

Phytohormone signaling pathway for eliciting leaf phenolic synthesis in honeysuckle (*Lonicera japonica* Thunb.) under coastal saline environment

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ABSTRACT

To date, it remains unclear how saline stress induces leaf phenolic synthesis in honeysuckle (*Lonicera japonica* Thunb.), a traditional Chinese medicinal crop. This study was to investigate hormone signaling for salt-induced leaf phenolic synthesis in honeysuckle from molecular, physiological and ecological levels by pot experiments and field trials. As a major ingredient of soil salinity in coastal land, NaCl was used in salt treatment for simulating coastal saline environment. NaCl stress significantly increased leaf abscisic acid (ABA) and salicylic acid (SA) concentrations, potentiated leaf phenolic synthesis by elevating phenylalanine ammonia-lyase (PAL) activity and PALs transcription, and enhanced leaf phenolic accumulation. Under NaCl stress, tungstate and paclobutrazol pretreatments inhibited leaf ABA and SA accumulation, respectively, and lowered PAL activity, PALs transcription, and phenolic accumulation. Thus, ABA and SA participated in transducing salt-induced leaf phenolic synthesis. Paclobutrazol pretreatment hardly influenced leaf ABA concentration in salt-treated plants, but tungstate pretreatment abated salt-induced leaf SA accumulation, indicating that ABA was an upstream signal of SA. Under NaCl stress, SA application restored leaf SA level with no effect on ABA level but incompletely restored PAL activity and phenolic accumulation in tungstate-pretreated plants, confirming the downstream role of SA and implying the existence of other downstream signals. Leaf ABA and SA concentrations were higher in plants in coastal saline plots than non-saline plots, and the roles of ABA and SA in mediating salt-induced leaf phenolic synthesis passed ecological test, according to a significant positive correlation of leaf ABA and SA levels with phenolic concentration. In conclusion, ABA acted as an upstream signal of SA to elicit leaf phenolic synthesis in honeysuckle under coastal saline environment.

1. Introduction

Under saline stress, plants are confronted with osmotic pressure, ionic toxicity and secondary oxidative stress, and take use of adaptive mechanisms to maintain ion, water, and reactive oxygen species (ROS) homeostasis (Munns and Tester, 2008; Hossain and Dietz, 2016; Van Zelm et al., 2020). Phenolic synthesis is an salt adaptive response for helping control ROS level in plant tissues, but less attention has been paid to its induction mechanism compared with stomatal closure, osmolyte synthesis, and Na⁺ exclusion (Cheynier et al., 2013; Sah et al.,

2016; Zhu, 2016).

Plant phenolics comprising flavonoids, phenolic acids, and tannins are a major kind of secondary metabolites. Recent studies attached importance to bioactive phenolic ingredients in plants for producing functional foods, cosmetics and medicines (Shahidi and Ambigaipalan, 2015; Van Hung, 2016; Dra et al., 2019; Gunes et al., 2019; Hazrati et al., 2019; Al Juhaimi et al., 2020), and also investigated phenolic synthesis without concerning environmental disturbance (Liang et al., 2013; Park et al., 2015; Jia et al., 2017; Xing et al., 2018; Wang et al., 2019; Waseem et al., 2019). Phenolics are capable to scavenge ROS, and

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phenolic accumulation can strengthen antioxidant defense under environmental stresses (Grace and Logan, 2000; Cheynier et al., 2013). To date, phenolic accumulation has been widely reported in plants exposed to salt stress, but phenolic synthesis is rarely emphasized, let alone its signaling pathway. As a traditional viewpoint, phenolics play a secondary role in antioxidant protection in contrast to the primary antioxidant system involving superoxide dismutase, ascorbate peroxidase, ascorbate, etc. (Ferrerres et al., 2011; Fini et al., 2011; Agati et al., 2013). Thus, present studies mainly focus on signal transduction for the major salt tolerance mechanisms such as Na⁺ transportation, osmotic regulation and primary antioxidants rather than phenolic synthesis (Jayakannan et al., 2013; Osakabe et al., 2014; Dinnyeny, 2015; Hossain and Dietz, 2016; Per et al., 2017; Yan et al., 2018). However, phenolic synthesis becomes very important from the view of plant nutrition, and notably, saline stress can improve the value of medicinal crops by elevating accumulation of bioactive phenolic compounds (Rouphael et al., 2018; Yan et al., 2016a). So far, synergic variation of plant hormones with phenolic concentration has been reported in some plants under salt stress (Zhu et al., 2016; El-Esawi et al., 2017; Ibrahim et al., 2019; Khaleghnezhad et al., 2019; Mousavi and Shabani, 2019; Perin et al., 2019), but it still lacks sufficient evidence whether these hormones participate in mediating salt-induced phenolic synthesis. In a word, the signaling pathway for salt-induced plant phenolic synthesis remains far from clear.

Plant hormones are a series of endogenous signal molecules and regulate many physiological metabolisms. In the numerous plant hormones, abscisic acid (ABA) is defined as a stress hormone because of its crucial role in eliciting adaptive behaviors. For instance, salt-induced leaf stomatal closure and root Na⁺ exclusion depend on ABA signaling pathway (Osakabe et al., 2014; Sah et al., 2016; Yan et al., 2018). Likewise, salicylic acid (SA) also can induce salt adaptive responses such as osmolyte synthesis and prevention of K⁺ loss (Jayakannan et al., 2013; Per et al., 2017). At present, the promotional effects of ABA and SA on plant phenolic accumulation have been reported (Yang et al., 2012; Liang et al., 2013; Alonso et al., 2016; Shakirova et al., 2016; El-Esawi et al., 2017; Golkar et al., 2019; Khaleghnezhad et al., 2019; Mousavi and Shabani, 2019), whereas the inconsistent results in some studies imply that the signal transduction for phenolic synthesis is species specific (Li et al., 2010; Buran et al., 2012; Felicijan et al., 2016). Recently, ABA and SA were definitely demonstrated as signal molecules to elicit phenolic synthesis, respectively, in the leaves of *Aristolelia chilensis* upon drought stress and in the leaves of *Camellia sinensis* with elevated CO₂ exposure (Gonzalez-Villagra et al., 2019; Li et al., 2019). However, it remains not explicit whether ABA and SA mediate salt-induced plant phenolic synthesis, and moreover, the cross-talk between SA and ABA is largely unknown.

As a traditional Chinese medicinal crop, honeysuckle (*Lonicera japonica* Thunb.) is rich in bioactive phenolics such as chlorogenic acid, and has been widely used for producing various Chinese medicines including some important antiviral medicines. In our recent studies, salt tolerant honeysuckle cultivar was screened for planting in coastal saline land, and the field trials proved its capacity to desalinate coastal soil (Yan et al., 2015, 2016b). Most importantly, soil salinity improved its medicinal quality by elevating phenolic and chlorogenic acid accumulation (Yan et al., 2016a). Thus, it is of great advantage to utilize honeysuckle for exploiting coastal abandoned saline land. It should be stressed that leaf phenolic accumulation resulted from elevated phenolic synthesis in honeysuckle under coastal saline environment, however, hormone signals for eliciting leaf phenolic synthesis were still unknown (Yan et al., 2017). To date, almost all studies on signal transduction are conducted by using pot experiments, and lack ecological proof and practical value. This study was intended to explore the roles of ABA and SA in salt-induced leaf phenolic synthesis in honeysuckle from molecular and physiological levels by pot experiments and verify their function from the ecological level by field trials in coastal saline land. We hypothesized that ABA cooperated with SA to induce leaf phenolic

synthesis in honeysuckle under saline stress. Our study can provide insight into phenolic synthetic mechanism in plants, and may present a valuable guidance for agricultural practice in coastal saline land.

2. Materials and methods

2.1. Room pot experiments

Bear root honeysuckle plants were placed in plastic pots containing Hoagland nutrient solution and grown (pH 5.7) in climatic chambers (Qiushi, China). The nutrient solution was continuously aerated and refreshed once every two days. Photosynthetic active radiation intensity was controlled at 200 μmol m⁻² s⁻¹ for 12 h (07:00 to 19:00) every day, and day/night temperature and humidity were set at 25/18 °C and 65 % in the chambers. Forty days later, uniform plants were separated to seven groups. Tungstate sodium and paclobutrazol are widely used as specific inhibitors, respectively, for ABA and SA biosynthesis (Jiang and Zhang, 2002; Ren and Dai, 2012; Guajardo et al., 2016; Li et al., 2019). As a major ingredient of soil salinity in coastal land, NaCl is commonly used to conduct salt treatment for simulating coastal saline environment (Yan et al., 2016a, b; Yan et al., 2017). In the first group, control plants were pretreated by deionized water without subsequent exposure to NaCl stress. In the second group, plants were pretreated by tungstate sodium without subsequent exposure to NaCl stress. In the third group, plants were pretreated by paclobutrazol without subsequent exposure to NaCl stress. In the fourth group, plants were exposed to 200 mM NaCl for six days after pretreatment with water. In the fifth group, plants were exposed to 200 mM NaCl for six days after pretreatment with tungstate sodium. In the sixth group, plants were exposed to 200 mM NaCl for six days after pretreatment with paclobutrazol. In the seventh group, plants were pretreated by tungstate sodium, applied with exogenous SA, and then exposed to 200 mM NaCl for six days. One day before NaCl treatment, the attached leaves were immersed in tungstate sodium (1 mM) and paclobutrazol (0.1 mM) solutions for 5 min to inhibit ABA and SA synthesis, and tungstate-pretreated leaves were immersed in SA (50 μM) solution for 5 min for SA application. Tungstate sodium, paclobutrazol and SA were bought from sigma. Five replicate plants in each group were randomly selected to sample newly expanded leaves for parameter measurements.

2.2. Field trials

Field plots were established in Dongying Halophyte Arboretum in Shandong province, China (37°24'N, 118°39'E). Climate conditions, soil background and plot design have been described in our previous studies (Yan et al., 2016b, 2017). Generally, there were four replicate non-saline and saline plots, and the electronic conductance of tilth soil was 486 μS cm⁻¹ and 910 μS cm⁻¹, respectively. Three plants were randomly selected in each plot to collect newly expanded leaves in growth seasons.

2.3. Measurements of ABA and SA concentrations

ABA and SA concentrations were simultaneously analyzed according to Liu et al. (2012). ABA and SA were extracted from fresh leaf tissues, and liquid chromatography mass spectrometry was used to determine their concentrations. The extraction procedure, liquid chromatography process and analyses of mass spectrum have been illuminated in our recent study (Yan et al., 2018). In this study, ABA and SA were quantified by monitoring 263/153 and 137/93 transition based on MS/MS method.

2.4. The in situ visualization of phenolics

Small fresh leaf pieces were sampled and embedded in 3 % agarose. Transverse sections (40 mm) were prepared by using a vibrating blade microtome (Leica VT1200, Germany), immersed in absolute methanol

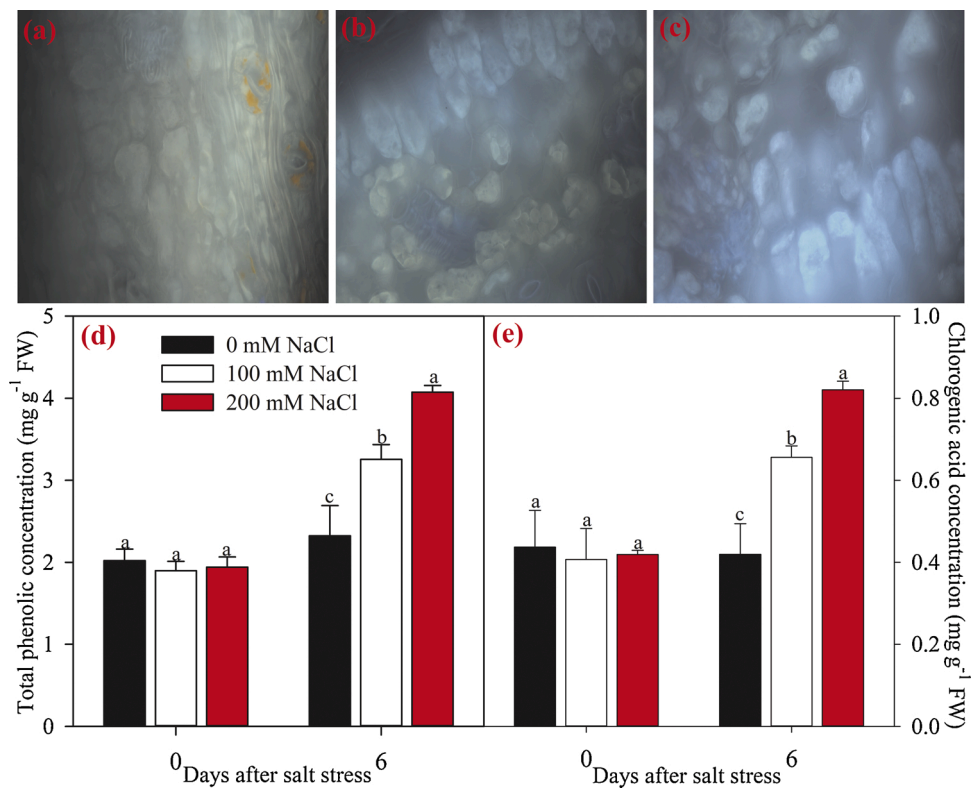


Fig. 1. Changes in leaf total phenolic (d) and chlorogenic acid (e) concentrations in honeysuckle after salt stress and *in situ* visualization of leaf phenolics with enlargement of 100 times in control plants without NaCl stress (a) and plants exposed to 100 mM (b) and 200 mM (c) NaCl stress for 6 days. FW indicates fresh weight. Data are the mean of five replicates (\pm SD) in the figure. Significant differences at $P < 0.05$ are represented by different letters on error bars.

for 1 min for clearing the chlorophyll, and then mounted in 90 % glycerol solution. The leaf sections were viewed under a fluorescence microscope (Olympus BX53, Japan) with UV light (365 nm excitation). After excitation by UV light, phenolics, particularly the caffeic acid

esters, can specifically emit blue fluorescence at 400–430 nm (Hutzler et al., 1998).

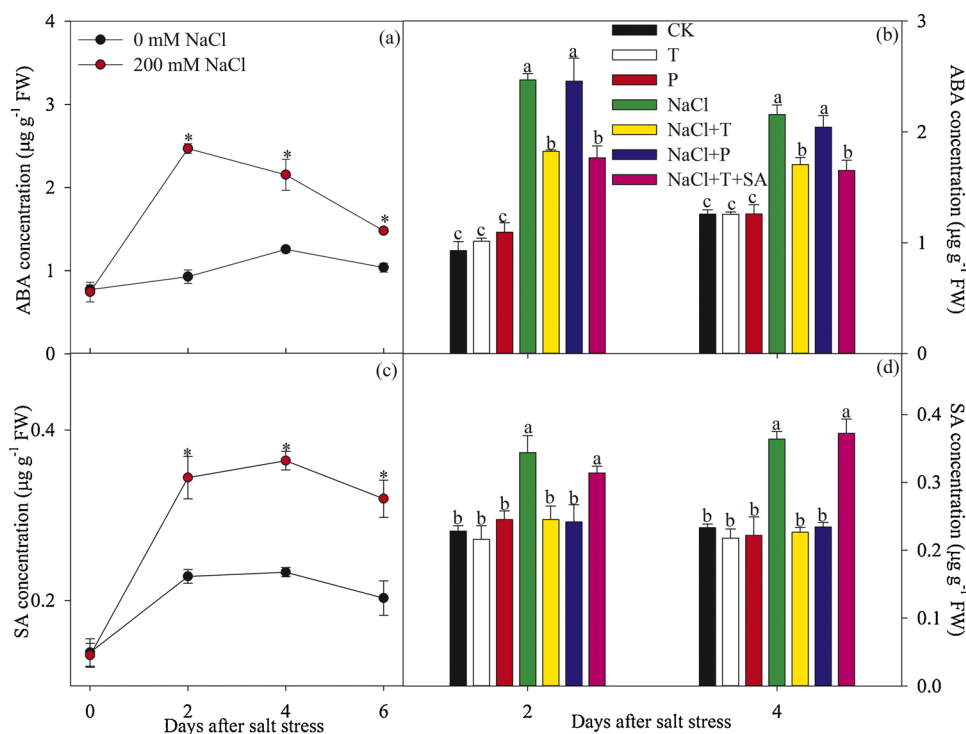


Fig. 2. Variation of leaf ABA and SA concentrations during salt stress (a, c) and leaf ABA and SA concentrations in tungstate-pretreated and paclobutrazol-pretreated honeysuckle under salt stress (b, d). FW indicates fresh weight. Data are the mean of five replicates (\pm SD) in the figure. Significant differences at $P < 0.05$ are represented by different letters on error bars and asterisks. CP indicates control plants without exposure to NaCl stress; T indicates tungstate-pretreated plants without exposure to NaCl stress; P indicates paclobutrazol-pretreated plants without exposure to NaCl stress; N indicates plants exposed to 200 mM NaCl stress; NT indicates plants exposed to 200 mM NaCl stress after tungstate pretreatment; NP indicates plants exposed to 200 mM NaCl stress after paclobutrazol pretreatment; NTS indicates plants exposed to 200 mM NaCl stress after tungstate pretreatment and SA application. The symbols, CP, T, P, N, NT, NP and NTS are also used in the following figures.

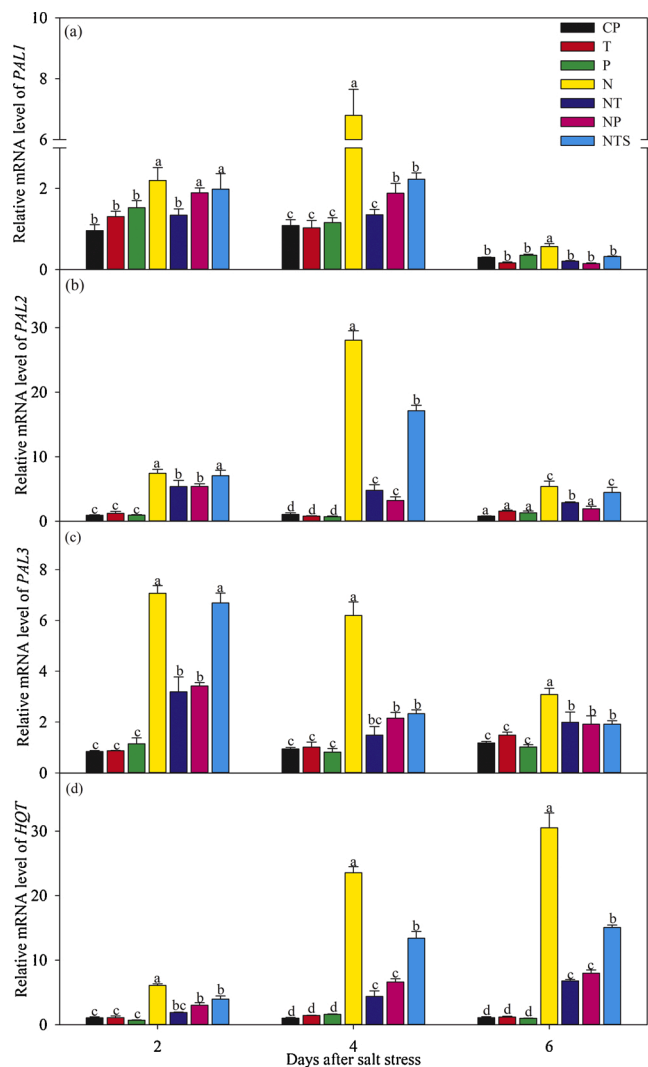


Fig. 3. Gene transcription of leaf phenylalanine ammonia-lyase (PAL, a-c) and hydroxycinnamoyl-CoA quinase: hydroxycinnamoyl transferase (HQT, d) in tungstate-pretreated and paclobutrazol-pretreated honeysuckle under salt stress. The genes, *PAL1*, *PAL2* and *PAL3* belong to PAL gene family. Data are the mean of five replicates (\pm SD) in the figure. Significant differences at $P < 0.05$ are represented by different letters on error bars.

2.5. Measurements of total phenolic and chlorogenic acid concentrations and phenylalanine ammonia-lyase (PAL) activity

Fresh leaves were homogenized in 60 % methanol after grinding with liquid nitrogen. The mixture was centrifuged to collect supernatant for measuring total phenolic and chlorogenic acid concentrations (Yan et al., 2017). Fresh leaves were homogenized in borate buffer (pH 8.8) and centrifuged after grinding with liquid nitrogen, and the supernatant was used for measuring PAL activity (Yan et al., 2017).

2.6. Real time quantitative PCR analysis

The experiments of real time quantitative PCR were carried out for analyzing transcription of PAL and hydroxycinnamoyl-CoA quinase: hydroxycinnamoyl transferase (HQT) by using the same internal reference gene, primers and equipment in our recent study (Yan et al., 2016a).

2.7. Statistical analysis

The presented data are the mean of five replicate plants in pot experiments or samples collected from four replicate plots. All kinds of data were compared through one-way ANOVA by using SPSS 16.0 (SPSS Inc., Chicago, IL, USA) through LSD test, and difference was considered significant at $P < 0.05$. SPSS 16.0 was also used to perform regression analysis.

3. Result

3.1. Effects of salt stress on leaf total phenolic and chlorogenic acid concentrations

Leaf total phenolic and chlorogenic acid concentrations were markedly elevated after 6 days of 100 mM NaCl stress, and the increase was up to 75.4 % and 95.5 % after 6 days of 200 mM NaCl stress (Fig. 1 d and e). As shown in Fig. 1a–c, the specific blue fluorescence gradually increased with the aggravation of NaCl stress, verifying salt-induced leaf phenolic accumulation.

3.2. Effects of ABA and SA synthetic inhibitors on their concentrations under salt stress

Leaf ABA and SA concentrations were remarkably elevated after 2 days of NaCl stress, and they were maintained at a significantly higher level in salt-treated plants than control plants in following days (Fig. 2a and c). Tungstate and paclobutrazol did not obviously influence leaf ABA and SA concentrations, however, salt-induced increase in leaf ABA and SA concentrations were significantly dampened in tungstate-pretreated and paclobutrazol-pretreated plants (Fig. 2b and d). Tungstate pretreatment also abated salt-induced increase in leaf SA concentration, whereas salt-induced increase in leaf ABA concentration was not affected by paclobutrazol pretreatment (Fig. 2b and d). Notably, SA application restored leaf SA concentration but had no effect on leaf ABA concentration in tungstate-pretreated plants under salt stress (Fig. 2b and d).

3.3. Effects of ABA and SA synthetic inhibitors on genes transcription under salt stress

Tungstate and paclobutrazol did not obviously influence the transcription of *PAL1*, *PAL2*, *PAL3* and *HQT*, but their transcription was remarkably elevated by NaCl stress (Fig. 3). Tungstate and paclobutrazol pretreatments depressed salt-induced elevation in transcription of *PAL1*, *PAL2*, *PAL3* and *HQT*, and the depression was partly mitigated by SA application in tungstate-pretreated plants (Fig. 3).

3.4. Effects of ABA and SA synthetic inhibitors on PAL activity and phenolic concentration under salt stress

Tungstate and paclobutrazol did not obviously influence PAL activity, total phenolic and chlorogenic acid concentrations in the leaves (Fig. 4). NaCl stress significantly increased leaf PAL activity by 61.4 %, 41.2 % and 110.4 %, respectively, at day 2, 4 and 6, and consistently, leaf total phenolic and chlorogenic acid concentrations were remarkably increased by 75.4 % and 95.5 % after 6 days of NaCl stress (Fig. 4). Salt-induced elevation in leaf PAL activity, total phenolic and chlorogenic acid concentrations was significantly depressed in tungstate-pretreated and paclobutrazol-pretreated plants (Fig. 4). The depression in tungstate-pretreated plants was greater and could be partly alleviated by SA application (Fig. 4).

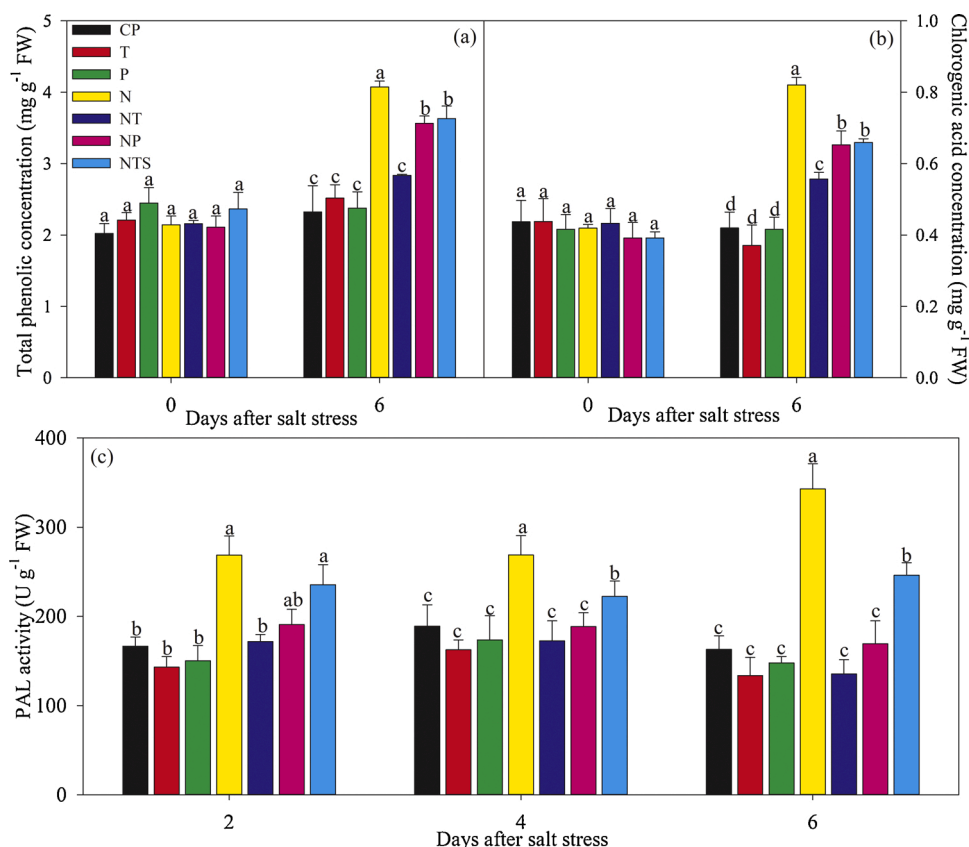


Fig. 4. Leaf total phenolic (a) and chlorogenic acid (b) concentrations and phenylalanine ammonia-lyase (PAL, c) activity in tungstate-pretreated and paclobutrazol-pretreated honeysuckle under salt stress. Data are the average value of five replicates (\pm SD) in the figure. Significant differences at $P < 0.05$ are represented by different letters on error bars.

3.5. Correlation of leaf ABA and SA concentrations with total phenolic concentration

In growth seasons of 2014 and 2015, leaf ABA and SA concentrations were higher in plants in saline plots than non-saline plots, and insignificant difference arose in some individual months (Fig. 5a and b). The regression analysis suggested a significant positive correlation of leaf ABA and SA concentrations with total phenolic concentration (Fig. 5c and d).

4. Discussion

Consistent with our previous study (Yan et al., 2017), salt stress enhanced leaf total phenolic and chlorogenic acid concentrations in honeysuckle and was beneficial to its medicinal quality (Fig. 1d and e). In this study, salt-induced leaf phenolic accumulation was confirmed by *in situ* visualization through fluorescence microscopy (Fig. 1a–c), and leaf phenolic synthesis was also elevated by salt stress according to increased PAL activity and transcription of *PALs* and *HQT* (Figs. 3 and 4c). As the crucial hormone signals, ABA and SA are commonly accumulated in plants for defensive purposes upon environmental stresses, and phenolic synthesis and accumulation can be enhanced by exogenous applying ABA and SA in some plants (Alonso et al., 2016; El-Esawi et al., 2017; Golkar et al., 2019; Khaleghnezhad et al., 2019; Mousavi and Shabani, 2019). Therefore, higher leaf ABA and SA concentrations appeared in parallel with elevated phenolic synthesis in salt-treated honeysuckle, implying that ABA and SA potentially participated in eliciting phenolic synthesis under salt stress.

However, it remains not definite whether ABA and SA can serve as signal molecules to induce phenolic synthesis in plants under salt stress. As expected, salt-induced leaf ABA accumulation was effectively

depressed in honeysuckle by tungstate (Fig. 2b). The declined leaf ABA concentration inhibited phenolic synthesis by abating salt-induced increase in PAL activity (Fig. 4c), which also could be verified from molecular level by lower transcription of *PALs* and *HQT* in tungstate-pretreated plants than non-pretreated plants upon salt stress (Fig. 3). Correspondingly, salt-induced leaf total phenolic and chlorogenic acid accumulation was dampened in tungstate-pretreated plants (Fig. 4a and b). Thus, salt-induced leaf phenolic synthesis in honeysuckle depended on ABA signaling pathway. As with ABA, SA was also proved to be engaged in triggering leaf phenolic synthesis under salt stress from physiological and molecular levels, because the declined SA accumulation reduced leaf phenolic and chlorogenic acid concentrations and inhibited PAL activity and transcription of *PALs* and *HQT* under salt stress (Figs. 3 and 4). Therefore, ABA and SA mediated salt-induced leaf phenolic synthesis in honeysuckle. In fact, the induction of physiological adaptive responses relies on signaling networks rather than a single hormone (Gollack et al., 2014), and particularly, Li et al. (2019) also reported that SA cooperated with NO to induce leaf flavonoid synthesis in tea plant under elevated CO₂. In this study, tungstate pretreatment reduced not only ABA accumulation but also SA accumulation in the leaves under salt stress (Fig. 2b and d), suggesting that ABA-dependent SA production was responsible for phenolic synthesis, and unchanged leaf ABA level in paclobutrazol-pretreated plants upon salt stress also corroborated that ABA was an upstream signal (Fig. 2b). Overall, salt-induced leaf phenolic synthesis was mediated by ABA and SA signaling in honeysuckle, and ABA acted as an upstream signal of SA. It was noteworthy that tungstate pretreatment led to greater reduction of total phenolic and chlorogenic acid concentrations in salt-treated plants than paclobutrazol pretreatment, and so was in PAL activity (Fig. 4). This result was in accordance with the role of ABA as upstream signal, and it also indicated that apart from SA, there might be other unknown

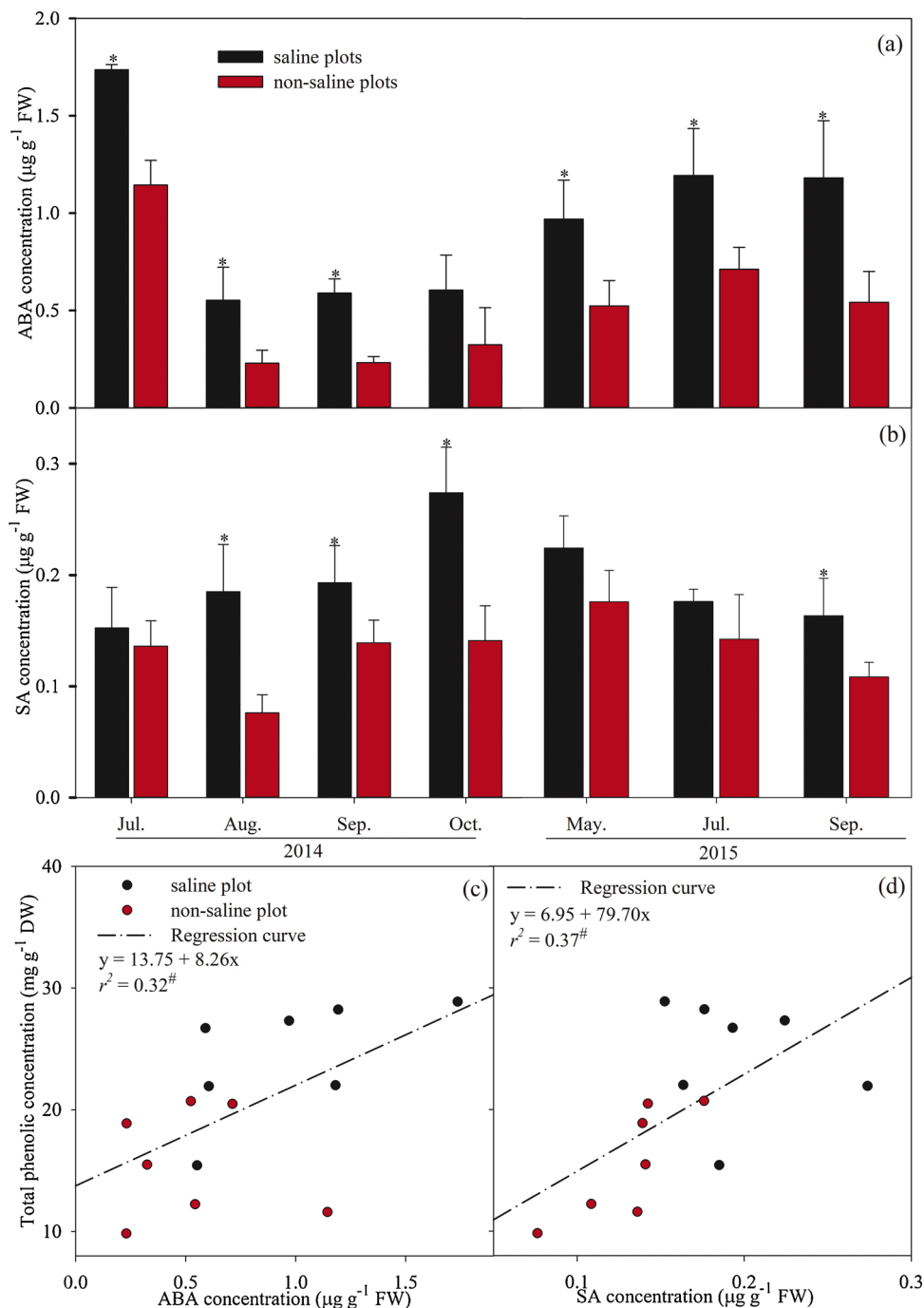


Fig. 5. Leaf abscisic acid (ABA, a) and salicylic acid (SA, b) concentrations in honeysuckle in non-saline and saline plots, and the regression analysis of leaf total phenolic concentration with ABA (c) and SA (d) concentrations. FW and DW indicate fresh and dry weight. Data are the mean of four replicate plots (\pm SD) in the figure. Significant difference at $P < 0.05$ is represented by asterisks on error bars, and significant correlation at $P < 0.05$ was indicated by #.

downstream signals to mediate ABA-dependent phenolic synthesis under salt stress. Moreover, this inference was underpinned by the fact that SA application incompletely restored leaf phenolic synthesis and concentration in tungstate-pretreated plants under salt stress (Fig. 4). Besides ABA and SA, other signal molecules such as brassinosteroid, ethylene, and methyl jasmonate have been reported to elicit plant phenolic synthesis (Yang et al., 2012; Liang et al., 2013; Coban and Gokturk Baydar, 2016), and they are proper candidate signals for deeply exploring salt-induced leaf phenolic synthesis in honeysuckle in future study. ABA and SA were preferentially selected in this study, because of their low price and convenience for application in agricultural practice.

To date, the results about signal transduction for physiological

metabolisms are rarely examined by field trial and lack an ecological proof. In agreement with pot experiments, leaf ABA and SA concentrations in honeysuckle were also enhanced by soil salinity in the field trials (Fig. 5a and b). Hormones production might be interrupted by environmental factors such as precipitation, light and temperature. Consequently, leaf ABA and SA levels exhibited seasonal changes, and possibly due to the dilution effects of environmental factors, the differences of leaf ABA and SA concentrations between plants in saline and non-saline plots became insignificant in some individual months (Fig. 5a and b). It should be emphasized that a significant positive correlation of leaf ABA and SA levels with total phenolic concentration was demonstrated in honeysuckle under coastal saline environment (Fig. 5c and d), and this

finding conformed to the signaling transduction mechanism revealed by hydroponic experiments. In other words, the viewpoint that salt-induced leaf phenolic synthesis was mediated by ABA and SA in honeysuckle passed ecological test and had important practical value.

In agreement with our hypothesis, ABA acted as an upstream signal of SA to elicit leaf phenolic synthesis in honeysuckle under salt stress, according to the analysis at molecular and physiological levels. This finding was verified from ecological angle under coastal saline environment, providing a critical guidance for agricultural practice in coastal abandoned saline land.

CRedit authorship contribution statement

Kun Yan: Methodology. **Lanxing Bian:** Methodology. **Wenjun He:** Methodology. **Guangxuan Han:** Methodology. **Zishan Zhang:** Methodology. **Zheng Sun:** Methodology. **Likun Liang:** Methodology. **Hongxin Jia:** Methodology. **Guangmei Wang:** Methodology.

Declaration of Competing Interest

The authors report no declarations of interest.

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