



Strigolactone Enhances Alkaline Tolerance in Soybean Seeds Germination by Altering Expression Profiles of ABA Biosynthetic and Signaling Genes

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Abstract

Soybean is an important oil crop used as a plant protein and oil resource by humans, worldwide. However, abiotic stresses have significantly inhibiting soybean seed germination and yield. Plant hormones can improve stress tolerance by regulating gene expression and physiological processes. In this study, the effect of exogenous SL (strigolactone) was studied on germination and post-germination attributes of soybean grown under alkaline stress. Alkaline stress showed significant delayed response of the germination process and also reduced the radicle length. The qRT-PCR (real-time quantitative PCR) analysis showed that alkali stress increased the expression levels of *GmABF4*, *GmAAO3*, *GmAB15*, *GmNCED5*, and *GmNCED9* genes in ABA (Abscisic acid) pathways. The exogenous application of SL promoted soybean radicle length. The SL-treated plants were much more tolerant to alkali stress exhibiting higher germination rates, longer radicles, increased fresh weight, and higher catalase enzyme activity as compared to control plants. After alkaline stress, the expression of SL biosynthetic and signaling genes, such as *GmD27*, *GmCCD8*, *GmMAX2* and *GmDAD2*, was substantially increased under SL exogenous application while expression patterns of ABA biosynthetic and signaling genes, such as *GmAB15*, *GmNCED9*, and *GmAAO3*, were decreased. In conclusion, these results exhibited a positive role of SL on seed germination attributes via alleviation of ABA-mediated germination inhibition, improved antioxidant system as well as efficient biosynthesis, perception, and signaling of SL hormone.

Keywords Strigolactones · Abscisic acid · Soybean · Alkaline stress

Introduction

Land alkalization is an important agriculture-based environmental issue and an emerging threat worldwide that strongly influence crop yield and quality (Yang et al. 2009). The total area of saline-alkaline soils is about 831 millions/

ha in china and more than 70% is affected specifically in north-eastern China (Wang et al. 2008). Alkaline stresses are high pH, composed of soda salt, such as Na_2CO_3 and NaHCO_3 , which by releasing HCO_3^- or CO_3^{2-} ions exert a damaging effect on plants (Yang et al. 2008). Furthermore, alkaline stress inhibits absorption of some anions, such as NO_3^- , Cl^- , H_2PO_4^- , disturbs the ionic balance as well as results in iron deficiency and impaired root elongation and growth (Liu and Guo 2011; Zhang et al. 2019).

Strigolactones (SLs) are biosynthetic derivatives of carotenoids, synthesized as a result of sequential oxidative cleavage with two carotenoid cleavage dioxygenases named CCD7 and CCD8. Recent research revealed a significant connection between SLs and ABA in multiple physiological mechanisms especially under stress conditions (Lopez-Raez et al. 2010). As ABA and SL share the same biosynthetic origin from carotenoids; therefore, their interactive studies at the regulatory and biosynthetic levels are currently in progress (Haider et al. 2018). The roles of SLs in hypocotyl

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growth, root development, seedlings photomorphogenesis, secondary growth as well as in different steps of symbiotic relationship in response to drought and salt stresses and mycorrhizal colonization have been recognized so far (Al-Babili and Bouwmeester 2015; Roman et al. 2016). Furthermore, SLs also contribute in the process of seed germination as well as in early seedling development of model plant *Arabidopsis* (Toh et al. 2012). In arbuscular mycorrhizal *Sesbania cannabina* seedlings, the elevated levels of H₂O₂ due to higher NADPH oxidase activity further regulate SL production which improves plant tolerance against salt stress (Kong et al. 2017). Whereas, in another study reported that fungal symbiosis induced the SL levels in tomato and lettuce plants by altering levels of ABA and SL corresponding marker genes expression, such as *NCED9*, *CCD7* and *CCD8*, and ultimately results in drought stress tolerance (Ruiz-Lozano et al. 2016).

Recently, we have identified some SL biosynthetic and signaling genes in soybean genome. And these genes were positively induced under salt and alkaline stress conditions (Qiao et al. 2020). However, the effect of SLs on soybean seed germination attributes and its possible mechanism under alkaline stress is still unknown. Therefore, in this study, we reported first time the effect of SL exogenous application on soybean seed germination and post-germination attributes, its role in the alleviation of alkali stress with special emphasis on ABA and SL biosynthetic and signaling pathway genes.

Materials and Methods

Plant Materials and Growth Conditions

The soybean (DN50) seeds were disinfected with 10 ml of 30% H₂O₂ and 75 ml of 95% ethanol and rinsed five times with distilled water. The seeds were uniformly sown in Petri dishes lined with wet filter paper. The seeds were germinated in dark conditions with 22–28 °C room temperature environmental conditions.

Plant Treatments

For alkaline treatment, the soybean seeds were transferred in distilled water with 0 mM, 75 mM or 100 mM NaHCO₃ solution. For SL treatment, the seeds were transferred in distilled water with 0.5, 1 or 2 μM SL solution. For +NaHCO₃/+SL treatments, the soybean seeds were transferred in distilled water with 100 mM NaHCO₃ and then subjected with 0, 0.5, 1 or 2 μM SL solution, respectively. All treated seeds were placed in dark environmental conditions.

Phenotypic Analysis

The germination rates were calculated upon emergence of radicle through the seed coat as well as length of radicle was measured. The germinated seeds were collected as three biological replicates and stored at –80 °C for further assays.

Determination of Catalase Activity

Soybean seeds treated with 100 mM NaHCO₃ and +NaHCO₃/+SL (100 mM NaHCO₃ and 1 μM SL) solution. The treatments seeds were harvested at 24 h and 48 h to investigate the CAT activity. Catalase activity was determined by measuring the conversion rate of H₂O₂ to H₂O and oxygen molecules, as described by Chance and Maehly (Chance and Maehly 1955).

Total Plant RNA Extraction, cDNA Synthesis and Gene Expression Analysis

Total RNA was extracted using Omini plant RNA isolation kit (Cwbio, Beijing) and transcribed in to cDNA using First-Strand cDNA Synthesis kit (Toyobo, Japan) according to the protocol. All the primers were designed using Primer 5.0 software. The internal reference gene *GADPH* and SL biosynthetic and signaling gene primers used in this study are listed in Table S1. For gene expression analysis, the soybean seeds were treated with +NaHCO₃/+SL (100 mM NaHCO₃ and 1 μM SL). Samples were collected at different time intervals, such as 0, 6, 12, and 24 h. qRT-PCR was performed on the ABI 7500 fluorescence sequencer.

Statistical Analyses

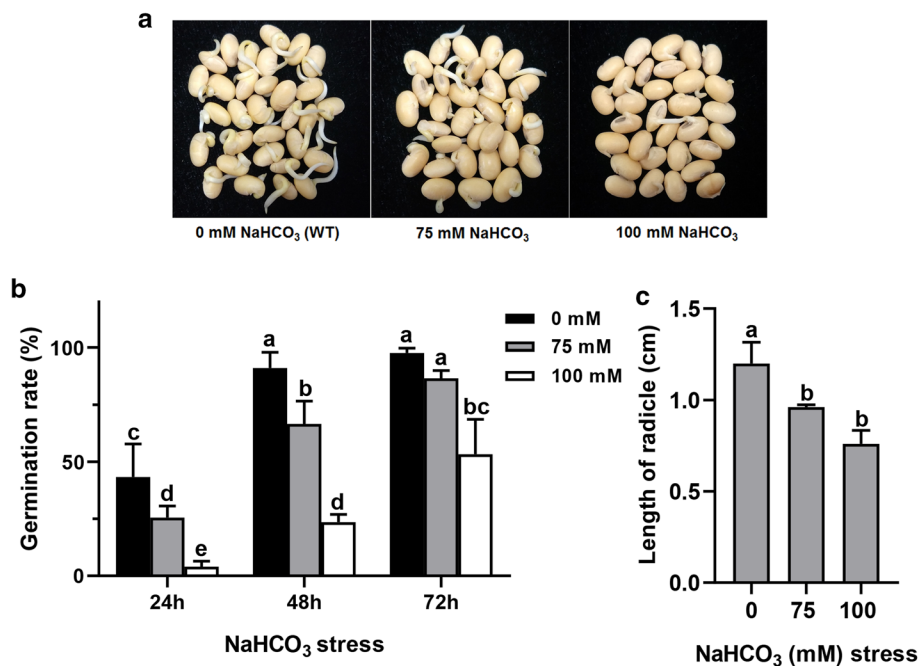
The length of radical and germination rates were calculated by Least Significant Difference test (LSD) and different letters indicate statistical differences among means (*p* value < 0.05). The three biological and technical replicates qRT-PCR data were calculated using the 2^{-ΔΔC_t} method and LSD.

Results

Exogenous NaHCO₃ Application Delayed Seed Germination Rates in Soybean

The germination analysis revealed that alkali treatment delays the seed germination process (Fig. 1a, b). It was observed that plants treated with 75 mM and 100 mM alkali exhibited less germination percentage as compared

Fig. 1 NaHCO_3 hinders soybean seed germination. Different alkali concentrations (0 mM, 75 mM and 100 mM) were used while ultra-pure water was used as a control. **a** The images are shown for soybean after sowing 72 h. **b** Germination rate analysis. **c** Radicle lengths of germinated soybean seeds. Each bar indicates mean of three replicates \pm standard errors. The different letters on bars indicate significant differences using Least Significant Difference test at p value < 0.05

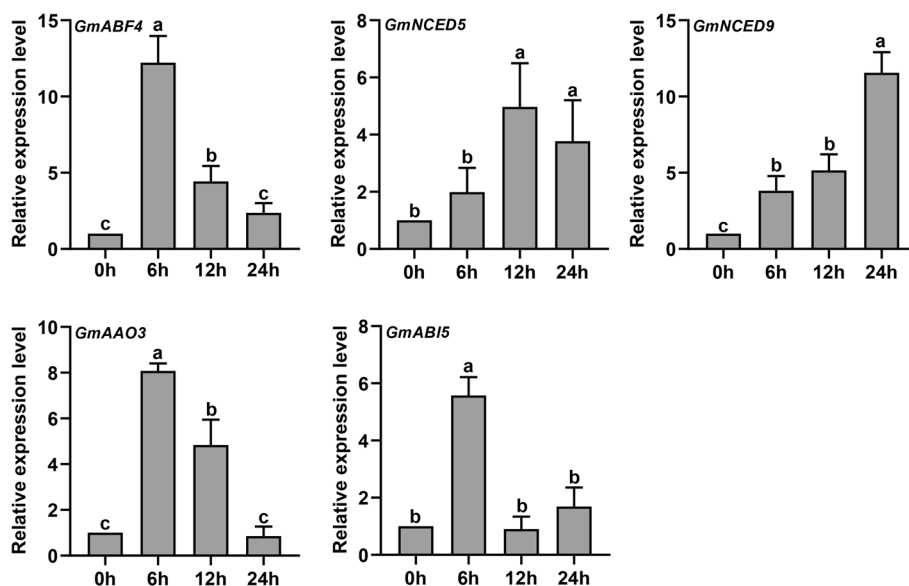


to control. The soybean germination rate was increased with the passage of time, and as 2- to sixfold increase in germination rates was observed after 72 h of different alkali treatments than 24 h and 48 h time period. To further verify the inhibition effect of alkali, post-germination analysis such as radicle length of germinated seeds was calculated (Fig. 1 c). Exogenous NaHCO_3 treatment had a remarkable inhibition effect on radicle length with 26% and 34% decrease under 75 mM and 100 mM alkali treatments as compared to control. These results showed that the soybean germination process was significantly delayed through under alkaline stress.

Alkali Treatment Upregulated the ABA Biosynthetic Genes Expression During Germination Stage

To analyze the link between the inhibitory effect of NaHCO_3 on seed germination and effects on the ABA pathway genes, we investigated the transcript patterns of ABA biosynthetic and signaling genes in a time course manner (Fig. 2). The qRT-PCR results revealed that all ABA pathway genes were significantly induced under alkaline stress. For example, the transcript levels of *GmABF4*, *GmABI5* and *GmAAO3* by 12, 4.5, and eightfold under alkali stress, respectively. The alkali treatment also enhanced the expression levels of *GmNCED5*

Fig. 2 Expression patterns analysis of soybean ABA biosynthetic and signaling genes in response to alkali stress. The seed were supplemented by 100 mM NaHCO_3 for 0, 6, 12 and 24 h. The gene transcript data results were assessed using the $2^{-\Delta\Delta C_t}$ method. Each bar indicates mean of three replicates \pm standard errors. The different letters on bars indicate significant differences using Least Significant Difference test at p value < 0.05



and *GmNCED9* at 12 h and 24 h, respectively. Collectively, the transcript pattern analysis of germinating soybean seedlings showed that the biosynthesis and signaling genes of ABA were enhanced by alkali treatment.

SL Improved the Delayed Germination Phenotype of Alkali Treated Seeds

Studies on SLs have been revealed its role in seed thermo-inhibition in *Arabidopsis* (Toh et al. 2012). To further confirm its role in seed germination process of soybean, the effect of SL treatment on the phenotype of germinating soybean was determined (Fig. 3a, b). The results showed that the effect was non-significant on soybean seed germination after SL treatments.

To further evaluate the role of SL in alkaline stress, the 0.5, 1, and 2 μM SL treatments were applied along with 100 mM NaHCO_3 and germination rate of soybean was observed (Fig. 3c, d). The results showed that no significant difference was observed among different treatments after 24 h and 48 h. However, after 72 h of sowing, significant increase in germination rate was observed by the application of 0.5 μM and 1 μM SL under alkaline stress. The SL-treated plants were more tolerant to alkali stress and highest germination rates were observed under 1 μM SL (69%) which

was statistically similar with 0.5 μM SL (60%) application after 72 h.

Furthermore, the effects of SL, NaHCO_3 , and + NaHCO_3 /+SL applications were observed on post-germination attributes, such as on radicle length and fresh weight (Fig. 4 a, b). The results showed that SL treatment of 0.5 μM SL increased the length of radicle (31%) as compared to control plants after 72 h of treatment. The effect on SL was not-significant on fresh weight. When plants were subjected under alkali stress, 1 μM and 2 μM SL treatments increased the radical length, as compared to plants only treated with 100 mM NaHCO_3 . Also, the application of 1 μM SL under NaHCO_3 application showed significant increase in fresh weight of soybean seedlings as compared to plants only treated with 100 mM NaHCO_3 . These results suggest that SL application can enhance germination rates, radicle length, and fresh weight of soybean under alkaline stress condition.

Relative Expression Levels of SL and ABA Biosynthetic and Signaling Genes under + NaHCO_3 /+SL Treatment

To assess the possible roles of SL and ABA biosynthetic and signaling genes in soybean germination stage under + NaHCO_3 /+SL treatment, we further analyzed the

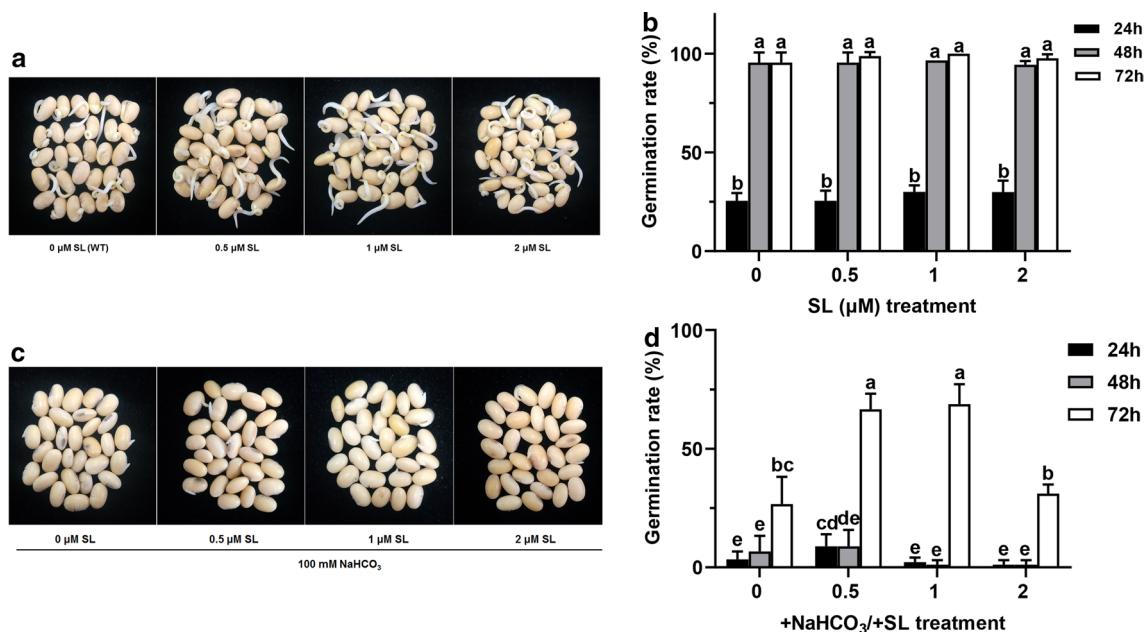


Fig. 3 Soybean seed germination analysis under SL or + NaHCO_3 /+SL treatments. Different SL concentrations (0, 0.5, 1 and 2 μM) and 100 mM NaHCO_3 condition were used while ultrapure water was used as a control. **a** The images are shown for soybean sowing under different concentrations of SL treatments. **b** Germination rate analysis under SL application. **c** The images are shown for

soybean after sowing 72 h with different SL concentrations under 100 mM NaHCO_3 condition. **d** Germination rate analysis with different SL concentrations under 100 mM NaHCO_3 condition. Each bar indicates mean of three replicates \pm standard errors. The different letters on bars indicate significant differences using Least Significant Difference test at p value < 0.05

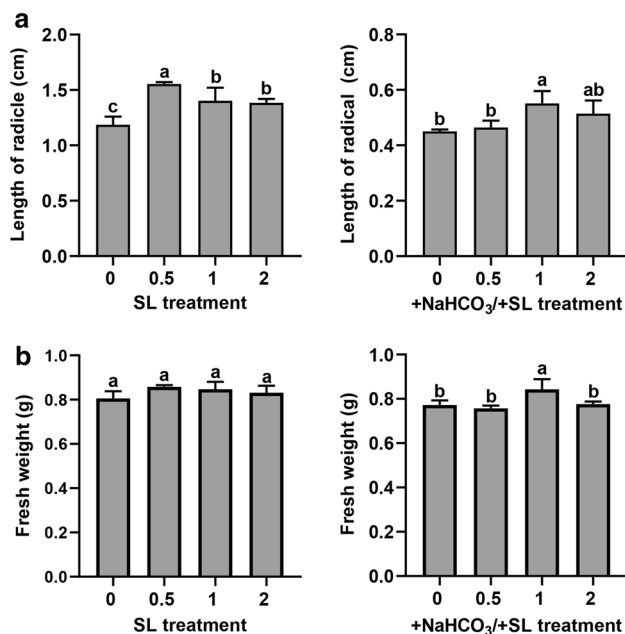


Fig. 4 SL promotes radicle length (a) and fresh weight (b) of soybean seedlings under SL or +NaHCO₃/+SL treatments. Different SL concentrations (0, 0.5, 1 and 2 μM) were used while ultra-pure water was used as a control. Each bar indicates mean of three replicates ± standard errors. The different letters on bars indicate significant differences using Least Significant Difference test at *p* value < 0.05

expression patterns of some important pathway genes by qRT-PCR analysis (Fig. 5). After treatment of SL under alkaline stress, the significant increase in transcript expression level of various SL biosynthetic genes, such as *GmD27* (fourfold), *GmCCD8* (threefold), *GmMAX2* (fivefold) and *GmDAD2* (12-fold), was observed at 6 h. However, the expression levels of *GmCCD7* and *GmD14* were decreased. In case of ABA signaling genes, increase in *GmABF4* (two-fold) expression level was noted at 12 h. Whereas expression levels of *GmABI5*, *GmNCED9* and *GmAAO3* decreased as compared to control. However, the expression level of *GmNCED5* showed a mixed pattern, with 13% decrease at 6 h followed by 24% increase at 12 h. The results showed that SL and ABA biosynthetic and signaling genes were significantly regulated under +NaHCO₃/+SL treatment, suggested that SL and ABA signaling may cross-talk in soybean seed germination under alkali stress.

Effect of +NaHCO₃/+SL Application on Catalase Activity During Seed Germination

To elucidate the oxidative status of soybean germinating seedlings after exogenous treatments of NaHCO₃ and SL, the catalase activity (CAT, an oxidative stress parameter) was investigated (Fig. 6). The results showed that CAT enzyme activity was significantly increased after 24 h and

48 h of exogenous alkali treatment as compared to control. The combined treatment of +NaHCO₃/+SL caused a significant increase in the CAT activity as compared to NaHCO₃. There was a fourfold and eightfold increase in CAT activity at 24 h and 48 h of +NaHCO₃/+SL, respectively, as compared to NaHCO₃ alone. The results indicated the potential role of SL for protection against oxidative damage in alkaline environment.

Discussion

SLs were primarily discovered as an “ecological signal” for the development of symbiotic relationship among plants and useful microbes as well as for seed germination in parasitic weeds (Xie et al. 2010). But in recent years, SLs have drawn more attention due to their significant regulatory roles in several physiological and molecular processes during the plant adaptations under abiotic stresses (Mostofa et al. 2018; Niu et al. 2021). Recently, the role of SLs and their interaction with other plant hormones such as ABA have been investigated to improve salt and drought resistance (Haider et al. 2018; Ren et al. 2018). In this study, the effect of exogenously applied SL on seedling growth and germination rate of soybean under alkali stress conditions was investigated. Alkali stress greatly reduces seed germination rates, radicle elongation as well as plant biomass due to increasing concentration of carbonate salts (Li et al. 2010). Similar effects were observed in our study, as 75 mM and 100 mM alkali stress significantly reduced the germination rates and radicle lengths (Fig. 1). In the past, soybean alkali tolerance-related key genes and proteins have been identified (Kan et al. 2015; Qiao et al. 2020). But, the detailed molecular mechanisms about the inhibitory effect of alkali on soybean seed germination and post-germination attributes are not fully explained, especially the interconnection between alkali effects and strigolactone, ABA pathways at soybean germination stage.

SLs and ABA share common biosynthetic origin from carotenoids; therefore, it might be anticipated to study the interaction of these two hormones especially under alkaline stress conditions. In our study, the transcript pattern analysis of ABA biosynthetic and signaling genes under alkali stress conditions clearly showed upregulation of these genes at different time points (Fig. 2). Studies on *Arabidopsis* biosynthesis and signaling mutants *nced5* and *abi5* showed faster germination phenotypes as compared to wild plants (Frey et al. 2012; Piskurewicz et al. 2008), which strengthen the fact that alkali stress reduces the germination rate of soybean via efficient biosynthesis and signaling of ABA hormone which is a key regulator of germination inhibition. Similarly, overexpression of *ABA2*, *ABI4* and *ABI5* lead toward delayed germination phenotypes in different plant spp, such

Fig. 5 Expression patterns analysis of soybean SL and ABA biosynthetic and signaling genes in response to combine application of SL and alkali. The seedlings were supplemented + NaHCO₃/+SL (100 mM NaHCO₃ and 1 μM SL) for 0, 6, 12 and 24 h. The gene transcript data results were assessed using the 2^{-ΔΔC_t} method. Each bar indicates mean of three replicates ± standard errors. The different letters on bars indicate significant differences using Least Significant Difference test at *p* value < 0.05

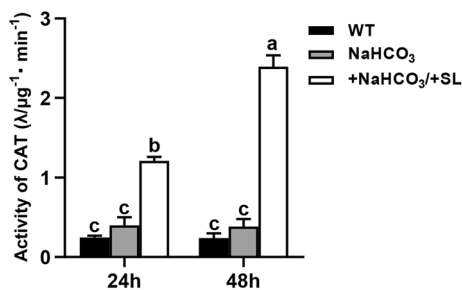
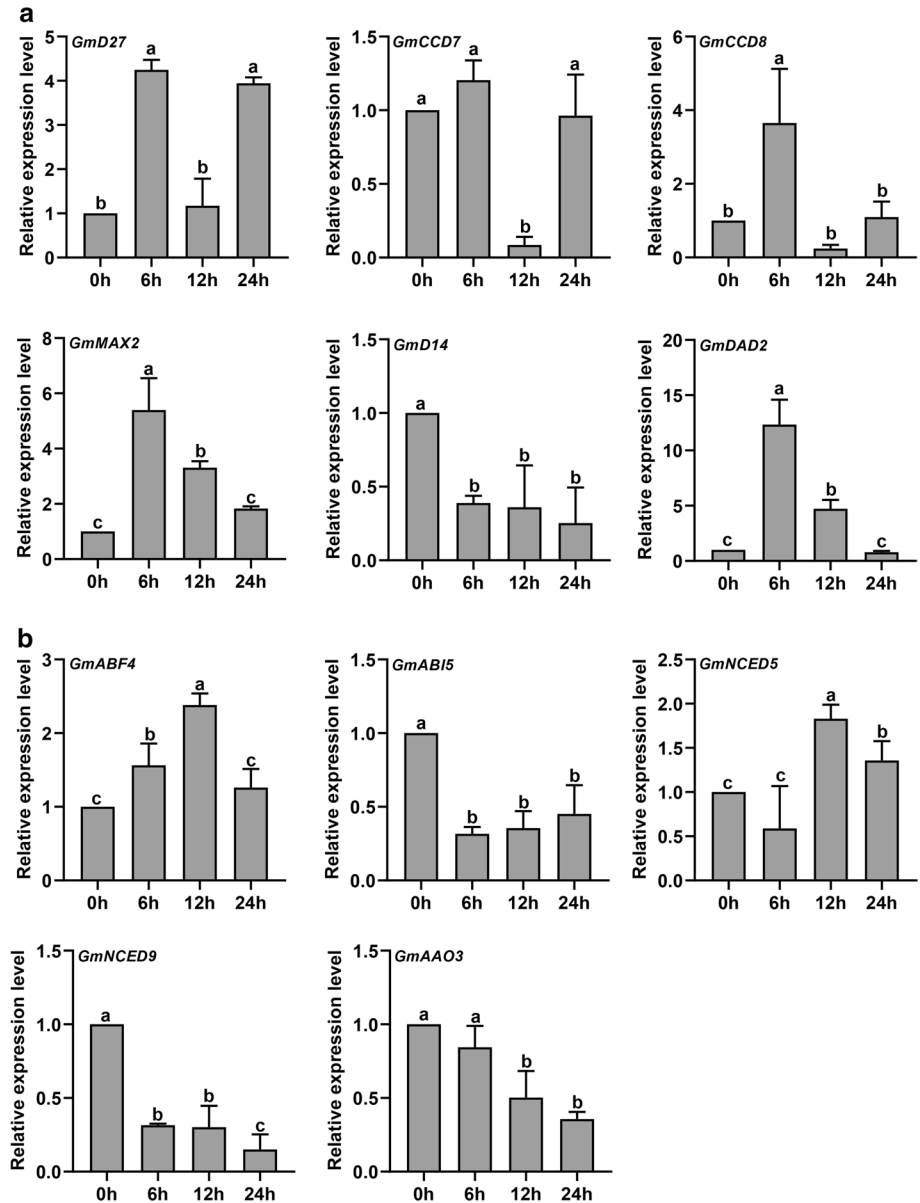


Fig. 6 CAT enzyme activity analysis in germinating soybean seedlings in response to NaHCO₃ and +NaHCO₃/+SL treatments. Each bar indicates mean of three replicates ± standard errors. The different letters on bars indicate significant differences using Least Significant Difference test at *p* value < 0.05

as tobacco, potato and *Arabidopsis* (Shu et al. 2013; Zhu et al. 2020). *ABI5* further induces the expression of polygalacturonase inhibiting protein 1 and 2 genes (*PGIP1* and *PGIP2*) activity which ultimately blocks seed coat rupturing and constrains seed germination (Kanai et al. 2010).

To further characterize the responses of SL application on soybean seed germination process under alkaline stress, a phenotypic analysis was performed and results revealed that SL application promoted the germination of soybean seeds under alkali treatment (Fig. 3). The +NaHCO₃/+SL-treated plants exhibited better germination rates, longer hypocotyl lengths and increased fresh biomass as compared to plants only supplemented with SL as well as with alkali (Fig. 3–4). Previously, SL has shown its promotive role in seed germination of parasitic plant spp., such as *Striga* and *Orobanche*

(Toh et al. 2012), via alteration in hormonal levels like GR24 application decreased the ABA levels through transcription repression of *NCED9*, an ABA biosynthetic gene and increased the GA levels. But, the role of SLs toward abiotic stress responses still requires more research attention especially in case of alkaline responses. In this regard, recent available reports portray SLs, a positive regulator of abiotic stress responses such as stomatal closure induction (Lv et al. 2018; Zhang et al. 2018), establishment of Arbuscular mycorrhizal symbiotic relationships under drought stress as well as increased activity of NADPH oxidase to produce H_2O_2 for better signaling (Ruiz-Lozano et al. 2016). ABA-SLs cross-talk to ameliorate salt stress was observed (Ren et al. 2018). Therefore, we proposed about SL and ABA signaling cross-talk in soybean seed germination under alkali stress.

To further emphasize the pathway mechanisms between these two hormones toward alkali stress responses, the qRT-PCR analyses were conducted at soybean seed germination stage and expression patterns of different SL biosynthetic and signaling genes as well as ABA biosynthetic-signaling genes were observed (Fig. 5). Intriguingly, our results showed that exogenous $+NaHCO_3/+SL$ application significantly enhanced the expression of SL biosynthetic genes at 6 h time point such as *GmD27*, an isomerase DWARF27 gene which converts trans- β -carotene into 9-cis- β -carotene (Alder et al. 2012); *GmCCD7* and *GmCCD8*, are carotenoid cleavage enzymes which cleave the product of *GmD27* into 9-cis- β -apo-10'-carotenal and caralactones respectively after cleavage (Booker et al. 2004). As reported by Kim et al. 2013, both *GmCCD7* and *GmCCD8* are functional enzymes involved in carotenoid metabolism of soybean root nodules, but *GmCCD8* displayed higher expression at 7- and 24-day-old nodules than *GmCCD7* which showed better expression in 2-day-old nodules as well as it also appeared in his experiments that *GmCCD8* is actively involved in cleavage of Zeaxanthin (carotenoid) than *GmCCD7*. Our data also implied that, *GmD27* and *GmCCD8* exhibited higher expression levels in comparison of *GmCCD7* under $+NaHCO_3/+SL$ treatment which might be due to active involvement of *GmD27* and *GmCCD8* genes in both SL biosynthesis and cleavage reactions. SL signal perception gene also displayed enhanced expression such as *GmDAD2*, as well as SL signal transduction gene *GmMAX2/D3* F-box protein which interacts with D14 in cytosol to enhance expression of other downstream genes for SL responses showed enhanced expression (Wang and Smith 2016). In contrast, the *GmD14* gene showed down-regulate expression under alkali and SL treatment. These data suggest that exogenous SL application at soybean seed germination stage could promote SL biosynthesis, signal perception and signal transduction under alkaline stress conditions.

Previously, we showed that *GmD27a* gene showed no alteration in gene expression both at 6 h and 12 h after alkali

treatment (Qiao et al. 2020), but in contrast under combined application of alkali stress and SL exogenous application, a significant increase in *GmD27* expression pattern was observed in our study, which clearly indicates improved SL biosynthesis under alkali stress conditions. Also, we investigated the expression levels of ABA biosynthetic/signaling genes again. However, our results showed that the expression patterns of *GmABI5*, *GmAAO3* and *GmNCED9* genes were decreased under combine application of $+NaHCO_3/+SL$. While *GmNCED5* expression decreased at 6 h time point and increased at 12. Similarly, higher *NCED6* and *NCED9* transcripts levels were observed in developing *Arabidopsis* seeds, as compared with *NCED2*, *NCED3* and *NCED5* genes.

Intriguingly, *ABI5* gene, a basic leucine-zipper transcription factor, not only inhibits activity of polyglactouronase via *PGIP1* and *PGIP2* genes to block seed coat rupturing, but also showed interactions with several plant hormone signals to control seed germination phenomenon. In this regard, study has reported that regulatory proteins of jasmonic acid called as JAZs (Jasmonate Zim Domain) could repress transcription of *ABI5* and alter seed germination in bread wheat (Ju et al. 2019). Similarly, involvement of *BES1*, a BR insensitive 1-EMS-suppressor 1 and *BZR1*, the brassinazole resistant 1 proteins to suppress the *ABI5* activity and DELLA proteins of GA signaling in induction of *ABI5* activity have been reported so far, former results in seed germination promotion and later involved in inhibition (Hu et al. 2019; Zhao et al. 2019). Further, the active involvement of *AAO3* gene in ABA biosynthesis in germinating seeds as compared to other *AAO4* and *AAO1* genes has been reported by Seo et al. (2004), whereas *AtNCED9* and *AtNCED6* genes are highly expressed in endosperm and embryo and production of ABA in both tissues, further regulate seed dormancy (Lefebvre et al. 2006). After phenotypic analysis of *nced* triple mutants in *Arabidopsis* (*nced5*, *nced6*, *nced9*) by Frey et al. (2012), he reported the active involvement of the *NCED5* gene along with *NCED6* and *NCED9* genes in induction of seed dormancy. It is expected in our study that, the decreased transcript expression levels of *GmNCED5*, *GmNCED9*, *GmAAO3* and *GmABI5* might be responsible for low levels of ABA biosynthesis which in turn inhibits seed dormancy and promote seed germination under $+NaHCO_3/+SL$ treatments. These results suggest that exogenous SL application at soybean seed germination stage could overcome harmful effects of alkali stress and promote seed germination via inhabiting ABA biosynthesis and signaling at this stage.

To further investigate the role of SL for protection against oxidative damage in alkaline environment, we calculated CAT enzyme activity (Fig. 6), a main component of ROS scavenging cascade. Our results displayed a substantial increase in CAT activity under combine

application of SL and alkali as compared to plants which only experienced alkali stress, which clearly showed positive role of SL in adjusting antioxidant enzyme activity under alkali stress conditions. Many other researchers also reported same observation in their studies, such as the induced antioxidant enzyme activities in wheat and rice under SL, ABA and salicylic acid exogenous applications (Agarwal et al. 2005; Sedaghat et al. 2017). These evidences proposed that, in most of the features which we studied, SL treatment has a significant positive influence on seed germination traits, antioxidant enzyme system as well as on SL biosynthesis, perception and signaling while negatively associated with ABA biosynthesis and signaling when applied under alkali stress conditions.

Conclusion

Under alkali stress conditions, the supplementation of exogenous SL resulted in greater germination rates and improved post-germination trait such as radicle lengths which revealed that SL could relieve the damage of high alkali stress in soybean germinating seedlings. SL positively contributes to alleviate inhibitory effects of ABA on seed germination by altering expression of different ABA biosynthetic and signaling genes under alkali stress conditions. SL application can also minimize oxidative damage under stress conditions by enhancing CAT enzyme activity. Further, increased expression levels of SL pathway genes also displayed its efficient biosynthesis, perception and signaling at seedling stage which can improve the adaptability of soybean seeds to highly alkaline environment.

Author Contributions XM, Z-u-N and XJ performed the experiment, analyzed data; Z-u-N and SA wrote the manuscript; CC, NA and SA edited the manuscript; LY, CC, and Z-u-N provided ideas, designed the research; all authors read and approved the final manuscript.

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Data Availability The data which support the findings of this work will be available from Chen Chao and Nisa, ZU.

Declarations

Conflict of interest The manuscript has been read and approved by all authors. The authors declare no conflict of interest.

Ethical Approval This article does not contain any studies with human participants or animals performed by any of the authors.

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