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**Research Article**

# Molecular Characterization of Silicon (Si) Transporter Genes, Insights into Si-acquisition Status, Plant Growth, Development, and Yield in Alfalfa

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## ABSTRACT

Silicon (Si) has the potential to improve plant growth and stress tolerance. The study aimed to explore Si-involving plant responses and molecular characterization of different Si-responsive genes in alfalfa. In this study, the exogenous supplementation of Si enhanced plant growth, and biomass yield. Si-acquisition in alfalfa root and shoot was higher in Si-supplemented compared to silicon deficient (-Si) plants, implying Si-acquisition has beneficial on alfalfa plants. As a consequence, the quantum efficiency of photosystem II (Fv/Fm) was significantly increased in silicon-sufficient (+Si) plants. The quantitative gene expression analysis exhibited a significant upregulation of the *Lsi1, Lsi2, Lsi3, NIP5;1*, and *NIP6;1* genes in alfalfa roots, while *BOR1, BOR4, NIP2,* and *NIP3* showed no significant variation in their expression. The MEME results further noticed the association of four motifs related to the major intrinsic protein (MIP). The interaction analysis revealed that *NIP5;1* and *Lsi1* showed a shared gene network with *NIP2, BOR1,* and *BOR4,* and *Lsi2, Lsi3* and *NIP3-1,* respectively. These results suggest that members of the major intrinsic proteins (MIPs) family especially *Lsi1, Lsi2, Lsi3, NIP5;1,* and *NIP6;1* genes helped to pass water and other neutral solutes through the cell membrane and those played significant roles in Si uptake and transport in plants. Together, these insights might be useful for alfalfa breeding and genome editing approaches for alfalfa improvement.

**(Key words**: Alfalfa, Gene characterization, Gene network, Silicon transporter, Trait improvement)

## Ⅰ. INTRODUCTION

Silicon (Si) is a beneficial element that extensively studied in plant growth and multiple stress tolerance in plants (Kabir et al., 2021). Si is a crystalline structural element that makes up 27.2 % of Earth's crust after oxygen (Huff, 2001). Si and silica ($SiO_2$) react to form silicon monoxide (SiO). Si uptake can vary in different plant organs and diverse plant species. Lateral roots contribute to better Si uptake than hair roots in rice plants (Ma et al., 2001). Plant uptake Si as silicic acid (SA) that up to 0.1-10 % by dry weight (Imtiaz et al., 2016). Si uptake efficiency is higher in monocot primitive plant species compared to dicot species (Rahman et al., 2022b). Si is mostly uptaken by plants via the apoplastic route, though aquaporin involving a complex symplistic route is also involved in Si translocation in different aerial parts via xylem (Guerriero et al., 2016).

The role of Si has been well studied in multiple biotic and abiotic stress tolerance in diverse plant species (Rahman et al., 2018; Joudmand and Hajiboland, 2019; Kabir et al., 2021; Hajiboland, 2022). It has been reported that Si deficiency declines plant proper growth and reproduction (Epstein and Bloom, 2005). However, several recent studies confirmed that Si plays a significant role in boosting plant growth, enhancing photosynthesis, $N_2$ fixation, and crop improvement (Rahman et al., 2018; Rastogi et al., 2021). Si shows a combined benefit by interacting with growth regulators (e.g., gibberellic acid, methyl jasmonate), which leads to improved growth parameters, decreases free toxic radicals, and regulates candidate genes in plants (Moradi et al., 2022; Raza et al., 2022). However, the significance of Si in enhancing plant growth and agronomic traits has not been well established.

The accumulation and transport of Si highly depends on plant tissue and the distance complex molecular mechanisms in plants. Several members of the major intrinsic proteins (MIPs) family help pass water and other neutral solutes through the cell

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membrane and can play a role of heterotetramers (Park and Saier, 1996). Specially, Si has been reported to influx into plant cells through nodulin 26-like intrinsic proteins (NIPs) that belong to MIPs family (Ma et al., 2006). Most of the candidate genes of the NIP family are localized at the plasmamembrane and reported to be expressed in plants' entire cell surface (Maurel et al., 2015). The *NIP5;1* promotes Si uptake in sugar beet (Rahman et al., 2022b). However, members of NIPs candidates show higher permeability of beneficial and toxic metals rather than water transport efficiency (Sabir et al., 2020). Functions of other genes such as *Lsi1* (low silicon 1) and *Lsi2* belongs to a NIPs subfamily in aquaporin, *Lsi* genes are localized on exodermis and endodermis of rice root cells or proximal side of the same cells (Ma and Yamaji, 2008). In the same study, *Lsi1* shows the influx transport efficiency of Si, while *Lsi2* shows it reverse. *Lsi2* plays a role in Si transport from root cells to apoplastic (Ma and Yamaji, 2008), However, *Lsi1* and *Lsi2* are vital candidates for efficient uptake of Si in plants.

Alfalfa (*Medicago sativa* L.) is a widely cultivated forage legume crop called as 'queen of forage' (Rahman et al., 2022c). Alfalfa is an excellent source of hay and high protein content (Rahman et al., 2020). Alfalfa provides nitrogen ($N_2$) benefits to the soil that declines the supplementation dose and cost of chemical fertilizer to agricultural fields (Das et al., 2021). Silicon-based fertilizer could be excellent alternative, cost-effective and ecofriendly rather than commercial fertilizer for improving forage crops. Whether and how Si supplementation enhances growth, development, and yield attributes in plants is still an unsettled issue. Therefore, in this study, we performed in alfalfa. Subsequently, molecular characterization was also performed that disclosed their involvement in Si uptake and transportation in different plant organs, which led to alfalfa improvement.

## Ⅱ. MATERIALS AND METHODS

### 1. Plant growth and treatment

Viable seeds of alfalfa (*Medicago sativa* L.) were sterilized with 70% ethanol for 1 min, then washed with milli-Q water thrice. The seeds were transferred to the germination tray for 2 days; then alfalfa seedlings were transferred to Hoagland nutrient solution for two weeks (Hoagland and Arnon, 1950). A total two treatments were maintained: control plants without silicon (-Si) and control plants with 1 mM $KiSiO_3$ (+Si). Following two weeks of treatment, plants were harvested. The root and shoot samples were collected, whereas roots were washed properly with deionized water to remove the solution component. Finally, the collected samples were quickly frozen with liquid N2 and stored at -80°C for further analysis.

### 2. Determination of morpho-physiological parameters

The quantum efficiency of photosystem II (Fv/Fm) was measured by a portable fluorometer (LI-600 Porometer/Fluorometers, Korea), and alfalfa leaf greenness (SPAD value) was determined by machine (SPAD-502, Minolta, Japan). Alfalfa root-shoot length (cm) was determined by caliper, and plant dry weight (g) was a digital weight machine.

### 3. Determination of ICP-MS

Silicon (Si) accumulation in alfalfa samples was determined following the protocol used earlier (Haque et al., 2021). Alfalfa root and shoot samples were digested with a solution of $HClO_4/HNO_3$ (1:3 v/v). The Si concentration in the digested solution was determined using ICP-spectrometry (Agilent 7700, Japan). The Si content was calculated based on a standard known solution.

### 4. Expression analysis of Si-transporter genes

Expression of Si-transporter genes was analyzed using the q-RT PCR following the protocol used previously (Rahman et al., 2021; Haque et al., 2022). The gene-specific primers were used in this study (Table 1). Total RNA was isolated from alfalfa seedling tissue using an extraction kit (QIAGEN, USA) following the protocol used previously (Kabir et al., 2023). Then RNA was quantified using a Nanodrop Spectrophotometer. The cDNA was prepared from the total RNA to convert first-strand cDNA. The expression of Si-transporter genes at the mRNA level was evaluated by a thermal-cycler system (CFX96 Dx, Biorad-USA). The q-RT-PCR was set at 95°C for 3 min, 40 cycles at 95°C for 5 s, 60°C for 30 s. The expression level of candidate genes was calculated by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001), where *Actin* was used as internal control. A total of three

**Table 1. List of gene Medicago gene primers used for gene expression analysis**

| Gene name | Forward primer | Reverse primer | Gene ID/Accession |
|---|---|---|---|
| *Lsi1* | GCGGTGCGGTTTGATTTGAT | CGTGGTGAGGAGATTCCCAA | Medtr7g010650.1 |
| *Lsi2* | TGACATGGACAGCCAATGCT | ATCAAATCAAACCGCACCGC | Medtr7g010650.2 |
| *Lsi3* | TGGTTCCTCAGCTACACCAA | CAGCAGTCGGGTCCTCAATA | Medtr8g038580.1 |
| *AQP1* | TTGTTGCTGTCTCCGTTGGT | CCGAATGCTGGAACAGACGA | AJ251652 |
| *BOR1* | GGTGTAGCTGAGCCAACAGT | CCGGTAAGGCGTGTGAATCT | XM_024779336 |
| *BOR4* | CCAAGAAGACTCGTTGCACCT | TCCTTTTGTTGCGCCAGTTG | XM_039831144 |
| *NIP3* | GAGTGGTGGTGTCACGGTTC | GAGTGGTGGTGTCACGGTTC | AY539749 |
| *NIP2* | CGGTAGCTGAGGTGATAGGC | CTGCAGGATTGAAATGAGCA | AY539750.1 |
| *NIP5;1* | CAGGTGCTCATCTCAATCCA | TAGGGACAGTGACACCACCA | XM_003591968 |
| *NIP6;1* | GGTGTCACGGTTCCTTCAGT | GGCCCAGCAATGAGTATGTT | XM_003604170 |

biological replications were considered for each treatment.

### 5. MSA, motif characterization, phylogenetic tree and bioinformatics analyses

Multiple sequence alignments (MSA) of the protein information of Si transporter genes with Arabidopsis homologs were analyzed using Crustal omega (https://www.ebi.ac.uk/Tools/msa/clustalo/). The sub-cellular localization of the homologs was tracked using the CELLO v.2.5 analyzing tool. Furthermore, MEME Suite 5.1.1 (http://meme-suite.org/tools/meme) was applied to the category of the five conserved protein motifs using default parameters, and a total five numbers of motifs were obtained. The MyHits (https://myhits.sib.swiss/cgi-bin/motif scan) web tool was used to verify the motifs by cross-checking with various domains (Sigrist et al., 2010). The phylogenetic tree was constructed using MEGA software (version 11). Finally, the interactome of Si-responsive candidate was performed by the STRING network (http://string-db.org) and the method used previously (Rahman et al., 2022a), and those are visualized in Cytoscape (Szklarczyk et al., 2019).

### 6. Data analysis

The physiological and molecular data in alfalfa were examined by analysis of variance (ANOVA). The significant level of the analyzed data was considered at $p \leq 0.05$. The experimental data were presented in the graph using GraphPad Prism software (V.8.0.2). At least three replicates were subjected to each treatment.

## Ⅲ. RESULTS AND DISCUSSION

This study explored a significant output of molecular characterization of the Si-transporter genes in alfalfa plant. In this study, Si sufficient (+Si) significantly improved alfalfa growth, development, and other agronomic traits compared to Si deficient (-Si) plants. In the the next section, we presented whether and how Si acquisition enhances alfalfa growth and biomass yield. Subsequently, Si-responsive transporter genes and their roles in alfalfa improvement were also discussed.

### 1. Si-acquisition in alfalfa improved growth and biomass yield

A significant growth improvement was observed in Si-treated (+Si) alfalfa plants compared to control (-Si) plants (Fig. 1). The Fv/Fm performance was significantly improved in +Si plants than −Si plants (Fig. 2A). However, no significant difference was found in between the +Si and −Si plants in case of leaf greenness parameter (Fig. 2B), suggesting that Si-supplementation do not have any significant impact on leaf pigmentation. Due to photosynthetic performance, the plant biomass yield, such as root-shoot length and their dry weight, was substantially improved in +Si plants compared to −Si plants (Fig. 2C-F). A significant Si-acquisition in root and shoot of the +Si plant compared to −Si plant (Fig. 2G and H) suggests that Si is useful for alfalfa plants that improve shoot and root growth and biomass yield in response to Si. The role of Si in plant improvement and stress tolerance has been well documented in barley (Joudmand and Hajiboland, 2019) and


Fig. 1. Phenotypic changes of alfalfa seedlings. The 2-week-old alfalfa seedlings show phenotypic differences after suppling with and without silicon (Si) in alfalfa. Phenotypic differences of alfalfa seedling. -Si and +Si present plant grows in absence of Si, and alfalfa grows with Si.

sugar beet (Kabir et al., 2021). In the present study, we claimed Si-acquisition in alfalfa significantly improved plant growth, development, and biomass yield, which might be useful to alfalfa breeders and farmers to apply Si-based fertilizer in alfalfa improvement.

## 2. Molecular characterization and confirmation of Si-responsive candidates in alfalfa

Silicon (Si) responsive total of ten candidates showed a phylogenetic relationship (Fig. 3). In the phylogenetic tree, the relationship was separated into group I (*Lsi1, Lsi2*), and group II (*NIP5;1*). In another side, *NIP5;1* further branched within the same group I (*NIP3, NIP6;1*). However, group I was further separated into different clade named group III (*Lsi3, BOR1,* and *BOR4*), while *APQ1* and *NIP2* were brunched from the origin and divided into group IV. The MEME tools showed the five most conserved motifs in 10 Si responsive candidates of Medicago species. The three (Motif 1: GGWGSDTCAATGAATCCAGYAAGRACATTAGGWCCWG CWRTTGCWRCHWVMWMCTACAAAGGMATHTGGWTM TATHTGGTDGSMCCTATTMTWGGTGCHC, Motif 2:

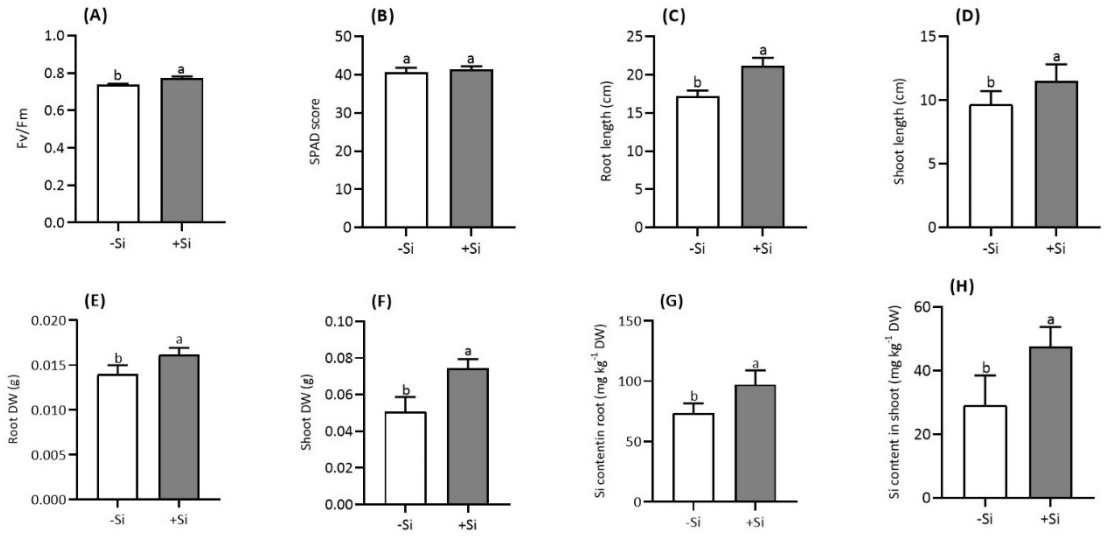


Fig. 2. Morpho-physiological difference in alfalfa plants after suppling with and without silicon (Si) in alfalfa. Regulation of morpho-physiological and Si acquisition in alfalfa. The quantum efficiency of photosystem II (Fv/Fm) (A); SPAD score (B); root length (C); shoot length (D); root dry weight (E); shoot dry weight (F), Si concentration in root (G), and Si concentration in shoot (H) of alfalfa seedlings. Different letters above the bar column show significant differences ($p \leq 0.05$) among the group means with standard error (SE). At least three individual replications were considered for each treatment.

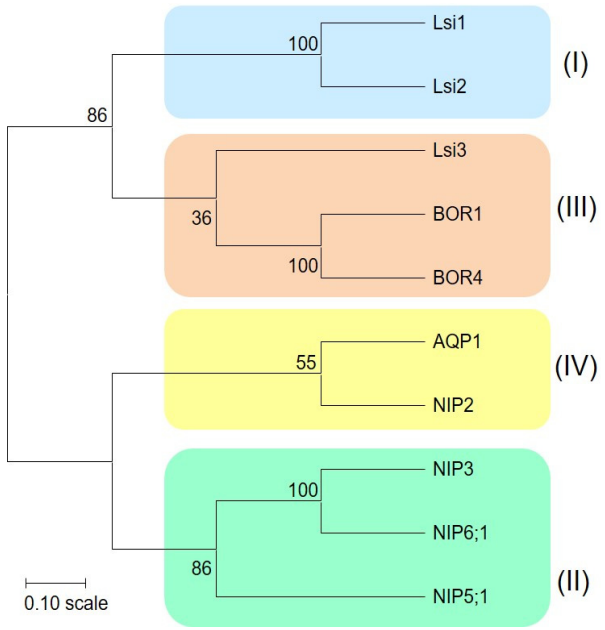


Fig. 3. A phylogenetic tree presents the relationship of different Si-responsive candidate genes in Medicago species. The tree was constructed by MEGA software according to previous study (Lee et al., 2018) using the neighbor-joining method.

CACATCTC YGGTGCWCATMTBAATCCGGCTGTYACCW TWGCWTTTGCYRCMKTWAARCAYTTYCCCTGGAAACA KGTHCCWKTKTAYATTGCWGCWCAAS, Motif 3:CCTTCW GKWKVADWYV KHCAAGCTTTWGYNWTWGARWTTRT SATCACCTTTAMYCTSRTGTTYRTYKTYACTGCHGTTGC CACYGACWCAARAGCBGTDG, Motif 4: TCKCNAAAAAG GTWKKWGCTGAGKTT ATAGGVACATAYWTHTTRRTRT TTGCYGGDABWGSNDCTGCWRTTGTGAACHAMAAKR WWVAWAACDHAGWMWC) matched with the member of NIP and *Lsi* and AQP candidate family, while Motif 5 (TTGCWGGARTHGCDGTTGGWDCNMCTGTYAYNMTVA ANATHMTNRTTGC WGGRCC) not matched and/or partially matched with the above candidate proteins (Fig. 4). The phylogenetic relationship and motif locations confirmation would be exciting and helpful for moving forward breeding and genome editing approach to alfalfa improvement.

### 3. Si-enhanced the candidate gene expression in alfalfa

Gene expression of Si transporters genes showed that a total six Si responsive genes were significantly upregulated in +Si

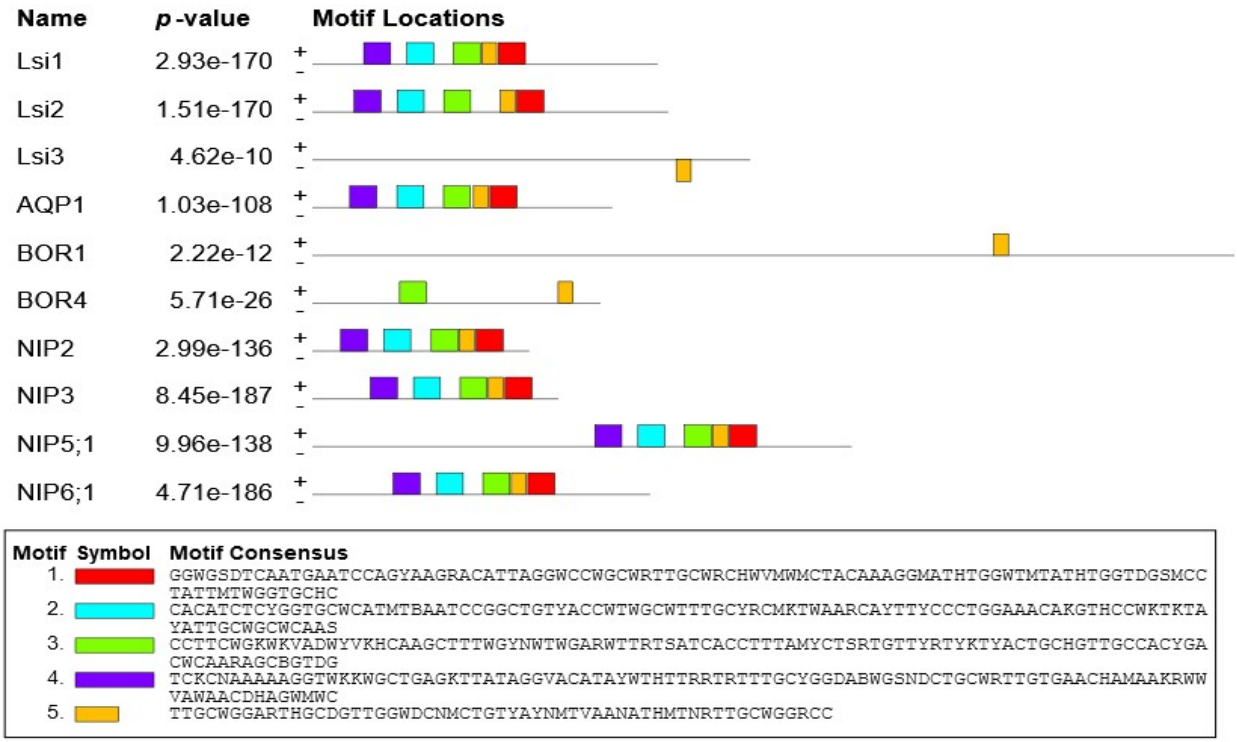


Fig. 4. Schematic representation of the 5 conserved motifs in different Si-responsive candidates Medicago. Scale bar corresponds to 0.1 amino acid substitution per residue. Different motifs, numbered 1-5, are displayed in different colored boxes.

plants (Fig. 5). The homologs of low silicon (*Lsi*) genes *Lsi1, Lsi2,* and *Lsi3* were highly expressed in alfalfa roots (Fig. 5 A-C). The *Lsi* genes belong to a Nod26-like major intrinsic protein (NIP) subfamily in aquaporin, *Lsi2* encodes an anion transporter (Ma and Yamaji, 2008). *Lsi1* is localized on root exodermis and endodermis, while *Lsi2* is localized on the proximal side of the same cells (Ma et al., 2008). *Lsi1* shows influx transport activity for Si, while *Lsi2* shows efflux transport activity. In this study, the upregulation of *Lsi1* suggesting the transport of Si into alfalfa root cells, while *Lsi2* effluxs Si from root cells to apoplastic. Therefore, *Lsi1, Lsi2,* and *Lsi3* are required for efficient uptake and transport of Si in alfalfa plants. Another candidate gene aquaporin 1 (*AQP1*) is significantly upregulated in +Si plants suggesting that *AQP1* is responsible for regulating water relation, cell turgor pressure, and hydraulic regulation in roots and leaves. We noticed that the high expression of *AQP1* is involved in the homeostasis of solutes and hydraulic regulation, which led to alfalfa for better

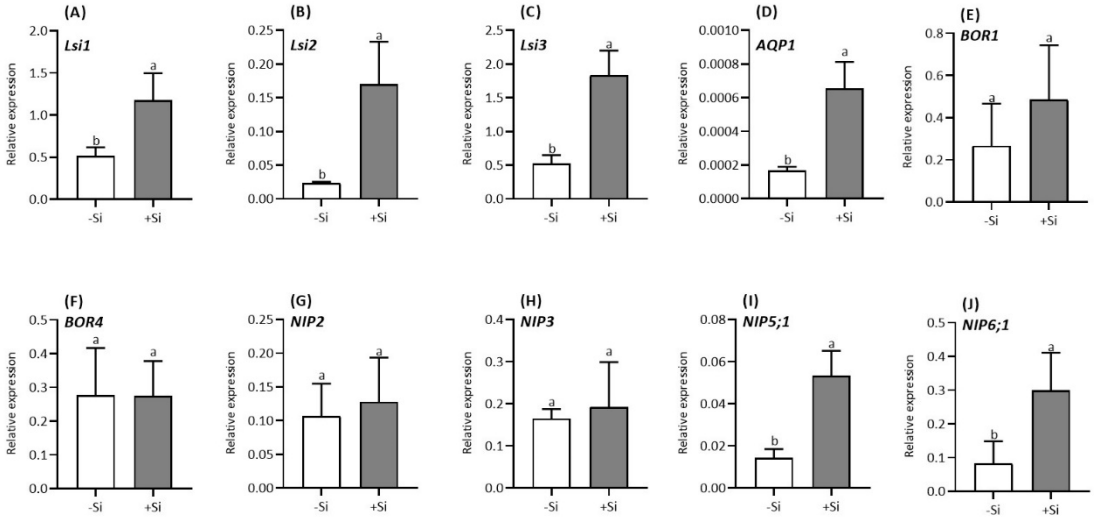


Fig. 5. Expression analysis of Si responsive genes after suppling with and without silicon (Si) in alfalfa. Relative expression of *Lsi1* (A), *Lsi2* (B), *Lsi3* (C), *APQ1* (D), *BOR1* (E), *BOR4* (F), *NIP2* (G), *NIP3* (H), *NIP5;1* (I) and *NIP6;1* (J) in alfalfa roots. The *MsActin* was used as internal control. The expression level of target genes was calculated following the $2^{-\Delta\Delta Ct}$ method that was mentioned detail in material and method section. Different letters above the bar column show significant differences ($p \leq 0.05$) among the group means with standard error (SE).

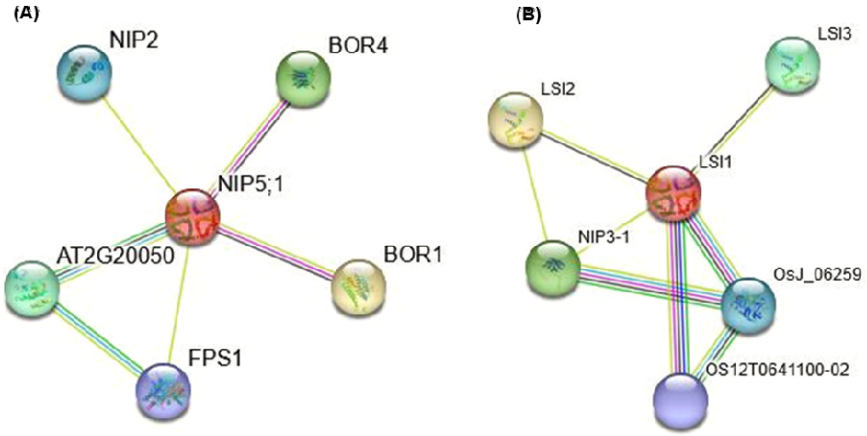


Fig. 6. Interactome analysis of Medicago *NIP5;1* and *Lsi1*candidates. Interactions of candidates and their predicted partners are presented by clusters. The gene networks are detected through STRING network. Interactome analysis of *NIP5;1* and its predicted partner (A), interactome analysis of *Lsi1*and its predicted partner (B).

water relations during plant growth and development.

However, *BOR1*, *BOR4, NIP2,* and *NIP3* did not show any significant variation in Si sufficient (+Si) and Si deficient (−Si) plants (Fig. 5E-H), while *NIP5;1* and *NIP6;1* both showed high expression in +Si plant roots (Fig. 5I and J), suggesting that *NIP5;1* and *NIP6;1* are responsible for Si uptake in alfalfa. Our findings wherein are consistent with a previous study, where *NIP5;1* was localized in plasmamembrane and involved in Si acquisition in sugar beet (Rahman et al., 2022b). The findings together suggest that the candidate genes of the Nod26-like major intrinsic protein (NIP) subfamily in aquaporin significantly impact Si regulation and transport alfalfa plants. Further, we analyzed the predicted partner of critical candidates. The interaction of these candidates was supported by the interactome analysis, in which *NIP5;1* showed a shared gene network with *NIP2, BOR1, BOR4,* and other metal-responsive candidates. The *Lsi1* exhibited a relationship with *Lsi2, Lsi3* and *NIP3-1* (Fig. 6A, B). NIP subfamily of aquaporin proteins consists of several members including *NIP1;1, NIP1;2, NIP5;1, NIP6;1,* and *NIP7;1* (Xu 2015). NIP genes are located in the plasmamembrane (PM) and reported to be expressed on cell surface (Maurel et al., 2015). Furthermore, *NIP3;1* involved in metal uptake and translocation from root-to-shoot in Arabidopsis (Xu 2015). Previous studies have documented that NIP genes were involved in enhancing Si uptake into the stele of roots while it was located in the PM (Gomes et al., 2009). In our study, we found the *NIP5;1,* and *NIP6;1* were significantly expressed in alfalfa roots. So as a consequence, we claimed that the overexpression of *NIP5;1, NIP6;1* are involved in Si-uptake and translocation processing in alfalfa, which lead to Si-acquisition and alfalfa development. These genes information and their interactome analysis will be helpful for forward breeding and genome editing approaches for improving alfalfa and other forage legume crops.

## Ⅳ. CONCLUSIONS

This study implies the positive impact of Si for enhancing plant growth, development, and yield attributes by regulating Si-acquisition and upregulation of Si-transporter genes in alfalfa roots. The qPCR analysis showed that genes *Lsi1, Lsi2, Lsi3, NIP5;1,* and *NIP6;1* were highly expressed in alfalfa plants supplemented with Si, suggesting that members of major intrinsic proteins (MIPs) family help to pass water and other neutral solute through the cell membrane. Those played significant roles in Si uptake and transport in plants. This study further confirmed the molecular and computational characterization of the candidate genes, which will encourage us to improve alfalfa plants by through forward breeding and genome editing approach.

## Ⅴ. ACKNOWLEDGEMENTS

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