Original article

Salt stress in apple seedlings was mitigated by *n*-propyl dihydrojasmonate, a synthetic analog of jasmonic acid

L. Wang¹, S. Wang¹, H. Tomiyama¹, P. Opio¹, H. Ohara², K. Ohkawa¹, T. Saito¹ and S. Kondo¹¹ Graduate School of Horticulture, Chiba University, Matsudo, Japan² Center for Environment, Health and Field Sciences, Chiba University, Kashiwa, Japan

Summary

The effects of *n*-propyl dihydrojasmonate (PDJ) application, which is a synthetic analog of jasmonic acid (JA) against sodium chloride (NaCl) stress in apple seedlings, were examined. The malondialdehyde (MDA) and proline concentrations, endogenous JA and abscisic acid (ABA) concentrations, antioxidant activities, Na⁺ and K⁺ concentrations, along with 9-*cis*-epoxycarotenoid deoxygenase 1 (*MdNCED1*), ABA 8'-hydroxylase 1 (*MdCYP707A1*), allene oxide synthase 1 (*MdAOS1*), jasmonate resistant (*MdJAR*), jasmonate ZIM-domain 2 (*MdJAZ2*), Δ 1-pyrroline-5-carboxylate synthetase (*MdP5CS*), ornithine aminotransferase 1 (*MdOAT1*), proline dehydrogenase (*MdPDH*), salt overly sensitive 1 (*MdSOS1*) and Na⁺/H⁺ exchanger 1 (*MdNHX1*) gene expressions in the leaves were analyzed. NaCl treatment without PDJ application (PDJ-NaCl⁺) significantly decreased chlorophyll concentrations and superoxide (SOD), peroxidase (POD) and catalase (CAT) activities, but increased the ion leakage, MDA, proline, and Na⁺ concentrations, as well as the hydrogen peroxide (H₂O₂) and endogenous JA and ABA concentrations in the leaves. Foliar application of PDJ before NaCl treatment (PDJ+NaCl⁺) decreased Na⁺ concentrations, ion leakage, MDA, proline accumulation and H₂O₂ generation, but increased K⁺ concentrations, antioxidant capacity, and chlorophyll concentrations in the leaves. In the PDJ+NaCl⁺ group, although the JA and ABA concentrations and *MdAOS1*, *MdJAR*, and *MdNCED1* gene expressions were increased, the *MdJAZ2* and *MdCYP707A1* gene expressions were decreased. In addition, the PDJ+NaCl⁺ group showed higher *MdPDH*, *MdSOS1*, and *MdNHX1* gene expressions and lower *MdP5CS* gene expressions than the PDJ-NaCl⁺ group at 7 and 10 days after treatment. The present data suggested that PDJ application could induce rapidly endogenous JA and ABA, enhance SOD, POD, and CAT activities, decrease the Na⁺ uptake, and mitigate NaCl stress.

Keywords

abscisic acid, antioxidant activity, jasmonic acid, *Malus domestica*, NaCl stress, proline

Significance of this study

What is already known on this subject?

- PDJ application can enhance chilling tolerance.

What are the new findings?

- PDJ application mitigates NaCl stress by inducing endogenous JA and ABA accumulation, enhancing antioxidant activities, and reducing Na⁺ uptake in apple seedlings.

What is the expected impact on horticulture?

- PDJ mitigates NaCl stress in fruit trees and results in stable yield and fruit quality.

an unfavorable abiotic environment leads plants to develop strategies to respond appropriately. These strategies include the alteration of phytohormones such as jasmonic acid (JA) and abscisic acid (ABA) and amino acids such as proline levels (Figure 1). JA can reinforce stress resistance in plants exposed to NaCl by enhancing the activities of antioxidant enzymes and triggering the expression of stress response genes (Ruan et al., 2019). Pedranzani et al. (2003) showed that salt-tolerant tomato (*Solanum lycopersicum*) showed higher levels of JA concentrations and AOS gene expressions. The JA signaling mechanism against abiotic stress involves the activation of JA by the *JAR* gene, which conjugates JA and isoleucine to jasmonoyl-L-isoleucine (JA-Ile), and ultimately activates the downstream responses via the degradation of jasmonate ZIM-domain (JAZ) repressor proteins (Figure 1A) (Ruan et al., 2019).

Plants accumulate ABA through the functions of two key enzymes, 9-*cis*-epoxycarotenoid deoxygenase (NCED) and ABA 8'-hydroxylase (CYP707A) (Waśkiewicz et al., 2013) (Figure 1B). Over-expression of the *OsNCED5* gene enhanced salt tolerance by controlling ABA biosynthesis and genes related to abiotic stress in rice (*Oryza sativa* L.) (Huang et al., 2019). Sales et al. (2017) found that the inhibition of *CYP707A1* attenuated the catabolism of ABA and increased endogenous ABA concentrations under NaCl stress and decreased Na⁺ intake and mitigated salinity damage in apple seedlings. Generally, crosstalk exists between ABA and JA with regard to their signaling behavior (Yang et al., 2019). De Ollas et al. (2013) found that JA transient accumulation was needed for the ABA response to salt tolerance in citrus (*Citrus reticulata* Blanco).

Various antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT) and peroxidase (POD) have been induced against salt stress (Ahmad et al., 2016). As a free radical scavenger, proline is synthesized by the enzyme

Introduction

Sodium chloride (NaCl) in soil causes ion toxicity, the formation of reactive oxygen species (ROS), membrane dysfunction, oxidative damage, and the attenuation of multiple metabolic activities (Annunziata et al., 2017). Exposure to

FIGURE 1. A summary of a schematic representation of the biosynthesis and degradation of JA, ABA, and proline (A, B, C) and the Na⁺ ion transport in plant cells (D). The genes analyzed in our study are underlined. (A) JA-related genes: *AOS*, allene oxide synthase; *JAR*, jasmonate resistant; *JAZ*, jasmonate ZIM-domain; (B) ABA-related genes: *NCED*, 9-cis-epoxycarotenoid deoxygenase; *CYP707A*, ABA 8'-hydroxylase; (C) proline-related genes: *P5CS*, Δ^1 -pyrroline-5-carboxylate synthetase; *OAT*, ornithine aminotransferase; *P5CR*, Δ^1 -pyrroline-5-carboxylate reductase; *PDH*, proline dehydrogenase; (D) Na⁺ ion-transport-related genes: *SOS1*, salt overly sensitive 1; *NHX*, Na⁺/H⁺ exchanger; *HKT*, Na⁺ and/or K⁺ permeable transporter.

Δ^1 -pyrroline-5-carboxylate synthetase (*P5CS*) and ornithine- δ -aminotransferase (*OAT*), and degraded through oxidation by proline dehydrogenase (*PDH*) (Cao et al., 2020; Figure 1C). It has been shown that proline accumulation under salt stress in plant tissues is attributed to the high expression of the *P5CS* gene and decreased expression of the *PDH* gene (Cao et al., 2020). This resulted in protection against NaCl-induced ROS and lipid peroxidation through the increase of antioxidant enzyme activities (Reddy et al., 2015). ABA induced proline production in salt-stressed plants by activating *P5CS* gene expression (Guan et al., 2019). ABA regulates the intracellular Na⁺ dynamic balance via inducing the expression of the *salt overly sensitive 1* (*AtSOS1*) and *Na⁺/H⁺ exchanger 1* (*AtNHX1*) genes in *Arabidopsis thaliana* (Figure 1D). Both genes encode the plasma membrane Na⁺/H⁺ transporter and Na⁺/H⁺ exchanger protein, functioning as a Na⁺ efflux carrier from the cell and in Na⁺ compartmentalization in vacuoles (Yamaguchi et al., 2013).

A synthetic analog of JA, *n*-propyl dihydrojasmonate (PDJ), has been widely used in Japan for the promotion of apple coloration (Kondo et al., 2000). PDJ could inhibit *Botrytis cinerea* pathogen infection in apples (Suktawee et al., 2019). PDJ treatment also improved chilling tolerance in apple fruits through the induction of scavenging activity against reactive oxygen (Kondo, 2009). Therefore, PDJ application may mitigate biotic and abiotic stresses, including salinity.

In the present study, the endogenous JA and ABA and

proline concentrations, SOD, POD, CAT, and ascorbate peroxidase (APX) antioxidant activities, Na⁺ and K⁺ concentrations, and their respective gene expressions were examined to clarify whether PDJ can mitigate NaCl stress in apple seedlings.

Materials and methods

Plant materials and treatment

Ninety-day-old 'Tsugaru' apple seedlings [*Malus sylvestris* (L.) Mill. var. *domestica* (Borkh.) Mansf.] were used. The germinated apple seeds were sown in plastic trays (26 × 52 × 6 cm) with a capacity of 72 seeds per tray with a spacing of 4 × 4 cm, containing moist vermiculite as a substrate. The trays were set under the greenhouse for growth; a half liter of water was added in the bottom of each tray every day to keep it moist, and 0.2% (v/v) Hyponex® solution was applied as a nutrient (Hyponex Japan Co., Osaka, Japan). The seedlings were grown under natural light conditions with an average daytime temperature range in the greenhouse of 25 to 30°C, and a range of 15 to 20°C at night.

Treatment consisted of an application of 0.8 mM of PDJ solution containing 0.5% Approach BI® (Maruwa Biochemical Co., Tokyo, Japan) on both sides of the leaves. After 24 h, 100 mM NaCl was applied to seedlings by dipping into 1 L NaCl solutions per tray. The apple seedlings were divided into four groups as follows: (1) a group without any treatment (untreated control); (2) a group treated

with 0.8 mM of PDJ solution, but without NaCl (PDJ⁺NaCl⁻); (3) a group treated with 100 mM NaCl solution, without PDJ application (PDJ⁻NaCl⁺); and (4) a group administered a combination of 100 mM NaCl and PDJ (PDJ⁺NaCl⁺). The concentrations of PDJ and NaCl were based on a previous study by Sales et al. (2017). A total of 240 seedlings were sampled from each group, divided into three replications, and healthy fresh leaves were collected at 1, 4, 7, and 10 days after the beginning of the NaCl treatment, frozen with liquid nitrogen, and kept at -80°C until further analysis.

Determination of ion leakage and lipid peroxidation

Ion leakage was determined according to Jambunathan (2010). The fourth leaf from the tip of each seedling (six replications per each treatment) was sampled, and ion leakage was determined using an electrical conductivity meter (LAQUAtwin-pH-22, Horiba, Japan). The relative ion leakage was expressed as a percentage of conductivity before and after the boiling of leaves in respective aqueous extracts. The amount of malondialdehyde (MDA) was measured according to Heath and Packer (1968). MDA was determined spectrophotometrically at OD₄₅₀, OD₅₃₂, and OD₆₀₀ using the UV-VIS spectrophotometer (2J1-0010, Hitachi, Japan).

Measurement of hydrogen peroxide (H₂O₂), proline concentrations, and antioxidative enzyme activity

The concentrations of H₂O₂ in fresh leaves (0.5 g, three replications) were measured according to the method of Shi et al. (2005). The absorbance was at 390 nm, and the final concentration was calculated according to the standard curve derived from different concentrations of H₂O₂. The proline concentrations were assayed based on the method of Sales et al. (2017). The fresh leaf tissues (0.5 g, three replications) were homogenized with 10 mL sulfosalicylic acid 3% (w/v) and then centrifuged at 15,000 *g* for 10 min. After reaction with ninhydrin solution, the compound was measured at 520 nm by a spectrophotometer (U-2910; Hitachi, Tokyo, Japan). A standard curve was obtained using L-proline. For antioxidant enzyme analysis, the leaf tissues (three replications of 0.5 g) were homogenized with 15 mL phosphate buffer (50 mM, pH 7.8) containing 0.5 mM EDTA and 2% (w/v) polyvinylpyrrolidone and centrifuged at 15,000 *g* for 15 min. The antioxidant enzyme activities of SOD, POD, CAT, and APX were measured according to the method of He et al. (2014).

Determination of chlorophyll, Na⁺ and K⁺ concentrations

After the extraction of chlorophyll in the leaves using N, N-dimethylformamide, the chlorophyll concentration was measured at absorbances of 663 and 645 nm using a spectrophotometer (U-2910; Hitachi, Tokyo, Japan). The chlorophyll *a* (Chl *a*), chlorophyll *b* (Chl *b*), and total chlorophyll concentrations were calculated according to Burton et al. (1986). The Na⁺ and K⁺ concentrations in the leaves were determined according to Sales et al. (2017). The leaf samples (0.5 g dry weight, three replications) were mixed with 15 mL nitric acid, then converted to ashes at 450°C and finally ground into powder. The residues were dissolved in 0.1 N hydrochloric acid and filtered. The solutions were analyzed by multitype ICP emission spectrometers (ICPE-9000; ICP-AES Multitype ICP Emission Spectrometer, Shimadzu, Japan).

Analysis of JA and ABA

JA in the leaves was analyzed according to Suktawee et al. (2019). Leaf samples (1 g FW, three replications) were homogenized in 10 mL saturated NaCl solution, 20 mL diethyl

ether containing 0.005% butylated hydroxytoluene (BHT) and 500 µL of 1 M citric acid, and 100 µL [(±)-2-(2,3-²H₂) JA (100 mg L⁻¹)] as an internal standard. The final extract was dissolved with methanol and analyzed using gas chromatography/mass spectrometry-selective ion monitoring (GC-MS-SIM) (QP 5000; Shimadzu, 25 m × 0.25 mm I.D. column) as described by Kondo et al. (2009). The ABA was extracted using the method described by Sales et al. (2017). Leaf samples (1 g FW, three replications) were homogenized with 20 mL cold 80% (v/v) methanol with 100 mg L⁻¹ BHT and 0.5 g polyvinylpyrrolidone as a stabilizer with 0.2 µg ABA-*d*₆ as an internal standard. The extract was purified with high performance liquid chromatography (flow rate, 1.5 mL min⁻¹; detection at 254 nm; ODS-Mightysil RP-18 column, 250 mm × 4.6 mm i.d.) and finally measured with GC-MS-SIM (QP5000; Shimadzu, Kyoto, Japan).

Total RNA isolation, cDNA synthesis and quantitative reverse transcription polymerase chain reaction (qRT-PCR) analysis

Total RNA was extracted using cetyltrimethylammonium bromide as reported by Kondo et al. (2012). The first-strand cDNA was synthesized from 1 µg of total RNA according to the instruction manual of ReverTra Ace[®] qPCR RT Master Mix (Toyobo, Osaka, Japan). qRT-PCR was performed on a StepOnePlus[™] system (Applied Biosystems, U.S.A.) using the Thunderbird[™] SYBR[®] qPCR Mix (Toyobo, Osaka, Japan). The primers except for the *MdUbiquitin-11* gene were designed using Beacon Designer 7 (Premier Biosoft Int.) software (Supplemental Table 1). The *MdUbiquitin-11* gene was used as an internal control gene. The results were calculated using the 2^{-ΔΔCT} method (Yu et al., 2007). Three biological replicates were performed for each set of treatment.

Statistical analysis

All data were statistically analyzed with SPSS statistics software (IBM SPSS statistics 20, IBM Corp., Armonk, NY) and means separated using Duncan's multiple range test at the P ≤ 0.05 level.

Results

Chlorophyll, MDA, ion leakage and H₂O₂ concentrations

At 10 days after treatment (DAT), the concentrations of Chl *a*, Chl *b*, and total chlorophyll were lowest in the PDJ⁻NaCl⁺ group compared to the other groups (Figure 2a). The Chl *a*/Chl *b* ratio was highest in the PDJ⁺NaCl⁺ group, followed by the PDJ⁺NaCl⁻ and PDJ⁻NaCl⁻ groups, and the untreated control group was the lowest.

The ion leakage and MDA and H₂O₂ concentrations in the leaves exhibited a similar tendency. These increased in the PDJ⁻NaCl⁺ and PDJ⁺NaCl⁺ groups through NaCl treatment (Figures 2b–d). The PDJ⁺NaCl⁺ group showed lower concentrations compared to the PDJ⁻NaCl⁺ group at 7 and 10 DAT. Ion leakage and MDA production at 7 and 10 DAT were not significantly different between the PDJ⁻NaCl⁻ group and the untreated controls. The PDJ⁻NaCl⁻ group showed increased H₂O₂ concentrations compared to the untreated control at 1 and 4 DAT.

Endogenous JA concentrations and expressions of JA-related genes

The endogenous JA concentrations in the PDJ⁻NaCl⁺ and PDJ⁺NaCl⁺ groups were significantly higher from 4 to 10 DAT compared to the untreated controls (Figure 3a). JA in the

FIGURE 2. Changes of Chl *a*, Chl *b*, and total Chl (a), ion leakage (b), MDA (c), and H₂O₂ (d) concentrations. Chlorophyll concentrations were obtained only at 10 DAT in this study. The data represent the average value ± SE of six replications for the ion leakage and of three replications for the other indexes. Different letters indicate significant differences as determined by Duncan's multiple range test at $P \leq 0.05$.

PDJ⁺NaCl⁺ group was 1.8-fold that in the PDJ⁺NaCl⁻ group at 1 DAT. The JA concentrations in the PDJ⁺NaCl⁻ group increased at 1 and 4 DAT. Both the *allene oxide synthase 1* (*MdaOS1*) and *jasmonate resistant* (*MdJAR*) genes were up-regulated by NaCl treatment compared with the untreated control group at 4, 7, and 10 DAT (Figure 3b, c). Compared to the PDJ⁺NaCl⁻ group, the *MdaOS1* and *MdJAR* gene expressions in the PDJ⁺NaCl⁺ group showed higher levels at 7 and 10 DAT, respectively. On the other hand, the expressions of the *jasmonate ZIM-domain 2* (*MdJAZ2*) gene in the PDJ⁺NaCl⁺ group were significantly lower than those in the untreated control (Figure 3d). In the PDJ⁺NaCl⁺ group, the expressions of the *MdJAZ2* gene were lower than that in the PDJ⁺NaCl⁻ group at 1 and 4 DAT.

Endogenous ABA concentrations and expressions of ABA-related genes

NaCl treatment (PDJ⁺NaCl⁺ and PDJ⁺NaCl⁻) significantly increased the ABA concentrations compared to other treatments at 4, 7, and 10 DAT (Figure 4a). The ABA concentrations in the PDJ⁺NaCl⁺ treatment were higher than those in the PDJ⁺NaCl⁻ group at 1 DAT but lower at 7 and 10 DAT. The ABA concentrations in the PDJ⁺NaCl⁻ group were increased at 1 DAT but significantly declined at 7 and 10 DAT, compared to the PDJ⁺NaCl⁺ group. The *9-cis-epoxycarotenoid deoxygen-*

ase 1 (*MdNCED1*) gene expressions in both the PDJ⁺NaCl⁺ and PDJ⁺NaCl⁻ groups were up-regulated at 7 DAT (Figure 4b). The *MdNCED1* gene expressions in the PDJ⁺NaCl⁻ group were highest compared to the other treatments at 10 DAT. The expression of the *ABA 8'-hydroxylase 1* (*MdCYP707A1*) gene in the PDJ⁺NaCl⁻ and PDJ⁺NaCl⁺ groups showed a similar tendency as the *MdNCED1* gene (Figure 4c).

Antioxidant enzyme activities

The SOD, CAT, and POD enzyme activities in the PDJ⁺NaCl⁺ group increased at 4 DAT but declined significantly at 10 DAT compared to the untreated control group (Figure 5a–c). Foliar application of PDJ before the NaCl treatment (PDJ⁺NaCl⁺ group) increased the antioxidant enzyme activities of SOD, CAT, POD, and APX compared to those of the PDJ⁺NaCl⁻ group at 7 DAT (Figure 5).

Proline concentrations and expressions of proline-related genes

The proline concentrations in the PDJ⁺NaCl⁺ group significantly increased at 4, 7, and 10 DAT and followed by the PDJ⁺NaCl⁻ group (Figure 6a). In general, the expressions of both the *Δ1-pyrroline-5-carboxylate synthetase* (*MdP5CS*) and *ornithine aminotransferase 1* (*MdOAT1*) genes were significantly upregulated in the PDJ⁺NaCl⁺ and PDJ⁺NaCl⁻ groups compared to the untreated group at 4 to 10 DAT (Figure 6b, c). In contrast, the *proline dehydrogenase* (*MdPDH*) gene expression was downregulated in the PDJ⁺NaCl⁺ group (Figure 6d).

Na⁺ and K⁺ concentrations and expressions of ion-transport-related genes

The Na⁺ concentrations in the PDJ⁺NaCl⁺ and PDJ⁺NaCl⁻ groups increased by 6.8- and 2.5-fold, respectively, compared to those in the untreated control group at 10 DAT (Figure 7a). The PDJ⁺NaCl⁻ treatment did not influence the Na⁺ concentrations compared to the untreated control, but it enhanced the K⁺ levels at 7 and 10 DAT. The K⁺ concentration in the PDJ⁺NaCl⁺ group was highest, followed by those in the PDJ⁺NaCl⁻ and PDJ⁺NaCl⁺ groups, and the untreated control group was lowest at 10 DAT. The highest K⁺/Na⁺ ratio was the PDJ⁺NaCl⁺ group, and the lowest was the PDJ⁺NaCl⁻ group. The expressions of both the *salt overly sensitive 1* (*MdSOS1*) and *Na⁺/H⁺exchanger 1* (*MdNHX1*) genes in the PDJ⁺NaCl⁺ group increased at 4 DAT (Figure 7b, c). The expressions of the *MdSOS1* and *MdNHX1* genes in the PDJ⁺NaCl⁺ group were increased significantly compared to the other treatments at 7 and 10 DAT.

Discussion

In our study, a decrease in chlorophyll concentrations was observed in the PDJ⁺NaCl⁺ group, but there was no significant difference in the PDJ⁺NaCl⁻ group, when compared with the control group. Rezai et al. (2013) found that MeJA application inhibited the degradation of chlorophyll in pepper (*Capiscum annuum* L.) under NaCl stress. During the process of chlorophyll degradation, Chl *b* is first converted to Chl *a*, then degraded by chlorophyllase (Fang et al., 1998). This process may explain the increased ratio of Chl *a*/Chl *b* and the decrease in the total chlorophyll concentrations in NaCl-treated leaves. Membrane lipids are considered to be significantly vulnerable to the salinity, and NaCl-induced stress generates ROS, disrupting lipid membrane integrity and ion homeostasis (Reddy et al., 2015). Our study revealed that MDA, the final product of lipid peroxidation, and the ion leakage rate were significantly inhibited in the PDJ⁺NaCl⁺ plants. Qiu et

FIGURE 3. Endogenous JA concentrations (a) and gene expressions of *MdAOS1* (b), *MdJAR* (c), and *MdJAZ2* (d). The data represent the average value $\pm$ SE ($n=3$). The *MdAOS1*, *MdJAR*, and *MdJAZ2* were plotted compared to the control (*Ubiquitin gene*) expression. Different letters indicate significant differences as determined by Duncan's multiple range test at $P \leq 0.05$.

al. (2014) reported that exogenous JA treatment ameliorated NaCl injury in wheat (*Triticum aestivum* L.) by decreasing the concentrations of MDA and H₂O₂ and the ion leakage rate. Meanwhile, at 7 DAT, the APX enzyme activity in the PDJ⁺NaCl⁻ group was higher than that in the PDJ⁻NaCl⁺ group. These results suggested that the PDJ⁺NaCl⁻ treatment retarded the MDA production and ion leakage. Therefore, PDJ may mitigate membrane injury caused by NaCl stress.

It has been shown that ABA induced stomatal closure and up-regulated the expression of stress-related genes to increase salt tolerance (Waśkiewicz et al., 2013). Our results showed that the ABA concentrations and *MdNCED1* gene expression were increased in the PDJ⁺NaCl⁻ group. This was consistent with Kim et al. (2009), who found that JA application induced the increase of endogenous ABA concentrations quickly under NaCl stress and stronger salt tolerance in chard plant (*Beta vulgaris* L.). Huang et al. (2019) also reported that *OsNCED5* transgenic plants presented stronger salt tolerance than wild-type plants owing to higher endogenous ABA concentrations. The JA concentrations in our study continually accumulated in the PDJ⁺NaCl⁻ group, as did the *MdAOS1* gene expression. Pedranzani et al. (2003) also showed that salt-tolerant tomato showed higher levels of JA concentrations and *AOS* gene expressions. The accumulation of endogenous JA could activate the expression of the *JAR* gene and enhance the conversion of JA to JA-Ile, resulting in the induction of the proteolysis of JAZ (Ruan et al., 2019). The higher expression of the *MdJAZ2* gene in the PDJ⁺NaCl⁻ group implied the higher accumulation of JA-Ile, whose function is in activating JA signaling pathways directly. Liu et al. (2019) reported that overexpression of the *PnJAZ1* gene confers salinity tolerance in moss (*Pohlia nutans*) via crosstalk with the ABA signaling pathway. De Ollas et al. (2013) showed that both JA and ABA accumulated in salt-stressed plants. Thus, it is reasonable to presume that the application of PDJ initiated

FIGURE 4. Endogenous ABA concentrations (a) and gene expressions of *MdNCED1* (b) and *MdCYP707A1* (c). The data represent the average value $\pm$ SE ($n=3$). Different letters indicate significant differences as determined by Duncan's multiple range test at $P \leq 0.05$.

FIGURE 5. Antioxidant enzymes of SOD (a), CAT (b), POD (c), and APX (d) activity in apple leaves. The data represent the average value $\pm$ SE ($n=3$). Different letters indicate significant differences as determined by Duncan's multiple range test at $P \leq 0.05$.

FIGURE 6. Proline concentrations (a) and gene expressions of *MdP5CS* (b), *MdOAT1* (c), and *MdPDH* (d) in apple leaves under different treatments. The data represent the average value $\pm$ SE ($n=3$). Different letters indicate significant differences as determined by Duncan's multiple range test at $P \leq 0.05$.

the JA and ABA response, hence modulating multiple defense systems to overcome the adverse impacts caused by salt.

H_2O_2 is an important signaling molecule that is involved in many metabolic processes, and its excessive generation frequently triggers oxidative stress, lipid peroxidation and MDA accumulation (Liu et al., 2020). In our study, the high concentrations of H_2O_2 and MDA in the PDJ⁻NaCl⁺ group suggested the occurrence of oxidative stress. In contrast, the lower level of H_2O_2 and MDA in the PDJ⁺NaCl⁺ group implies a mitigation effect of PDJ application on oxidative injury caused by NaCl stress. This result may be associated with the higher SOD, POD, and CAT enzyme activities in the PDJ⁺NaCl⁺ group. Shu et al. (2016) reported that the accumulation of endogenous ABA mediated H_2O_2 generation, which consequently induced the antioxidant defense systems and

enhanced cucumber (*Cucumis sativus* L.) salt tolerance. The increase of these enzyme activities was essential for enhancing salt tolerance in wheat (*Triticum aestivum* L.) and grapes (*Vitis vinifera* L.) (Qiu et al., 2014; Haider et al., 2019). Our results were consistent with those of a previous report by Kondo et al. (2009), who found that exogenous application of PDJ enhanced the chilling tolerance of the fruits by increasing the radical scavenging ability. Additionally, Shan and Yang (2017) also found that exogenous JA induced other antioxidant metabolites such as ascorbic acid and glutathione synthesis with neutralized toxic effects of NaCl stress. Thus, the reduction of H_2O_2 and the enhancement of SOD, POD, and CAT enzyme activities in the PDJ⁺NaCl⁺ group led to higher radical scavenging activity to minimize the oxidative injury caused by NaCl stress.

FIGURE 7. Na⁺ and K⁺ concentrations (a), *MdSOS1* (b), and *MdNHX1* (c) gene expressions in apple leaves under different treatments. The data represent the average value ± SE (*n* = 3). Different letters indicate significant differences as determined by Duncan's multiple range test at *P* ≤ 0.05.

Proline plays an essential role in stabilizing the membrane structure and enhancing the cell osmotic potential. It accumulates in many plant species in parallel with increased external salinity (Cao et al., 2020). In our study, the proline concentrations and the expressions of *MdP5CS* and *MdOAT1* genes were significantly increased in both NaCl treated groups, with a reversed expression trend of the *MdPDH* gene only in the PDJ+NaCl⁺ group. Cao et al. (2020) found that proline accumulation was attributed to a decrease expression of *OsPDH* and an increase of the *OsP5CS* gene, which are regulated by ABA-dependent signals under NaCl stress. This result may support our result which showed that NaCl treatment induced proline and ABA accumulation. The lower proline concentrations in the PDJ+NaCl⁺ group may imply less injury to the seedlings due to NaCl stress.

It has been shown that plants increase Na⁺ uptake and reduce K⁺ uptake under salt stress (Garcia-deblás et al., 2003). High Na⁺ concentrations in plants retard the uptake of K⁺ ions and accelerate their efflux, resulting in increased ion leakage and membrane lipid peroxidation in the leaves, as well as the occurrence of oxidative stress (Zhang et al., 2018). In our study, the Na⁺ ion uptake in the PDJ+NaCl⁺ group was increased significantly, while K⁺ ion decreased, implying the occurrence of Na⁺ toxicity. Nevertheless, the application of PDJ prior to NaCl stress caused lower Na⁺ uptake and higher K⁺/Na⁺ ratio when compared with the NaCl-only treated group. This result may show that PDJ regulates the absorption of Na⁺ in plant tissues. Previous studies found that the overex-

pression of *MdNHX1* or *SOS1* genes decreased Na⁺ efflux, enhancing salt resistance in apple and tobacco plant (Sun et al., 2017; Yue et al., 2012). The up-regulation of the *MdSOS1* and *MdNHX1* genes in the PDJ+NaCl⁺ group was concomitant with a reduced Na⁺ concentration. This process was most likely regulated by endogenous ABA (Osakabe et al., 2014).

In conclusion, PDJ application prior to NaCl treatment could mitigate the NaCl stress by inducing the rapid accumulation of endogenous JA and ABA, enhancing antioxidant enzymes activities, regulating proline concentrations, reducing Na⁺ uptake, and retarding the increase of MDA and ion leakage (Figure 8). Therefore, PDJ could be used to mitigate NaCl stress in fruit trees to stabilize yield and fruit quality.

References

- Ahmad, P., Abdel Latef, A.A., Hashem, A., Abd Allah, E.F., Gucel, S., and Tran, L.-S.P. (2016). Nitric oxide mitigates salt stress by regulating levels of osmolytes and antioxidant enzymes in chickpea. *Front. Plant Sci.* 7, 347. <https://doi.org/10.3389/fpls.2016.00347>.
- Annunziata, M.G., Ciarmiello, L.F., Woodrow, P., Maximova, E., Fuggi, A., and Carillo, P. (2017). Durum wheat roots adapt to salinity remodeling the cellular content of nitrogen metabolites and sucrose. *Front. Plant Sci.* 7, 2035. <https://doi.org/10.3389/fpls.2016.02035>.
- Burton, K.W., King, J.B., and Morgan, E. (1986). Chlorophyll as an indicator of the upper critical tissue concentration of cadmium in plants. *Water, Air, Soil Poll.* 27(1–2) 147–154. <https://doi.org/10.1007/BF00464777>.
- Cao, X., Wu, L., Wu, M., Zhu, C., Jin, Q., and Zhang, J. (2020). Absciscic acid mediated proline biosynthesis and antioxidant ability in roots of two different rice genotypes under hypoxic stress. *BMC Plant Biol.* 20, 1–14. <https://doi.org/10.1186/s12870-020-02414-3>.
- De Ollas, C., Hernando, B., Arbona, V., and Gómez-Cadenas, A. (2013). Jasmonic acid transient accumulation is needed for abscisic acid increase in citrus roots under drought stress conditions. *Physiol. Plant.* 147, 296–306. <https://doi.org/10.1111/j.1399-3054.2012.01659.x>.
- Fang, Z., Bouwkamp, J.C., and Solomos, T. (1998). Chlorophyllase activities and chlorophyll degradation during leaf senescence in non-yellowing mutant and wild type of *Phaseolus vulgaris* L. *J. Exp. Bot.* 49, 503–510. <https://doi.org/10.1093/jexbot/49.320.503>.
- Garcia-deblás, B., Senn, M.E., Bañuelos, M.A., and Rodríguez-Navarro, A. (2003). Sodium transport and HKT transporters: The rice model. *Plant J.* 34, 788–801. <https://doi.org/10.1046/j.1365-3113X.2003.01764.x>.
- Guan, C., Huang, Y.-H., Cen, H.-F., Cui, X., Tian, D.-Y., and Zhang, Y.-W. (2019). Overexpression of the *Lolium perenne* L. delta1-pyrroline 5-carboxylate synthase (*LpP5CS*) gene results in morphological alterations and salinity tolerance in switchgrass (*Panicum virgatum* L.). *PLoS ONE* 14, e0219669. <https://doi.org/10.1371/journal.pone.0219669>.
- Haider, M.S., Jogaiah, S., Pervaiz, T., Yanxue, Z., Khan, N., and Fang, J. (2019). Physiological and transcriptional variations inducing complex adaptive mechanisms in grapevine by salt stress. *Environm. Exp. Bot.* 162, 455–467. <https://doi.org/10.1016/j.envexpbot.2019.03.022>.
- He, J., Ren, Y., Chen, X., and Chen, H. (2014). Protective roles of nitric oxide on seed germination and seedling growth of rice (*Oryza sativa* L.) under cadmium stress. *Ecotoxicol. Environm. Safety* 108, 114–119. <https://doi.org/10.1016/j.ecoenv.2014.05.021>.
- Heath, R.L., and Packer, L. (1968). Photoperoxidation in isolated chloroplasts: I. Kinetics and stoichiometry of fatty acid peroxidation. *Arch. Biochem. Biophys.* 125, 189–198. [https://doi.org/10.1016/0003-9861\(68\)90654-1](https://doi.org/10.1016/0003-9861(68)90654-1).

FIGURE 8. Schematic diagram of the regulation of NaCl tolerance in apple seedlings by PDJ treatment. Dark arrows indicate increases, and blank arrows indicate decreases.

Huang, Y., Jiao, Y., Xie, N., Guo, Y., Zhang, F., Xiang, Z., Wang, R., Wang, F., Gao, Q., and Tian, L. (2019). *OsNCED5*, a 9-*cis*-epoxycarotenoid dioxygenase gene, regulates salt and water stress tolerance and leaf senescence in rice. *Plant Sci.* 287, 110188. <https://doi.org/10.1016/j.plantsci.2019.110188>.

Jambunathan, N. (2010). Determination and detection of reactive oxygen species (ROS), lipid peroxidation, and electrolyte leakage in plants. In *Plant Stress Tolerance*, R. Sunkar, ed. (Springer), p. 291–297. https://doi.org/10.1007/978-1-60761-702-0_18.

Kim, S.K., Sohn, E.Y., and Joo, G.J. (2009). Influence of jasmonic acid on endogenous gibberellin and abscisic acid in salt-stressed chard plant. *J. Environm. Biol.* 30(3), 333–338.

Kondo, S. (2009). Roles of jasmonates in fruit ripening and environmental stress. *Acta Hort.* 884, 711–716. <https://doi.org/10.17660/ActaHortic.2010.884.96>.

Kondo, S., Tomiyama, A., and Seto, H. (2000). Changes of endogenous jasmonic acid and methyl jasmonate in apples and sweet cherries during fruit development. *J. Am. Soc. Hortic. Sci.* 125, 282–287. <https://doi.org/10.21273/JASHS.125.3.282>.

Kondo, S., Sugaya, S., Sugawa, S., Ninomiya, M., Kittikorn, M., Okawa, K., Ohara, H., Ueno, K., Todoroki, Y., and Mizutani, M. (2012). Dehydration tolerance in apple seedlings is affected by an inhibitor of ABA 8'-hydroxylase CYP707A. *J. Plant Physiol.* 169, 234–241. <https://doi.org/10.1016/j.jplph.2011.09.007>.

Liu, S., Zhang, P., Li, C., and Xia, G. (2019). The moss jasmonate ZIM-domain protein PnJAZ1 confers salinity tolerance via crosstalk with the abscisic acid signalling pathway. *Plant Sci.* 280, 1–11. <https://doi.org/10.1016/j.plantsci.2018.11.004>.

Liu, T., Ye, X., Li, M., Li, J., Qi, H., and Hu, X. (2020). H₂O₂ and NO are involved in trehalose-regulated oxidative stress tolerance in cold-stressed tomato plants. *Environm. Experim. Bot.* 171, 103961. <https://doi.org/10.1016/j.envexpbot.2019.103961>.

Osakabe, Y., Yamaguchi-Shinozaki, K., Shinozaki, K., and Tran, L.S.P. (2014). ABA control of plant macroelement membrane transport systems in response to water deficit and high salinity. *New Phytol.* 202, 35–49. <https://doi.org/10.1111/nph.12613>.

Pedranzani, H., Racagni, G., Alemano, S., Miersch, O., Ramírez, I., Peña-Cortés, H., Taleisnik, E., Machado-Domenech, E., and Abdala, G. (2003). Salt tolerant tomato plants show increased levels of jasmonic acid. *Plant Growth Regul.* 41, 149–158. <https://doi.org/10.1023/A:1027311319940>.

Qiu, Z., Guo, J., Zhu, A., Zhang, L., and Zhang, M. (2014). Exogenous jasmonic acid can enhance tolerance of wheat seedlings to salt stress. *Ecotoxicol. Environm. Safety* 104, 202–208. <https://doi.org/10.1016/j.ecoenv.2014.03.014>.

Reddy, P.S., Jogeswar, G., Rasineni, G.K., Maheswari, M., Reddy, A.R., Varshney, R.K., and Kishor, P.K. (2015). Proline over-accumulation alleviates salt stress and protects photosynthetic and antioxidant enzyme activities in transgenic sorghum [*Sorghum bicolor* (L.) Moench]. *Plant Physiol. Biochem.* 94, 104–113. <https://doi.org/10.1016/j.plaphy.2015.05.014>.

Rezai, S., Orojloo, M., Bidabadi, S.S., and Soleimanzadeh, M. (2013). Possible role of methyl jasmonate in protection to NaCl-induced salt stress in pepper cv. 'Green Hashemi'. *Int. J. Agric. Crop Sci.* 6, 1235.

Ruan, J., Zhou, Y., Zhou, M., Yan, J., Khurshid, M., Weng, W., Cheng, J., and Zhang, K. (2019). Jasmonic acid signaling pathway in plants. *Int. J. Molec. Sci.* 20, 2479. <https://doi.org/10.3390/ijms20102479>.

Sales, L., Ohara, H., Ohkawa, K., Saito, T., Todoroki, Y., Srilaong, V., and Kondo, S. (2017). Salt tolerance in apple seedlings is affected by an inhibitor of ABA 8'-hydroxylase CYP707A. *J. Plant Growth Regul.* 36, 643–650. <https://doi.org/10.1007/s00344-017-9665-y>.

Shan, C., and Yang, T. (2017). Nitric oxide acts downstream of hydrogen peroxide in the regulation of ascorbate and glutathione

metabolism by jasmonic acid in *Agropyron cristatum* leaves. *Biol. Plant.* **61**, 779–784. <https://doi.org/10.1007/s10535-017-0708-9>.

Shi, S., Wang, G., Wang, Y., Zhang, L., and Zhang, L. (2005). Protective effect of nitric oxide against oxidative stress under ultraviolet-B radiation. *Nitric Oxide* **13**, 1–9. <https://doi.org/10.1016/j.niox.2005.04.006>.

Shu, S., Gao, P., Li, L., Yuan, Y., Sun, J., and Guo, S. (2016). Absciscic acid-induced H₂O₂ accumulation enhances antioxidant capacity in pumpkin-grafted cucumber leaves under Ca(NO₃)₂ stress. *Front. Plant Sci.* **7**, 1489. <https://doi.org/10.3389/fpls.2016.01489>.

Suktawee, S., Shishido, M., Wang, S., Saito, T., Okawa, K., Ohara, H., Nimitkeatkai, H., Ikeura, H., and Kondo, S. (2019). *n*-Propyl dihydrojasmonates influence ethylene signal transduction in infected apple fruit by *Botrytis cinerea*. *Hortic. J.* **88**, 41–49 <https://doi.org/10.2503/hortj.UTD-001>.

Sun, M.H., Ma, Q.J., and Liu, X. (2017). Molecular cloning and functional characterization of *MdNHX1* reveals its involvement in salt tolerance in apple calli and *Arabidopsis*. *Sci. Hortic.* **215**, 126–133. <https://doi.org/10.1016/j.scienta.2016.11.031>.

Waśkiewicz, A., Beszterda, M., and Goliński, P. (2013). ABA: role in plant signaling under salt stress. In *Salt Stress in Plants*, P. Ahmad, M.M. Azooz, and M.N.V. Prasad, eds. (Springer), p. 175–196. https://doi.org/10.1007/978-1-4614-6108-1_8.

Yamaguchi, T., Hamamoto, S., and Uozumi, N. (2013). Sodium transport system in plant cells. *Front. Plant Sci.* **4**, 410. <https://doi.org/10.3389/fpls.2013.00410>.

Yang, J., Duan, G., Li, C., Liu, L., Han, G., Zhang, Y., and Wang, C. (2019). The crosstalks between jasmonic acid and other plant hormone signaling highlight the involvement of jasmonic acid as a core component in plant response to biotic and abiotic stresses. *Front. Plant Sci.* **10**. <https://doi.org/10.3389/fpls.2019.01349>.

Yu, L., Liu, J., Zhuang, Z., Yang, L., Zhang, R., Ye, X., and Cheng, J. (2007). Quantitative analysis of real-time PCR expression production by REST and 2-($\Delta\Delta CT$). *J. Trop. Med.* **10**, 956–958.

Yue, Y., Zhang, M., and Zhang, J. (2012). *SOS1* gene overexpression increased salt tolerance in transgenic tobacco by maintaining a higher K⁺/Na⁺ ratio. *J. Plant Physiol.* **169**(3), 255–261. <https://doi.org/10.1016/j.jplph.2011.10.007>.

Zhang, Y., Fang, J., Wu, X., and Dong, L. (2018). Na⁺/K⁺ balance and transport regulatory mechanisms in weedy and cultivated rice (*Oryza sativa* L.) under salt stress. *BMC Plant Biol.* **18**, 375. <https://doi.org/10.1186/s12870-018-1586-9>.

Received: Sep. 29, 2020

Accepted: Dec. 16, 2020

Addresses of authors:

Liwei Wang¹, Shanshan Wang¹, Hiroyuki Tomiyama¹, Peter Opio¹, Hitoshi Ohara², Katsuya Ohkawa¹, Takanori Saito¹ and Satoru Kondo^{1,*}

¹ Graduate School of Horticulture, Chiba University, Matsudo 271-8510, Japan

² Center for Environment, Health and Field Sciences, Chiba University, Kashiwa 277-0882, Japan

* Corresponding author;

E-mail: s-kondo@faculty.chiba-u.jp

Tel./Fax: +(81)-47-308-8800

SUPPLEMENTAL INFORMATION

SUPPLEMENTAL TABLE S1. Primers used for qRT-PCR analysis in this study.

Gene	Forward/reverse primer (5'–3')	Accession No.
<i>MdNCED1</i>	(F) TACTACTACTACTACTACTACA (R) GAAGGTTGTTGATAGGAA	XM_009356579
<i>MdCYP707A1</i>	(F) ACCGAGGAGATTACCATA (R) CTTCAATGCCAGGAACATA	XM_008358695
<i>MdAOS1</i>	(F) GAACCTGTTATCCACTCT (R) GGAAGACTCGTAGAAGAA	XM_008366758.3
<i>MdJAR</i>	(F) CTGCCTTCAATCAACATT (R) AATCAACAACCTCCAACCTT	XM_008377840.2
<i>MDJAZ2</i>	(F) ATTCTTGTCTTCCGTCTCT (R) ACCTTGCCTATCGTGATT	NM_001328912.1
<i>MdP5CS</i>	(F) TTATCGGACTTGTGAATA (R) ACAGGTATCTTAGTTGAC	XM_008389606.3
<i>MdOAT1</i>	(F) CGAGTATCTAACAAGCAT (R) ACACGAGACAATAATAGC	XM_017327930.2
<i>MdPDH</i>	(F) AAGCCAATGTGCCTCTAT (R) AGGAGTAGGTCAAGTAATCG	XM_008359589.3
<i>MdSOS1</i>	(F) ACCTGCTGACTGGCCCT (R) ATGTCCAGTAAGCAGCTTGG	XM_008391743.1
<i>MdNHX1</i>	(F) GGTGTTGCGATTGATGAC (R) AGTGGTTGGTTCTGATGA	NM_001328858.1
<i>Ubiquitin11</i>	(F) TCGCTGGAAAGCAGCTCGAAGA (R) GCTTCCGGCAAAGATCAGACG	XM_008360582.2