A FUNGAL PROTEASE NAMED AsES TRIGGERS ANTIVIRAL IMMUNE RESPONSES AND EFFECTIVELY RESTRICTS VIRUS INFECTION IN ARABIDOPSIS AND *NICOTIANA BENTHAMIANA* PLANTS

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- **Background and Aims** Plants have evolved complex mechanisms to fight against pathogens. Within this mechanisms, pattern-triggered immunity (PTI) relies on the recognition of conserved microbe- or pathogen-associated molecular patterns (MAMPs or PAMPs, respectively) by membrane bound receptors. Indeed, PTI restricts virus infection in plants and, in addition, BRI1-associated kinase 1 (BAK1), a central regulator of PTI, plays a role in antiviral resistance. However, the compounds that trigger antiviral defences, along with their molecular mechanisms of action, remain mostly elusive. Herein, we explore the role of a fungal extracellular subtilase named AsES in its capacity to trigger antiviral responses.
- **Methods** In this study, we obtained AsES by recombinant expression, evaluated and characterized its capacity to trigger antiviral responses against *tobacco mosaic virus* (TMV), by performing time course experiments, analyzing gene expression, virus movement and callose deposition experiments.
- **Key Results** The results in this study provide direct evidence that the exogenous treatment with recombinant AsES increases a state of resistance against TMV infection, both in Arabidopsis and *Nicotiana benthamiana* plants. Also, the antiviral PTI response exhibited by AsES in Arabidopsis is mediated by the BAK1/SERK3 and BKK1/SERK4 co-receptors. Moreover, AsES requires a fully active salicylic acid (SA) signalling pathway to restrict the TMV movement by inducing callose deposition. Additionally, treatment with PSP1, a biostimulant based on AsES as the active compound, showed an increased resistance against TMV in *N. benthamiana* and tobacco plants.
- **Conclusions** AsES is a fungal serine protease which triggers antiviral responses relying on a conserved mechanism by means of the salicylic acid signalling pathway

and could be exploited as an effective and sustainable biotechnology strategy for viral disease management in plants.

Key words: protease, *tobacco mosaic virus* (TMV), plant immunity, BAK1, BKK1, salicylic acid (SA), callose.

INTRODUCTION

Plants have evolved an efficient immune system to prevent disease by defending themselves from many pathogens, such as fungi, bacteria and viruses. To survive to those biotic stresses, plants need to mount an effective immune response, which involves, unlike mammals, successful non-motile mechanisms (Serrano *et al.* 2016; Nejat and Mantri 2017).

Regarding the pathogens infecting plants, viruses are obligate intracellular parasites absolutely dependent on the host cell machinery to multiply and spread. They are capable of infecting all living organisms and are responsible for approximately 47% of plant diseases (Anderson *et al.* 2004). When viruses surpass the plant defence mechanisms and, therefore, succeed on plant infection, cause many physiological alterations leading to disease symptoms and damages, such as stunted growth, reduced vigour, decreased market esthetical values of the products and/or yield loss (Boualem *et al.* 2016). On the other hand, plants counteract pathogen infection by using different mechanisms. The first layer of defence is represented by pattern-triggered immunity (PTI), which is triggered upon perception of conserved structural microbes' motifs, termed microbe- or pathogen-associated molecular patterns (MAMPs/ PAMPs), through cell-surface-associated pattern recognition receptors (PRRs). PTI activation leads to a cascade of signalling events that involve changes in ion fluxes across the plasma membrane, production of reactive oxygen species (ROS), activation of mitogen-activated protein kinases (MAPKs), accumulation of salicylic acid (SA), differential expression of defence-related genes, callose deposition and stomatal closure (Bazzini *et al.* 2007; Boller and Felix 2009; Dodds and Rathjen 2010; Pieterse *et al.* 2012; Manacorda *et al.* 2021). Altogether, these events effectively repel most non-adapted pathogens, while contributing to basal immunity during infection (Couto and Zipfel 2016).

A second layer of defence involves intracellular immune receptors called R proteins, which directly or indirectly recognize effectors secreted by pathogens into the host

intracellular space and activate effector-triggered immunity (ETI). Although PTI and ETI seems to act independently, different researchers have recently suggested an intricate connection between these two immune signalling pathways, which reveals a mutual potentiation (Peng *et al.* 2018; Lu and Tsuda 2021; Ngou *et al.* 2021; Yuan *et al.* 2021). Virtually, all viral proteins, including replicase, movement proteins (MPs) and coat proteins (CPs), can act as avirulence (Avr) determinants (effectors recognized by R proteins) necessary for successful infections, and are almost invariably virulence factors in a susceptible host (Conti *et al.* 2012; Gouveia *et al.* 2017).

An additional intracellular detection system in plants specific for viruses involves RNA silencing, which is activated upon double-stranded (ds) RNA perception as a defence-inducing signal (Niehl and Heinlein 2019). This mechanism is initiated by type III RNases, DICER-LIKE (DCL), which binds and cleaves the dsRNA into 21 and 24 nucleotides (nt) called small interfering RNAs (siRNA) (Yang and Li 2018; Guo *et al.* 2019). Subsequently, siRNAs associate with an endogenous enzymatic RNA-induced silencing complex (RISC) responsible for recognizing target RNAs and for acting on their specific degradation. The ARGONAUTE protein, within the RISC complex, is involved in the degradation of viral chains whose sequence is complementary to that of siRNAs (Bologna and Voinnet 2014; Zhang *et al.* 2015; Boualem *et al.* 2016).

Plant immune responses against viruses seems to rely mostly on RNA silencing and ETI (Zvereva and Pooggin 2012; Mandadi and Scholthof 2013). In recent years, several studies have demonstrated that plants deploy the innate immune system to fight viruses, in a similar manner to that used for non-viral pathogens (Conti *et al.* 2017). In fact, PTI has a role against plant viruses (Yang *et al.* 2010; Kørner *et al.* 2013; Niehl *et al.* 2016; Zvereva *et al.* 2016; Nicaise and Candresse 2017). In line with this, researchers have reported the participation of the PRR co-receptors kinase BAK1/SERK3 (for BRASSINOSTEROID

INSENSITIVE1 (BRI1)-ASSOCIATED RECEPTORKINASE1, also named SOMATIC EMBRYOGENESIS RECEPTOR- LIKE KINASE 3, SERK3), BAK1-related SERK family member SERK4 (also called BAK1-LIKE, BKK1) and SERK family-related kinase NIK1 in virus resistance (Yang *et al.* 2010; Kørner *et al.* 2013; Zorzatto *et al.* 2015; Macho and Lozano-durán 2019).

In the context of infection, pathogens secrete numerous compounds of different nature to establish the disease. Among these compounds, proteases and specifically subtilases have gained great attention regarding their role in plant-pathogen recognition and immune priming (Ramírez *et al.* 2013; Figueiredo *et al.* 2014, 2018). *Acremonium strictum* elicitor subtilase (AsES) is an extracellular serine protease (GenBank accession number JX684014.2) obtained and purified from the opportunistic fungus *A. strictum* (Chalfoun *et al.* 2013; Racedo *et al.* 2013).

Previous studies have demonstrated that AsES is an elicitor capable of inducing a defence response by activating calcium influx, oxidative burst, cell-wall reinforcement by callose and lignin depositions, SA accumulation, upregulation of some defence-related genes, among others, both in strawberry and Arabidopsis (Chalfoun *et al.* 2013; Hael-Conrad *et al.* 2018; Perato *et al.* 2020; Caro *et al.* 2020). Moreover, AsES confers protection against pathogens of different lifestyles, such as the hemibiotrophic fungus *Colletotrichum acutatum* and the necrotrophic pathogen *Botrytis cinerea* (Chalfoun *et al.* 2013; Hael-Conrad *et al.* 2015). Recently, we have demonstrated that AsES is a conserved subtilase that elicits typical PTI-like defence responses in a BAK1/SERK3-dependant manner and that its proteolytic and eliciting activities are unrelated (Caro *et al.* 2020).

BAK1 is a central player in different aspects of plant immunity and seems to participate in resistance against diverse RNA viruses (Yang *et al.* 2010; Kørner *et al.* 2013). Here we explored if AsES acts as a broad spectrum elicitor capable of triggering antiviral responses,

and also addressed the role of BAK1 and its closest homolog BKK1 on the responses obtained. The results from this research provide direct evidence that Arabidopsis plants pretreated with AsES, and subsequently infected with Crucifer-infecting *tobacco mosaic virus* (TMV-cg) (Yamanaka *et al.* 1998), present an increased level of resistance against viral infection. Consistently, mutant plants for the co-receptors BAK1 and BKK1 exhibited increased susceptibility to virus infection, despite AsES treatment.

Thus, AsES antiviral responses relies on a conserved mechanism dependent on an active SA signalling pathway that involves different defence events oriented to restrict TMV spread in *Nicotiana benthamiana* plants. Moreover, PSP1, a biostimulant based on AsES, conferred increase resistance against TMV in N. *benthamiana* and *N. tabacum* (tobacco) plants.

MATERIALS AND METHODS

Plant material and growth conditions

The *Arabidopsis thaliana* lines used in this study were derived from Columbia (Col-0) ecotypes. The mutant lines used were *bak1-5 bkk1-1* (Schwessinger *et al.* 2011) and *fls2 efr cerk1*, a triple mutant for the corresponding pattern recognition receptor genes (Xin *et al.* 2016). *N. benthamiana* wild-type plants were employed for viral movement assays and the transgenic *NahG* were included in the virus tracking and callose experiments. *NahG* gene encodes a salicylate hydroxylase that metabolizes SA to catechol and, makes the plants unable to accumulate SA. *N. tabacum* (cv. Xhanti NN) plants were used for the local necrotic assay. Arabidopsis plants were grown as one plant per pot in a growth chamber under standard conditions at 21 °C. Relative humidity (RH) was maintained at 60 to 70%, with a 16/8 h light/dark cycle. *N. benthamiana* and *N. tabacum* plants were grown in a greenhouse under controlled temperatures within a 20/26°C range and a 16/8 h light/dark cycle.

AsES recombinant protein production

AsES protein was expressed in *E. coli*, extracted from its periplasm and subsequently purified by affinity chromatography using the 10× His-tag, following the protocol described by Caro et al. (2020). The protein content was quantified according to Bradford (1976) using bovine serum albumin as a standard. For biological assays, the fraction containing AsES protein was desalted and concentrated using Vivaspin ultrafiltration columns (Sartorius) with milli-Q water and 10% glycerol. The *E. coli* strain BL21 (DE3) carrying the pMAL-p5x vector (empty vector), after undergoing the same purification procedure than AsES, was used as a negative control of defence induction.

AsES treatment and TMV-Cg viral inoculation

Arabidopsis plants (1.06 stage) (Boyes *et al.* 2001) were sprayed with a 60 nM solution of AsES protein or with the empty vector solution. After 48 h of pretreatment, the third expanded leaf of each Arabidopsis plant (1.08 stage) was dusted with carborundum. Subsequently, 5 µl of semi-purified TMV-Cg virus diluted in 20 mM phosphate buffer (pH 7) were added and the surface of the leaf was gently abraded. Samples of the systemic leaves 8, 11 and 13 (those located immediately above of the inoculated leaf) were taken at 5 and 12 dpi. The leaves were frozen in individual tubes in liquid nitrogen and stored at −80 °C until RNA extraction.

AsES treatment and TMV-GFP viral inoculation

The abaxial side of 4 week-old *N. benthamiana* leaves was infiltrated with a 60 nM AsES solution (aprox. 500 µL) or the empty vector solution, as control, for 48 h. Afterwards, carborundum was dusted on the same pretreated leaf and 20 µl of semi-purified TMV-GFP viral inoculum (complete virus and GFP regulated by a duplicated *CP* promoter) (Lindbo 2007) was added before gently abrading the surface of the leaf. The TMV-GFP viral

inoculum that produces approximately 100 local lesions was employed to infect *N. benthamiana* plants.

PSP1 treatment and TMV-U1 viral inoculation

PSP1 (trademark Howler in Argentina, Summit Agro S.A.) was a gift from ANNUIT S.A. PSP1 was diluted to a 2% solution and sprayed on the adaxial side of N. benthamiana and tobacco plants and 48 h later, inoculated with TMV, strain U1. TMV-U1 viral inoculum was diluted to produce approximately 80-100 local lesions per leaf. In the case of tobacco, the number of local lesions was determined by counting the lesions evidenced by a hypersensitive response (HR). Acibenzolar-S-methyl (ASM) is sold under the trade names BOOST®, ACTIGARD® and BION® (Syngenta, Switzerland) and it was used according to the manufacturer's instructions.

RNA extraction and RT-qPCR analysis

Total RNA was isolated from frozen-ground Arabidopsis leaf tissue using Trizol Reagent (Invitrogen) and then treated with DNAse I (Invitrogen) for removal of genomic DNA contamination. The RNA concentration was determined by using an ND-1000 spectrophotometer (NanoDrop Technologies). The purity of total RNA was determined by the OD_{260nm}:OD_{280nm} ratio.

The first-strand cDNA was synthesized for messenger-RNA detection by using MMLV (Invitrogen) from 1.5 µg of pure RNA, according to the manufacturer's instructions. All RT-qPCR experiments were carried out in a StepOne Plus Real-Time PCR System (Applied Biosystems). All experimental conditions were performed with a minimum of eight biological replicates and two technical replicates, as recommended by minimum information for publication of quantitative real-time PCR experiments (MIQE) requirements (Bustin *et al.* 2009) (see Supplementary data Table S1 for more details). Elongation factor *EF1α*

(At5g60390) was used as the internal reference gene; previously selected by our group for its invariant expression under viral-infection conditions. The primers used for RT-qPCR experiments are listed in Supplementary data Table S2. RT-qPCR data analysis and primer efficiencies were obtained using LinReg PCR software (Ruijter *et al.* 2009). Data analysis was performed according to the Pfaffl algorithm (Pfaffl 2001). Relative expression ratios and statistical analysis were performed using fgStatistics software interface (http://sites.google.com/site/fgStatistics/). The cut-off for statistically significant differences was set as $p < 0.05$.

Local and systemic movement assays

TMV-GFP cell-to-cell movement was determined in *N. benthamiana* plants pretreated with AsES or control solutions. The diameter of the infection was determined on 20 random infection foci in each plant, by using Vernier Caliper foci under a 365 nm UV light lamp (UV LED 7 w/220 V). Systemic movement analyses consisted of determining the presence of infection fluorescence in distal tissues through direct visualization under a 365 nm UV light lamp (UV LED 7 w/220 V) at 5 and 7 dpi. Twelve biological replicates (n= 12) for each treatment were used and the results were expressed as the mean ± SE of at least three independent experiments. The asterisks indicate statistically significant differences between the control plants and the AsES proteins tested ($p <0.05$).

Callose quantification

Callose depositions were evaluated through a histochemical staining technique in leaves from 5-week old *N. benthamiana* plants that had been hand infiltrated either with the empty vector solution (control) or AsES solution (60 nM). After 48 h, a group of control and AsES pretreated plants were also infected with TMV as previously described. Evaluations were performed at 5, 7 and 9 dpi on local and systemic tissues. Harvested leaves were stained

with 0.01% aniline blue (Sigma) according to Yun et al. (2006). Images were taken by fluorescence microscope with UV filter (excitation, 365/10 nm; emission, 460/50 nm). The callose deposits were quantified using ImageJ software (https://imagej.nih.gov/ij/). One leaf was harvested per plant. At least nine biological replicates were used for each treatment.

RESULTS

AsES protein induces TMV-Cg resistance in Arabidopsis thaliana

To investigate whether AsES acts as a broad spectrum elicitor and, therefore, the induced activation of the immune signalling responses are also effective to restrict virus infection, we analysed the effect of AsES treatment by infecting Arabidopsis plants with TMV-Cg. The exogenous application of AsES resulted in a significant reduced accumulation (more than 30-fold reduction, Log_2 scale <-5) of the virus in relation to the control (empty vector)-treated plants at early and late stages of the infection, 5 and 12 days post-infection (dpi), respectively (Fig. 1 A). Arabidopsis Col-0 plants infected with TMV-Cg displayed mild symptoms associated with growth retardation, leaf yellowing and a delay in floral transition (Dardick *et al.* 2000; Pereda *et al.* 2000; Rodriguez *et al.* 2014; Zavallo *et al.* 2015). Consistently, regarding the phenotypic aspects, the infected plants sprayed with AsES showed no clear symptoms of infection in comparison to the control-infected plants; actually, the AsES treated-infected plants mimicked the aspect of mock non-infected plants (Fig. 1B). Importantly, upon AsES treatment, the *fls2 efr cerk1* mutant (*fec*) still presented a significant reduction of viral accumulation (Supplementary data Fig. S1). This finding confirms that AsES, instead of any contaminant that may be present because of the bacterial-recombinant origin of AsES, is responsible for the antiviral response.

Thus, these results suggest that AsES induces antiviral immunity to TMV-Cg.

The co-receptors BAK1 and BKK1 mediate TMV-Cg resistance induced by AsES protein

The induction of plant PTI responses by different PAMPs are often associated with common co-receptor kinases (Saijo *et al.* 2018; DeFalco and Zipfel 2021). Previous results have reported that the co-receptor SERK3/BAK1, a central regulator in PTI responses, is associated with resistance against three different RNA viruses (Yang *et al.* 2010; Kørner *et al.* 2013) and that it also mediates AsES-induced immune responses (Caro *et al.* 2020). In line with this, we investigated the potential role of SERK3/BAK1 and SERK4/BKK1 on the activation of antiviral responses induced by AsES. The assay consisted of analysing viral accumulation in systemic tissues of TMV-infected Arabidopsis mutants pretreated with AsES for 48 h before the infection. The selected mutant was the SERK family co-receptor kinase double mutant *bak1-5 bkk1-1*.

Despite the results obtained for Col-0 plants, at 5 and 12 dpi the *bak1-5 bkk1-1* mutant showed no significant differences with the control-infected plants regarding viral accumulation levels (Fig. 2A). Furthermore, with respect to TMV-infection symptoms, at 12 dpi AsES-treated *bak1-5 bkk1-1* mutant plants displayed similar disease symptoms to the control-infected plants, as evidenced by growth reduction and a shortened floral stem (Fig. 2B). This finding suggests a BAK1/BKK1-AsES dependent signalling on antiviral defence. As expected, and based on previous reports (Yang *et al.* 2010; Kørner *et al.* 2013; Niehl and Heinlein 2019), a comparison between Col-0 control-TMV and *bak1-5 bkk1-1* control-TMV plants revealed a significantly higher viral accumulation on the mutant; which indicates the mutant is more susceptible to TMV-Cg infection (Supplementary data Fig. S2).

AsES activates a plethora of defence-related genes during TMV-Cg infection

To explore the role of the potential defence-related signalling pathways involved in AsES-induced resistance to TMV, we analysed the expression profile of a set of genes by quantitative reverse transcription PCR (RT-qPCR). The study included *PR1*, *WRKY70*, *ERF6* (ETHYLENE RESPONSE FACTOR6), *PDF1.2 (PLANT DEFENSIN 1.2)*, *WRKY8* and *ERF104* (ETHYLENE RESPONSE FACTOR 104). *PR1* and *WRKY70* are both genes involved in the SA pathway (Li *et al.* 2006; Spoel *et al.* 2009), whereas *ERF6* is a target of the MAPK network and regulator of ROS signaling (Sewelam *et al.* 2013). On the other hand, *PDF1.2* is an ethylene (ET)/ jasmonic acid (JA) responsive gene (Mur *et al.* 2006; Memelink 2009) and *WRKY8* is an abscisic acid (ABA) and SA responsive gene involved in defence against TMV-Cg (Chen *et al.* 2010, 2013). Finally, *ERF104* is an ET-responsive gene involved in basal immunity and regulated by WRKY8 during viral infection (Chen *et al.* 2013). The analyses were performed on systemic leaves of Col-0 and *bak1-5 bkk1-1* plants treated with AsES for 48 h, following infection with TMV-Cg and sampled after 5 and 12 days post infection.

As a result, Col-0 AsES-infected plants displayed significantly induced levels of *PR1* expression at 5 dpi in relation to the control-infected plants (Fig. 3A). Nevertheless, *PR1* expression decreased in AsES-treated infected plants at 12 dpi (Fig. 3A). *WRKY70* showed a similar pattern as *PR1*, with an increased expression at 5 dpi and a significant decrease at 12 dpi in AsES-treated infected plants in relation to the control- infected Col-0 plants (Fig. 3B). Although the mRNA level of *PDF1.2* was significantly reduced at 5 dpi, its expression level at 12 dpi was similar to that of the control plants (Fig. 3C). On the other hand, *ERF6* showed a significantly increased expression at 5 dpi, but no differences at 12 dpi (Fig. 3D). Concomitantly, *WRKY 8* expression showed an upregulation at 5 dpi and downregulation at 12 dpi (Fig. 3E). The *ERF104* gene showed a significant reduction at 5 dpi and no changes at

12 dpi (Fig. 3F). It is worth noting that the expression profile of the analysed genes exhibited in Col-0 plants because of an induction by AsES throughout infection was lost in the *bak1-5 bkk1-1* mutant plants (Fig. 3 A-F).

RNA silencing is one of the mechanisms implicated in viral counter-defence in plants. With this in mind, we evaluated the expression levels of the main components of the silencing machinery, *AGO1*, *AGO2* and *DCL1* (Qu *et al.* 2008), in TMV-Cg infected plants pretreated with AsES for 48 h before the infection. No significant differences were detected regarding the mRNA accumulation of *AGO1*, *AGO2* and *DCL1* in the plants during the TMV-Cg infection (Supplementary data Fig. S3).

AsES treatment increases TMV resistance in Nicotiana benthamiana *plants*

To evaluate whether other plant species are also sensitive to AsES protection against virus infection, we tested control and AsES-treated *N. benthamiana* plants for their resistance against infection with TMV, which was tagged with green fluorescent protein (GFP) (Lindbo 2007). Viral infection foci of *N. benthamiana* plants pretreated with AsES protein or the empty vector (control) for 48 h before TMV-GFP inoculation were assessed by direct observation of GFP fluorescence at 3, 4, 5 and 7 dpi, respectively (Fig. 4A).

Plants treated with AsES showed a significant reduction of infection foci in comparison with the control plants (Fig. 4A). The evaluation of primary infection foci number revealed that AsES-inoculated leaves accumulated on average70 % less TMV-GFP than the control plants (Fig. 4B). The observed reduction on the viral local infection level in this plant species of the Solanaceae family suggests that AsES broadly elicits antiviral immune responses in plants.

AsES treatment restricts TMV movement

To deeper characterize AsES-resistance effect against TMV we evaluated viral local and systemic movement as the infection progresses. The AsES treatment showed no significant differences regarding local cell-to-cell movement on inoculated leaves at 3, 4 and 5 dpi in relation to the control-infected plants (Fig. 5A). Nevertheless, AsES-inoculated leaves displayed significant differences at 7 dpi (Fig. 5A).

The TMV systemic movement analysis showed consistent results. AsES-treated-plants neither presented any detectable TMV-GFP fluorescence on distal tissue at 5 dpi, in contrast to the control-inoculated plants, in which the systemic infection symptoms were very clear (Fig. 5B). At 7 dpi, although AsES-inoculated plants presented systemic symptoms, these symptoms occurred mainly in the leaves located above the inoculated leaf (same side of the plant). Conversely, the signal observed in the control plants was significantly stronger than in the AsES-inoculated plants; moreover, the systemic symptoms reached proximal as well as more distant leaves (Fig. 5B). Taking into account that SA acts as the initiating signal for systemic resistance against TMV (Zhu *et al.* 2014) and that AsES treatment activates SA responsive genes during the TMV infection in Arabidopsis and *N. benthamiana* (Fig. 3 A-B and Supplementary data Fig. S4 respectively), we evaluated the role of this hormone on the delayed observed for TMV systemic movement upon AsES treatment. Our results indicate that the overall effect of reduction on the TMV systemic movement, was lost when the evaluation was performed on the SA-deficient *N. benthamiana NahG* transgenic plants (Gaffney *et al.* 1993; Delaney *et al.* 1994) (Supplementary data Fig. S5 A-B).

Taken together, our results demonstrate that AsES restricts TMV movement, probably locally but clearly at the systemically stage, and requires for this a fully active SA signalling pathway.

Early callose deposition at the inoculation site correlates with a delay in the systemic spread of the virus in AsES-treated plants

Callose accumulation was evaluated by aniline blue staining at the inoculation site of non-infected and infected *N. benthamiana* plants, previously treated with AsES. The AsES treated plants presented a significant increase in the callose deposits in comparison to the control plants at 5 dpi (Fig. 6 A-B). Upon TMV inoculation, an increase in the callose deposits was detectable in both control and AsES treated-infected plants in relation to non-infected plants at 5 dpi (Fig. 6 A-B).

Remarkably, the overall quantity of callose deposits observed at the local infection site was significantly higher for AsES/TMV plants than for the individual treatments, AsES or TMV infected plants. This indicates that upon infection, the defence response associated to callose accumulation induced by TMV is highly potentiated by the AsES treatment. No differences regarding callose accumulation on the distal tissues were observed at 5, 7 and 9 dpi of AsES and AsES/TMV plants in comparison to their respective controls (Supplementary data Fig. S 6 A).

Researchers have demonstrated that SA plays a crucial role on the callose depositions during the regulation of the plasmodesmata permeability conditioning the local and systemic spread of the virus (Benitez-Alfonso *et al.* 2010; Wang *et al.* 2013; Huang *et al.* 2019). With this in mind, we evaluated the role of this hormone in the callose accumulation triggered by AsES in the context of the viral infection. The assessment consisted of treating *N. benthamiana NahG* transgenic plants and WT plants (as controls) with AsES before inoculating them with TMV. Unlike the WT plants, AsES or AsES/TMV transgenic infected plants showed no increased levels of callose deposits at the inoculation site at 5 dpi (Fig. 6 C-D). Similar results were obtained in distal tissues of AsES and AsES/TMV transgenic infected plants at 5, 7 and 9 dpi (Supplementary data Fig. S 6 C-D).

Altogether, these results suggest that AsES-mediated defence response against TMV requires a fully active SA signalling pathway that would lead to higher callose accumulation levels.

The biostimulant PSP1 triggers immune responses against TMV in N. benthamiana *and tobacco plants*

PSP1 is a biostimulant based on the elicitor AsES for disease management in monocot and dicot crops (Chalfoun, Durman, González-Montaner, *et al.* 2018; Chalfoun, Durman, Budeguer, *et al.* 2018). Considering that AsES triggers robust immune responses against TMV, we evaluated whether this effect was also observed for PSP1. In a similar manner to the assays performed with AsES, we evaluated PSP1 antiviral activity in *N. benthamiana* plants according to PSP1 manufacturer's instructions for field trial applications using TMV-GFP as inoculum, Data showed similar results, a statistical reduction of primary infection foci (Fig. 7 A-B).

In addition, to present a more comprehensive analysis of PSP1 functionality, we considered adding a cultivated species and a different test approach. In order to do this, tobacco plants carrying the *N* gene (Whitham *et al.* 1996) were used in a local necrotic lesion assay to test the antiviral effect of PSP1 upon TMV infection. The aerial parts of tobacco NN plants were sprayed with PSP1 as suggested by the manufacturer and after an induction period of 48 h, plants were infected with TMV. A chemical elicitor of plant immunity, Acibenzolar-S-methyl (ASM), which is a functional analog of salicylic acid, was also used as control (Louws *et al.* 2001; Kundu *et al.* 2011). Consistently, results showed that plants pretreated with PSP1 have a reduced amount of local necrotic lesion when compared to control plants (Supplementary data Fig. S7 A-B).

DISCUSSION

During the constant battle of plants against microbes, the perception of compounds of different nature known as "elicitors" trigger physiological and morphological responses in plants with the aim of restricting the pathogen colonization. To date, most described elicitors activate plant immunity against bacterial and fungal pathogens (Wiesel *et al.* 2014; Boutrot and Zipfel 2017; Malik *et al.* 2020) and the compounds associated to resistance against viruses are phytohormones, such as brassinosteroids (BRs) (Deng *et al.* 2016), SA and JA (Shang *et al.* 2011), carbohydrates (Iriti and Varoni 2015; Jia *et al.* 2016), glycolipids (Ipper *et al.* 2008; Chiu *et al.* 2018) or antimicrobial compounds (Luo *et al.* 2010). In this research, we report for the first time the capacity of a fungal subtilase named AsES to induce antiviral responses in different plant species, presenting an alternative and environmentally friendly strategy to minimize the scope of chemical disease management in plants.

The experiments with the well-established TMV-Cg/Arabidopsis system demonstrated obvious differences between the AsES-treated and control plants regarding TMV-Cg accumulation within systemically infected leaves. Moreover, the results obtained by RT-qPCR were consistent with the phenotypic data from AsES-treated infected plants, i.e. lack of symptoms. Thus, these data confirmed that AsES is an effective elicitor of plant immunity by inducing TMV-Cg resistance in Arabidopsis.

In recent years, rapid progress has been made in elucidating the mechanism of action of different MAMPs/PAMPs, as well as in the characterization of the pathogens against which they are effective. Within these mechanisms, BAK1, a member of the SERK family, seems to participate in the activation of the immune responses related to some of these compounds and, furthermore, may be the member of this family making the largest contribution to PTI (Ma *et al.* 2016). In a previous study, AsES induced hallmark PTI readouts, namely seedling

growth inhibition and activation of defence-related genes, in a BAK1-dependent manner (Caro *et al.* 2020).

In this study, we showed that the increased TMV resistance induced by AsES is lost in absence of BAK1 and its closest homolog BKK1. Our data is consistent with previous research that BAK1 and BKK1 contributed to antiviral resistance in plants (Yang *et al.* 2010; Kørner *et al.* 2013) (Supplementary data Fig. S2). For instance, a *bak1* Arabidopsis mutant presented increased susceptibility to different RNA viruses, namely TMV-U1 and *oilseed rape mosaic virus* (ORMV) (from the genus *Tobamovirus*) and *turnip crinkle virus* (TCV) (from the genus *Carmovirus*) (Kørner *et al.* 2013). In addition, a mutation in SERK4/BKK1 led to enhanced TCV accumulation (Yang *et al.* 2010). Another study have demonstrated that silencing *BAK1*, increased the susceptibility to TMV-GFP in *N. benthamiana* plants (Deng *et al.* 2016).

Nevertheless, a BAK1 dependency on plant defence induction is not necessarily associated with antiviral defence. For instance, plants pretreated with the very well-known flagellin-derived peptide flg22, which binds to the receptor kinase FLS2 and, immediately upon ligand perception heteromerizes with BAK1 (Chinchilla *et al.* 2007; Heese *et al.* 2007; Schulze *et al.* 2010), developed disease symptoms on infection by ORMV (Niehl *et al.* 2016).

Thus, we hypothesize that the antiviral defences associated with SERK3/SERK4 co-receptor strongly depends on specificity of perception upon ligand binding as well as on the temporal dynamics of the signalling events associated with antiviral immunity.

During plant infection, TMV directs transcriptional changes as means to enhance phloem loading and, consequently, systemic spread (Chen *et al.* 2013; Zavallo *et al.* 2015; Tsuda and Somssich 2015; Collum *et al.* 2016). Likewise, PAMPs perception by PRRs induce rapid, robust and selective transcriptional reprogramming, which is central for

launching an effective primary immune response (Li *et al.* 2016). Since AsES modulates the expression of defence-related genes (Chalfoun *et al.* 2013; Hael-Conrad *et al.* 2015, 2018; Perato *et al.* 2020; Caro *et al.* 2020), it could also act as a positive modulator of defence responses in the context of a viral infection, and, therefore, increase the resistance to, for example, TMV infection.

The involvement of SA-mediated defence responses during TMV infection (Zhu *et al.* 2014; Collum *et al.* 2016; Lee *et al.* 2016), as well as its participation on the activation of both local and systemic acquired resistance (SAR) in distal tissues, reduces the effects of secondary attacks (Alazem and Lin 2015). In previous studies, the AsES treatment induced *PR1* overexpression and SA accumulation in a time-dependent manner in strawberry plants (Chalfoun *et al.* 2013; Hael-Conrad *et al.* 2018). In addition, according to the results obtained in AsES-pretreated Arabidopsis plants, SA, JA and ET signalling pathways participate in the defence response induced, with a concomitant *PR1* upregulation (Hael-Conrad *et al.* 2018; Caro *et al.* 2020). Recently, our group has demonstrated that TMV negatively modulates the SA-signalling components, thus favouring the long distance movement and the consequent overall infection (Venturuzzi *et al.* 2021).

Altogether, these previously reported results agree with the ones obtained in the present study. Indeed, herein, the AsES treatment increased the expression of the SA-responsive genes *PR1* and *WRKY70* during the course of the infection. In addition, at 5 dpi the *PDF1.2* transcript level in the AsES-TMV infected plants was significantly lower than in the control-infected plants. Therefore, and based on the very well-known antagonistic role between the SA and JA signalling pathways (Kunkel and Brooks 2002; Mur *et al.* 2006), our data support the idea that AsES potentiates the activation of the SA signalling pathway and, therefore, the resistance observed against TMV-Cg.

ROS are constantly produced during a normal growth and development of plants, and also participate in signal recognition and transduction concerning plant responses to biotic and abiotic stresses (O'brien *et al.* 2012; Baxter *et al.* 2014; Waszczak *et al.* 2018). Considering that altered ROS levels commonly occur during viral infections (Conti *et al.* 2017) and that enhanced levels of ROS reduce TMV accumulation in *N. benthamiana* plants (Conti *et al.* 2012), herein we evaluated the expression pattern of the ROS responsive gene *ERF6* (Sewelam *et al.* 2013) on AsES treatment during TMV-Cg infection. The assays revealed that AsES upregulates *ERF6* at an early stage of infection; which suggests that ROS participates on AsES-induced virus defence signalling. ROS and plant hormones interact (Mittler *et al.* 2011; Xia *et al.* 2015); therefore, the induction of genes associated with ROS and plant hormones by AsES suggests a positive combination when considering the responses observed against viral infections.

On the other hand, WRKY8 transcription factor participates in responses to stressful stimuli and plant basal defence (Chen *et al.* 2010), with a demonstrated positive role in mediating plant defence responses against TMV-Cg (Chen *et al.* 2013). In this study, during viral infection, AsES significantly upregulated *WRKY8* expression in the mutant plants in relation to the control-infected plants. These results are consistent with previous research in which TMV-Cg infection inhibited WRKY8 expression (Chen *et al.* 2013). Thus, since WRKY8 seems to have a role as an activator of anti-TMV-Cg defences as well as an inhibitor of virus transport in vascular bundles, the reduced viral accumulation upon AsES treatment may be explained by the upregulation of WRKY8.

Moreover, our results showed that *WRKY8* upregulation at early times of infection negatively correlates with a significant low expression level of the ET responsive TF *ERF104* upon AsES treatment. Chen et al. (2013) have reported similar results for TMV-Cg infection, demonstrating that WRKY8 negatively regulates *ERF104.* In previous studies, researchers

have suggested that ET plays a negative role in viral infections (Love *et al.* 2007; Chen *et al.* 2013). All these data and the fact that ET-responsive genes expression is lower in AsES-treated plants during infection supports the idea that AsES activates antiviral defence by alleviating the repression of the SA signal transduction.

The RNA silencing pathway, which is another well-known component of the antiviral immunity, modulates and tunes responses to certain stresses by employing hormones such as SA and ABA (Yang and Li 2018; Alazem *et al.* 2019). As mentioned before, in this study AsES activated the SA pathway during TMV-Cg infection. With all this in mind, we evaluated whether the antiviral defence responses observed upon AsES treatment were related to the RNA silencing as the main operating defence mechanism. Our data demonstrated that AsES does not alter the transcript levels of key components of the silencing machinery such as *AGO1*, *AG02* and *DCL1* during the TMV-Cg infection. Thus, RNA silencing would not be the main mechanism involved in countering viral infection elicited by AsES.

A successful infection for plant viruses depends on the ability to access the vascular phloem and to move systemically into distal tissues. For this purpose, the virus must first accumulate within cells of the vascular system, then translocate trough the phloem and finally reach the distant susceptible tissues. Our tracking assay using TMV-GFP indicates that AsES significantly reduces the number of initial infection foci in *N. benthamiana* plants. Once the initial infection established, differences regarding cell-to-cell and systemic movement were evident on AsES-treated plants. Previous research has reported a positive correlation between the ability of TMV to move from cell-to-cell and the speed of systemic movement (Dardick *et al.* 2000). In the present study, a reduction in the TMV-cell-to-cell movement was linked to a delayed in TMV systemic movement upon AsES treatment. Likewise, a preceding study with a culture filtrate (F8) derived from a fungus from the genus *Trichosporon* showed

similar results. The treatment with the culture filtrate also inhibited initial, but not later, stages of viral infection in *N. benthamiana* (Chiu *et al.* 2018).

Callose deposits together with other early defence mechanisms are thought to restrict the viral movement to the site of infection (Wu *et al.* 2018) . In the present study, the callose deposits in AsES-treated TMV-infected plants displayed a significant increase at the local, but not distal, tissues at early stages of infection. Therefore, callose accumulation within the local site of infection, among the plethora of defence responses that are triggered by AsES, may explain the delay on the cell-to-cell movement. Furthermore, and based on other results obtained in this research, the delay on the progression of the infection is likely to be due to a delay on the load of the virus into the phloem, rather than because of the obstruction of the way out to systemically spread into the sink tissues. It is also important to note that the callose accumulation induced by AsES is much stronger in the presence of the virus. This may indicate that AsES produces an enhanced state of defence by preparing the plant (priming) to better respond to the moment of the pathogen attack. Similar results were recently reported by Li *et al*., (2021), when observed that a polypeptide extract derived from the fungus *Penicillium chrysogenum* induces callose deposition priming around plasmodesmata, restricting therefore TMV movement in *N. benthamiana* plants.

Herein, we also demonstrated the need of a fully active SA-mediated signalling pathway for triggering the defence response events induced by AsES and associated with viral immunity. This makes sense when considering that SA is one of the triggering signals regulating PD closure (Wang *et al.* 2013; Huang *et al.* 2019) and that AsES induces SA signalling events, before (Chalfoun *et al.* 2013; Hael-Conrad *et al.* 2018; Caro *et al.* 2020) and during the TMV infection. Similarly, in tobacco and tomato, SA treatment increased resistance to TMV by reducing viral accumulation and delaying the appearance of disease symptoms (Nie 2006; Liao *et al.* 2015).

In conclusion, this study demonstrates that AsES is a broad spectrum elicitor subtilase capable of activating antiviral PTI responses mediated by the SA signalling pathway in different plant species. AsES could be a suitable candidate to be exploited as a promising biotechnological and sustainable strategy for activating antiviral immunity in plants with a more robust defence and a better performance in the presence of the pathogen.

SUPPLEMENTARY DATA

Supplementary data are available online at https://academic.oup.com/aob and consist of the following. **Fig S1:** RT-qPCR experiments validating AsES antiviral resistance in *fls2 efr cerk1* mutant plants. **Fig S2:** RT-qPCR analysis of TMV mRNA accumulation in the double mutant *bak1-5 bkk1-1*. **Fig S3:** RT-qPCR analysis of components of the silencing machinery upon infection with TMV-Cg. **Fig. S4:** RT-qPCR analysis of a SA responsive gene in *N. benthamiana*. **Fig S5:** Analysis of the effect of AsES on TMV movement in *N. benthamiana*, *NahG*. **Fig S6:** Callose depositions induced by AsES during TMV infection on distal tissues of *N. benthamiana* plants. **Fig. S7:** PSP1, a biostimulant based on AsES, induces antiviral defence in *tobacco* plants.**Table S1**: MIQE checklist. **Table S2:** Primer sets used in this study.

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FIGURE LEGENDS

Fig. 1 AsES increases TMV-Cg resistance in Arabidopsis plants. (A) Quantitative real-time PCR analysis of TMV-Cg mRNA accumulation in Arabidopsis plants treated with AsES. Relative mRNA Cg*CP* level was determined in Col-0 plants, pretreated with an AsES solution (60 nM), at 5 and 12 days post infection (dpi). The transcript level is referenced to control-infected plants in Log2 scale. The mean value of eight biological replicas ± SE is shown. The asterisks indicate significant differences (* P-values < 0.05). **(B)** Phenotypic aspect of Col-0 Arabidopsis plants pretreated with the AsES solution and challenged with TMV-Cg. Arabidopsis Col-0 plants were treated with the AsES (60 nM) or empty vector (control) solutions for 48 h and then infected with TMV-Cg. Pictures were taken at 12 dpi. Each experiment was independently repeated at least three times; the results shown here are representative for each repeat.

Fig. 2 BAK1 and BKK1 mediateTMV-Cg-resistance induced by AsES elicitor protein. (A) Quantitative real-time PCR analysis of TMV mRNA accumulation in Arabidopsis plants treated with AsES. Relative mRNA Cg*CP* level was determined at 5 and 12 days post infection (dpi) on AsES pretreated (60 nM) in Col-0 and *bak1-5 bkk1-1* mutant plants. The transcript level is referenced to control-infected plants. The mean value of eight biological replicas ± SE is shown. The asterisks indicate significant differences (* P-values < 0.05). **(B)** Phenotypic aspect of *bak1-5 bkk1-1* mutant Arabidopsis plants pretreated with AsES and challenged with TMV-Cg. Arabidopsis *bak1-5 bkk1-1* mutant plants were treated with the AsES (60 nM) or empty vector(control) solutions for 48 h and then infected with TMV-Cg. Pictures were taken at 12 dpi. Each experiment was independently repeated at least three times; the results shown here are representative for each repeat.

Fig. 3 AsES treatment upregulates transcriptional level of SA-responsive genes to counteract viral infection. (A) to **(F)** Relative mRNA levels of *PR1*, *WRKY70*, PDF1.2, *ERF6*, *WRKY8* and *ERF104* were determined at 5 and 12 days post infection (dpi) on TMV-Cg infected Col-0 and *bak1-5 bkk1-1* mutant plants, previously elicited for 48 h by AsES treatment. The expression levels were referenced to control-infected plants (empty vector-treated) in Log_2 scale.. Elongation factor *EF1α* was used as the internal reference gene. Data correspond to the mean ± standard error of eight biological replicates. The asterisks indicate significant differences in relation to the control-infected plants (*P-values < 0.05).

Fig. 4 AsEs induces antiviral defences in *N. benthamiana* plants. (A) *N. benthamiana* leaves were treated with the empty vector (control) or the AsES (60 nM) solutions for 48 h and then inoculated with TMV-GFP. Pictures were taken under a UV light at 3, 4, 5 and 7 days post infection (dpi). Representative pictures for each treatment and time-points are presented. **(B)** The infection level was determined as the initial Nº of GFP spots per infiltrated leaf. The mean value of 12 biological replicas ± SE is shown. The asterisks indicate significant differences (*P-value <0.05; **P-values <0.01, Student's t-test).

Fig. 5 AsES restricts cell-to-cell movement and mediates resistance to systemic infection of TMV-GFP. (A) Cell-to-cell movement evaluation of TMV-GFP particles in *N. benthamiana* plants pretreated with the empty vector (control) or AsES (60 nM) solutions at different time points. The average value obtained from 12 biological replicas ± SE is shown. The asterisks indicate significant differences (* = P-values <0.05, Student's t-test). **(B)** Local and Systemic movement of TMV-GFP viral particles at 5 and 7 days post infection (dpi) in *N. benthamiana* plants preinfiltrated with the empty vector (control) or AsES solutions. A representative picture corresponding to local and distal tissues for control or AsES treatment is presented.

Fig. 6 Contribution of callose deposition to AsES-Induced resistance against TMV in local tissues. (A) WT **(C)** *NahG* mutant *N. bentahmiana* plants were infiltrated either with the empty vector (control) or AsES (60 nM) solutions, whereas a group of the treated plants were also infected with TMV after a 48 h of pretreatment. Callose depositions were evaluated in treated leaves at 5 days post infection (dpi), stained with aniline blue and analysed by fluorescence microscope. **(B), (D)** Callose quantification on WT or *NahG N. bentahmiana* plants respectively. Data represent the mean ± SE of three independent experiments with nine plants per experiment. The asterisks indicate significant differences (* P-value <0.05; *** P-values <0.001, Student's t-test).

Fig. 7 PSP1, a biostimulant based on AsES, induces antiviral defenses in *N. benthamiana* plants. (A) *N. benthamiana* leaves were treated with water (control) or a 2% solution of PSP1 for 48 h and then inoculated with TMV-GFP. Pictures were taken under a UV light at 4 days post infection (dpi). Representative pictures for each treatment are presented. (B) The infection level was determined as the initial Nº of GFP spots per leaf. The mean value of 12 biological replicas ± SE is shown. The asterisks indicate significant differences (*P-value <0.05; **P-values <0.01, Student's t-test).

Figure 1

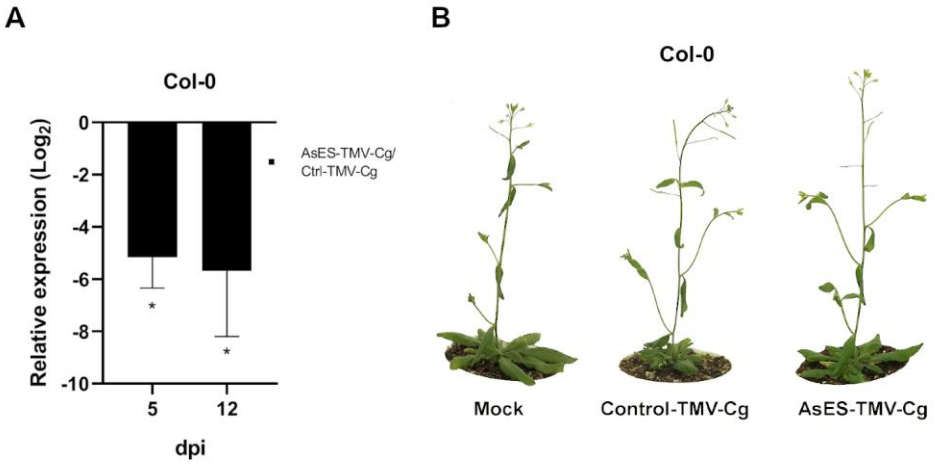

Figure 2

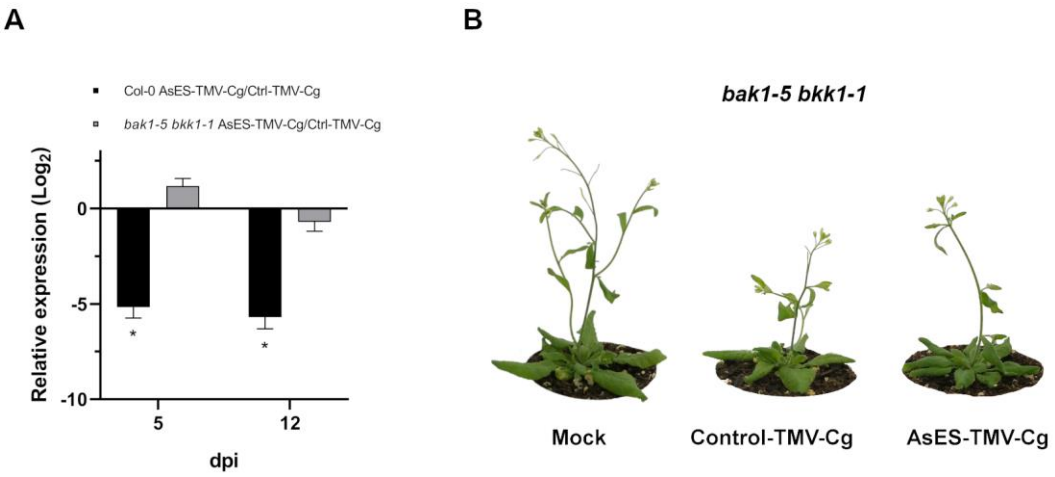

Figure 3

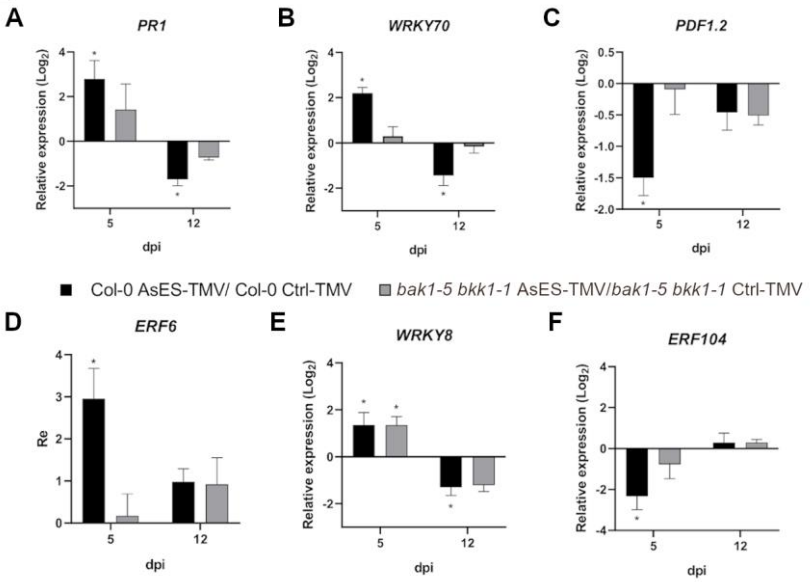

Figure 4

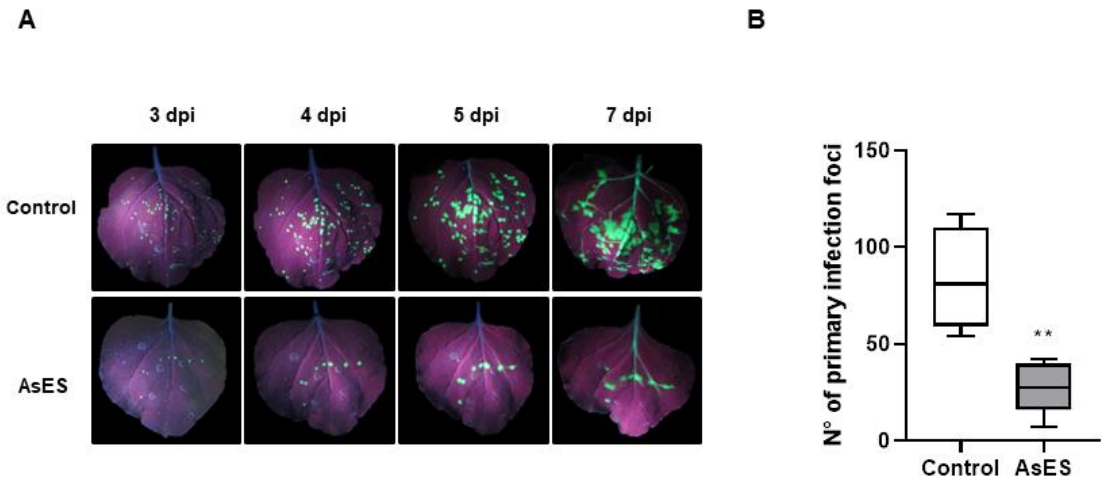

Figure 5

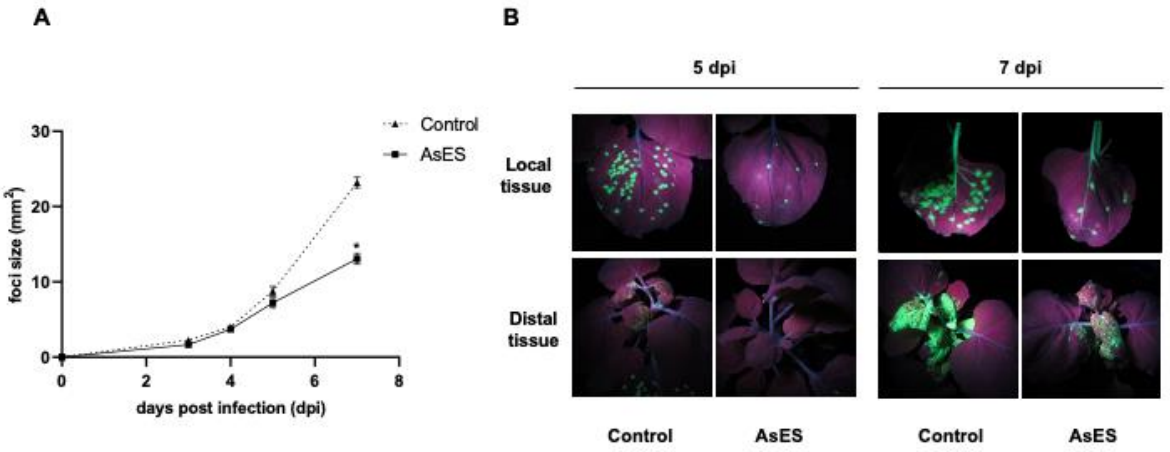

Figure 6

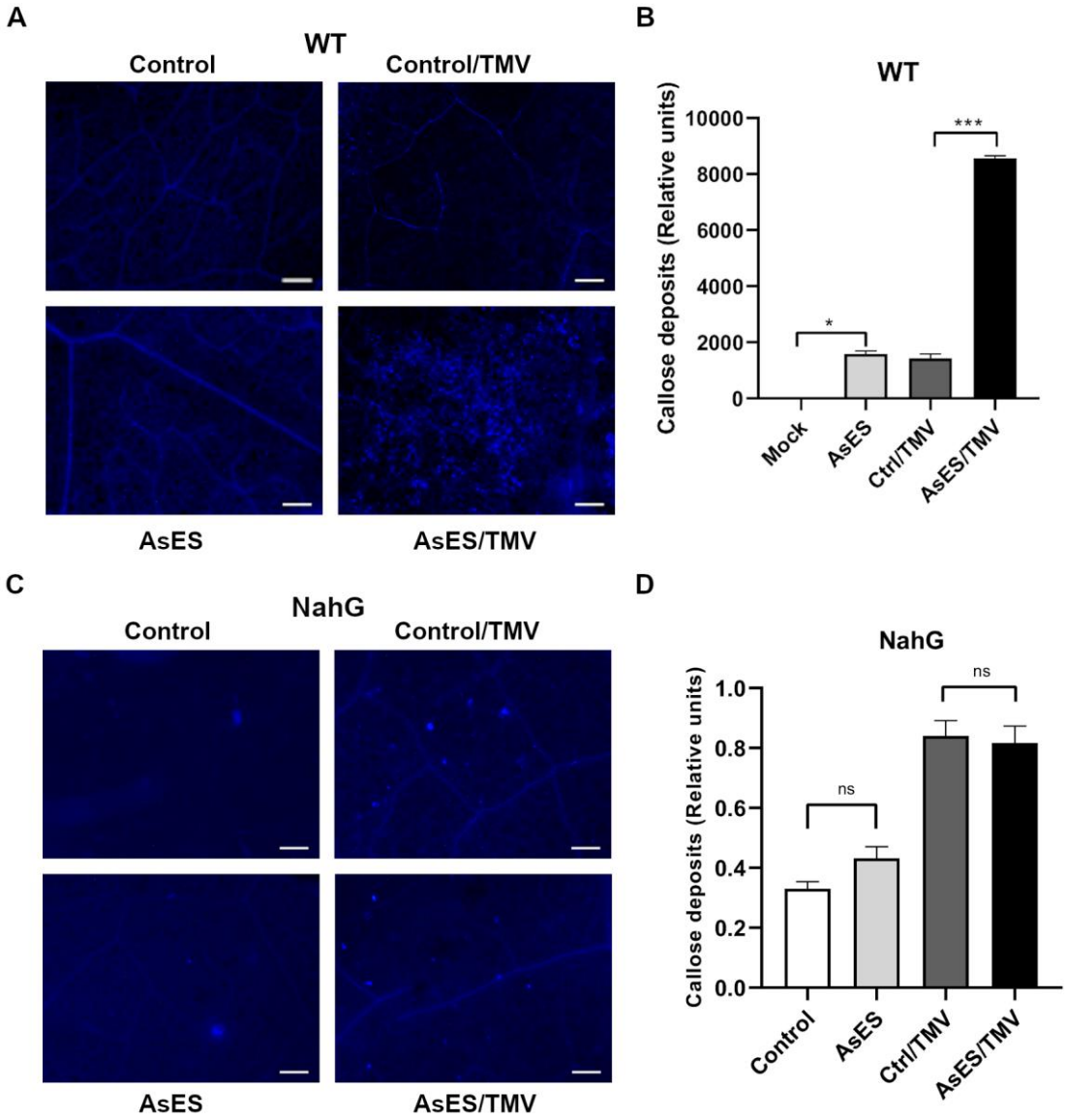

Figure 7