

Jasmonate mediates salt-induced nicotine biosynthesis in tobacco (*Nicotiana tabacum* L.)



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ABSTRACT

Jasmonate (JA), as an important signal, plays a key role in multiple processes of plant growth, development and stress response. Nicotine and related pyridine alkaloids in tobacco (*Nicotiana tabacum* L.) are essential secondary metabolites. Whether environmental factors control nicotine biosynthesis and the underlying mechanism remains previously unreported. Here, we applied physiological and biochemical approaches to investigate how salt stress affects nicotine biosynthesis in tobacco. We found that salt stress induced the biosynthesis of JA, which subsequently triggered the activation of JA-responsive gene expression and, ultimately, nicotine synthesis. Bioinformatics analysis revealed the existence of many NtMYC2a-recognized G-box motifs in the promoter regions of *NtLOX*, *NtAOS*, *NtAOC* and *NtOPR* genes. Applying exogenous JA increased nicotine content, while suppressing JA biosynthesis reduced nicotine biosynthesis. Salt treatment could not efficiently induce nicotine biosynthesis in transgenic *anti-CO11* tobacco plants. These results demonstrate that JA acts as the essential signal which triggers nicotine biosynthesis in tobacco after salt stress.

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Jasmonate (JA) is an essential hormone in higher plants where it acts as an endogenous growth regulator. Previous research indicates that JA acts as an important regulatory signal to influence multiple physiological processes, such as stomatal closure, plant resistance to cold, inhibition of Rubisco biosynthesis and so on. JA signaling is also involved in plant defense responses, including defense against mechanical damage and herbivorous insects. JA and its methyl jasmonate (MeJA) are a class of fatty acid derivatives. The biosynthesis of JA and MeJA was elucidated by [Vick and Zimmerman \(1983\)](#) and [Hamberg and Hughes \(1988\)](#). With α -linolenic acid as substrate, molecular oxygen is inserted by a lipoxygenase (13-LOX) at carbon atom 13 leading to the formation of a fatty acid hydroperoxide 13 *S*-hydroperoxy-(9Z,11E,15)-octadecatrienoic acid. This compound is dehydrated by the allene oxide synthase (AOS) to an unstable allene oxide which can be either

hydrolyzed non-enzymatically to α - and γ -ketols or cyclized to racemic 12-oxo-phytodienoic acid (OPDA). In the presence of an allene oxide cyclase (AOC), preferential formation of the (9*S*, 13*S*) enantiomer of OPDA occurs. The AOC-catalyzed step is regarded as the crucial step in octadecanoid and jasmonate biosynthesis because only this enantiomeric form is the substrate for the naturally occurring (+)-7-*iso*-JA, which is formed after reduction of OPDA by a specific OPDA reductase 3 (12-oxophytodienoate reductase 3) and three cycles of β -oxidation. Treatment of plants with products of the AOS branch, the octadecanoids and jasmonates, has been shown to lead to accumulation of mRNAs coding for LOX, AOS, AOC and OPR3 suggesting a feedforward regulation in JA biosynthesis. Also, biotic or abiotic stresses, which result in endogenous increases of octadecanoids and jasmonates, are usually accompanied by a transcriptional up-regulation of AOS, AOC and OPR3.

Tobacco (*Nicotiana tabacum*) generates an array of alkaloids that play essential roles in the plant defense response against herbivore and insect attack ([Kessler and Baldwin, 2002](#); [Steppuhn et al.,](#)

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2004). Nicotine is the main alkaloid produced by cultivated tobacco (*N. tabacum* L.), constituting approximately 0.6%–3% of the tobacco leaf dry weight. Nicotine is synthesized in the root from ornithine and arginine by way of putrescine. Putrescine is either metabolized to higher polyamines, such as spermidine and spermine, or conjugated with cinnamic acid derivatives or fatty acids in all higher plants; however, it is also converted into N-methylputrescine in plants that produces nicotine or tropane alkaloids. Thus, putrescine N-methyltransferase (PMT; EC 2.1.1.53) participates in the first committed step of alkaloid biosynthesis (Chattopadhyay and Ghosh, 1998; Chou and Kutchan, 1998). N-Methylputrescine is then oxidized by a diamine oxidase (EC1.4.3.6) and cyclized spontaneously to the 1-methyl-*N*-pyrrolinium cation, which is condensed with nicotinic acid or its derivative. Quinolinic acid phosphoribosyltransferase (EC2.4.2.19) serves as the entry-point enzyme in the pyridine nucleotide cycle, which supplies nicotinic acid. After biosynthesis in the tobacco root, nicotine is translocated to the leaf via the xylem and stored in the leaf vacuole with the help of a tonoplast-localized transporter. Nicotine can be demethylated in both leaves and roots, but is primarily demethylated in senescing leaves (Wagner et al., 1986; Chou and Kutchan, 1998). The accumulation of nicotine in tobacco is affected by environmental factors, cultural practices, and plant hormone levels. For example, the application of nitrogen fertilizer or jasmonate markedly increases nicotine biosynthesis (De Luca and St Pierre, 2000; Shoji et al., 2000; Goossens et al., 2003; Paschold et al., 2007). Zhang et al. (2012) reported that the tobacco transcription factors NtMYC2a and NtMYC2b formed a nuclear complex with NtJAZ1 to regulate jasmonate-induced nicotine biosynthesis, suggesting that NtMYC2a, NtMYC2b, and NtJAZ1 are involved in the regulatory pathway that controls nicotine biosynthesis.

To understand how environmental factors affect nicotine biosynthesis in tobacco, we investigated the effects of multiple environmental stresses on nicotine content in tobacco. Our results show that salt stress increases the level of transcription factor *NtMyc2a* and the biosynthesis of nicotine, while blocking jasmonate biosynthesis and signaling after salt stress reduces plant nicotine biosynthesis. However, application of exogenous JA significantly induces nicotine accumulation. These results suggest that JA acts as an important signal that mediates salt-induced nicotine biosynthesis in tobacco.

1. Materials and methods

1.1. Plant growth and treatment

Sterilized tobacco (*Nicotiana tabacum* cv. Wisconsin 38) seeds were germinated and grown to seedlings under continuous illumination on half-strength Gamborg B5 medium solidified with 2% gellan gum and supplemented with 0.3% sucrose at 24 °C. One-week-old plants were transferred to soil containing 3/5 peat soil, 1/5 vermiculite, 1/5 perlite, and grown for one week in the greenhouse at 24 °C (16 h light/8 h dark, 1200 $\mu\text{mol m}^{-2}\text{s}^{-1}$ light intensity) before salt treatment. For salt treatment, 2-week-old seedlings were sprayed with 1/4 Hoagland solution or a 100 m mol L⁻¹ NaCl solution of Hoagland for salt stress. The plants were irrigated every three days. For MeJA treatment, 2-week-old plants were sprayed with water, 10 n mol L⁻¹, 50 n mol L⁻¹, 100 n mol L⁻¹ or 500 n mol L⁻¹ MeJA solution. After various treatment times, tobacco leaves were collected for further analysis.

1.2. Nicotine content measurement

After treatment, 0.5 g of tobacco roots was collected and frozen in liquid nitrogen and lyophilized, before homogenization and

resuspension in 4 ml of 0.1 mol L⁻¹ H₂SO₄. The homogenate was sonicated for 60 min and centrifuged at 2000 g for 15 min. The supernatant pH was neutralized by adding 0.4 ml 25% NH₄OH, and the mixture was loaded onto an Extrelut-1 column (Merck) and eluted with 6 ml of chloroform. The eluent was dried at 37 °C, and the dry residues were dissolved in ethanol and analyzed by gas chromatography (Agilent 6890) and quadrupole mass spectrometry with electron impact ionization (Agilent 5973, Network, US). The column temperature was held at 100 °C for 10 min, and then increased to 260 °C, over a 35-min period, at a gradient of 8 °C/min. Synthetic nicotine (Zhengzhou Tobacco Research Institute) was used as a standard. Nicotine content analysis was performed at Zhengzhou Tobacco Research Institute.

1.3. Jasmonate content analysis

To measure jasmonate contents, 2-week-old plants were treated with salt solution for different times. After treatment, root tissue was harvested and jasmonate was analyzed by ELISA using monoclonal antibodies of jasmonate according to Hu et al. (2013). Jasmonate antibody was purchased from Shanghai YUANYAN biological technology company. Weight about 1 g tobacco roots tissue materials which was salt stress with different times, then using liquid nitrogen frozen ground into a powder. After the liquid nitrogen was volatilization, add 10 ml 80% methanol (contain 100 μg antioxidants butylatrd hydroxytoulene) is extracted for the night, after NH₄OH was add to supernatant fluid to pH 8.0, freeze-drying, then dissolved the extraction into 0.2 mol L⁻¹ acetic acid for JA content measurement.

1.4. RNA extraction and quantitative real-time RT-PCR (qRT-PCR)

Total RNA was extracted from 3-week-old tobacco seedling after different treatment using TRIzol reagent (Invitrogen), and qRT-PCR was performed as previously described (Zhang et al., 2012). Briefly, first-strand cDNA was synthesized from 1.5 μg DNase-treated RNA in a 20 μL reaction volume using M-MuLV reverse transcriptase (Fermentas) with oligo (dT)₁₈ primer. qRT-PCR was performed using SYBR Green I Master Mix with a Roche LightCycler 480 real-time PCR machine, according to the manufacturer's instructions. At least three biological replicates for each sample were used for qRT-PCR analysis, and at least two technical replicates were analyzed for each biological replicate. The *ACTIN2* gene was used as an internal control of gene expression. Gene-specific primers used to detect the transcripts are listed as below:

NtMYC2a-F: 5'-cctcattgatggtgctcgtg-3', NtMYC2a-R: 5'-ttcgggttcttctgctcagc-3';
 NtLOX-F: 5'-ggacttgaaggatggtggtgc-3', NtLOX-R: 5'-tca-cattaaacgtagcatctcct-3';
 NtAOS-F: 5'-gccaaacgcgacattatgat-3', NtAOS-R: 5'-ccaaaaatccttccggca-3';
 NtAOC-F: 5'-acagcttctacttcggcgat-3', NtAOC-R: 5'-tagcaactcagcggcagat-3';
 NtOPR-F: 5'-cctatgggcaactgaagca-3', NtOPR-R: 5'-cga-gatcagcatcaccttgt-3'.

1.5. Protein isolation and immunoblot analysis

Proteins were extracted from whole seedling with extraction buffer containing 50 m mol L⁻¹ Tris-Cl, pH 7.5, 150 m mol L⁻¹ NaCl, 1 m mol L⁻¹ PMSF, 1 \times Complete Protease Inhibitor Cocktail (Roche), 5% glycerol, 1 m mol L⁻¹ EDTA, and 1 m mol L⁻¹ DTT. The protein concentration was determined using a commercial Bradford assay kit following the manufacturer's instructions (Bio-Rad). The samples were mixed with equal volume of 10% sodium dodecyl sulfate (SDS) sample buffer and boiled for 3 min before separation

on a 10% SDS-PAGE gel. The fractionated proteins were then transferred to the PVDF membrane and immunoblot assays were performed as previously described (Zhang et al., 2012), using anti-PMT1 and anti-Actin (Abmart, Shanghai, China) antibodies at dilutions of 1:3000 and 1:2000, respectively. The anti-PMT1 antibody was prepared by immunizing rabbit with the synthesized peptide from the N-termination of tobacco PMT1 protein (MEVISTNTNGSTI).

2. Results

2.1. Salt stress induced the accumulation of nicotine

For salt stress, we watered two-week-old plantlets with 1/4 Hoagland with 50 mM NaCl, and then measured nicotine content at different times. We found that salt stress induced nicotine accumulation in the tobacco root system (Fig. 1A). Compared with the control plants, nicotine levels increased 1 day after treatment, and rapidly accumulated 3 days after treatment in salt-stressed plants. Salt stress-induced nicotine content reached a peak after 7 days of treatment, and sustained a high level for 9 days. NtPMT1 is the key enzyme responsible for nicotine biosynthesis in tobacco (Chattopadhyay and Ghosh, 1998; Chou and Kutchan, 1998). Using NtPMT1 antibodies to analyze NtPMT1 levels in tobacco after salt stress, we found that salt stress increased NtPMT1 protein levels 24 h after treatment, and detected sustained high protein levels 5–7 days after treatment (Fig. 1B). These results indicate that salt stress significantly induced nicotine biosynthesis and NtPMT1 protein accumulation in tobacco.

2.2. Salt treatment induced the transcriptional increase of MYC2a gene

It has been reported that MYC2a transcriptional factors are involved in jasmonate-mediated biosynthesis of nicotine (Zhang et al., 2012). To understand the role of MYC2a in salt-induced nicotine biosynthesis, we applied quantitative RT-PCR to detect transcriptional changes of MYC2a. As shown in Fig. 2, *NtMYC2a* transcript levels increased after 6 h of salt stress, reaching a peak after 36 h, with prolonged stress sustaining high levels of *NtMYC2a* transcripts. In contrast, *NtMYC2a* transcription levels did not change in control plants. These data suggest that salt treatment can significantly induce the transcriptional increase of *NtMYC2a*.

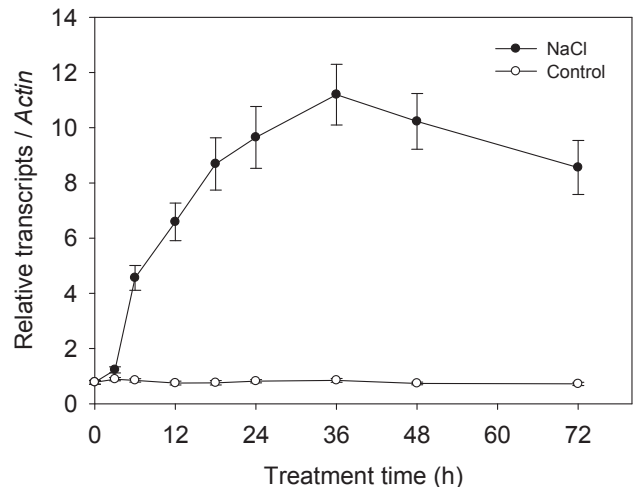


Fig. 2. Salt stress induced the transcriptional increase of *NtMYC2a*. Two-week-old seedlings were treated with 50 m mol L⁻¹ salt solution for indicated time periods. Transcript levels were measured by qRT-PCR. Data represent the means of three replicate experiments (\pm SE).

2.3. Salt treatment induced the accumulation of tobacco jasmonate

Previous results indicated that jasmonate acts as an important signal mediating the biosynthesis of nicotine in tobacco. Following jasmonate stimulation the transcription factor NtMYC2a specifically recognizes G-box (5'-CA(C/T)(G/A)T(T/A)-3') sequences in the promoter of genes associated with nicotine biosynthesis. Here we first applied a bioinformatics approach to analyze promoter sequences of genes involved in jasmonate biosynthesis, including *NtLOX*, *NtAOS*, *NtAOC*, *NtOPR*. As shown in Fig. 3A, we identified several G-box elements in the promoter regions of these genes, suggesting that NtMYC2a may control their gene expression during salt stress. To test this possibility, we examined the expression of these genes after salt stress. As shown in Fig. 3B, we found that salt stress indeed induced substantial changes in the expression of these genes. At the same time, we also analyzed JA content in tobacco seedlings after salt stress. Salt treatment stimulated the accumulation of jasmonate, and JA content reached a peak after 7 days of salt stress (Fig. 4). These results indicate that salt treatment can increase jasmonate biosynthesis by inducing jasmonate biosynthesis-related gene transcription.

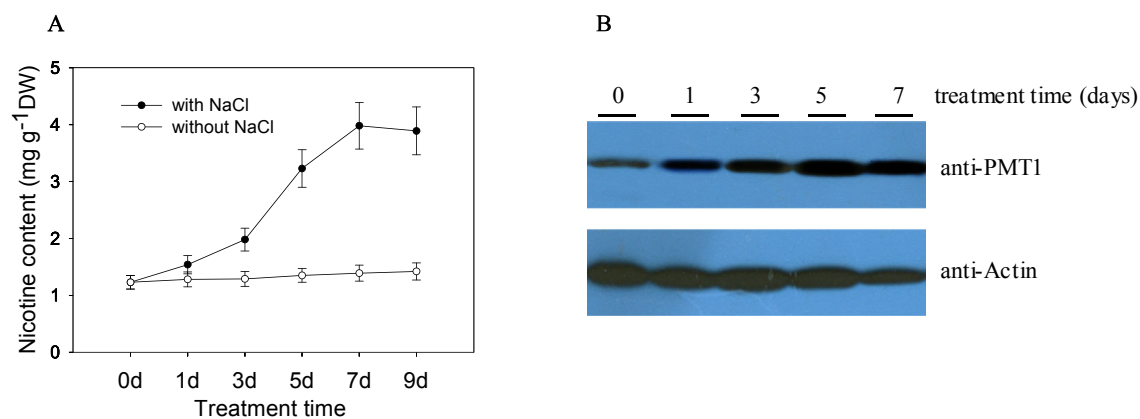


Fig. 1. Salt stress induced the accumulation of nicotine and NtPMT1. Two-week-old seedlings were treated with 50 m mol L⁻¹ salt solution and the nicotine content was measured at indicated times (A); NtPMT1 protein levels were also examined using anti-PMT1 antibody and western blotting analysis. anti-Actin was used as a loading control. Data represent the mean of three replicate experiments (\pm SE).

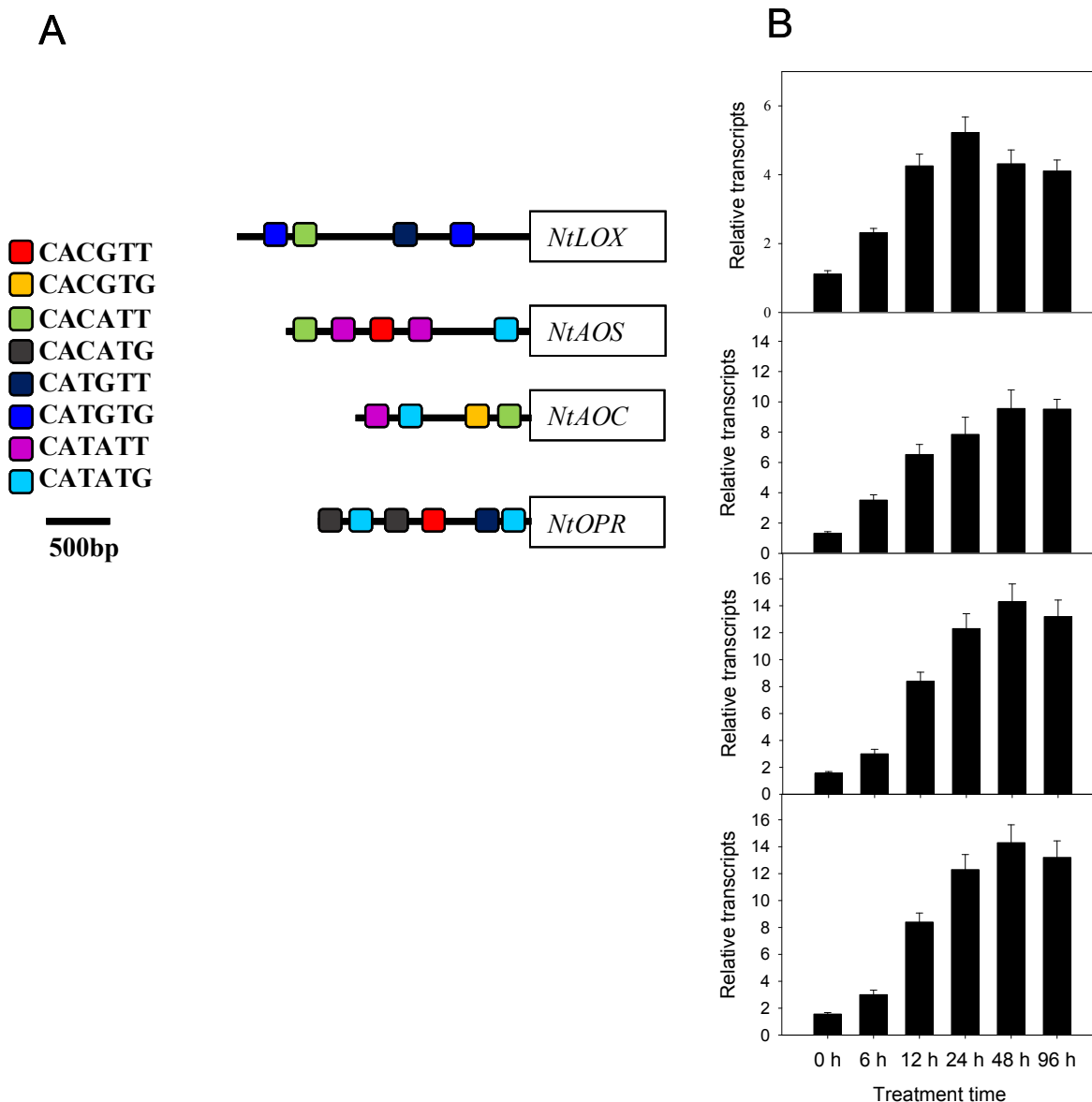


Fig. 3. Salt stress induced increased expression of *NtLOX*, *NtAOS*, *NtAOC* and *NtOPR*. (A) Bioinformatics were used to identify the G-box region in the promoter of *NtLOX*, *NtAOS*, *NtAOC* and *NtOPR*. Different colored boxes in the promoter region of these genes represent different motif sequences shown in the left panel. (B) Salt stress induced an increase in transcript levels of *NtLOX*, *NtAOS*, *NtAOC* and *NtOPR*. Two-week-old tobacco seedlings were treated with 50 mol L⁻¹ salt solution; the transcriptional levels of *NtLOX*, *NtAOS*, *NtAOC* and *NtOPR* were measured at indicated times. Data represent the means of three replicate experiments (\pm SE).

2.4. Exogenous application of JA induced the increase of nicotine in the tobacco root tissue

Previous results indicate that exogenous application of JA can induce the accumulation of nicotine in tobacco suspension cells (Shoji et al., 2008). Our above results showed that salt stress induced JA and nicotine biosynthesis. To test whether salt-induced nicotine biosynthesis depends on JA signaling, we examined whether nicotine content of wild-type plants was affected by exogenous application of jasmonate. As shown in Fig. 5, nicotine biosynthesis was induced in plants sprayed with methyl jasmonate (MeJA; 10, 50, 100, 500 nM). Nicotine content was most effectively induced 3–6 days after treatment with 100 nM MeJA. This result suggests that jasmonate acts as a signal factor that induces nicotine biosynthesis in tobacco plants.

2.5. Inhibiting jasmonate signal reduced salt-induced nicotine biosynthesis

Having found that both salt and MeJA treatment directly induced nicotine biosynthesis, we then asked whether suppressing the accumulation of JA reduces nicotine biosynthesis under salt stress. Sodium diethyldithiocarbamate (DIECA) is an inhibitor of LOX, a key enzyme in jasmonate biosynthesis. We treated tobacco seedlings with DIECA for 6 h following salt stress. With or without jasmonate inhibition, salt-stressed plants had greater nicotine content than control plants; nicotine content in plants after DIECA treatment was lower than in untreated salt-stressed plants (Fig. 6). This data indicates that suppressing JA biosynthesis positively controls salt-induced nicotine biosynthesis in tobacco.

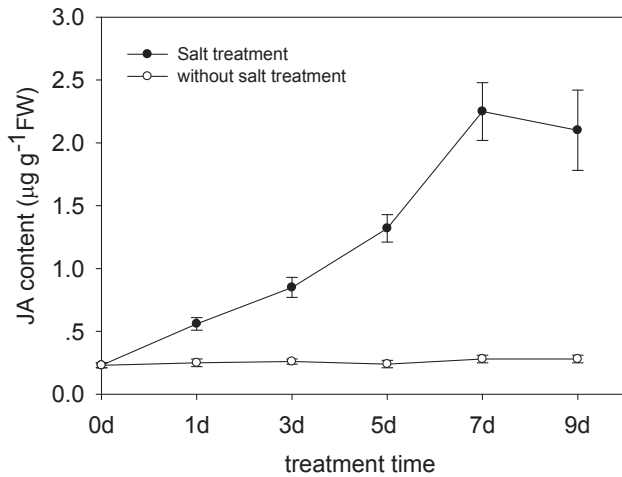


Fig. 4. Salt stress induced jasmonate biosynthesis. Two-week-old seedlings were treated with a 50 mmol L⁻¹ NaCl solution. Endogenous jasmonate levels were measured at various time. Data represent the means of three replicate experiments (\pm SE).

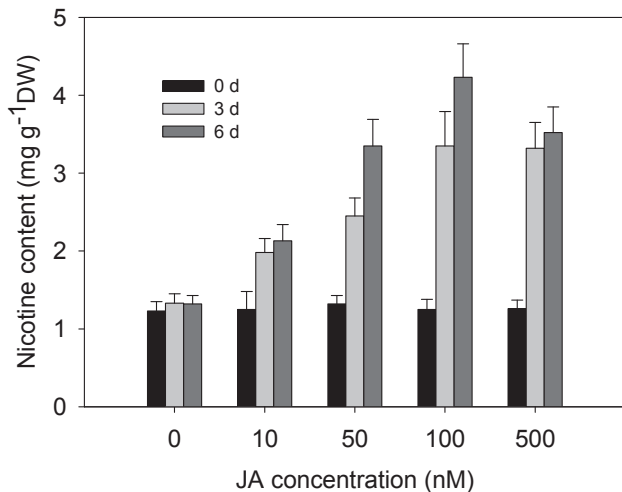


Fig. 5. Exogenous application of JA induced an increase in nicotine biosynthesis in tobacco root tissue. Two-week-old seedlings were sprayed with MeJA at indicated times, and nicotine content was measured in root tissue after 3 and 6 days of MeJA treatment. Data represent the means of three replicate experiments (\pm SE). The means denoted by different letters indicate significant differences according to Tukey's test ($p < 0.05$).

NtCOI1 is a receptor of the JA signal and perceives JA stimulation in plants. Silencing NtCOI1 will lead to less nicotine after JA or less. To further understand the role of NtCOI1 in salt-induced nicotine biosynthesis, we generated the transgenic line expressing antisense of *NtCOI1* gene, these transgenic lines were termed *anti-NtCOI1* lines. In three individual transgenic *anti-NtCOI1* lines, we found that salt stress could not effectively induce nicotine accumulation, compared with the non-transgenic control lines (Fig. 7). This result indicates that NtCOI1 perception of the JA signal plays an essential role in salt-induced nicotine biosynthesis in tobacco.

3. Discussion

The plant hormone JA plays important roles in multiple processes of plant growth and development (Vick and Zimmerman, 1983; Hamberg and Hughes, 1988; Zhang et al., 2012). Previous

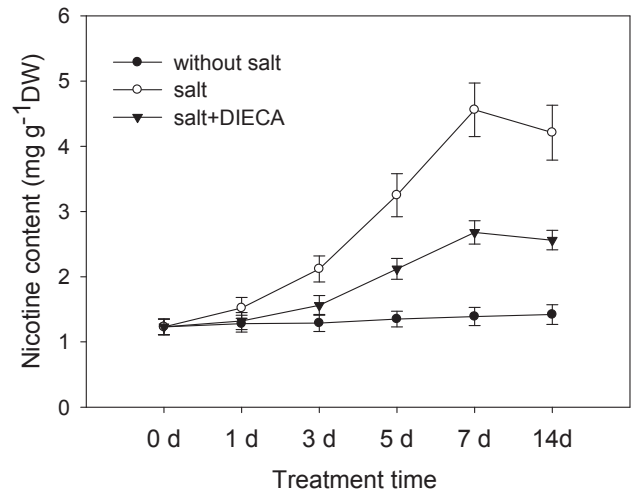


Fig. 6. The LOX enzyme inhibitor DIECA suppressed salt-induced nicotine biosynthesis. Two-week-old tobacco seedlings were treated with 100 μ mol L⁻¹ DIECA for 6 h, following 50 mmol L⁻¹ salt stress for indicated time, and nicotine content was measured. Data represent the means of three replicate experiments (\pm SE).

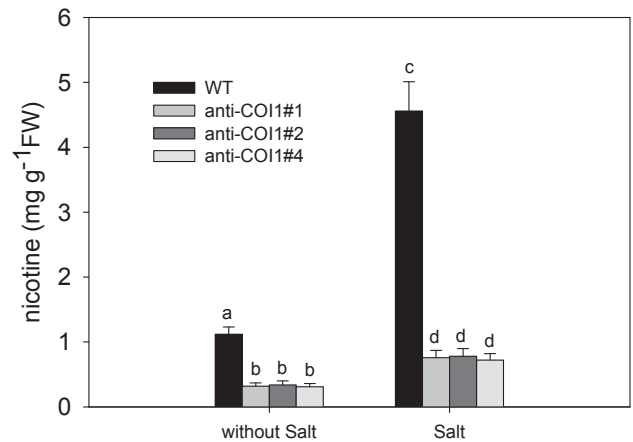


Fig. 7. Salt stress fails to induce nicotine biosynthesis in transgenic anti-COI1 tobacco seedlings (*anti-COI1#1*, *anti-COI1#2*, *anti-COI1#4*). Two-week-old plants from three different *col1* mutant lines (*col1#1*, *col1#2*, *col1#4*) and wild-type plants were treated with 50 mM salt solution for 6 days. Nicotine content was measured. Data represent the means of three replicate experiments (\pm SE). The means denoted by different letters indicate significant differences according to Tukey's test ($p < 0.05$).

results indicated that mechanical damage can induce the biosynthesis of JA. In tobacco plants, JA also participates in nicotine biosynthesis (Zhang et al., 2012). Our results show that salt stress leads to an increase in nicotine biosynthesis, accompanied by the accumulation of *NtPMT1* gene transcripts (Fig. 1). At the same time exogenous application of jasmonate can significantly induce nicotine biosynthesis in tobacco root tissue (Fig. 5), while the LOX enzyme inhibitor DIECA suppresses salt-induced nicotine biosynthesis (Fig. 6). These results suggest that jasmonate is an important signal molecule involved in regulation of nicotine biosynthesis after salt stress. In the jasmonate signaling pathway, MYC2 is an essential component that regulates the expression of a series of jasmonate-responsive genes (Hamberg and Hughes, 1988; Zhang et al., 2012). In the absence of jasmonate signaling, JAZ repressors bind to MYC2 transcription factors to suppress the activity of MYC2. Following jasmonate signal stimulation, JAZ repressors are degraded by ubiquitin proteins and MYC2 protein activity is

restored. MYC2 specifically recognizes G-box *cis*-acting sequences found in target promoters to activate gene expression (Zhang et al., 2012). In this paper we found that salt stress increased the transcriptional levels of *NtMYC2a*; therefore, we examined the expression of *NtLOX*, *NtAOS*, *NtAOC*, and *NtOPR*, genes which contain G-box sequences in their promoters and are known to be involved in JA biosynthesis in tobacco (Fig. 3A). We found salt stress treatment increased the transcriptional levels of *NtLOX*, *NtAOS*, *NtAOC* and *NtOPR*, indicating that salt stress induced the expression of *NtMYC2* which subsequently activated the transcription of these genes and nicotine biosynthesis (Fig. 3B). We also noticed that short-term salt treatment (12 h to 1 day) quickly led to a set of physiological responses, including increased JA and nicotine biosynthesis (Figs. 1 and 4), while increasing the transcriptional levels of *NtMYC2a* and JA biosynthesis-related genes (Fig. 2). However, after long-term salt treatment (7 days), accumulation of JA and nicotine, and the transcriptional levels of *NtMYC2a* and JA biosynthesis-related genes dropped slightly compared with those following 5 days of treatment (Figs. 1, 2 and 4), suggesting that JA and nicotine biosynthesis contribute to the short-term adaptation of tobacco response to salt stress. Once tobacco acclimatizes to salt stress, such as at 7 days of treatment, JA and nicotine biosynthesis may be regulated by a negative feedback loop, though the details of the regulatory mechanism require more investigation in the future.

A previous study indicated the COI1 protein, as the JA signal receptor in *Arabidopsis*, perceives JA stimulation. *Arabidopsis coi1* mutants show insensitivity to JA stimulation, and transgenic antisense *NtCOI1* tobacco plants also could not efficiently accumulate nicotine after JA treatment (Shoji et al., 2008). Our studies show that salt stress can induce an increase in jasmonate content to induce nicotine biosynthesis (Figs. 1 and 4), and inhibition of jasmonate biosynthesis inhibits the salt stress-induced biosynthesis of nicotine (Fig. 6). However, in transgenic antisense *NtCOI1* plants, salt stress could not efficiently induce nicotine biosynthesis (Fig. 7). This result indicates that tobacco *NtCOI1* protein, as the jasmonate receptor, is involved in the nicotine biosynthesis induced by salt stress. Our above results showed that suppressing JA biosynthesis by DIECA compromised salt-induced nicotine biosynthesis. It seems that perceiving the JA signal by JA receptor *NtCOI* also plays an essential role in nicotine biosynthesis under salt stress.

In summary, our results show that JA acts as an essential signal mediating nicotine biosynthesis in tobacco under salt stress. During this process, salt treatment induced the expression of MYC2a transcription factors. MYC2a transcription factors then activated the expression of JA biosynthesis-related genes to induce JA generation. At the same time, the JA signal was sensed by COI1 proteins, inducing JAZ repressor protein degradation to release MYC2a activity, which then activated *NtPMT1* expression, finally inducing nicotine biosynthesis.

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