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The Role of Potassium-independent Asparaginase (ASPGA1) Serine Phosphorylation in Enzymatic Activity and *Arabidopsis thaliana* Defense Responses

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by

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The Thesis of Nazanin Ahmadian is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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ABSTRACT OF THE THESIS

The role of Potassium-independent Asparaginase (ASPGA1) Serine Phosphorylation in Enzymatic Activity and *Arabidopsis thaliana* Defense Responses

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Master of Science in Biology

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Professor Alisa Huffaker, Chair
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Plants defend against pathogens through metabolic reprogramming and *de novo* synthesis of various proteins and defense metabolites, which requires mobilization of nitrogen through nitrogen-releasing enzymes. Plant-type L-asparaginase (ASPG) is an essential nitrogen-releasing enzyme, which catalyzes the conversion of asparagine to aspartic acid, releasing free ammonia. However, little is known about the activation mechanism of this enzyme in relation to plant immune pathways. Here, we defined the variable-loop serine site phosphorylation of the enzyme asparaginase after AtPep1-induced immune responses. Furthermore, we set out to examine the

role of this serine phosphorylation in enzyme activation, and the subsequent effect on *Arabidopsis thaliana* immune responses against the necrotrophic fungus *Botrytis cinerea.* Asparaginase activity assays of phosphomimetic (*aspga/aspgb*: ASPGA1.1 ^{S169D}-YFP, *aspga/aspgb*: ASPGA1.2 ^{S89D}-YFP) and phosphoabolishing (*aspga/aspgb*: ASPGA1.1 ^{S169A}-YFP , *aspga/aspgb*: ASPGA1.2 ^{S89A}-YFP) transgenic *Arabidopsis* lines demonstrated that variable-loops serine phosphorylation increases autoproteolytic cleavage and enzyme activity in both isoforms of ASPGA1. It also was observed that double mutant *Arabidopsis* plants (*aspga/aspgb)* lacking both potassium-dependent (ASPGB1) and potassium-independent (ASPGA1) enzymes are susceptible to fungal attack. Additionally, our analysis further revealed that the phosphoabolishing transgenic *Arabidopsis* line *aspga/aspgb*: ASPGA1.1 ^{S169A}-YFP is similarly susceptible to *B. cinerea.* Taken together, this data shows the variable-loop serine phosphorylation plays a role in asparaginase enzymatic activity and implicates ASPGA1 as an essential influencer of defense output in *A. thaliana.*

1. Introduction

In their natural environment plants are constantly under attack from herbivores and pathogens, which are major contributors to crop loss and a threat to food security (Strange and Scott, 2005). Similar to the mammalian immune system, plants mount immune responses through activation of dynamic signaling pathways and production of defense metabolites (Iriti and Faoro, 2007). Plants also ward off their attackers directly through physical barriers, such as wax and thorns, and accumulation of toxins, including protease inhibitors that can disrupt metabolism (Arnaiz *et al*., 2018). In addition, plants can defend indirectly by recruiting natural enemies of their attackers to their location (War *et al*., 2012; Huffaker *et al*., 2013). For example, emission of plant volatile terpenoids attracts parasitic organisms, previously observed with wasps that parasitize caterpillars by laying their eggs on them. These herbivore-induced volatiles also warn neighboring plants of the presence of enemies (Wei *et al*., 2007).

Plants recognize attacking herbivores and pathogens using plasma membrane-bound pattern recognition receptors (PRRs) that bind to herbivore-associated molecular pattern molecules (HAMPs) (Steinbrenner *et al*., 2020; Iriti and Faoro, 2007) and pathogen-associated molecular patterns (PAMPs), such as lipopolysaccharide, peptidoglycan, and bacterial flagellin (Grennan, 2006). Following activation, numerous endogenous defense signals are released, including AtPep1, an amplifier of innate immunity cleaved through metacaspase activity from the larger precursor protein AtPROPEP1 (Huffaker *et al*., 2006; Hander *et al*., 2019). AtPep1 activates neighboring cell-surface receptors PEPR1/PEPR2 (Yamaguchi *et al*., 2006; Yamaguchi *et al*., 2010) through the recruitment of the SERK (Somatic Embryogenic Receptor Kinases) coreceptors, causing a spatio-temporal

amplification of the immune response (Postel *et al*., 2010; Huffaker 2015, Gou and Li, 2020). Upon activation, the PEP receptors trigger widespread downstream signaling events, including the kinase cascades that ultimately leads to transcriptional and metabolic reprograming (Huffaker *et al*., 2006, Yamaguchi *et al*., 2010, Huffaker *et al*., 2013, Ross *et al*., 2014).

This metabolic reprogramming is essential for plant immune responses and requires *de novo* synthesis of proteins and defense metabolites (Sun *et al*., 2020). These are metabolically expensive processes that require mobilization of nitrogen for incorporation into newly synthesized molecules, such as pyrrolizidine alkaloid and cyanogenic glucosides (Macel, 2011; Zagrobelny, 2004). Therefore, nitrogen availability may be a limiting factor for plant immunity and resistance to pathogens. The amino acid asparagine is a primary molecule used by plants for nitrogen storage and transport, due to having the highest nitrogen to carbon ratio among all amino acids and its unreactive nature (Lea *et al*., 2007). In defending cells requiring nitrogen, conversion of asparagine to aspartate releases nitrogen in the form of ammonia (NH_3) that can be assimilated into other metabolic pathways (Curtis *et al*., 2018).

Asparaginase (ASPGA) is an essential nitrogen-releasing enzyme, which catalyzes the conversion of asparagine to aspartic acid (Michalska *et al*., 2006), releasing free ammonia. In plants asparaginase are especially necessary for nitrogen supply in sink tissues such as developing leaves and roots and in germinating seeds. *Arabidopsis thaliana* genome includes two asparaginase genes, *ASPGA1* with two isoforms ASPGA1.1 and ASPGA1.2, and *ASPGB1*(Van Norman and Benfey, 2009). ASPGB1 is activated by potassium and its catalytic efficiency has been reported to be 50-fold higher than potassium-independent ASPGA1 (Bruneau *et al*., 2006; Gaufichon *et al*., 2016).

The enzymatic activity of L-asparaginases depends on the liberation of a nucleophilic threonine residue through an autoproteolytic cleavage process (Michalska *et al*., 2006). Plant-type L-asparaginases are heterotetramers composed of two β -subunits sandwiched by two α-subunits, with each α/β heterodimer arising from the autoproteolytic cleavage of the precursor protein (Fig 1). The autoproteolytic cleavage occurs in the conserved sequence GlyThrVal and liberates the nucleophilic threonine at the N-terminal end of the β -subunit (Michalska *et al*., 2006). The α-subunit is the most variable between ASPGA1 and ASPGB1, and the variable loop at the C-terminal end of the ASGPA1 α-subunit contains a serine residue (S169 in ASPGA1.1, S89 in ASPGA1.2) (Gabriel *et al*., 2012), which we have recently discovered to be phosphorylated within minutes of peptide-triggered immune responses in Arabidopsis suspension cells. While existing studies have established the necessity of the autoproteolytic cleavage process in liberating the nucleophilic threonine residue, they have not previously observed this site-specific phosphorylation, nor identified post-translational modification as a potential regulator of cleavage.

We hypothesized that (1) the serine site phosphorylation might affect the autoproteolytic cleavage process and activity in ASPGA1, (2) altered activation of ASPGA1 might affect nitrogen availability in the form of NH_3, (3) and changes in nitrogen content could impact immune output and disease resistance in plants. In order to investigate our hypotheses, ASPGA1 isoforms, ASPGA1.1 and ASPGA1.2, containing phosphomimetic (aspartate substitution) and phospho-abolishing (alanine substitution) sites were transiently expressed in *Nicotiana benthamiana* in order to investigate cleavage time and enzymatic activity. Similarly, asparaginase activity for both isoforms, ASPGA1.1 and ASPGA1.2, was measured in T-DNA insertional mutant lines of *A. thaliana* plants expressing the

aforementioned alanine and aspartate substitutions in the ASPGA1 serine residue. Faster cleavage time and increased enzymatic activity were observed for the phosphomimetic aspartate-substituted ASPGA1.1 and ASPGA1.2 after transient expression in both heterologous systems and stable complementation of T-DNA insertion lines. To examine immune output, T-DNA insertion lines were challenged with the necrotrophic fungal pathogen *Botrytis cinerea.* The phospho-abolishing alanine-substituted ASPGA1.1 and ASPGA1.2 lines showed compromised resistance compared to wild type plants.

2. Materials and Methods

2.1 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. Sterilization of Arabidopsis seeds were carried out in a sealed chamber containing chloric gas (50 mL 100% bleach, 1.5 mL HCl 36.5%) for 2-4 hours. Seeds were plated aseptically on half-strength Murashige and Skoog (MS) containing 0.8% phytoagar. After stratifying for 2-5 days at 4°C, seeds were germinated in a light- and humidity- controlled growth chamber (22°C, 12h light/12h dark). After 2 weeks seeds were transplanted to soil. *N. benthamiana* seeds were planted in soil and grown at 22°C under 16h light / 8h dark conditions. 5- to 6- week old plants were utilized for all *N. benthamiana* assays.

2.2 Construction of plasmid constructs and transgenic plant materials

The *ASPGA1.1* and *ASPGA1.2* coding sequences were from Arabidopsis cDNA using gene-specific primers and standard PCR. Alanine and aspartic acid substitutions were made to generate phosphoabolishing and phosphomimetic PCR fragments ASPGA1.1$^{S169A,\ S169D}$and ASPGA1.2$^{S89A,\ S89D}$, using BioLabs Q5 Site-Directed Mutagensis kit. The primers used are listed in Table 1. The PCR fragments were initially cloned into the pENTR D-TOPO vector and later recombined into expression vector pXCSG with a C-terminal fluorescent YFP tag. All constructs were sequenced in order to verify lack of unintended mutations prior to transformation into *Agrobacterium tumefaciens* strain GV3101-pMP90RK. The constructs were subsequently expressed in plants either through transient transformation of *N. benthamiana* or floral dip transformation of Arabidopsis.

2.3 *Agrobacterium tumefaciens* growth and preparation

Agrobacterium strain GV3101 containing the plasmid construct to be expressed was initially streaked on LB medium containing 1.5% agar with selective antibiotics and grown at 28°C for 2 days. A single colony from the streaked plate was used to inoculate a liquid culture of LB containing appropriate antibiotics. The OD_{600} of the culture was measured within the range of 0.6-1.25 (20-24 hours after inoculation). Cells were harvested via centrifugation (5000xg, 5 minutes) and subsequently used for agroinfiltration or floral dip.

2.4 Arabidopsis floral dip transformation

aspga/aspgb double knockout plants were transformed using the floral dip method (Clough and Bent 1998) with modifications. Briefly, *A. tumefaciens* strain

GV3101 containing the plasmid construct to be transformed was grown in 50 mL of Luria-Bertani medium (LB) containing selective antibiotics to a final OD_{600} of 0.5-0.8. The bacterial culture was resuspended in a 120 mL solution containing 5% sucrose and 0.03% Silwet L-77. Arabidopsis flowers and bolts were submerged in the bacterial solution and gently agitated for approximately 10 seconds. After dipping, flowers were covered and stored in low-light conditions for 16-24 hours before being returned to normal growth conditions.

2.5 Agroinfiltration and transient transformation of *Nicotiana benthamiana*

Harvested *Agrobacterium tumefaciens* cells were resuspended in infiltration buffer (10 mM $MgCl_2$, 10 mM MES, 200 μM acetosyringone) to a final OD_{600} of 0.45 and allowed to equilibrate in buffer for 1 hour at 22°C prior to infiltration. A blunt-end syringe was used to infiltrate the third and fourth leaves of 5- to 6- week old *Nicotiana benthamiana* plants. The leaves were sampled 24 hrs after infiltration for relevant bioassays 24 hours after infiltration unless otherwise noted.

2.6 Subcellular localization of L-asparaginase

N. benthamiana leaves transiently transformed with YFP-tagged constructs of genes under the control of the 35S promoter were sampled and visualized using a confocal microscope (Nikon Eclipse TE2000-U) at 20x magnification. The wavelengths for YFP excitation and emission were 514 nm and 527 nm, respectively.

2.7 Luminol-based Reactive Oxygen Species (ROS) detection and measurement

Detection of ROS responses in Arabidopsis were performed as previously described (Smith and Heese 2014), with modifications. Two leaf disks from independent Arabidopsis plants were placed in each well in a 96-well

microtiter plate filled with 100 µl ddH2O. Leaf disks were selected from 4-to 5- week old plants and floated adaxial side up in wells. The plate was then incubated overnight at 22℃ under continuous light in order to reduce wounding response. Prior to elicitation, 100 µl of 2x elicitation solution (20 µg/mL HRP, 34 µg/mL Luminol, as well as 100 µM AtPep1) was added to each well using a multichannel pipette. The plate was placed without delay into a BioTek Synergy H1 microplate reader (BioTek) to measure ROS production over the course of 40 minutes.

2.8 Protein extraction

3x Lamellae buffer (0.24 M Tris-Hcl pH 6.8, 6% SDS, 30% glycerol, 16% Beta-mercaptoethanol, 0.006% bromophenol blue, 10M urea) was added to plant tissue previously frozen in liquid nitrogen and ground into powder. The samples containing 1:15 (m/v) tissue to buffer ratio were boiled at 95℃ for 5 minutes, and then centrifuged (3 minutes, 13,000xg). The supernatant was collected for Western blotting

2.9 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 using a TBS-Tween (TBST) solution containing 5% non-fat milk prior to incubation with TBST containing 2% BSA and a 1:1000 dilution of primary antibody (α-GFP rabbit or α-HA mouse, Sigma) overnight at 4℃. After at least five washes with TBST for 5 min each, the blot was incubated with TBST containing a 1:1000 dilution of secondary antibody (α-rabbit-HRP conjugate or α-mouse-HRP conjugate, respectively,

Sigma) for at least 2 hours. After at least five more TBST washes, the blot was visualized using the SuperSignal™ West Pico Chemiluminescent Substrate (Thermo Scientific) and a Bio-Rad Molecular Imager Chemidoc™ XRS+ imaging system. The blot was stained with Ponceau in order to visualize protein loading.

2.10 RNA extraction / RT-qPCR

Total RNA was isolated from snap-frozen and powdered tissue samples using Trisure (Bioline) according to manufacturer's instructions. 6 ug of the isolated RNA was treated with DNAseI (Invitrogen) in order to remove genomic DNA contamination and run on an agarose gel to ensure RNA integrity. 2 ug of this RNA was reverse-transcribed in order to synthesize cDNA using M-MLV reverse transcriptase (Invitrogen) according to manufacturer's instructions. cDNA integrity was tested via PCR prior to further use. Quantitative PCR was performed using a Bio-Rad CFX96 real-time PCR detection system (Bio-Rad) in conjunction with SsoAdvanced™ Universal SYBR Green Supermix (Bio-Rad). The expression levels of *PDF 1.2* gene in different lines were normalized to that of a reference gene, *ACTIN2* (At3g18780).

2.11 Asparaginase purification

Arabidopsis seedlings were grown on half-strength MS medium for 7 days and were moved to a 100 μM AtPep1 for a given amount of time. Plant tissue was snap-frozen in liquid nitrogen and ground into powder. The crude extract of asparaginase was prepared by homogenization of the ground powder in 20 mM Tris-HCl buffer, pH 8.0 containing 10% glycerol, 50 mM KCl, 12.5 mM β-

mercaptoethanol, and 1mM PMSF. The homogenate was centrifuged at 10,000xg for 10 minutes and the supernatant was used for analysis (Mohamed *et al*., 2015).

2.12 Asparaginase activity assay

Asparaginase was purified from 300mg of 7-days old Arabidopsis seedlings using the purification method explained above. The obtained crude asparaginase extract was added to 20 mM Tris-HCl buffer, pH 8.0 solution containing 0-10 µmol L-asparagine. Asparaginase activity was measured using a conductivity meter (Mettler Toledo 51343055). Conductivity was measured at 37 °C. Upon hydrolysis of L-asparagine by L-asparaginase, conductivity of a solution increases due to release of ammonium and aspartate ions.

2.13 Bradford assay

Total protein concentrations in the crude extract were measured by adding 1.5 mL Bradford reagent (50mg Coomassie Brilliant Blue G-250 in 50 ml of methanol and 100 ml 85% (w/v) phosphoric acid (H_3PO_4)) to 30 µL of crude extract. Concentrations were calculated in comparison to values obtained using a Bovine Serum Albumin (BSA) standard curve.

2.14 Phosphoproteomics

Arabidopsis thaliana T87 suspension-cultured cells treated for 10 minutes with water or 100nM AtPep1 were harvested into liquid nitrogen. Total proteins were extracted from the samples and CeO_2 affinity capture was used to enrich the phosphopeptides, which were then separated by nano-LC three-phase capillary column using salt gradients with an LTQ Velos linear trap tandem mass spectrometry. Data-dependent acquisitions and positive ion modes were used for quantification. Prior to

scanning, peptides were separated into three mass classes, and five data-dependent MS/MS scans were performed after each MS scan. Data was extracted and searched using Spectrum Mill (Agilent). Peptide abundance and phosphorylation levels were quantified by spectral counting with counts for each protein representing the total number of peptides that matches to that protein.

2.15 *Botrytis cinerea* assay

B. cinerea B05.10 was grown on 2x V8 plates (36% V8 juice, 0.2% $CaCO_3$, 2% Bacto-agar) for 8 days at room temperature. The grown spores were harvested by scrubbing the plates and resuspending the spores in an autoclaved BD Difco Potato Dextrose Broth (with 0.1% Tween) to a concentration of 5 x 10^5 spores/mL (Mengiste et al., 2003; Veronese et al., 2006). The suspension was incubated for 2 hours at 25-28˚C with gentle shaking before inoculation.

For inoculation, leaves from four-week old Arabidopsis seedlings were punctured using a small needle, and a single 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 5 days, after which the inoculated leaves were detached, and the lesion area was measured using ImageJ.

2.16 Statistical analyses

Statistical analyses were conducted using Excel 2016 (Microsoft Inc.) and vassarstats.net. Student's unpaired t-test was utilized to make pairwise comparisons, and unpaired ANOVAs were used for multiple comparisons between groups. The Tukey test was utilized to analyze significance of differences between trials with multiple variables.

3. Results:

3.1 ASPGA1 is phosphorylated upon AtPep1 treatment

In order to identify proteins that contribute to Arabidopsis immunity, *A. thaliana* T87 suspension-cultured cells were treated with water or 100nM AtPep1. Phosphopeptides were quantified and analyzed using mass spectrometry. ASPGA1 isoforms, ASPGA1.1 and ASPGA1.2, were found to be phosphorylated.

AT5G08100.1 (ASGPA 1.1) and AT5G08100.2 (ASPGA1.2) genes encode 315 and 235 amino acid precursor peptides, respectively. Both isoforms contain a conserved sequence where the precursor peptide undergoes autoproteolytic cleavage, resulting in α and β subunits. The self-cleavage liberates a nucleophilic threonine residue at the N-terminal end, which is part of the active site in the fully formed $\alpha_2\beta_2$ heterotetrametric enzyme. (Michalska *et al*., 2006). Upstream of the autoproteolytic cleavage site is the variable loop sequence, which contains a conserved 10-residue sequence in both isoforms. This variable loop sequence was phosphorylated after AtPep1 treatment in both ASPGA1.1 and ASPGA1.2 on serine 169 and serine 89, respectively (Fig 2).

3.2 AtPep1-induced defense signaling is not compromised in *aspga/aspgb* double mutant

In order to determine whether ASPGA1 plays a role in Arabidopsis immunity, Pep-signaling pathway was investigated in asparaginase double knockout mutant *aspga/aspgb.*14-day old *aspga/aspgb*, Col-0 and *pepr1/pepr2* seedlings were incubated in 1μM AtPep1 solution for 24 hrs. Subsequently, the expression of AtPep1-responsive marker gene *PLANT DEFENSIN 1.2 (PDF1.2)* was measured. Plant defensins are small peptides that inhibit the growth of a broad range of fungi

(Thomma *et al.,* 2002). Relative *PDF1.2* expression was measured using RT-qPCR. Both Col-0 and *aspga/aspgb* had increased *PDF1.2* expression after AtPep1 treatment, while *pepr1/pepr2* had diminished expression (Fig 3.A). To further examine whether *aspga/aspgb* double mutant is compromised in defense responses, ROS production was measured in *aspga/aspgb,* Col-0 and *pepr1/pepr2* lines. Upon elicitation with AtPep1, *aspga/aspgb* showed similar ROS emission levels as Col-0, while *pepr1/pepr2* showed diminished ROS accumulation as expected (Fig 3.B).

3.3 Asparaginase activity increases with AtPep1 treatment.

In order to examine the effect of AtPep1 signaling on ASPGA1 activity, 14-day old Col-0 and *aspga/aspgb* seedlings were treated with 1 µM AtPep1 and incubated at different timepoints (0, 1.5 and 4 hrs). After extraction and purification, L-asparaginase activity was measured *in vitro* in presence of 0, 5 and 10 µmol L-asparagine. Conductivity meter was used to measure the activity of the extracted enzyme by gauging the production of ammonia and aspartate ions. Asparaginase activity was found to significantly increase after 4hrs of AtPep1 treatment in wild type Col-0 (Fig 4.A, B). *Aspga/aspgb* did not show any significant difference in asparaginase activity compared to Col-0 WT.

To investigate the role of serine site phosphorylation on L-asparaginase autoproteolytic cleavage process, C-terminal YFP-tagged phosphomimetic (ASPGA1.1 ^{S169D} and ASPGA1.2^{S89D}) and phosphoabolishing (ASPGA1.1 ^{S169A} and ASPGA1.2^{S89A}) constructs were generated. These variants were transiently expressed in *Nicotiana benthamiana* using *Agrobacterium tumefaciens.* After 24 hours, L-

asparaginase was extracted from *N. benthamiana* leaves. Asparaginase activity was measured using conductivity meter at various amounts of L-asparagine (0,6,8,10 μmol). Both phosphomimetic isoforms, ASPGA1.1 ^{S169D} and ASPGA1.2^{S89D} show increased enzymatic activity compared to wildtype ASPGA at different L-asparagine concentrations (up to 10-fold increase in activity in ASPGA1.1 ^{S169D} and 2-fold increase in ASPGA1.2^{S89D} at 10 μmol) (Fig 5.A, C). While activity plateaus at 6 μmol for ASPGA1.1 ^{S169D}, ASPGA1.2^{S89D} shows continuous increase in activity with increasing L-asparagine. The ASPGA1.1 and ASPGA1.2 proteins expressed in *N. benthamiana* were detected by Western blot 24 hrs and 48 hrs after infiltration. As expected, the phosphomimetic variants in both ASPGA1.1 and ASPGA1.2 showed higher accumulation of cleaved β -subunit compared to wild type ASPGA. Meanwhile, the phosphoabolishing variants showed a lower amount of cleaved enzyme compared to wild type ASPGA. The bands were visualized at 40kDa, which corresponds to the cleaved β -subunit (13.5kDa) plus the 26 kDa C-terminal YFP tag (Fig 5.B, D).

3.4 Stable transgenic Arabidopsis lines expressing ASPGA1.1 ^{S169D} show increased enzymatic activity

In order to better understand the effects of serine site phosphorylation on enzymatic activity of L-asparaginase, stable transgenic lines expressing an ASPGA-YFP construct under the control of the Cauliflower Mosaic Virus (CaMV) p35S promoter were created. Western blotting was used to determine L-asparaginase in *Arabidopsis* transgenic lines compared to *Arabidopsis* double mutant *aspga/aspgb.* As expected, the phosphomimetic lines (ASPGA1.1 ^{S169D} and ASPGA1.2^{S89D}) showed

higher accumulation of cleaved β -subunit (indicated by a more prominent band) compared to wild type ASPGA. Meanwhile, the phosphoabolishing lines (ASPGA1.1 ^{S169A} and ASPGA1.2^{S89A}) had a lower amount of cleaved enzyme compared to wild type ASPGA. The bands were visualized at 40kDa, which corresponds to the cleaved β -subunit (13.5kDa) plus the C-terminal YFP tag (26kDa) (Fig 6.A).

A conductivity meter was used to measure asparaginase enzymatic activity in wildtype Col-0, ASPGA1.1 and ASPGA1.2 transgenic lines in presence of 0,5,10 µmol L-asparagine. While ASPGA1.1 ^{S169D} and ASPGA1.2 ^{S89D} lines showed significantly increased enzymatic activity compared to that of Col-0, phosphoabolishing ASPGA1.1 ^{S169A} and ASPGA1.2 ^{S89D} lines showed reduced activity at 5 and10 µmol L-asparagine. ASPGA1.1-wt and ASPGA1.2-wt had enzymatic activity comparable to that of Col-0, as expected. (Fig 6.B, C).

3.5 Phosphoabolishing transgenic line ASPGA1.1 ^{S169A} shows increased susceptibility to *Botrytis cinerea*

In order to determine the susceptibility of phosphomimetic and phosphoabolishing transgenic lines to necrotrophic fungus *Botrytis cinerea*, 21-day old Col-0, *pepr1/pepr2, aspga/aspgb* and ASPGA1.1-YFP transgenic lines were infected with *B. cinerea.* Lesion area was measured five days after infection. The double mutant *aspga/aspgb* line shows a two-fold increase in lesion area, indicating susceptibility to *B. cinerea* (Fig 7.A). Similarly, ASPGA1.1 ^{S169A} line also had a two-fold increase in lesion area compared to lesion area observed in Col-0, suggesting increased susceptibility of the phosphoabolishing line to *B. cinerea.* Meanwhile, the measured lesions areas in phosphomimetic ASPGA1.1 ^{S169D} line were comparable to

that of Col-0, which suggests that the phosphomimetic line does not confer increased resistance to *B. cinerea* (Fig 7.B).

3.6 Arabidopsis transgenic lines do not display altered ROS emission

To further examine whether Arabidopsis transgenic lines are compromised in defense responses, ROS production was measured in wildtype Col-0, ASPGA1.1 and ASPGA1.2 lines. The *pepr1/pepr2* line was used as negative control. Upon elicitation with AtPep1, the phosphomimetic and phosphoabolishing lines showed similar ROS emission levels as Col-0, while *pepr1/pepr2* showed diminished ROS accumulation as expected (Fig 8.A, B).

4. Discussion:

4.1 ASPGA1 plays a role in Arabidopsis immunity

This study aimed to better understand the potential regulatory role of variable loop serine phosphorylation of K^+-independent asparaginase, ASPGA1, in its autoproteolytic cleavage and subsequent effect on enzyme activity and immune output in *A. thaliana.* Characterization of this protein was initiated as part of an investigation into proteins contributing to Arabidopsis immunity, as ASPGA1 was found to be among the proteins phosphorylated after AtPep1 treatment (Fig 2). Together this evidence, along with the observed increase in susceptibility of Arabidopsis double knockout line *aspga/aspgb* to *Botrytis cinerea* (Fig 7.A) and increase in ASPGA1 activity in wild type Col-0 plants after AtPep1 treatment (Fig 4.A) indicated that ASPGA1 promotes immune output in Arabidopsis.

The AtPep1 signaling pathway following the activation of the PRR induces transcriptional changes in numerous plant defense genes, such as the *PLANT*

DEFENSIN 1.2 (PDF1.2) gene, and also triggers the release of various endogenous defense responses. Production of reactive oxygen species (ROS) is one of the earliest of these responses that results from the heterodimerization of PEPR and BAK1(Lin *et al*., 2014: Kadota *et al*., 2014). ROS production occurs within minutes of elicitation, therefore alterations of the early signaling pathway components should result in changes in ROS output. Lack of altered *PDF1.2* expression and ROS output in asparaginase double knockout mutant *aspga/aspgb* as well as the phosphomimetic and phosphoabolishing Arabidopsis transgenic lines (Fig 3, Fig 8) indicates that ASPGA1 does not alter early signaling pathway components in these phenotypic responses.

4.2 ASPGA1 phosphorylation promotes autoproteolytic cleavage and enzyme activity

It was previously established that deletion of residues 169-173 in the variable loop region of ASPGA1 causes disruption in the autoproteolytic cleavage process (Gabriel *et al*., 2012). Therefore, the serine 169 phosphorylation in this region (13 residues upstream of the conserved autoproteolytic cleavage site) after AtPep1 treatment suggested that the phosphorylation might serve a regulatory role. The observed reduction in self-cleavage in the phosphoabolishing mutants along with increased cleavage and significant rise in activity in the phosphomimetic mutants of both isoforms (Fig 5) indicate that the variable loop serine phosphorylation promotes cleavage and activation of the precursor peptide.

Transient expression of the C-terminal YFP tagged constructs shows that the phosphoabolishing ASPGA1.1^{S169A} and ASPGA1.2^{S89A} are hindered in their autoproteolytic cleavage process, while phosphomimetic ASPGA1.1^{S169D} and ASPGA1.2^{S89D} show increased cleavage. However, the cleavage is not completely

abolished and still occurs albeit on a slower timescale, which suggests that the serine site phosphorylation serves to enhance activation of the autoproteolytic cleavage of ASPGA precursor. As the activity of asparaginase is dependent on the cleavage of the precursor peptide and liberation of the nucleophilic threonine, alterations to the autoproteolytic cleavage mechanism affects the enzymatic efficiency of the enzyme. This is evident from the increased enzymatic activity of the phosphomimetic ASPGA after transient expression in *N. benthamiana* and also in *A. thaliana* transgenic lines (Fig 6).

4.3 Phosphoabolishing Arabidopsis line is more susceptible to *Botrytis cinerea*

Botrytis cinerea is a necrotrophic fungus that attacks damaged or wounded plant tissue and kills its plant hosts to colonize the dead tissue. Plants defend against *B. cinerea* infection through *de novo* synthesis of nitrogen containing secondary metabolites, such as alkaloids, cyanogenic glycosides, glucosinolates and non-protein amino acids (Singh S.K, 2018). It has been previously shown that *A. thaliana* plants grown in abundant supply of ammonia are more resistant to *B. cinerea* (Soulie *et al*., 2020). Therefore, nitrogen availability is essential in defense against *B. cinerea.* The increased susceptibility of the phosphoabolishing line ASPGA1.1 ^{S169A} (Fig 7) indicates insufficient defense responses in this line. Additionally, the phosphoabolishing line shows a similar resistance level as *aspga/aspgb,* indicating that in prolonged periods of pathogen infection the phosphoabolishing line suffers from similarly reduced pathogen resistance.

4.4 Reduced enzymatic activity of ASPGA1 may reduce nitrogen availability

Arabidopsis L-asparaginases are essential nitrogen-releasing enzymes, which play a necessary role in nitrogen availability in developing plants and germinating seeds. As nitrogen is incorporated in many of the defense proteins and metabolites, reduced nitrogen availability could lead to insufficient defense responses and vulnerability to pathogenic attacks. Taken together, the role of ASPGA1 and the data collected in this work suggest that available nitrogen may be reduced in the double knockout mutant *aspga/aspgb* and the phosphoabolishing ASPGA1.1 ^{S169A} line. This is reflected in the results obtained from the *B. cinerea* assays. The phosphoabolishing ASPGA1.1 ^{S169A} line shows reduced resistance to *B. cinerea* that are comparable to *aspga/aspgb*. These results seem to strongly indicate that both lines suffer from insufficient nitrogen supplies during prolonged periods of pathogen infection. A more conclusive demonstration of this could be achieved by investigating levels of asparagine and aspartate in infected Arabidopsis transgenic plants. This would give more insight into nitrogen metabolism and availability in the phosphoabolishing and phosphomimetic lines during activated defense responses.

5. Future directions in ASPGA1 characterization

The data obtained here suggests that the variable loop serine phosphorylation promotes the autoproteolytic cleavage process and subsequent activation of ASPGA1. However, the mechanism by which the phosphorylation assists the self-cleavage remains to be determined. Additionally, the data indicates that ASPGA1 is phosphorylated after AtPep1 treatment, however, the connection between ASPGA1 phosphorylation and AtPep1 signaling cascade has yet to be shown. Further

examination of the protein(s) involved in ASPGA1 phosphorylation may help to elucidate the activation process of ASPGA1 in relation to AtPep1 signaling pathway. Furthermore, additional investigation of phosphomimetic and phosphoabolishing transgenic Arabidopsis lines would aid in understanding the effects of ASPGA1 serine phosphorylation in Arabidopsis resistance to various pathogens. As ASPGA1 activity levels directly influence the rate of asparagine catabolism and aspartate production, asparagine levels are suspected to be affected in the phosphomimetic and phosphoabolishing transgenic lines, as well as the double knockout mutant *aspga/aspgb* line. Therefore, investigating the amino acid metabolism and as well as measuring the levels of asparagine and nitrogen-containing defense metabolites such as glucosinloates would further clarify the role of ASPGA1 in Arabidopsis growth and immunity.

6. Conclusions

The effect of variable loop serine phosphorylation in the autoproteolytic cleavage process and activity of ASPGA1 isoforms was investigated. The data collected in this work suggests that ASPGA1 serine phosphorylation promotes the autoproteolytic cleavage process and significantly increases enzyme activity in both isoforms. Additionally, the results indicate that abolishing the serine phosphorylation reduces enzymatic efficiency of ASPGA1, leading to reduced resistance to *Botrytis cinerea.* The identification of ASPGA1 phosphorylation helps provide more insight into a potential mechanism by which Pep signaling pathway regulates defense responses through nitrogen-releasing enzymes. It is possible that other nitrogen-metabolizing enzymes are similarly regulated through the Pep signaling pathway, or even differentially expressed, as described previously (Bazin *et al*., 2020). Understanding the

mechanism by which Pep signaling induces ASPGA1 phosphorylation may also provide more insight into the effect of activated immunity on activity levels of other nitrogen-metabolizing enzymes. Altogether continued study of ASPGA1 could open a new door for investigating altered amino acid metabolism and nitrogen utility against pathogen attacks.

Figures

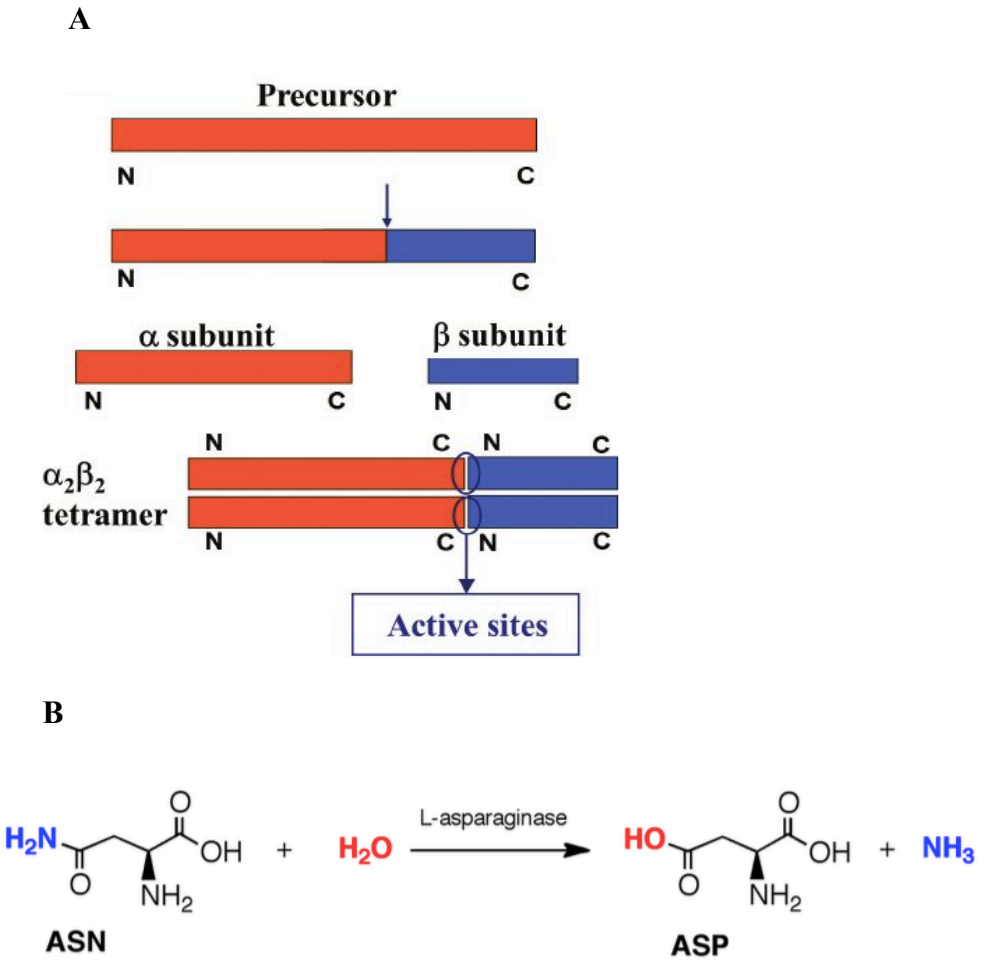

Figure 1. ASPGA1 autoproteolytic cleavage and function.
Adapted from:
A) Marco Betti, Margarita García-Calderón, Carmen M. Pérez-Delgado, Alfredo Credali, Peter Pal'ove-Balang, Guillermo Estivill, Miroslav Repčák, José M. Vega, Francisco Galván, Antonio J. Márquez. (2014). Reassimilation of ammonium in *Lotus japonicus*. *Journal of Experimental Botany*, Volume 65, Issue 19, October 2014, Pages 5557–5566.
B) Nguyen, H. A., Su, Y., & Lavie, A. (2016). Structural Insight into Substrate Selectivity of Erwinia chrysanthemi L-asparaginase. *Biochemistry*, *55*(8), 1246–1253.

phosphopeptide	Unique peptide	Protein Coverage	Accession	Water treatment				Atpep1 treatment				P-value
				Rep1	Rep2	Rep3	Rep4	Rep1	Rep2	Rep3	Rep4	
VQLDYTVPsP	1	0.046809	AT5G08100.1 AT5G08100.2	0	0	0	0	4.246885617	6.056935191	2.963314171	8.552856654	0.004100199

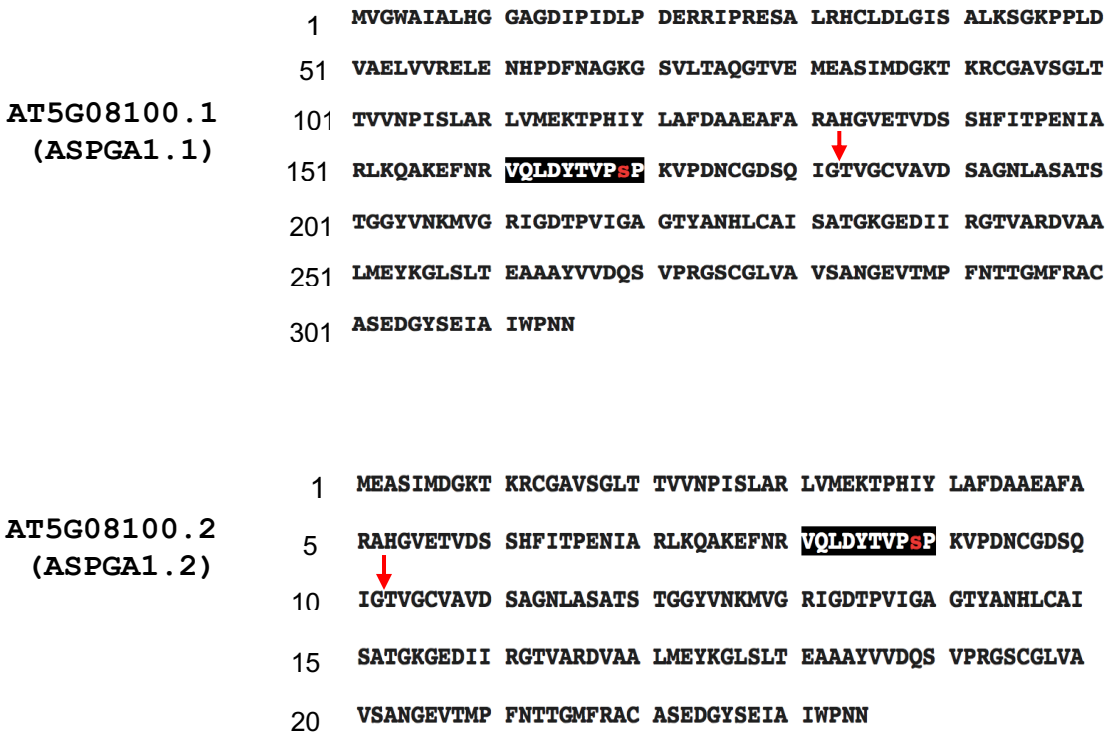

Figure 2. ASPGA1 variable loop serine residue is phosphorylated upon AtPep1 treatment. *Arabidopsis thaliana* T87 suspension-cultured cells were treated with either water or 100nM AtPep1. Proteins extracted from the samples were analyzed using mass spectrometry. A) ASPGA1.1(AT5G08100.1) and ASPGA1.2 proteins (AT5G08100.2) are phosphorylated on a serine residue in a conserved sequence upon AtPep1 treatment. B) ASPGA1.1 and ASPGA1.2 peptide sequences containing the conserved sequence. The *arrow* marks the autoproteolytic cleavage site giving rise to the a- and b-subunits, s represents phosphorylated Ser169 in ASPGA1.1, and Ser89 in ASPGA1.2.

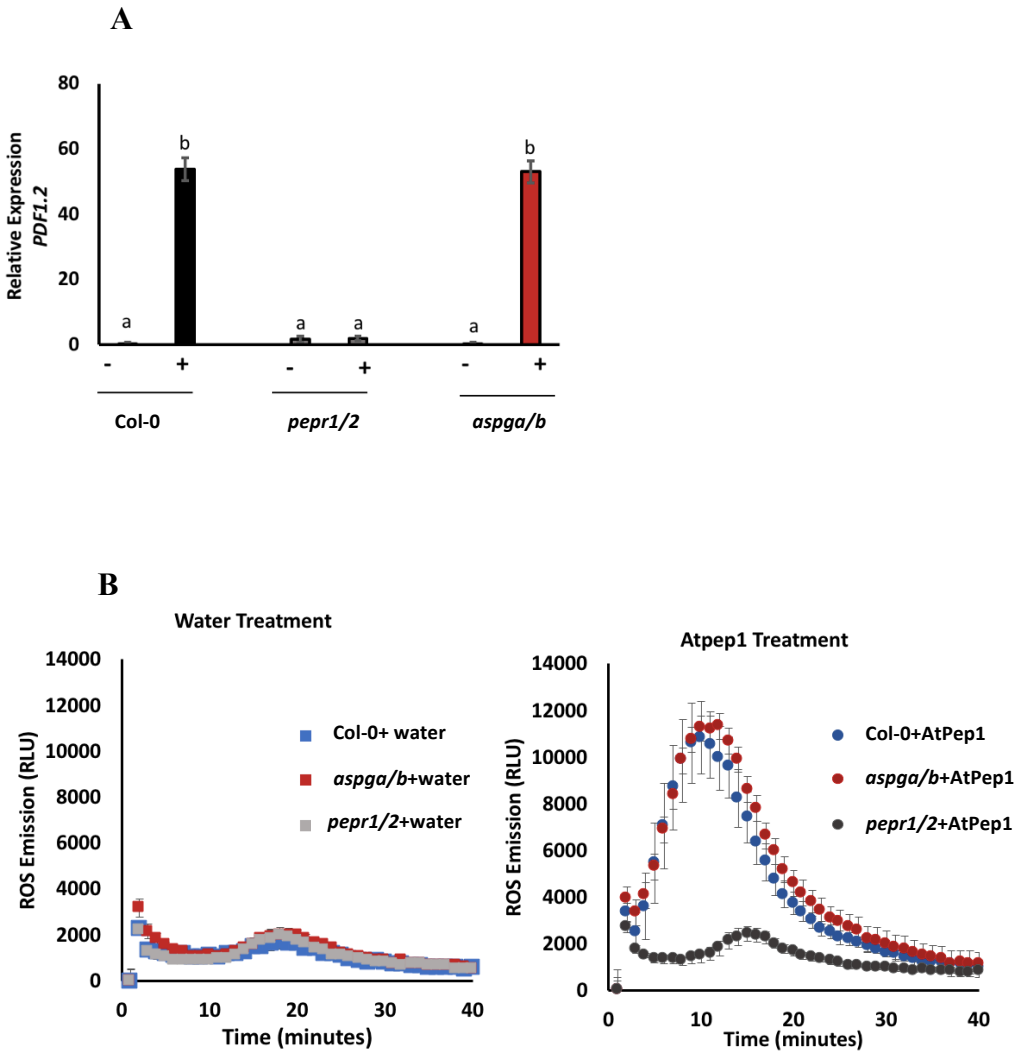

Figure 3. Asparaginase double knockout *aspga/aspgb* does not display altered sensitivity to AtPep1 treatment compared to wildtype Col-0. 14-day old seedlings of T-DNA insertion *Arabidopsis* double mutant line *aspga/aspgb* and wildtype Col-0 were treated with 1µM AtPep1 A) Relative *PDF1.2* expressions were measured in 14-day old seedlings incubated with 1µM AtPep1 using RT-qPCR (N=3 biological replicates).) ROS emission was measured using leaf disks from 21-day old plants *aspga/aspgb* and Col-0, 24 hrs after water or AtPep1 treatment. Error bars indicate standard error of the mean. Asterisks "**" and different letters indicate statistically significant difference at P<0.01. (one-way ANOVA followed by Tukey's test corrections for multiple comparisons).

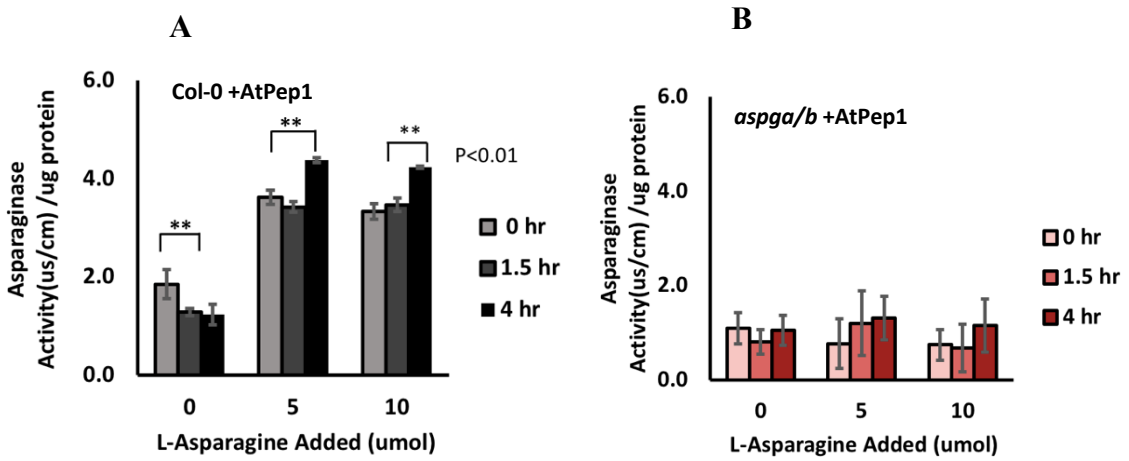

Figure 4. Asparaginase activity is increased with AtPep1 treatment. 14-day old seedlings of T-DNA insertion *Arabidopsis* double mutant line *aspga/aspgb* and wildtype Col-0 were treated with 100μM AtPep1 A, B) Conductivity meter was used to measure asparaginase activity in lant). Error bars indicate standard error of the mean. Asterisks "**" and different letters indicate statistically significant difference at P<0.01. (one-way ANOVA followed by Tukey's test corrections for multiple comparisons).

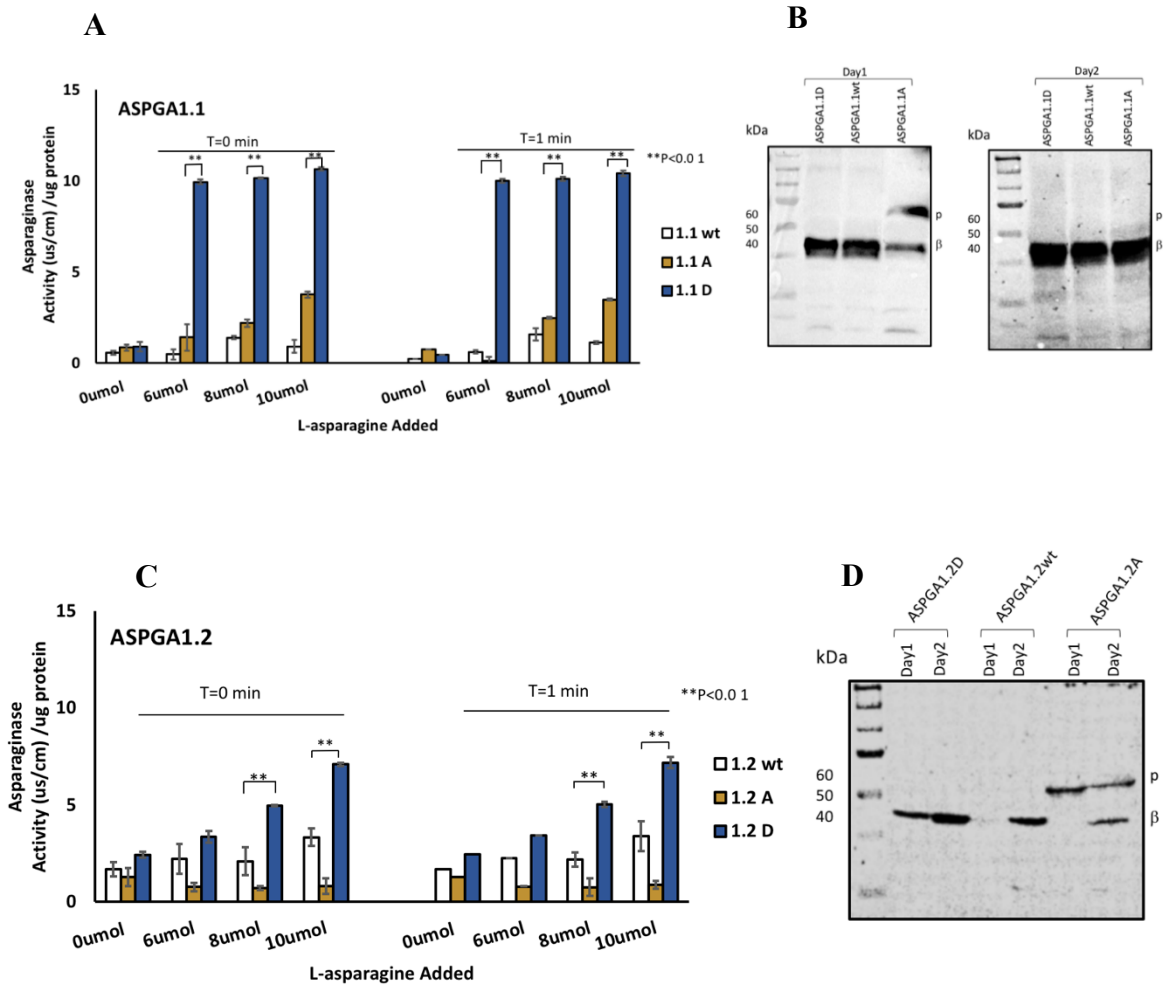

Figure 5. Phosphomimetic isoforms ASPGA1.1 ^{S169D} and ASPGA1.2^{S89D} show higher enzymatic activity. C-terminal YPF-tagged ASPGA1.1 and ASPGA1.2 were transiently expressed in *Nicotiana benthamiana.* Conductivity meter was used to measure A) ASPGA1.1 activity 24hrs post inoculation and C) ASPGA1.2 activity 48hrs post inoculation. Western blotting was used to check levels of B) ASPGA1.1 and D) ASPGA1.2 24hrs and 28hrs after inoculation. *p* polypeptide precursor; *β* indicates subunit. Error bars represent a standard error of the mean, N=5 plants per genotype, with 3 technical replicates per plant. Error bars indicate standard error of the mean. Asterisks "**" indicate statistically significant difference at P<0.01 (one-way ANOVA followed by Tukey's test corrections for multiple comparisons).

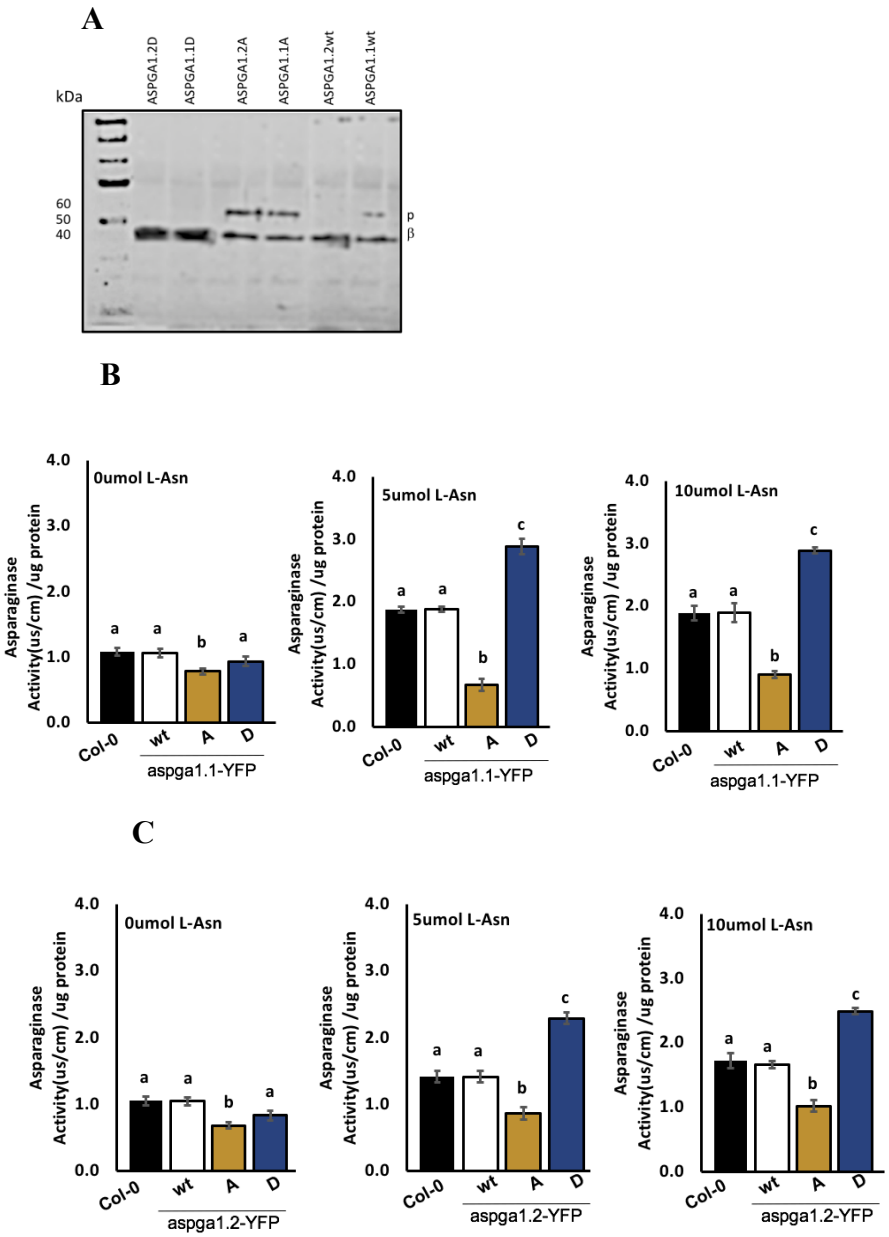

Figure 6. ASPGA1.1 ^{S169D} -YFP transgenic line shows increased enzymatic activity in presence of L-asparagine. A) western blotting was used to check asparaginase levels in *Arabidopsis* transgenic lines compared to T-DNA insertion *Arabidopsis* double mutant line *aspga/aspgb*. *p* polypeptide precursor; *β* indicates subunit. B, C) Conductivity meter was used to measure asparaginase enzymatic activity in wildtype Col-0 and *Arabidopsis* transgenic lines. (N=5 plants per genotype, with 3 technical replicates per plant). Error bars indicate standard error of the mean. Different letters represent significant differences (one-way ANOVA followed by Tukey's test corrections for multiple comparisons; P <0.01)

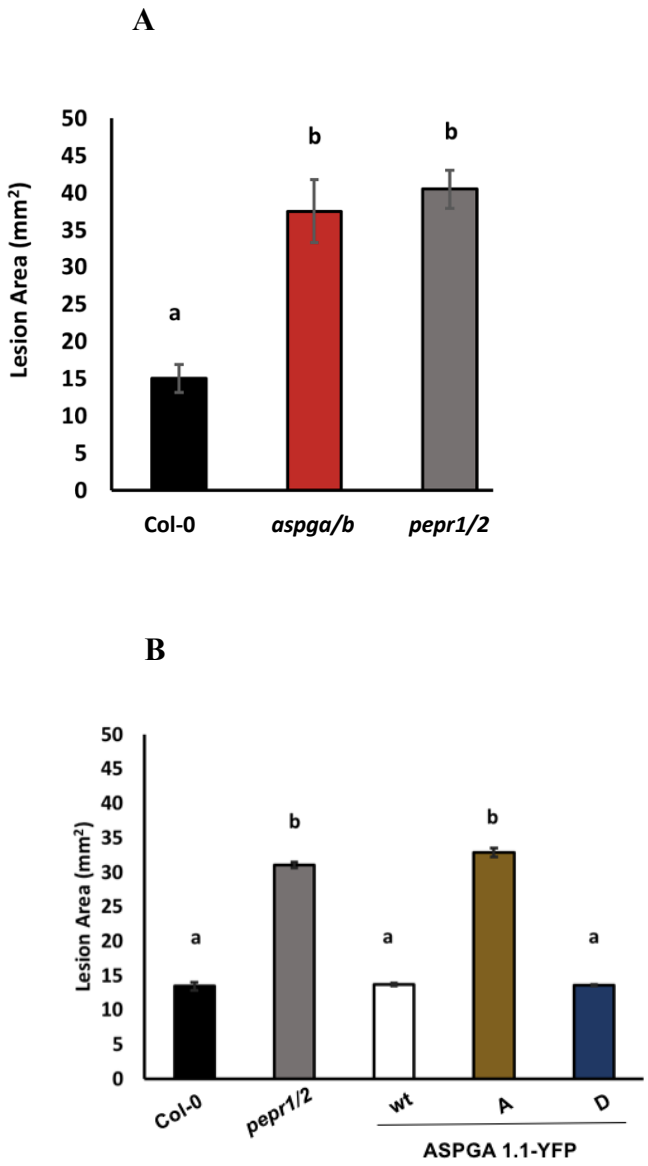

Figure 7. ASPGA1.1 ^{S169A} **-YFP transgenic line displays increased susceptibility to *Botrytis cinerea.*** 21-day old plants A) Col-0, *pepr1/pepr2, aspga/aspgb* B) ASPGA1.1-YFP transgenic Arabidopsis lines were infected with *Botrytis cinerea* and lesion area was measured after 5 days. (N=5 plants per genotype, with 3 technical replicates per plant). Error bars indicate standard error of the mean. Different letters represent significant differences (one-way ANOVA followed by Tukey's test corrections for multiple comparisons; $P < 0.01$)

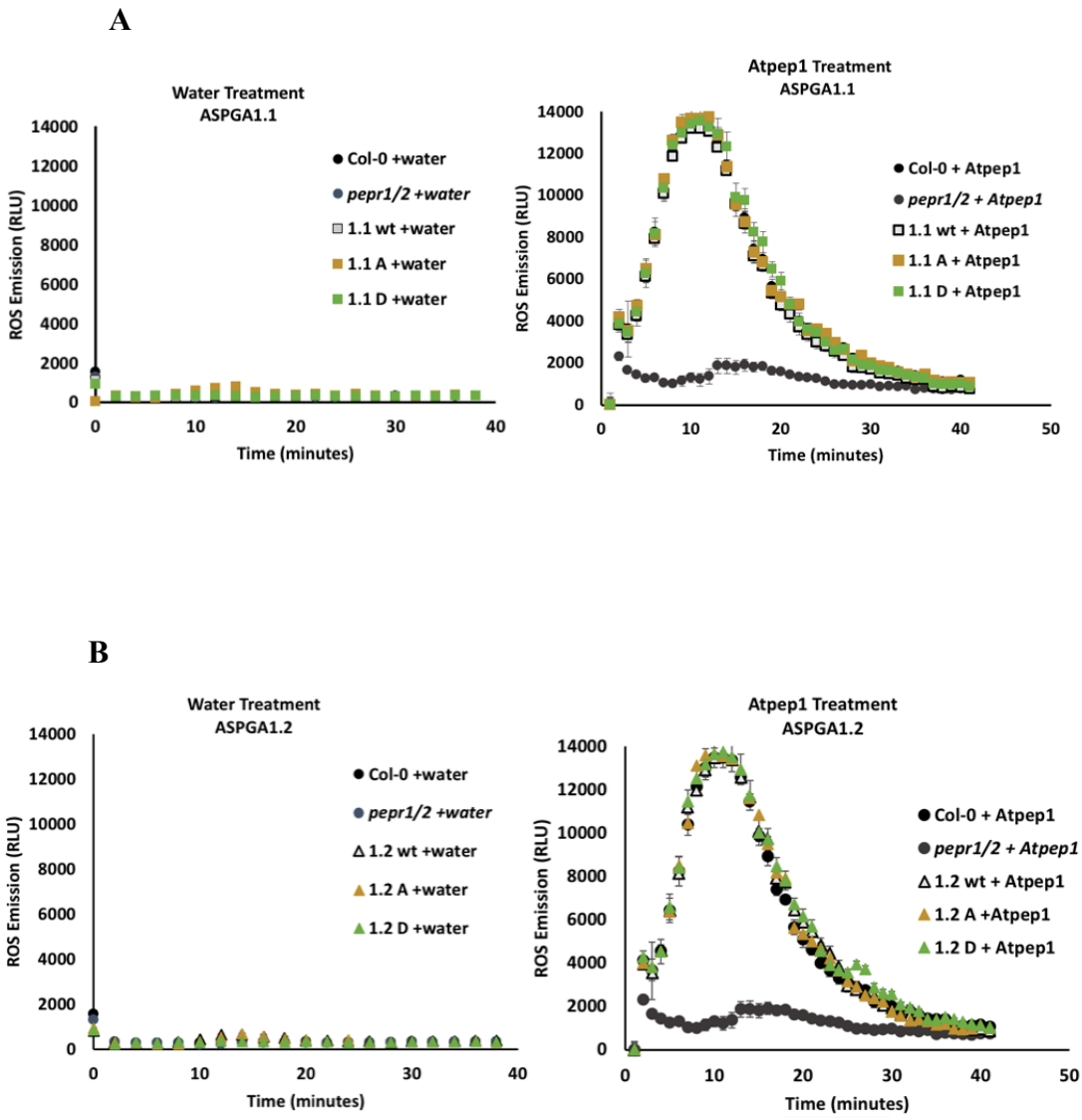

Figure 8. Arabidopsis transgenic lines do not display altered ROS emission.
21-day old Col-0, A) ASPGA1.1-YFP and B) ASPGA1.2 Arabidopsis transgenic seedlings were treated with 1μM AtPep1. ROS emission was measured using leaf disks from 21-day old plants *aspga/aspgb* and Col-0, 24 hrs after water or AtPep1 treatment. Error bars indicate standard error of the mean.

Table 1. Primers utilized in this work. A list of primers used in gene expression analysis, cloning, and creation of transgenic constructs.

Name	Locus	Primer Sequence	Purpose
ASPGA1.1	AT5G08100.1	5'-CACCATGGTGGGGTGGGCGATTG-3' Fw	cDNA amplification
		5'-ATTGTTTGGCCAGATTGCGATCTCAG-3' Rv	
ASPGA1.2	AT5G08100.2	5'-CACCATGGAAGCTTCCATTATGGACGG-3'Fw	cDNA amplification
		5'-ATTGTTTGGCCAGATTGCGATCTCAG-3' Rv	
ASPGA1.1A ASPGA1.2A	AT5G08100.1 AT5G08100.2	5'-TCCAGTTGGATTACACAGTCCCTGCTCCGAAAGTACCG-3'Fw	Phosphomimetic mutation
		5'-CGGTACTTTCGGAGCAGGGACTGTGTAATCCAACTGGA-3'Rv	
ASPGA1.1D ASPGA1.2D	AT5G08100.1 AT5G08100.2	5'-AGTCCAGTTGGATTACACAGTCCCTGATCCGAAAGTACCGG-3'Fw	Phosphoabolishing mutation
		5'-CCGGTACTTTCGGATCAGGGACTGTGTAATCCAACTGGACT-3'Rv	
ACTIN gene	AT2G37620.1	5'-TCCCTCAGCACATTCCAGCAGAT-3' Fw	Gene expression analysis
		5'- AACGATTCCTGGACCTGCCTCATC-3' Rv	
PDF 1.2 gene	AT5g44420	5'-CTTATCTTCGCTGCTCTTGT-3' Fw	Gene expression analysis
		5'- CGTAACAGATACACTTGTGTGC-3' Rv	
ASPGA1.1 ASPGA1.2	AT5G08100.1 AT5G08100.2	5'- GTAAAACGACGGCCAG-3' Fw	Cloning into pENTR-D-TOPO
		5'- TTTTTCTTTGGCATATGGCAG-3' Rv	

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