Biochemistry and Biophysics Reports 26 (2021) 100952

Contents lists available at ScienceDirect

Biochemistry and Biophysics Reports

journal homepage: www.elsevier.com/locate/bbrep

G protein and PLDδ are involved in JA to regulate osmotic stress responses in *Arabidopsis thaliana*

Ning Yang *, Yue Zhang, Lu Chen, Wei Wang, Ruirui Liu, Run Gao, Yaping Zhou, Hui Li

College of Life Sciences, Northwest Normal University, Lanzhou, 730070, PR China

ARTICLE INFO

Keywords:
Jasmonic acid
Phospholipase Dδ
G protein
Lipoxygenase
Osmotic stress

ABSTRACT

Jasmonic acid (JA) is regarded as an endogenous regulator which plays an important role in regulating plant growth, development and stress response. Using the seedlings of *A. thaliana* ecotype Col-0 (wild-type, WT), phospholipase Dδ (PLDδ) deficient mutant (*pldδ*), the G protein α subunit (GPA1) deficient mutant (*gpa1-4*), 9-Lipoxygenase (9-LOX) deficient mutants (*lox1* and *lox5*) as materials, the effects of JA responding to osmotic stress and the functions of G protein and PLDδ in this response were investigated. **The results showed that GPA1 involved in the regulation of JA to PLDδ under osmotic stress. Both GPA1 and PLDδ participated in the regulation of JA on the seed germination and osmotic tolerance.** Exogenous MeJA reduced the EL and MDA in WT, but increased the EL and MDA in *gpa1-4* and *pldδ*, indicating that GPA1 and PLDδ were involved in the protection of JA on the membrane. The genes expression levels, and the activities of PLDδ and LOX1 were significantly induced by osmotic stress. The LOX activity and JA content in *pldδ* seedings were lower obviously than those in WT, but were markedly increased and were higher than WT after applying phosphatidic acid (PA). These results demonstrated that JA responded to osmotic stress by regulating G protein and PLDδ in *A. thaliana*. PLDδ was located upstream of 9-LOX and involved in the JA biosynthesis.

1. Introduction

Osmotic is one of the most important limiting factors for plant growth and agricultural production around the world [1]. Plant constantly experiencing osmotic stress may possess or develop some unique physiological adaptation mechanisms to reduce the damage caused by stress [2]. Understanding the mechanisms of plant abiotic stress tolerance is valuable to develop stress tolerant crop plant for sustaining crop productivity in future.

Jasmonic acid (JA) is a signal molecule which plays role in response to biotic and abiotic stresses [3]. It has also been shown that JAs influence plant growth and development, including the flower promotion, fruit development, as well as inhibition of seed and pollen germination [4]. Qiu and his companions reported that exogenous JA **enhances** the tolerance of wheat seedlings to salt stress [5]. As the inhibitors of JA biosynthesis, Ibuprofen (IBU) and salicylhydroxamic acid (SHAM) block JA biosynthesis by inhibiting lipoxygenase, and provide valuable information for understanding the role of JA in plants [6,7].

PLDδ is the most abundant PLD in *A. thaliana* except PLDα1 and is the one of the major sources of endogenous PA [8]. Some **researchers** conducted on several different plant species have shown that PLD genes **are** upregulated and PLD activities **are** increased under water-deficit [9]. PLDδ is also activated in response to high salinity and rapid dehydration [10,11]. Compared with wild-type plants, PLDδ knockout (KO) plants exhibit less tolerance to freezing injuries whereas PLDδ overexpression (OE) plants exhibit more tolerance [12]. PLDδ-antisense *A. thaliana* plants **do not** display overt changes in phenotype, but PLDδ-KO plants **are** more susceptible to salt stress [13].

Heterotrimeric G-proteins, which have been identified in a number of plant species and play roles in several plant signal pathways, are composed of distinct α, β and γ subunits. Pharmacological studies have identified a role for heterotrimeric G-proteins in signaling pathways regulated by a number of phytohormones as well as by biotic and abiotic environmental signals such as pathogens, ozone, and light [14]. Zhao and Wang reported that the α-subunit (Gα) of heterotrimeric G-protein interacts with *A. thaliana* PLDα1 through a motif analogous to the DRY motif in G-protein-coupled receptors [15]. Analysis of gene identification suggested that the Gα has a role in modulating the expression of JA-inducible genes [14].

Lipoxygenase (LOX) plays an important role in JA synthesis [16]. At

* Corresponding author.
E-mail address: xbsd-yn@163.com (N. Yang).

https://doi.org/10.1016/j.bbrep.2021.100952
Received 2 November 2020; Received in revised form 4 February 2021; Accepted 5 February 2021

present, in *A. thaliana* genome, it is speculated that a total of six genes encode LOX protein, which is named LOX1-6 in turn. LOX proteins are divided into 9-LOX (AtLOX-1 and AtLOX-5) and 13-LOX (AtLOX2, AtLOX3, AtLOX4 and AtLOX6) according to the oxidic position of fatty acid C atom [11]. Vellosillo and co-workers found that 9-LOX is involved in lateral root development and defense responses in *A. thaliana* [17]. Remans reported that LOX1 is the highest expressed member of the LOX gene family in roots [18]. Keunen demonstrated that a strongly **upregulated** transcription level of the cytosolic LOX1 gene exposures after Cd in *A. thaliana* roots [19].

Previous studies showed that JA level increases when plants exposed to various stress conditions [20]. JA may act directly as a ligand for heterotrimeric G-protein in *A. thaliana* [21]. PLD is also regulated by the heterotrimeric G protein [22]. 13-LOX is widely considered to be involved in the JA biosynthesis [23]. However, to date, the relevance between 9-LOX and JA biosynthesis is still unclear. In addition, whether or not JA responds to osmotic stress by regulating G protein and PLD, and what the PLD function is in LOX-induced JA biosynthesis were never reported before. In this study, **we found that JA responded to osmotic stress by regulating G protein and PLDδ.** PLDδ was located upstream of 9-LOX and involved in the JA biosynthesis.

2. Materials and methods

2.1. Plant material and treatments

The WT and T-DNA insertion mutants of *pldδ* (SALK_092,469), *gpa1-4* (SALK_001846), *lox1* (SALK_012,188) and *lox5* (SALK_050,933) were obtained from the *Arabidopsis* Biological Resource Center (ABRC). Seeds of different genotype were kept in the dark at 4 °C for 3 days to break dormancy, then sterilized with Ethanol (75%, v/v) for 30 s, NaClO (0.5%, v/v) for 30 s, followed by washing with sterile water three times. The seeds were sown on MS medium containing 3% (w/v) sucrose and 0.5% (w/v) agar, then incubated at 22 °C with 16 h light/8 h dark photoperiod.

Osmotic stress treatment was followed as described previously with some modifications [24]. For mannitol treatment, 15-day-old seedlings were transferred to MS medium plate containing 0 or 0.3 mol L^{-1} manntiol for different times.

For MeJA treatment, 15-day-old seedlings were transferred to MS medium plate containing 0.25 μmol L^{-1} MeJA for 24 h. For JA synthesis inhibitors treatment, 15-day-oldseedlings were transferred to MS medium plates containing 5 μmol L^{-1} IBU and 100 μmol L^{-1} SHAM for 24 h.

For drought treatments, 20-days-old plants were subjected to progressive drought by withholding water for 14 days.

2.2. Total RNA extraction, reverse transcription PCR and RT-qPCR analysis

Total RNA was extracted from plants with RNAiso Plus reagents (Takara) according to the manufacturer's instructions. The RT-qPCR primers were designed based on *PLDδ* (At3g15730), *LOX1* (At3g62130), *LOX5* (At1g48420) and *Actin* gene (AY825362). The *Actin* gene was used as an internal control. All primers were shown in Supplementary Table 2. Relative expression was calculated as $2\text{-}\triangle\triangle^{Ct}$.

2.3. Measurement of LOX activity, JA content, G protein activity and PLD activity

LOX activity was measured according to Axelrod et al. [25] with minor modification. Each 0.2 g of leaf was homogenized with 1 mL of 0.1 mol L^{-1} PBS (pH 6.8) containing 1% Triton X-100 and 4% polyvinylpyrrolidone (PVP), and centrifuged at 15,000×*g* for 30 min. The supernatant was collected as a crude enzyme solution, and stored at −80 °C for subsequent experiments. Reaction system (3 mL): 2.9 mL 0.1 mol L^{-1} PBS (pH 6.8), 75 μL crude enzyme solution, 25 μL reaction substrate (10 mmol L^{-1} sodium linoleate mother liquor: 70 mg sodium linoleate solid, after mixing 70 μL of Triton X-100 and 4 mL of ddH_2O, titrating with 0.5 mol L^{-1} NaOH until the solution was clarified. Make up to 25 mL and store at −20 °C). One unit of LOX is defined as the amount of enzyme which causes an increase in absorption at 234 nm of 0.001min^{-1} (3 min period) at 25 °C when linoleic acid is used as the substrate.

Methods of JA content, GTP hydrolysis activity and the PLD activity were shown in the Supplementary material.

2.4. Seed germination test

For the germination assays, approximately 50 seeds were plated on MS medium containing 3% sucrose with different treatments. To break the dormancy, fully desiccated seeds were incubated at 4 °C for 3 days in the dark before germination and subsequently grown at 22 °C with 16 h light/8 h dark photoperiod in a growth chamber. Seed germination was observed for 10 days.

2.5. Determination of electrolyte leakage (EL) and malondialdehyde (MDA)

EL was measured by the method of Walker et al. [26]. The leaves (0.2 g) were quickly washed three times and then incubated in centrifuge tube containing 5 mL distilled deionized water at 25 °C for 2 h. The conductivity in the bathing solution was determined (E1). Then, the samples were heated in boiling water for 30 min and cooled to room temperature and conductivity was read again (E2). Conductivity was measured using a conductance bridge (DDS-11A, Yamei Electron Instrument Factory, Wuxi, China). Electrolyte leakage, EL [%] was calculated as E1/E2 × 100.

Levels of lipid peroxidation were assessed by measuring the amount of malondialdehyde (MDA) in tissue. Fresh leaf samples were homogenized in 10% trichloroacetic acid (TCA). The homogenate was centrifuged at 15,000×*g* for 20 min at 4 °C. The supernatant was collected and mixed with 0.6% thiobarbituric acid (TBA) in 10% TCA. Samples were heated at 95 °C for 15 min in a water bath, and then cooled on ice. The samples were centrifuged at 10,000×*g* for 10 min and the absorbance of solutions at 532, 600 and 450 nm was recorded.

MDA content ($\mu g \cdot g^{-1}$) = 0.45 × (A532 - A600) - 0.56 × A450.

2.6. Statistical analysis

Each experiment was carried out with three biological replicates. The results were expressed as the means ± SE. The data were analyzed using SPSS (version 17.0, IBM SPSS, Chicago, IL, USA), and error bars were calculated based on Tukey's multiple range test ($p < 0.05$). Figures were created by Origin2017 and Adobe Photoshop CS5.

3. Results

3.1. Osmotic stress affected the JA, G protein, PLD and LOX

Osmotic treatment increased the endogenous JA content, GTP hydrolysis activity, PLD activity and LOX activity (Fig. 1A–D). The JA content, PLD activity and LOX activity increased after 6 h, and GTP hydrolysis activity increased after 24 h. These indicated that JA, PLD and LOX responded to osmotic stress more rapidly than G protein. RT-qPCR was used to investigate the relative expression levels of *PLDδ* and *LOX* (*LOX1*, *LOX5*) genes under osmotic stress (Fig. 1E–G). *PLDδ* were significantly higher than the control except for 12 h. *LOX1* increased significantly, peaked at 48 h and decreased at 72 h. *LOX5* was inhibited in the early stage of stress (6–24 h), and promoted significantly in the late stage of stress (48–72 h). **Therefore, the seedlings were treated with mannitol for 48 h in the further experiment.**

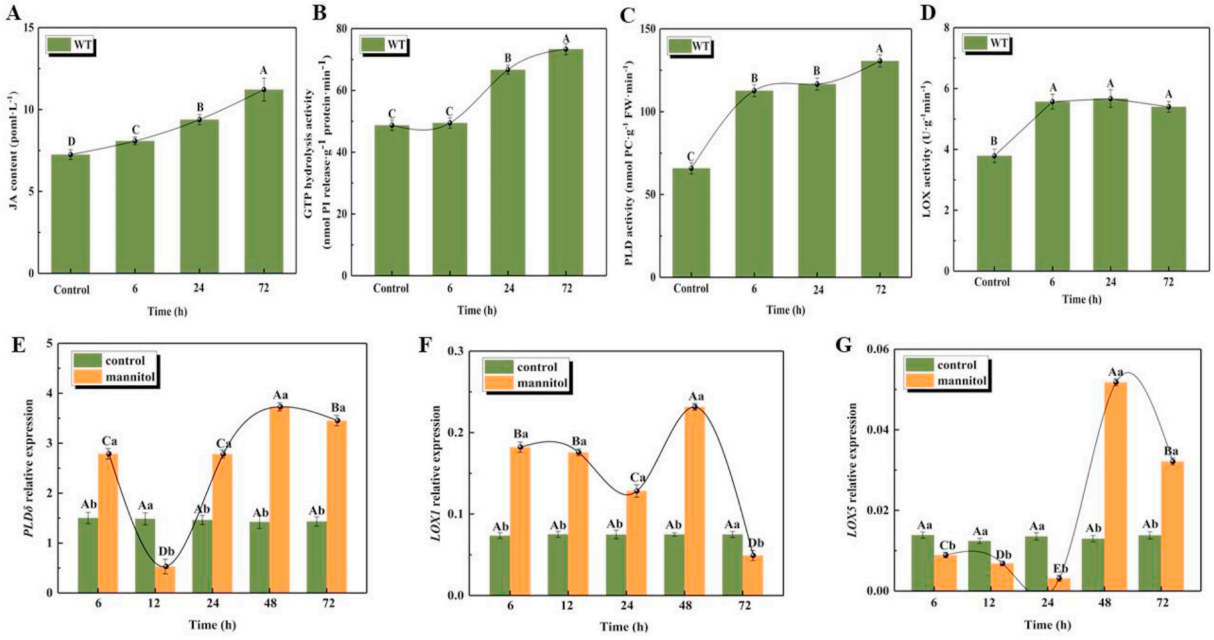

Fig.1. Osmotic stress affected the JA, G protein, PLD and LOX. (A–D) The JA content, GTP hydrolysis activity, PLD activity and LOX activity were tested under osmotic stress in WT. (E–G) The relative expression levels of *PLDδ* and *LOX* (*LOX1, LOX5*) genes were tested under osmotic stress in WT. Note: Capital letters indicate significant differences between groups, and lowercase letters indicate significant differences within groups. The 16-day-old seedlings was tested after mannitol treatment for 48 h. $P \leq 0.05$. The same below. (The gene expression of material treated with mannitol was compared with that of control).

3.2. Effects of JA on the seed germination, EL and MDA content under osmotic stress

Osmotic stress inhibited the seed germination. The germination in *gpa1-4* and *pldδ* were significantly lower than WT, and the inhibitory effect of *gpa1-4* was the most remarkable (Fig. 2A–B). Compared to the single mannitol treatment, the co-treatment of mannitol + MeJA inhibited the germination in WT and *pldδ* except for *gpa1-4*. JA synthesis inhibitors promoted the germination in WT, *gpa1-4* and *pldδ*, among which only WT could recover to the control (Fig. 2A, C).

EL and MDA were indicators of membrane injury under osmotic stress. Osmotic stress increased the EL and MDA content in WT, *gpa1-4*

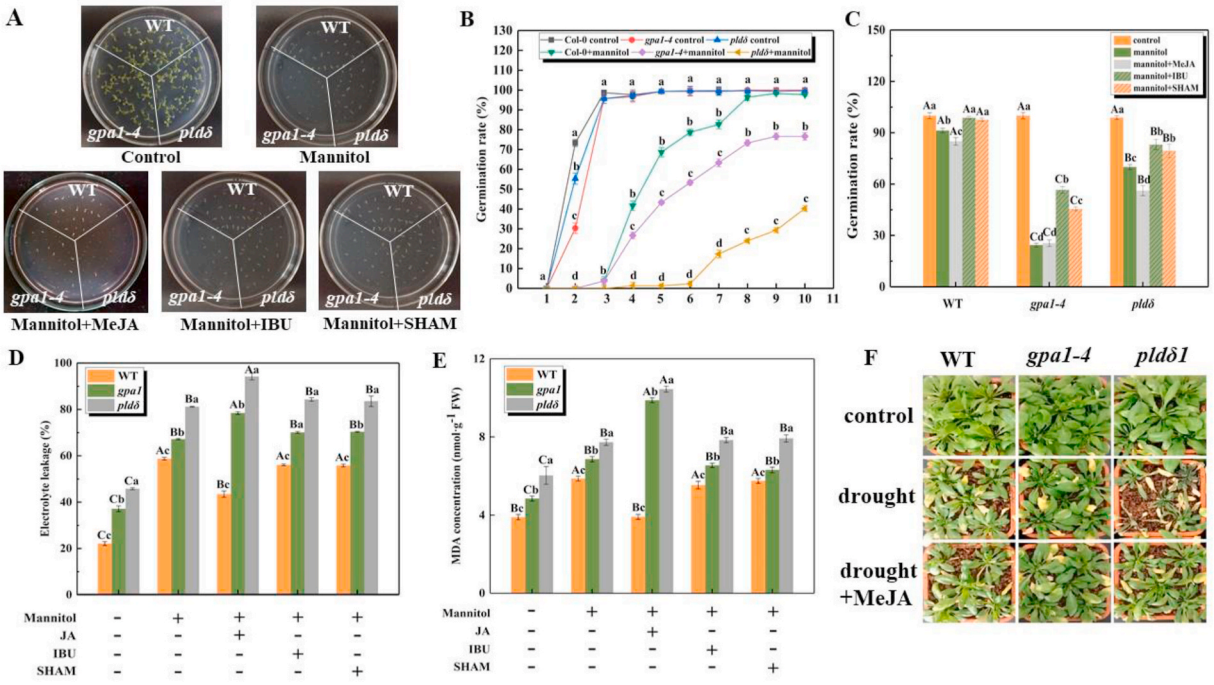

Fig.2. Effects of JA on the seed germination, EL and MDA content under osmotic stress. (A, C) After exogenous JA, IBU and SHAM applying, the germination rates were measured under the mannitol treatment in WT, *gpa1-4* and *pldδ*. (B) The germination rates were measured under the mannitol treatment in Col-0, *gpa1-4* and *pldδ*. Note: lowercase letters indicate significant differences between groups. (D–E) After exogenous JA, IBU and SHAM applying, the Electrolyte leakage and the MDA concentrations were measured under the mannitol treatment in WT, *gpa1-4* and *pldδ*. (F) After exogenous JA applying, drought tolerance of WT, *gpa1-4* and *pldδ* plants. $P \leq 0.05$.

and *pldδ*. Both EL and MDA content in *gpa1-4* and *pldδ* were significantly higher than that in WT under osmotic stress, indicating that the *gpa1-4* and *pldδ* mutants were more sensitive to osmotic stress. JA synthesis inhibitor had no effect on the EL and MDA in all seedlings (Fig. 2D–E). Exogenous MeJA reduced the EL and MDA in WT, but increased both of them in *gpa1-4* and *pldδ*.

The effect of exogenous MeJA under drought treatment was also investigated, shown as Fig. 2F. When 20-days-old plants were subjected to progressive drought by withholding water for 14 days, the tolerance of *gpa1-4* and *pldδ* to drought stress was significantly weaker than that of WT, and the inhibitory effect of *pldδ* was the most remarkable, showing typical symptoms of wilting and yellowing of leaf margin. Compared to control, the co-treatment of drought + MeJA mitigated the wilting in WT, *pldδ* and *gpa1-4*.

3.3. GPA1 involved in the regulation of JA to PLD, and PLDδ was located upstream of 9-LOX to participate in the JA biosynthesis under osmotic stress

The PLD activity and *PLDδ* gene expression in WT and *gpa1-4* were measured to investigate the role of the GPA1 and the PLDδ in JA regulation under osmotic stress (Fig. 3A–B). From the results, the PLD activity in *gpa1-4* was lower obviously than that in WT. Osmotic stress increased the PLD activity in WT, but it had no increase effect on the PLD activity in *gpa1-4*. Exogenous MeJA and JA synthesis inhibitors reduced the PLD activity in WT, but increased the PLD activity in *gpa1-4*. Gene expression revealed that the trend of *PLDδ* gene expression was consistent with the trend of PLD activity in WT, but was not consistent in *gpa1-4*. The results showed that GPA1 involved in the process that JA regulated the PLD activity under osmotic stress.

The LOX activity decreased gradually in the late stage of osmotic stress (Fig. 3C). 80 μM PA was added for compensation experiment. Compared to the single mannitol treatment, the mannitol + PA co-treatment decrease the LOX activity in WT but increased it in *pldδ* (Fig. 3D). The JA contents in *pldδ* were always lower than that in WT under osmotic stress. Application of exogenous PA increased JA content in *pldδ* effectively (Fig. 3E).

To determine the role of signal relationship between PLDδ and 9-LOX in JA synthesis under osmotic stress, the effect of exogenous PA on JA content in WT, *lox1* and *lox5* were evaluated (Fig. 3F). Under osmotic stress, the JA contents of *lox1* and *lox5* were significantly lower than that of WT. Compared to the single mannitol treatment, the mannitol + PA co-treatment increased the JA contents in WT, but didn't alter in *lox1* and *lox5*. This demonstrated that 9-LOX was involved in the JA synthesis under osmotic stress, and PLDδ/PA was located upstream of 9-LOX to participate in the JA biosynthesis.

4. Discussion

Osmotic is one of the most important limiting factors for plant growth and agricultural production [1]. JA is an important signal molecule against biotic and abiotic stresses. Creelman and Mullet asserted that a rapid JA accumulation was observed in water-deficient soybean leaves [27]. Lots of evidences have demonstrated that G protein and PLD were involved in the regulation of abiotic stress response [28,29]. Our results showed that osmotic stress increased endogenous JA content, GTP hydrolysis activity, PLD activity and LOX activity markedly, indicating that JA, G protein, PLD and LOX responding to osmotic stress positively (Fig. 1).

Seed germination is regulated by a number of signals and mechanisms. JA can inhibit the seed and pollen tube germination in *A.thaliana* [4]. G proteins and PLD are also thought to be involved in the seed germination [30,31]. In this study, osmotic stress inhibited the seed germinations of WT, *gpa1-4* and *pldδ* in different degrees, among which

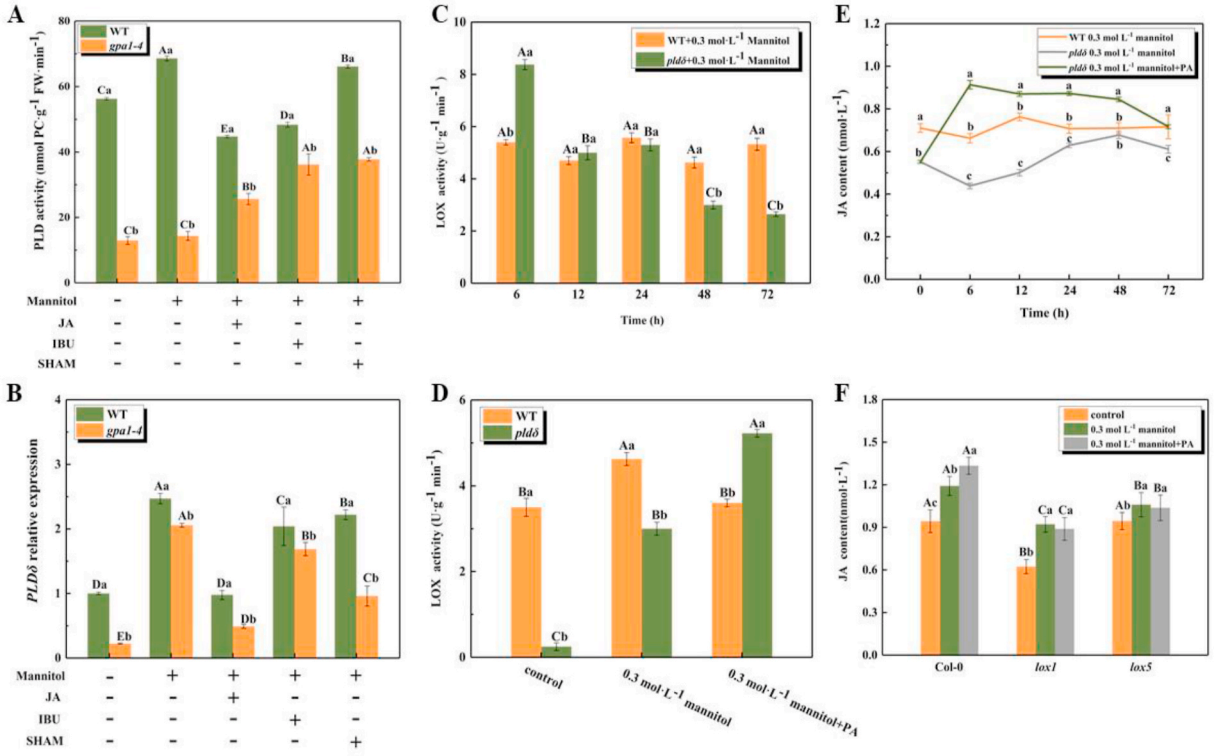

Fig.3. GPA1 involved in the regulation of JA to PLD, and PLDδ was located upstream of 9-LOX to participate in the JA biosynthesis under osmotic stress. (A–B) After exogenous MeJA, IBU and SHAM applying, the PLD activities and the *PLDδ* relative expression levels were measured under the mannitol treatment in WT and *gpa1-4*. (C) The LOX activity was measured under the mannitol treatment in WT and *pldδ*. (D–E) After exogenous PA applying, the LOX activity and the JA content was measured under the mannitol treatment in WT and *pldδ*. (F) After exogenous PA applying, the JA content was measured under the mannitol treatment in WT, *lox1* and *lox5*. $P \leq 0.05$.

the inhibitory effect of *gpa1-4* was the most obvious. These reflected that both GPA1 and PLDδ were involved in the seed germination under osmotic stress, and GPA1 played a major role. Exogenous MeJA decreased the seed germinations of *pldδ* and *gpa1-4*, and JA synthesis inhibitors increased the seed germinations of WT, *gpa1-4* and *pldδ*. These indicated that JA regulated the seed germination by G protein and PLDδ (Fig. 2A–C). MDA is the production of lipid peroxidation, which reduces the level of antioxidants, leading to membrane system damage and even cell death. It was reported that exogenous JA can enhance tolerance of wheat seedlings to salt stress by decreasing MDA content and enhancing activities of antioxidant enzymes [5]. In this study, we found that exogenous MeJA decreased the MDA content efficiently in WT, **but increased it in *gpa1-4* and *pldδ* mutants**. **These demonstrated that GPA1 and PLDδ participated in the protection of JA** on the **cell membrane** (Fig. 2D–E). The tolerance of *gpa1-4* and *pldδ* to drought stress was significantly weaker than that of WT, and the inhibitory effect of *pldδ* was the most remarkable (Fig. 2F), compared to control, the co-treatment of drought + MeJA mitigated the wilting in WT *pldδ* and *gpa1-4*.

GPA1 is the main functional component of G-protein. When the G protein-coupled receptor receives the extracellular first messenger, the receptor is activated to further activate the G protein, which can activate its downstream effector to produce an intracellular second messenger [32]. Compared to the single osmotic treatment, both exogenous MeJA and endogenous JA inhibitors reduced PLD activity in WT and increased PLD in *gpa1-4*. *PLDδ* gene expression was completely consistent with PLD activity in WT, while *PLDδ* gene expression was inconsistent with PLD activity in *gpa1-4*. This result indicated that GPA1 involved in the process that JA regulated the PLD activity under osmotic stress. When G protein can't work properly, the lack of correlation between *PLDδ* gene expression and PLD enzyme activity is not surprising, because gene expression is characterized by only one isoenzyme of one gene family, whereas enzyme measurements typically include all expressed members of such a family. Moreover, enzyme activities can be affected by a number of feedback regulations, so that a good correlation is not always found (Fig. 3A–B).

In this study, LOX activity was lower in *pldδ* than WT (48–72 h) (Fig. 3C). After adding exogenous PA, LOX activity increased significantly in *pldδ*, but decreased significantly in WT (Fig. 3D). **The decrease of LOX activity in WT may be caused by excessive intracellular total PA content.** Therefore, we inferred that PLDδ regulated LOX by moderating PA. Wang reported that PA participates in the JA biosynthesis [33]. In accordance with this, we found exogenous PA increased the JA content in *pldδ*. This reflected that PLDδ participates in the JA biosynthesis (Fig. 3E). 13-LOX is widely considered to be involved in JA synthesis [34]. Our study showed that 9-LOX was also involved in the JA synthesis under osmotic stress, and PLDδ/PA was located at the upstream of 9-LOX to participate in the JA biosynthesis (Fig. 3F).

This research preliminarily confirmed the relevance of G protein, PLDδ and JA responding to osmotic stress. Our date suggested that *GPA1* gene participates in the PLD regulation. Both GPA1 and PLDδ are involved in the regulation of JA on the seed germination, cell membrane protection and **osmotic stress tolerance**. PLDδ/PA is located at the upstream of 9-LOX and participates in the 9-LOX-induced JA synthesis. **However, the mechanism that underlies the way that osmotic stress is communicated to the G protein, and in turn to PLD is still unclear. These are worthy of further exploration (Fig. 4).**

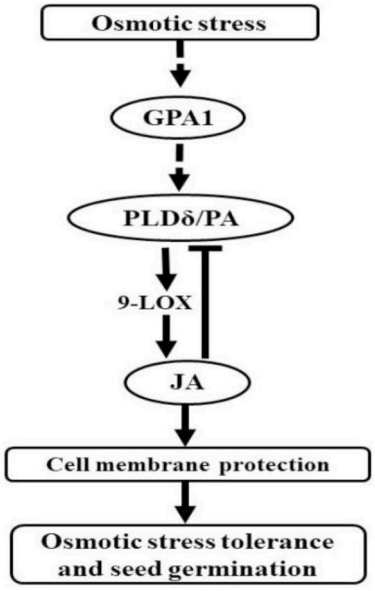

Fig.4. A schematic model of JA responded to osmotic stress by regulating G protein and PLDδ in *A. thaliana*. Solid arrow: activation; Dotted arrow: unknown mechanisms; Short lines have no arrow: inhibiting.

Author statement

Ning Yang: Supervision, Writing review & editing, Project administration. **Yue Zhang:** The experimental operation, Data curation, Writing-Original draft preparation. **Lu Chen:** The experimental operation, Visualization, Investigation. **Wei Wang:** Visualization, Investigation. **Ruirui Liu:** The experimental operation, Visualization, Investigation. **Run Gao:** Visualization, Investigation. **Yaping Zhou:** Visualization, Investigation. **Hui Li** : Visualization, Investigation.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgment

This project is supported by the National Natural Science Foundation of China (31960061 and 31660116). The authors have no conflicts of interest to declare.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrep.2021.100952.

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