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Translocation of phospholipase $A_2\alpha$ to apoplasts is modulated by developmental stages and bacterial infection in *Arabidopsis*

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Phospholipase A_2 (PLA_2) hydrolyzes phospholipids at the *sn*-2 position to yield lysophospholipids and free fatty acids. Of the four paralogs expressed in *Arabidopsis*, the cellular functions of $PLA_2\alpha$ *in planta* are poorly understood. The present study shows that $PLA_2\alpha$ possesses unique characteristics in terms of spatiotemporal subcellular localization, as compared with the other paralogs that remain in the ER and/or Golgi apparatus during secretory processes. Only $PLA_2\alpha$ is secreted out to extracellular spaces, and its secretion to apoplasts is modulated according to the developmental stages of plant tissues. Observation of $PLA_2\alpha$-RFP transgenic plants suggests that $PLA_2\alpha$ localizes mostly at the Golgi bodies in actively growing leaf tissues, but is gradually translocated to apoplasts as the leaves become mature. When *Pseudomonas syringae* pv. *tomato* DC3000 carrying the avirulent factor *avrRpm1* infects the apoplasts of host plants, $PLA_2\alpha$ rapidly translocates to the apoplasts where bacteria attempt to become established. *$PLA_2\alpha$ promoter::GUS* assays show that $PLA_2\alpha$ gene expression is controlled in a developmental stage- and tissue-specific manner. It would be interesting to investigate if $PLA_2\alpha$ functions in plant defense responses at apoplasts where secreted $PLA_2\alpha$ confronts with invading pathogens.

Keywords: phospholipase A_2, translocation, apoplast, bacterial infection, subcellular localization

INTRODUCTION

Phospholipase A_2 (PLA_2) is widespread throughout nature and stereospecifically catalyzes the hydrolysis of phospholipids at *sn*-2 to produce lysophospholipids and free fatty acids, which are important mediators or precursors in signal transduction pathways in animal cells (Schaloske and Dennis, 2006; Burke and Dennis, 2009). There is evidence that plant PLA_2s are also involved in diverse biological and physiological processes such as senescence, wound healing, elicitor and stress responses, defense against pathogens, and the induction of secondary metabolite accumulation (Wang, 2001, 2004; Ryu, 2004; Scherer et al., 2007; Seo et al., 2008; Kirik and Mudgett, 2009; Mansfeld, 2009; Froidure et al., 2010; Liao and Burns, 2010).

There are four PLA_2 paralogs in *Arabidopsis*: *$PLA_2\alpha$*, *$PLA_2\beta$*, *$PLA_2\gamma$*, and *$PLA_2\delta$*. The paralogs *$PLA_2\gamma$* and *$PLA_2\delta$* are expressed solely in pollen, localized in the endoplasmic reticulum (ER)/Golgi bodies and ER, respectively, and mediate pollen germination and tube growth (Kim et al., 2011). *$PLA_2\beta$* is localized in the ER and expressed in different tissues such as young seedlings, elongating flower stems, and pollen, and it mediates cell elongation, shoot gravitropism, stomatal opening, and pollen development (Lee et al., 2003; Kim et al., 2011). Although *$PLA_2\alpha$* appears to be ubiquitous in diverse organs (Ryu et al., 2005; Mansfeld and Ulbrich-Hofmann, 2007; Kim et al., 2011), its temporal and spatial expression dynamics in different tissues and its subcellular translocation during different developmental stages are unknown.

In this study, we report that $PLA_2\alpha$ in *Arabidopsis* moves from the ER/Golgi apparatus to the apoplasts as the leaves become mature, and that *$PLA_2\alpha$* gene expression is controlled in both a developmental stage- and organ-dependent manner. Several lines of evidence suggest that secretory proteins or proteins enhancing secretory pathways play important roles in plant defense responses (Wang et al., 2005; Kwon et al., 2008; Sup Yun et al., 2008). Thus, we examined if the secretion of $PLA_2\alpha$ to apoplasts is modulated by pathogen infection. Interestingly, translocation of $PLA_2\alpha$ to apoplasts was rapidly enhanced in response to the inoculation of *Pseudomonas syringae* pv. *tomato* DC3000 carrying *avrRpm1* (*Pst-avrRpm1*). These observations suggest that $PLA_2\alpha$ proteins secreted into apoplasts in response to bacterial infection may play a role in host defense responses.

MATERIALS AND METHODS

PLANT MATERIALS AND REAGENTS

Arabidopsis thaliana (Col-0) plants were grown in soil pots at 22°C, 60% relative humidity, with a 16-h photoperiod and a photon flux density of 110 μmol m^{-2} s^{-1}.

GUS STAINING

For histochemical localization studies, a *$PLA_2\alpha$-promoter::GUS* (*$ProPLA_2\alpha$::GUS*) construct was cloned by incorporating the *$PLA_2\alpha$* sequence upstream of the ATG start codon (from −1175 bp to +3 bp) into the *Hind*III and *Bam*HI sites of the pBI101 vector. The resulting plasmids were inserted into *Agrobacterium tumefaciens* strain EHA105, which was transformed into *Arabidopsis* using the floral dip method as described previously (Bechtold and Pelletier, 1998). Histochemical GUS assays to show tissue-specific *$PLA_2\alpha$* expression at different developmental stages were performed as previously described (Jefferson et al., 1987). Tissues from *$ProPLA_2\alpha$::GUS*-transformed plants were immersed in GUS solution [1 mM X-gluc, 100 mM sodium phosphate buffer (pH 7.0), 0.5 mM $K_3Fe(CN)_6$, 0.5 mM $K_4Fe(CN)_6$, 10 mM EDTA, and 0.1% (v/v) Triton X-100] and incubated for 12 h at 37°C due to its weak staining. After GUS staining, 100% ethanol was used to remove the chlorophyll.

SUBCELLULAR LOCALIZATION OF $PLA_2\alpha$-RFP IN *ARABIDOPSIS*

To investigate the dynamics of *$PLA_2\alpha$* subcellular localization, transgenic *Arabidopsis* plants carrying *Pro35S::$PLA_2\alpha$-RFP* were generated (Lee et al., 2010). Leaf tissues from the *$PLA_2\alpha$-RFP* transgenic *Arabidopsis* plants were viewed at different developmental stages using a laser scanning confocal microscope (Meta system, Zeiss) after incubation in water or 1 N KNO_3 for 5 min to trigger plasmolysis. RFP-fluorescence was excited at 543 nm and the emitted fluorescence was collected with a band-pass filter at 560–615 nm.

CO-LOCALIZATION ASSAY OF $PLA_2\alpha$-RFP AND ST-GFP

To investigate whether $PLA_2\alpha$-RFP proteins are co-localized with a Golgi marker ST-GFP, transgenic *Arabidopsis* plants expressing both $PLA_2\alpha$-RFP and ST-GFP were generated (Lee et al., 2010). Close-to-mature leaves of 3-week-old transgenic plants were observed with a laser scanning confocal microscope (Meta system, Zeiss). RFP and GFP fluorescence was detected using at 543/560–615 nm and 488/505–530 nm excitation/emission filter sets, respectively.

BACTERIAL INOCULATION OF PLANTS

Pseudomonas syringae pv. *tomato* DC3000 carrying *avrRpm1* (*Pst-avrRpm1*) were obtained from Y. J. Kim (Korea University, Seoul, Korea). Plants were inoculated by spreading a bacterial suspension (1×10^8 CFU ml^{-1} in 0.015% v/v Silwet L-77 and 10 mM $MgCl_2$) onto the adaxial leaf surfaces of *Arabidopsis* carrying *Pro35S::$PLA_2\alpha$-RFP*. Plants designated as NT were given no treatment, whereas mock plants were treated with 0.015% v/v Silwet L-77 and 10 mM $MgCl_2$. All the data presented in this study were obtained from at least three independent replicates.

RESULTS

HISTOCHEMICAL ANALYSIS OF GUS ACTIVITY OF THE *$PLA_2\alpha$* PROMOTER

Although RT-PCR analysis shows that *$PLA_2\alpha$* transcripts are present in different parts of *Arabidopsis* tissues (Kim et al., 2011), there is little information regarding $PLA_2\alpha$ gene expression at different developmental stages. To elucidate the cell type-specific expression patterns of the *$PLA_2\alpha$* gene, transgenic *Arabidopsis* lines were generated that expressed the *beta-glucuronidase* (*GUS*) reporter gene under the control of the *$PLA_2\alpha$* promoter (**Figure 1**). GUS activity was detected in the cotyledons, the shoot apex,

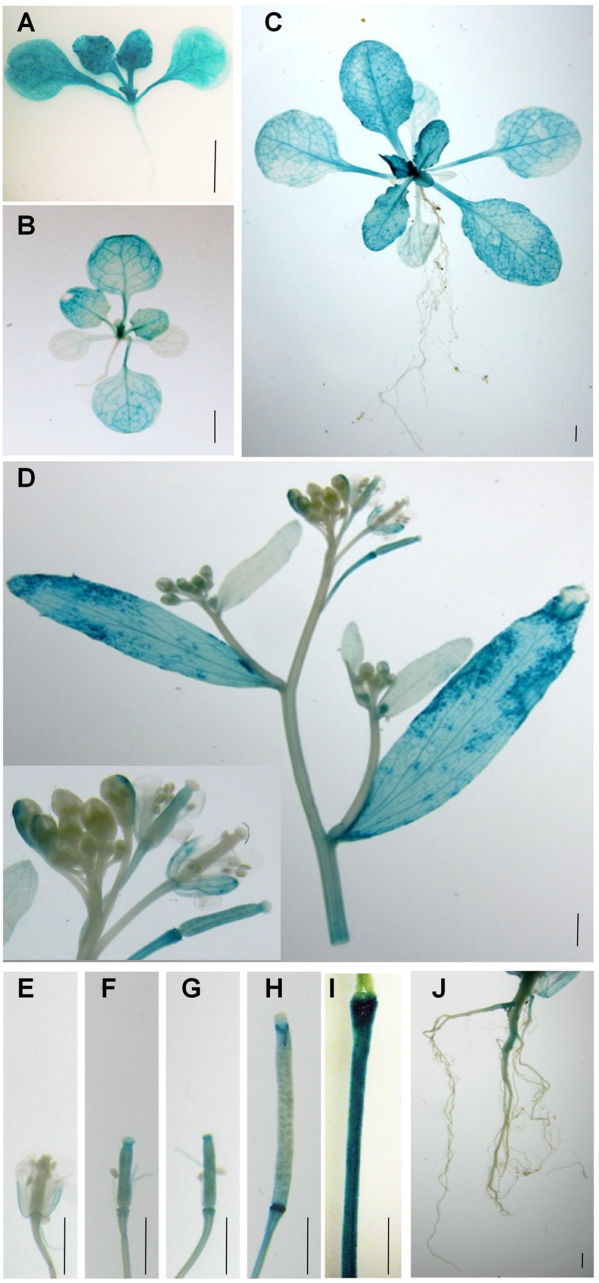

FIGURE 1 | Spatial and temporal expression of *$PLA_2\alpha$*. Spatiotemporal expression patterns of the *$PLA_2\alpha$* gene in transgenic *Arabidopsis* plants harboring the *$PLA_2\alpha$* promoter fused with the *GUS* gene. Promoter activity was visualized by histochemical GUS staining. **(A)** Seven-day-old plant. **(B)** Fourteen-day-old plant. **(C)** Three-week-old plant. **(D)** Flower cluster, cauline leaf, and stem of a 5-week-old plant. **(E–H)** Carpels and developing siliques of a 5-week-old plant. **(I)** Pedicel of the control transgenic plants harboring the *35S* promoter fused with the *GUS* gene. **(J)** Root of a 6-week-old plant. Bars = 2 mm.

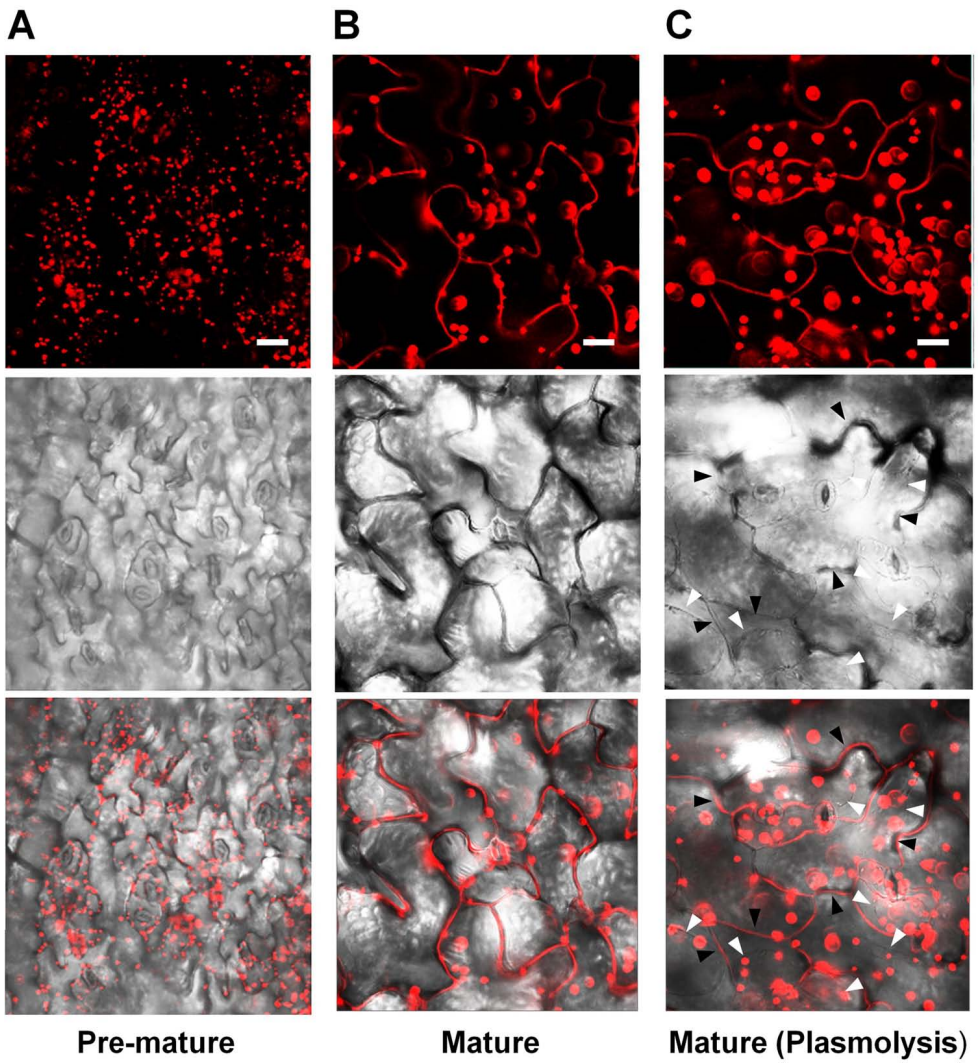

FIGURE 2 | Subcellular localization of $PLA_2\alpha$ at different leaf ages in $PLA_2\alpha$-RFP transgenic *Arabidopsis* plants. (A,B) Epidermal cells in pre-mature leaf tissues **(A)** and mature leaf tissues **(B)** from *Pro35SPLA$_2\alpha$-RFP* transgenic *Arabidopsis* plants (4-week-old) were viewed using confocal microscopy. **(C)** Mature leaf tissues were incubated in 1 N KNO_3 to induce plasmolysis before being viewed with confocal microscopy. The slightly decreased clarity of images in **(C)** appears to result from the diffusion of $PLA_2\alpha$-RFP into the expanded apoplasts due to plasmolysis. White arrows in **(C)** indicate the plasmolyzed plasma membrane, whereas black arrows indicate extracellular spaces. Fluorescent (top), bright field (middle), and merged images (bottom) are presented. Bars = 20 μm.

the hypocotyl, and the vascular tissues of 7-day-old germinated seedlings (**Figure 1A**). Strong GUS activity was detected in the shoot apex in 14-day-old seedlings and 3-week-old plants, and was preferentially expressed in young leaves rather than old leaves (**Figures 1A–C**). No GUS activity was detected in the roots at this stage. In 5-week-old plants, GUS expression was found in the cauline leaves, sepals, styles, and pedicel of reproductive tissues (**Figure 1D**). The apical end of the pedicel is particularly dark-stained, apparently due to its thickened cell tissues based on a comparison with the GUS staining of the control transgenic plants harboring the *35S* promoter fused with the *GUS* gene (**Figure 1I**). In plants transformed with *ProPLA$_2\alpha$::GUS*, GUS expression was also detected in the developing siliques (**Figures 1E–H**) and in the main roots of flowering plants (**Figure 1J**). Taken together, these data indicate that *PLA$_2\alpha$* gene expression is controlled in a unique developmental stage- and tissue-specific manner.

SUBCELLULAR LOCALIZATION OF $PLA_2\alpha$

Lee et al. (2010) reported that fluorescence signals for $PLA_2\alpha$-fusion proteins were observed at the Golgi apparatus of root hair cells. However, Froidure et al. (2010) showed time-dependent localization of $PLA_2\alpha$ using a transient expression system incorporating *N. tabacum*. The YFP reporter fused with $PLA_2\alpha$ was detected in cytoplasmic vesicles around the nucleus 36 h after agroinfiltration to tobacco leaves, and was detected at the extracellular spaces outside the cells at a later time point (48 h after agroinfiltration). To resolve these inconsistencies, we investigated in more detail the subcellular localization of $PLA_2\alpha$ by analyzing

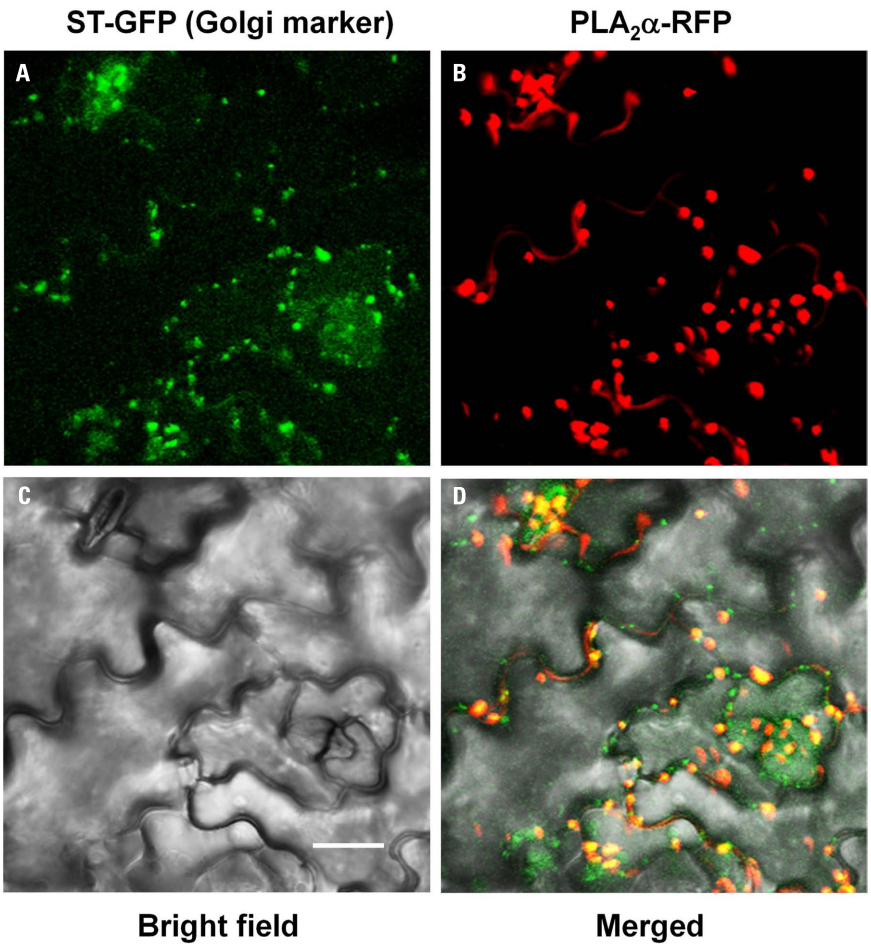

FIGURE 3 | Co-localization of $PLA_2\alpha$-RFP with ST-GFP, a Golgi body marker. Close-to-mature leaves of transgenic *Arabidopsis* plants expressing both $PLA_2\alpha$-RFP and ST-GFP were observed with a laser scanning confocal microscope. $PLA_2\alpha$-RFP signals are mostly overlapped with ST-GFP, a Golgi body marker. ST-GFP **(A)**, $PLA_2\alpha$-RFP **(B)**, bright field **(C)**, and merged image **(D)** are presented. Bar = 20 μm.

the fluorescence of fusion proteins in transgenic plants carrying *Pro35S::PLA2α-RFP*. The leaves of 4-week-old *PLA2α-RFP* transgenic seedlings were viewed using a laser scanning confocal microscope. The results showed that the subcellular localization of $PLA_2\alpha$ was dependent on the developmental stages of leaf tissue. $PLA_2\alpha$-RFP fusion proteins were present primarily at the Golgi apparatus in pre-mature young leaves (**Figure 2A**), whereas in mature leaves, they were detected primarily in the apoplasts (**Figure 2B**). Even after cell plasmolysis was induced by treatment with 1 N KNO_3 for 5 min, the $PLA_2\alpha$-RFP signal remained in the extracellular spaces or diffused into the gap between the cell wall and the plasma membrane that is induced by plasmolysis (**Figure 2C**). These results indicate that $PLA_2\alpha$ is indeed localized in the apoplasts of mature leaves.

CO-LOCALIZATION OF $PLA_2\alpha$ WITH A GOLGI MARKER

As secretion of proteins to apoplasts is known to occur through ER and Golgi bodies, $PLA_2\alpha$-RFP signals were mostly detected at the Golgi bodies in pre-mature young leaves (**Figure 2A**). However, the fluorescent spots become gradually bigger as the leaves become mature, leading us to suspect that they may be other cellular organelles. To investigate if the big $PLA_2\alpha$ fluorescent spots are real Golgi bodies, we performed co-localization assay of $PLA_2\alpha$ with a Golgi body marker, sialyltransferase (ST). Close-to-mature leaves of transgenic *Arabidopsis* plants expressing both $PLA_2\alpha$-RFP and ST-GFP were observed with a laser scanning confocal microscope. As shown in **Figure 3**, big spots of $PLA_2\alpha$-RFP signals are mostly overlapped with the spots of a Golgi body marker (ST-GFP), confirming that $PLA_2\alpha$ is localized in Golgi apparatus before secretion to the apoplasts. We found that the apparent big size spots result from aggregation of several Golgi bodies and strong brightness of RFP-fluorescence. Aggregation of Golgi bodies appears to be gradually enhanced as the leaves become mature.

$PLA_2\alpha$ TRANSLOCATES TO APOPLASTS IN RESPONSE TO THE INOCULATION OF AVIRULENT BACTERIA

As leaves become mature, $PLA_2\alpha$ is secreted into the apoplast, where it generates its lipid products, lysophospholipids and free fatty acids. The lipid products have been suggested to function as bio-active molecules that mediate a variety of cellular processes.

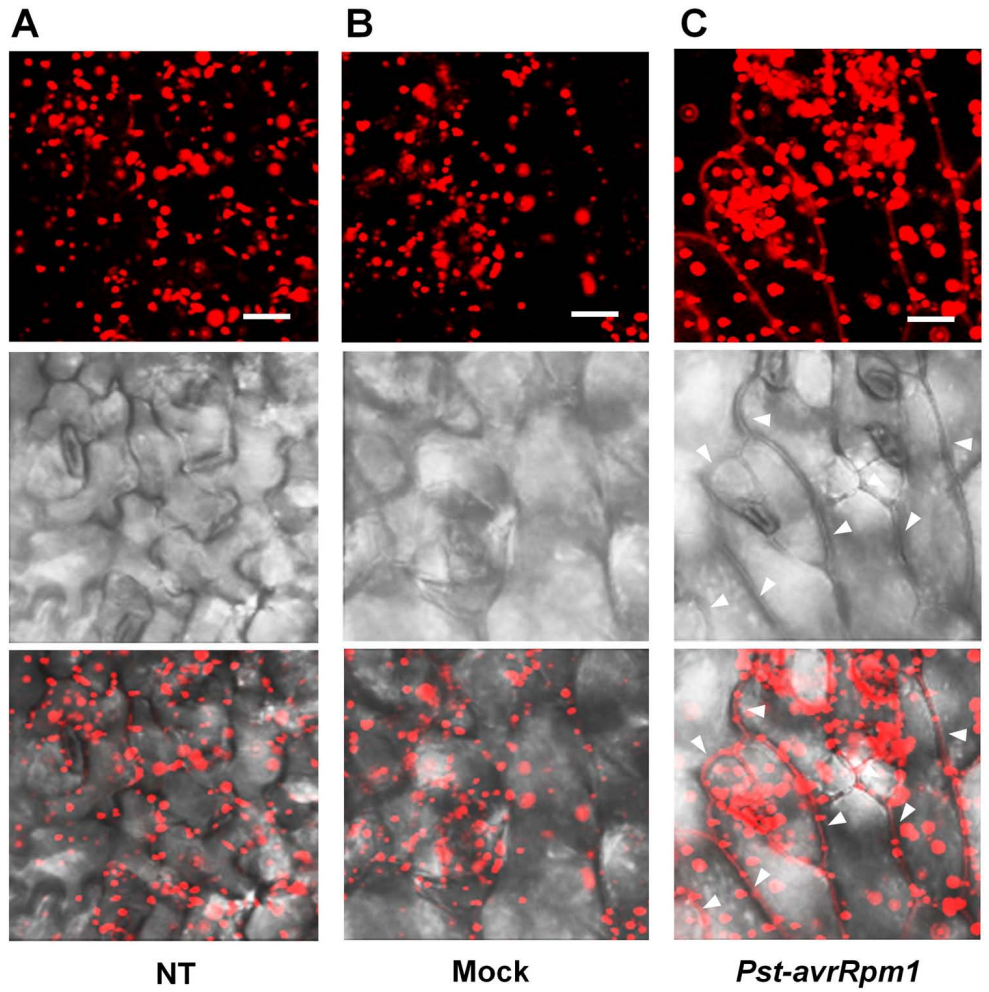

FIGURE 4 | Translocation of $PLA_2\alpha$ to apoplasts was enhanced by the inoculation of bacteria, *Pst-avrRpm1*, in pre-mature young leaves. (A–C) Images showing increased fluorescence intensity and vesicle sizes followed by the translocation of $PLA_2\alpha$ to apoplasts at 3 h post-inoculation of *Pst-avrRpm1* **(C)** compared to the no-treatment control **(A)** and 0.015% Silwet/10 mM $MgCl_2$-treated mock **(B)** in pre-mature young leaves where $PLA_2\alpha$ is normally localized primarily in Golgi bodies. Fluorescent (top), bright field (middle), and merged images (bottom) are presented. Bars = 20 μm.

Apoplasts are an important site for the interaction of plant cell defense mechanisms with invading bacteria, which attempt to become established in the apoplasts. If $PLA_2\alpha$ positively participates in defense responses to pathogen attack, we hypothesized that its translocation to the apoplasts would be enhanced when pathogens are inoculated. As speculated, the translocation of $PLA_2\alpha$ to the apoplasts was enhanced at 3 h post-inoculation of avirulent bacteria, *Pst-avrRpm1*, in young leaves (**Figure 4C**) and in close-to-mature leaves (**Figure 5C**), as compared to non-treated controls (NT) and 0.015% Silwet/10 mM $MgCl_2$-treated mocks (**Figures 4A,B and 5A,B**).

DISCUSSION

$PLA_2\alpha$ is expressed in a tissue- and developmental stage-specific manner in *Arabidopsis* plant tissues. Relatively strong activities of the *$PLA_2\alpha$* promoter were observed in actively growing seedlings and young leaves. Expression decreased slightly as leaves became mature. This expression pattern of *$PLA_2\alpha$* is different from that of *$PLA_2\beta$*, which is expressed at a very low level in the mature leaves (Lee et al., 2003). This pattern of expression is also observed in the cauline leaves of the inflorescence stems, which display strong expression of *$PLA_2\alpha$* but low expression of *$PLA_2\beta$*. At the young seedling and reproductive organ developmental stages, both *PLA_2* paralogs display similar expression patterns; strong expression in actively growing tissues and reproductive organs such as sepals, pedicels, and styles of open flowers, but low expression in petals, stigmas, and ovaries. Expression of both *PLA_2* paralogs was detected in developing siliques but not in maturing seeds. However, *$PLA_2\alpha$* was not expressed in pollen tissues, in contrast to the strong expression of *$PLA_2\beta$*. In the root, *$PLA_2\alpha$* was expressed at the late stages of growth, whereas *$PLA_2\beta$* was expressed in roots from seedling stages (Lee et al., 2003). These results suggest that $PLA_2\alpha$ and $PLA_2\beta$ may play a role in plant growth and development in harmony with holding their own cellular roles at the different cellular localization and expressing tissues.

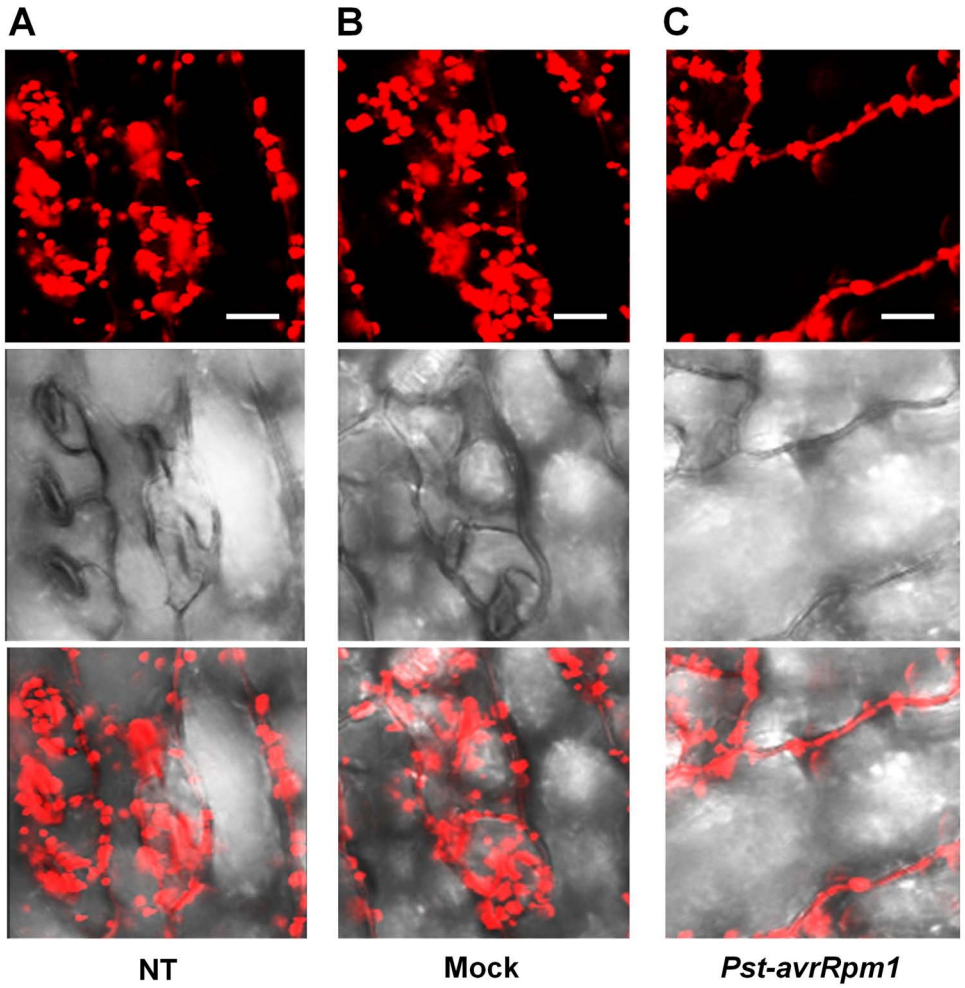

FIGURE 5 | Enhanced translocation of $PLA_2\alpha$ to apoplasts in response to the inoculation of *Pst-avrRpm1* in close-to-mature leaves. (A–C) Significant enhancement of $PLA_2\alpha$ translocation to apoplasts was observed at 3 h post-inoculation of *Pst-avrRpm1* **(C)** compared to the no-treatment control **(A)** and 0.015% Silwet/10 mM $MgCl_2$-treated mock **(B)** in close-to-mature leaves where $PLA_2\alpha$ is already detected at a low level in the apoplasts. Fluorescent (top), bright field (middle), and merged images (bottom) are presented. Bars = 20 μm.

Arabidopsis PLA_2 genes encode proteins with N-terminal signal peptides, which are predicted to be secreted via ER and Golgi bodies to apoplasts and/or vacuoles. $PLA_2\beta$ has a KTEL sequence at its C-terminus, which is similar to the canonical ER-retention signal KDEL, and was shown to be localized in the ER (Seo et al., 2008). The $PLA_2\gamma$ and $PLA_2\delta$ isoforms, which are solely expressed in pollen, are localized in the ER and/or Golgi (Kim et al., 2011). $PLA_2\beta$, $PLA_2\gamma$, and $PLA_2\delta$, which share high sequence homologies with each other, are expressed during pollen growth and development and play critical roles during pollen germination and tube growth (Kim et al., 2011). In contrast to $PLA_2\gamma$ and $PLA_2\delta$, $PLA_2\beta$ is expressed in tissues such as actively growing leaves and elongating stems, and regulates shoot cell elongation and stem gravitropism, likely as a downstream component of auxin signaling (Lee et al., 2003). In addition, $PLA_2\beta$ is expressed in guard cells in response to light and modulates light-induced stomatal opening (Seo et al., 2008).

$PLA_2\alpha$ localizes primarily at Golgi bodies in actively growing young leaves but translocates to apoplasts as the leaves become mature. In root tissues, $PLA_2\alpha$ localizes at Golgi bodies (Lee et al., 2010). Localization of $PLA_2\alpha$ in Golgi bodies was confirmed by co-localization assay with a Golgi body marker. Studies in animal cells indicate that lysophospholipids, which are generated by PLA_2, modulate retrograde trafficking and the cisternal structure of the Golgi complex by modifying membrane tubule formation (de Figueiredo et al., 1998; Brown et al., 2003). As in animal cells, PLA_2 at Golgi bodies in plant cells may play an important role in the intracellular trafficking of proteins. Membrane fusion, formation, and intracellular trafficking at the Golgi bodies are prominent processes during active growth stages of plant tissues. Consistent with this hypothesis, Golgi-localized $PLA_2\alpha$ in root hairs appeared to act in the trafficking of PIN proteins (Lee et al., 2010). In actively growing young leaf tissues, $PLA_2\alpha$ localized at Golgi bodies may facilitate growth and development by mediating vesicular trafficking.

Once leaf tissues are mature, $PLA_2\alpha$ translocates to the apoplasts by way of Golgi bodies. Among the four *Arabidopsis* PLA_2 paralogs, only $PLA_2\alpha$ translocates to the apoplasts of mature leaves. The reason for $PLA_2\alpha$ movement from ER and Golgi bodies to apoplasts in the mature leaves is unknown. It could be speculated that as leaves are mature the demand for $PLA_2\alpha$ activity diminishes in ER and Golgi bodies since the demand for vesicular trafficking for active growth decreases. If so, $PLA_2\alpha$ may rather translocalize to the apoplasts, probably, in order to fulfill some other mission in the apoplasts of the mature leaves. Translocation of $PLA_2\alpha$ is supported by its unique enzyme characteristics. The optimal pH range for $PLA_2\alpha$ activity is quite broad compared with other PLA_2 paralogs, from pH 6 to 11 (Lee et al., 2005), so that $PLA_2\alpha$ may fully be functional not only in the ER and Golgi bodies but also in the apoplasts. In contrast, $PLA_2\beta$, which is localized in the ER, has a narrow range of optima from pH 6 to 7, whereas $PLA_2\gamma$ and $PLA_2\delta$, which are localized in the ER/Golgi bodies and ER of pollens, respectively, have optima at pH 7–9 and pH 8–9, respectively (Lee et al., 2005).

Structurally, apoplasts are formed by a continuation of the cell walls of adjacent cells and the associated extracellular spaces. The apoplast is important for a plant's interaction with the environment and microbes. The apoplast is also a site for cell-to-cell communication. Therefore, we examined the dynamics of $PLA_2\alpha$ secretion to apoplasts in response to bacterial attack. When *Pst-avrRpm1* infects host plants, the pathogens cannot enter into the cytoplasm but remain in the apoplasts and attempt to become established. Our results indicate that $PLA_2\alpha$ rapidly translocates to apoplasts in response to pathogen invasion. These data prompt us to speculate that $PLA_2\alpha$ may play a certain role in the apoplasts, where host cells confront invading pathogens. Thus, it would be of interest to investigate the role of $PLA_2\alpha$ in a variety of cellular processes, including host defense responses to pathogen attacks.

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