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Small RNA 2 from the beneficial fungus *Trichoderma atroviride* targets plant gene *At-EDA9 of Arabidopsis thaliana* to establish a mutualistic relationship

Tesis que presenta

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La tesis "**The small RNA 2 from the beneficial fungus *Trichoderma atroviride* targets plant gene *At-EDA9* of *Arabidopsis thaliana* to establish a mutualistic relationship**" presentada para obtener el Grado de Maestra en Ciencias en Biología Molecular fue elaborada por **Emily Daniela Gallardo Andrade** y aprobada el 3 de diciembre de 2024 por los suscritos, designados por el Colegio de Profesores de la División de Biología Molecular del Instituto Potosino de Investigación Científica y Tecnológica, A.C.

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Acta de examen

Dedicatoria

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Abbreviations

EDA9	*Embryo sac Development Arrest 9*
ET	Ethylene
ETI	Effector-Triggered Immunity
ETS	Effector-Triggered Susceptibility
EVs	Extracellular Vesicles
HR	Hypersensitive Response
ISR	Induced Systemic Resistance
JA	Jasmonate
LRR	Leucine-Rich Repeats
MAMPs	Microbe-Associated Molecular Patterns
NB-LRR	Nucleotide Binding Leucine-Rich Repeats
PAMPs	Pathogen-Associated Molecular Patterns
PDF1.2	Plant Defensin 1.2 Gene
PGDH	3-Phosphoglycerate Dehydrogenase
PPSB	Phosphorylated Pathway of Serine Biosynthesis
PR-1A	Pathogenesis-Related 1A Gene
PRRs	Pattern Recognition Receptors
PSAT	3-Phosphoserine Aminotransferase
PSP	Phosphoserine Phosphatase
PTI	PAMP-Triggered Immunity
ROS	Reactive Oxygen Species
SA	Salicylic Acid
SAR	Systemic Acquired Resistance
SER	L-Serine

Glossary

Argonaute (AGO) Proteins: Proteins that are part of the RNA-induced silencing complex (RISC), which plays a role in RNA silencing and regulation of gene expression.

Cross-Kingdom RNA Silencing: A process where small RNAs produced by one organism (e.g. fungus) used to regulate gene expression in another (e.g. plant), impacting immune responses and development.

Dicer-Like Proteins (DCL): Enzymes involved in the biogenesis of small RNAs, which are key regulators in RNA interference mechanisms.

Effector Molecules: Proteins or other molecules secreted by pathogens to manipulate host cell processes, often to suppress plant immunity and facilitate infection.

ETI (Effector-Triggered Immunity): A stronger and more specific immune response activated when plant resistance proteins recognize pathogen effectors. It often leads to a hypersensitive response to restrict pathogen spread.

Growth Promotion: The increase in plant growth and development often induced by beneficial microorganisms like *Trichoderma* through mechanisms such as enhanced nutrient uptake or hormone modulation.

Hemibiotrophic Pathogen: A pathogen that initially lives in harmony with the host (biotrophically) and later switches to a destructive necrotrophic phase. *Pseudomonas syringae* is an example.

Hypersensitive Response (HR): A localized cell death response in plants that occurs at the site of pathogen attack, effectively containing the pathogen and preventing its spread.

ISR (Induced Systemic Resistance): A form of systemic immunity in plants triggered by beneficial microbes like *Trichoderma*, usually mediated by phytohormones such as jasmonic acid (JA) and ethylene (ET).

Mutant Line: A genetically modified plant where a specific gene is disrupted or altered to study its role in plant growth, development, or defense.

Necrotrophic Pathogen: A type of pathogen that kills host tissue and then feeds on the dead material. *Botrytis cinerea* is an example of a necrotrophic pathogen.

Overexpressing Line: A genetically modified plant line in which a particular gene is expressed at higher-than-normal levels to study its effects on physiology and resistance.

PAMP (Pathogen-Associated Molecular Patterns): Molecules commonly found in pathogens that are recognized by plants as signals of infection, triggering immune responses.

Phosphoglycerate Dehydrogenase (PGDH): An enzyme involved in the phosphorylated pathway of serine biosynthesis. It plays a crucial role in regulating plant metabolism and stress responses.

PTI (PAMP-Triggered Immunity): The first layer of plant immune response, triggered upon recognition of PAMPs by plant receptors, leading to defense mechanisms such as reactive oxygen species (ROS) production.

Reactive Oxygen Species (ROS): Highly reactive molecules containing oxygen, produced as part of plant defense. They can damage cellular components but also signal for defense activation.

Rhizosphere: The region of soil that surrounds and is influenced by plant roots. It is rich in microbial activity and plant-microorganism interactions.

SAR (Systemic Acquired Resistance): A whole-plant immune response that provides long-term resistance against a broad spectrum of pathogens. It is primarily regulated by salicylic acid (SA).

Serine Biosynthesis Pathway: A metabolic pathway crucial for producing serine, an amino acid involved in the synthesis of other compounds such as cysteine and glutathione, and essential for plant stress response.

sRNA (Small RNA): Short, non-coding RNA molecules that play a role in gene regulation by targeting mRNA for degradation or by preventing translation.

***Trichoderma* spp.:** A genus of filamentous fungi known for its role in promoting plant growth and providing biocontrol against plant pathogens by secreting enzymes and antimicrobial compounds.

Resumen

El ARN pequeño 2 del hongo benéfico *Trichoderma atroviride* hace blanco en el gen *EDA9 de Arabidopsis thaliana* para establecer una relación mutualista

En la rizosfera, las plantas interactúan con diversos microbiomas que pueden incluir microorganismos como bacterias, oomicetos y hongos. Como organismos sésiles, las plantas han desarrollado variados mecanismos de reconocimiento y sistemas de respuesta de defensa para sobrevivir en interacción con microorganismos fitopatógenos y beneficiosos. En este estudio, evaluamos el rol regulatorio del RNA pequeño 2 (*Ta*_sRNA2) del hongo benéfico *Trichoderma atroviride,* que media un silenciamiento por entre reinos al hacer blanco en *At-EDA9* de *Arabidopsis thaliana*. Nuestros hallazgos muestran que *Ta*_sRNA2 silencia *At-EDA9* al dirigirse a su transcrito, alterando la respuesta de *Arabidopsis* ante el estrés biótico. La sobreexpresión de *At-EDA9* mejoró la resistencia al patógeno necrotrófico *Botrytis cinerea*, mientras que las líneas sobreexpresantes presentaron una mayor susceptibilidad al patógeno hemibiotrófico *Pseudomonas syringae*. Además, se observó que la sobreexpresión de *At-EDA9* parece interferir con la promoción de crecimiento mediada por *Trichoderma.* Por otro lado, la mutante *eda9* no mostró inducción de biomasas ni en la condición control ni durante la interacción con *Trichoderma,* lo que podría indicar que *At-EDA9* desempeña un papel en la promoción de crecimiento inducida por el hongo.

Este mecanismo de silenciamiento entre reinos eleva el papel de *Ta*_sRNA2 en la modulación de las respuestas inmunes de la planta y la formación de biomasa. El silenciamiento selectivo de *At-EDA9* por *Ta*_sRNA2 sugiere que *Trichoderma* media finamente el metabolismo de la planta para optimizar la relación mutualista. Nuestros hallazgos mejoran la comprensión del uso de *Trichoderma* en métodos agrícolas sostenibles y optimizados para la respuesta al estrés, mejorando la producción de cultivos.

Palabras clave: Inmunidad vegetal, *Trichoderma,* Mecanismo de silenciamiento, Interacción entre reinos, Fitopatógenos.

Abstract

The small RNA 2 from the beneficial fungus *Trichoderma atroviride* targets plant gene *EDA9* of *Arabidopsis thaliana* to establish a mutualistic relationship

In the rhizosphere, plants interact with diverse microbiomes that may include microorganisms such as bacteria, oomycetes and fungi. As sessile organisms, plants have evolved a variety of recognition mechanisms and defense response systems to survive deleterious interactions with pathogens or beneficial microorganisms. In this study, we investigated the regulatory role of the small RNA 2 (*Ta*_sRNA2) from the beneficial fungus *Trichoderma atroviride* which mediates cross-kingdom RNA silencing by targeting *At-EDA9* in *Arabidopsis thaliana*. Our findings show that *Ta*_sRNA2 silences *At-EDA9* by targeting its transcript, thereby altering *Arabidopsis thaliana´s* responses to biotic stress. We observed that the overexpression of *At-EDA9* enhanced resistance against the necrotrophic pathogen *Botrytis cinerea,* while the overexpressing lines exhibited increased susceptibility to the hemibiotrophic pathogen *Pseudomonas syringae.* Additionally, it was observed that *At-EDA9* overexpression appears to interfere with the growth promotion mediated by *Trichoderma.* In contrast, the *eda9* mutant did not show biomass induction under control condition or after its interaction with *Trichoderma,* suggesting that *At-EDA9* may play a role in the growth promotion induced by the fungus.

This RNA-mediated silencing mechanism highlights the role of the *Ta*_sRNA2 in modulating plant immune responses and biomass formation. The selective silencing of *At-EDA9* by this small RNA suggests that *Trichoderma* fine tunes plant metabolism to optimize the mutualistic relationship. Our findings improve our understanding of the use of *Trichoderma* in sustainable and optimized agricultural practices in stress response, enhancing crop production.

Keywords: Plant Immunity, *Trichoderma*, Silencing mechanism, Cross-kingdom interaction, Phytopathogens.

Introduction

In the rhizosphere, plants interact with a plethora of organisms, including animals and a wide range of microorganisms such as bacteria, fungi, and oomycetes. These complex interactions drive the evolution of plant defense mechanisms against biotic and abiotic stresses. Plants, for instance, upregulate the production of phytochemical compounds, activate cellular signaling pathways, detect microbial patterns or danger signals, trigger the activation of pattern recognition receptors (PRRs), and promote the expression of specialized pathogen-related (PR) genes expression. Similarly, phytopathogens have also gained a counter-defense response against adaptative strategies of the plants, producing enzymes to penetrate their physical barriers and secreting specific proteins to suppress the plant's chemical and immune defenses (De-la-Peña et al., 2012).

Once the plant’s physical and chemical barriers such as the cell wall and phytoanticipins are breached, its immune response to biotic stress operates in multiple layers. First, pathogen- or microbial-associated molecular patterns (PAMPs or MAMPs) are recognized by pattern-recognition receptors (PRRs), prompting PAMP triggered immunity (PTI). PTI is characterized by the local production of reactive oxygen species (ROS), activation of mitogen-activated protein kinase (MAPK) cascades, and the induction of transcription factors that activate pathogenesis-related (PR) genes (Groen et al., 2013; Mitra & Suprasanna, 2024). During PTI, microbial pathogens are effectively prevented from colonizing the plant further.

However, some phytopathogens have evolved strategies to evade PTI by secreting effector molecules that can suppress the plant’s initial immune response, facilitating tissue colonization. These specialized effectors promote plant susceptibility by inactivating defense enzymes in the apoplast or disrupting the recognition of PAMPs by plant membrane receptors. If PRRs fail to recognize these molecular patterns, the pathogen can block local immune responses by inhibiting signaling cascades that activate defense genes. As a result, pathogen effector molecules suppress the plant’s defense signaling, promoting pathogen spread (Kloppholz, Kuhn, and Requena, 2011). Once the PTI threshold is

surpassed, and the signaling cascade is blocked, resistance gene activation is impaired leading to effector-triggered susceptibility (ETS). In response, the plant may trigger a second layer of defense known as effector-triggered immunity (ETI), which is mediated by the recognition of pathogen effectors through nucleotide-binding leucine-rich repeat (NB-LRR) proteins. This recognition can trigger a hypersensitive response (HR) at the infection site, halting pathogen growth and limiting further tissue colonization (Llorens et al., 2020).

This local response is accompanied by the systemic activation of defense mechanisms, leading to systemic acquired resistance (SAR), which protects throughout the plant against subsequent pathogen attacks. SAR is primarily regulated by salicylic acid (SA) and plays a crucial role in defending against biotrophic and hemibiotrophic pathogens, such as *Pseudomonas syringae*. In contrast, induced systemic resistance (ISR), is regulated by the phytohormones jasmonic acid (JA) and ethylene (ET), which are key in defense against necrotrophic pathogens such as *Botrytis cinerea,* as well as in facilitating beneficial interactions with microorganisms, including fungi of the *Trichoderma* genus (Cao et al., 1997; Radhika et al., 2015; Weiberg et al., 2013).

Trichoderma spp*.* is a filamentous ascomycete fungus commonly found in soil. Its reproductive strategy in nature is a low frequency of sexual reproduction via ascospores and a high frequency of asexual reproduction through conidiation, which promotes adaptive variation. For instance, certain mutations have enabled *Trichoderma* spp. to colonize diverse habitats, compete for space, decompose organic matter (such as dead host), and feed on other fungi through mycophagy (Chaverri & Samuels, 2013). *Trichoderma* is also known for secreting antibiotics that protect plant roots by inhibiting the growth of harmful microorganisms in the rhizosphere (Lanzuise et al., 2022). Its primary mode of action in the rhizosphere involves antagonism and direct or indirect competition with other microorganisms. By colonizing plant roots, *Trichoderma* modulates microbial interactions in the rhizosphere and acts as a biological control agent, providing an eco-friendly alternative to traditional hazardous agrochemicals. Additionally, *Trichoderma*

promotes plant growth by enhancing nutrient uptake, stimulating root development, and modulating plant hormone production, particularly auxins gibberellins, and cytokinin (Santoyo et al., 2024).

Cross-kingdom RNA is a crucial mechanism by which organisms regulate gene expression through interspecies interactions. This process enables organisms to influence other species' metabolic and immune responses by transferring small RNA (sRNA) that target and silence specific genes in the host. For instance, pathogens and symbiotic microorganisms use RNAi to modulate plant defenses and metabolism, contributing to the evolutionary arms race in host-pathogen interactions (Tang et al., 2022). Certain phytopathogens, such as *B. cinerea* secrete sRNAs that hijack the plant´s RNA-induced silencing complexes (RISCs) to suppress plant defense genes, thereby enhancing their ability to infect the host (He et al., 2023).

Interestingly, in the context of mycoparasitism, the mapping of sRNAs from *Trichoderma* have shown that some of these matched genes related to defense and growth, leading to some authors to suggest that *Trichoderma* may employ RNAi-based mechanisms to modulate biomass and plant immunity (Ramírez-Valdespino et al., 2019). *Trichoderma* may influence metabolic pathways and suppress plant immune response through cross-kingdom RNA silencing during this interaction (Hernández-Hernández et al., submitted). The biogenesis of these sRNAs involves the processing of RNA precursors by Dicer-like (DCL) proteins, which are then loaded into RISCs containing Argonaute (AGO) proteins to guide gene silencing. *Trichoderma* seems to regulate plant gene expression through this mechanism, promoting growth and enhancing resistance against phytopathogens that could otherwise compromise the plant's fitness.

Various metabolic pathways are critical in modulating plant response to infection, with one key process being photorespiration. This complex pathway, closely linked to photosynthesis, begins with the Rubisco-mediated conversion of 2-phosphoglycolate. Photorespiration helps prevent the accumulation of harmful intermediates, while maintaining the availability of essential metabolites for plant

defense, including the amino acid serine. Serine plays a central role in plant immune responses by regulating reactive oxygen species (ROS), maintaining cellular redox balance, and supporting the synthesis of defense-related compounds. Serine biosynthesis is vital for the plant's stress response and overall defense mechanisms as a precursor of glycine, cysteine, glutathione and tryptophan. Glutathione is a key antioxidant involved in modulating ROS, reducing harmful peroxides and maintaining cellular redox homeostasis (Benstein et al., 2013). In addition, to supporting the folate cycle and generating NADPH and other reducing agents during biotic stress, serine contributes to the synthesis of defense-related compounds such as glucosinolates, particularly indole glucosinolates. These compounds, regulated by MPK3 and MPK6 kinases, produce antimicrobial products such as isothiocyanates upon hydrolysis, which enhance plant resistance to microbial attack (Clay et al., 2006; Cole et al., 2017; Poveda, 2021). Serine is synthesized through phosphorylated photorespiration, via the conversion of glyoxylate and glycine from the Calvin cycle, a process in which the phosphoglycerate dehydrogenase (PGDH) family plays a pivotal role. Research by Casatejada-Anchel et al. (2021) revealed differential expression patterns among PGDH isoforms, with PGDH1 showing the highest functional activity, suggesting its critical role in maintaining physiological processes related to serine biosynthesis. PGDH1 is also essential for embryonic development, associated with the gene *At-EDA9* (*EMBRYO SAC DEVELOPMENT ARREST 9*). Mutations in this gene lead to lethal defects in early embryo stages (Igamberdiev & Kleczkowski, 2018; Toujani, Muñoz-Bertomeu, Flores-Tornero, Rosa-Téllez, Anoman, Alseekh, et al., 2013).

To protect crops from significant damage caused by these stresses, a comprehensive understanding of plant immunity and cross-kingdom interactions between plants and microorganisms is essential. In this context, small RNA libraries were generated from *Trichoderma* and Arabidopsis during their interaction and under control conditions. The *T. atroviride* small RNA (*Ta*-sRNA2) was selected for further investigation due to its accumulation at 24-, 48-, 72-, and 96-hours post-interaction with *Arabidopsis thaliana.* Potential Arabidopsis targets of *Ta*_sRNA2 were predicted to assess its role in gene regulation during the

Trichoderma-Arabidopsis interaction (Jijón-Moreno et al., unpublished data). Notably, *Ta*_sRNA2 showed strong complementarity to the Arabidopsis gene *At-EDA9,* suggesting its involvement in gene regulation during the symbiotic interaction between the fungus and the plant.

This study aims to deepen our understanding of the molecular mechanisms underlying the interaction between the beneficial fungus *T. atroviride* and Arabidopsis*.* Given the crucial role of serine biosynthesis via the phosphorylated pathway, particularly involving the PGDH family in plant stress responses, it is likely that *Trichoderma*-Arabidopsis interaction modulates serine-related genes, including *At-EDA9.* By investigating the transient silencing *At-EDA9* mediated by *Ta*_sRNA2, we seek to uncover the regulatory dynamics through which *Trichoderma* influences Arabidopsis gene expression. Additionally, studying the phenotypic effects of *eda9* insertional mutant and *At-EDA9* overexpressing lines will help elucidate how this gene contributes to growth promotion and systemic resistance against foliar pathogens during *Trichoderma* interactions. To further investigate this relationship, a vector for *Ta*_sRNA2 overexpressing was generated using an inducible promoter system to assess the impact of *Ta*_sRNA2 accumulation on plant growth and defense activation in the mutualistic *Trichoderma*-Arabidopsis interaction. Moreover, generating a mutant-resistant version of *At-EDA9* (*EDA9*-mr) with point mutations that prevent *Ta*_sRNA2 silencing will provide further insight into the silencing mechanism mediated by *Trichoderma.* Characterizing the role of *Trichoderma atroviride*'s *Ta*-sRNA2 and its putative target, *At-EDA9* in *Arabidopsis thaliana* is crucial for developing strategies to enhance plant resistance against pathogens and improve crop productivity.

Results

*Ta*_sRNA2 likely silences the *At-EDA9* transcript through sequence complementarity

To investigate the role of *Ta*_sRNA2 in regulating the accumulation of its potential target gene *At-EDA9* during their interaction, *Nicotiana benthamiana* leaves were infiltered with *Agrobacterium tumefaciens* carrying vectors with strong promoter p35S, pEG-TasRNA2 for the sRNA2 expression, pEG-*EDA9* for the target expression and pEG100 containing the strong promoter as control. Forty-eight hours post-infiltration (hpi), the infiltrated tissue was harvested, flash-frozen in liquid nitrogen, and processed for further analysis. Total RNA was extracted, and its integrity was assessed as described in the material and methods section. Complementary DNA (cDNA) was then synthesized and used to measure *At-EDA9* expression in the presence or absence of *Ta*_sRNA2 via RT-qPCR (**¡Error! No se encuentra el origen de la referencia.**). The results showed significant downregulation of the *At-EDA9* transcript during interaction with *Ta*_sRNA2, compared to the control condition with the empty vector (without *Ta*_sRNA2). The differences suggest a potential RNA-mediated silencing mechanism driven by sequence complementarity on the *At-EDA9* transcript.

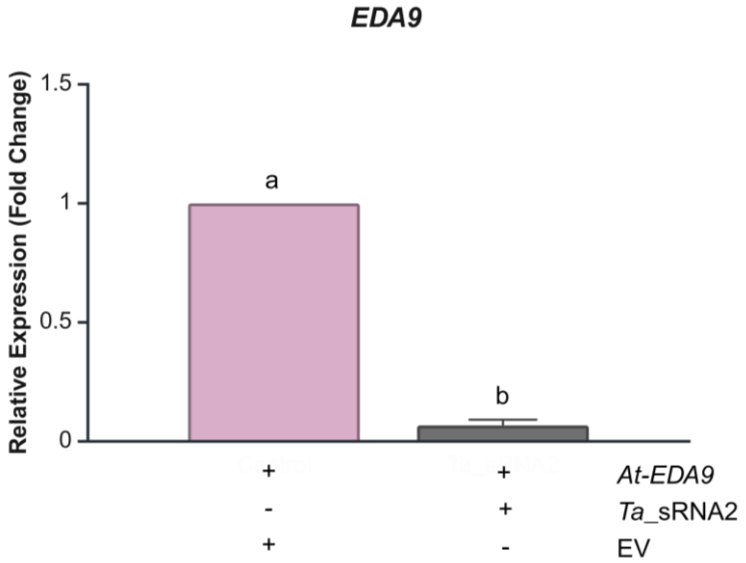

FIGURE 1. *TA-SRNA2* POTENTIALLY SUPPRESSES THE RELATIVE EXPRESSION OF *AT-EDA9* DURING THEIR INTERACTION.

Relative accumulation of *At-EDA9* transcript under control conditions and in response to transient co-expression with *Ta*_sRNA2 from *T. atroviride* in *Nicotiana benthamiana.* The pink bar represents the relative *At-EDA9* transcript levels in the presence of an empty vector as control (without *Ta*_sRNA2), while the gray bar shows *At-EDA9* accumulation when co-expressed with *Ta_sRNA2.* Statistical significance between treatments was determined using one-way ANOVA, with significant differences observed at p-value<0.01.

The mutated sequence of *At-EDA9* resistant mutant (*EDA9-mr*) suppresses the silencing driven by *Ta*_sRNA2

To investigate the silencing mechanism mediated by *Ta*_sRNA2 through sequence complementarity with its putative target gene *At-EDA9*, six silent point mutations were introduced into the RNA target site of *At-EDA9* to create a resistant version to *Ta*_sRNA2 (**¡Error! No se encuentra el origen de la referencia.**). *At-EDA9* was cloned and subject to two rounds of PCR using different primer sets containing the mutations. The shorter fragments generated from the mutagenesis were treated with *Dpn* I restriction enzyme to eliminate any remaining wild-type gene template. These short fragments were then fused through a joint PCR, using primers targeting the full-length gene. The resulting fragment was subcloned into pJET1.2/blunt Cloning Vector and transformed into *E. coli.* A restriction enzyme digestion profile was performed to confirm the assembly of the construct, with the electrophoresis patterns matching the expected sizes (See Table 2 for enzyme details and expected fragment sizes). Finally, Sanger sequencing confirmed the successful introduction of the six intended point mutations, as verified by sequence alignment between the wild-type *At-EDA9* fragment and the mutated *At-EDA9-mr* colonies (**¡Error! No se encuentra el origen de la referencia.**). All designed point mutations were correctly introduced.

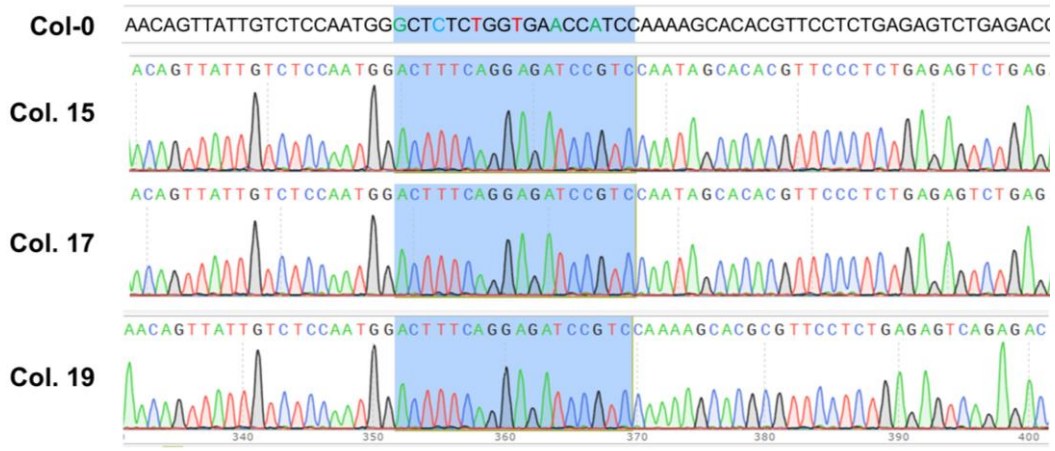

FIGURE 2. SANGER SEQUENCING OF POINT MUTATIONS IN THE PUTATIVE TARGET (*AT-EDA9*) OF *TA*_SRNA2.

Nucleotide sequence alignment of the *At-EDA9* fragment, highlighting the Col-0 sequence at the top and the sequences from the positive colonies (Col. 15, 17, 19) displaying the induced point mutations. Chromatograms for each sequence illustrate the corresponding nucleotide variations in the selected colonies.

At-EDA9 overexpressing lines presented differences in mRNA levels

To further evaluate the role of *At-EDA9* in plant immunity and biomass production, transgenic (T1) lines were generated by Jijón-Moreno et al. In this study, transcript accumulation of *At-EDA9* was evaluated by RT-qPCR of three transgenic (T2) overexpressing lines in the Col-0 background, driven by the *35S* promoter (*35S*::*EDA9*-mCh #3, *35S*::*EDA9*-mCh#11 and *35S*::*EDA9*-mCh#13) with *ACT2* as the reference gene. As shown in **¡Error! No se encuentra el origen de la referencia.**, all transgenic lines exhibited significant overexpression of *At-EDA9* compared to the Col-0 wild-type. However, the relative accumulation of *At-EDA9* transcripts varied among the lines. Lines *35S::EDA9*-mCh #11 and #13 showed markedly higher expression levels with a 36 and 66-fold increase, respectively, compared to *35S*: *EDA9*-mCh #3, which exhibited only a 1.3-fold increase. These results led to the selection of lines *35S*: *EDA9*-mCh #11 and #13 for further studies on plant-pathogen-beneficial microorganism interactions, as they showed different expression levels of *At-EDA9* overexpression. The distinct expression levels of these lines will also provide insights for exploring the dose-dependent effects of *At-EDA9* on the plant defense responses.

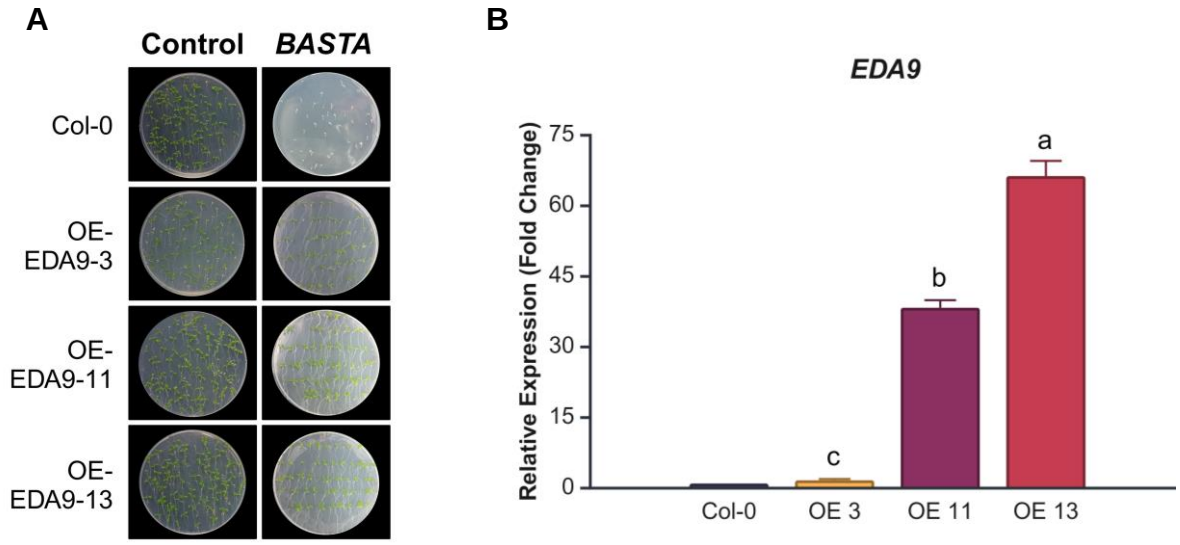

Figure 4. Relative Expression Levels of *At-EDA9* in Overexpressing *At-EDA9* Transgenic Lines.

A. Seedlings of *A. thaliana* overexpressing lines of *At-EDA9* on MS medium selected with BASTA. **B**. mRNA levels of *At-EDA9* were assessed in three transgenic lines (*35S*::*EDA9*-mCh #3, *35S*::*EDA9*-mCh #11, and *35S*::*EDA9*-mCh #13) in the Col-0 background using RT-qPCR, with *ACT2* as the reference gene.

*Ta*_sRNA2 overexpressing vector

Transgenic lines overexpressing *Ta*_sRNA2 under the 35S promoter were not viable seedlings. This observation suggests that one of the target genes silenced by *Ta*_sRNA2 plays an essential role in early plant development. As stated before, *At-EDA9* is a critical factor, given its involvement in key developmental processes during early stages. To further investigate the role of *Ta*_sRNA2 in modulating its putative target gene *At-EDA9*, a vector containing a strong inducible promoter for the overexpression of *Ta*_sRNA2 was constructed (Figure 3). This approach minimizes the impact on plant viability by inducing the presence of *Ta*_sRNA2 in the later stages of the plant´s life cycle.

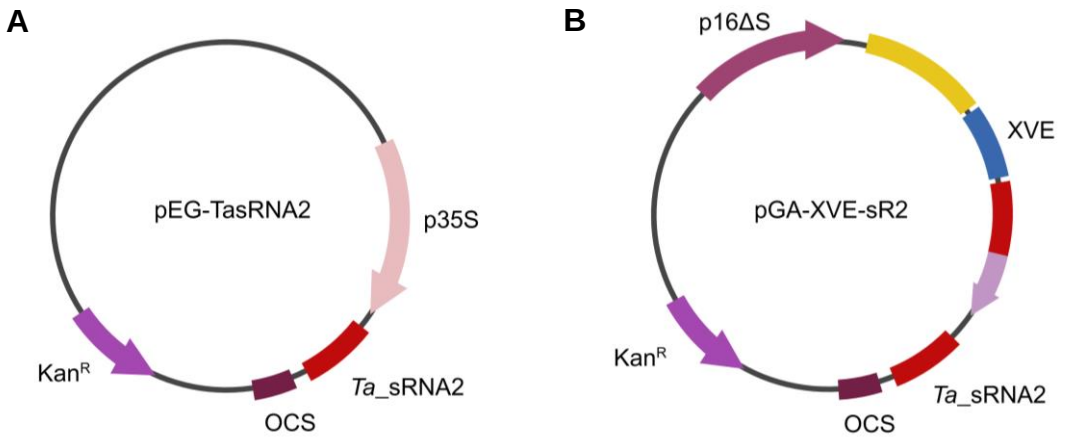

FIGURE 3. CONSTRUCTS OF THE VECTORS FOR THE OVEREXPRESSION OF THE *TA*_SRNA2

Schematic representation of the original pEG-TasRNA2 vector and the constructed pGA-XVE-sR2 for the inducible expression of *Ta*_sRNA2. **A.** The original construct pEG-TasRNA2 generated by Jijón-Moreno et al. contains constitutive p*35S* promoter driving the expression of *Ta*_sRNA2, the ocs terminator, and with a Kanamycin resistance gene for selection. **B.** pGA-XVE-sR2 construct featuring the β-estradiol-inducible promoter, comprising the sequence of the promoter p16ΔS along with XVE inducible system, enabling controlled expression of the small RNA 2. Both constructs retain the terminator and Kan^R cassette.

At-EDA9 enhances resistance to *Botrytis cinerea* but is not involved in priming triggering by *Trichoderma atroviride*

To evaluate the role of *Ta*-sRNA2 in plant immunity, particularly its potential target *At-EDA9,* we evaluated the *At-EDA9* overexpressing lines (*At-EDA9 35S::EDA9*-mCh #11/ #13) and the *eda9* mutant during the interaction of *A. thaliana, T. atroviride* and *Botrytis cinerea.* Four medium-sized leaves from each plant (n=12) were inoculated with 5 µL of a *B. cinerea* suspension at $1x10^5$ conidia/mL in Phosphate-buffered saline (PBS) after prior root treatment with *T. atroviride.* Three dpi, fungal-induced damage was documented with photographs and analyzed using ImageJ software to calculate the damage area relative to the total leaf area.

As shown in Figure 4, both *At-EDA9* overexpressing lines exhibited significantly enhanced resistance to *B. cinerea* compared to the Col-0 leaves, whereas the *eda9* mutant showed increased susceptibility. This suggests that *At-EDA9* accumulation enhances resistance against necrotrophic pathogens. Additionally,

plants pre-treated with *T. atroviride* in both, overexpressing lines and Col-0 demonstrated effective systemic resistance, unlike plants treated with inoculation buffer alone. This mutation in *eda9* may not compromise resistance against necrotrophic pathogens in control or through resistance gain mediated by *Trichoderma.*

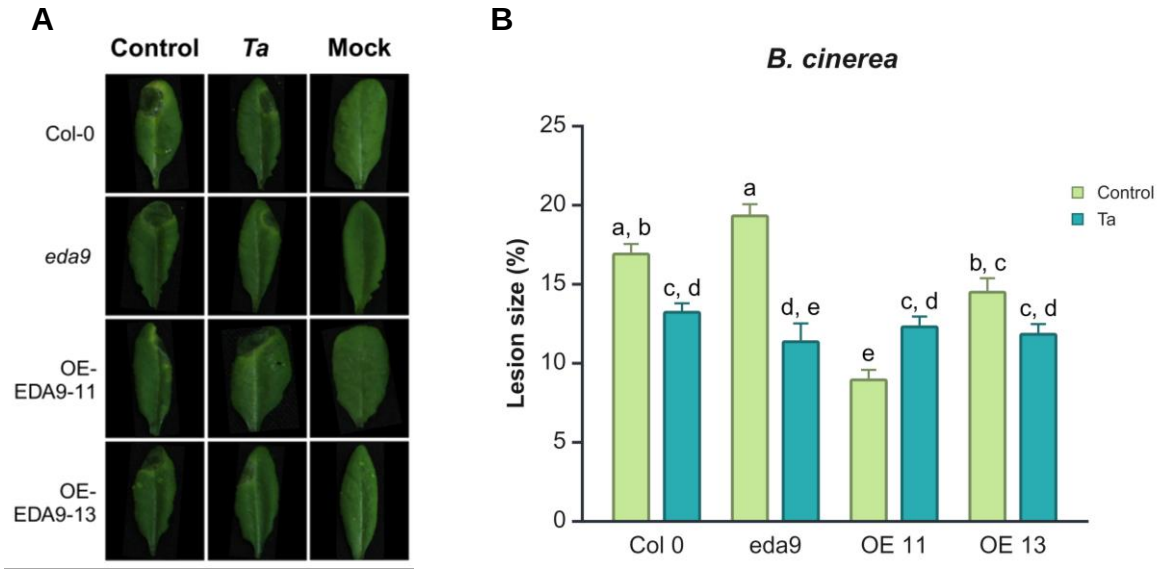

FIGURE 4. *AT-EDA9* POSITIVELY CONTRIBUTES TO DISEASE RESISTANCE AGAINST THE NECROTROPHIC PATHOGEN *B. CINEREA* BUT DOES NOT PLAY A ROLE IN PRIMING TRIGGERING BY *T. ATROVIRIDE*

A. Representative photos of infected leaves at 3 dpi, comparing mock, control and *Trichoderma*-treated conditions. **B**. Representative leaves from each plant (n=18) were inoculated with *B. cinerea* following pre-treatment with *Trichoderma*. Lesion size was evaluated at three dpi and quantified using ImageJ software to measure the damaged area induced by the fungus as a percentage of the total leaf area. The figure includes overexpressing lines (*At-EDA9 35S::EDA9*-mCh #11/ #13), Col-0 and *eda9* mutant. Different letters denote statistically significant differences between groups ($p<0.05$).

At-EDA9 plays a negative role in plant immunity against *Pseudomonas syringae*, with *T. atroviride* potentially boosting resistance mechanisms

To investigate the role of *Ta*_sRNA2 on its putative target, *At-EDA9,* during the interaction between *A. thaliana* and the beneficial fungus *T. atroviride*, we evaluated the *At-EDA9* overexpressing and mutant lines in response to the hemibiotrophic pathogen *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst* DC3000).

Plants pre-treated with *T. atroviride* were infiltrated with a bacterial suspension of *Pst* DC3000 and left to interact for 3 days, after which samples were collected to assess colony-forming units (CFU). In untreated plants, the overexpressing lines *35S*::*EDA9*-mCh #11 and #13 exhibited significantly increased susceptibility to *Pst* DC3000 compared to the *eda9* mutant and Col-0 lines, which showed no significant difference in susceptibility (Figure 5Figure 5). The resistance patters in plants pre-treated with *T. atroviride* remained consistent, with the overexpressing line *35S*::*EDA9*-mCh #13 being the most susceptible. However, the *eda9* mutant, when interacting with *T. atroviride,* displayed enhanced resistance, as indicated by a reduced CFU count compared to the *eda9 line* treated with inoculation buffer alone. Interestingly, the overexpressing line *35S*::*EDA9*-mCh #11 pre-treated with *Trichoderma* showed a marked improvement in resistance compared to the same line without pretreatment. This result also correlated with gene dosage, as the overexpressing line #13, which had higher *At-EDA9* transcript accumulation, demonstrated increased susceptibility to *Pseudomonas* compared to the other lines.

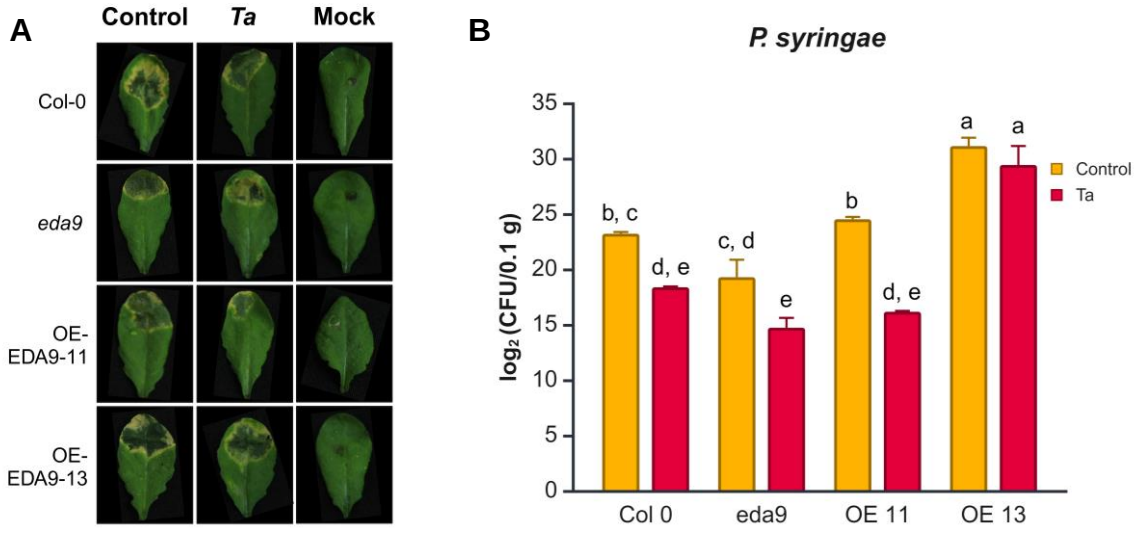

FIGURE 5. *AT-EDA9* PLAYS A NEGATIVE ROLE IN PLANT IMMUNITY AGAINST THE HEMIBIOTROPHIC PATHOGEN PSEUDOMONAS SYRINGAE, WITH *TRICHODERMA ATROVIRIDE* POTENTIALLY AMPLIFYING RESISTANCE MECHANISMS

Arabidopsis plants in interaction with *T. atroviride* were infiltrated with *P. syringae* pv. *Tomato* DC3000. **A.** At six dpi, representative photos of each plant line were taken. **B**. At three dpi with *P.*

syringae, leaf tissue samples were collected, and CFUs were calculated by plating the bacterial suspension obtained on King's B media. The figure displays CFU counts from the overexpressing lines (*At-EDA9 35S::EDA9*-mCh #11/ #13), Col-0 and *eda9* mutant. Differences in resistance patterns following pre-treatment with *T. atroviride* or buffer inoculation are illustrated. Groups labeled with different letters indicate statistically significant differences ($p<0.05$).

At-EDA9 positively contributes to plant growth and has a role in the growth promotion mediated by *T. atroviride*

To explore the role of *Trichoderma* in influencing its putative target *At-EDA9*, during growth promotion in Arabidopsis, we conducted growth assays using *35S*:: *EDA9*-mCh #11 and #13, as well as *eda9* mutant, during their interaction with *T. atroviride*.

Sixteen-day-old plants were root-treated with *T. atroviride* and eighteen dpi; the plants were carefully washed to remove the substrate attached to the roots. The plants were then dried for 24 hours and weighted on an analytical scale to determine dry biomass.

As shown in Figure 6, the *At-EDA9* overexpressing lines exhibited a significant biomass increase compared to Col-0 plants, both in control conditions and interacting with *Trichoderma*. In contrast, the *eda9* mutant showed no significant biomass increase in either condition. A similar pattern was observed during interaction with *Trichoderma*, where the mutant line did not exhibit notable growth promotion compared to treated Col-0 plants. Interestingly, both overexpressing lines did not promote additional growth when pre-treated with *T. atroviride. This* suggests that the overexpression of *At-EDA9* in these plant lines may interfere with the growth promotion mediated by *Trichoderma*.

A **B**

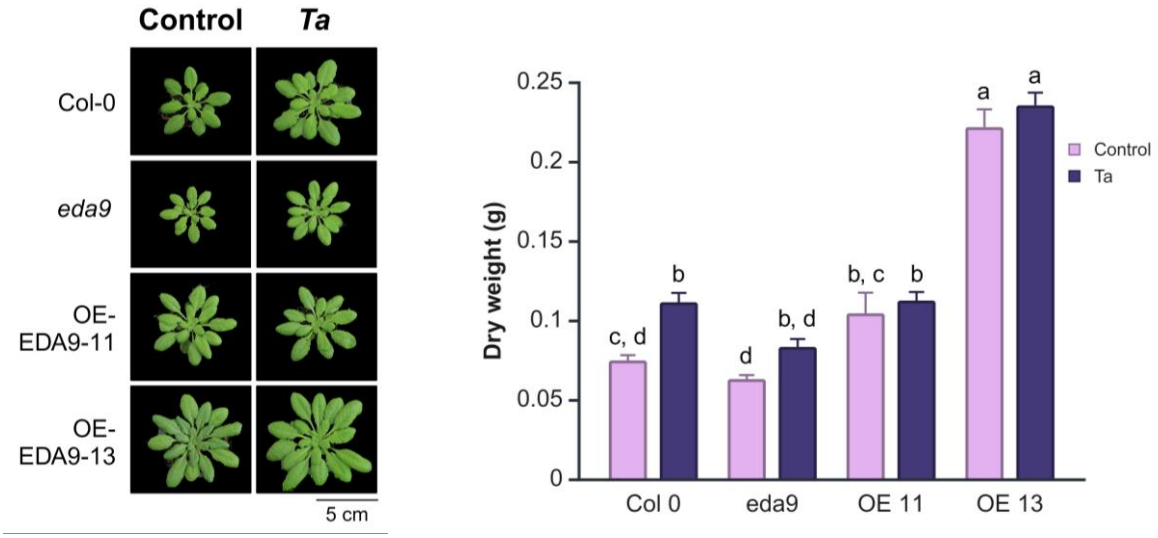

FIGURE 6. *AT-EDA9* POSITIVELY CONTRIBUTES TO PLANT GROWTH AND HAS A ROLE IN THE GROWTH PROMOTION MEDIATED BY *T. ATROVIRIDE*

Sixteen-day-old Arabidopsis plants (n=22) were root-inoculated with a *T. atroviride* conidial suspension. At 18 dpi, plant biomass was evaluated by determining dry weights. **A.** Representative photos of the rosette of the overexpressing and *eda9* mutant plant lines were captured. **B**. The figure compared the dry weights of overexpressing lines (*At-EDA9 35S*::*EDA9*-mCh #11/ #13), Col-0 and *eda9* mutant line following treatment and in control. Groups with different letters indicate statistically significant differences ($p<0.05$).

Discussion

Interactions between plants and microorganisms are crucial for regulating plant immunity and growth. In this study, we focused on the role of small RNA 2 (*Ta*_sRNA2) derived from *T. atroviride* in modulating the expression of the *At-EDA9* gene in *A. thaliana*.

Our transient co-expression assays in *N. benthamiana,* revealed a significant reduction in *At-EDA9* transcript levels when interacting with *Ta*_sRNA2, indicating a direct sequence complementarity-mediated silencing mechanism. This finding supports the growing body of evidence suggesting that cross-kingdom RNA interference is a conserved strategy employed by both deleterious and beneficial microorganisms to modulate host gene expression. Similar cross-kingdom silencing has been observed in *Verticillium daliae,* where fungal small RNAs target host immunity genes (Zhang et al., 2016). These results are consistent with previous reports highlighting the role of small RNAs in microorganism-plant interactions. For instance, pathogenic small RNAs from *B. cinerea* have been shown to hijack the plant's RISC complex to enhance pathogen efficacy (He et al., 2023; Liu et al., 2021; Weiberg et al., 2013). Interestingly, in mycoparasitism, the mapping of sRNAs from *Trichoderma* show that some of these matched genes are related to defense and growth, leading to some authors suggesting that *Trichoderma* may use RNAs to promote mutualistic interactions (Hernández-Hernández et al., submitted, Rebolledo-Prudencio et al., 2022).

The involvement of *At-EDA9* in the phosphorylated pathway of serine biosynthesis makes it a critical component of the plant´s immune response. Serine plays a vital role in maintaining cellular redox homeostasis and serves as a precursor for the biosynthesis of amino acids such as cysteine and glycine, which are central to managing oxidative stress in plants (Benstein et al., 2013; Toujani, Muñoz-Bertomeu, Flores-Tornero, Rosa-Téllez, Anoman, & Ros, 2013). Our results demonstrated that *At-EDA9* overexpression significantly enhanced resistance to the necrotrophic pathogen *B. cinerea,* while *eda9* mutant exhibited increased susceptibility. This observation supports the idea that serine and its associated

metabolites, such as glutathione, are critical for mitigating damage caused by ROS during infection (Noctor et al., 2012; Piasecka et al., 2015). However, the role of *At-EDA9* in SAR and ISR appears to be more complex. While *At-EDA9* overexpression conferred resistance to *B. cinerea,* the priming of systemic resistance was unclear after interaction with *Trichoderma.* This suggests that *At-EDA9* may be more prominent in local immune responses against necrotrophic pathogens. Still, other factors in the plant´s immunity may influence its involvement in ISR. ISR, primarily mediated by JA and ET, is a well-established pathway activated by microorganisms like *Trichoderma* (Contreras-Cornejo et al., 2011; Martínez-Medina et al., 2013). The absence of a clear priming response in the *At-EDA9* overexpressing lines suggests that *At-EDA9* accumulation is more directly associated with local defense responses rather than influencing complex signaling networks involved in ISR.

Dosage-dependent effects of *At-EDA9* conferred resistance to *B. cinerea* but had the opposite effect on defense against the hemibiotrophic pathogen *P. syringae.* Higher *At-EDA9* transcript levels were correlated with increased susceptibility to *P. syringae*, particularly evident in the overexpressing lines with the highest *At-EDA9* expression. This increased susceptibility may result from imbalanced metabolic processes mediated by *At-EDA9* accumulation. Excessive serine accumulation and other photorespiratory metabolites can negatively impact immune responses to hemibiotrophic pathogens. Overproduction of serine through photorespiratory pathways might disrupt the levels of ROS and nitrogen species, which could impair the plant´s defense strategy against pathogens like *P. syringae* (Bloom et al., 2002; Rojas et al., 2014; Torres et al., 2006). Conversely, the *eda9* mutant line pre-treated with *Trichoderma* exhibited enhanced resistance to *P. syringae,* suggesting that the lower levels may help fine-tune the plant immunity in response to this pathogen. The dual role of *At-EDA9* in mediating resistance to necrotrophic and hemibiotrophic pathogens underscores the complexity of plant immune regulation. The differential effects of *At-EDA9* suggest that it may act as a regulatory node in balancing plant immune responses. Previous studies have shown that plants manage the trade-off between growth and immunity through regulatory molecules

involved in metabolic pathways, particularly those related to amino acid biosynthesis (González-lópez et al., 2021; Guo et al., 2018).

In addition to its role in defense, *At-EDA9* plays a significant role in plant growth, particularly through its interaction with *Trichoderma.* Both *At-EDA9* overexpressing lines exhibited a notable increase in biomass compared to wild-type plants, suggesting that *At-EDA9* is involved in growth regulation, potentially mediated by serine accumulation, essential for producing key metabolites involved in plant development (Ros et al., 2014). Interestingly, the lack of further biomass increases in *At-EDA9* overexpressing lines when interacting with *Trichoderma* suggests that the growth-promoting effects of *Trichoderma* may require a reduction of *At-EDA9* levels to initiate. Alternatively, previous studies have shown that *Trichoderma* promotes growth through mechanisms independent of small RNAs, such as modulating hormone signaling pathways, including auxins, gibberellins, and cytokinins (Contreras-Cornejo et al., 2009; Nieto-Jacobo et al., 2017; Salas-Marina et al., 2011).

The findings of this study have important implications for understanding the role of *Ta*_sRNA2 in silencing *At-EDA9*, opening potential applications in crop protection and improvement. However, the dosage-dependent effects of *At-EDA9* raise concerns about unintended susceptibility to certain pathogens, as seen with *P. syringae.* For instance, enhancing defense against one pathogen may inadvertently increase vulnerability to another, a challenge well documented in crop improvement strategies (Campos et al., 2016; Yang et al., 2012). Future research should focus on unveiling the molecular mechanisms by which *Ta*_sRNA2 modulates *At-EDA9* concerning plant growth and immunity, particularly in serine biosynthesis and its role in different pathogen defense strategies. Additionally, studying the effects of *Trichoderma*-mediated *Ta*_sRNA2 on *At-EDA9*'s regulation of root colonization in Arabidopsis will offer a deeper insight into the molecular mechanisms underlying mutualistic relationships.

Critical low levels of *At-EDA9* transcripts result in embryo lethality and effects on male gametophyte development (Casatejada-Anchel et al., 2021; Toujani, Muñoz-Bertomeu, Flores-Tornero, Rosa-Téllez, Anoman, Alseekh, et al., 2013). To address this, we have constructed the vector pGA-XVE-sR2 containing the sequence for *Ta*_sRNA2 under the control of a β-estradiol-induced promoter, enabling the generation of transgenic *Arabidopsis thaliana* lines where *Ta*_sRNA2 overexpression can be precisely regulated in both timing and level. This approach ensures normal plant growth and viability while allowing us to study the effects of *At-EDA9* downregulation. By varying *Ta*_sRNA2 expression levels, we can further investigate the dosage-dependent effects observed in this study, where *At-EDA9* transcript accumulation showed contrasting outcomes in resistance to different phytopathogens. This approach will help clarify how *Ta*_sRNA2 fine-tunes *At-EDA9* expression and its role in plant immunity. Additionally, a mutated version of *At-EDA9* in *N. benthamiana* co-expression assays with *Ta*_sRNA2 will allow us to confirm the specificity of the RNA-meditated silencing mechanism. This could provide further insights into the post-transcriptional regulation mediated by *Trichoderma* in its interaction with the host plant.

Methods

Plant lines and strains

Arabidopsis thaliana plants used in this study include wild-type Ecotype Columbia-0 (Col-0), T-DNA insertional mutant of *At-EDA9* "SALK_011381" (*eda9)* sourced from ABRC (Joseph Ecker, 2001), and overexpressing lines of *At-EDA9* Col-0 *35S*::*EDA9*-mCh #3, #11 and #13 (Jijón-Moreno et al., unpublished results). *Nicotiana benthamiana LAB strain* plants were used for transient co-expression.

Plant material and growth conditions

Seeds were sown in plastic pots filled with a sterilized substrate composed of Vermiculite, Perlite, and Peat moss (3:1:1). To ensure uniform germination, seeds were stratified at 4 °C for 48 h before being transferred to a temperature- and light-controlled room at 23 °C with a 12-h light/dark cycle. After germination, seedlings were transplanted into individual plastic pots and grown for 14 days before *Trichoderma* treatment.

Plant-pathogen interaction assays

Four-week-old Arabidopsis plants were inoculated at the roots with a *Trichoderma atroviride* solution, previously cultivated on Potato Dextrose Agar (PDA) for 7 days. The conidia were collected and quantified using a Neubauer chamber, and the suspension was adjusted to a concentration of $1x10^6$ conidia/mL in sterile water.

- Four medium-sized leaves from each Arabidopsis plant were inoculated with 5 µL of *B. cinerea* spore suspension ($1×10^5$ conidia/mL) in sterile PBS. Three days post-inoculation (dpi), fungus damage was assessed. Images were captured using ScAnalyzer equipment and analyzed with ImageJ software to calculate the percentage of damaged area relative to the total leaf area.
- Arabidopsis leaves were infiltrated with *Pseudomonas syringae* pv. *tomato* DC3000 bacterial suspension ($5×10^5$ UFC/ml) in sterile PBS using a 3 mL needleless syringe. To maintain humidity plants were covered after

the infiltration. Leaf samples were collected with a cork borer immediately post-infiltration to quantify colony-forming units (CFU). The remaining plants were left for 3 days, after which additional samples were taken for CFU analysis. Leaf tissue of the samples (0.005 g) was cleaned to remove surface contaminants, ground to extract bacteria, and serial dilutions were plated on King´s B agar. Photographic evidence of the infiltrated leaves was also captured at 6 dpi using “ScAnalyzer”.

Growth promotion assay

Sixteen-day-old plants (n=22) were root-treated with 500 µL *T. atroviride* spore suspension (1×10^6 conidia/mL), with a buffer-only control group. The suspension was gently applied to the base of the plant rosette, and plants were grown for 18 days under controlled conditions as described above. After this period, growth was documented photographically using the ScAnalyzer) to compare treated plants with controls. Plants were then washed in a three-step process to remove substrate residues and dried on absorbent paper. Fresh weight was recorded using an analytical scale, followed by drying at 60°C for 24 h to measure dry weight.

Transient co-expression in *Nicotiana benthamiana*

Three strains of *Agrobacterium tumefaciens* were transformed with different constructs: pEG100-*Ta*_sRNA2, pEG-*EDA9*, and PEG100 empty vector (EV) for control. Three co-expression treatments were established: (1) *At-EDA9* with EV to evaluate target gene accumulation, (2) *Ta*_sRNA2 with EV as control, and (3) *Ta*_sRNA2 with *At-EDA9* to evaluate RNA silencing of the target gene. After 48 h of interaction, infiltered leaves were harvested and flash-frozen in before RNA extraction and RT-qPCR analysis to measure gene expression.

Quantitative reverse transcription polymerase chain reaction (RT-qPCR)

Total RNA was extracted using the cetyltrimethylammonium bromide (CTAB) method. Samples were homogenized, phase-separated using chloroform-isoamyl alcohol (24:1 vol/vol) solution (pH 8) and precipitated with isopropanol and

potassium acetate (10 µM) at -20°C for at least 4 hours, followed by a cleaning process with ethanol and finally resuspended in nuclease-free water. The total RNA was quantified using the microvolume plate (Take3) and an Epoch spectrophotometer to evaluate the quantity of the extracted RNA. Integrity was assessed through denaturing electrophoresis on a 1% agarose gel. To eliminate the contaminating DNA, the samples were treated with Turbo DNase, as recommended by the manufacturer´s instructions (Invitrogen). PCR conducted with specific primers was performed, and the absence of amplified products in the treated samples indicated that no detectable DNA remained. Additionally, to verify that the DNase treatment has not compromised RNA integrity, the samples were quantified and observed with denaturing electrophoresis loaded on a 1% agarose gel.

Subsequently, the RNA samples were used to synthesize cDNA driven by SuperScript II Reverse Transcriptase, as recommended by the manufacturer´s instructions (Invitrogen). The resulting cDNA was quantified and diagnosed via PCR using the primers of the housekeeping and target gene, its product was observed on a 2% agarose gel. The qPCR reactions were conducted using Maxima SYBR Green, with 300 ng/µL of cDNA as a template for all samples. The housekeeping gene *PP2A* from *N. benthamiana* was used as a reference for normalizing the target gene expression in the co-expressing system, while *ACT2* from *A. thaliana* for the quantification of the overexpressing lines, the primers used are annotated on Table 2. Data was annotated with the $2^{-\Delta\Delta ct}$ Method for relative gene expression analysis.

PCR-based Site-Directed Mutagenesis of *At-EDA9*

The cloned vector pJET-*EDA9*-2 generated by Jijón-Moreno et al. contained an 1860 bp fragment of cDNA of the *At-EDA9* gene from *A. thaliana* Col-0 plants. For which, internal oligonucleotides with 6 mismatches were designed for the *At-EDA9* over the target region that aligns to *Ta*-sRNA2 (Table 1). The mismatches were carefully chosen to be silent mutations, which do not alter the amino acid sequence, thereby avoiding loss of protein function. The mutagenesis relied on annealing two sets of mutagenic primers and the external *At-EDA9* primers (Table

1). This resulted in the amplification of two *At-EDA9* independent fragments of 1483 with primers A+C, and 440 bp with primers B+D, respectively. To eliminate the plasmid DNA of *At-EDA9* used as a template, the PCR products were treated with the restriction enzyme *Dpn* I, which cleaved adenine-methylated dam sites. The absence of the template DNA was confirmed through PCR with *At-EDA9* primers A+D. Subsequently, to obtain the full version of *At-EDA9* with the 6-point mutations (*EDA9*-mr), the A+D primers were used to fuse the fragments through overlapping PCR. The obtained fragment was evaluated by denaturing electrophoresis on a 1% agarose gel to confirm its expected size (1860 bp). The complete fragment was cloned into a pJET1.2/blunt Cloning Vector and used for *Escherichia coli* transformation. Various dilutions of the transformed bacteria were grown on LB medium with Carbenicillin as a selective antibiotic. Colonies were screened by PCR with the A+D *At-EDA9* oligonucleotides and sent for DNA sequencing to the National Laboratory for Agricultural, Medical and Environmental Biotechnology (LANBAMA) to verify the mutations on *At-EDA9*.

*Ta*_sRNA2 overexpressing vector

The vector pEG-TasRNA2, originally generated by Jijón-Moreno et al., was the backbone for constructing a controlled expression system for *Ta*_sRNA2 from *T. atroviride*. While this vector contained the necessary sequence for the overexpression of the sRNA2, a controlled expression is essential to obtain viable seedlings of *A. thaliana*. To develop an inducible system, we inserted the p16ΔS-sXVE-mCherryC-Bar-KC294590 promoter, responsive to β-estradiol, allowing precise temporal, dose-dependent and controlled expression of the gene of interest (Schlücking et al., 2013; Zuo et al., 2000). We designed oligonucleotides flanking the p16ΔS-sXVE sequence of the system, incorporating four restriction enzymes recognition sites (*Stu* I, *Nru* I, *BamH* I, *Xho* I) to position the promoter into the backbone accurately. The 2803 bp fragment was extracted from a 0.9% agarose gel following electrophoresis and purified for subsequent cloning. This fragment containing the promoter sequence was cloned into a pJET1.2/blunt Cloning Vector, followed by *Escherichia coli* transformation to enable amplification. The colonies

were diagnosed with a PCR of the plasmid DNA from transformed colonies to confirm successful insertion.

The positive colonies of the cloned p16ΔS-sXVE underwent a double digestion with *Stu* I and *Xho* I, to release the inducible promoter fragment. These same restriction enzymes were used to excise the constitutive p*35S* promoter previously inserted in pEG-TasRNA2. The digested plasmid and promoter were then ligated to generate the expression construct, which was transformed into Dh5α *E. coli* and selected on LB medium containing Kanamycin. To verify the correct insertion of the inducible promoter into pEG-TasRNA2, plasmid DNA was purified from selected colonies and analyzed by PCR using the designed primers that flank the promoter insertion site. For further verification, a restriction enzyme mapping was conducted on positive clones with enzymes detailed in

Name	**Gene**	**Sequence 5' → 3'**	**Length**
ACT2-RT2-Fw	Actin	GCTGTTGACTACGAGCAGGAG	21
ACT2-RT2-Rv	ACT2-RT2-Rv	AACGAGGGCTGGAACAAGAC	20
NbPP2A-FW	PP2A	GCTTGGTGCCCTTTGTATGC	20
NbPP2A-RV	NbPP2A-RV	CATTGCCCACTCTGGACCAA	20
AT-EDAD9-RT-F	*At-EDA9*	GTGTTGACTTAGTGAGCTTTGATG	24
AT-EDAD9-RT-R	At-EDAD9-RT-R	GGCAAAGGTTTCGTCGTTTAG	21

Name	**Added restriction enzymes**	**Sequence 5' → 3'**	**Length**
Prom-P16ΔS-Fw	Stu I	TTAAGGCCTTGGAACCATCTTTTGGGTTCCT	31
Prom-SUPERR-Rv	Nru I BamH I Xho I	TGACTCGAGATGGATCCAATCGCGAGGCTAGAGTCG ACTAGCTTCAG	47
pGA-XVE-Fw	-	TGATTGGACTTGTTTGGAGGTC	22

pGA-XVE-Rv	-	CCATTCCTTCAACACACTTTCC	22

Table 3, confirming the construct of the pGA-XVE-sR2 vector containing both the inducible promoter p16ΔS-sXVE and the backbone with the *Ta-sRNA2*. Internal primers were designed to sequence the inducible system region further to evaluate full fragment.

Conclusion

This study elucidates the regulatory role of the sRNA2 from *T. atroviride* influencing *A. thaliana* immunity and biomass formation via gene silencing of *At-EDA9.* Our findings reveal that the *Ta*_sRNA2 can drive a cross-kingdom RNA silencing mechanism to modulate the gene expression of *At-EDA9* of the host. As suggested in the literature, the overexpression of *At-EDA9* conferred resistance to the necrotrophic pathogen *B. cinerea,* suggesting its importance in serine biosynthesis and redox homeostasis, fundamental aspects modulated during infection to control ROS. Conversely, when challenged with the hemibiotrophic pathogen *P. syringae,* the overexpressing lines of *At-EDA9* showed increased susceptibility, potentially due to the accumulation of serine or subsequent secondary metabolites, possibly causing imbalanced ROS production and compromising defense.

The cross-kingdom interaction between *Ta_sRNA2* and *At-EDA9* emphasizes the role of *Trichoderma* in fine-tuning the plant defenses through the delivery of RNA. Our study suggests that the *Ta*_sRNA2 directly modulates the transcript levels of *At-EDA9*, aligning with its suggested metabolic adjustment ability to improve immunity and biomass production. By targeting this specific gene, sRNA2 may play a fundamental role in balancing serine levels and associated metabolites, generally related to systemic and local immune responses. This fine-tuned regulation of plant defense and development underscores the importance of cross-kingdom sRNA-mediated mechanisms to achieve a mutualistic relationship between *Trichoderma* and *Arabidopsis.* This understanding could suggest new approaches in plant defense strategies for crop improvement through its interaction with *Trichoderma.*

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Tables

TABLE 1. OLIGONUCLEOTIDES USED FOR MUTAGENESIS OF *AT-EDA9*.

Key	Name	Type	Sequence 5' → 3'	Length
A	At-*EDA9*-F2	Wild type	AACCTCGAGCCTAGGGCCGCCATGGCGGCCTAAACCATGTCAGCCACCGCCG	52
D	At-*EDA9*-R2	Wild type	TTATACGTATCGCGAGAGCTTGAGGAAAACGAACTCCT	38
B*	At-*EDA9*mut-FW	6-point mutation	GACTCTCAGAGGAACGTGTGCTTTTGGACGGATCTCCTGAAAGTCCATTGGAGACAATAACTG	63
C*	At-*EDA9*mut-RV	6-point mutation	CAGTTATTGTCTCCAATGGACTTTCAGGAGATCCGTCCAAAAGCACACGTTCCTCTGAGAGTC	63

TABLE 2. OLIGONUCLEOTIDES USED FOR RT-QPCR

Name	Gene	Sequence 5' → 3'	Length
ACT2-RT2-Fw	Actin	GCTGTTGACTACGAGCAGGAG	21
ACT2-RT2-Rv	ACT2-RT2-Rv	AACGAGGGCTGGAACAAGAC	20
NbPP2A-FW	PP2A	GCTTGGTGCCCTTTGTATGC	20
NbPP2A-RV	NbPP2A-RV	CATTGCCCACTCTGGACCAA	20
AT-EDAD9-RT-F	*At-EDA9*	GTGTTGACTTAGTGAGCTTTGATG	24
AT-EDAD9-RT-R	At-EDAD9-RT-R	GGCAAAGGTTTCGTCGTTTAG	21

Name	Added restriction enzymes	Sequence 5' → 3'	Length
Prom-P16ΔS-Fw	Stu I	TTAAGGCCTTGGAACCATCTTTTGGGTTCCT	31
Prom-SUPERR-Rv	Nru I BamH I Xho I	TGACTCGAGATGGATCCAATCGCGAGGCTAGAGTCGACTAGCTTCAG	47

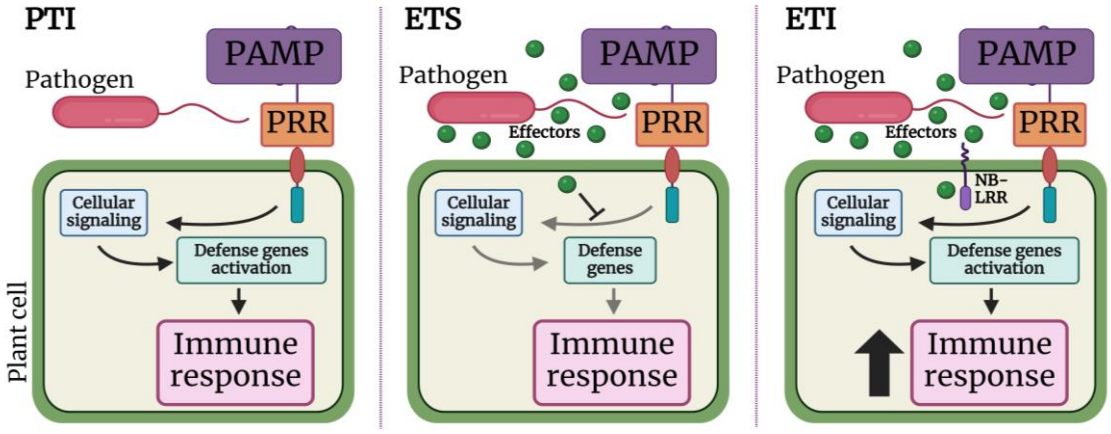
pGA-XVE-Fw	-	TGATTGGACTTGTTTGGAGGTC	22
pGA-XVE-Rv	-	CCATTCCTTCAACACACTTTCC	22

TABLE 3. RESTRICTION ENZYMES USED TO DIAGNOSE PGA-XVE-SR2

Cut	Enzyme	Buffer	Kb	T (h)	Tube	Inactivation (T°C)
Single	Sal I	O or 3.1	4.834	1h30	I	65°C
	Sac II	B	4.834	1h30	IV	
Double	Hind III	R or red	1.851	1h30	II	80°C
			2.983			
Triple	Sac I	Tango o yellow	0.177	1h	III	75°C
			0.625			
			4.032			

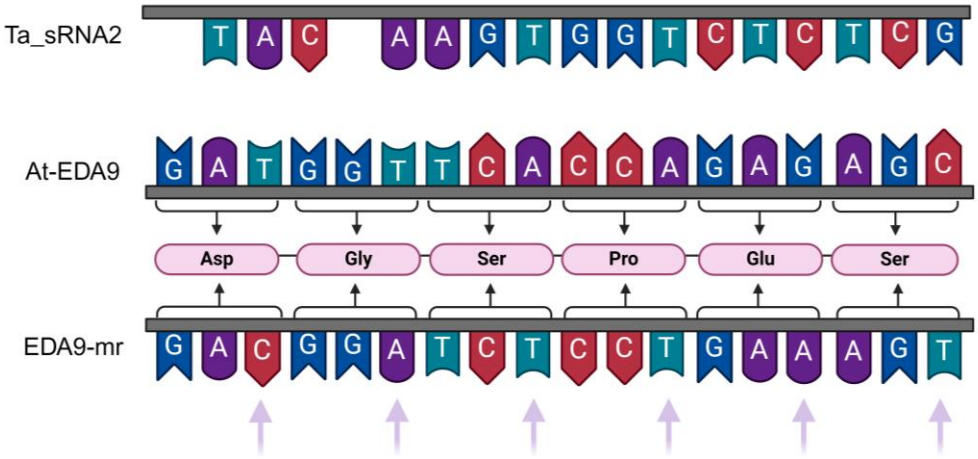
Supplementary Figures

SUPPLEMENTARY 1. SCHEMATIC REPRESENTATION OF THE PLANT IMMUNE RESPONSE DURING PATHOGEN INTERACTION.



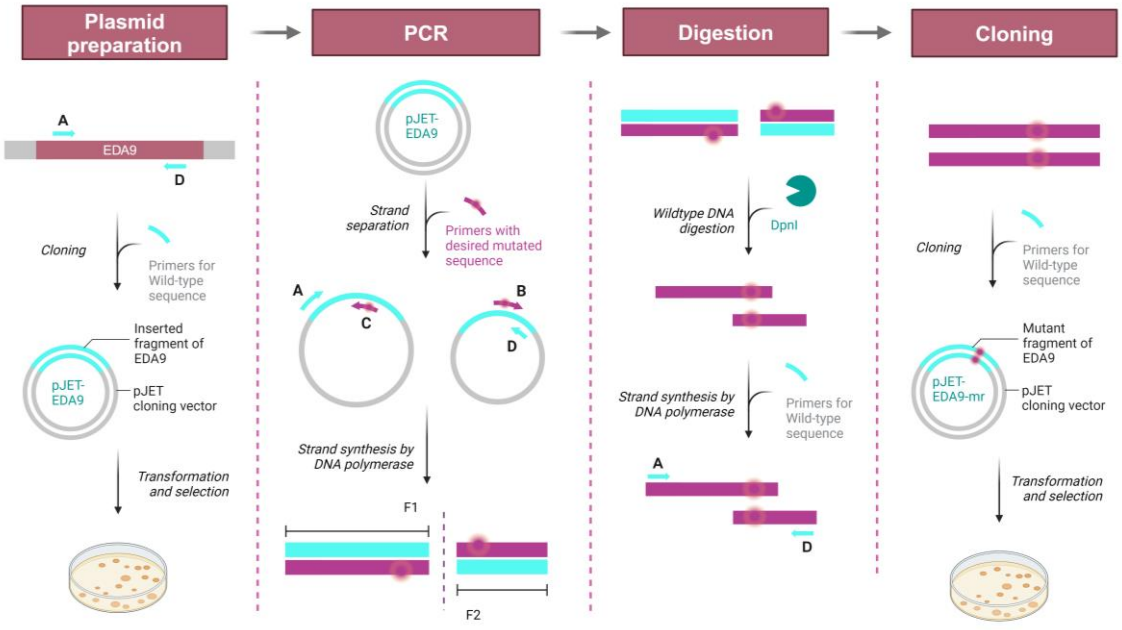
Supplementary 1. PTI. Pathogen Associated Molecular Patterns (PAMPs) are recognized by Pathogen Recognition Receptors (PRR) located on the plant cell membrane. This recognition initiates a signaling cascade that activates the expression of defense-related genes, ultimately triggering the plant's immune response. **ETS.** To facilitate infection and colonization, pathogens secrete effectors that inhibit plant immune signaling. These effectors suppress the activation of the plant's immune defenses, allowing the pathogen to evade detection and establish infection. **ETI.** Nucleotide Binding Leucine-Rich Repeat (NB-LRR) proteins in plants detect pathogen effectors, which initiates a robust signaling cascade. This leads to the activation of defense genes and a stronger, more targeted immune response against the pathogen.

SUPPLEMENTARY 2. POINT MUTATIONS DESIGNED FOR THE MUTANT SEQUENCE OF AT-*EDA9* RESISTANT TO *TA_SRNA2*



Supplementary 2. Schematic representation of the six-point mutations designed for the mutant *At-EDA9* sequence resistant to *Ta*_sRNA2. The illustration highlights silent nucleotide changes introduced in the *At-EDA9* target sequence to disrupt *Ta*_sRNA2-mediated silencing while preserving the original amino acid sequence.

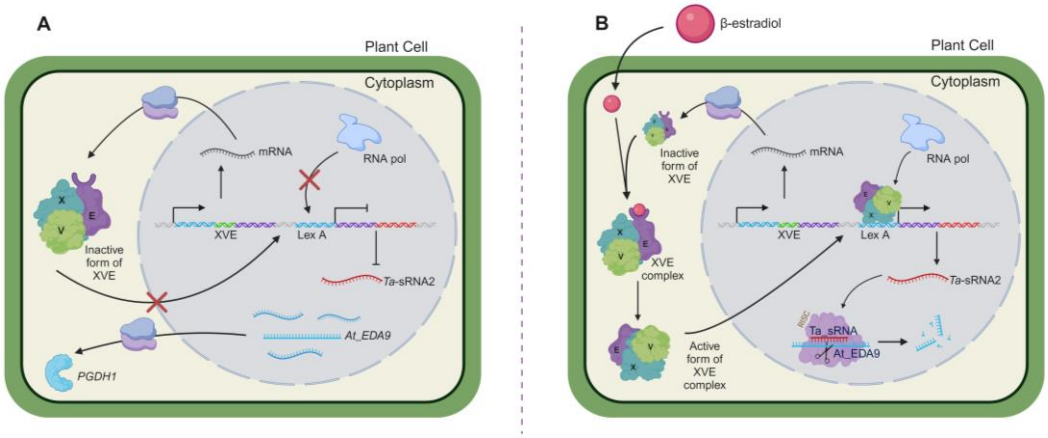
SUPPLEMENTARY 3. SITE-DIRECTED MUTAGENESIS STRATEGY BY PCR



Supplementary 3. Schematic illustration of the site-directed mutagenesis strategy employed to introduce specific nucleotide changes within the target gene sequence

of *At-EDA9* by PCR. The process involves designing forward and reverse primers containing the desired mutation (indicated by a purple fluorescent dot), which anneal to complementary sequences flanking the mutation site.

SUPPLEMENTARY 4. SCHEMATIC REPRESENTATION OF THE INDUCIBLE PROMOTER FOR *TA*_SRNA2



Supplementary 4. A. Schematic of the pGA-XVE-sR2 overexpression system of *Ta*_sRNA2 in the absence of the inducer β-estradiol. The XVE fusion protein (inactive form) remains in the cytoplasm, and transcription of *Ta*_sRNA2 is repressed due to the absence of the active XVE complex in the nucleus. The *Ta*_sRNA2, located downstream of the LexA binding site, is thus not transcribed, and regular levels of *At-EDA9* are observed. **B.** Upon the addition of the inducer β-estradiol, the XVE complex becomes activated and translocates into the nucleus, where it binds to the LexA operator sequence. This binding promotes RNA polymerase recruitment and transcription initiation, leading to the overexpression of *Ta*_sRNA2 and silencing of *At-EDA9*. The system will allow a controlled, inducible expression of *Ta_sRNA2* in response to β-estradiol, facilitating temporal regulation of gene expression of *At-EDA9* within plant cells.