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Foliar application of silicon improves stem strength under low light stress by regulating lignin biosynthesis genes in soybean (*Glycine max* (L.) Merr.)

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Foliar application of silicon improves stem strength under low light stress by regulating lignin biosynthesis genes in soybean (*Glycine max* (L.) Merr.)

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Graphical abstract
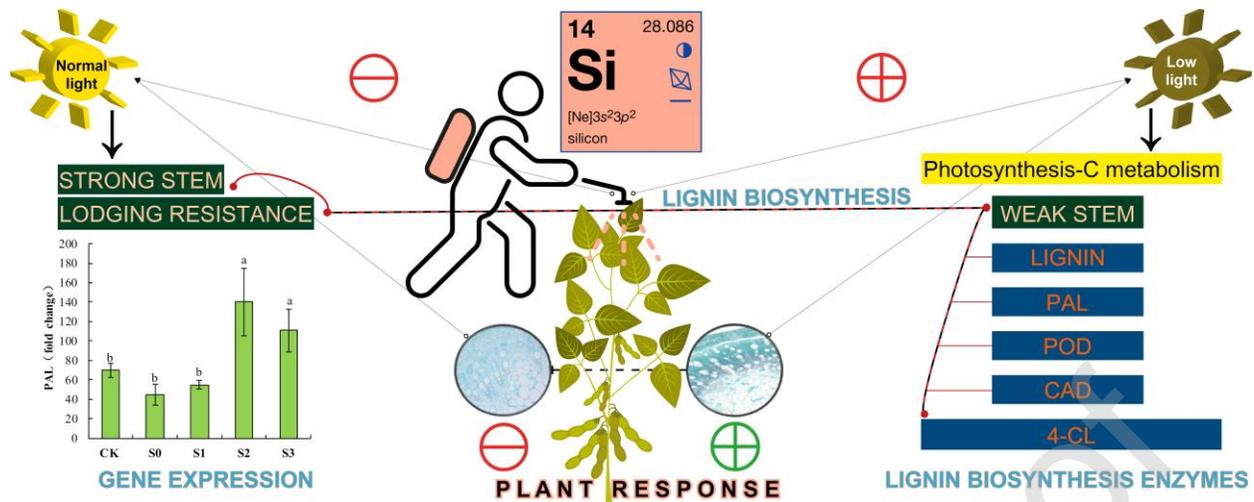
Highlights

- Si boosts lignin biosynthetic enzyme activity, mainly phenylalanine ammonia lyase.
- Si up-regulates lignin biosynthetic genes.
- Si enhances photosynthesis by increasing chlorophyll content and stomatal conductance under low light.
- Si decreases the drastic shading effects on stem stability.
- Si application in intercropping could be effective in decreasing lodging.

Abstract

In order to improve soybean's resistance to lodging, silicon (Si) solutions at concentrations of 0,100, 200,300 mg kg^{-1} were applied during the seedling stage. The Si accumulation in different parts of the plants, the photosynthetic parameters of leaves and chlorophyll content, the stem bending resistance, the expression of genes of lignin biosynthesis and associated enzyme activity and sap flow rates were measured at early and late growth stages. The potential mechanisms for how Si improve growth and shade tolerance, enhances lodging resistance and improves photosynthesis were analyzed to provide a theoretical basis for the use of Si amendments in agriculture. After application of Si at 200 mg kg^{-1}, the net photosynthetic rate of soybeans increased by 46.4% in the light and 33.3% under shade. The application of Si increased chlorophyll content, and fresh weight of leaves, reduced leaf area and enhanced photosynthesis by increasing stomatal conductance. The activity of peroxidase (POD), 4-coumarate:CoA ligase (4CL), cinnamyl alcohol dehydrogenase (CAD) and phenylalanine ammonia-lyase (PAL) increased

during pre-and post-growth periods, whereas Si also increased lignin accumulation and inhibited lodging. We concluded that Si affects the composition of plant cell walls components, mostly by altering linkages of non-cellulosic polymers and lignin. The modifications of the cell wall network through Si application could be a useful strategy to reduce shading stress in intercropping.

Keywords: silicon; lignin biosynthesis; lodging resistance; gene regulation

Introduction

Silicon (Si) is the second most abundant element in the soil after oxygen (Luyckx et al., 2017). Because of its strong affinity for other ions, Si is rarely present in pure form but is found as silica (SiO_2), silicic acid (H_4SiO_4), and silicate (Zhang et al., 2012). In the earth's crust, Si is deposited in the form of quartz (SiO_2), sand and sandstone. In living organisms, Si occurs as amorphous silica and soluble silicic acid (Deshmukh et al., 2013). In plants, Si reduces the angle between stems and leaves and alters canopy structure, changes plant morphology, making them erect and robust, increases chloroplast size, grana number and chlorophyll content, improves photosynthesis and promotes accumulation of dry matter (Feng et al., 2010; Tripathi et al., 2016). Silicon forms a "cuticle-double Si layer" in leaves. One layer is located in the middle of the cell wall and cuticle layer, and the other layer combines with cellulose in the cell wall to reduce water transpiration and invasion of diseases and pests (Ma and Yamaji, 2006) . Si also inhibits and alleviates the toxic effects of heavy metals, activates phosphorus in soil and promotes its absorption and utilization along with other nutrients by plants (Jianfeng and Takahashi, 1990; Jianfeng and Takahashi, 1991; Tripathi et al., 2015; Wang et al., 2019).

The biological role of Si in plant growth has been extensively investigated, but Si is still not recognized as an essential nutrient (Luyckx et al., 2017; Rastogi et al., 2019). In plants, Si occurs mainly as free monosilicic acid, amorphous silica gel, and polysilicic acid forms (Mitani et al., 2005). These are mainly found in the epidermal tissues, cell walls, intercellular spaces or outer cell structures such as roots and xylem (Ma et al., 2011; Mitani et al., 2005). The content of Si in plants is usually measured as percentage of SiO_2 in dried plant material. The majority of dicots such as cucumbers, melons, strawberries, and soybeans (*Glycine max* (L.) Merr.) absorb Si passively (Mitani and Ma, 2005). In some plants such as tomatoes and beans, Si solubility is low and the element is biogeochemically immobile (Liang et al., 2006). The Si content differs according to plant species and also varies with environmental conditions (Nishimura et al., 1989; Rodrigues et

al., 2015). For example, the Si content in the Gramineae family was 10-20 times higher than that in the Leguminosae and other dicotyledonous plants (Debona et al., 2017). Studies have found that Si increased the thickness at the base of rice stems, reduced internode length, improved the erectness of leaves, changed plant canopy structure, increased photosynthesis, and enhanced lodging resistance (Ma and Feng, 2004). Former studies proposed that Si improves photosynthesis through increasing stomatal conductance, photosynthetic electron transport rate and regulating Rubisco enzymes (Agarie et al., 1992; Maghsoudi et al., 2016; Zhang et al., 2018). Silicification of sclerenchyma cells and thickening of pachydermal tissues inhibited lodging in rice plants. During the reproductive growth phase, Si content increased bending resistance of the rice stalk (Dorairaj et al., 2017) and soluble sugar content of maize (Abdel Latef and Tran, 2016). Furthermore, Si affects the composition of plant cell walls, mostly by altering linkages of non-cellulosic polymers and lignin (Głazowska et al., 2018).

Low light due to shading is a common abiotic stress that can reduce the growth of intercropped plants, and those planted at high density in greenhouses, and agroforestry systems (Hussain et al., 2019a; Hussain et al., 2019b). Soybean is intercropped with maize on 667,000 hectares in southwest China and suffers from shade stress at the early vegetative stage (Liu et al., 2017b; Yang et al., 2018). One of the most serious effects of shade stress is lodging (Yang et al., 2018). In multiple cropping systems, decreased photosynthesis due to shading caused poor transport of carbohydrates to the stem resulting in low stem strength. In our former studies, we proved that structural carbohydrates such as lignin and cellulose and nonstructural carbohydrates such as sucrose and soluble sugars, are important mediators of stem strength (Hussain et al., 2019b; Hussain et al., 2019c). Low light condition had a significant impact on lignin biosynthesis and metabolism (Boerjan et al., 2003) through effects on activity of the enzymates phenylalanine ammonia-lyase (*PAL*), peroxidase (*POD*), 4-coumarate: CoA ligase (*4CL*) and cinnamyl alcohol dehydrogenase (*CAD),* which are involved in lignin biosynthesis and vary with genotype and environment (Koyama et al., 2012; Liu et al., 2017a). Comparisons of plants with low light-resistant and low light-susceptible genotypes confirmed that shade tolerant plants had higher lignin content (Wang et al., 2015). The enzymatic activities *(PAL, CAD, 4CL* and *POD*) were also higher in shade-resistant plants compared to shade-susceptible plants (Hussain et al., 2020a). Under low light stress, stems contain low lignin concentration, which weakens them and leads to lodging and severe yield loss (Liu et al., 2018). At present, Si amendment has been tested primarily on rice and

other grasses, and most of these studies were in monoculture environments. There have been few studies on the effects of Si on susceptibility to lodging in intercropped leguminous plants. While the biological role and importance of Si in plant growth has been extensively investigated, Si still has not been recognized as an essential nutrient even though there is little active Si that can be directly used by plants.

In consideration of Si's many beneficial attributes, we hypothesized that foliar application of Si might effectively improve soybean lignin biosynthesis under low light stress. The objectives of this study were to investigate the variations in photosynthetic activity and chlorophyll pigments, growth and biomass, sap flow, lignin content and associated enzyme activities, and the expression of lignin biosynthesis genes in soybeans grown under light and shade conditions with different Si application levels.

Materials and Methods
Experimental Design

The experiment was conducted at Sichuan Agricultural University, Wenjiang district, Chengdu, Sichuan, China in 2018. The experimental design included complete randomization of the Nandou-032-4, shade-sensitive soybean cultivar grown in pots. Five to ten seeds were sown in each pot (30 cm diameter, 20 cm deep). The soil was a loam with pH 6.7, total nitrogen 2.1g kg^{-1}, magnesium 21.3g kg^{-1}, available phosphorus 24.5mg kg^{-1}, available potassium 121mg kg^{-1}, and available nitrogen 130 mg kg^{-1}. The experimental setup was divided into 5 groups: (1) control (CK) with normal light of 1250 μmol/m^2/s; (2) shaded plants (low light at 350 μmol/m^2/s) with no Si (S0); (3) shaded plants with Si treatment at 100 mg/kg (S1); (4) shaded plants with Si treatment of 200 mg/kg (S2); and (5) shaded plants with Si treatment of 300 mg/kg (S3). The shading condition was provided by using black shade net and light intensity measured by HR350 (Hipoint Inc., Gaoxiong, Taiwan). The Si was applied as Na$_2$SiO$_3$·9H$_2$O through foliar spraying, with five pots for each treatment and three replicates. Physiological effects of salt usually require doses exceeding 50 mM and the amount of sodium in our treatments is very low, so effects due to salt are unlikely (Abdul

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Qados, 2011). The first foliar treatments were applied when the true leaves unfolded, and sprayings were done every 7-10 days after that, for a total of three times. The soybean seedlings were sampled at the vegetative (V6) and reproductive (R5) growth stages to determine the effects of growth phase on experimental parameters.

Leaf area and fresh leaf weight

Three plants from each treatment were harvested to measure the leaf area with a CI-203 CID Bio-Science Portable Instruments for Precision Plant Measurement Inc. (Camas, WA, USA). The fresh weight was measured using an electronic balance with an accuracy of 0.001mg.

Determination of photosynthetic parameters

The newest fully expanded trifoliate leaves of the soybean plants from each treatment were selected to measure photosynthetic rate (Pn), stomatal conductance (Gs), intercellular CO_2 concentration (Ci) and transpiration rate (Tr) using a Li-6400 portable photosynthesis system (LI-COR Inc., Lincoln, NE, USA) under steady light intensity from 09:00-11:00. Chlorophyll content was measured with a portable chlorophyll meter (SPAD-502, Konica Minolta, Inc., Tokyo, Japan).

Collection of xylem sap

Xylem sap was collected by exudation after the plants were decapitated at the stem about 2 cm above the root base. Soft rubber tubes were fixed over the decapitated stem and the xylem sap was collected by a micropipette for 1 h, after discarding the exudates obtained during the first few minutes.

Snapping Resistance

Snapping resistance was tested with a digital stem strength meter (YYD - Type 1, Zhejiang Top Instrument Co., Ltd.) (Hussain et al., 2020a).

Lignin content

Fresh first internode stem samples (0.3 g) were ground in liquid N_2 with a pestle in a mortar. The samples were transferred into a centrifuge tube containing 95% ethanol, thoroughly mixed and centrifuged at 5000 x g for 4 min. The supernatants were discarded, and the pellets were rinsed

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twice with 80% ethanol to remove soluble metabolites after which they were rinsed with acetone and dried in a drying oven at 60°C. Dried samples were incubated at 80°C for 2 h in a 4:1 (v/v) mixture of acetic acid: acetyl bromide, then cooled to room temperature and transferred to 50 mL volumetric flasks containing 2 M NaOH, 5 mL acetic acid and 7.5 M hydroxylamine hydrochloride, which were added to terminate the reaction. Acetic acid was added to each sample to maintain a constant volume. The sample's absorbance at 280 nm was measured by spectrophotometry (Shimadzu UV-2450, Japan) and lignin content was calculated by the following formula:

Lignin content (mg/g) = $0.075 \times (\Delta A_{280} - 0.0068) \div W \times T$

Where, ΔA_{280} is absorbance at 280 nm, W is sample weight in g, and T is the dilution factor.

Silicon content

Dried leaves (0.2 g) were microwave digested with 3 ml concentrated HNO_3 + 2 ml H_2O_2 for 1 h. Digested samples were diluted with 15 ml deionized H_2O, transferred into 25 ml plastic flasks, mixed with 1 ml concentrated HF and left overnight at room temperature. After the addition of 2.5 ml of 2% (w/v) H_3BO_3, the volume was adjusted to 25 ml with deionized H_2O, and Si was determined by inductively coupled plasma optical emission spectroscopy (ICP-OES) (PerkinElmer, NexION 2000) after a final 1:100 (v/v) dilution of the samples with deionized H_2O. For measuring soluble Si in the nutrient solution, aliquots were diluted 1:100 (v/v) with deionized H_2O and Si content was determined by ICP-OES as previously described (Jelena et al., 2013).

Activity of lignin biosynthetic enzymes

Five representative plants were selected from each pot. Middle sections from the second stem internode were frozen in liquid nitrogen and placed at -80 °C for later determination of enzyme activity. The activity of phenylalanine ammonia-lyase (PAL), 4-coumaric ligase (4CL), peroxidase (POD), and cinnamyl alcohol dehydrogenase (CAD) were determined by ELISA (JiangLai, ShangHai, China) (Liu et al., 2017a).

Gene expression of key enzymes in lignin biosynthesis

Gene expression of *PAL, POD, CAD*, and *4-CL* was assayed with the Quant Studio 6 Flex Real-Time PCR System (Thermo Fisher Scientific, USA). The primers designed for expression analysis are shown in Table 1. Young parts of the stem were removed at the V6 and R5 stages and

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immediately preserved in liquid nitrogen and transferred to -80° C. The samples were ground, RNA was extracted according to (Wen et al., 2020), and the relative expression of the *POD, CAD, 4-CL* and *PAL* genes was determined by real-time fluorescent quantitative PCR

Statistical analysis

Comparisons among different treatments were performed using Duncan's multiple range tests and SPSS (version 19.0 Chicago, USA). The graphical representation was made with Microsoft Power Point 2010, Adobe InDesign 2020 and SigmaPlot.

Results

Effect of silicon on photosynthetic parameters

In comparing the normal light (CK) with the low light treatment (S0) with no Si, we found that the net photosynthetic rate, stomatal conductance, and transpiration rate of soybean leaves decreased by 42.6%, 21.1%, and 80.8% respectively, whereas, the intercellular carbon dioxide concentration increased by 54.8% (**Fig. 1**). Compared to growth in shade without Si (S0), the foliar application of Si significantly increased the net photosynthetic rate, stomatal conductance, and transpiration rate of soybean leaves, while the intercellular carbon dioxide concentration decreased in S1, S2, and S3. Compared to S0, an Si application rate of 200 mg/kg (S2) was observed to be most effective in improving the net photosynthetic rate (113.2%), stomatal conductance (80.2%), and transpiration rate (688.7%), while the intercellular carbon dioxide concentration decreased. Comparing S2 with S3, the first three important photosynthetic parameters, net photosynthetic rate, stomatal conductance and transpiration rate, decreased, while the intercellular carbon dioxide concentration increased in S3.

Effects of silicon on chlorophyll content in soybean leaves

From **Fig. 2** it can be seen that under low light conditions, foliar application of Si significantly increased the chlorophyll content of leaves, but this decreased with later Si applications. At the 6th trifoliate stage (V6 stage), the chlorophyll content was maximally increased after treatment with 200 mg Si/kg.

Effect of silicon on leaf morphology

From S0 to S3 treatments, the plants exhibited a typical shade avoidance reaction. Under shade,

the leaf area increased significantly in S0 for enhancing the ability to obtain light, but the fresh leaf weight decreased significantly, perhaps due to higher evapotranspiration under high light condition (**Fig. 3**). In the shaded environment, Si application significantly reduced leaf area, increased the fresh weight of leaves and enhanced the absorption and utilization of light. The leaf area per plant showed the largest decrease of 53.8% in with S3 compared to S0. The fresh leaf weight per plant increased significantly with S1 (43.4%) and S2 (52.9%) relative to S0.

Bleeding sap

Sap flow reflects the active water absorption capacity of the roots and can be used to evaluate their physiological activity. As can be seen in Fig. 4, after shading in S0, the sap flow from the cut stems was significantly reduced. When exogenous Si was applied to shaded soybeans, however, the wound flux gradually increased with increasing Si concentration. The sap flow reached maximum in S2 treatment, 159.7% higher than S0, but did not increase after that. This observation suggests that an appropriate Si application can enhance soybean root activity and promote the absorption of water and nutrients.

Effect of silicon on lignin content of stems

The low light condition in S0 significantly reduced the lignin content and Si accumulation in the second internode. After the application of Si in S2, the Si content in the stem base was significantly increased 75.9%, while lignin concentration went up by 133.3% compared to S0 (**Fig. 5**). Correlation analysis of lignin content and breaking resistance at the base of stems with Si content (**Fig. 6**) showed that the lignin content and the snapping resistance of soybean stems were significantly positively correlated with Si content. The histochemical structure of stem cross-sections with regard to lignin localization was also analyzed (**Fig.7**).

Activity of key lignin synthesis enzymes

Activity of the four key enzymes involved in lignin synthesis was determined in the second internode of stem at the seedling stage (V6 stage). It was found that shading (S0) significantly reduced the activity of POD, CAD, 4CL and PAL compared to CK. After Si application, the enzyme activity first increased and then decreased with increasing Si concentration. POD enzyme activity followed the trend: S2>S1>S0>S3 (**Fig. 8**). The activity of CAD, 4CL and PAL enzyme was as follows: S2>S1>S3>S0. These results suggest that Si increases lignin content by promoting the activity of enzymes responsible for lignin synthesis. Higher lignin levels in the stems improves

their mechanical strength and makes them more resistant to lodging under low light conditions.

Gene expression of key enzymes in lignin synthesis

Real-time quantitative PCR was used to measure the expression of the four enzymes primarily involved in lignin biosynthesis at the vegetative (V6) and reproductive (R5) stages (**Fig. 9**; note that the y-axes for V6 and R5 data are different). The relative expression levels *of POD, CAD, 4CL* and *PAL* in the two growth stages were not significantly different between S0 and CK. However, the relative expression levels of the four genes in the later growth stage were significantly reduced compared with that in the early growth stage. After Si application, the relative expression levels of *POD, CAD*, and *PAL* genes were significantly increased in the V6 phase of soybean growth, and reached a maximum value with S2 treatment (S2>S3>S1>S0). The relative expression levels of the *4CL* genes did not differ significantly with Si treatments. At the R5 stage, the relative expression of the *POD* gene was significantly reduced compared with that of S0, while the S2 treatment showed the lowest value. The relative expression of *CAD, 4CL*, and *PAL* genes in general increased compared to S0 treatment. At the V6 growth stage, *POD, CAD,* and *PAL* were significantly higher in S2, with the exception of *4-CL*. However, at R5 *POD* and *CAD* expression were significant at S0 and S2 respectively while *4-CL* and *PAL* were significant at S1. The incremental expression of the four key lignin synthesis genes after Si treatment may be one of the main reasons for the increased lignin concentration in soybean stems.

Discussion

Photosynthesis and chlorophyll

With intercropping, the taller crop shades the lower growing one. In former study maize reduced the active photosynthetic radiation reaching the leaves of the shorter soybeans, which lowered the net photosynthetic rate and restricted normal growth and development (Fan et al., 2019; Yao et al., 2017). Previous studies have found that applying an appropriate amount of Si to different crops under abiotic stress can increase the chlorophyll content and improve the photosynthetic rate (Lu et al., 2018; Pei et al., 2010; Wang et al., 2019; Zhang et al., 2018). Silicon was also found to improve the net photosynthetic rate, stomatal conductance, and chlorophyll content of tobacco grown in soil contaminated with cadmium (Lu et al., 2018). The addition of Si increased the ratio of chlorophyll a/b and enhanced electron transport chain and photosynthesis of plants by activating photosynthesis related genes (Song et al., 2014). Si can enhance photosynthesis in rice by changing

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the leaf structure and the chlorophyll content (Wang et al., 2019). The results of current study showed that the net photosynthetic rate, stomatal conductance, and transpiration rate of soybean leaves decreased under low light conditions. The intercellular carbon dioxide concentration increased, which reduced the photosynthetic rate of the plants, consistent with previous studies (Chuang-Dao et al., 2011; Yang et al., 2017). In our experiments, the net photosynthetic rate, stomatal conductance, and transpiration rate of the leaves of soybean plants under normal light or shade were significantly increased and the intercellular carbon dioxide concentration was significantly reduced after the appropriate amount of Si was applied. Other studies have shown that Si had no effect on chlorophyll content (Gao et al., 2011). Si application can reduce intercellular carbon dioxide concentration by increasing the net photosynthetic rate, stomatal conductance, and transpiration rate of soybean leaves.

Acclimation to low light intensities involves a complex of changes in the photosynthetic apparatus collectively known as the shade syndrome. This involves specific alterations in the organization and/or abundance of protein complexes in the thylakoid membranes (Timperio et al., 2012), lower levels of photosystem II (PSII), ATP synthase, cytochrome b/f (Cyt b/f) complex, and components of the Calvin-Benson cycle, especially low rubisco content (Leong and Anderson, 1984), reduction in thickness of the mesophyll layer, and changes in number and structure of chloroplasts (Oguchi et al., 2003; Terashima et al., 2005). Together, these effects contribute to significant decrease in photosynthetic capacity in shaded leaves (Zivcak et al., 2014). The results of this study demonstrated that Si application could alleviate the shade syndrome, probably through a complex of responses leading to recovery of photosynthetic capacity, as reported earlier Si recovers photosynthetic process under stress (Ming et al., 2012). One of the obvious responses at the leaf level was an increase in fresh weight per leaf area in silicon-treated plants, which logically would be associated with an increase in thickness of the mesophyll layer. However, it must be emphasized that the exact molecular mechanism and the pathways leading to alleviation of the shade syndrome in Si treated plants is still unknown.

Leaf Morphology

Previous studies have found that the morphological and anatomical structures of plant leaves change greatly under low light conditions (Feng et al., 2019; Hussain et al., 2020b). Plants adapt to shading by reducing leaf thickness and increasing leaf surface area (Terashima et al., 2006; Toshiaki et al., 2005). This study showed that the leaf area of shaded soybeans increased and the

fresh leaf weight decreased, in agreement with the results of Evans and Poorter (2010) and Terashiman et al. (2006). This change was caused by a decrease in leaf thickness and an increase in specific leaf weight. However, some studies have found that plant leaves become smaller after exposure to low light conditions (Gyung-Tae et al., 2005; Toshiaki et al., 2005; Wanzhuo et al., 2014), which may be the result of different shading degrees. Previous studies reported that Si increased the length of rice leaf epidermal cells and the ductility of the cell wall (Gyu et al., 2002), increased leaf thickness, ATP content and the number of chloroplasts and mitochondria per cell, and thickened vascular bundles to promote growth under stressful condition (Ju et al., 2017; Xu et al., 2017). Si reversed some effects of shading, producing thicker leaves with greater mass per leaf area, consistent with the rest of the results. Si had similar effects on plants under drought (Gong et al., 2003) and salinity stress (Bradbury and Ahmad, 1990). The application of Si to shaded soybean seedlings in the current experiments significantly reduced the leaf area, increased the fresh weight and thickness, and improved the efficiency of leaf absorption and utilization of light energy.

Lodging resistance, lignin content, enzyme activity, and gene expression

Previous studies demonstrated how a plant's resistance to lodging was positively related to the content of lignin and Si. For example, in grasses the higher the lignin content at the base of the stem, the stronger the synthase activity, the larger the stem diameter and the stronger the resistance to lodging (Peng et al., 2014; Zheng et al., 2017). As expected, an increase in lignin content enhanced the mechanical strength of plant stem and reduced lodging (Bhuiyan et al., 2009; Ma, 2009; Tripathi et al., 2003). The increase of lignin content in plants is achieved by increasing the activity of the lignin biosynthesis enzymes and gene expression during lignin synthesis. In support of our findings, it has been reported that *PAL, 4-CL, CAD,* and *POD* enzyme activity were significantly and positively correlated with lignin content (Boudet et al., 2003; Hu et al., 2016; Wang et al., 2015). The content of lignin decreased with decreasing *PAL* activity and increased with *PAL* overexpression (Luo et al., 2008).

Studies on rice cell wall mutants showed positive correlation between biomass and Si levels with respect to cellulose, hemicelluloses and lignin content (Zhang et al., 2015). Increased Si stimulated deposition of cellulose and hemicelluloses in rice, suggesting further involvement of Si in the formation of cell walls (Zhang et al., 2015). Hemicelluloses were identified as ligands binding Si in cell walls of rice cell cultures (He et al., 2015; He et al., 2013; Zhang et al., 2015). In cell walls of the maidenhair fern, *Adiantum raddianum*, Si co-localized with epitopes of homogalacturonans

and 1,5-arabinans (Leroux et al., 2013). This is in agreement with our findings showing the histochemical structure and lignin content of stems from Si-treated soybean plants (**Fig.7**). Furthermore, it has been previously reported that Si application significantly increased the transcription of *PAL* and *4CL*, which enhanced the biosynthesis of monolignols (Fleck et al., 2011). The current study showed that the activity of *POD, CAD, 4CL* and *PAL* enzymes and the content of lignin were significantly reduced in shade-grown soybeans. However, Si supplementation increased the activity of these four key enzymes in the stem showing a single peak curve change, which first increased and then decreased, mirrored by similar changes in expression of the *POD, CAD*, and *PAL* genes. The lignin and Si content of the stem showed the same trend with maximum expression in S2. Differences in the relative expression of genes regulating the activity of 4CL were not significant. We conclude that appropriate concentrations of Si can promote the activity of key enzymes in lignin synthesis and increase the expression of related genes, thereby increasing the content of lignin in the stem and improving the lodging resistance ability of shaded, intercropped soybeans.

Conclusions

Application of Si at appropriate concentrations increases net photosynthetic rate (Pn), stomatal conductance (Gs) and transpiration rate (Tr) of soybeans, reduces intercellular carbon dioxide concentration (Ci) and increases chlorophyll content. At the same time Si can reduce the leaf area and increase the fresh weight and leaf thickness of soybeans under shading, thus enhancing photosynthesis. Si also increased the stem strength and lodging resistance index, which was significantly and positively correlated with the Si content of the stems (r = 0.896). The lignin content of soybean stems under shaded conditions was maximal at an Si treatment of 200 mg/kg. Therefore, suitable application of Si could enhance lodging resistance and nutrient transport in soybean plants. The application of Si increased the xylem and phloem thickness in stem cross sections stem under shading, increased the cross-sectional area of the stem base, and increased the xylem and phloem proportion. Si fertilization results in increased absorption and accumulation of Si in stems, which promotes *POD, CAD, 4 CL* and *PAL* gene expression and enzyme activity producing lignin and increasing stem strength and resistance to lodging. The hypothetical mechanism of Si-mediated lignin biosynthesis is shown in **Fig. 10**. Future genomic profiling, RNAseq transcriptome studies, proteomics and methylome analyses should be undertaken to

reveal more of the details and deepen our understanding of the benefits of Si application in low light environments.

Author Contributions

Sajad Hussain: Writing Original Draft, Conceptualization, Software, Visualization. **Li Shuxian:** Reviewing, Data Curation. **Maryam Mumtaz:** Valiadation. **Marian Brestic:** Investigation and Resources. **Nasir Iqbal:** Reviewing and Editing. **Iram Shafiq :** Conceptualization. **Muhammad Shoaib:** Discussion. **Wang Li:** Reviewing Methodology. **Xu Mei:** Chemical Analysis. **Chen Bin:** Data Curation. **Qin Sisi:** Investigation and Resources. **Marek Zivcak:** Reviewing and Editing. **Anshu Rastogi:** Reviewing and Editing. **Milan Skalicky:** Formal Analysis**: Vaclav Hejnak:** Investigation and Resources **Liu Weiguo:** Project administration and Supervision. **Yang Wenyu:** Reviewing and Supervision

Conflicts of Interest

All authors declared no conflict of interest.

Acknowledgments

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Table 1. Soybean primer information

GeneID	Primer sequence
GmPOD	5'-GATTAGGTGAGCGTTATG-3'
	5'-TTATGAGAAGATGGATGGA-3'
GmCAD	5'-TAATCACACCAGACAGAA-3'
	5'-GCAGCATTAGCAATATCA-3'
Gm4CL	5'-ACGATGACGATGAGTTAT-3'
	5'-TAGGATGAGTGAGAAGAAG-3'
GmPAL	5'-CAAGGATATTCAGGCATTAGATT-3'
	5'-CCACGAAGAGGAAGACAT-3'
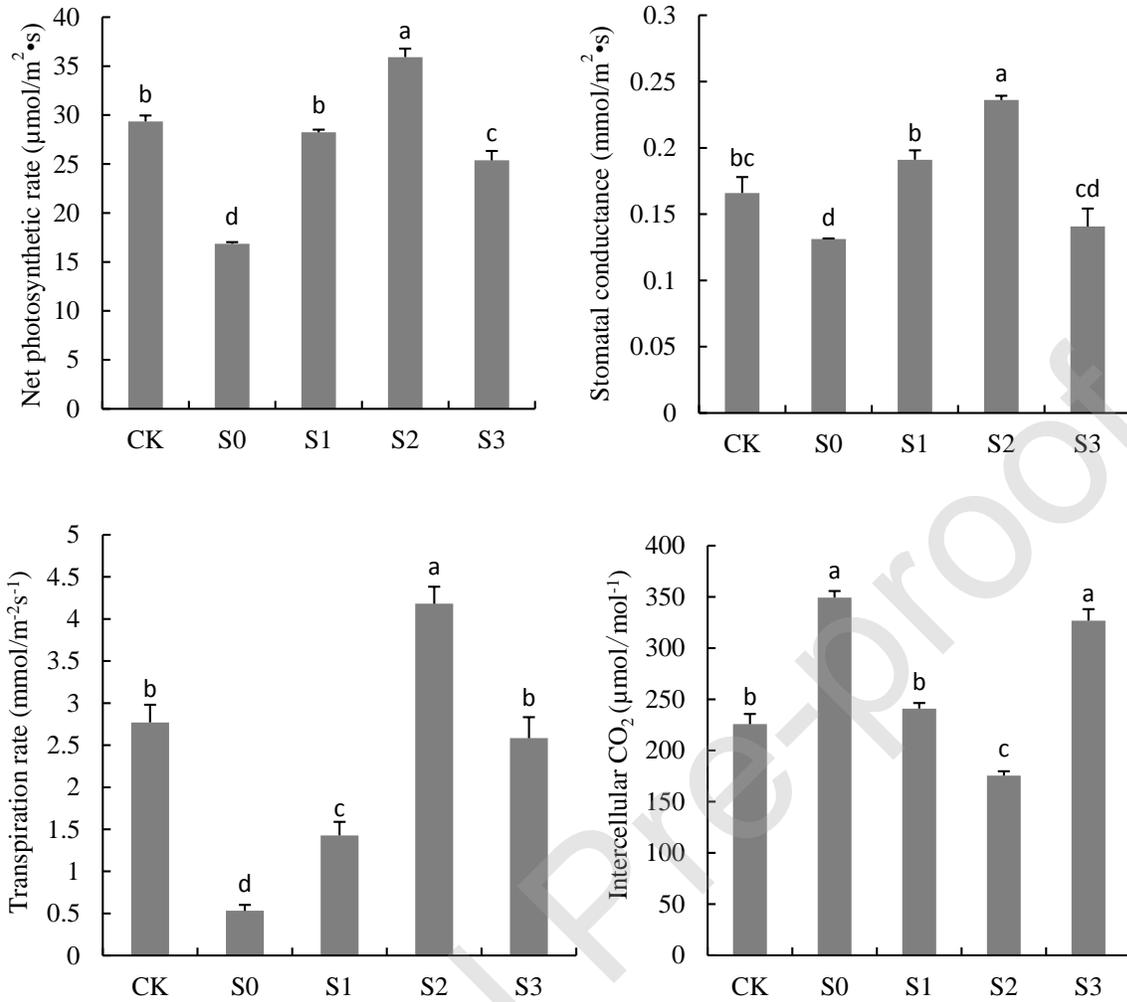
Figure 1. Effects of silicon on net photosynthetic rate, stomatal conductance, transpiration rate and intercellular carbon dioxide concentration of soybean leaves. CK = normal light; S = shade; S0, 0 mg Si/kg; S1, 100 mg Si/kg; S2, 200 mg Si/kg; S3, 300 mg Si/kg. Values are the mean of three replicates ±SD. Lowercase letters above error bars indicate significant difference ($p < 0.05$) between treatments.
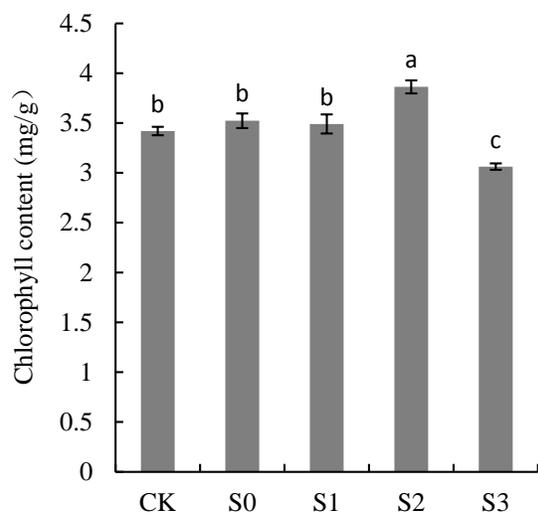
Figure 2. Effect of silicon on chlorophyll content in soybean leaves. CK = normal light; S = shade; S0, 0 mg Si/kg; S1, 100 mg Si/kg; S2, 200 mg Si/kg; S3, 300 mg Si/kg. Values are the mean of three replicates ±SD. Lowercase letters above error bars indicate significant difference ($p < 0.05$) between treatments.
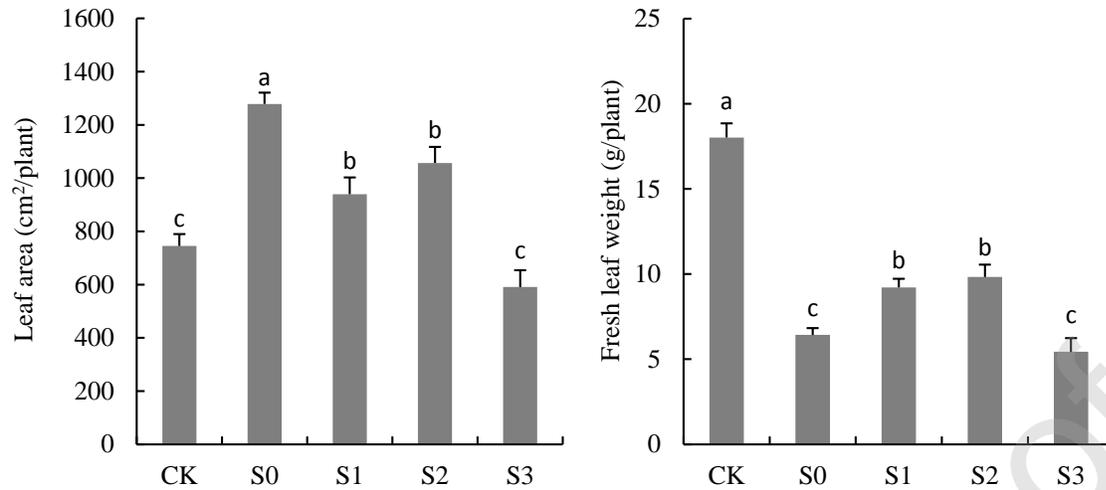
Figure 3. Effects of silicon on leaf area and fresh weight. CK = normal light; S = shade; S0, 0 mg Si/kg; S1, 100 mg Si/kg; S2, 200 mg Si/kg; S3, 300 mg Si/kg. Values are the mean of three replicates ±SD. Lowercase letters above error bars indicate significant difference ($p < 0.05$) between treatments.
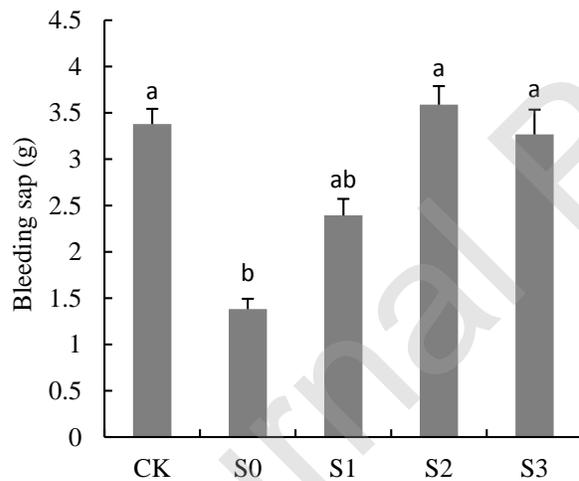
Figure 4. Effects of silicon on sap bleeding from roots. CK = normal light; S = shade; S0, 0 mg Si/kg; S1, 100 mg Si/kg; S2, 200 mg Si/kg; S3, 300 mg Si/kg. Values are the mean of three replicates ±SD. Lowercase letters above error bars indicate significant difference ($p < 0.05$) between treatments.

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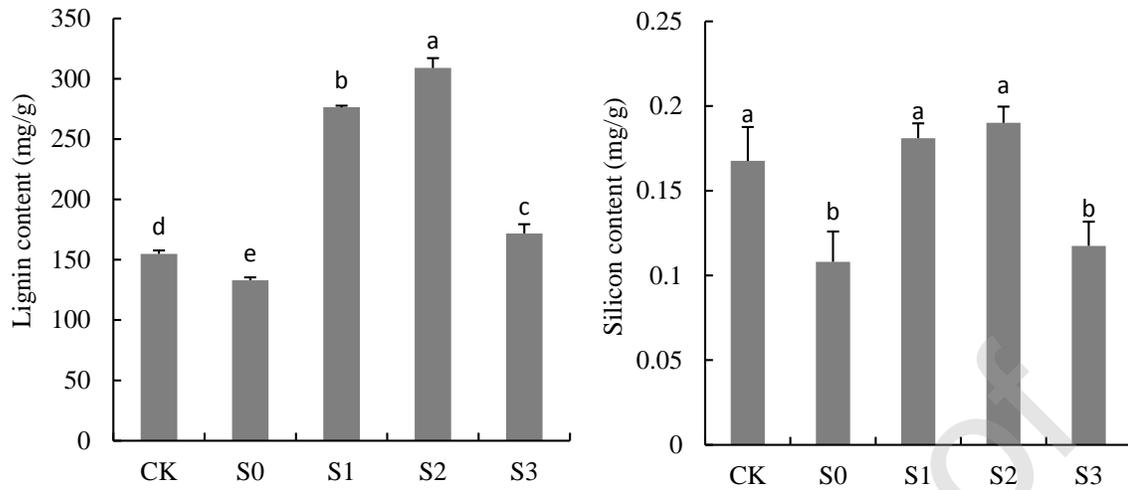
Figure 5. Effects of silicon on lignin and silicon contents in soybean stems. CK = normal light; S = shade; S0, 0 mg Si/kg; S1, 100 mg Si/kg; S2, 200 mg Si/kg; S3, 300 mg Si/kg. Values are the mean of three replicates ±SD. Lowercase letters above error bars indicate significant difference ($p < 0.05$) between treatments.
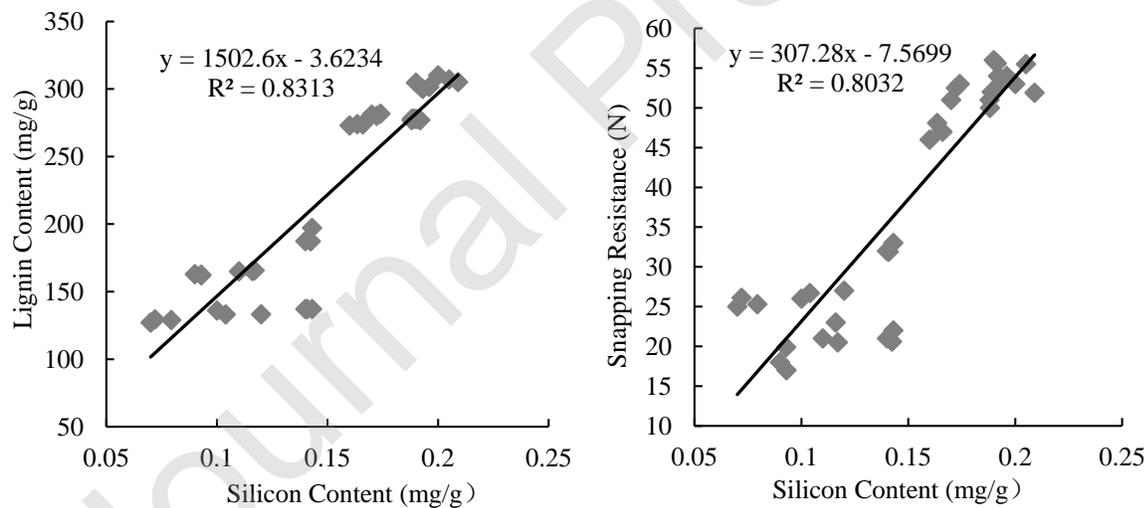
Figure 6. Relationship between lignin content, snapping resistance and silicon content in soybean stems.

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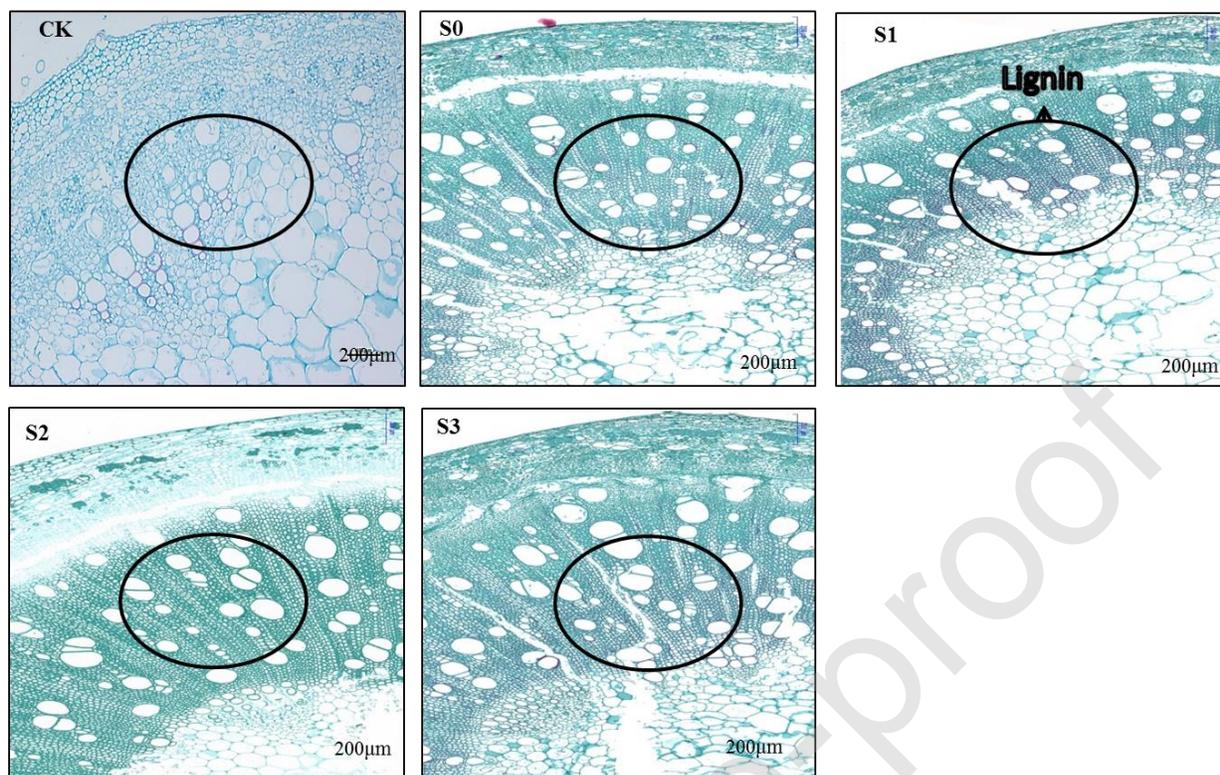
Figure 7. Histochemical analysis of the lignin content under CK = normal light; S = shade; S0, 0 mg Si/kg; S1, 100 mg Si/kg; S2, 200 mg Si/kg; S3, 300 mg Si/kg. The pink color in the red circle represents lignin content. Bar = 200 μm.
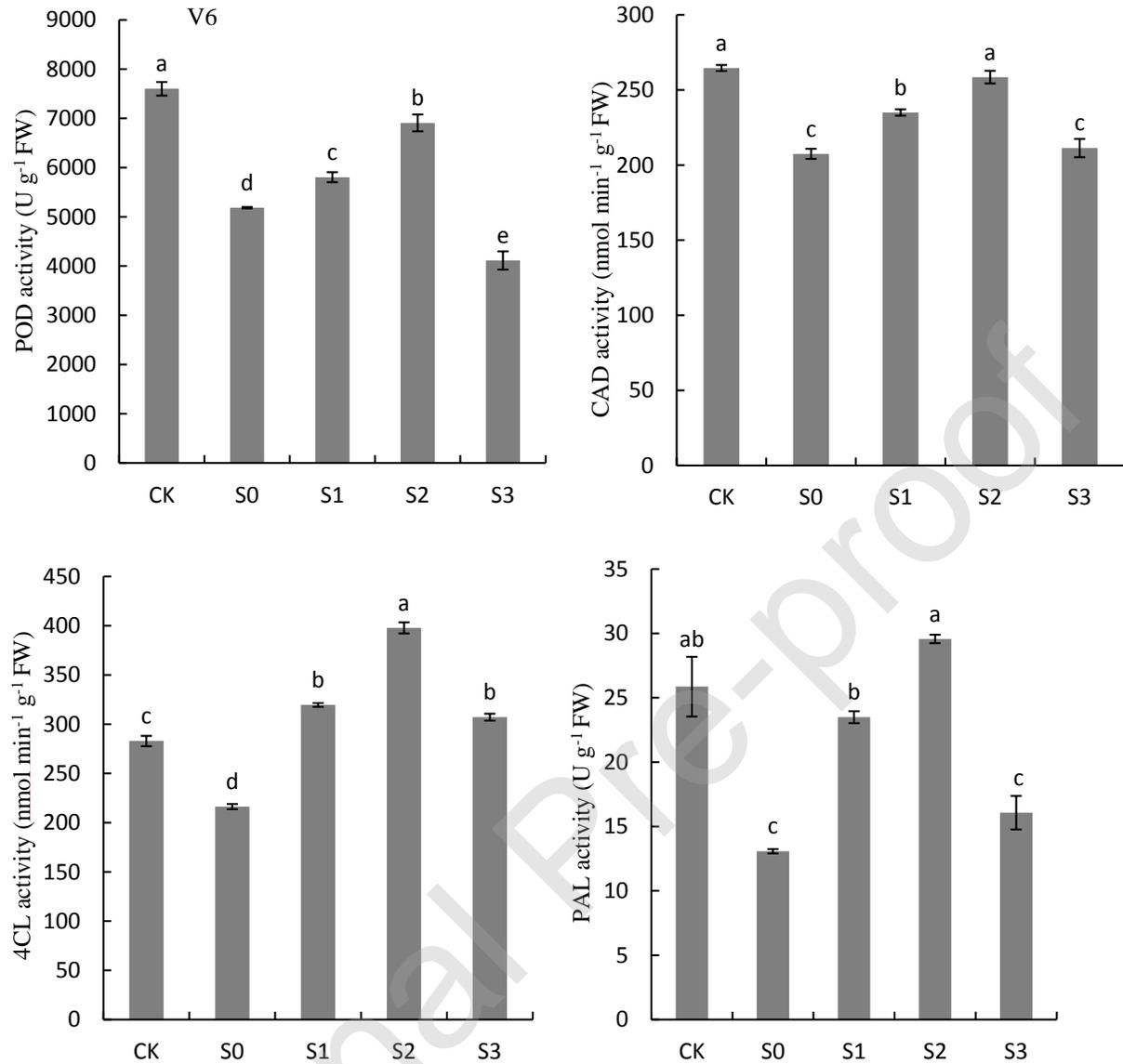
Figure 8. Effects of silicon on activities of key enzymes in lignin synthesis in soybean stems. CK = normal light; S = shade; S0, 0 mg Si/kg; S1, 100 mg Si/kg; S2, 200 mg Si/kg; S3, 300 mg Si/kg. Values are the mean of three replicates ±SD. Lowercase letters above error bars indicate significant difference ($p < 0.05$) between treatments.
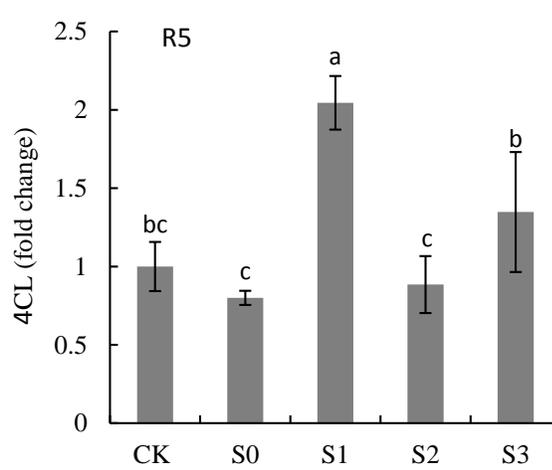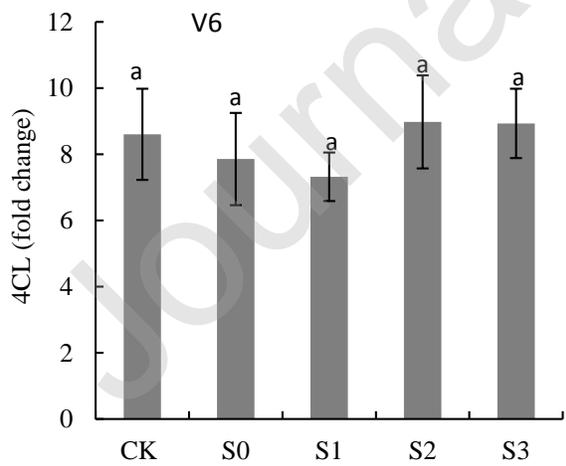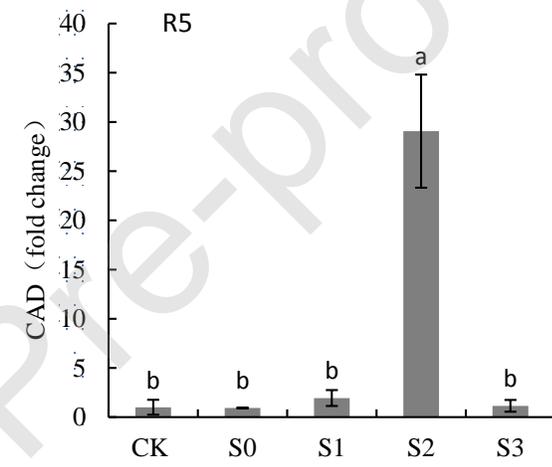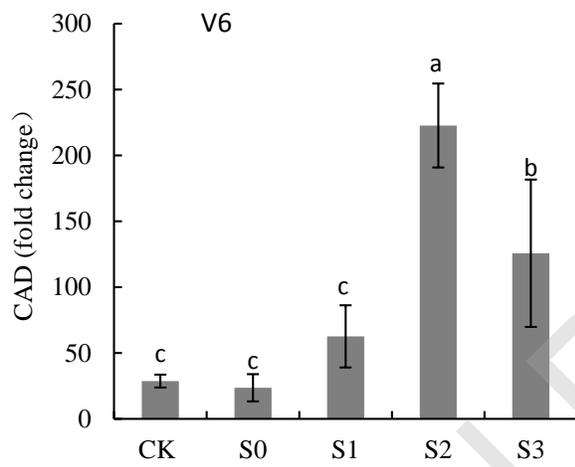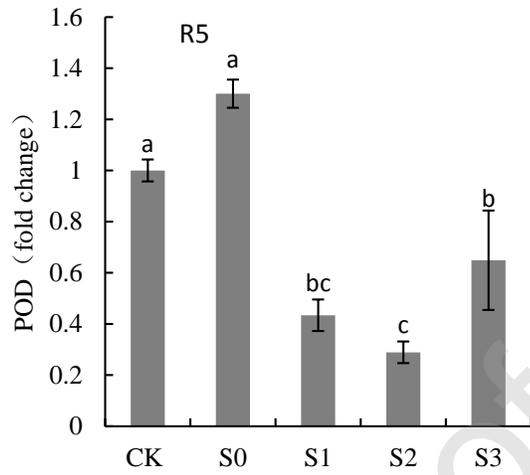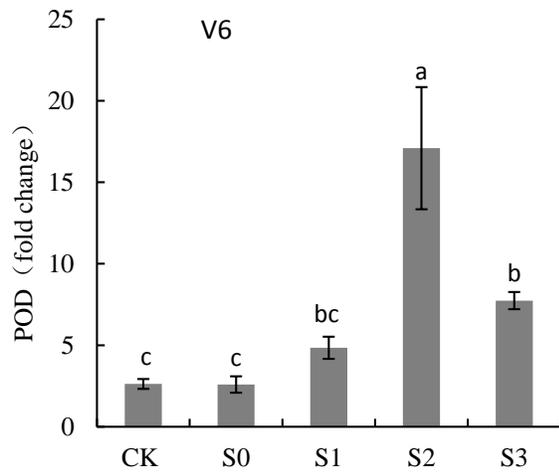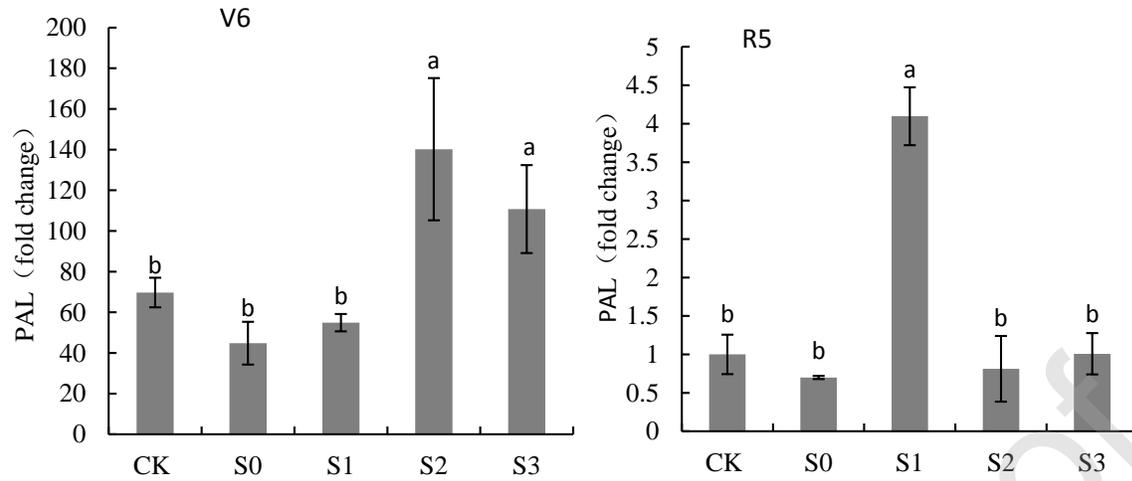
Figure 9. Effects of silicon on gene expression of key enzymes in lignin synthesis in soybean stems during the vegetative phase (V6) and the reproductive phase (R5). CK = normal light; S = shade; S0, 0 mg Si/kg; S1, 100 mg Si/kg; S2, 200 mg Si/kg; S3, 300 mg Si/kg. Values are the mean of three replicates ±SD. Lowercase letters above error bars indicate significant difference ($p < 0.05$) between treatments.
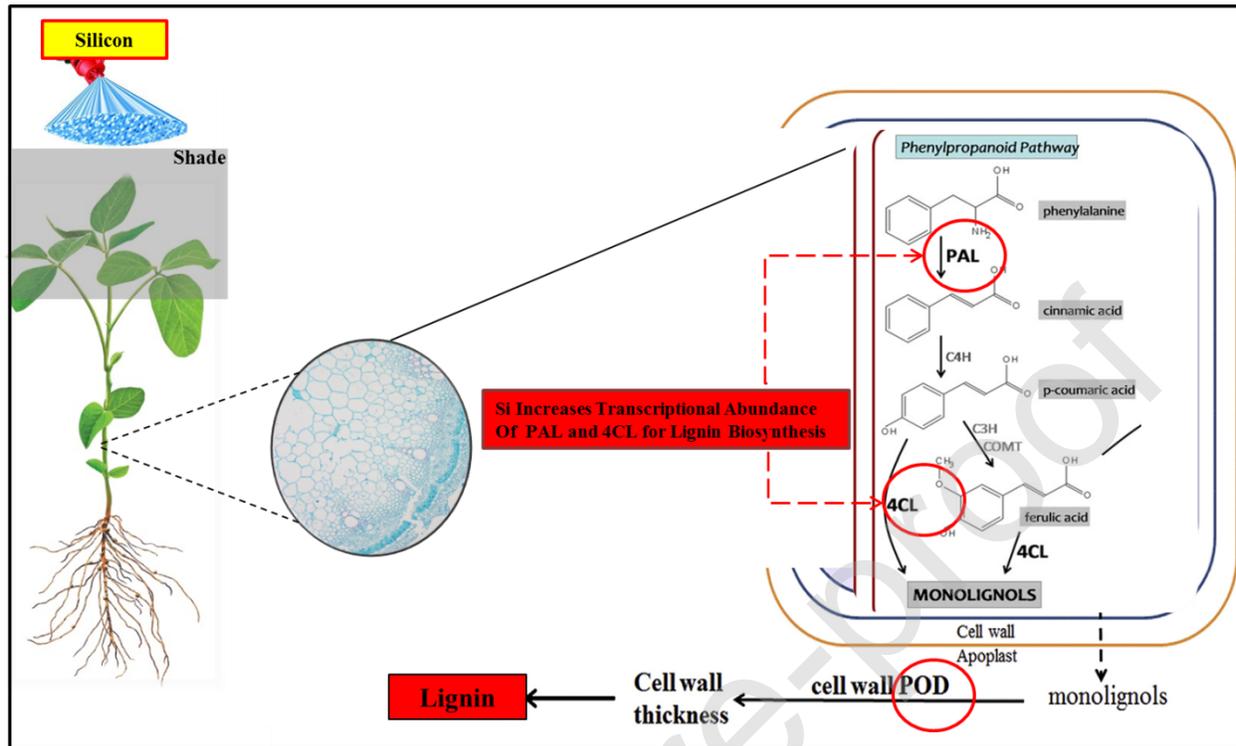
Figure 10. Hypothetical mechanism of Si promotion of lignin biosynthesis.

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