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Role of Potassium-Independent Asparaginase Phosphorylation During the Plant Immune

Response

A thesis submitted in partial satisfaction of the requirements for the degree Master of Science

in

Biology

by

Adelaida Estrada-Cardenas

Committee in charge:

       Professor Alisa Huffaker, Chair
       Professor Eric A. Schmelz, Co-Chair
       Professor Lisa McDonnell

2022

The Thesis of Adelaida Estrada-Cardenas is approved, and it is acceptable in quality and form for publication on microfilm and electronically.

University of California San Diego
2022

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ABSTRACT OF THESIS

Role of Potassium-Independent Asparaginase Phosphorylation During the Plant Immune

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Adelaida Estrada-Cardenas

Master of Science in Biology

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In agriculture, plants are constantly attacked by pests and disease, thus threatening food security, and increasing the chemical input of pesticides and fungicides meant to maximize crop yields. Analyses of the effects phosphorylation has on asparaginase enzymatic activity have suggested that phosphorylation promotes asparaginase activity (Ahmadian, N., 2020), increasing

catabolism of asparagine to release nitrogen in support of the resource-intense immune response. To elucidate whether there is a correlation between asparaginase phosphorylation and enhanced plant immune response, we developed stable transgenic lines expressing phosphomimetic and phosphoabolishing variants of asparaginase. When these plants were inoculated with the fungal pathogen *Botrytis cinerea,* a decrease in disease resistance was observed in the phosphoabolishing line. This decreased resistance indicates that asparaginase phosphorylation is needed to facilitate plant immune response. As a future testable hypothesis, we speculate that amino acid levels of ammonium and aspartate are affected by asparaginase phosphorylation and subsequently its enzymatic activity. Together this work created and initiated characterization of genetic resources to better develop our understanding of how primary metabolism and nitrogen availability affect plant immunity.

## Introduction

Plants play a major role in providing our food supply; however, they are constantly attacked by pests and pathogens, causing disease and losses that threaten our food security (Strange and Scott, 2005). During these attacks, plants launch an immune response to quickly identify and fight off pests and pathogens (Iriti and Faoro, 2007). Their immune responses include forming structural defenses, toxic specialized metabolites, and defensive proteins (Arnaiz et al., 2018). Another form of defense is the production of volatile terpenoids from damaged plant tissues, which attract natural enemies of the pest, such as predators and parasitoids, and serves as a warning to neighboring plants of imminent danger (War et al., 2012; Huffaker et al., 2013).

Unfortunately, these immune responses alone are unable to sufficiently protect plants in every case. To meet agricultural production needs and maximize crop yield, pesticides and fungicides are used to combat crop loss, which can have negative environmental impacts. For example, long-term, low-dose exposure to pesticides has been linked to endocrine health effects including cancer, birth defects, hormone disruption, immunosuppression, and diminished intelligence (Brouwer et al., 1999; Crisp et al., 1998; Hurley et al., 1998, Aktar et al. 2009). Research into plant immunity seeks to ultimately aid the design of plants that can protect themselves more effectively, thus minimizing the need for added fungicides and pesticides. Reducing pesticide and fungicide use in agricultural production by enhancing plant resistance to pests and disease can lead to more sustainable practices while still maximizing crop yield to protect our food security.

Plants recognize pest and pathogen attacks using a large diversity of pattern recognition receptors (PRRs) (Haney et al., 2014). For example, these receptors bind to

herbivore-associated molecular pattern molecules (HAMPs) commonly found in caterpillar oral secretions (Steinbrenner et al., 2020; Iriti and Faoro, 2007). They also bind to bacterial and fungal microbe-associated molecular patterns (MAMPs), such as lipopolysaccharide, peptidoglycan, and flagellin (Grennan, 2006). Recognition of exogenous molecular patterns by PRRs initiates a downstream signaling cascade that activates a variety of downstream immune responses (Huffaker et al., 2006).

Plants produce some endogenous signaling molecules upon pathogen attack and one such elicitor molecule is the Arabidopsis Plant Elicitor Peptide (PEP) 1 (AtPep1). Pep-signaling is critical in innate immunity activation after the detection of pests and disease and the signaling cascades are further amplified by these PEPs, and plants with compromised Pep-signaling are more susceptible to disease (Huffaker et al., 2006).

In Arabidopsis, upon activation, AtPep1 is derived from its precursor gene, PRECURSOR OF PEP1 (AtPROPEP1) (Huffaker et al., 2006). AtPep1 then binds to both of its cell surface receptors, PEP-RECEPTOR1 (AtPEPR1) and AtPEPR2, (Yamaguchi et al., 2010) via the recruitment of a coreceptor, BRI1-ASSOCIATED RECEPTOR KINASE1 (BAK1), to trigger cellular signaling that promotes the immune response (Postel et al., 2010; Huffaker 2015). AtPep1 subsequently activates the transcripts encoding the antimicrobial protein PLANT DEFENSIN 1.2 (PDF 1.2) and synthesis of a second messenger signal, reactive oxygen species (ROS). Plant defensins are small peptides that inhibit the growth of a broad range of fungi (Thomma et al., 2002), and the production of reactive oxygen species (ROS) is one of the earliest responses triggered after heterodimerization of PEPR and BAK1 (Lin et al., 2014; Kadota et al., 2014). Both PEPR and BAK1 then interact with BOTRYTIS-INDUCED KINASE1 (BIK1) causing an influx of ions, inhibiting the negative regulation on immune response of CPK28 and

releasing AtPROPEP1 which ultimately aids in the spatiotemporal amplification of defense responses (Hander et al., 2019; Lin et al., 2014; Bredow et al., 2021; Huffaker et al., 2007).

Mechanisms of Pep-signaling regulating immune output have been primarily elucidated in Arabidopsis thaliana, a commonly-used model plant due to its small genome, short life cycle, and large seed output (Van Norman, J. M., &amp; Benfey, P. N, 2009). As a member of the mustard family, A. thaliana can also be linked to many cultivated plant species such as mustard, cabbage, and radish (Van Norman, J. M., &amp; Benfey, P. N, 2009). Additionally, Arabidopsis is readily transformable using Agrobacterium tumefaciens to make mutant lines; therefore, making it a good model for genetic manipulation and targeted point mutations to examine protein function. For these reasons, the complexities of Pep-signaling have largely been examined in Arabidopsis, but the findings have been extended to diverse plant species as their function as immunoregulatory signals is conserved among higher plants (Huffaker 2011, Huffaker 2013, Poretsky 2020, Dressano et al., 2020).

Understanding the full network of cellular regulators that mediate plant immunity is an ongoing question of importance. Post-translational modification is a common regulatory mechanism for cellular signaling components, and so to identify previously undiscovered cellular proteins involved in immune signaling, our lab has performed a screen of phosphoproteomic changes in both Arabidopsis and maize after AtPep1 treatment as compared to controls. This allowed for detection of phosphorylated proteins, quantification of any changes in their phosphorylation state and mapping of the phosphorylation sites. This screen revealed many candidate proteins that were found to change in phosphorylation state, suggesting their potential role in plant immune responses (Dressano et al., 2020).

Among the proteins identified as differentially phosphorylated after AtPep1 treatment was the asparaginase, ASPGA1. Asparaginases are essential nitrogen-releasing enzymes in plants, and catalyze the conversion of asparagine to aspartic acid (Michalska et al., 2006), releasing free nitrogen as ammonia. Elicitor-induced immune responses are characterized by large-scale transcriptional changes, leading to increased biosynthesis of defensive proteins and metabolites (Sun et al., 2020). A steady supply of nitrogen is needed to produce newly synthesized proteins as well as nitrogen-containing defense metabolites such as pyrrolizidine alkaloid and cyanogenic glucosides (Macel, 2011; Zagrobelny, 2004). Although the direct link remains unclear, robust plant immunity likely requires release of nitrogen from storage amino acids. Asparagine is a dominant nitrogen-storage amino acid in plants, likely due to its highest nitrogen to carbon ratio among all proteinaceous amino acids (Lea et al., 2007). To release available nitrogen from asparagine, it is converted into aspartic acid by asparaginase, with nitrogen liberated in the form of ammonia ($NH_3$) (Curtis et al., 2018). In plants, asparaginase is essential for nitrogen supply in actively growing sink tissues such as developing leaves, roots and germinating seeds (Michalska et al., 2006). The *A. thaliana* genome includes two asparaginase genes, ASPGA1 (AT5G08100) and ASPGB1 (AT3G16150) (Van Norman and Benfey, 2009). ASPGB1 is activated by potassium, and its catalytic efficiency in the presence of potassium has been reported to be 50-fold higher than ASPGA1, which is potassium-independent (Bruneau et al., 2006; Gaufichon et al., 2016).

Plant asparaginase enzymes are inactive until autoproteolytic cleavage within their variable activation loop generates a nucleophilic catalytic group (Michalska et al., 2006; Gabriel et al., 2012). Plant type asparaginases are heterotetramers composed of two beta-subunits sandwiched by two alpha-subunits (Michalska et al., 2006). Each alpha and beta

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heterodimer arises from the autoproteolytic cleavage of the precursor protein (Michalska et al., 2006). The autoproteolytic cleavage liberates the nucleophilic threonine at the N-terminal end of the beta-subunit within the conserved sequence GlyThrVal (Michalska et al., 2006). Recently, we discovered that a serine residue (Serine 169) adjacent to the cleavage site in the ASPGA1 activation loop is phosphorylated within minutes of peptide-triggered immune responses in Arabidopsis suspension cells. This phosphorylation has not been previously observed. We hypothesized that phosphorylation proximal to the cleavage site could affect asparaginase cleavage and enzymatic activity, and potentially could lead to altered immune responses.

In studying contributions of ASPGA1 to plant immune responses, the lab has been aiming to address the following questions: (1) does serine site phosphorylation affect the autoproteolytic cleavage process and activity in ASPGA1, (2) does altered activation of ASPGA1 affect nitrogen availability in the form of $NH_3$, (3) and do changes in nitrogen content impact immune output and disease resistance in plants. As shown in Figure 2B and 2C, our earlier work examined how loss of asparaginase activity in the aspga1/aspga2 double knockout mutant affected two early immune response pathway defense markers. Upon AtPep1 treatment, accumulation of transcripts encoding the antimicrobial protein PDF 1.2 and production of second messenger signal ROS were not affected in aspga1/aspga2 and were comparable to Col-0 while levels of both were significantly diminished in pepr1/pepr2, a double mutant that is insensitive to AtPep1 treatment. This indicated that asparaginase activity was not required for initial activation of these responses. In contrast, when disease resistance of the aspga1/aspga2 double knockout was examined after inoculation with the necrotrophic fungal pathogen, *Botrytis cinerea* (Fig. 2D), it was observed that aspga1/aspga2 had increased

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susceptibility to the fungus compared to Col-0, similar to the immunocompromised *pepr1/pepr2* double mutant. This indicated that although initial activation of immunity did not appear to be dependent on asparaginase activity, effective resistance mediated by longer-term immune activation does require functional asparaginase enzyme.

To assess whether an induced immune response via AtPep1 treatment increases asparaginase activity, plants were treated with AtPep1 in conjunction with hydrolysis of L-asparagine by L-asparaginase using Col-0 and the asparaginase double knockout mutant aspga1/aspga2. As a result, it was found that Col-0 had an increase in asparaginase activity with increased AtPep1 incubation time; however, without the ability to supplement asparaginase function, there appears to be no significant difference in asparaginase activity with increased AtPep1 incubation time in aspga1/aspga2 suggesting AtPep1 signaling is associated with increased asparaginase activity (Fig. 2A). To further investigate how asparaginase phosphorylation affects asparaginase activity, two c-terminal YFP-tagged constructs were made and transiently expressed in *Nicotiana benthamiana* using Agrobacterium tumefaciens: phosphoabolishing (-YFP and -YFP) where the phosphorylation site serine residue was substituted with the alanine amino acid, and phosphomimetic (-YFP and -YFP) where the phosphorylation site serine residue was substituted with the aspartate amino acid. It has been observed that after 48 h of agroinfiltration, the asparaginase activity levels were increased in the leaves treated with phosphomimetic construct, which has constitutive phosphorylation while in the leaves expressing the phosphoabolishing construct had little to no phosphorylation, had decreased enzyme activity (Figure 3A) suggesting the role of phosphorylation on asparaginase activity. Additionally, western blot analysis was also performed using these agroinfiltrated samples expressing the phosphovariants in order to

observe the changes in cleavage of the protein (Fig. 3B). Samples were taken at 24 hours and 48 hours post inoculation. The phosphomimetic variant could be seen to have only the cleaved fragment, the beta subunit, in samples collected after 24 h and 48 h of agroinfiltration with the beta subunit band being more intense at 48hrs. The phosphoabolishing line had the non-cleaved fragment, the precursor peptide, at both 24 h and 48 h with a less intense beta subunit band appearing after 48 h. From this study, it has been understood that asparaginase phosphorylation had an effect on cleavage and in turn its activity.

To further confirm the phenotypes observed in our study, we generated stable expression lines of Arabidopsis overexpressing the phosphoabolishing and phosphomimetic versions of this gene. These transgenic lines were characterized by PCR and used for different assay to address the questions related to the role of asparaginase in immune responses.

In this study, I sought to further understand of the role asparaginase phosphorylation plays in plant immunity by addressing the following questions: How does the phosphorylation of the asparaginase variable loop affect asparaginase enzyme activity and cleavage? Does the liberation of nitrogen from asparagine generate a protective response to enhance plant resistance to disease? How does disease affect cleavage? To address these questions, I generated mutant lines in which the phosphorylated serine residue in asparaginase has been substituted through site-directed mutagenesis to either mimic or abolish phosphorylation. Additionally, I began initial characterization of these lines through a study of resistance to *B. cinerea*.

**Materials and Methods**

- Plant materials and growth conditions
    - *Arabidopsis thaliana* ecotype Columbia-0 (Col-0) was used as a wild-type reference plant. The Pep-insensitive receptor knockout *pepr1/pepr2* double

mutant (Yamaguchi *et al.*, 2010) was also used as a control for all relevant bioassays. Asparaginase double mutant *aspga/aspgb* has been previously described (Ivanov *et al.,* 2011) and was used as background for generating transgenic Arabidopsis lines and also as a control for all the experiments. Sterilization of Arabidopsis seeds was carried out in a sealed chamber containing chlorine gas (50 mL 100% bleach, 1.5 mL of 37% HCl) for 2-4 hours. Seeds were plated aseptically on half-strength Murashige and Skoog (MS) containing 0.8% phytoagar. After stratifying for 2-3 days at 4°C, seeds were germinated in a light- and humidity- controlled growth chamber (22°C, 12h light/12h dark).

- DNA Extraction, Genotyping, and Quantitative analysis of fungal DNA
  - Total DNA was extracted from snap-frozen and powdered samples using Edward's Buffer (200mM Tris-HCl pH 7.5, 250mM NaCl, 25mM EDTA, 0.5% SDS). To 50mg of the tissue, 300uL of the buffer was added and vortexed vigorously before centrifugation at max speed for 10 min. The supernatant was transferred to a fresh tube and an equal volume of 100% isopropanol was added and incubated for 5-10 minutes prior to centrifugation at 12K rpm for 10min. Precipitated DNA was washed with 70% ethanol in order to remove any remaining salts and centrifuged at 12K rpm for 10 min. The samples were then left to dry overnight, and resuspended in 50uL of autoclaved milliQ water. The extracted DNA was used for different experiments.
  - For genotyping the transgenic lines, asparaginase gene (AT5G08100) specific forward and YFP specific reverse primers were used. 2X GoTaq master mix was used for the PCR reaction and the following PCR conditions were used: 95°C for

5 min, 95°C for 30s, 58°C for 30s, 72°C for 1:30min. Step 2 to step 4 were repeated for 30 cycles, with a final extension of 72°C for 7 min. After the PCR reaction, the samples were loaded onto a 1% agarose gel and the transgenic lines were selected based on the PCR bands visualized using the Bio-Rad Molecular Imager ChemidocTM XRS+ imaging system..

- For the quantification of the fungal DNA from Botrytis cinerea infected samples, fungal specific cutinase gene (Z69264) primers were used and the ACTIN2 (At3g18780) gene was used as a housekeeping gene to normalize the CutA gene expression. The primer sequences used in the study are provided in Table 1. All the DNA samples were normalized and 50ng of the DNA was used for the qPCR. Quantitative PCR was performed with SsoAdvanced TM Universal SYBR(R) Green Supermix (Bio-Rad) and a StepOne Real-Time PCR System (Applied Biosystems). The data was analyzed using the 2-$\Delta\Delta$CT method.

- Immunoprecipitation of Proteins from Transgenic Overexpression Lines

  - ~700mg of snap-frozen and powdered plant tissue was ground in a mortar and pestle on ice with extraction buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P- 40, 50% glycerol, 10 mM dithiothreitol (freshly made), Roche Protease Inhibitor tablet) added in 1:2 tissue to buffer ratio. Samples were then incubated in the extraction buffer for 40-50min with end to end mixing at 4°C. The GFP-trap magnetic beads were prepared during this time by making a stock tube containing 10uL of beads per sample. The beads were washed three times with the extraction buffer while centrifuging at 1K rpm for 1 min at 4°C in between each wash. Extraction buffer was used to resuspend the beads before

aliquoting 100ul of the solution per sample. Following the incubation of the

tissue samples in extraction buffer, the samples were centrifuged (max speed for

30 min at 4°C), 40ul of the supernatant was saved as input protein sample, and

the remaining supernatant was added to the GFP-trap magnetic beads and

incubated at 4°C for 3hr with end-to-end mixing. Following centrifugation (1K

rpm for 1 min at 4°C), the beads were washed three times with the extraction

buffer while centrifuging at 1K rpm for 1 min at 4°C in between each wash.

2xSDS loading dye was used to resuspend the beads and also for the input

protein samples. The samples were boiled at 95°C for 10 minutes, and then

centrifuged (10 minutes, max speed). The supernatant was collected for Western

blotting.

- Western Blot

  ○ Extracted protein samples were separated on an 8% SDS-PAGE gel and

  transferred to a nitrocellulose membrane using a Trans-Blot Turbo Transfer

  System (Bio-Rad) according to the manufacturer's instructions. The membrane

  was blocked for an hour in 5% non-fat milk prepared in TBS-Tween (TBST)

  solution.. After the incubation, the blot was washed for three 10 min washes

  with TBST and incubated in a 1:10,000 dilution of primary antibody (α-GFP

  rabbit, Invitrogen, A6455) overnight at 4°C. After three washes with TBST for

  10 min each, the blot was incubated with a 1:5000 dilution of secondary

  antibody (α-rabbit-HRP conjugate, Sigma, A6145) for 1 hour. After three more

  10 min TBST washes and a single 10 min TBS wash, the blot was visualized

  using the SuperSignalTM West Pico Chemiluminescent Substrate (Thermo

Scientific) and a Bio-Rad Molecular Imager ChemidocTM XRS+ imaging system. The blot was stained with Ponceau stain in order to visualize protein loading.

- Botrytis cinerea Assay

  - *B. cinerea* B05.10 was grown on 2x V8 plates (36% V8 juice, 0.2% $CaCO_3$, 2% Bacto-agar) for 7-10 days at room temperature. The grown spores were harvested by scrapping the plates and resuspending the spores in an autoclaved BD Difco Potato Dextrose Broth (with 0.1% Tween) to a concentration of $1-5 \times 10^5$ spores/mL (Mengiste et al., 2003; Veronese et al., 2006). The suspension was incubated for 2 hours at 28°C with gentle shaking before inoculation. For inoculation, leaves from four-week old Arabidopsis seedlings were punctured using a small needle, and 5 μL aliquot of spore suspension was deposited on the adaxial surface of each wound site. Inoculated plants were kept under a transparent cover for 3-5 days, after which the inoculated leaves were detached, and the lesion area was measured using ImageJ software.

- Statistical analyses

  - Statistical analyses were conducted using Excel 2016 (Microsoft Inc.).

## Results

*Generation of Arabidopsis Lines Overexpressing Phosphovariants of ASPGA*

In order to produce stable Arabidopsis transgenic lines overexpressing the phosphoabolishing and phosphomimetic variants of ASPGA1.1 and ASPGA1.2, the double knockout mutant *aspga1/aspga2* plants were transformed via floral dipping with *Agrobacterium tumefaciens*. These constructs carried the glufosinate (BASTA) gene as a plant selection marker

in its backbone for its use in selection of transgenic lines. Seeds produced from the transformed

plants were considered generation $T0$. Plants that were likely to carry the transgene were selected

by growing them on half strength Murashige and Skoog media that included the antibiotic,

BASTA (16µg/l). Plants for each transgenic line that carried the transgene and selectable marker

were resistant to BASTA; therefore, successfully grew on the antibiotic media. The BASTA

resistant plants were then selected and subsequently genotyped by PCR and protein separation

via an SDS-PAGE gel, and western blotting. Transgene positive plants were further propagated

until generation $T_3$ or until each transgenic line had become homozygous. **Figure 4**,

demonstrates the entire work flow of the study.

*Genotyping of ASPGA1 Transgenic Lines*

To confirm the presence of the transgene in the *aspga1/aspga2* lines transformed with

*35S::ASPGA1.1-YFP*, *35S::ASPGA1.2-YFP*, and phosphovariants, we genotyped leaves from

BASTA-resistant plants using gene specific forward primer and YFP specific reverse primer.

The PCR products were separated on a 1% agarose gel as shown in **Figure 5**. Col-0 was used as

a negative control to identify any non-specific amplification products since there is no YFP-

tagged ASPGA1 to which the primers could anneal in this background. As expected, the Col-0

samples did not have a band of the anticipated size (656 bp) which would be produced through

amplification of the *ASPGA1-YFP* transgene. In Figure 5, the independent lines for

$ASPGA1.1^{S169D}$-YFP analyzed did not have an apparent band at our expected size. However, we

have screened additional $ASPGA1.1^{S169D}$-YFP samples and identified PCR positive transgenic

lines that could be used for further studies. The samples for $ASPGA1.2^{S89WT}$-YFP,

$ASPGA1.2^{S89A}$-YFP, $ASPGA1.2^{S89D}$-YFP, $ASPGA1.1^{S169WT}$-YFP and $ASPGA1.1^{S169A}$-YFP

also appeared to have a band at 656bp indicating the presence of the asparaginase transgene

fused to YFP.

*Immunoprecipitation And Western Blot Analysis Of ASPGA1 Transgenic Lines*

In order to identify the ASPGA1 transgenic lines with better protein expression, we analyzed T2 and T3 seedlings of all the transgenic lines through western blotting. The basta resistant seedlings were used for the protein extraction. The extracted proteins were then loaded onto SDS-PAGE gels to visualize the YFP-tagged proteins from these selected transgenic lines. Protein extraction of wild type Col-0 was used as control. As shown in **Figure 6,** the protein bands specific to the transgene were not detected as we could observe similar protein banding pattern in all the tested samples including the controls. This led to the interpretation of the blot to be inconclusive. However, as ASPGA1 was fused with a YFP-tag, we used GFP-trap agarose magnetic beads to immunoprecipitate the proteins with YFP tag. As expected, this method has greatly reduced the background protein banding and as shown in **Figure 7**, we could identify the protein bands corresponding to the precursor ASPGA1.1 protein (60kDa), ASPGA1.2 (51.5 kDa) and its beta subunit ( 40 kDa). The immunoprecipitated sample for $ASPGA1.2^{S89A}$-YFP appears to have a very faint band that could potentially be the beta subunit around 40kDa. The immunoprecipitated sample for $ASPGA1.2^{S89D}$-YFP appears to have a very faint band that could potentially be its corresponding precursor peptide around 51.5kDa. Both $ASPGA1.2^{S89A}$-YFP and $ASPGA1.2^{S89D}$-YFP appear to have a faint free YFP band around 26.4 kDa. The immunoprecipitated samples for $ASPGA1.1^{S169WT}$-YFP, $ASPGA1.1^{S169D}$-YFP, and $ASPGA1.2^{S89WT}$-YFP have an apparent beta subunit band around 40kDa. From the western blot, it was also observed that the immunoprecipitated sample of $ASPGA1.1^{S169A}$-YFP and $ASPGA1.2^{S89A}$-YFP had less cleavage of precursor protein

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compared to their wild type and phosphomimetic counterparts. This suggests that the lack of phosphorylation of serine residue in the phosphoabolishing variants could have reduced the autoproteolytic cleavage as anticipated in our earlier assays.

*Phosphoabolishing ASPGA1.1 and ASPGA1.2 Transgenic Lines Show increased Susceptibility to Botrytis cinerea*

To determine whether increased enzymatic activity and cleavage resulting from asparaginase phosphorylation affects plant immune responses, we tested the disease resistance of transgenic lines overexpressing *35S::ASPGA1.1-YFP*, *35S::ASPGA1.2-YFP*, and all phosphovariant lines, by inoculating them with the necrotrophic fungus *Botrytis cinerea* (**Fig. 8**). Col-0 was used as a wild-type reference plant, and as additional controls we examined the immunocompromised *pepr1/pepr2* double mutant, and the *aspga1/aspga2* double mutant. Both *pepr1/pepr2* and *aspga1/aspga2* displayed larger lesions compared to the lesion area observed in Col-0, indicating a decreased resistance to *B. cinerea*. Similarly, the $ASPGA1.1^{S169A}$-YFP and $ASPGA1.2^{S89A}$-YFP transgenic lines had increased lesion size compared to Col-0, implying a similarly decreased resistance to *B. cinerea*. In contrast, lesion area in both phosphomimetic lines $ASPGA1.1^{S169D}$-YFP and $ASPGA1.2^{S89WT}$-YFP were not significantly different from Col-0. Together this suggests that the loss of phosphorylation-mediated ASPGA1 regulation in the phosphoabolishing lines was associated with an enhanced disease susceptibility phenotype similar to that observed in the *aspga1/aspga2* mutant that lacks all asparaginase activity.

In order to quantify the relative levels of pathogen load in all transgenic lines, we extracted DNA from *Botrytis cinerea*-infected leaf samples and measured the average level of *Botrytis cinerea* DNA, by using fungal specific CUTINASE (CutA) primers. For each genotype

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infected leaf samples were collected and pooled 5 days after inoculation (n = 3, 10 leaves per biological replicate). Following extraction and purification, relative presence of CutA-encoding DNA was measured using qPCR (**Fig. 9**). As expected, we detected increased levels of CutA in the immunocompromised *pepr1/pepr2* plants. In contrast, we detected slightly less CutA encoding DNA in *aspga1/aspga2* compared to Col-0. For both $ASPGA1.2^{S89D}$-*YFP* and $ASPGA1.2^{S89A}$-*YFP*, CutA levels were higher than that of Col-0. Detectable CutA-encoding DNA for both $ASPGA1.1^{S169D}$-YFP and $ASPGA1.1^{S169A}$-YFP; were similar to that of Col-0, as were CutA levels in $ASPGA1.1^{S169WT}$-YFP and $ASPGA1.2^{S89WT}$-YFP.

# Figures
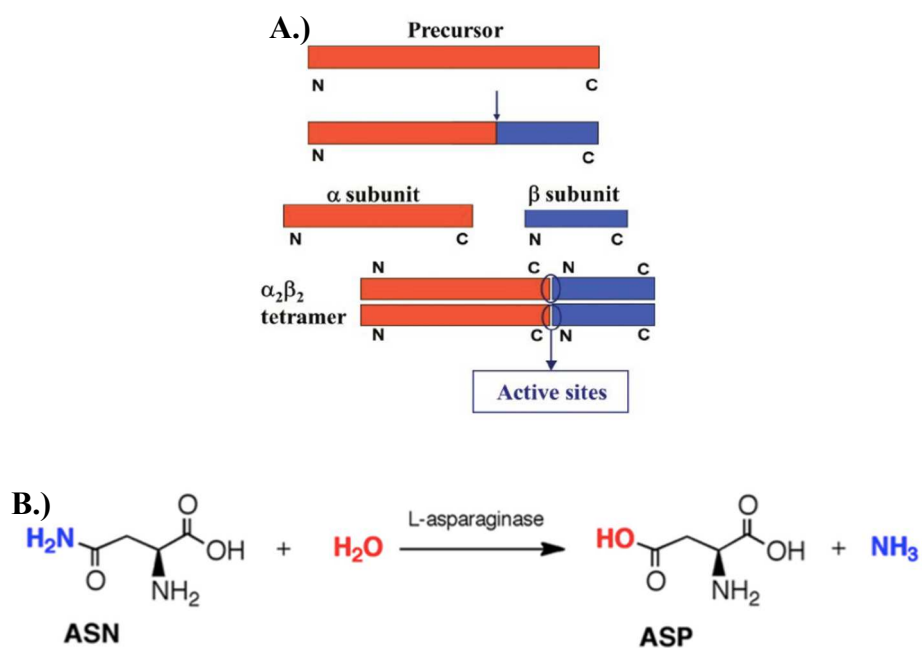




**Figure 1.) ASPGA1 autoproteolytic cleavage and function.** In order to form the catalytically active form of the enzyme, the precursor protein cleaves itself into two subunits *viz*., alpha and beta. These subunits then diamerize to form the active site of the enzyme. Active form of asparaginase then binds to asparagine (ASN) and converts it to aspartate (ASP) with the release of free ammonia ($NH_3$). As adapted by Betti et al., 2014 and Nguyen et al., 2016.
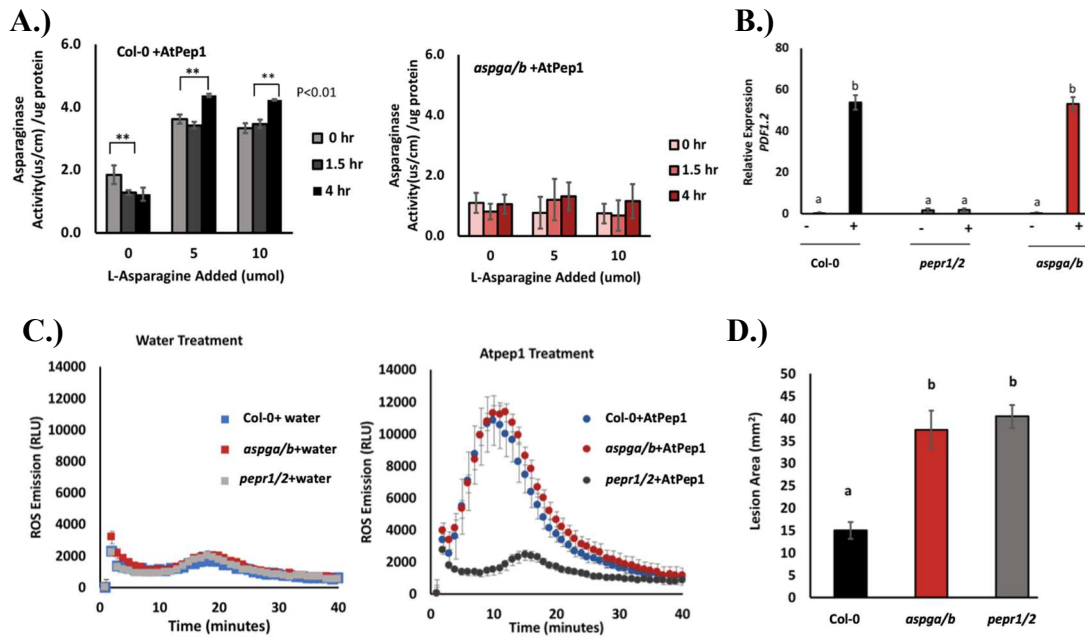
**Figure 2.) Exogenous AtPep1 treatment of Asparaginase double knockout aspga/aspgb shows no effect on early defense response pathways.** 14-day old seedlings of T-DNA insertion *Arabidopsis* double mutant line *aspga/aspgb* and wildtype Col-0 were treated with 1μM AtPep1. **A.)** Conductivity meter was used to measure asparaginase activity in plant. **B.)** Relative *PDF1.2* expressions were measured in 14-day old seedlings incubated with 1μM AtPep1 using RT-qPCR (N=3 biological replicates). **C.)** ROS emission was measured using leaf disks from 21-day old plants *aspga/aspgb* and Col-0, 24 h after water or AtPep1 treatment. **D.)** 21-day old plants of Col-0, *pepr1/pepr2, aspga/aspgb* were infected with *Botrytis cinerea* and lesion area was measured after 5 days of infection. (N=5 plants per genotype, with 3 technical replicates per plant). Error bars indicate standard error of the mean. Asterisks "**" and different letters indicate statistically significant differences at P<0.01. (one-way ANOVA followed by Tukey's test corrections for multiple comparisons).
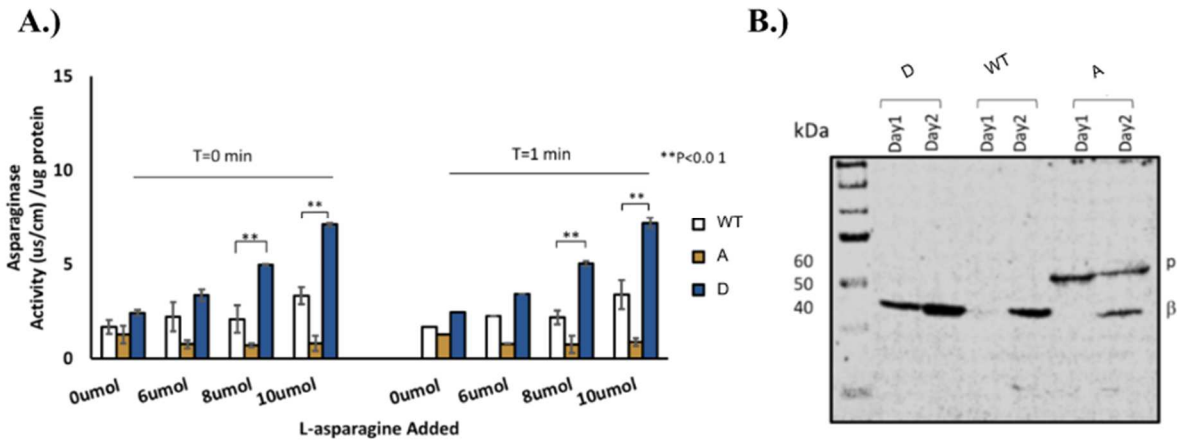
**A.)**



**B.)**



**Figure 3.) Transient expression of phosphovariants in *Nicotiana benthamiana*.** C-terminal YPF-tagged ASPGA1 was transiently expressed in *Nicotiana benthamiana.* Conductivity meter was used to measure **A.)** ASPGA1 activity 48 h post inoculation at both 0min and 1min of being added to a 20 mM Tris-HCl buffer, pH 8.0 solution containing 0-10 μmol L-asparagine. Western blotting was used to check levels of **B.)** ASPGA1 24 h and 48 h after inoculation. *P:* polypeptide precursor; b indicates beta subunit; D: phosphomimetic variant, WT: wild type asparaginase gene; A: phosphoabolishing variant. N=5 plants per genotype, with 3 technical replicates per plant. Error bars indicate standard error of the mean. Asterisks "**" indicate a statistically significant difference at $P<0.01$ (one-way ANOVA followed by Tukey's test corrections for multiple comparisons).
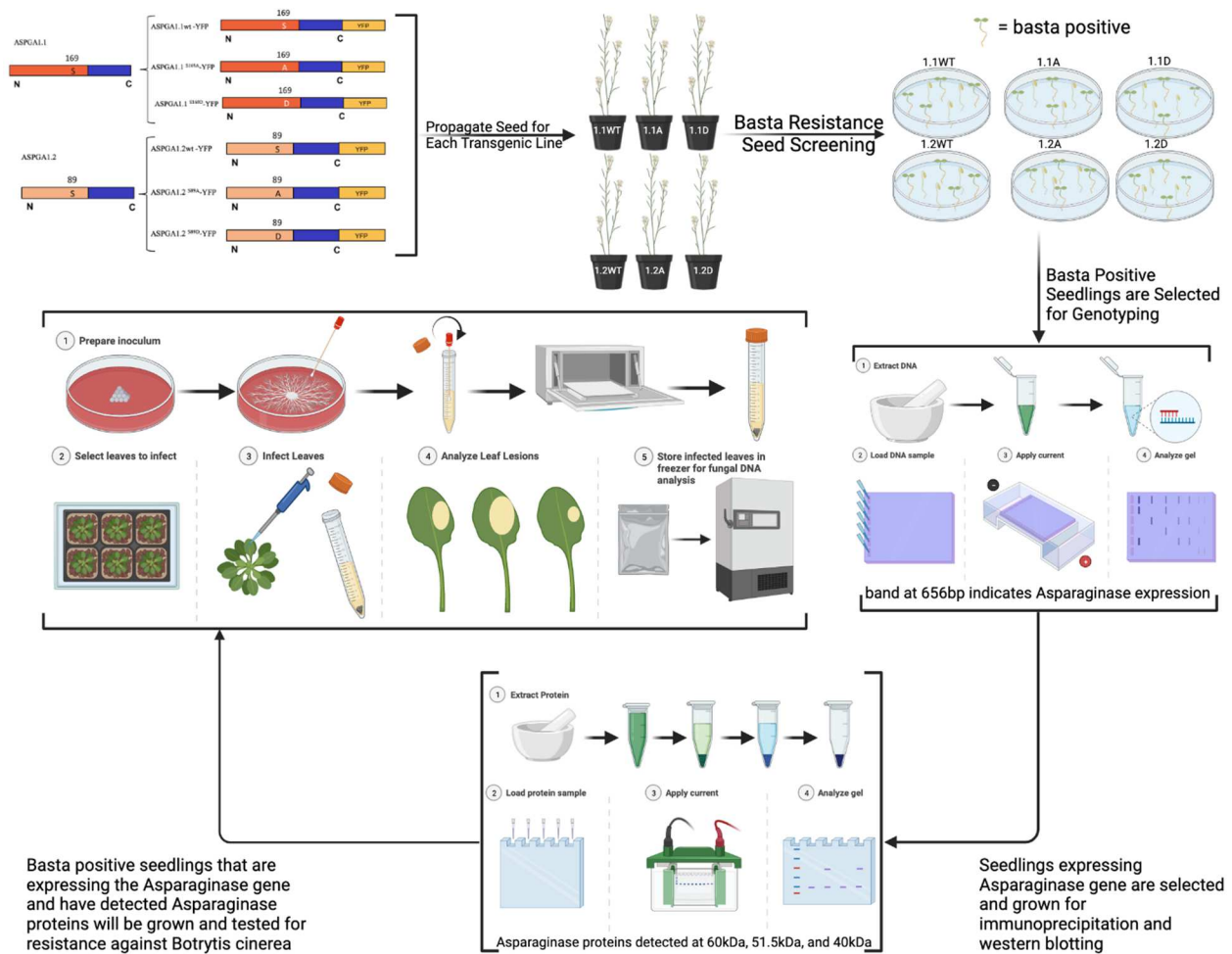
**Figure 4.) Workflow for Testing the Effect of Phosphorylation on Plant Disease.**
*Arabidopsis thaliana T$_3$* seeds were grown and harvested after about 3 months. Seeds were
screened in the presence of Basta and the resistant plants were further genotyped by PCR and
immunoprecipitation followed by western blotting to detect the transgene and its protein
expression respectively. The selected transgenic lines were then used for the bioassay to assess
their role in pathogen resistance.
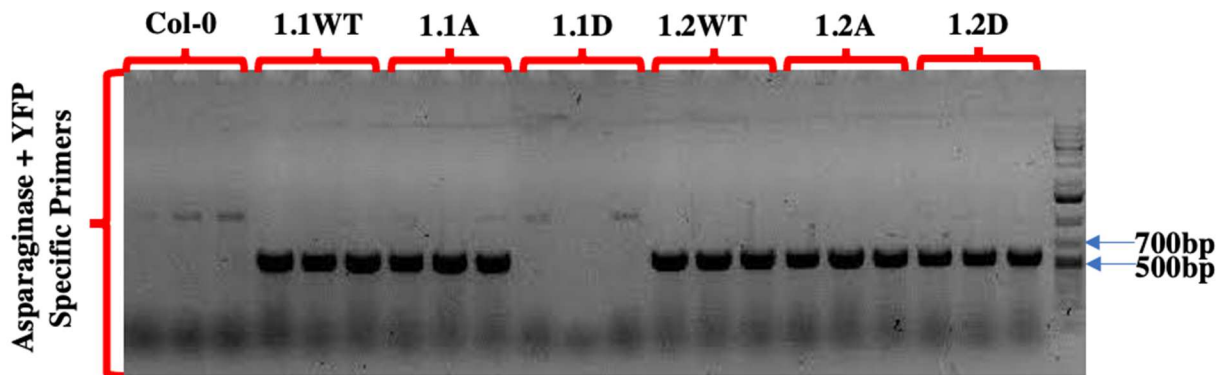
**Figure 5.) Genotyping of ASPGA1.1-YFP and ASPGA1.2-YFP Transgenic Lines.** DNA was extracted from leaves of transgenic plants expressing ASPGA1.1–YFP and ASPGA1.2–YFP in the *aspga1/aspga2* background. Detection of the Asparaginase gene as separated on a 1% agarose gel following PCR. An asparaginase forward primer and YFP reverse primer were used to detect the YFP-tagged asparaginase gene. Three independent events per line were analyzed. PCR product of 656bp denotes the presence of transgene. WT: wild type asparaginase gene; A: phosphoabolishing variant; D: phosphomimetic variant.
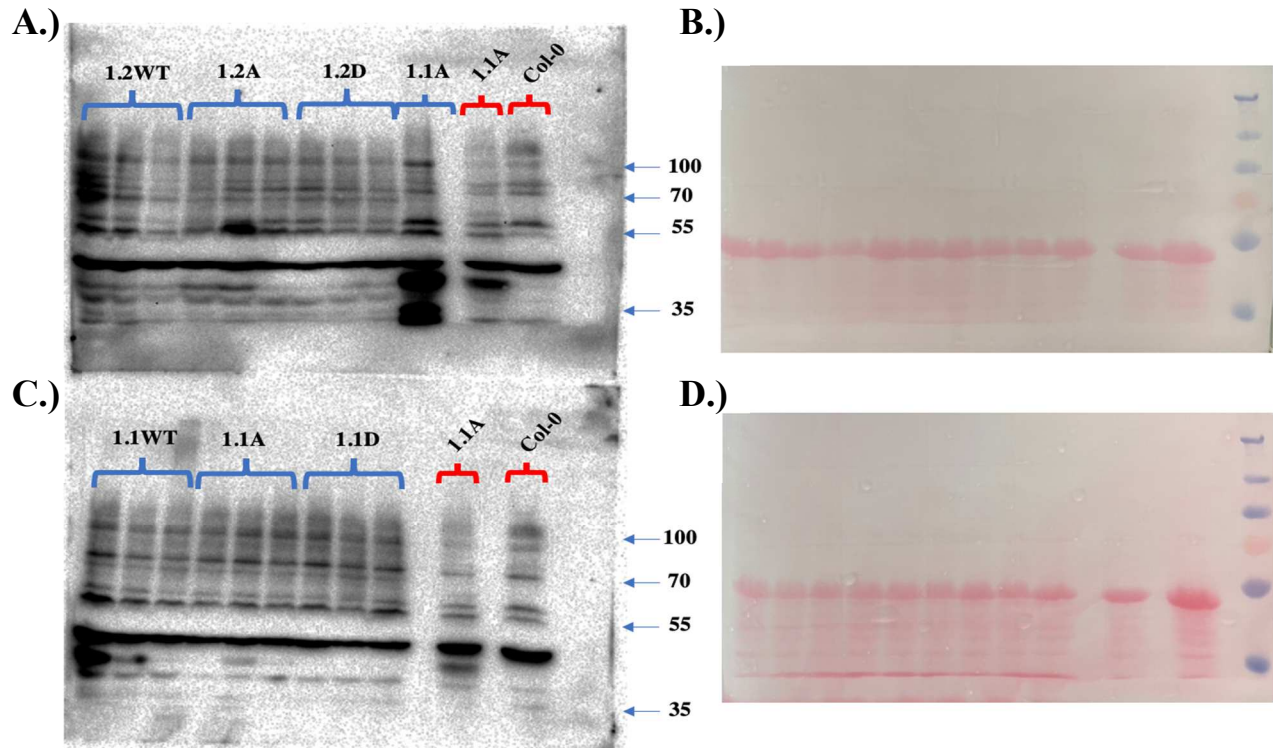
**Figure 6.) Western blot of protein extracted from ASPGA transgenic lines.** Proteins were extracted from leaves of transgenic plants expressing **A.)** ASPGA1.1–YFP; ASPGA1.1A-YFP and ASPGA1.1D-YFP and **C.)** ASPGA1.2–YFP; ASPGA1.2A-YFP and ASPGA1.2D-YFP . Protein extracted from Col-0 plants seedlings were used as controls. Anti-GFP antibody was used to detect all three proteins. One independent event per line was analyzed. ASPGA1.1–YFP precursor peptide, 60 kDa; ASPGA1.2–YFP precursor peptide, 51.5 kDa; Beta subunit, 40 kDa; Free YFP, 26.4kDa as denoted by the PageRuler Plus Prestained Protein Ladder. **B , D.)** Ponceau staining was used for the verification of protein loading.
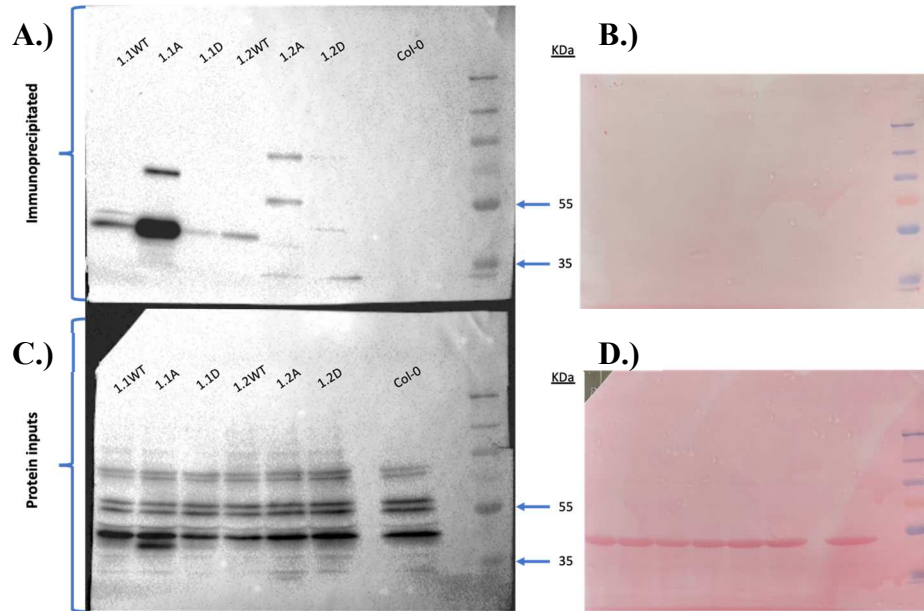
**Figure 7.) Immunoprecipitation and western blot analysis of ASPGA1.1-YFP Transgenic Lines. Seedlings of the transgenic lines selected on Basta were used for the protein extraction. The extracted protein was applied to GFP-trap agarose magnetic beads to concentrated YFP-tagged proteins. The concentrated proteins were then used for the western blot analysis using a 8% SDS-PAGE gel A.) Western blot image of ASPGA1.1 and ASPGA1.2 lines after immunoprecipitation C.) Western blot of inputs protein samples before immunoprecipitation. B, D.)** Ponceau staining was used for the verification of protein loading. Anti-GFP antibody was used to detect all three proteins. One independent event per line was analyzed. ASPGA1.1–YFP precursor peptide, 60 kDa; ASPGA1.2–YFP precursor peptide, 51.5 kDa; Beta subunit, 40 kDa; Free YFP, 26.4kDa as denoted by the PageRuler Plus Prestained Protein Ladder.
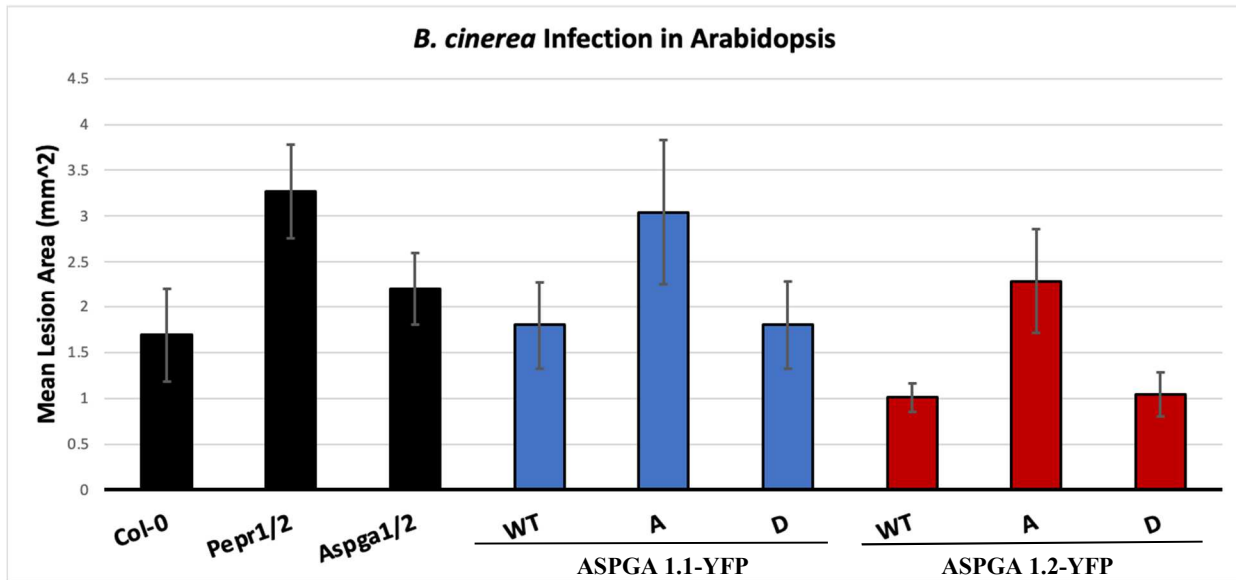
**Figure 8.) Loss of phosphorylation at S169 and S89 in ASPGA1.1-YFP and ASPGA1.2-YFP is associated with increased susceptibility to *Botrytis cinerea*.** 4-5 week old plants were used for the assay. Col-0, *aspga1/aspga2*, and *pepr1/pepr2*, ASPGA1.1-YFP and ASPGA1.2-YFP transgenic *Arabidopsis* lines were inoculated with *B. cinerea* and lesion area was measured with ImageJ after 5 days of infection (n=30, 10 plants used per genotype, with 3 leaves inoculated per plant). Error bars represent standard error of the mean. WT: wild type version of the gene; A: phosphoabolishing variant; D: phosphomimetic variant.
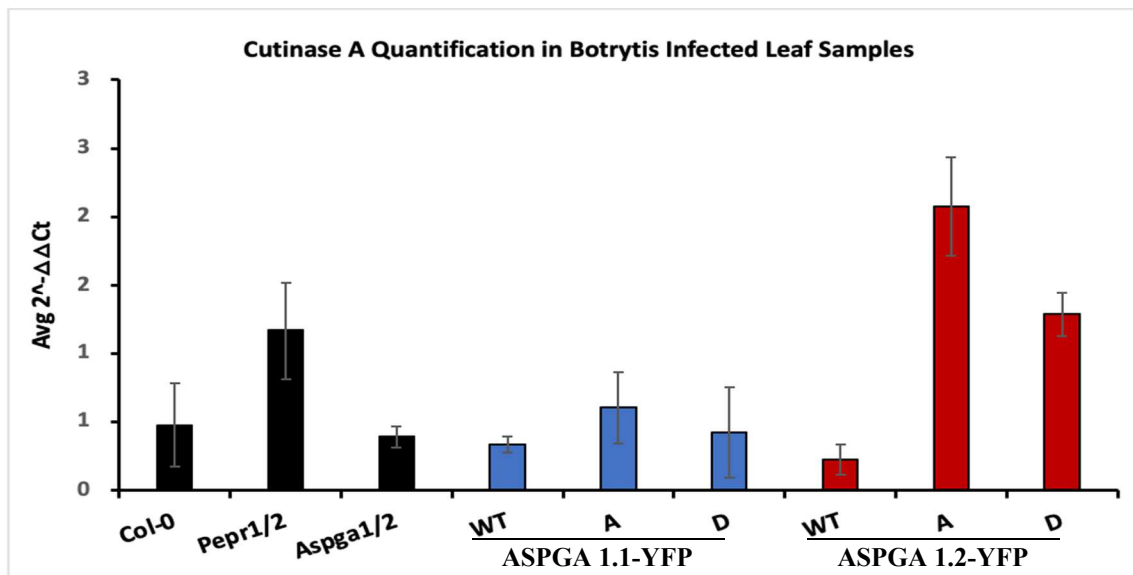


**Figure 9.) Fungal DNA quantification of *B. cinerea* infected samples by qPCR.** Infected leaf samples were pooled for each genotype (n=3, 10 leaves per biological replicate). Fungal DNA was extracted from the infected samples and fungal specific Cutinase (CutA) gene specific primers were used to quantify the fungal DNA by qPCR. Error bars represent standard error of the mean. WT: wild type version of the gene; A: phosphoabolishing variant; D: phosphomimetic variant.

**Table 1.)** List of primers used in this study.

| Name | Primer Sequence | Purpose |
|------|-----------------|---------|
| ACTIN gene primers | Fw: 5'-CTTGCACCAAGCAGCATGAA-3' | Gene expression analysis |
| | Rv: 5'-CCGATCCAGACACTGTACTTCCTT-3' | |
| CUTINASE gene primers | Fw: 5'-GATGTGACGGTCATCTTTGCCC-3' | |
| | Rv: 5'-AGATTTGAGAGCGGCGAGG-3' | |
| YFP reverse primer | 5'-CGTCGCCGTCCAGCTCGACCA-3' | Genotyping of transgenic lines |
| ASPGA1 gene forward primer | 5'-CCGAAAGTACCGGACAATTGCGG-3' | |

## Discussion

Because plant immune signaling involves protein phosphorylation, we identified candidate proteins involved in immunity through a screen of phosphoproteome changes occurring within minutes after treatment with the immunoregulatory hormone AtPep1 (Huffaker *et al*., 2006). In this screen, the potassium-independent asparaginase ASPGA1 was among the candidate proteins identified as differentially phosphorylated during the immune response. Our subsequent studies have aimed to deduce whether ASPGA1 plays a role in plant immunity and to determine if the observed phosphorylation on the ASPGA1 variable loop serine affects enzyme function and/or immune output in *A. thaliana* (Ahmadian, N., 2020).

To assess whether asparaginase enzyme activity was required for AtPep1-induced immune responses, we examined the *aspga1/aspga2* double knockout which has no asparaginase activity. Markers of plant immunity such as production of ROS second messenger molecules and expression of the gene encoding the antimicrobial protein PDF1.2 were quantified after

AtPep1 treatment (Huffaker *et al*., 2005; Kadota *et al*., 2014), revealing that levels of both markers increased in *aspga1/aspga2* similarly to WT Col-0 (**Figure 2B and 2C**). This indicated that ASPGA1 is not required for initial activation of immune responses. In contrast, when longer-term immune responses were probed through assessment of *Botrytis cinerea* infection several days after inoculation, *aspga1/aspga2* had increased susceptibility compared to Col-0 (**Fig. 2D**). Thus, while asparaginase activity is not necessary to initiate immunity, it does appear to be required for disease resistance in the longer term. Because (1) ASPGA1 was phosphorylated after AtPep1 treatment and (2) loss of asparaginase was associated with a loss of disease resistance, we wondered whether asparaginase enzyme activity is affected during AtPep1-induced immune activation. Wild type Col-0 treated with AtPep1 showed significantly increased asparaginase activity after 4 hours (**Fig. 2A**). Given that AtPep1 induces asparaginase activity and that loss of this activity in *aspga1/aspga2* compromises disease resistance, asparaginase appears to be a necessary component of plant immune responses.

As AtPep1 stimulated asparaginase activity, we hypothesized that the AtPep1-induced phosphorylation of ASPGA1 might affect enzyme activity and possibly also immunity and disease resistance. When expressing the ASPGA1 phosphovariants in a heterologous system, the phosphoabolished form was delayed in the autocleavage event that generates a catalytically active form of the enzyme (**Fig. 3B**). Further, plants expressing phosphoabolished ASPGA1 had reduced asparaginase activity as compared to those expressing wild type ASPGA1 (**Fig. 3A**). These findings support a role for immune-induced phosphorylation of the ASPGA1 regulatory loop in promoting autocleavage and thereby activating asparaginase activity. Interestingly, initial characterization of the ASPGA1 phosphovariants in stably transformed Arabidopsis plants revealed that the phosphoabolished form of ASPGA1 has enhanced disease susceptibility when

challenged with *Botrytis cinerea* (**Fig. 8**). Given that the phosphoabolishing variant of ASPGA1 was slower to autocleave, had reduced enzyme activity and was more susceptible to fungal infection similar to *aspga1/aspga2,* it seems likely that phosphorylation of ASPGA1 during the immune response stimulates enzyme activity that is necessary to mount a protective response.

Once activated, ASPGA1 breaks down asparagine to aspartic acid and ammonia; The free nitrogen that comes from this ammonia could then fuel resource-intensive immune responses (Gaufichon et al., 2016). However, asparagine breakdown may also play a directly protective role aside from facilitating increased metabolic activity. Asparagine has been shown to be a particularly important source of nitrogen for *Botrytis cinerea*. *In vitro* assays using media supplemented with various amino acids due showed that asparagine supported more rapid mycelial growth than other amino acids, and no significant mycelial growth was observed in the absence of a nitrogen source (Seifi et al., 2014). The same study examined activity of the enzyme which produces asparagine, asparagine synthetase, after infection by *B. cinerea* in wild type tomato plants which are susceptible to *B. cinerea* as compared to abscisic acid-deficient *sitiens* tomato mutant that is resistant. This revealed that in wild type plants there was a strong upregulation of asparagine synthetase 16 hours post infection with increased asparaginase levels followed by a subsequent depletion 48 hours post infection. In contrast, asparagine synthetase activity was not increased in the *sitiens* mutant 16 hours post inoculation. The authors conclude that *B. cinerea* may trigger host asparagine synthesis as a virulence strategy since this amino acid most effectively fuels its growth. It is possible that host activation of asparaginase is a defense response that protects against disease by degrading asparagine that could promote fungal growth.

It is also possible that asparaginase enzyme activity affects immune responses and resistance through generation of the enzyme product aspartate. A previous study by Brauc et al.

(2011) focused on the activity of cytosolic aspartate aminotransferase (AAT2), which transfers an amino group to aspartate to generate glutamate, in the context of *B. cinerea* infection. After observing that *AAT2* gene expression was altered during *B. cinerea* infection, fungal resistance was examined in lines constitutively expressing the *AAT2* gene. While the effects of *AAT2* overexpression on amino acid content were somewhat variable, it was found that higher levels of *AAT2* expression correlated with enhanced susceptibility to *B. cinerea* (Brauc et al., 2011). Interpretation of the mechanism by which this susceptibility might occur was complicated by the fact *ATT2* overexpression affected the relative levels of numerous amino acids. Together these studies support a role for asparagine and aspartate metabolism as a factor contributing to *B. cinerea* resistance, but additional examination will be required to better understand how this occurs, and further studies of ASPGA1 may help shed light on potential mechanisms.

Beyond the *B. cinerea* pathosystem, asparaginase activity is likely to contribute more broadly to plant immunity and resistance. Nutritional content of plant tissues is also critical for plant-herbivore interactions, but the role of asparaginase in this context has not been well studied. Recently, the effects of asparaginase activity on herbivore resistance was examined in cotton, *Gossypium hirsutum* (Gul et al., 2020). Cotton lines constitutively expressing asparaginase were found to have enhanced resistance to the insect pest *Bamesia tabaci,* silverleaf whitefly (Gul et al., 2020). *B. tabaci* is a devastating agricultural pest with a very broad host range of more than 500 species. It is a notorious vector for many viral diseases, including a broad range of geminiviruses that affect diverse plant species. Given this breadth, the study of how asparaginase facilitates plant immune response is relevant not just to fungal pathogens, but to insect pests and viral pathogens. Furthermore, associations between asparaginase activity and resistance have been demonstrated across diverse species, including tomato, Arabidopsis and

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cotton, indicating that understanding the role of asparaginase could provide insights broadly applicable across plant species.

## Future Directions

Overall, in accordance with these earlier studies, we have observed that asparaginase is a positive contributor to plant defenses. Namely, we have found that the immunoregulatory hormone AtPep1 stimulates ASPGA1 activity and triggers phosphorylation of ASPGA1 that is associated with increased autoproteolytic cleavage. Our data also suggests that abolishing this key phosphoregulatory site decreases asparaginase activity and cleavage and increases susceptibility to *B. cinerea*. However, the mechanisms by which ASPGA1 facilitates the immune response remain to be determined. Effects of phosphorylation-mediated activation of ASPGA1 on levels of asparagine, aspartic acid and other amino acids during the immune response are as yet unknown. Nor has it been determined whether the phosphovariant forms of ASPGA1 alter basal amino acid levels. Moreover, the breadth of ASPGA1 phosphorylation as a regulatory mechanism is not yet understood. While ASPGA1 phosphorylation has been demonstrated after AtPep1 treatment, it has not been examined during the course of infection or in response to other hormones or elicitor molecules that trigger immune responses. Additionally, how ASPGA1 phosphorylation affects resistance to other pathogens, and to insect pests will be important to determine. More broadly, it would be interesting to understand if other stresses that require extensive metabolic activity also cause changes in phosphorylation or activity of ASPGA1. Ultimately, this research has provided genetic tools to answer these questions and many others relating to plant resistance to stress and the intersection of primary metabolism with immunity.

## Conclusions

In my thesis I continued the investigation of ASPGA1, a phosphorylated protein candidate that was identified through phosphoproteomic screening by measuring the phosphopeptides in Arabidopsis after Atpep1 treatment to not only detect changes in protein phosphorylated state, but also map the phosphorylation site. The data collected in this work suggests that asparaginase phosphorylation is necessary for a fully protective immune response. Furthermore, the results indicate that the loss of asparaginase phosphorylation decreases its enzymatic activity, thus, decreasing its autoproteolytic cleavage and ultimately leading to reduced resistance against *Botrytis cinerea*. The identification of ASPGA1 phosphorylation provides insight not only into how other nitrogen releasing enzymes can potentially respond to fungal infection and alteration of their phosphorylation site amino acid residue, but also how other nitrogen releasing enzymes can potentially become activated in the AtPep1-signaling pathway as part of mounting an immune response. Further investigation of ASPGA1 phosphorylation can lead us into new territory by providing a greater understanding of how the AtPep1-signaling pathway can affect other nitrogen releasing enzymes in order to enhance plant immunity against pests and disease.

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