APR | translational |
| | 328−348 | 159 | −20.15 | 88.89 | ATPS | cleavage |
| | 1182−1202 | 156 | −25.20 | 86.67 | CbL | cleavage |
| | 11−64 | 191 | −21.25 | 94.44 | SULTR2;1 | cleavage |
| | 85−105 | 159 | −33.40 | 80 | SULTR2;2 | translational |
| | 124−144 | 84 | −20.33 | 66.67 | SULTR3;1 | cleavage |
| | 86−107 | 138 | −21.78 | 73.68 | SULTR3;3 | translational |
| 865-5p | 1830−1850 | 175 | −21.45 | 83.33 | SULTR3;1 | cleavage |
| | 1082−1102 | 178 | −20.82 | 82.35 | SULTR4;1 | cleavage |
| | 1043−1063 | 182 | −20.32 | 88.24 | SULTR4;2 | cleavage |
| 843 | 774−795 | 82 | −21.07 | 75 | CbL | translational |
| | 1387−1406 | 129 | −21.57 | 88.89 | SULTR1;2 | translational |
| | 1671−1691 | 118 | −20.61 | 69 | SIR | translational |
| | 136−156 | 123 | −29.12 | 93.75 | SULTR4;1 | translational |
| 863-3p | 1240−1262 | 141 | −20.36 | 83.33 | SULTR1;2 | translational |
| | 941−962 | 178 | −24.70 | 89.47 | SULTR3;2 | cleavage |
| 2111a-5p | 297−318 | 108 | −20.58 | 87.50 | SULTR1;2 | translational |
| | 1775−1793 | 123 | −20.73 | 87.50 | SULTR3;2 | translational |
| 5661 | 859−878 | 125 | −22.24 | 72.22 | ATPS | translational |
| | 671−693 | 114 | −21.50 | 70 | SULTR3;1 | translational |
| 396a | 153−174 | 126 | −21.54 | 78.57 | ATPS | translational |
| | 1234−1255 | 102 | −20.45 | 68.42 | SIR | translational |
| | 1502−1523 | 98 | −20.84 | 80 | SULTR1;1 | translational |
| | 1884−1905 | 131 | −20.77 | 75 | SULTR3;1 | translational |
| | 360−384 | 122 | −25.27 | 68.18 | SULTR3;2 | translational |
| | 1903−1923 | 141 | −22.79 | 66.67 | SULTR3;5 | translational |
| 5650 | 1200−1220 | 102 | −22.79 | 78.60 | SULTR3;1 | translational |
| 838 | 1653−1673 | 143 | −24.58 | 72.22 | SULTR1;1 | translational |
| | 1752−1772 | 174 | −20.29 | 88.24 | SULTR2;1 | cleavage |
| | 845−865 | 163 | −22.76 | 88.89 | SULTR2;2 | translational |
| 159a | 60−81 | 154 | −21.83 | 73.68 | APR | translational |
| | 1197−1217 | 122 | −21.75 | 73.33 | ATPS | translational |
| | 293−313 | 134 | −20.74 | 86.67 | SULTR3;2 | translational |

important oilseed crop and an essential source of vitamins, calcium, fat, and proteins. The demand for sulfur is higher in *B. juncea* (40 kg/hectare) compared to other crops.[9] The recent decades witnessed the decline in S content of various crops including *B. juncea*, resulting in the decrease of crop quality and yield. Hence, to overcome this deficiency, S-fertilizers are used; however, they are not always a remedy.[10]

In addition to this, the expression profile of various associated genes was modulated at the post-transcriptional level by small noncoding RNAs, known as microRNAs (miRNAs).[11] These miRNAs were processed from precursor molecules (pre-miRNA) with a hairpin structure.[12] The mature miRNAs are 21 nucleotides short and are incorporated into a ribonucleoprotein complex called an RNA-induced silencing complex (RISC). The miRNA guides the RISC to mRNAs containing a complementary sequence (target site) to the miRNA.[13] Most miRNA-targeted plant mRNAs are cleaved by RISC, but in the case of animals, it has also been demonstrated that RISC suppresses translation without the cleavage of target mRNA.[14] Many plant miRNAs regulate the expression of transcription factors, but a subset of miRNA-targeted genes are involved in metabolism.[15] However, the characterization of miRNAs involved in the regulation of S uptake and assimilatory pathways of mustard remains elusive.

This study was performed to identify the miRNAs targeting the genes and transporters of S uptake and the assimilation pathway under high and insufficient S conditions. The three cultivars Pusa Jaikisan, Varuna, and Pusa Bold were selected on the basis of their S-utilization efficiency. The ability of a plant to maintain growth on S-limited conditions is termed as sulfur efficiency (SE). *B. juncea* cultivar Pusa Bold is a highly S-efficient genotype; Varuna is moderate; and Pusa Jai Kisan is S-inefficient.[8] Pusa Bold has both high-affinity and low-affinity sulfate transporters, and Pusa Jai Kisan has only low-affinity sulfate transporters.[8] The predicted miRNAs and targets were validated using expression studies, and the relationship of their differential expression patterns was correlated with the target mRNAs. The targets cleaved by miR395a, miR865−5p, miR838, and miR863−3p were validated through 5′ RACE in cultivars of *B. juncea* and show that they are the authentic targets of their respective targeting miRNAs. The study identified the miRNAs involved in sulfur uptake and the assimilation pathway of *B. juncea*.

## RESULTS

**_In Silico_ Characterization and Validation of miRNAs Involved in S Uptake and the Assimilation Pathway.** *In silico* analysis, using the miRanda database tool, provides a set of miRNA target genes of sulfur uptake and the assimilation pathway of mustard (*B. juncea*). The top-scoring miRNA−target pairs were selected based on maximum complementarity and



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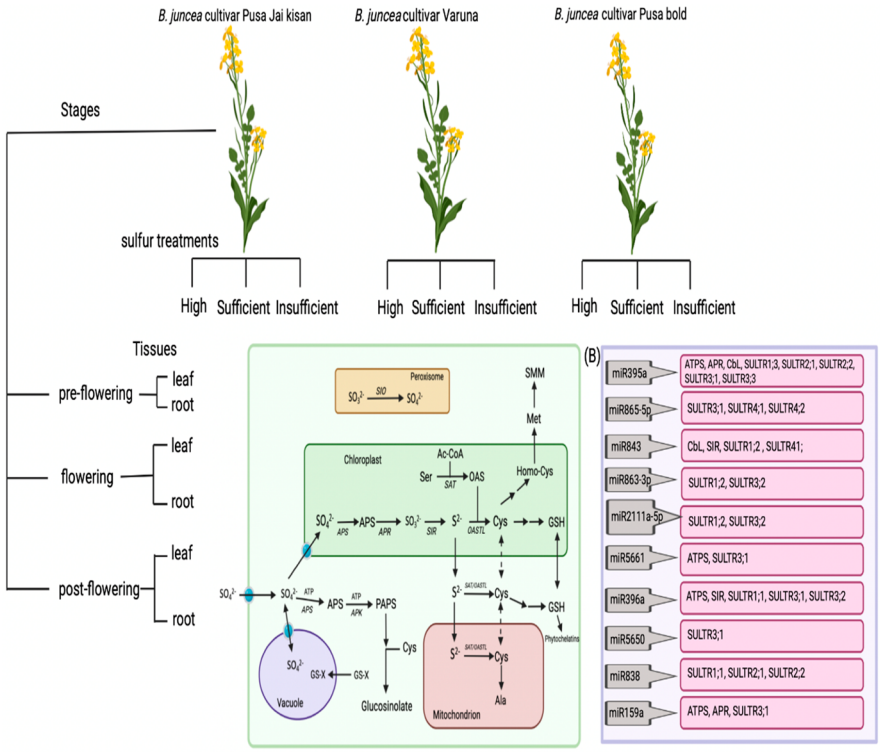


**Figure 1.** MiRNAs, targeting genes, and transporter of S uptake and the assimilation pathway under high, sufficient, and insufficient sulfur conditions in different stages and tissues of three cultivars of *B. juncea* (ATPS: ATP sulfurylase, APR: adenosine 5′-phosphosulfate reductase, CbL: cystathionine *β*-lyase, CgS: cystathionine *γ*-synthase, SIR: sulfite reductase, SULTR: sulfur transporters, Cys: cysteine, SAT: serine acetyltransferase, and OASTL: *O*-acetyl-serine lyase).

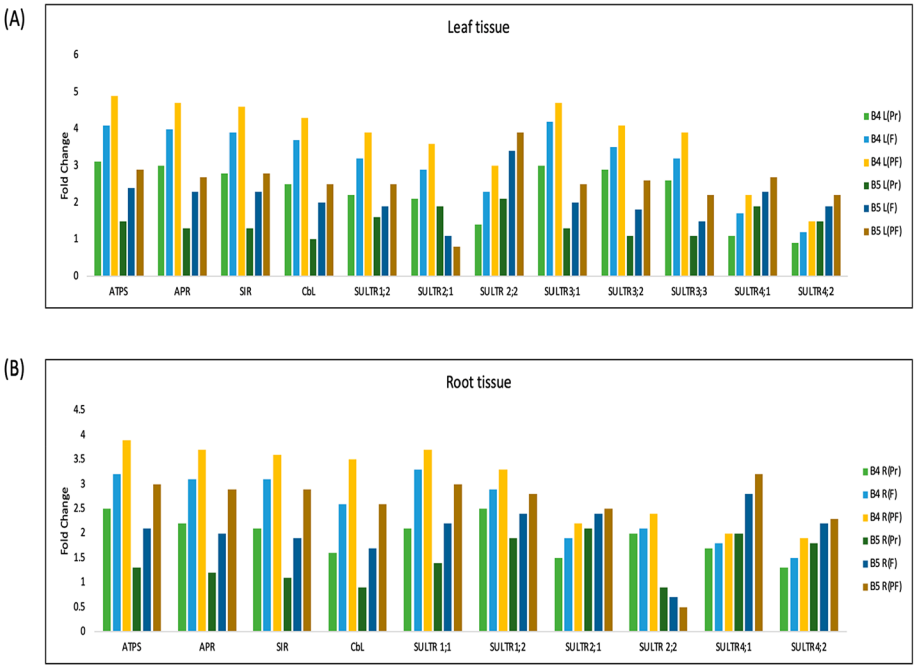


**Figure 2.** Fold change in expression of genes and sulfur transporters (SULTRs) quantified by real-time PCR in *B. juncea* cultivars Pusa Bold (B) under high (B4) and insufficient (B5) sulfur treatments in (A) leaf and (B) root tissues at preflowering (Pr), flowering (F), and postflowering (F).

optimal minimum free energy (MFE), as summarized in Table 1.

As a total, 14 targets of miRNAs involved in sulfur uptake and the assimilation pathway were identified. The top selected genes involved in sulfur assimilation pathways include ATPS, APR, SIR, and CbL. Similarly, transporters involved in sulfur uptake were SULTR1;1, SULTR1;2, SULTR2;1, SULTR2;2, SULTR3;1, SULTR3;2, SULTR3;3, SULTR4;1, and SULTR4;2. SULTR3;1 is targeted by the maximum number of miRNAs, viz., miR395a, miR865-5p, miR5661, miR396a, and


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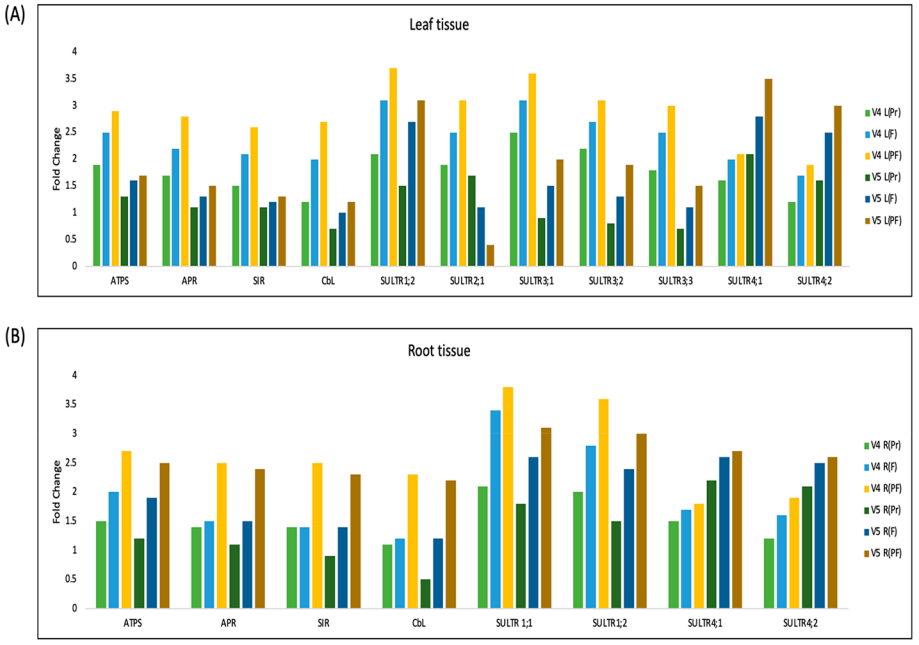


**Figure 3.** Fold change in expression of genes and sulfur transporters (SULTRs) quantified by real-time PCR in *B. juncea* cultivars Varuna (V) under high (V4) and insufficient (V5) sulfur treatments in (A) leaf and (B) root tissues at preflowering (Pr), flowering (F), and postflowering (F).

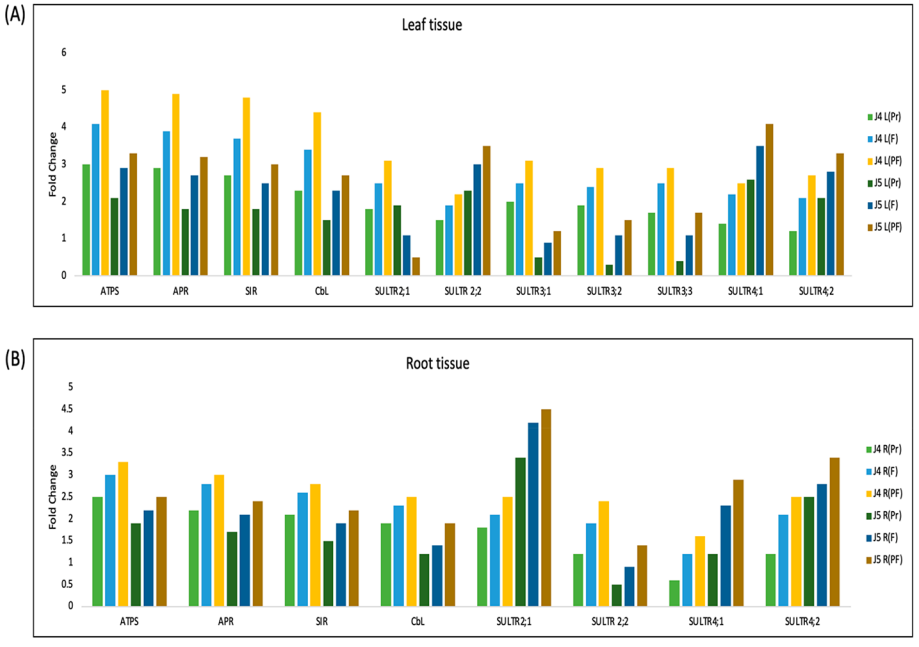


**Figure 4.** Fold change in expression of genes and sulfur transporters (SULTRs) quantified by real-time PCR in *B. juncea* cultivars Pusa Jai Kisan (J) under high (J4) and insufficient (J5) sulfur treatments in (A) leaf and (B) root tissues at preflowering (Pr), flowering (F), and postflowering (F).

miR5650. Among S-assimilation genes, ATPS was targeted by the maximum number of miRNAs, viz., miR395a, miR5661, miR396a, and miR159. Figure 1 shows the miRNAs, targeting genes, and transporter of S uptake and the assimilation pathway under high, sufficient, and insufficient sulfur conditions in different stages and tissues of three cultivars of *B. juncea*.

**Differential Expression and Correlation of miRNAs with Their Target mRNAs.** RT-PCR assays were performed to validate mRNA expressions targeted by miRNAs in different tissues at preflowering, flowering, and postflowering stages of three selected cultivars of *B. juncea*, namely, Pusa Jai Kisan, Pusa Bold, and Varuna, grown under sulfur treatments (high and insufficient). The samples were named as T1 (S-high) and T2 (S-insufficient). The amplified PCR product was resolved on 2% agarose gel, and the amplicons of mRNAs were detected. Real-time PCR was performed for analysis of expression of the mature miRNAs and target mRNAs. Fold change in expression of mRNAs (target genes and sulfur transporters) of leaf and root tissues is shown in Figures 2, 3, and 4.

Similarly, real-time analysis of miRNA levels quantified by real-time PCR of leaf and root tissues is shown in Figures 5, 6, and 7. All the selected ten miRNAs and their target mRNAs



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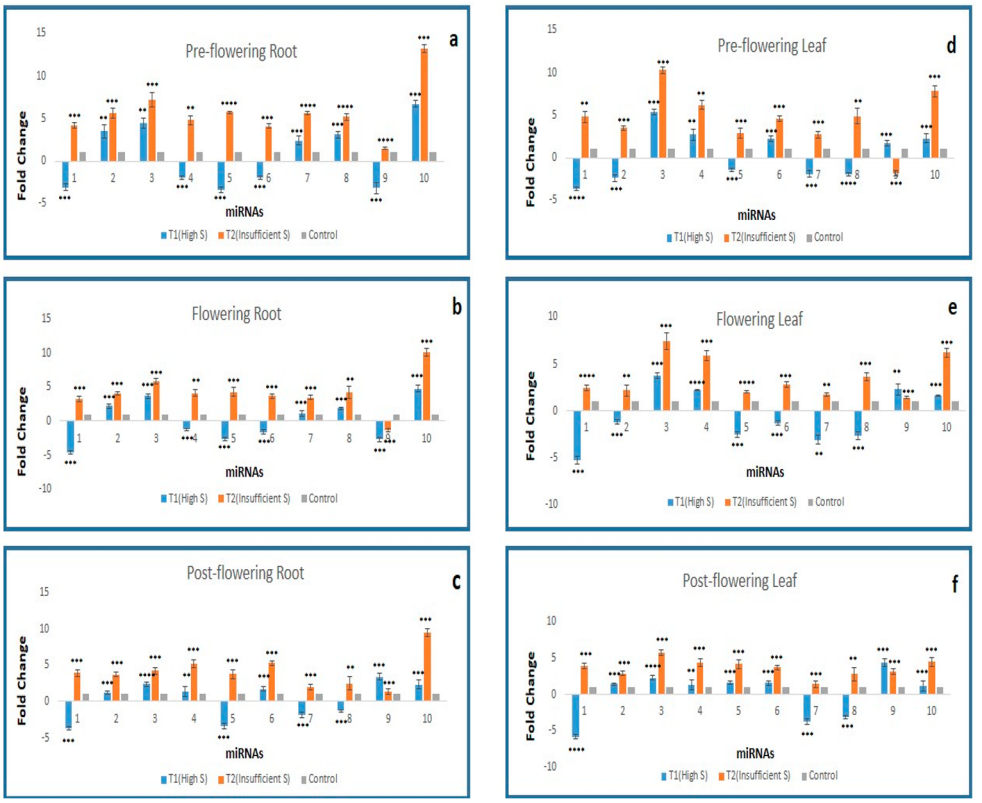


**Figure 5.** Real-time analysis of selected miRNA levels quantified by real-time PCR at preflowering, flowering, and postflowering stages in root (a,b,c) and leaf (d,e,f) tissues of the *B. juncea* cultivar Pusa Jai kisan. The expression levels of miRNAs were normalized to the expression level of U6 snRNA. Relative expression was calculated using the 2^ equation, where Ct = (Ct − Ctu6). 1, miR395a; 2, miR865-5p; 3, miR843; 4, miR863; 5, miR2111a-5p; 6, miR5661; 7, miR396a; 8, miR5650; 9, miR838; and 10, miR159a. ANOVA followed by Dunnett's test was applied to all miRNAs compared to U6. The error bar shows the standard error. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$.

showed a differential expression profile in different tissues of three stages under S treatments in *B. juncea* cultivars.

The correlation between miRNAs and their target mRNAs in all three cultivars was scrutinized using the Pearson correlation test in MetaboAnalyst software (http://www.MetaboAnalyst.ca/). This revealed that in *B. juncea* cultivar Pusa Jai Kisan miR395a was positively correlated with target SULTR 2;1 (Pearson coefficient 0.33). Similarly, miR865-5p and miR838 were positively correlated with their target SULTR4;1, SULTR 4;2 (Pearson coefficient 0.35 and 0.43), and SULTR 2;2 (Pearson coefficient 0.39), respectively. However, negative correlation was noticed in the expression pattern of miR395a with its target SULTR 2;2. Other miRNAs were also found to be negatively correlated with their targets, as shown in Table 3.

In *B. juncea* cultivar Varuna, miR395a was positively correlated with target SULTR 2;1 (Pearson coefficient 0.31), and miR865-5p was found to positively correlate with targets SULTR4;1 and SULTR4;2 (Pearson coefficient 0.42 and 0.51) and negatively correlate with target SULTR3;1 (Pearson coefficient −0.79). The miR843 had four targets CbL, SIR, SULTR1;2, and SULTR4;1. It was positively correlated with SULTR4;1 (Pearson coefficient 0.52) but not correlated with CbL, SIR, and SULTR1;2 (Pearson coefficients −0.19, −0.23, and −0.01). The miR396 was positively correlated with target SULTR1;1 (Pearson coefficient 0.62) (Table 3).

For the expression pattern of miRNAs in *B. juncea* cultivar, Pusa Jai Kisan, in leaf tissue (Figure 5), miR395a, miR865-5p, miR2111a-5p, miR396a, and miR5650 had shown a significantly decreased expression in S-high conditions compared to S-insufficient conditions. Only miR838 showed low expression in S-insufficient conditions at the preflowering stage. The expression of miR5661 was decreased in S-high conditions, and the expression of miR838 increased in S-insufficient conditions at the flowering stage compared to the preflowering stage. However, the expression of miR865-5p, miR2111a-5p, and miR5661 increased at the postflowering stage in S-high conditions. The expression of miR395a, miR396a, and miR5650 showed a decrease in the S-high condition expression profile, compared to S-insufficient conditions at all three stages. Expression of miR843, miR863, and miR159a remains higher in S-high as well as S-insufficient conditions at all three stages, although their expression was significantly higher in the S-insufficient conditions when compared to the S-high conditions.

The expression patterns of miRNAs in *B. juncea* cultivar Pusa Jai Kisan in the root tissue (Figure 5) showed a decrease in the expression of miR863, miR2111a-5p, miR5661, and miR838 in S-high conditions compared to S-insufficient conditions at preflowering and flowering stages. The expression of miR863, miR5661, and miR838 increased, but the expression level of miR396a and miR5650 declined at the postflowering stage in S-high conditions. The expression studies of miR395a and miR2111a-5p showed a decrease in the expression levels from the preflowering to postflowering stage in S-high conditions. However, the expression of miR865-5p, miR843, and miR159a remains higher in S-high conditions, but their expression levels were significantly higher in S-insufficient conditions in all three stages.



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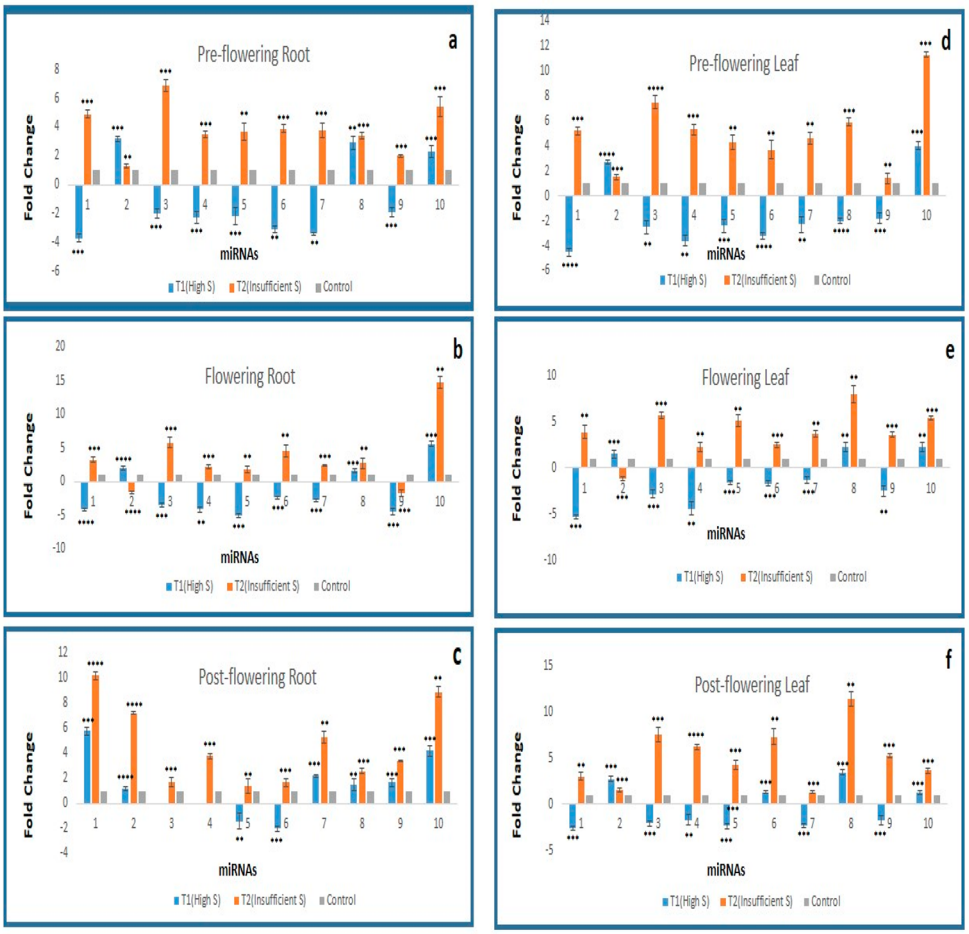


**Figure 6.** Real-time analysis of selected miRNA levels quantified by real-time PCR at preflowering, flowering, and postflowering stages in root (a,b,c) and leaf (d,e,f) tissues of the *B. juncea* cultivar Pusa Bold. The expression levels of miRNAs were normalized to the expression level of U6 snRNA. Relative expression was calculated using the 2^ equation, where Ct = (Ct − Ctu6). 1, miR395a; 2, miR865-5p; 3, miR843; 4, miR863; 5, miR2111a-5p; 6, miR5661; 7, miR396a; 8, miR5650; 9, miR838; and 10, miR159a. ANOVA followed by Dunnett's test was applied to all miRNAs compared to U6. The error bar shows the standard error. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$.

The results of miRNAs expression studies in *B. juncea* cultivar Pusa Bold in leaf tissue(Figure 6) showed that the expression of miR395a, miR843, miR863, miR2111a-5p, miR5661, miR396a, miR5650, and miR838 decreased in S-high conditions, compared to S-insufficient conditions at the preflowering stage. Expression of miR865-5p decreased in S-insufficient conditions at the flowering stage and increased at the postflowering stage. Furthermore, expression of miR838 increased in S-high conditions at the flowering stage and decreased at the postflowering stage, whereas the expression pattern of miRNAs in *B. juncea* cultivar Pusa Bold in root tissue (Figure 6) showed a decreased expression of miR395a, miR843, miR863, miR2111a-5p, miR5661, and mir396a in S-high conditions, compared to S-insufficient conditions at all three stages. The expression of miR5650 increased in both S-high and S-insufficient conditions at all three stages.

The analysis of miRNA expression patterns in *B. juncea* cultivar Varuna in leaf tissue (Figure 7) showed decreased expressions of miR865-5p, miR843, miR2111a-5p, miR5661, and miR396a in S-high conditions, compared to S-insufficient conditions at the preflowering stage. Even so, increased expressions of miR395a, miRmiR843, miR5650, miR838, and miR159a were noticed in both S-high and S-insufficient conditions at the preflowering stage. Expression of miR395a was decreased in S-high conditions at flowering and postflowering stages. High expression of miR843 and miR2111a-5p was noticed at the postflowering stage compared to the preflowering or flowering stages in S-high conditions. The expression of miR5650, miR838, and miR159a remains higher in all three stages, but their expression was significantly higher in S-insufficient conditions compared to S-high conditions.

The expression pattern of miRNAs in the root tissues of *B. juncea* cultivar Varuna (Figure 7) showed low expression of miR395a, mir863, miR2111a-5p, miR5661, and miR838 in S-high conditions compared to S-insufficient conditions at preflowering and flowering stages. Expression of miR863 and miR5661 increased at the postflowering stage compared to preflowering or flowering stages in S-high conditions, whereas the expression of miR396a decreased at the postflowering stage in S-high conditions. Interestingly, the expression levels of all selected miRNAs in the floral tissues were higher in S-insufficient conditions except miR838 and miR843.

The fold change in expression levels of miRNAs was lower in the postflowering stage compared to the preflowering or flowering stages in S-high as well as S-insufficient conditions in both root and leaf tissues. On the contrary, all the target mRNAs involved in the sulfur assimilation pathway (ATPS, APR, SIR, and CbL) were found to show higher expression


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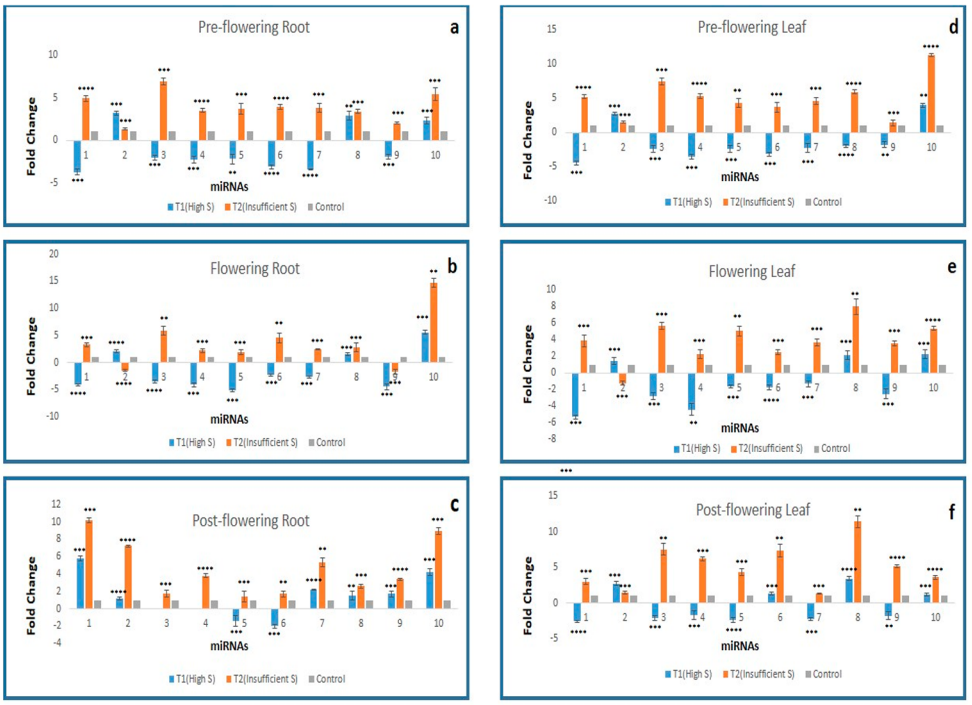


**Figure 7.** Real-time analysis of selected miRNA levels quantified by real-time PCR at preflowering, flowering, and postflowering stages in root (a,b,c) and leaf (d,e,f) tissues of the *B. juncea* cultivar Varuna. The expression levels of miRNAs were normalized to the expression level of U6 snRNA. Relative expression was calculated using the $2^{\wedge}$ equation, where Ct = (Ct − Ctu6). 1, miR395a; 2, miR865-5p; 3, miR843; 4, miR863; 5, miR2111a-5p; 6, miR5661; 7, miR396a; 8, miR5650; 9, miR838; and 10, miR159a. ANOVA followed by Dunnett's test was applied to all miRNAs compared to U6. The error bar shows the standard error. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, $****P < 0.0001$.

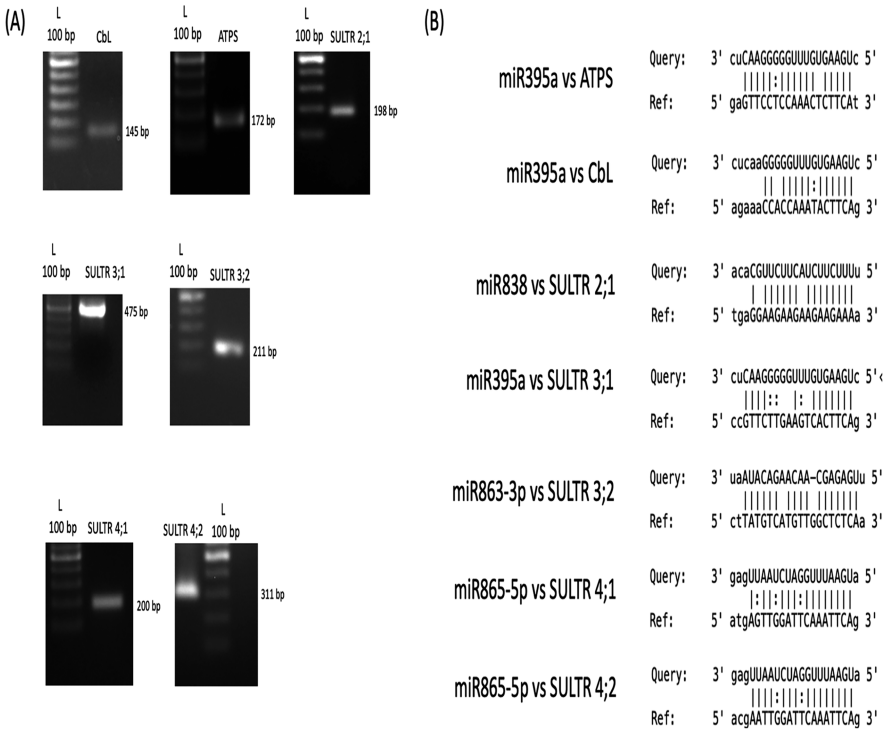


**Figure 8.** Validation of miRNA targets. 5′ RACE analysis was carried out for a predicted target of miRNAs. (A) Amplified cleaved product of targets (CbL: 145bp, ATPS: 172bp, SULTR 2;1:198bp, SULTR 3;1:351bp, SULTR 3;2:198bp, SULTR 4;1:200bp, and SULTR 4;2:311bp) (L: Ladder 100bp) and (B) predicted miRNAs and their target using a miRanda database tool (target cleaved by miRNAs).

levels at high S conditions compared to S-insufficient conditions in leaf and root tissues. Expression levels of sulfate transporters except for SULTR4;1 and SULTR4;2 were higher in high S conditions than S-insufficient conditions. The expression levels of all selected miRNAs were, however, found to be highest in cultivar Pusa Jai Kisan, followed by Varuna and Pusa Bold.



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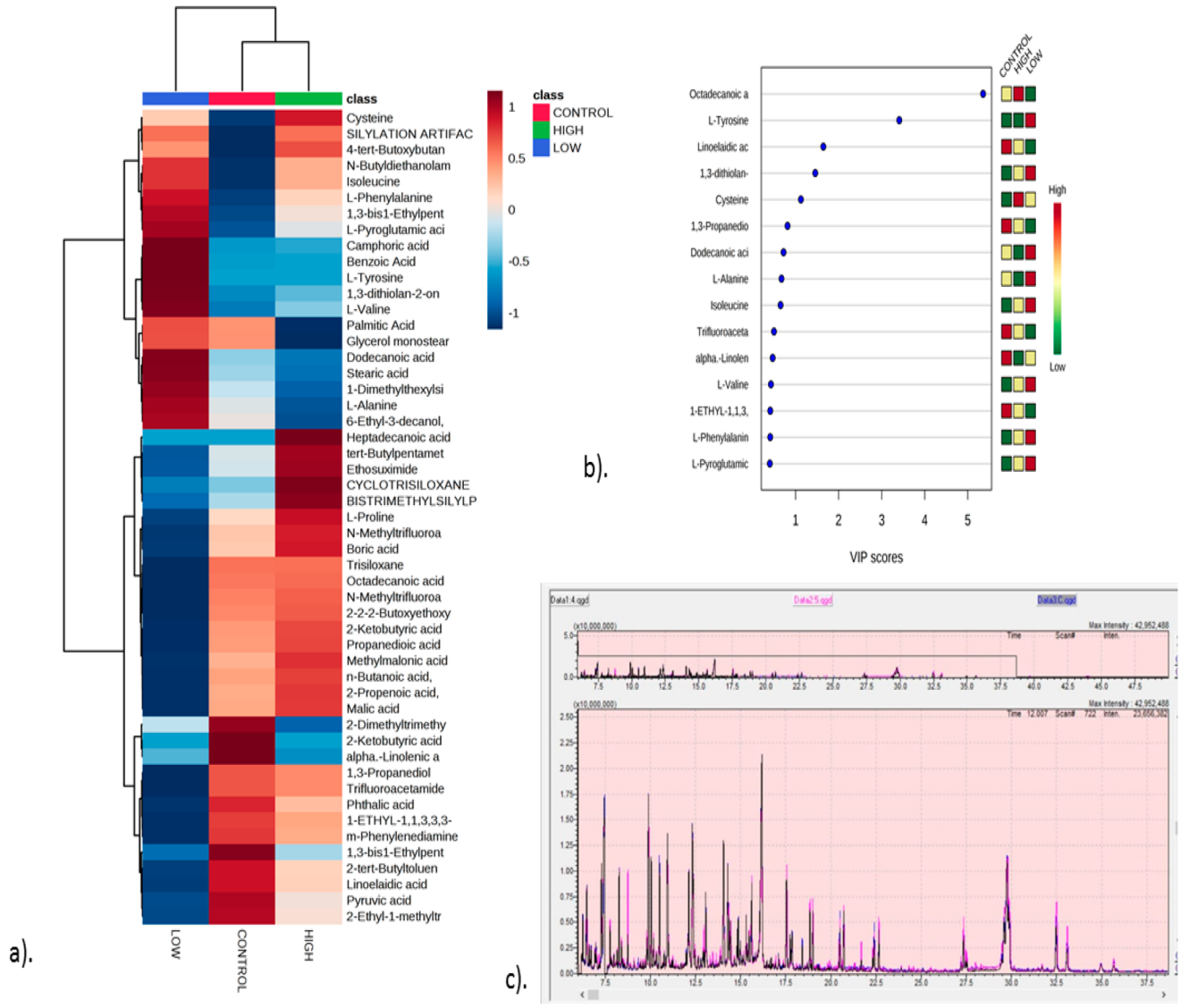


**Figure 9.** Hierarchically clustered (a) heatmap metabolite content of selected metabolites. Color scale represents different concentrations of the metabolites with dark brown being the highest and dark blue the lowest (b). Important features identified by PLS-DA. The colored boxes on the right indicate the relative concentrations of the amino acids (c). Chromatogram showing metabolites in *B. juncea* cultivar Pusa Bold.

Additionally, based on the correlation data between miRNAs and their targets, two miRNAs (miR395a and miR865-5p) were selected for further studies as they were found to target multiple genes including the key gene (ATPS) and vacuolar transporters involved in sulfur uptake and the assimilation pathway in *B. juncea*.

**Target mRNA Validation with 5′ RLM-RACE.** To validate the cleaved target, predicted using the psRNA Target server, the 5′ RACE assay was carried out to show that genes ATPS and CbL and sulfur transporters SULTR2;1, SULTR3;1, SULTR3;2, SULTR4;1, and SULTR4;2 have a site of cleavage guided by their target miRNAs. 5′ RACE cleaved products are shown in gel pictures (Figure 8). The 5′ RACE gene-specific outer and inner primers are listed in Table S3.

**Leaf Metabolite Screening by Gas Chromatography–Mass Spectrometry.** *B. juncea* cultivars Pusa Jai Kisan, Pusa Bold, and Varuna were grown in a greenhouse under two S treatments, and leaves were harvested at the postflowering stage for metabolite analysis. Chromatographic peaks obtained from GC-MS studies of collected leaves extracted in methanol and then dissolved in *N*-(*tert*-butyldimethylsilyl)-*N*-methyltrifluoroacetamide were identified using the NIST14s and Wiley8 databases. All metabolite data were normalized using the log transformation method. Partial least-squares discriminant analysis (PLS-DA) was used for supervised analysis, and a heatmap was generated using MetaboAnalyst (http://www.MetaboAnalyst.ca/). The metabolites from all treated samples were identified with >350 resolved peaks shown as an overlay in Figures 9,10, and 11.

The most relatively identified compounds include free amino acids, fatty acids, alcohols, esters, and other derivatized compounds. GC-MS showed changes in free amino acids and metabolite concentrations in cultivar Pusa Bold, and free amino acids, including cysteine and proline concentrations, were higher in high-S treatment compared to S-insufficient treatment. However, alanine, valine, and isoleucine concentration was higher in S-insufficient treatment compared to other treatments. In cultivar, Varuna, free amino acids cysteine, valine, serine,


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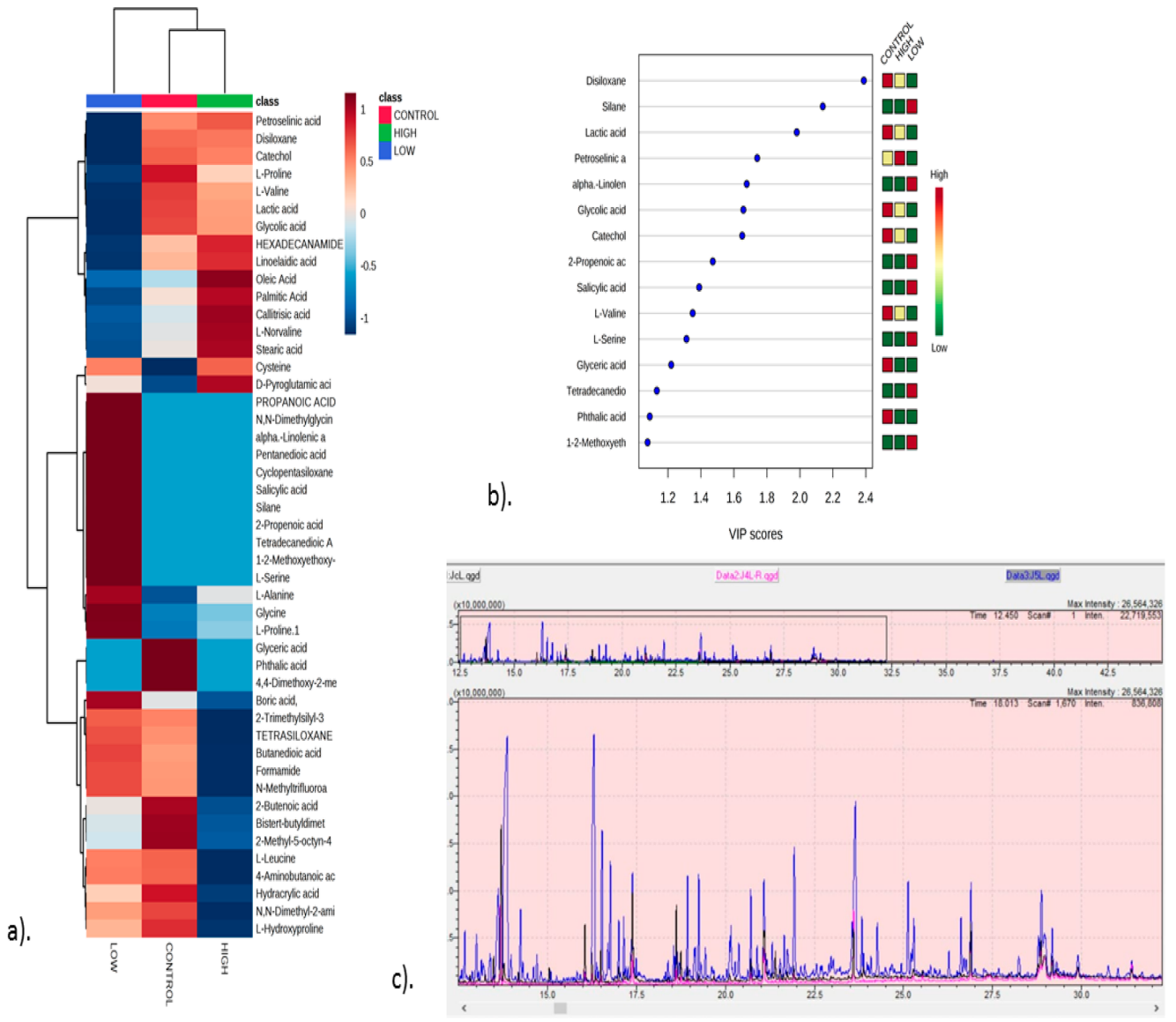


**Figure 10.** Hierarchically clustered (a) heatmap metabolite content of selected metabolites. Color scale represents different concentrations of the metabolites with dark brown being the highest and dark blue the lowest (b). Important features identified by PLS-DA. The colored boxes on the right indicate the relative concentrations of the amino acids (c). Chromatogram showing metabolites in *B. juncea* cultivar Pusa Jai kisan.

isoleucine, and alanine concentrations were higher in S-insufficient treatment than S-high treatment. In cultivar, Pusa Jai Kisan, the cysteine level was higher in S-high treatment, whereas alanine and serine levels were higher in S-insufficient treatment compared to other tissues.

**Sulfur Estimation by the CHNS Analyzer.** In *B. juncea* cultivars Pusa Bold, Varuna, and Pusa Jai Kisan, S content was measured in tissue samples collected at preflowering, flowering, and postflowering stages from plants grown in S-sufficient (Jc, Bc, and Vc), S-high (J1, B1, and V1), and S-insufficient conditions (J2, B2, and V2), and the one-way ANOVA test was performed using SPSS software (Table 2). The increase in S content was observed at the postflowering stage in all three selected cultivars. When leaf samples were compared with the root and stem, they showed higher S content.

## DISCUSSION

*Brassica juncea* is a third important oilseed crop after soybean and palm in the world, and it demands a high amount of sulfur. Sulfur is mostly taken up by the plants from soil as inorganic sulfate and are assimilated into cysteine, which is thought to be the natural sulfur cycle's principal entry point.[7] On the other hand, decreasing trends in emission of S from the industrial sources resulting in limiting inputs of S from deposition and therefore acquisition of sulfur by plants have become an increasingly relevant challenge for the agricultural industry.[6] The importance of S for plant growth, and for crop yield, in addition to its importance for animal and human diets, has resulted in a greater focus on studies into sulfate uptake, transport, and assimilation processes.

At present, more than two thousand miRNA genes among diverse species have been identified, annotated, and characterized.[15] In *Arabidopsis thaliana* and *Brassica napus*, miR395 expression has been found to be induced under S depletion.[16,17]


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ACS Omega 2022, 7, 13228−13242

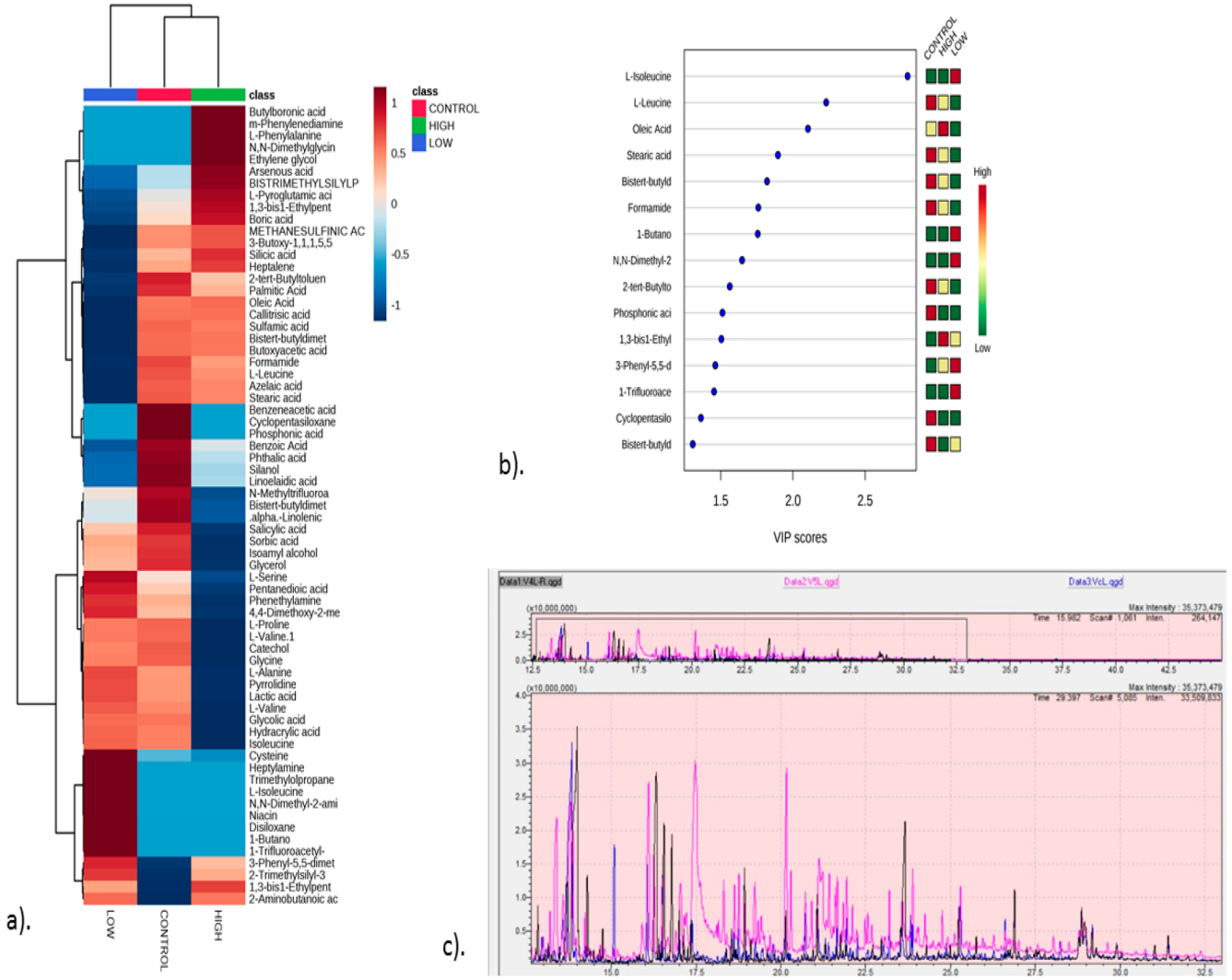


**Figure 11.** Hierarchically clustered (a) heatmap metabolite content of selected metabolites. Color scale represents different concentrations of the metabolites with dark brown being the highest and dark blue the lowest (b). Important features identified by PLS-DA. The colored boxes on the right indicate the relative concentrations of the amino acids (c). Chromatogram showing metabolites in *B. juncea* cultivar Varuna.

**Table 2. Percentage of S Content in *B. juncea* Cultivars Varuna, Pusa Jai Kisan, and Pusa Bold Grown under S- Sufficient (Vc, Jc, and Bc), S-High (V1, J1, and B1), and S-Insufficient (V2, J2, B2) Conditions**[a]

| | | samples (S %) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| stages | tissues | Vc | V1 | V2 | Jc | J1 | J2 | Bc | B1 | B2 |
| preflowering | L | 1.251 | 1.293 | 1.11 | 1.173 | 1.19 | 1.223 | 1.31 | 1.328 | 1.116 |
| | S | 1.09 | 1.19 | 1.013 | 1.108 | 1.098 | 0.84 | 1.17 | 1.198 | 1.015 |
| | R | 1.203 | 1.243 | 0.984 | 1.132 | 1.186 | 1.215 | 1.281 | 1.304 | 0.989 |
| flowering | L | 1.31 | 1.283 | 1.122 | 1.19 | 1.219 | 1.32 | 1.354 | 1.392 | 1.118 |
| | S | 1.22 | 1.15 | 1.019 | 1.113 | 1.16 | 0.88 | 1.202 | 1.225 | 1.019 |
| | R | 1.301 | 1.257 | 1.113 | 1.159 | 1.195 | 1.28 | 1.324 | 1.35 | 1.007 |
| postflowering | L | 1.339 | 1.308 | 1.139 | 1.206 | 1.224 | 1.342 | 1.376 | 1.41 | 1.148 |
| | S | 1.28 | 1.19 | 1.022 | 1.17 | 1.183 | 1.302 | 1.265 | 1.297 | 1.028 |
| | R | 1.303 | 1.281 | 1.124 | 1.194 | 1.205 | 1.313 | 1.354 | 1.392 | 1.12 |

[a]The one-way ANOVA test was performed using SPSS software.

To date, however, no information is available for the miRNAs that may regulate the expression of genes involved in the S uptake and assimilation pathways of *B. juncea*. The ability of a plant to maintain growth on S-limited conditions is termed as sulfur efficiency (SE). *B. juncea* cultivar Pusa Bold is a highly S-efficient genotype; Varuna is moderate; and Pusa Jai Kisan is S-inefficient.[8] Pusa Bold has both high-affinity and low-affinity sulfate transporters, and Pusa Jai Kisan has only low-affinity sulfate transporters. With the advent of novel next-generation technology, computational approaches have introduced and


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ACS Omega 2022, 7, 13228−13242

current software tools could identify miRNAs from EST/GSS sources and predict their secondary structures, targets, and homologues.[18] The computational predicted data need to be validated experimentally. Thus, combined computational and experimental approaches would provide more relevant data to understand sulfur uptake and assimilation pathways.

In the present study, we attempted to identify miRNA-targeting genes of S uptake and an assimilation pathway using computational and experimental methods in mustard cultivars. Their relationship pattern has been analyzed with the expression levels of their target mRNAs, S content, and metabolic profile at preflowering, flowering, and postflowering stages in different tissues under sulfur treatments (S-insufficient and S-high). Using the miRanda database tool, a total of 18 miRNAs belonging to 10 highly conserved families were found to be involved in regulating S uptake and assimilation pathway genes. Based on the query coverage and alignment score, the miRanda database tool results predicted ten miRNA targeting genes and transporters of the pathway. These miRNAs are conserved and include miR395a, miR865-5p, miR843, miR863-3p, miR211a-5p, miR5661, miR396a, miR5650, miR838, and miR159. These miRNAs have been found to target multiple genes in S uptake and assimilation in mustard cultivars.

Real-time PCR data revealed the variation in the expression level of miRNAs and target mRNAs within tissues (leaf and root) under different S treatments in these cultivars. This section discusses the correlation between miRNAs and their target mRNAs studied using the Pearson correlation test in MetaboAnalyst software (http://www.MetaboAnalyst.ca/) (Table 3). In Pusa Bold and Pusa Jai Kisan, expression levels of miR395a were found to be positively correlated with their target mRNA SULTR2;1, which might play a role in overexpression of SULTR2;1 in S-insufficient conditions in leaf and root tissues. The SULTR2;1 transporter is located in xylem parenchyma cells and pericycles in the root.[19] It was found that in the sulfur-starved conditions in the root the SULTR2;1 transporter induction facilitates the transport of sulfate from the root to the shoot.[19] Apart from this, miR395a was negatively correlated with its other targets, viz., ATPS and CbL, in all three cultivars. ATPS catalyzed the first step in the sulfur assimilation pathway.[20] Jones-Rhoades et al. (2004) showed a negative correlation between ATPS and miR395. Our study observed that S-insufficient conditions induced miR395a that might result in repression of ATPS mRNA. In addition, miR395 was negatively correlated with target APR in both Pusa Bold and Pusa Jai Kisan.

Similarly, in the case of other miRNAs, the expression of miR865-5p was positively correlated with its target SULTR4;1 and SULTR4;2 in Varuna, Pusa Jaikisan, and weakly correlated in Pusa Bold. SULTR4;1 transporters have been localized to the vacuolar membrane[22] and reported to be expressed in root and leaf tissues. Its proposed role is in the efflux of sulfate from the vacuole.[5] An increase in expression of SULTR4;1 in S-insufficient conditions indicates that to fulfill demand and supply of sulfur in cells it facilitates efflux of sulfur from the vacuole to the cytosol. The SULTR4;2 is also located in vacuoles. The SULTR4;1 gene was reported to be expressed under both sulfur-sufficient and -deficient conditions in roots,[19] whereas SULTR4;2 gene expression was highly induced by low sulfur content in the root tissues.[21] On the contrary, negative correlation was found among other targets of miR865-5p, viz., SULTR3;1, in all three cultivars. SULTR3;1 is a chloroplast-localized sulfate transporter, responsible for transport of sulfate into chloroplast.[22] In addition, SULTR4;1 was also targeted by miR843 and found to be positively correlated in Varuna and Pusa Bold. The miR843 expression was, however, found to be negatively correlated with its other targets in all three cultivars.

Expression of miR396a was negatively correlated with its target ATPS, SIR, SULTR3;1, and SULTR3;2 mRNAs in Pusa Jai Kisan and Varuna. Another target of miR396a, SULTR1;1, was however not correlated in Pusa Bold and positively correlated in Varuna. The SULTR1;1 was located in the root and known to be a high-affinity sulfate transporter. It was observed to be overexpressed in the S-starvation conditions. Another microRNA, miR838 expression, was positively correlated with SULTR2;2 in Pusa Jai Kisan. The SULTR2;2 is a low-affinity sulfate transporter located in root and leaves. It facilitates the movement of sulfate from the vascular bundle to palisade cells in the leaf.[19] Therefore, miR838 might play a role in its overexpression in S-insufficient conditions. Another significant microRNA, miR159a expression, was negatively correlated with its target mRNAs, ATPS, APR, and SULTR3;2, in all the three cultivars. miR159a belongs to the miR159 family that in *Arabidopsis thaliana* targets several members of the transcription factor family MYB.[23] Besides this, the expressions of miR863, miR2111a-5p, and miR5661 were negatively correlated or not correlated with their target mRNAs in all three cultivars.

**Table 3. Pearson Coefficient of miRNAs with Their Target mRNAs**

| | | Pearson coefficient value | | |
|---|---|---|---|---|
| miRNA | target | Pusa Bold | Varuna | Pusa Jai kisan |
| miR395a | ATPS | −0.41 | −0.34 | −0.692 |
| | APR | −0.41 | −0.28 | −0.66 |
| | CbL | −0.33 | −0.31 | −0.66 |
| | SULTR2;1 | **+0.43** | **+0.25** | **+0.33** |
| | SULTR2;2 | −0.22 | −0.08 | −0.10 |
| | SULTR3;1 | - | - | - |
| | SULTR3;3 | - | - | - |
| mir865-5p | SULTR3;1 | −0.41 | −0.79 | −0.67 |
| | SULTR4;1 | **+0.13** | **+0.42** | **+0.35** |
| | SULTR4;2 | **+0.21** | **+0.51** | **+0.43** |
| miR843 | CbL | −0.63 | −0.19 | −0.61 |
| | SIR | −0.67 | −0.23 | −0.61 |
| | SULTR1;2 | −0.64 | +0.10 | - |
| | SULTR4;1 | **+0.49** | **+0.52** | - |
| miR863 | SULTR1;2 | −0.49 | −0.32 | - |
| | SULTR3;2 | −0.26 | +0.09 | 0.00 |
| miR2111a-5p | SULTR1;2 | −0.63 | +0.11 | - |
| | SULTR3;2 | −0.10 | −0.09 | 0.00 |
| miR5661 | ATPS | −0.43 | −0.24 | −0.38 |
| | SULTR3;1 | −0.32 | −0.35 | −0.18 |
| miR396a | ATPS | −0.53 | −0.32 | −0.76 |
| | SIR | −0.49 | −0.38 | −0.85 |
| | SULTR1;1 | +0.07 | **+0.62** | - |
| | SULTR3;1 | - | −0.74 | −0.76 |
| | SULTR3;2 | −0.38 | −0.74 | −0.74 |
| miR5650 | SULTR3;1 | +0.084 | −0.10 | −0.73 |
| miR838 | SULTR1;1 | −0.14 | −0.66 | −0.24 |
| | SULTR2;1 | −0.04 | | - |
| | SULTR2;2 | +0.24 | | **+0.38** |
| miR159a | ATPS | −0.58 | −0.53 | −0.82 |
| | APR | −0.57 | −0.50 | −0.80 |
| | SULTR3;2 | −0.52 | −0.35 | −0.69 |



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These observations of the present study suggest that the expression of most of the selected miRNAs is higher in S-insufficient conditions. The miRNAs upregulated under S-insufficient conditions may downregulate their target mRNAs. A positive correlation between miRNAs and mRNAs has been found in several studies; for example, Khan et al. (2020) revealed a positive correlation of miR5083 with its target NAC1 gene, an important transcription factor involved in artemisinin biosynthesis in *Artemisia annua*. Thus, it was interesting to discover a positive correlation of miRNA-targeting genes and sulfate transporters in three cultivars.

GC-MS data revealed that in Pusa Bold the concentration of free cysteine (Cys) was higher in S-high conditions when compared with S-insufficient conditions. Its accumulation is limited by feedback regulation of SAT.[24] Limitation of SAT might be the reason for the accumulation of free Cys because the metabolic coupling is present between nitrogen and sulfur assimilation.[9] Similarly, in Pusa Jai Kisan, free Cys was higher in S-high conditions, but in Varuna free Cys was higher in S-insufficient conditions. Varuna responds well in S-sufficient conditions, Pusa Bold in S-insufficient conditions, and Pusa Jai Kisan in S-high conditions. It might be the role of their transporters that define their S-utilization efficiency, which in turn is regulated by the miRNAs. Based on this study, a suitable variety can be selected to manipulate the miRNA-regulating S-assimilation and uptake pathway to enhance sulfur utilization under a limited supply of sulfur.

## METHOD

***In Silico* Identification of miRNA-Targeting Genes.** The candidate target genes involved in the sulfur uptake and assimilation pathway of *B. juncea* were obtained by searching for homologous genes of *A. thaliana* in the Brassica Database and retrieved from the NCBI GenBank (http://ncbi.nlm.nih.gov/), as the sequence information on *B. juncea* is very limited. Moreover, miRNAs for selected target genes were predicted using the psRNA target analyzer under preloaded small RNAs/user-submitted transcripts (http://plantgrn.noble.org/psRNATarget/), a small RNA target-analyzing server for plant studies. A total of 18 miRNAs belonging to 10 highly conserved families were found, and 100 conserved miRNAs were obtained from the miRBase database (http://www.mirbase.org/; release 16, September 2010).[25] These miRNAs came from *A. thaliana*, *B. rapa*, and *B. napus*. All the miRNAs acquired through *in silico* analysis were targeted against genes involved in sulfur uptake and the assimilation pathway using the miRanda database tool with the detailed statistical study of minimum free energies (MFEs).[26] The prediction was made using miRanda with default parameters (Gap Open Penalty: e0.9; Gap Extend: e0.4; Score Threshold: 50.0; Energy Threshold: _0.20 kcal/mol). A list of conserved miRNA sequences and genes involved in sulfur uptake and the assimilation pathway are given in Table S1.

**Primer Designing.** For the selected miRNAs, miRNA-specific stem-loop primers for reverse transcription reactions were designed, consisting of approximately 50 nt.[27] We used publicly available gene sequences from *Arabidopsis* and related Brassica species to design the gene-specific primers. The recently available *B. rapa* genome portal (http://brassicadb.org/brad) and the Brassica genome gateway (http://brassica.bbsrc.ac.uk) were used to obtain genome survey sequences (GSS) and the Brassica specific expressed sequence tags (EST) of the candidate target genes, with *Arabidopsis* complementary DNA sequence (CDS) as a query.[28] To ensure gene amplification in *B. juncea*, the *Arabidopsis* and Brassica sequences of each candidate gene were aligned together, and the primers were designed from the consensus regions of the aligned sequences, preferably spanning an intron. IDT primer quest tools (http://eu.idtdna.com/primerquest/home/index) were used to designed gene-specific primers from CDS and analyzed in OligoAnalyser (https://eu.idtdna.com/calc/analyser), according to the manufacturer's instructions. Integrated DNA Technology, USA, synthesized the primers. The sequence of real-time PCR primers of miRNAs and their target mRNAs along with internal control primers are given in Table S2 (a and b).

**Plant Material and Growth Conditions.** Seeds of three selected mustard genotypes Pusa Jai Kisan, Pusa Bold, and Varuna [*Brassica juncea* (L.) Czern. and Coss.], procured from Indian Agricultural Research Institute, New Delhi, India, were sown in pots with sand and germinated under controlled conditions in an artificial climate chamber programmed for a constant temperature of 20 °C, RH 75%, and a 16 h light period (280−300 $\mu$mol) in polyhouse at Jamia Hamdard, New Delhi, India. The pots were irrigated with Hoagland solution containing 3 mM $KNO_3$, 2 mM $Ca(NO_3)_2$, 1 mM $NH_4H_3PO_4$, 50 mM KCl, 25 mM $H_3BO_3$, 2 mM $MnCl_2$, 2 mM $ZnCl_2$, 0.5 mM $CuCl_2$, 0.5 mM $(NH_4)_6Mo_7O_{24}$, and 20 mM NaFeEDTA at regular intervals. The pH of the solution was adjusted to 5.5 with KOH. Sulfate was supplied as $MgSO_4$ at two levels, viz., 50 $\mu$M $SO_4^{2-}$ (S-insufficient) and 4 mM $SO_4^{2-}$ (S-high), whereas 1 mM $SO_4^{2-}$ was provided as a control (S-sufficient). $Mg^{2+}$ was maintained at 1 mM by the addition of $MgCl_2$ in the treatments.[8] The study complies with local and national regulations.

**Total RNA Isolation and Complementary DNA Synthesis.** The tissues were collected at three stages: preflowering (leaf and root), flowering (leaf and root), and postflowering (leaf and root). Total RNA from 100 mg of each tissue sample was isolated using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA), according to the method described by Simms et al. (1993).[29] The concentration and purity of RNA samples were measured using a NanoDrop spectrophotometer (ND1000). The genetic integrity was evaluated by subjecting RNA to 1.5% denaturing agarose gel electrophoresis. miRNA-specific cDNAs were synthesized using the RevertAid H Minus cDNA synthesis kit (Fermentas, USA) from 1 $\mu$g of total RNA, and cyclic conditions were kept as described by Varkonyi-Gasic et al. (2007).[30] Similarly, mRNA-specific cDNAs were synthesized from 1 $\mu$g of total RNA using the Verso cDNA synthesis kit (Thermo Scientific, USA), and cyclic conditions were kept as 42 °C for 90 min and 85 °C for 5 min for enzyme inactivation.

**Expression Analysis of miRNAs and Predicted Target Using qRT-PCR.** Quantitative real-time PCR (qPCR) was performed in triplicate using SYBR green chemistry on a real-time thermal cycler (Light Cycler 480, Roche, USA). The qPCR assay was designed according to the MIQE (minimum information for publication of quantitative real-time PCR experiment) guidelines.[31] To normalize the data, $\beta$-actin and U6 snRNA were used as internal controls for target genes and miRNAs, respectively. Reaction mixtures contained 10 $\mu$L of SYBR Green Mix, 2 $\mu$L of diluted cDNA, double-distilled water, and a final primer concentration of 0.5 $\mu$M. The following amplification conditions were applied: an initial denaturation step of 95 °C for 30 s; 40 cycles at 95 °C for 30 s; 55 °C for 30 s, and an initial denaturation step of 95 °C for 10 min; 40 cycles at 95 °C for 15 s; and 60 °C for 60 s for mRNAs and miRNAs,


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ACS Omega 2022, 7, 13228−13242

respectively. The specificity of amplification was checked by melting curves.

Fold variations in expressions of miRNAs and mRNAs among RNA samples were calculated using the $\Delta\Delta C_T$ method.[32] For the treated sample, fold change relative to the control sample was calculated as $2^{-\Delta\Delta C_T}$, where $\Delta\Delta C_T$ = ($C_T$ of the treated sample − $C_T$ of the reference gene − ($C_T$ of control sample − $C_T$ of reference gene). The standard deviation and standard error were calculated from three replicates.

**Pearson Correlation.** The correlation between miRNAs and their target mRNAs was inspected using the Pearson correlation test on MetaboAnalyst (http://www.MetaboAnalyst.ca/). A perfect negative correlation was defined as $r = -1$ and a perfect positive correlation as $r = +1$.[23] Correlation values between −0.3 and +0.3 were taken as no correlation between miRNA and its target mRNA.[23,33]

**5′ RLM-RACE.** Cleavage sites of target mRNAs for few conserved miRNAs were experimentally mapped by the RLM-RACE assay. The FirstChoice RLM-RACE kit was used according to manufacturer's instructions. Nested gene-specific primers were used for amplification of the target gene. The amplicon was gel purified and sequenced to validate the cleavage site.[34]

**Extraction Procedure for GC-MS.** Leaves were sampled at the postflowering stage. For each sample, there were three technical replicates. The air-dried Brassica leaves were pulverized in a mortar and pestle into a fine powder. One gram powder of each sample was extracted three times, with 5 mL of methanol at room temperature, and sonicated for 15 min and centrifuged. The extract was concentrated to 1 mL by vacuo. After centrifugation, the solvent portion was collected and filtered using syringe filters (0.45 $\mu$m) and dissolved in *N*-(*tert*-butyldimethylsilyl)-*N*-methyltrifluoroacetamide (Alfa Aesar, Fisher scientific, USA). Samples were then immediately analyzed using GC-MS for untargeted metabolites.[35]

**Sulfur Content.** The estimation of sulfur in treated and control samples of plant tissues was carried out by direct determination using the CHNS analyzer (model VERIOEL-III, GMBH, Germany). The dry matter was converted into a fine powder with a mortar−pestle and tightly packed in preweighed aluminum boats. The tungsten oxide was added to plant material for complete digestion. Sulfanilic acid was used as a standard in the boat instead of biological material.[3637,]

## ■ ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.2c00676.

Table S1: miRNAs with their sequences and targeting genes. Table S2a: miRNAs with their primer sequences. Table S2b: mRNAs with their primer sequences. Table S3: 5′-RLM-RACE primers used in this study (PDF)

## ■ AUTHOR INFORMATION


### Corresponding Author

**Malik Zainul Abdin** − *Centre for Transgenic Plant Development, Department of Biotechnology, School of Chemical and Life Science, Jamia Hamdard, New Delhi 110062, India;* orcid.org/0000-0002-7522-7336; Email: mzabdin.ctpd2@gmail.com, mzabdin@jamiahamdard.ac.in

### Authors

**Sadia Iqrar** − *Centre for Transgenic Plant Development, Department of Biotechnology, School of Chemical and Life Science, Jamia Hamdard, New Delhi 110062, India*

**Kudsiya Ashrafi** − *Centre for Transgenic Plant Development, Department of Biotechnology, School of Chemical and Life Science, Jamia Hamdard, New Delhi 110062, India*

**Shazia Khan** − *Centre for Transgenic Plant Development, Department of Biotechnology, School of Chemical and Life Science, Jamia Hamdard, New Delhi 110062, India*

**Monica Saifi** − *Centre for Transgenic Plant Development, Department of Biotechnology, School of Chemical and Life Science, Jamia Hamdard, New Delhi 110062, India*

**Nazima Nasrullah** − *Centre for Transgenic Plant Development, Department of Biotechnology, School of Chemical and Life Science, Jamia Hamdard, New Delhi 110062, India*

Complete contact information is available at: https://pubs.acs.org/10.1021/acsomega.2c00676


### Author Contributions

M.Z.A. and S.I. conceived and designed the research. S.I. carried out bioinformatics analysis and laboratory work and also drafted the manuscript. K.A. helped in RNA isolation and CHNS analysis. N.N. analyzed the GCMS data. M.S. and S.K. helped with data analysis. All authors read and approved the manuscript.

### Notes

The authors declare no competing financial interest.

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## ■ ABBREVIATIONS:

ATPS: ATP sulfurylase
APR: adenosine 5′-phosphosulfate reductase
CbL: cystathionine $\beta$-lyase
CgS: cystathionine $\gamma$-synthase
GC-MS: gas chromatography−mass spectrometry
SIR: sulfite reductase
SULTR: sulfur transporters

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