Identification of *Arabidopsis* Phospholipase A Mutants With Increased Susceptibility to *Plasmodiophora brassicae*

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Clubroot, caused by the obligate parasite *Plasmodiophora brassicae*, is one of the most devastating diseases of canola (*Brassica napus*) in Canada. The identification of novel genes that contribute to clubroot resistance is important for the sustainable management of clubroot, as these genes may be used in the development of resistant canola cultivars. Phospholipase As (PLAs) play important roles in plant defense signaling and stress tolerance, and thus are attractive targets for crop breeding. However, since canola is an allopolyploid and has multiple copies of each PLA gene, it is time-consuming to test the functions of PLAs directly in this crop. In contrast, the model plant *Arabidopsis thaliana* has a simpler genetic background and only one copy of each PLA. Therefore, it would be reasonable and faster to validate the potential utility of PLA genes in *Arabidopsis* first. In this study, we identified seven homozygous *atpla* knockout/knockdown mutants of *Arabidopsis*, and tested their performance following inoculation with *P. brassicae*. Four mutants (*pla*1-iα, *pla*1-iγ3, *pla*1-iii, *ppla*-iiiβ, *ppla*-iiiδ) developed more severe clubroot than the wild-type, suggesting increased susceptibility to *P. brassicae*. The homologs of these *Arabidopsis* PLAs (*AtPLAs*) in *B. napus* (*BnPLAs*) were identified through Blast searches and phylogenetic analysis. Expression of the *BnPLAs* was subsequently examined in transcriptomic datasets generated from canola infected by *P. brassicae*, and promising candidates for further characterization identified.

Keywords: clubroot, *Arabidopsis*, canola, phospholipase A, PLA genes, resistance

INTRODUCTION

Clubroot, caused by the obligate parasite *Plasmodiophora brassicae*, is one of the most devastating diseases of canola (oilseed rape; *Brassica napus*) in Canada and other regions. The infected host plants develop characteristic root galls, which interfere with water and nutrient uptake, leading to aboveground symptoms including yellowing and wilting of the leaves, stunting, premature ripening, and losses in seed yield and quality (Pageau et al., 2006; Hwang et al., 2012). The deployment of clubroot resistant (CR) cultivars has been the main strategy to manage clubroot in western Canada (Hwang et al., 2014). Unfortunately, the effectiveness of resistance can be quickly lost as a result of the selection pressure imposed on pathogen populations by short rotations with CR cultivars carrying similar sources of resistance. In Canada, “new” pathotypes of *P. brassicae* able to overcome the resistance in most CR canola cultivars were identified in 2013, only 4 years...
after the introduction of the resistance trait (Strelkov et al., 2016). Since then, resistance-breaking pathotypes, many of which exhibit novel virulence patterns, have documented in many fields (Strelkov et al., 2018; Hollman et al., 2021). Given these rapid shifts in the virulence of pathogen populations, it is necessary to identify additional sources of resistance, including novel gene targets in canola (Zhou et al., 2020a), to aid in sustainable clubroot management. These could be used in rotations with existing CR canola cultivars, helping to reduce selection pressure on pathogen populations and contributing to resistance stewardship (Hwang et al., 2019).

Phospholipase A (PLAs), which catalyze the hydrolysis of membrane phospholipids into free fatty acids and lysophospholipids, are attractive targets for crop breeding due to their important roles in plant defense signaling and stress tolerance (Yang et al., 2007; Canonne et al., 2011; Chen et al., 2013). PLAs are involved in the plant response to biotic stress by inducing jasmonic acid (JA), oxylinip and phytoalexin biosynthesis (Canonne et al., 2011; Ruelland et al., 2015). In addition, PLA activity was suggested to be important in elicitor-induced oxidative burst (e.g., Verticillium dahliae extract) (Chandra et al., 1996). Recent transcriptomic studies indicate the involvement of phospholipases in the host response to *P. brassicae*. For example, the expression of many genes encoding phospholipases was increased in the roots of *P. brassicae*-infected *Arabidopsis* at 24 days after inoculation (dal) (Irani et al., 2018). Expression profiling of *B. napus* transcriptome datasets enabled the identification of differentially expressed genes, including PLAs, following *P. brassicae* inoculation (Galindo-González et al., 2020; Zhou et al., 2020b).

Since PLAs appear to contribute to the response of host plants to pathogens, functional validation of their role against *P. brassicae* will provide valuable information regarding their potential utility in the development of CR canola cultivars. However, since the model plant *Arabidopsis thaliana* (*Arabidopsis* thereafter) possesses over 20 PLAs and canola has several homologs of each of these genes (Iqbal et al., 2020), it is time-consuming to test the functions of *BnPLAs* directly in canola. In contrast, *Arabidopsis* has a simpler genetic background (Cheng et al., 2014) and only one copy of each PLA gene (Chen et al., 2013), and as a crucifer, also serves as a host of *P. brassicae*. Therefore, it would be more feasible and faster to validate the roles of PLA genes in clubroot resistance in *Arabidopsis*, to select promising candidates for further characterization in canola.

In this study, we identified and selected seven T-DNA insertion *pla* knockout/knockdown mutants of *Arabidopsis* from the *Arabidopsis* Biological Resource Center, and compared their performance with the wild-type plants following inoculation with *P. brassicae*. We believe that, this study would be first step in characterizing the role of PLAs in clubroot resistance, and will serve as the foundation of additional work in this area. The results showed that four mutants (*pla*-1iix, *pla*-1iix, *pla*-1iii, *plpa*-iiiiii) developed more severe clubroot than the wild-type, suggesting increased susceptibility to *P. brassicae*. After identifying the homologs of the seven AtPLAs in canola (*BnPLAs*), and examining their expression profiles in published transcriptomic datasets generated from *B. napus* inoculated with *P. brassicae*, several *BnPLAs* were identified as promising candidates for further characterization in canola.

**MATERIALS AND METHODS**

**Plant Materials and Growth Conditions**

The *Arabidopsis* mutants *pla*-1iix (SALK_08694C), *pla*-1iix (SALK_012432C), *pla*-1iix (SALK_033291), *splaa*-α (SALK_099415C), *plpa*-iiiiii (SALK_057212C) and *plpa*-iiiiii (SALK_029470), derived from the wild-type ecotype Columbia, were obtained from the *Arabidopsis* Biological Resource Center (abrc.osu.edu; Table 1). The seeds were multiplied by growing into the next generation, and the homozygosity of T-DNA insertion of all the *pla* mutants was confirmed by PCR using two gene-specific primers (LP and RP) and a T-DNA border primer (Table 2). The seed was multiplied in a growth chamber set at 22/18°C with a photoperiod of 16 h day/8 h night. For inoculation with *P. brassicae*, all of the *pla* mutants and Columbia were grown in Sunshine LA4 potting mix (SunGro Horticulture, Vancouver, BC, Canada) in a greenhouse under 16 h light (natural light supplemented by artificial lighting) at 22°C. Three to five cold stratified (at 4°C for 3 days) seeds were placed in each pot (6 cm × 6 cm × 6 cm) and were thinned to one plant per pot after 1 week.

**Pathogen Material and Inoculation**

Inoculations were conducted with a single-spore isolate of *P. brassicae* pathotype 3H, as classified on the Canadian Clubroot Differential set (Strelkov et al., 2018), which had been stored at −20°C in infected root galls of the *B. napus* cultivar “Brutor.” The resting spore inoculum suspension was prepared following Strelkov et al. (2006). Briefly, 5 g of the frozen galls were homogenized in 100 mL water and filtered through eight layers of cheesecloth to remove plant debris. The resting spore concentration was measured with a hemocytometer and adjusted to 1.0 × 10^7 resting spores/mL with sterile distilled water. Two-week old seedlings were inoculated by modifying the method of Ludwig-Müller et al. (2017). Briefly, 2 mL of the inoculum suspension was added to the soil near the base of each plant with pipette. The non-inoculated control plants were treated with same amount of water using the same method.

The severity of clubroot was evaluated at 3 to 4 weeks after inoculation when the aboveground parts of inoculated plants were yellowing and purpling. Treatments were replicated six times with 24 plants per replicate. Clubroot severity was evaluated on a 0–3 scale (Figure 2A), where: 0 = no galls, 1 = galls mainly on the lateral roots, 2 = obvious galls on both the primary and lateral roots with a moderately reduced root system, and 3 = large galls on the primary roots with a significantly reduced root system. The individual severity ratings were used to calculate a disease index (DI) for each replicate using the formula described by Strelkov et al. (2006): DI (%) = [(n_1 + 1 × n_2 + 2 × n_3 × 3)/(N × 3)] × 100, where n_1, n_2, and n_3 are the number of plants in each severity class and N are the total number of plants tested. An one-way Anova followed by the Dunnett’s test in R (r-project.org) was used to compare
TABLE 1 | Arabidopsis thaliana (At) PLAs included in this study and their homologs in Brassica napus (Bn).

| Germplasm   | Gene | At gene ID | Bn gene ID | Bn peptide ID | Identity (%) | Coverage (%) | E-value |
|-------------|------|------------|------------|---------------|--------------|--------------|---------|
| CS855673    | PLA1-γ1 | AT1G06800 | BnaC05g04710D | CDY10228 | 86.65 | 99.22 | 0 |
|             |       |            | Bna110g27930D | CDY54880 | 85.39 | 83.88 | 0 |
| SALK_086894C| PLA1-α1 | AT1G06250 | BnaC05g04300D | CDY10187 | 83.08 | 94.80 | 0 |
|             |       |            | Bna110g28020D | CDY57813 | 82.84 | 94.80 | 0 |
|             |       |            | Bna08g01730D  | CDY86530 | 81.59 | 94.80 | 0 |
|             |       |            | Bna08g28540D  | CDY15570 | 81.48 | 95.51 | 0 |
| SALK_012432C| PLA1-γ2 | AT1G06930 | BnaC05g04710D | CDY10187 | 83.08 | 94.80 | 0 |
|             |       |            | Bna110g28020D | CDY57813 | 82.84 | 94.80 | 0 |
|             |       |            | Bna08g01730D  | CDY86530 | 81.59 | 94.80 | 0 |
|             |       |            | Bna08g28540D  | CDY15570 | 81.48 | 95.51 | 0 |
| SALK_009415C| sPLA2-α1 | AT1G51440 | BnaA06g02350D | CDY22842 | 83.85 | 98.48 | 0 |
|             |       |            | BnaC06g04560D | CDY49813 | 82.50 | 98.48 | 0 |
| SALK_003291 | PLA1-γ3 | AT1G30370 | BnaA07g07350D | CDY09593 | 87.15 | 99.81 | 0 |
|             |       |            | BnaC08g01730D | CDY86530 | 81.59 | 94.80 | 0 |
|             |       |            | BnaA09g28540D | CDY15570 | 81.48 | 95.51 | 0 |
| SALK_009415C| sPLA2-α1 | AT1G51440 | BnaA06g02350D | CDY22842 | 83.85 | 98.48 | 0 |
|             |       |            | BnaC06g04560D | CDY49813 | 82.50 | 98.48 | 0 |
| SALK_003291 | PLA1-γ3 | AT1G30370 | BnaA07g07350D | CDY09593 | 87.15 | 99.81 | 0 |
|             |       |            | BnaC08g01730D | CDY86530 | 81.59 | 94.80 | 0 |
|             |       |            | BnaA09g28540D | CDY15570 | 81.48 | 95.51 | 0 |

TABLE 2 | Primers used for T-DNA homozygous mutant lines identification.

| Gene ID     | Mutant line | Right genomic primer                  | Left genomic primer                  | T-DNA border primer          |
|-------------|-------------|--------------------------------------|--------------------------------------|-------------------------------|
| At1g06800   | WiscDsLox434H9 | CGATTTCGATCCATTTTCAAGTTATTCTCCATTCGGCATTTG | TTATTTCTCATTCCGGATTG |                              |
| At1g06250   | SALK_086894C | TCTACAGCCATATGAATGGGCATTCGGTTCCCAGATTCTTTG | ATTCGGTTCCCAGATTCTTTG |                              |
| At1g51440   | SALK_012432C | ATGACTATGTCACGTCTCCCGTTATTACCCATCCACGATCCC | TTATTTACTCATCCCAGATCCC |                              |
| At1g30370   | SALK_03291   | TGTGGAAGAGGTGTTTCTTGGAAATGCAAGTTCAATTGTTCG | ATAAACCGTTCAGGTGGTTTCTTGG |                              |
| At2g06925   | SALK_099415C | ATAAACCGTTCAGGTGGTTTCTTGG | ATAAACCGTTCAGGTGGTTTCTTGG |                              |
| At3g54950   | SALK_057212C | ATAAACCGTTCAGGTGGTTTCTTGG | ATAAACCGTTCAGGTGGTTTCTTGG |                              |
| At3g63200   | SALK_029470  | ATAAACCGTTCAGGTGGTTTCTTGG | ATAAACCGTTCAGGTGGTTTCTTGG |                              |
|             | For SALK lines (LBb1.3) | GCGTGGACCGCTTGCTGCAACT |                              |                              |
|             | For WISCDSLOX (P745) | AAGTCGCCAGATGTGTTATTAAGTGTGTC |                              |                              |

For SALK lines (LBb1.3)
For WISCDSLOX (P745)

the mean DIs of each Arabidopsis mutant to the wild-type, with differences regarded as significant at p < 0.05.

Identification of AtPLA Homologs in Brassica napus

Homologs of the seven AtPLAs from Arabidopsis were identified in B. napus through BLAST and phylogenic analysis. Specifically, peptide sequences of the seven AtPLA obtained from the Arabidopsis (TAIR 11) database were blasted against the B. napus genome (Chalhoub et al., 2014) using BLASTP (E-value ≤1e–10, coverage > 60%, identity > 60%, and the top 20 hits). Homologs of the AtPLAs also were identified in B. rapa and B. oleracea using the same parameters, to improve the accuracy of the subsequent phylogenic analysis. The sequences of selected peptides of the three Brassica species were aligned with the seven AtPLA peptide sequences using Muscle in MEGA7 (Kumar et al., 2016). A rooted phylogenic tree of the selected PLA peptides in the four species, using WRKY2 (AT5G56270) in Arabidopsis as the root, was generated with fasttress (Price et al., 2009) and visualized in MEGA7 to identify the AtPLA homologs in B. napus. In addition we have identified pylogenic relationship between all the available Arabidopsis PLAs and Brassica species. The expression profiles of the BnPLAs were examined in two published transcriptomic datasets generated from B. napus infected by P. brassicae (Galindo-González et al., 2020; Zhou et al., 2020b; Supplementary Figure 2).

RESULTS

We identified homozygous T-DNA insertion knockout/knockdown mutants representing each PLA subtype, pla1-γ1 (WiscDsLox434H9), pla1-α1 (SALK_086894C),
pla₁⁻iy₁ (SALK_012432C), spla₂⁻α (SALK_099415C), ppla-iiiβ (SALK_057212C), pla₁⁻iii (SALK_033291), and ppla-iiiδ (SALK_029470), using two gene-specific primers and the T-DNA left border primers (Alonso et al., 2003; Figure 1A). Insertion sites of pla₁⁻iy₁, pla₁⁻iia, pla₁⁻iy₃, ppla-iiiβ, and pla₁⁻iii were located in the exon regions of each PLA genes, while ppla-iiiδ contained its mutant sites within the promoter region (Figure 1B). Furthermore, while the spla₂⁻α mutant lines were reported to have multiple insertion sites within the promoter and exon regions of the gene, the exact map location was not indicated on the TAIR web site (Seo et al., 2008; TAIR-ABRC). Furthermore, this T-DNA mutant line (SALK_099415C) previously been confirmed as a complete gene knockout line as no detectable mRNA was observed (Seo et al., 2008).

**FIGURE 1** | Identification and characterization of Arabidopsis T-DNA insertion lines. (A) Identification of T-DNA insertion lines by PCR. LP and RP primers are located at the left and right sides of T-DNA insertion, respectively. LBb1.3 and P745 are used as flanking primers for SALK and WISC lines, respectively. In each gel picture #1 is the PCR sample with primers LP + RP and #2 is the PCR sample with primers LB1.3/P745 + RP. (B) Schematic diagrams of the positions of T-DNA insertions in PLA single mutant alleles. Start and stop codons are indicated.
To validate the roles of the selected PLAs in the host response to clubroot, we first evaluated the disease severity of the seven homozygous Arabidopsis pla mutants and the wild-type Arabidopsis, following inoculation with P. brassicae pathotype 3H. Four of the mutants (pla1-iiα, pla1-iγ3, pla1-iii, and ppla-iiiδ) had significantly greater DIs than the wild-type (Figure 2B), indicating increased susceptibility to P. brassicae pathotype 3H. Among these, the pla1-iiα and pla1-iγ3 appeared the most susceptible, with DIs of 75.0 and 74.7%, respectively, followed by ppla-iiiδ (69.8%) and pla1-iii (62.1%). The reactions for the remaining mutants were similar to the wild-type, which developed an DI of 50.4%.

We identified homologs of the seven PLAs in B. napus by BLAST and phylogenetic analysis. We first aligned the peptide sequences of seven AtPLA to the three Brassica species (B. rapa, B. oleracea, and B. napus) using BLASTP (see text footnote 2) to select potential PLA homologs with high identity (%) and coverage (%) and low E-values. After that, we performed phylogenetic analysis using Mega7 (Kumar et al., 2016) and fasttree (Price et al., 2009), and filtered Brassica PLAs that were not properly clustered with the targeted AtPLAs. In total, 21 homologs of BnPLAs were identified in B. napus, which were evolved from either B. rapa or B. oleracea (Figure 3), indicating duplication and loss of PLAs during the divergence of Arabidopsis and Brassica, but a conserved evolution of PLAs in Brassica. All the BnPLAs had >80% identity and coverage, and E-values near 0, to each of corresponding AtPLA (Table 1).

**DISCUSSION**

Plant phospholipids are major structural components of biological membranes (Adigun et al., 2021). Accumulating evidence indicates that phospholipid-based signal transduction
and phospholipid-derived products mediated by PLAs are important for plant growth, development, and responses to abiotic and biotic stress (Ryu, 2004; Grienenberger et al., 2010; Canonne et al., 2011; Chen et al., 2013; Adigun et al., 2021). Upon perception of invading pathogens, PLAs can be activated to hydrolyze phospholipids and galactolipids at their sn-1 or sn-2 positions to generate free fatty acids and lysophospholipids. These functions as precursors of second messengers to mediate downstream defense reactions, including the production of oxylinins and JA, and stimulation or inhibition of key signaling enzymes (e.g., MAKP, protein kinase and H⁺-ATPase) (Ryu, 2004; Canonne et al., 2011). PLAs are also involved in plant growth and development by auxin-related cell elongation in plants (Ryu, 2004), which might be associated with root galling in P. brassicae-infected plants. In addition, a recent expression analysis of drought-related lipid genes in soybean (Glycine max) suggested the involvement of PLAs (PLA₁-II and -III, sPLA, pPLA-I, -II, and -III) in response to water deficit (Ferreira, 2017). Since infection by P. brassicae interferes with root water uptake and leads to drought stress symptoms in the aboveground canopy (Ludwig-Müller, 2009), PLAs might be involved in a clubroot-related drought response in plants. While little is known regarding the role of PLAs in the host response to clubroot, recently published transcriptomic profiles from P. brassicae-infected B. napus allowed us to identify PLAs showing differential expression following inoculation (Fu et al., 2019; Galindo-González et al., 2020; Zhou et al., 2020b). Therefore, study of the role(s) of PLAs in the host response to clubroot could provide insights into the utility of PLAs in clubroot resistance breeding.

In plants, PLAs are classified into three main subtypes based on their catalysis site: phospholipase A₁ (PLA₁), secretory phospholipase A₂ (sPLA₂), and patatin-like PLA (pPLA). Arabidopsis has about 30 PLAs, including 12 PLA₁s, four sPLA₂s, and 13 pPLA (Eastmond, 2006; Chen et al., 2013; Li and Wang, 2014). Furthermore, in canola, each PLA has several homologs (Supplementary Figure 1) and it is difficult to study their roles in clubroot resistance directly given the complexity of the canola genome. To improve understanding of the roles of PLAs in clubroot resistance and to help to identify candidate PLA genes in canola contributing to resistance, we selected seven pla knockout/knockdown Arabidopsis mutants and compared their susceptibilities with the wild-type plants, following inoculation with P. brassicae. pathotype 3H. This is a common pathotype found across much of the canola producing regions of western Canada, and is highly virulent on canola that does not carry any clubroot resistance (Strelkov et al., 2018; Hollman et al., 2021).

Phospholipase A₁s specifically hydrolyze the sn-1 position in phospholipids. They further divided into groups I, II, and III PLA₁s and phosphatidic acid-specific PLA₁ (PA-PLA₁) (Kim and Ryu, 2014). Class I, II, and III PLA₁s are predicted to localize to the chloroplast, cytosol and mitochondria, respectively, based on the presence of N-terminal stretches (Ryu, 2004; Seo et al., 2009; Chen et al., 2011). While there is no clear evidence of PLA₁s in plant immunity, expression of several genes (e.g., PLA₁-Iγ1, PLA₁-Iγ2, and PLA₁-III) encoding PLA₁ proteins was induced in Arabidopsis response to pathogen attack (e.g., B. cinerea and P. syringae) (Grienenberger et al., 2010). In addition, increased expression of several PLA₁s (VviPLA₁-Iβ1, VviPLA₁-Iγ1, and VviPLA₁-IIβ) in grapevine (Vitis vinifera L.) was found within 24 h following inoculation with the biotrophic oomycete Plasmopara viticola (Laureano, 2018). Three of the four pla₁ mutants (pla₁-iia, pla₁-iγ3, and pla₁-iii) in the current were more susceptible to P. brassicae than the wild-type Arabidopsis, indicating the importance of PLA₁ in host defense to this pathogen.

Class I PLA₁s are involved in JA production (Wasternack and Song, 2017), including the two genes PLA₁-Iγ1 and PLA₁-Iγ3 in this study (Ellinger et al., 2010). However, the genes may mediate different pathways for JA production, with PLA₁-Iγ1 contributing to wound-induced JA production and PLA₁-Iγ3 contributing to JA production under non-wounded conditions (Ellinger et al., 2010). Transcription of PLA₁-Iγ1 in Arabidopsis was induced upon B. cinerea and P. syringae inoculation (Grienenberger et al., 2010), and expression of PLA₁-Iγ1 in grapevine was upregulated within 24 h of inoculation with P. viticola (Laureano, 2018). A homolog of AtPLA₁-Iγ1 in B. napus was downregulated (BnaC05g04710D) during the late infection stage in a compatible (susceptible) interaction with P. brassicae pathotype 3A (Zhou et al., 2020b), while the same gene was upregulated during early infection of a resistant B. napus genotype by pathotype 5X (Galindo-González et al., 2020; Supplementary Figure 2). Galindo-González et al. (2020) also identified another BnPLA₁-Iγ1 (BnaA10g27920D), which was upregulated in both susceptible and resistant B. napus, but with greater upregulation in the resistant reaction. In this study, pla₁-iγ1 and pla₁-iγ3 mutants showed greater susceptibility than the wild-type, but only pla₁-iγ3 showed a significant difference, suggesting that PLA₁-Iγ3 but not PLA₁-Iγ1 plays an important role in the response of Arabidopsis to P. brassicae. Thus, homologs of PLA₁-Iγ3 in canola may contribute to enhanced clubroot resistance. Given the fact that BnPLA₁-Iγ1 (BnaC05g04710D) was downregulated in a clubroot susceptible reaction and upregulated in a resistant reaction, this gene might also be associated with canola defense to clubroot.

There are four class II PLA₁s in Arabidopsis (PLA₁-IIα, -IIβ, -IIγ, and -IIδ) (Chen et al., 2013) but little is known regarding the role of class II PLA₁s in response to pathogens. Nonetheless, the expression of PLA₁-IIδ increased in grapevine following challenge by P. viticola (Laureano, 2018), suggesting the involvement of class II PLA₁s in the defense reaction. Our results showed that the pla₁-iia Arabidopsis mutant was more susceptible to clubroot than the wild-type, suggesting a role for PLA₁-IIα in the host response to P. brassicae. In addition, a homolog of this gene in B. napus (BnaA10g28020D) was downregulated during the late stage of susceptible reaction to clubroot (Zhou et al., 2020b), which is consistent with the present results. Since a PLA₁-II (GmPLA₁-IIα) in soybean was upregulated under drought conditions (Ferreira, 2017), PLA₁-IIα-mediated clubroot defense might include mitigating water stress. Collectively, these results suggest the importance of PLA₁-IIα in clubroot resistance, and the gene BnaA10g28020D might be a good candidate for further characterization of its role.

Only one class III PLA₁ is found in Arabidopsis (PLA₁-III) (Chen et al., 2013). PLA₁-III is important for plant
development and stress tolerance. The expression of PLA\(_1\)-III was modulated in Arabidopsis upon B. cinerea (Griebenberger et al., 2010) challenge, respectively. Expression of an AtPLA\(_1\)-III homolog in CR Chinese cabbage (B. rapa) was induced (BraA07g010560.3C) during the early infection stage (4 dai) following inoculation with a virulent pathotype of P. brassicaceae (Fu et al., 2019). A homolog of AtPLA\(_1\)-III was downregulated in B. napus (BnaC05g23260D) during the late stage of the clubroot-susceptible response (Galindo-González et al., 2020; Zhou et al., 2020b; Supplementary Figure 2), while two other BnPLA1-III homologs (BnaA07g07350D and BnaA09g26150D) were downregulated in both resistant and susceptible reactions, with greater downregulation in the latter (Galindo-González et al., 2020). These findings are consistent with our results, since the pla1-iii mutant was more susceptible to clubroot than the wild-type, indicating the importance of PLA1-III in plant defense to clubroot. Similarly, PLA1-III in soybean was regulated under drought conditions, with a downregulation in moderate and severe drought but an upregulation in extreme drought (Ferreira, 2017), further suggesting a role in mitigating drought stress.

sPLA\(_2\)-s, which specifically hydrolyze the sn-2 position of phospholipids, are the only PLA\(_2\)-s identified in plants (Kim and Ryu, 2014). Only four AtsPLA isoforms are found in Arabidopsis (sPLA\(_2\)-\(\alpha\), \(\beta\), \(\gamma\), and \(\delta\)), with sPLA\(_2\)-\(\alpha\) suggested to play a negative role in defense to pathogen attack by subcellular localizing to the cell nucleus and physically binding to MYB30 to repress defense in Arabidopsis (Froidurea et al., 2010; Canonne et al., 11; Kim and Ryu, 2014). sPLA\(_2\) have been suggested to be involved in the auxin signaling pathway, which promotes cell elongation (Ryu, 2004). For instance, overexpression of AtsPLA\(_2\)-\(\beta\) in Arabidopsis resulted in elongation of the leaf petioles and inflorescence stems, while silencing of the gene resulted in the opposite phenotype (Lee et al., 2003). Expression of BnsPLA\(_2\)-\(\alpha\) was increased in susceptible hosts and decreased in resistant hosts, following inoculation with P. brassicaceae (Galindo-González et al., 2020; Zhou et al., 2020b; Supplementary Figure 2), suggesting their negative roles in clubroot resistance. Considering that auxin is a crucial hormone for gall development in roots of P. brassicaceae-infected plants (Jahn et al., 2013), sPLA\(_2\)-\(\alpha\) might be involved in auxin-related pathways to promote gall development. Nonetheless, in this study, clubroot severity on the atspla\(_2\)-\(\alpha\) mutant was not significantly different from that of the wild-type.

Patatin-like PLAs, which hydrolyze phospholipids and galactolipids at both the sn-1 and sn-2 positions (Chen et al., 2011), are involved in defense signaling in plants upon infection by many pathogens. A member of pPAs in pepper (Capsicum annuum), CaPLP1, plays a positive role in plant innate immunity (Kim et al., 2014). Silencing of CaPLP1 increased plant susceptibility to the bacterium Xanthomonas campestris pv. vesicatoria and was associated with compromised defense responses, including reactive oxygen species production, hypersensitive cell death and expression of a SA-dependent gene CaPR1. Overexpression of this gene in Arabidopsis increased plant resistance to P. syringae and Hyaloperonospora arabidopsidis, which was associated with an enhanced oxidative burst, expression of SA- and JA-dependent genes, and cell death (Kim et al., 2014). Another pPLA, pPLA-IIa/PLP2, which contributes JA- and oxylipin-mediated cell death, positively regulated Arabidopsis resistance to the obligate parasite cucumber mosaic virus and the fungus V. dahlia by inducing oxylipins and JA biosynthesis, respectively. This gene, however, negatively regulated resistance to B. cinerea and P. syringae in Arabidopsis (la Camera et al., 2005, 2009; Zhu et al., 2021). In addition, the expression of several pPLAs (VvipPLA-1, VvipPLA-II\(\beta\), VvPlA-II\(\beta\), and VvIPiLA-II\(\beta\)) was induced in grapevine after inoculation with P. viticola (Laureano, 2018). Transcriptomic studies of B. napus inoculated with P. brassicaceae pathotype 3A indicated that expression of two copies of BnPLP2 increased earlier during the infection process, but not at later stages, in both the resistant and susceptible reactions (Zhou et al., 2020b). When B. napus was inoculated with P. brassicaceae pathotype 5X, expression patterns of the two genes were generally similar in both the resistant and susceptible reactions, except that the genes were also upregulated in the susceptible host during the late infection stage (Galindo-González et al., 2020; Supplementary Figure 2), suggesting a negative role of these genes in clubroot resistance. In addition, pPLAs are involved in the regulation of auxin-related responses. A study investigating the role of pPLAs in the regulation of auxin responses found delayed upregulation of auxin-responsive gene expression in all nine ppla mutants studied (Labusch et al., 2013). The authors also demonstrated that knocking out/down pPLA-III\(\beta\) in Arabidopsis affected auxin-related phenotypes, including shortened main roots and more lateral roots, and knocking out of pPLA-II\(\beta\) resulted in slightly longer roots and hypocotyls (Labusch et al., 2013).

Other pPLA enzymes, including PLAIVA/PLP1, are also positively associated with plant auxin signaling to modulate root development (Rietz et al., 2010). In addition, pPLAs are involved in response to water deficit in plants (Ferreira, 2017). Therefore, pPLAs could regulate the host response to clubroot via their involvement in defense pathways and drought- and auxin-related pathways. In this study, both ppla-ii\(\beta\) and ppla-iii\(\beta\) appeared to develop more severe symptoms than the wild-type Arabidopsis, but DI was significantly more severe only for ppla-ii\(\beta\) (Figure 2B), indicating a positive effect of pPLA-III\(\beta\) in clubroot resistance. In contrast, B. napus inoculated with pathotype 3A or 5X of P. brassicaceae showed reduced expression of BnpPLA-III\(\beta\) in both the resistant and susceptible reactions during early infection, and was downregulated in only the resistant hosts during late infection (Galindo-González et al., 2020; Zhou et al., 2020b; Supplementary Figure 2). These contrasting findings may reflect host-pathotype specific interactions, and suggest that functional validation of AtPLA-III\(\beta\) in response to additional P. brassicaceae pathotypes may improve understanding of its role in host defense.

**CONCLUSION**

To the best of our knowledge, this is the first study investigating the host response of PLAs to clubroot. We selected seven Arabidopsis atpla knockout/knockdown mutants, and identified
four mutants (pla1-iiia, pla1-iv3, pla1-iii, ppla-iiiib) that were more susceptible to P. brassicae than the wild-type plants. These results indicate that PLAs may play positive roles in host defense to clubroot. We identified homologs of the seven PLAs in B. napus and its parental species (B. rapa and B. oleracea) and explored their expression patterns following P. brassicae inoculation using available transcriptomic datasets; this information was used to select candidate genes that can be further characterized in canola. The pathways mediated by these PLAs are unknown, but could be related to JA biosynthesis, and auxin- and drought-responses. However, additional studies will be needed with more PLA mutants in Arabidopsis to confirm their role in clubroot resistance and elucidate the precise mechanism(s) of each PLA gene.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding authors.

AUTHOR CONTRIBUTIONS

SS, S-FH, and GC conceived, designed and supervised the experiment, and edited the manuscript. QZ and KJ performed the experiments and wrote the first draft of the manuscript. All authors contributed to the preparation of the final article.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2022.799142/full#supplementary-material
