Plant Science Research 45 (1&2) : 52-60, 2023

Plant Science Research

ISSN 0972-8546

Cell death in *Arabidopsis* mediated by *AtATG6* provides immunity against *Magnaporthe oryzae*

Reecha Mohapatra, Arya Kumar Dibyananda Naik, Sujit Kumar Bhutia and Binod Bihari Sahu[Ψ]
Laboratory of Plant Immunity, National Institute of Technology Rourkela, Odisha, India, 769008

ARTICLE INFO

Article history:
Received : 3 October 2023
Revised : 6 November 2023
Accepted : 2 December 2023

Keywords:

Magnaporthe oryzae
non-host resistance
rice blast
hypersensitive response
programmed cell death

ABSTRACT

Rice succumbs to *Magnaporthe oryzae*, causing rice blast, while *Arabidopsis*, acting as a nonhost, deploys an active defense mechanism against the pathogen. Despite extensive gene and QTL identification, no cure for rice blast has been found. *Arabidopsis* employs non-host resistance (NHR) with a hypersensitive response (HR) involving localized programmed cell death, crucial for pathogen limitation and resistance. Recognizing the pivotal link between cell death and disease resistance, our study delves into the relationship between AtATG6 and HR-associated cell death, focusing on its role in resisting *Magnaporthe oryzae*. In our experiments, both wild-type Col-0 and ATG6 mutant *Arabidopsis* were exposed to *Magnaporthe oryzae*, with trypan blue staining and electrolyte assays gauging cell viability dynamics. Microscopic representation of the oxidative burst examined the correlation between reactive oxygen species (ROS) generation and cell death. As revealed by the relative expression patterns of defense genes (PR1, WRKY53, and WRKY29), the mutant ATG6 operates by subverting the defense mechanism. Raman spectra analysis uncovered compromised plant immunity, manifesting as variations in carotenoid levels. This study illuminates the intricate interplay of AtATG6, cell death, and disease resistance in defense against *Magnaporthe oryzae* by *Arabidopsis*.

1. Introduction

Plants encounter biotic stress from a diverse array of microorganisms throughout their lifecycle, with only a few posing harm. The evolution of defense mechanisms in plants, shaped by the co-evolution of pathogenic microorganisms and plants, involves the recognition features on the plant's surface (Burdon & Thrall, 2009; Dodds & Rathjen, 2010; Schulze-Lefert & Panstruga, 2011). The vigilance in plant defense limits the compromise of innate defenses by pathogens, resulting in disease induction by only a few (U. Lipka, Fuchs, & Lipka, 2008). Plants exhibit induced resistance that operates at locations distant from the initial infection, providing long-term resistance triggered as an "alert signal" after the first encounter with pathogens. Conversely, successful breaches of inherent defenses during compatible interactions allow pathogen colonization, while active resistance prevents colonization during incompatible interactions (Dangl & Jones, 2001; Gill, Lee, & Mysore, 2015; Thordal-Christensen, 2003).

The decline in staple crop production, particularly rice (*Oryza sativa* L.), is attributed to various biotic and abiotic stresses globally, with fungal diseases causing significant production losses. The rice blast disease, caused by the highly damaging hemibiotrophic fungus *Magnaporthe oryzae*, remains a persistent challenge despite advanced disease management strategies. Developing disease-resistant cultivars through the incorporation of nonhost genes is a viable long-term solution (Devanna *et al.,* 2022; Reddy *et al.,* 2021).

Nonhost resistance (NHR), a broad-spectrum resistance against all genetic variants of a specific disease in a particular

[Ψ] *Corresponding author; Email:* binodbiharisahu@gmail.com

plant species, relies on self- and non-self-recognition within the plant immune system (Hadwiger, 2015; V. Lipka *et al.,* 2005; Senthil-Kumar & Mysore, 2013). Understanding the mechanisms of NHR and utilizing them for sustainable farming is a priority. NHR involves pre-formed physical and chemical barriers at the pre-penetration stage, with the identification of NHR genes and their molecular features being crucial for developing disease-resistant crop varieties (da Cunha, McFall & Mackey, 2006).

The intricate plant immune system encompasses various responses, including pathogen-induced cuticular wax synthesis, reactive oxygen species (ROS) production, and hypersensitive cell death. Plant recognition of pathogen-associated molecular patterns (PAMPs) initiates PAMP-triggered immunity (PTI), the first line of defense. Effector-triggered susceptibility (ETS) occurs when pathogens suppress PTI, leading to susceptibility in hosts. The zig-zag model illustrates the interplay between effector-triggered immunity (ETI) and PTI, involving cellular processes such as MAP kinase signaling, ROS production, and hypersensitive response (HR) (Dodds & Rathjen, 2010; Jones & Dangl, 2006; Zurbriggen, Carrillo, & Hajirezaei, 2010).

The genetically tractable Arabidopsis system provides insights into innate immunity and programmed cell death (PCD) pathways, including autophagy. Recent data from autophagy-deficient Arabidopsis suggest a significant role for autophagy in controlling plant immune responses, although its precise role remains unclear (Talbot & Kershaw, 2009; Yoshimoto *et al.,* 2009). Autophagy-related (ATG) genes, including ATG6, are involved in this catabolic process, impacting plant stress responses and pathogen-induced cell death (Hayward & Dinesh-Kumar, 2011; Hofius *et al.,* 2009).

ATG6, part of a complex with class III phosphatidylinositol T-kinase (PI3K)/Vps34, plays a role in vacuolar protein sorting (VPS) and autophagy in yeast. Plant ATG6 deficiency reduces autolysosome production, leading to increased susceptibility to stressors. ATG6's involvement in autophagy suggests its potential role in agricultural improvements and immunity-associated plant cell death (Furuya *et al.,* 2005; Patel & Dinesh-Kumar, 2008).

While the molecular mechanisms of autophagy and ATG6-associated pathways have received limited attention in crop plants, understanding these processes in nonhost model organisms may hold the key to enhancing disease resistance and agricultural productivity (Edinger & Thompson, 2004; Greenberg & Yao, 2004; Levine & Klionsky, 2004). In the present study, we delved into the molecular mechanisms through which AtATG6 orchestrates autophagy and oversees hypersensitive response-programmed cell death (HR-PCD) in the innate immune response within the nonhost, specifically *Arabidopsis*. Our findings reveal that ATG6 plays a crucial role in conferring immunity, linked with plant cell death, and holds the potential to be a key factor in advancing agricultural practices.

2. Materials and Methods

2.1 Plant growth and maintenance:

The *Arabidopsis thaliana* wild ecotype Col-0 (N1093) and the ATG6 mutant (N678948; homozygous for its T-DNA insertion) were sourced from the Nottingham *Arabidopsis* Stock Centre (NASC) and cultivated in a plant growth chamber. Ten-day-old seedlings were grown on flats containing a mixture of agropeat and vermiculite soil (3:1) under controlled conditions: light maintained at ~100 µE/m^2/s, temperature at 22°C, humidity at 65%, with a 14-hour light: 10-hour dark cycle. The growth medium was supplemented with ½ strength Hoagland growth media. Leaves from 21-day-old seedlings were utilized for the infection assay. Seeds from the respective ecotype and mutant were harvested and stored at 4°C for future use.

2.2 Pathogen culture conditions:

M. oryzae spores were collected from the National Rice Research Institute (ICAR-NRRI) and grown on freshly prepared oatmeal agar (OMA) plates at 28^0 C until sporulation (~7-9 days) for use during infection assay. The spore blocks from the old stock were transferred to the freshly prepared OMA medium in every 7-10 days. Antibiotic streptomycin (100µg/mL) was used to avoid any bacterial contaminations.

2.3 Infection assay:

The detached leaf assay for infection was performed by taking three leaves of 21 days old seedlings of soil grown *Arabidopsis*. Leaves of wild type and mutant ecotype of *Arabidopsis* were inoculated on right side of leaves beneath mid rib with 10 µL of conidia (approximately 10^5 spore/mL) extract of *M. oryzae* in 0.01% tween 20 solution. As a control, 10 µL drop of 0.01% tween 20 solution was put on the leaves. Three upper rosette leaves from each seedling were detached and kept on the moist filter paper in petriplates to maintain 100% humidity that is suitable for infection by the rice blast fungus. It was further covered by the Petri dish and kept under dark for a day and was exposed to light thereafter until disease progression was experimented. The phenotypes were observed at 1 DPI and 3 DPI. Three experimental set-ups were used for each ecotype.

2.4 Trypan Blue Staining:

The inoculated leaves were harvested for staining with

trypan blue to observe cell viability at desired time points. The inoculated leaves were kept dipped in alcoholic lactophenol for around 24 hours in the cups of 24 well microtiter plate until chlorosis. Leaves in the microtiter plate were stained with trypan blue (250 µg/mL) made in lactophenol (phenol: glycerol: lactic acid: water 1:1:1:1, v/v) for 15 minutes. It was further destained with lactophenol for ~1 hour; mounted in 50% glycerol and examined under bright field microscope (Vogel & Somerville, 2000).

2.5 Electrolyte leakage assay:

Two leaf discs of each ecotype (both control and treated) were cut and immediately put into well plates containing 2mL of sterile distilled water. Covered the plate with lid and incubated for 30minutes in a growth chamber. Then the water was replaced in each well with 2mL fresh sterile water. Again, it was incubated for different time intervals in the growth chamber. Calibrated electrolytic conductivity meter was used to check the conductivity of the solution. Then after, 100uL water sample from each well were put on the conductivity meter (LAQUAtwin-EC-33, HORIBA Scientific) and measured for the electrolyte conductivity at the determined time points (Jamra *et al.,* 2021).

2.6 DAB staining:

Diaminobenzidine (DAB) staining was performed to stain the reactive oxygen species (ROS) in early hour of infection (Daudi & O'Brien, 2012). Firstly, the treated leaves were soaked in DAB staining solution (1mg/mL) for 12hr. Then it was replaced by distilled water and kept for 12hr. Further, the leaves were dipped in solution of ethanol: acetic acid (96: 4) for chlorosis. After destaining, leaves were observed under brightfield microscope and images were recorded in an inverted light microscope (Magnus, Magcam-DC5 and OLYMPUS) for screening.

2.7 RNA (Ribonucleic acid) extraction and RT-qPCR analysis

The extraction of RNA from the control and treated leaves of *Arabidopsis* accessions were done using the TRIzol reagent following manufacturer's instructions. RNA concentration and purity was measured by the help of Nanodrop and 1.2% agarose gel electrophoresis, respectively. DNase I treatment was employed to remove any genomic DNA (Deoxyribonucleic acid) which was further confirmed by performing -RT-qPCR reaction (Actin primer set was used, gDNA amplification size is 220 bp and cDNA amplification size is 134 bp). Two µg of RNA samples were further processed to synthesize cDNA using cDNA synthesis kit. The level of expression of *PR1, WRKY53* and *WRKY29* marker genes were recorded and normalized to the expression level of *AtACTIN2* as internal control (Table 1).

2.8 Raman Spectroscopy

Fresh inoculated leaflets were collected at 3dpi and positioned on the stage mount onto a glass slide and used to take Raman spectral reading. Treated leaves were punched from the selected infected areas. The leaf samples were placed on the glass slide. The Raman measurement conditions were 800–1800 cm^{-1} of spectral range, 10 s of acquisition time, 20mW laser power, 532 nm visible light band, 1200gr/mm grating, 100µm slit, 300µm hole and 20x magnification objective (micro spot with 10µm ø). The calibration was performed daily by recording the Raman signal of a silicon wafer. In total, 3 biological replicates of spectral data sets were obtained from each control and infected plants. Raman spectra shown in this work correspond to the raw baseline corrected results along with smoothing of line graphs using Origin Pro 8.5 software. (Butler *et al.,* 2016; Vallejo-Pérez *et al.,* 2021).

2.9 Statistical analysis

Origin Pro 8.5 was used to calculate the Raman shifts obtained as raw data from the measurement of the spectra for baseline correction and deducing the graph. Graph Pad Prism 8.0.1 was used to prepare the fold changes in the expression of the differentially expressed genes under stress.

Table 1. List of primers used in qRT-PCR

Gene	Forward Primer	Reverse Primer
AtACT2	TCGGTGGTTCCATTCTTGCT	GCTTTTTAAGCCTTTGATCTTGAGAG
PR1	AAAACTTAGCCTGGGGTAGCGG	CCACCATTGTTACACCTCACTTTG
WRKY53	ACACCACCATTAGCCTCGCC	ACGCGGGGAAAGTTGTGTCA
WRKY29	CGGAG*ATG*GAGACAAGTGGCTT	TGTGAGGATCGTTTGTGTGGAGAA

3. Results

3.1 Arabidopsis *mutant* ATG6 *exhibits breach in immunity against* M. oryzae

The autophagy protein 6 encoded by *AT3G61710* functions for autophagosome assembly, mitophagy and protein targeting to vacuole that is meant for inducing programmed cell death in *Arabidopsis* (Feng, De Rycke, Dagdas, & Nowack, 2022; Lai, Wang, Zheng, Fan, & Chen, 2011; Lee et al., 2018). During pathogen attack, the plants sense the invader and counteract with several defence mechanisms according to the severity of infection. This defence response includes cell death restricting the pathogen spread in the host tissues. With the purpose of finding out the role of *AtATG6* in disease resistance against rice blast we used the homozygous T-DNA insertion line to check its possible involvement relating to disease resistance.

To study the interaction and pathogenicity of *M. oryzae* in wild type *Arabidopsis* Col-0 and mutant *ATG6,* trypan blue staining was performed. Unlike in Col-0, the pathogenicity of *M. oryzae* in *ATG6* with a susceptible response as early as 1 dpi was observed as evidenced from appressoria and hyphae formation. Trypan blue stain confirmed increased number of appressoria, heavy mycelia growth of the pathogen along with higher cell death due to hyphal penetration in *ATG6* 3 dpi leaves as compared to Col-0 (Fig. 1). This showed attempted but failed pathogen penetration inside the cell. This depicts the rise in hypersensitive cell death in *ATG6* at the entry site the pathogen during infection. Hence, this result confirms the contribution of *AtATG6* in offering nonhost resistance to the plant.

3.2 Ion conductivity enhanced in ATG6 *due to impaired immunity*

During pathogen attack the cell membrane integrity is somehow disrupted or completely lost due to cell death. This triggers higher chance of ion leakage from the cell. The computation of cell death from detached leaves of Col-0 and *ATG6* challenged with *M. oryzae* at 1dpi, 2dpi and 3 dpi using an ion leakage test was consequently of interest. Upon normalizing the measured ion leakage values (µ&!$^{-1}$) to the corresponding water control, the treated leaves of *ATG6* demonstrated increased cell death in comparison to Col-0 (Fig. 2). The infection by *M. oryzae* caused *ATG6* to have weakened immunity at increasing time points. The data are consistent with the trypan blue staining assay and can be correlated.

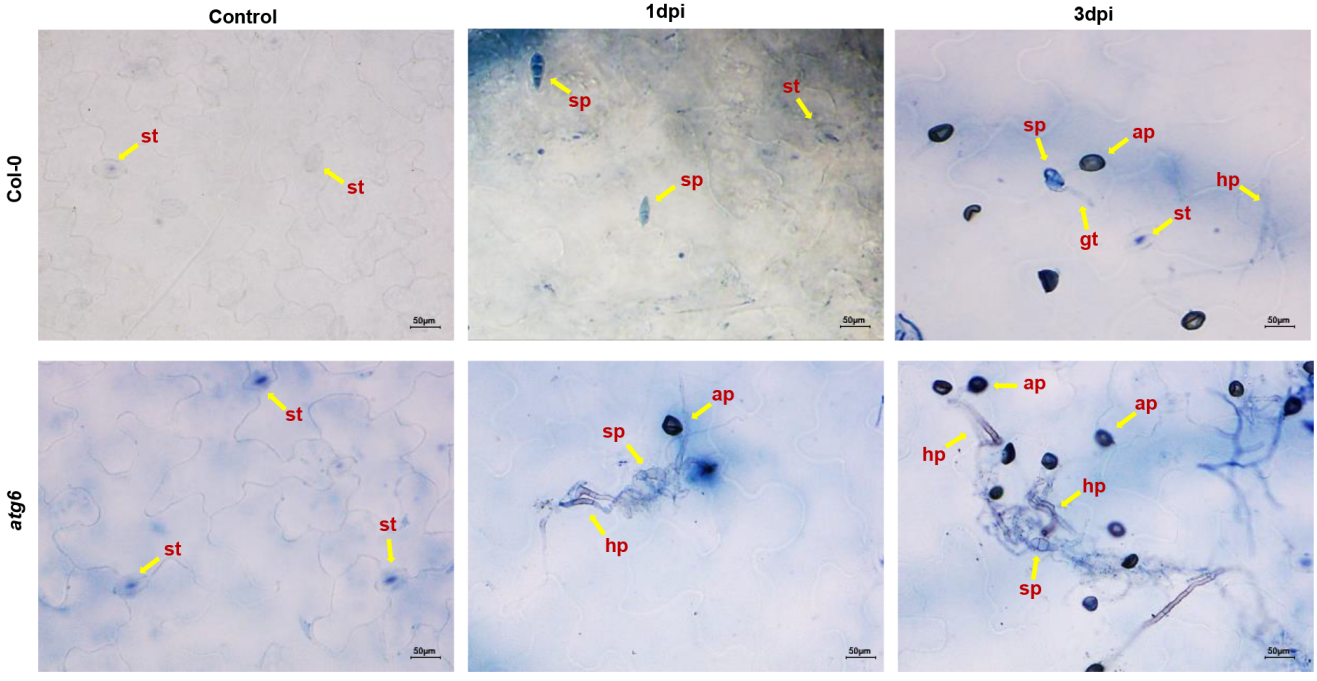

Figure 1: Trypan staining of infected leaves of Arabidopsis. Differential cell death in Col-0 (WT) and *atg6* (mutant) using trypan blue staining at 1 and 3 dpi. st, stomata; sp, spore/conidia; ap, appressoria; gt, germ tube. Scale bar =50µm

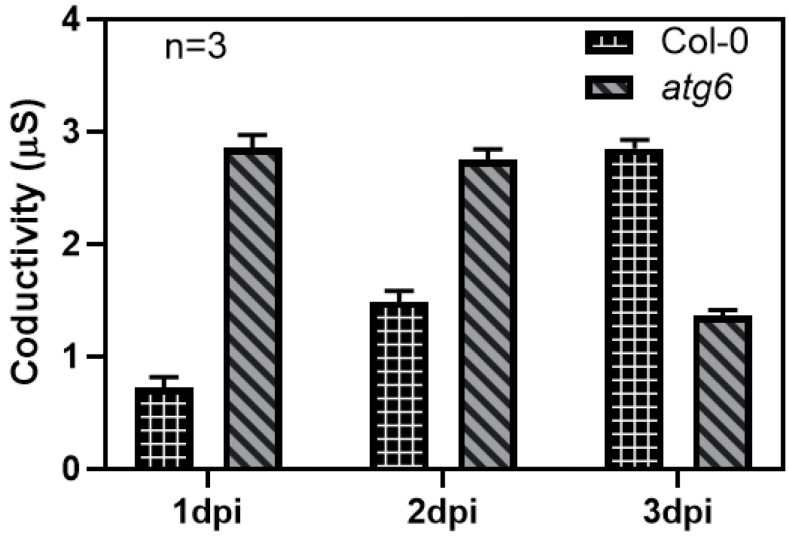

Figure 2: Electrolyte conductivity of infected leaves of Arabidopsis. Differential electrolyte leakage in Col-0 (WT) and *atg6* (mutant) measured at 1, 2 and 3 dpi. Equal area was used to measure the leakage. Three independent biological replication was used to calculate the SD.

3.3 ATG6 undergoes oxidative burst upon infection with M. oryzae

At the site of plant-pathogen contact, the production of ROS by plants serves as an early defence mechanism against biotic stress (Torres, 2010). As a result, DAB staining was performed to track the build-up of H_2O_2 as a yellowish-brown stain. Compared to Col-0, *ATG6* exhibited a noticeably higher level of H_2O_2 generation, as we discovered. Thus, an increase in the hypersensitive response brought upon pathogen invasion is associated with increased H2O2 generation (Fig. 3).

3.4 Differential expression of defence related genes

Activation of various molecular and physiological changes in plants are responses after sensing of challenges from intruding pathogen. Following pathogen infection, host synthesises several signalling cascades at various levels of defence. These signalling molecules includes various

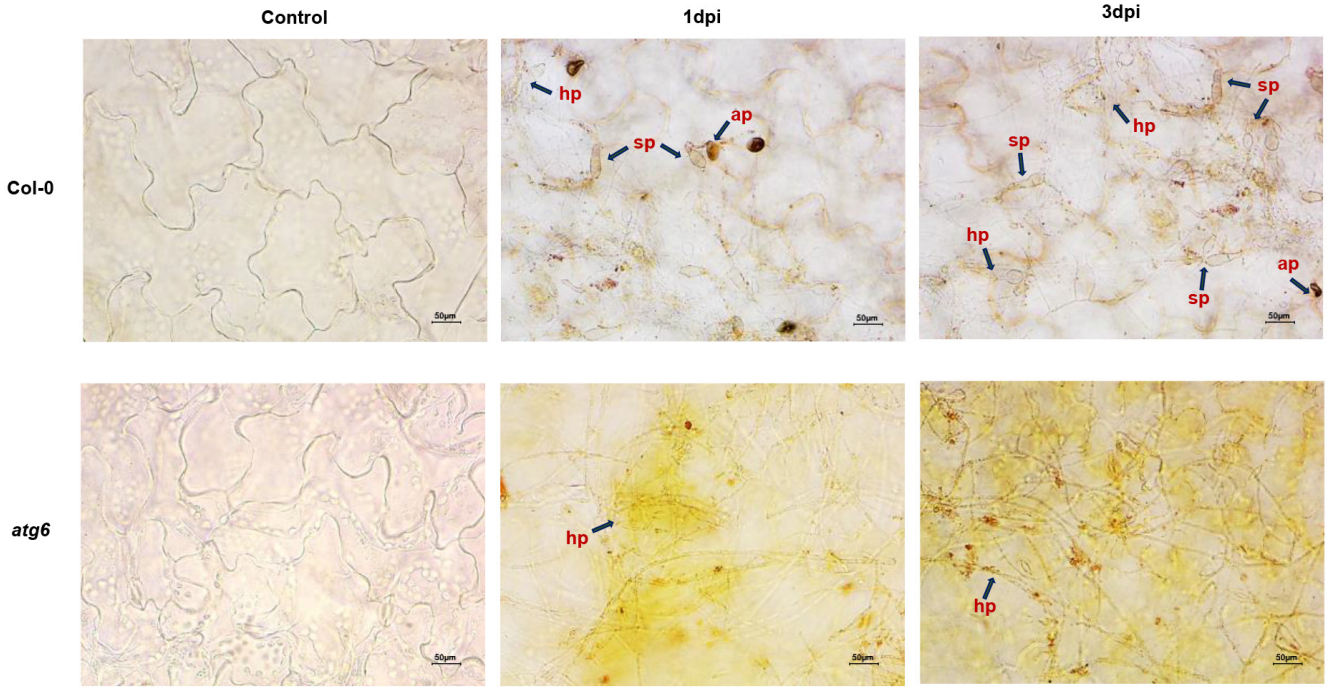

Figure 3: DAB staining of infected leaves of Arabidopsis. Differential ROS generation in Col-0 (WT) and *atg6* (mutant) using DAB staining at 1 and 3 dpi. St, stomata; sp, spore/conidia; ap, appressoria; hp, hyphae. Scale bar =50µm

hormones like salicylic acid (SA), jasmonic acid (JA), and ethylene as initial mode of defence. At molecular level, many defence related genes are activated in host plants further triggering the plant immune responses such as pathogen-triggered immunity (PTI) and effector-triggered immunity (ETI) (Gill *et al.,* 2015; He *et al.,* 2007; Rezaei, Mahdian, Babaeizad, Hashemi- Petroudi, & Alavi, 2019). Among the defence marker genes, *PR1* and *WRKY53* are responsible for early defence that is PTI, whereas *WRKY29* is involved in hypersensitive responses in later stages of infection (Hönig, Roeber, Schmülling, & Cortleven, 2023; Jiao *et al.,* 2022; Yi, Shirasu, Moon, Lee & Kwon, 2014). The relative expression level of *PR1* and *WRKY53* in *ATG6* in contrast to Col-0 increased after pathogen attack while expression of *WRKY29* is like or slightly higher than in Col-0 which depicts the elevated PTI responses in *ATG6* unlike in Col-0.

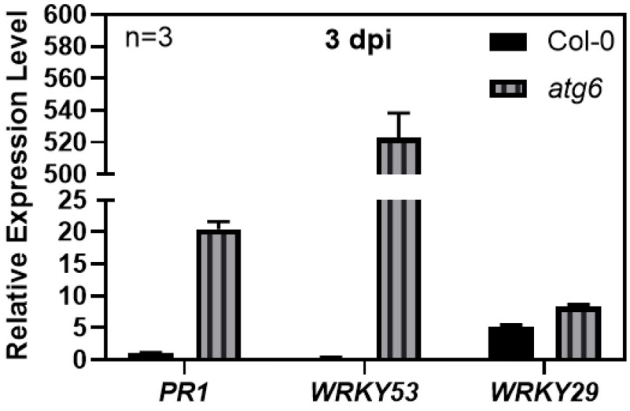

Figure 4: Differential expression of defence markers using qRT-PCR. Representation of the differential expression of genes *PR1*, *WRKY53* and *WRKY29* in Col-0 (WT) and *atg6* (mutant). Actin was used as internal control. Three independent biological replication was used to calculate the SD.

3.5 Spectral differences between Col-0 (WT) and ATG6 *indicate the differential expression of biomolecules involved in ROS chelation and other responses*

The Raman spectra obtained from *Arabidopsis* ecotypes of water control and infected leaves exhibited peaks associated to cellular components, and most prominent vibrational bands were associated to carbohydrates, carotenoids, chlorophyll, and phenolic compounds (Butler *et al.,* 2016; Chen, Zeng, Larkum, & Cai, 2004; Qin, Chao, & Kim, 2012). The plant-pathogen interaction is a complex biological system which can manipulate the plant metabolism and evade defence responses. Thus, the biochemical alterations were induced during the *M. oryzae* invasion, and these shifts were detectable in the Raman spectra (Mandrile *et al.,* 2019; Picaud, Le Moigne, Gomez de Gracia, & Desbois, 2001). The observed shift in peaks suggests degradation of carotenoids in the mutant *ATG6* due to breach in immunity as compared to Col- 0 (Figure-5).

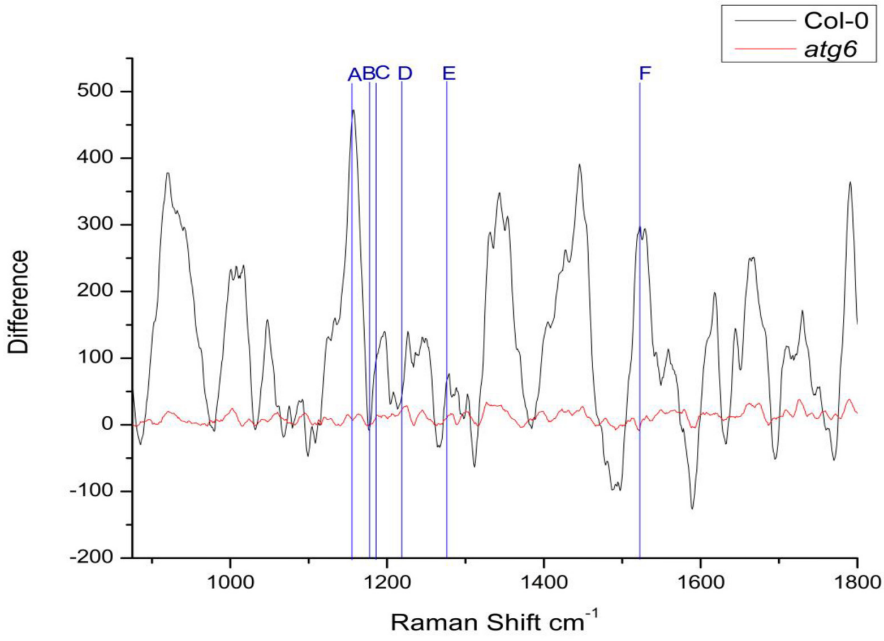

Figure 5. Raman spectra of the Arabidopsis infected with *M. oryzae* conidia. The difference in Raman spectra was calculated by deducting the value of control from the Infected. The raw data obtained from Raman Spectra was corrected with its base line. Vertical lines represent the specific compounds of carotenoids. A- 1155, B- 1180, C- 1185, D- 1218, E- 1276, G- 1521 (Vallejo-Pérez, M. R., et al. (2021); Zeng, J., et al. (2021))

5. Discussion

In the present investigation, we have observed that a mutation in AtATG6 leads to a breach in the plant immune system, resulting in hypersensitive cell death at the sites of pathogen entry (Patel & Dinesh-Kumar, 2008; Xu *et al.,* 2017). This increase in cell death at the infection zones subsequently induces electrolyte leakage, as ATG6 compromises cell membrane integrity (Kacprzyk, Dauphinee, Gallois, Gunawardena, & McCabe, 2016). Microscopic examinations reveal heightened ROS generation in the challenged leaves of ATG6, suggesting the crucial role of ROS as a signaling molecule in defense reactions (Gechev, Van Breusegem, Stone, Denev, & Laloi, 2006; Suman *et al.,* 2021). Consequently, compromised immunity in ATG6 contributes to elevated expression levels of defense genes such as PR1, WRKY53, and WRKY29 (Hönig *et al.,* 2023; Jiao *et al.,* 2022; Yi *et al.,* 2014). Changes in the Raman spectra indicate significant degradation of plant compounds like carotenoids and chlorophylls in pathogen-challenged plants compared to their controls (Zeng *et al.,* 2021).

Collectively, our results lead to the conclusion that cell death mediated by AtATG6 plays a crucial role in disease resistance against *M. oryzae*, contributing to the safeguarding of plant immunity. It is imperative to unravel the core mechanisms of AtATG6 and associated defense genes in conferring resistance to the host plant, elucidating the interconnected pathways involved.

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