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# Arabidopsis Argininosuccinate Lyase and Argininosuccinate Synthase are important for resistance against *Pseudomonas syringae*

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## Abstract

**B**ackground: Arginine is one of the important amino acids and is involved in a variety of plant responses against biotic and abiotic stresses. Two important genes Argininosuccinate Synthase (*AS*) and Argininosuccinate Lyase (*AL*) are associated with the production of arginine from ornithine. Here we report molecular characterization of *Arabidopsis thaliana* artificial micro RNA (amiRNA) mutants for the genes *AL* and *AS* in response to infection with *Pseudomonas syringae* DC3000.

**Methods:** Quantitative real time PCR (qRT-PCR) and Promoter::GUS plant lines were used for expression analysis. Artificial micro RNAs (amiRNA) mediated gene silencing was used to generate mutant plant lines. Flood inoculation technique was used for infection test essays with *Pseudomonas syringae*.

**Results:** Expression analysis of *A. thaliana* plants harboring promoter *AS*::GUS construct showed strong promoter activity upon *P. syringae* infection. Quantitative real time PCR (qRT-PCR) analysis showed that *AL* and *AS* expression was strongly induced upon infection with *P. syringae*. Infection essays for *P. syringae* showed enhanced susceptibility to virulent (*Pto*) as well as avirulent ( $\Delta avrPto/\Delta avrPtoB$ ) strains of *P. syringae*. However, mutant plants infiltrated with infiltration medium containing 1 mM L-arginine regain their resistance in comparison with wild type (Col-0) plants.

**Conclusion:** Our findings suggest that genes related to arginine metabolism play a key role in plant defenses during *P. syringae* infection on *A. thaliana*. This study revealed that proper functioning of arginine related genes is required to deploy defense response against *P. syringae*. Decrease in the expression of these genes improves conditions for the growth of pathogen.



## Introduction

Plants are exposed to a number of infectious organisms, however successful disease progression depends upon specific molecular interactions between host and pathogens. Plants are equipped with specialized defense strategies deployed locally or systemically. Most of these defense reactions are associated with basal defense also called non-host resistance. This is the most prevalent form of resistance at species level [1,2]. Several cellular pathways are required to be activated for this type of resistance in plants e.g. induction of pathogen responsive genes, generation of reactive oxygen species, production of nitric oxide and callose deposition to reinforce the cell walls [3-6]. Different amino acids pathways are involved in plant-pathogen interactions as well as in developmental responses to several stress responses [7-9].

Nitric oxide (NO) is a free radical gas, which acts as signal during the induction of defense responses in plants [10,11]. In *A. thaliana* arginine is the main source of nitric oxide during plant-pathogen interactions [12]. Arginine is synthesized in plants using ornithine as precursor. This is two-step process including Argininosuccinate Synthase (AS) which converts citrulline and aspartate into argininosuccinate then Argininosuccinate Lyase (AL) further converts argininosuccinate into arginine and fumarate [14]. Decrease in arginine content in *A. thaliana* results in decreased NO activity [12].

Arginine plays a central role during biotic as well as abiotic stress. Arginine provide precursor for many signal molecules like nitric oxide and polyamines during stress responses. Polyamines are important molecules during cell proliferation and programmed cell death. Nitric oxide has direct role in plant defense responses. Arginine also serves as precursor for proline biosynthesis. Proline is an important osmolyte produced during stress responses.

Due to importance of arginine in plant NO synthesis and defense response, we report the molecular characterization of genes coding for two key enzymes Argininosuccinate Synthase (AS) and Argininosuccinate Lyase (AL) associated with the production of arginine amino acid. Our findings suggest that proper functioning of these two genes is required to interfere with the population growth of *P. syringae* on *A. thaliana* leaves.

## Methods

### *Culture of Arabidopsis thaliana and Pseudomonas syringae*

Wild type *A. thaliana* (Col-0) plants were used in this study. Seeds were planted in 2X2 inch plastic pots. Day length cycle was set at 16 h light followed by 8 h dark and at 25°C in green house. These plants were used for

transformation. *A. thaliana* -*P. syringae* interactions were studied in sterile culture on a Murashige and Skoog's (MS) medium. Plates were incubated at cycle of 16 h light followed by 8 h dark and 25°C. After 15 days seedlings were inoculated with *P. syringae* using seedling flood inoculation technique [16].

### *Plasmid construction and plant transformation*

To clone promoter *AS::GUS* line, genomic DNA was extracted from *A. thaliana* using QIAamp DNA Mini Kit (Qiagen, Hilden, Germany), briefly ~ 418 bp fragment upstream from start codon of At4g24830 gene was amplified. Primers, pAS-Fr and pAS-Rv (Table 1) were used for promoter amplification. Sequences for the *SgsI/AscI* and *HindIII* restriction sites were included in primer design. Primers were designed using NCBI primer design tool

(<http://www.ncbi.nlm.nih.gov/tools/primerblast>).

Binary vector pMDC139 [17] was digested with *SgsI/AscI-HindIII* restriction enzymes. Amplified promoter sequence was cloned in binary vector.

Stable artificial micro RNAs (amiRNA) for *AL* and *AS* genes were constructed according to Schwab *et al.* [18]. Briefly sequences of four oligonucleotides for each *AL* and *AS* amiRNAs were identified. WMD Web Micro-RNA Designer was used to design oligonucleotides (<http://wmd3.weigelworld.org/cgi-bin/webapp.cgi>).

These oligonucleotides (Table 1) were used to amplify single amiRNA. Native *A. thaliana* miR319a precursor was used for site-directed mutagenesis. Modified pZZP3425 vector [19] was used to clone amplified amiRNAs. Presence of the inserts was confirmed by sequencing the binary vectors. *Agrobacterium* mediated plant transformation (floral dip technique) was used to insert binary vectors into the *A. thaliana* genome [20]. Screening of transformed plants was done on MS medium supplemented with kanamycin (50 µg mL<sup>-1</sup>). Homozygous T3 (third generation) lines were collected after segregation analysis. Silencing efficiencies of amiRNAs were tested in selected homozygous lines by qRT-PCR.

### *Infection test assays for Pseudomonas syringae*

Flood inoculation technique was used to infect *A. thaliana* seedlings according to Ishiga *et al.* [16]. Briefly fifteen day-old seedlings were inoculated under sterile hood. Inoculum was consists of 40 mL bacterial suspension prepared in sterile distilled H<sub>2</sub>O containing 0.025% Silwet (L-77) referred as control. L-Arginine was added in 1mM concentration [12] in infiltration medium and was referred as treatment. We use virulent strain of *P. syringae* DC3000 (*Pto*) as well as avirulent strain of *P. syringae* DC 3000 ( $\Delta$ avr*Pto*/ $\Delta$ avr*PtoB*). After 3 min of seedling submergence, bacterial inoculum was removed from plates by decanting. Petri plates were then sealed again and placed in the growth chamber. Internal

bacterial population was determined after 1, 2 and 3 days post inoculation (dpi). Four seedlings were harvested from each plate which represents one biological replicate; three biological replicates from each plant line were taken for infection essays. Weight of one replicate was recorded and seedlings were surface sterilized three times with 70% ethanol. One washing was given with sterile dH<sub>2</sub>O. Samples were homogenized in 1.5 ml tubes and plated on LB medium containing the rifampicin (50µg/ml). Colony forming units (CFU) were counted from serial dilutions (10<sup>-2</sup> to 10<sup>-6</sup>) and normalized against the weight of the sample.

#### Histochemical GUS assays

To study the expression patterns of *AL* and *AS* in *A. thaliana* leaves during *P. syringae* infection, GUS reporter gene was delivered under the control of native promoters for *AS* and *ARGAH2* genes. Promoter::GUS lines were infected with *P. syringae* and GUS activity was determined. X-Gluc. (5-bromo-4 chloro-3-indolyl β-D-glucuronic acid) reagent was used for GUS staining assay. Five week old plants were inoculated as described above. One day post inoculation (dpi) rosette leaves were harvested and immersed in X-Gluc. solution. Petri dishes containing leaves in X-Gluc. solution were subjected to vacuum for 3 min and then incubated at 37°C for four hours, after that these leaves were destained using 70% ethanol. Images of the leaves were taken with microscope (Axiovert 200M, Zeiss) containing a digital camera (AxioCam, Zeiss). Two *pAS::GUS* [15] and *pARGAH2::GUS* [21], T3 generation plant lines were studied for characterization of expression patterns.

#### Sample collection, RNA isolation and quantitative RT-PCR

Leaves were harvested for RNA isolation from *A. thaliana* plants. Samples were taken from plants infected with *P. syringae* at 1, 2 and 3 dpi. Harvested leaves were shock-frozen in liquid nitrogen. *A. thaliana* (Col-0) plants were used as control to compare amiRNA lines and non-infected plants of same age were used as control for *P. syringae* infected plants. Three independent biological replicates were used from each plant line to collect samples. Total RNA was extracted from *A. thaliana* leaves using RNase Plant Mini Kit (Qiagen, Germany). Quantity and quality of the RNA was analyzed using Agilent 2100 bio-analyzer (Agilent Technologies, USA). Random primers [oligo(dN)<sub>6</sub>] were used to reverse-transcribe RNA into cDNA using SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA, USA). Real-Time PCR System “ABI PRISM 7300 Sequence Detector” (Applied BioSystems, USA) was used to perform Quantitative RT-PCR. Program for PCR consists of 50°C for 2 min 95°C for 5 min and

consecutive 40 cycles of 95°C for 15 s, 60°C for 30 s and 72°C for 1 min stages. 18S rRNA gene was used as internal references. Primer sequences are given in Table. 1. Fold changes were calculated using 2<sup>-ddCt</sup> method [22].

Gene I.D	Direction	Sequence of primer (5'-3')
At4G24830 <i>pAS::GUS</i>	Fwd.	caagctgaaagggaatgaaactttaactcttaattct
	Rev.	aggcgccttttcgactattgtctg
At4G24830 amiRNA	1	gatgtgttctaattgcaggctgttctctctttgtattcc
	2	gaacagcctgacattagaacacatcaagagaatcaatga
	3	gaaccgcctgacattgaaacttccacagctgtgatag
	4	gaagtgttcaaatgtcaggegttctacatatattctct
At5G10920 amiRNA	1	gattatataagaactccagcgcgtctctctttgtattcc
	2	gacgcctggagttctataatacaagagaatcaatga
	3	gacgagctggagttctacatatattccacagctgtgatag
	4	gaatatatagaactccagcgtctctacatatattctct
At4G24830 ( <i>AS</i> ) qRT-PCR	Fwd.	aaagcacgggattggcgcca
	Rev.	gcggatcaaacctctctctg
At2G10920 ( <i>AL</i> ) qRT-PCR	Fwd.	gcagctcgaaactgatgctggt
	Rev.	cctcggaaagccacagacc
At4G08900 ( <i>ARGAH1</i> ) qRT-PCR	Fwd.	agcttggaggcctgtggac
	Rev.	gccttgtcccctctctctg
At4G08870 ( <i>ARGAH2</i> ) qRT-PCR	Fwd.	ggaaggtggctatgcgcggc
	Rev.	acactccgtgcgcaatccc
18S rRNA qRT-PCR	Fwd.	tgacacggggagtagtaca
	Rev.	agtctgtaattggaatgagtacaactaa

**Table 1:** Sequence of the primers used in this study for *AS* promoter amplification, amiRNA amplification and qRT-PCR.

## Results

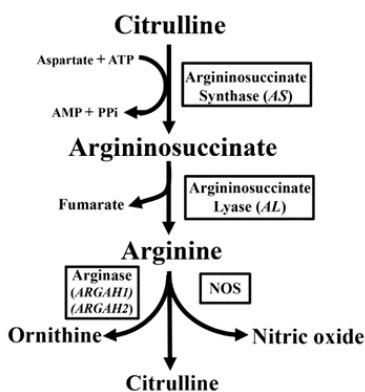
Native Promoter of argininosuccinate synthase and arginase 2 were cloned with GUS gene. Activities of *pAS::GUS* and *pARGAH2::GUS* constructs were studied in *P. syringae* infected and control *A. thaliana* leaves. Strong promoter activity was found in *pAS::GUS* lines upon infection with virulent strain of *P. syringae* (*Pto*) (Figure 2C) same was the case with avirulent strain (*ΔavrPto/ΔavrPtoB*) (Figure 2B). Promoter activity of *pARGAH2::GUS* was limited to few regions of leaves infected with *Pto* (Figure 2F) whereas *ΔavrPto/ΔavrPtoB* infection resulted in a very weak promoter activity (Figure 2E). Mock infiltrated control leaves of *pAS::GUS* and *pARGAH2::GUS* lines did not express any promoter activity (Figure 2A & D).

Expression of four genes playing key role in arginine metabolic pathway was determined by qRT-PCR during *P. syringae* infection on *A. thaliana* leaves. Two genes *AS* and *AL* associated with anabolic enzymes in arginine metabolism (Figure 1) were found significantly up-regulated at 1, 2 and 3 dpi. However, in case of virulent

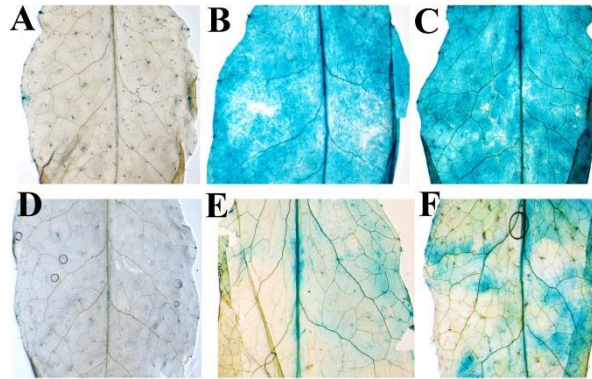
strain of DC3000 (*Pto*) gene expression was significantly higher at 2 and 3dpi as compared to 1dpi (Figure 3A). In case of  $\Delta avrPto/\Delta avrPtoB$  infection expression was higher at initial time point and decreased at 2 and 3dpi (Figure 3B). Gene expression of *ARGAH1* and *ARGAH2*, catabolic genes of arginine pathway (Figure 1) was not changed upon infection with both strains of *P. syringae* as compared to control samples of *A. thaliana* (Figure 3A & B).

To elucidate the involvement of enzymes in arginine metabolism, amiRNA-based gene silencing was done. Mutant plant lines for *AS* and *AL* were developed. Gene silencing by amiRNA was done because dominant null mutation may prove to be lethal for these genes. Homozygous T3 lines were selected by segregation analysis using kanamycin as described in the methods section. Expression of *AS* and *AL* in selected lines was determined using qRT-PCR. Mutant lines were selected based on their gene expression profiles. Two mutant lines *as5-2* and *as9-2* shows 60% decrease in gene expression for *AS*. In case of *AL* two mutant lines *al3-4* and *al4-7* showed more than 50% decrease in expression (Figure 3C). These mutant lines were subjected to infection essays with virulent as well as avirulent strains of *P. syringae* DC3000.

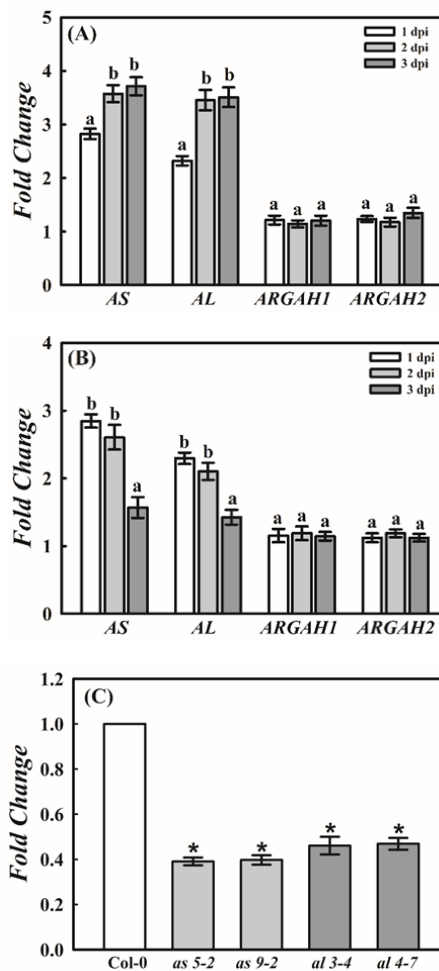
Infection essays were performed using virulent strain DC3000 *Pto* and avirulent strain DC3000  $\Delta avrPto/\Delta avrPtoB$ . In case of DC3000 *Pto* growth rate was significantly higher in all the tested amiRNA plant lines as compared to wild type (Col-0) plants during all three time points tested (Figure 4A). Whereas in case of DC3000  $\Delta avrPto/\Delta avrPtoB$  growth rate was significantly higher in mutant plants at 1 dpi and there was no significant difference at later time points (Figure 4C). Addition of L-arginine (1 mM) in infiltration medium reduced the growth rate of both *P. syringae* strains in mutant plants but did not affect the control plants significantly. However, overall growth rate was reduced in both control and mutant lines compared to non-treated plants (Figure 4 B & D).



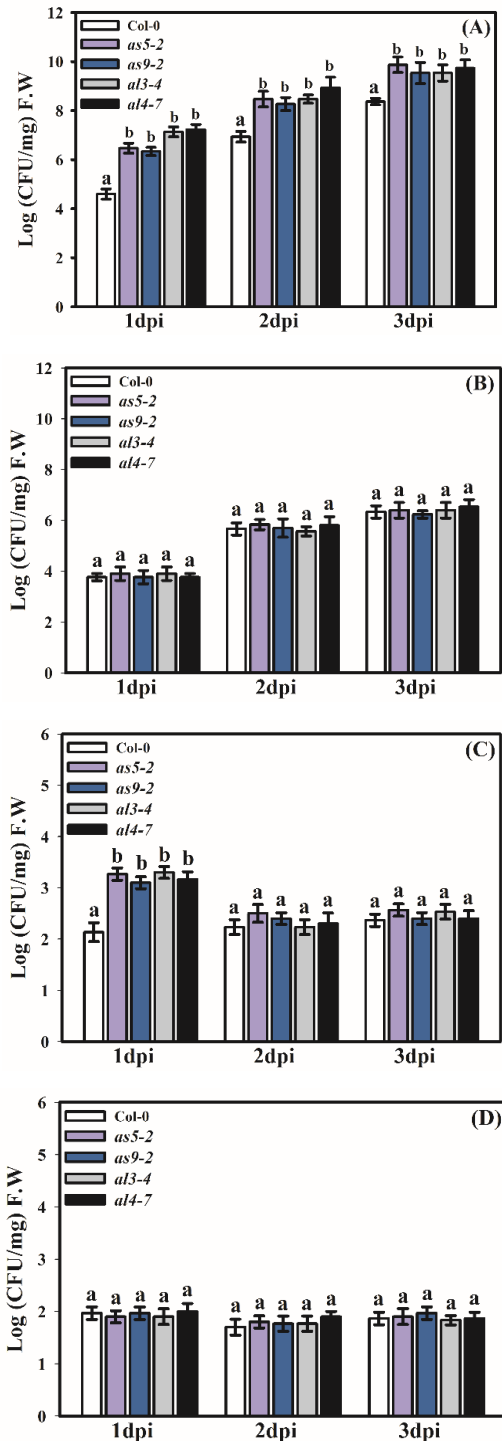
**Figure 1:** Arginine metabolism in *Arabidopsis thaliana*. Important enzymes are shown for each reaction. (modified from PMN: <https://plantcyc.org/>).



**Figure 2:** GUS staining upon *P. syringae* infection in leaves of pAS::GUS line. **A)** Mock treated leaves, **B)** *P. syringae* DC3000 ( $\Delta avrPto/\Delta avrPtoB$ ), **C)** *P. syringae* DC3000 (*Pto*); and pARGAH2::GUS line **D)** Mock treated leaves, **E)** *P. syringae* DC 3000 ( $\Delta avrPto/\Delta avrPtoB$ ), **F)** *P. syringae* DC3000 (*Pto*). Results were confirmed by two independently transformed plant lines for each GUS construct.



**Figure 3:** (A) Gene expression of *AS*, *AL*, *ARGAH1* and *ARGAH2* in *A. thaliana* leaves upon *P. syringae* DC3000 (*Pto*) infection. (B) Gene expression of *AS*, *AL*, *ARGAH1* and *ARGAH2* in *A. thaliana* leaves upon *P. syringae* DC3000 ( $\Delta avrPto/\Delta avrPtoB$ ) infection. (C) Gene expression of *AS* and *AL* in mutant *A. thaliana* lines. Gene silencing mutants were generated using amiRNAs for *AS* (*as5-2*, *as9-2*) and *AL* (*al3-4*, *al4-7*). Expression was determined in leaves of 15 day old plants by qRT-PCR. \* indicate significant differences from the control (Col-0), (t-test,  $p < 0.05$ ). **Fold Change**; Relative transcript levels were analysed by qRT-PCR using  $2^{-\Delta\Delta Ct}$  method. Values are means  $\pm$ SE,  $n = 3$  (biological replicates), for each gene, different letters indicate significant difference among time points tested ( $p < 0.05$ , one-way ANOVA, LSD).



**Figure 4:** Growth rate of *P. syringae* (DC3000) in *A. thaliana* wild type (Col-0) and amiRNA gene silencing plant lines. Gene silencing lines were generated for *AS* (*as5-2*, *as9-2*) and *AL* (*al3-4*, *al4-7*) genes. (A) Growth rate of *P. syringae* DC3000 (*Pto*) (B) Growth rate of *P. syringae* DC3000 (*Pto*) coinfiltrated with 1mM L-arginine. (C) Growth rate of *P. syringae* DC3000 (*ΔavrPto/ΔavrPtoB*) (D) Growth rate of *P. syringae* DC3000 (*ΔavrPto/ΔavrPtoB*) coinfiltrated with 1mM L-arginine. Growth rate determined at 1, 2 and 3 dpi was expressed as log (CFU/mg) fresh weight of plant leaves, values are means  $\pm$  SE, n = 3 (biological replicates), different letters indicate significant difference among different plant lines within a time point (one-way ANOVA, p < 0.05, LSD).

## Discussion

This study was performed to reveal the potential role of relevant genes for important enzymes of arginine metabolic pathway and their relevance in *A. thaliana*-*P. syringae* interactions. Presented data clearly indicate the remodeling of gene expression for these genes during *P. syringae* infection in *A. thaliana*. Our study shows importance of arginine anabolic genes and enzymes for successful plant defense against *P. syringae*. Induced gene expression of *AS* and *AL* upon *P. syringae* infection could be the result of defense related strategies employed by *A. thaliana*. Currently very little information is available about arginine cycle in *A. thaliana* and only a few studies are centered to this area of research [14]. Even, very less is known regarding its role in plant-pathogen interactions. However, it has been demonstrated that arginine cycling is modulated during compatible plant-nematode interactions [8,15]. Arginine is an important amino acid and centers many metabolic pathways involving plant defense reactions against abiotic [23] and biotic stresses [24,25]. Arginine cycle genes are also found to be modulated during mutualistic relationships of symbiotic fungi with plants [26].

To reveal the potential role of two important genes of arginine pathway, amiRNA-based gene silencing mutants of *A. thaliana* were generated. Argininosuccinate Synthase was suggested to be rate limiting enzyme of arginine biosynthesis [27]. Based upon these findings, two genes *AS* and *AL* were targeted for silencing in arginine metabolic pathway. Silencing of these genes reduced the expression significantly. Subsequently these mutants were subjected to infection assays with *P. syringae*. Silencing of *AS* and *AL* genes increased the growth rate of *P. syringae* on mutant plant lines. This phenomenon was reversed when L-arginine was added in the infiltration medium. Arginine is found to be essential for the synthesis of nitric oxide (NO) in *A. thaliana* [12] which is an important molecule as far as plant defense responses are concerned [10]. In our studies decrease in expression of *AS* and *AL* results in an increase of the growth of *P. syringae* on *A. thaliana*. This change can be traced back to reduced NO production and compromised plant defense. This hypothesis is also supported by the fact that decrease in the *AL* activity leads to the reduced production of NO [28].

Expression of two arginine catabolic genes associated with arginase enzyme was also studied. Arginase mostly functions to mobilize stored nitrogen from arginine in plants [13]. Arginase expression is also reported to be enhanced by wounding [29], as well as during pathogen infection [30]. Arginase and Nitric Oxide Synthase (NOS) use the same substrate, which is arginine [23]. Increase in the expression of arginine

synthesis genes and not in arginase genes suggests cycling of arginine towards NOS for the synthesis of NO because arginase negative mutants are suggested to accumulate more NO as compared to wild type plants [31].

Our data displays that silencing the two important genes in arginine biosynthesis increases the growth rate of *P. syringae* in *A. thaliana*. This could be the result of decrease in arginine production because these two genes are associated with the rate limiting enzymes of arginine cycle. Compromised defense seems to be due to decreased production of NO, which uses arginine as precursor. Addition of arginine in infiltration medium hindered with the growth of *P. syringae* in mutant plant lines as well as on wild type plants. This finding suggests that sufficient arginine is required for NO-mediated plant defense and its decrease improves the conditions for the growth of pathogen.

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## Conflict of Interest Statement

The authors declare that there is no conflict of interest regarding the publication of this paper.

## Authors' Contribution

Shahbaz Anwar, Muhammad Amjad Ali, Amjad Abbas and Krzysztof Wiczeorek all designed the study, carried out the lab work, drafted the manuscript and corrected the reviewed copy.

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