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The fungal subtilase AsES elicits a PTI-like defence response in *Arabidopsis thaliana* plants independently of its enzymatic activity

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SUMMARY

Acremonium strictum elicitor subtilisin (AsES) is a 34-kDa serine-protease secreted by the strawberry fungal pathogen *A. strictum*. On AsES perception, a set of defence reactions is induced, both locally and systemically, in a wide variety of plant species and against pathogens of alternative lifestyles. However, it is not clear whether AsES proteolytic activity is required for triggering a defence response or if the protein itself acts as an elicitor. To investigate the necessity of the protease activity to activate the defence response, AsES coding sequences of the wild-type gene and a mutant on the active site (S226A) were cloned and expressed in *Escherichia coli*. Our data show that pretreatment of *Arabidopsis* plants with inactive proteins, i.e. inhibited with phenylmethylsulphonyl fluoride (PMSF) and mutant, resulted in an increased systemic resistance to *Botrytis cinerea* and expression of defence-related genes in a temporal manner that mimics the effect already reported for the native AsES protein. The data presented in this study indicate that the defence-eliciting property exhibited by AsES is not associated with its proteolytic activity. Moreover, the enhanced expression of some immune marker genes, seedling growth inhibition and the involvement of the co-receptor BAK1 observed in plants treated with AsES suggests that AsES is being recognized as a pathogen-associated molecular pattern by a leucine-rich repeat receptor. The understanding of the mechanism of action of AsES will contribute to the development of new breeding strategies to confer durable resistance in plants.

Keywords: enzymatic activity, eliciting activity, plant defence, PAMP, subtilase.

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INTRODUCTION

Plants have evolved a sophisticated immune system to defend themselves from invading pathogens and pests that otherwise would cause devastating ecological and economic effects on ecosystems and agriculture (Boutrot and Zipfel, 2017).

Plants use a multilayered recognition system to protect against microbial infection, where the first tier involves pattern-recognition receptors (PRRs) localized at the cell surface that function as 'radars', detecting potentially dangerous compounds of a different nature, termed PAMPs or MAMPs (pathogen- or microbe-associated molecular patterns, respectively). PRR activation leads to a basal immune response called PAMP-triggered immunity (PTI) that produces a massive transcriptional reprogramming and biosynthesis of a complex mixture of metabolites that restrict microbial colonization (Yu *et al.*, 2017). On the other hand, pathogens have evolved a battery of virulence factors known as effectors, some of which are delivered inside host cells, to interfere with PTI and dampen basal defences (Cui *et al.*, 2015; Dangl *et al.*, 2013). A second layer of defence includes intracellular receptors called nucleotide-binding/leucine-rich-repeat (NLR) receptors, which recognize pathogen effectors and induce a faster and stronger resistance response than PTI, termed effector-triggered immunity (ETI) (Jones and Dangl, 2006). This response is often accompanied by a localized programmed cell death called the hypersensitive response (HR) and is effective against race-specific host-adapted pathogens (Spoel and Dong, 2012; Zebell and Dong, 2015). In addition, distally located tissues that are not in contact with any PAMP or MAMP may acquire a higher level of resistance (referred to as systemic acquired resistance, SAR) that can prime the plant against future pathogen attacks (Liu *et al.*, 2010; Mauch-Mani *et al.*, 2017). However, not all microbial defence activators conform to the common distinction

between PAMPs and effectors (Thomma *et al.*, 2011). For example, HR is usually associated with NLR-triggered immunity (Cui *et al.*, 2015) but there are several PAMPs (e.g. bacterial flagellin and oomycete elicitins) that can also induce HR (Taguchi *et al.*, 2003). Although it is known that PTI and ETI share many signalling components (Kadota *et al.*, 2019), PTI is often considered a weaker variant of ETI, less robust and transient (Jones and Dangl, 2006; Tsuda and Katagiri, 2010). Nevertheless, there are examples of PAMPs that systemically enhance resistance and are not associated with HR or necrotic disease symptoms on perception (Boutrot and Zipfel, 2017; Mishina and Zeier, 2007). Besides atypical examples of strong PTI responses, examples of weak ETI responses have been observed as well (Thomma *et al.*, 2011; Wirthmueller *et al.*, 2007).

Subtilisin-like proteases (also known as subtilases) belong to a large protein family widely distributed in archaea, bacteria and eukaryotes (Schaller *et al.*, 2012). Subtilases have a conserved catalytic triad characterized by the amino acids aspartic acid, histidine and serine (Dodson and Wlodawer, 1998), and have been classified according to MEROPS (http://merops.sanger.ac.uk) into the S8 family, within the SB clan of serine proteases and grouped into the subfamily S8A, with subtilisin being the type example of this subfamily (Rawlings *et al.*, 2006). With a broad spectrum of biological functions, these proteins have gained increasing attention with regard to their participation in responses to biotic and abiotic stresses and more recently in plant pathogen recognition and immune priming (Figueiredo *et al.*, 2014, 2018).

Acremonium strictum elicitor subtilisin (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). It is synthesized as a 388-residue inactive precursor composed of a signal peptide (SP), an N-terminal propeptide (I9) and a peptidase domain (S8). The inactive precursor undergoes autocatalytic maturation to release an active enzyme in a stepwise manner. First, the SP mediates the enzyme secretion outside the cytoplasmic membrane, where it is cleaved by a signal peptidase. Then, the I9 domain, which temporarily inhibits the enzyme activity and acts as a chaperone, is removed, resulting in an active protein (S8 domain) that consists of 283 amino acids. The mature protein (S8) is the one that retains proteolytic activity and, in addition, induces plant defence responses (Chalfoun *et al.*, 2013). It has been demonstrated that AsES triggers an immune response in strawberry plants (*Fragaria* × *ananassa*) conferring protection against anthracnose and grey mould diseases caused by the hemibiotrophic fungus *Colletotrichum acutatum* and the necrotrophic pathogen *Botrytis cinerea*, respectively (Chalfoun *et al.*, 2013; Salazar *et al.*, 2006). Among the defence reactions induced by AsES in strawberry are calcium influx, a biphasic burst of reactive oxygen species (ROS), HR, accumulation of phenolic compounds (PCs), cell wall reinforcement by callose and lignin depositions, salicylic acid (SA) accumulation, and the up-regulation of defence-related genes (Hael-Conrad *et al.*, 2017). AsES activity is not limited to a specific cultivar since it also confers protection to different strawberry cultivars against different virulent isolates of *C. acutatum* (Chalfoun *et al.*, 2013). Other highly homologous subtilases, such as proteinase K and Carlsberg, were also tested for their capacity to trigger an immune response in strawberry plants, but the eliciting activity was only found in AsES, suggesting that the proteolytic activity is responsible for defence induction (Chalfoun *et al.*, 2013).

It was further demonstrated that AsES eliciting activity is not limited to strawberry plants. In *Arabidopsis thaliana*, the defence response observed was characterized by a ROS burst and callose accumulation (Chalfoun *et al.*, 2013). It was also shown that AsES protects against *B. cinerea* through the activation of *A. thaliana* defence responses and that the signalling pathways of the hormones SA, jasmonic acid (JA) and ethylene (ET) were involved (Hael-Conrad *et al.*, 2015). Recently, it was shown that a biostimulant based on AsES, named PSP1 (plant stimulation and protection), reduces late-season disease development in field-grown soybean (Chalfoun *et al.*, 2018a). Chalfoun *et al.* (2018b) also reported that PSP1 protects against *Corynespora cassiicola* in soybean, red stripe (*Acidovorax avenae*) in sugarcane and head blight (*Fusarium graminearum*) in wheat.

With the aim of determining whether AsES proteolytic activity is required for triggering a defence response, we evaluated the eliciting property of an inhibited AsES protein and a mutant affected in its enzymatic activity. In this work, we provide direct evidence showing that proteolytic activity is not required to induce a PTI-like defence response.

RESULTS

Heterologous expression and purification of AsES and AsES^{S226A}

Constructs for recombinant AsES and the enzymatically inactive mutant AsES^{S226A} were made according to the schemes shown in Fig. 1A. Target proteins were fused to the MalE signal sequence to achieve export to the periplasm favouring correct protein folding, and to maltose-binding protein (MBP) with the goal of enhancing solubility (Fig. 1A). The expected molecular weights of Prodomain (Pro)-AsES (I9 + S8), AsES (S8) and AsES (S8)^{S226A} are listed in Table 1. Detection of target proteins was performed by SDS-PAGE/western blot sampled from the periplasmic extract (Figs 1B and S1A). The highest protein expression level was obtained, in both cases, when cells were induced by low isopropyl-β-D-thiogalactopyranoside (IPTG) concentrations (0.05 mM) and then incubated at lower temperatures, first at 28 °C (4 h) and then 4 °C overnight.

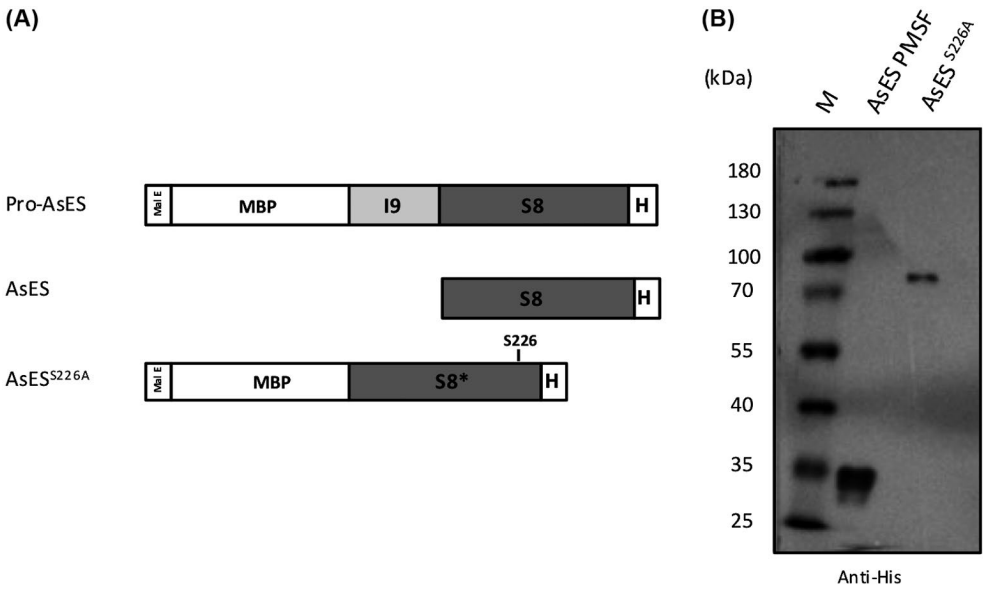

Fig. 1 Expression and purification of AsES and its derivatives. (A) Schematic representation of the structures of the immature form (Pro-AsES), its mature form (AsES) and a mutant version of it (AsES^{226A}). The fused malE signal peptide (MalE), the maltose-binding protein (MBP), the N-terminal propeptide (I9), the mature domain (S8) and the fused His-tag (H) are indicated. (B) Western blot analysis shows His-tagged AsES and AsES^{S229A} proteins purified to homogeneity with Ni-NTA resin. Molecular mass markers (M) in kilodaltons (kDa) are indicated on the left. Western blot analyses were performed using anti-His antibodies.

Table 1 Expected molecular weights of protein species described in this study

Proteins	Expected molecular weight (kDa)
Pro-AsES*,†	87.3
AsES†	34
AsES^{S226A*,†}	77.3

*These proteins have a fused MBP-tag.

†These proteins have a fused His-tag.

MBP-I9 + S8 protein was expressed as a zymogen with an apparent molecular weight of 87 kDa (Fig. S1B). The Pro-protein undergoes a stepwise processing resulting in a single band protein of approximately 34 kDa (Figs 1B and S1A,B) that corresponded to a soluble and pure mature AsES containing only the catalytic domain (S8). This result indicates that Pro-AsES has undergone a maturation process similar to that which occurs with AsES protein purified from *A. strictum* (WT-AsES). In order to prevent autolytic degradation and the one produced by other proteases (Bajorath *et al.*, 1988a,b), the enzymatic activity of recombinant AsES (S8) was deliberately inhibited with phenylmethylsulfonyl fluoride (PMSF) once the maturation process was completed (Fig. S2A,B and Table S2).

The protein band detected at 77 kDa corresponds to soluble, pure and enzymatically inactive AsES^{S226A} (MBP-S8^{S226A}) (Fig. 1B, Table S2).

AsES and AsES^{S226A} protect against *B. cinerea* infection

It was previously reported that WT-AsES protects against *B. cinerea* in *Arabidopsis* plants in a time- and dose-dependent manner (Hael-Conrad *et al.*, 2015). With the aim of confirming that recombinant AsES and AsES^{S226A} have a similar activity, 4-week-old *Arabidopsis* plants were pretreated and 48 h later were challenged with *B. cinerea*. The protective effect was evaluated in *A. thaliana* ecotype Col-0 and in the triple receptor mutant *fls2 efr cerk1* (*fec*) to rule out any side contaminant effect of flagellin or elongation factor-Tu that may be present due to the bacterial origin of the recombinant proteins assessed. Forty-eight hours after infection, Col-0 and *fec* pretreated plants presented a significant reduction (over 60%) in lesion area compared to control (empty vector) plants for both treatments (Fig. 2A–C). This protective effect was extended to 72 h for both treatments with a lesion area reduction of approximately 50% (Fig. 2A–C).

Taken together, these results indicate that AsES and AsES^{S226A} have a protective effect against *B. cinerea* in *Arabidopsis* plants, similarly to what was observed for WT-AsES (Fig. 2A–C) (Hael-Conrad *et al.*, 2015). By contrast and as expected, MBP protein does not have any protective effect (Fig. 2A,B).

AsES and AsES^{S226A} induce PTI marker gene expression

To characterize the defence response activated by AsES and AsES^{S226A}, the expression of the marker genes *PR-1* (Spoel *et al.*, 2009) and *WRKY70* (Li *et al.*, 2006) for their well-known regulatory

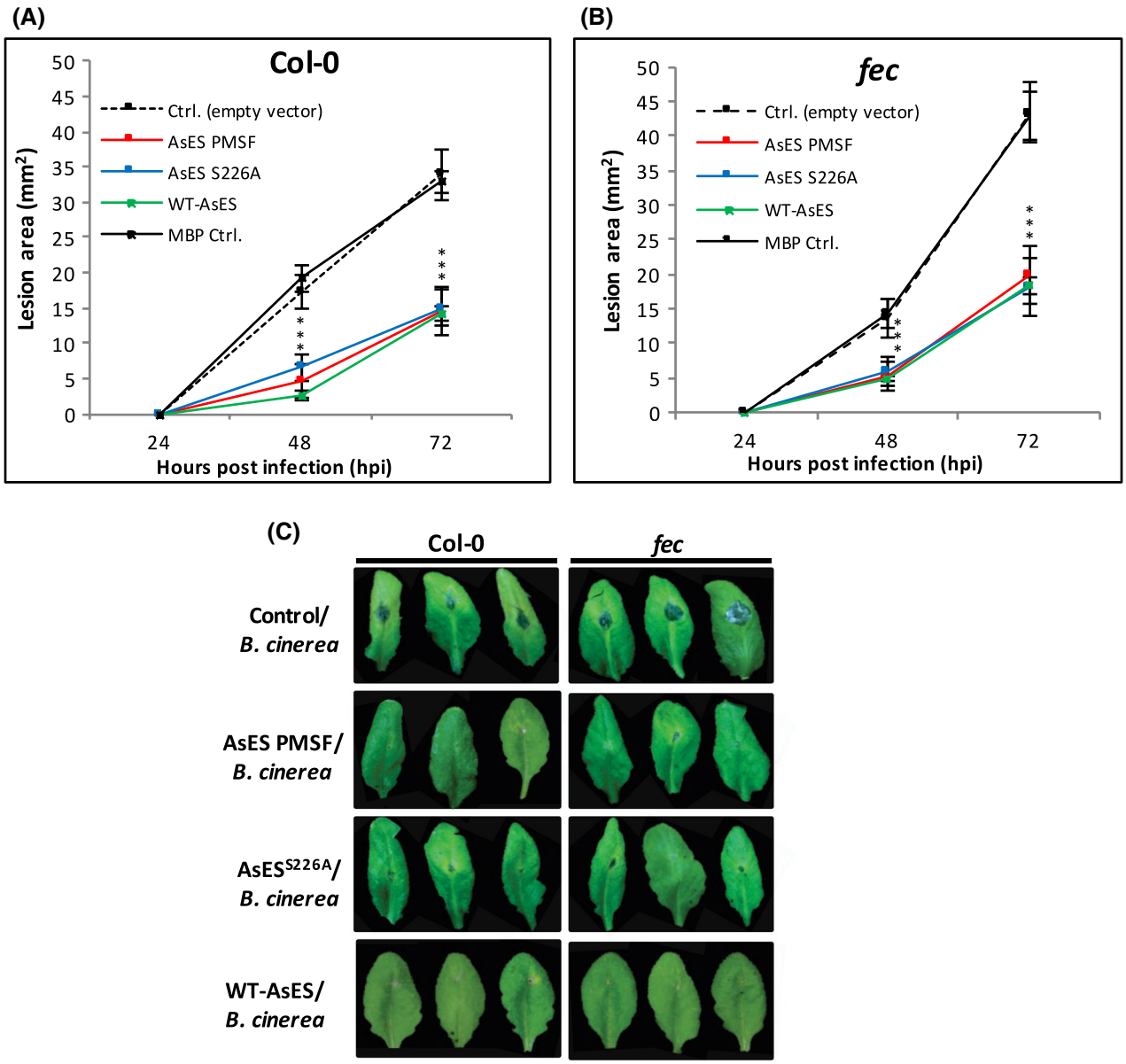

Fig. 2 Protective effect of AsES PMSF and AsES^{S226A} against *Botrytis cinerea* in *Arabidopsis*. Time-course of the development of the lesion area caused by *B. cinerea* in *Arabidopsis* plants, ecotype Col-0 (A) and in the triple mutant *fls2 efr cerk1* (*fec*, background Col-0) (B). Control (empty vector), AsES PMSF (60 nM) and AsES^{S226A} (60 nM) were applied 48 h prior to infection (hpti) and the lesion area was evaluated at 48 and 72 h post-infection (hpi). WT-AsES (60 nM) was used as a positive control of protection and maltose-binding protein (MBP), (60 nM) as a negative control for AsES^{S226A}. Mean values ± SE were obtained from three independent experiments ($n = 8$). Asterisks indicate a statistically significant difference between the mock, AsES PMSF, AsES^{S226A} and WT-AsES treated plants, according to Student's *t*-test ($P < 0.05$). (C) Appearance of *Botrytis* lesions on Col-0 and *fec* pretreated leaves with AsES PMSF (60 nM), AsES^{S226A} (60 nM) or WT-AsES (60 nM) compared to control plants at 72 hpi. A representative image of each treatment is presented.

role in the SA signalling pathway and *ERF6* for its central role in the JA/ET-mediated signalling pathway (Moffat *et al.*, 2012) were evaluated. Since major transcriptional changes induced by WT-AsES were observed at 4 and 48 hours post-treatment (hpt) (Chalfoun *et al.*, 2013; Hael-Conrad *et al.*, 2015), we focused on those time points to perform all gene expression analysis on treatment with AsES or AsES^{S226A}.

Quantitative reverse transcription PCR (RT-qPCR) analysis showed that *PR-1* was significantly up-regulated at 4 hpt in Col-0 and *fec* leaves after the treatment with AsES and AsES^{S226A} and as compared to mock plants (Fig. 3A). At 48 hpt, although the level of expression of *PR-1* decreased for both treatments, it was still slightly up-regulated when compared to mock-treated plants (Fig. 3A). The expression of *WRKY70* was significantly induced at

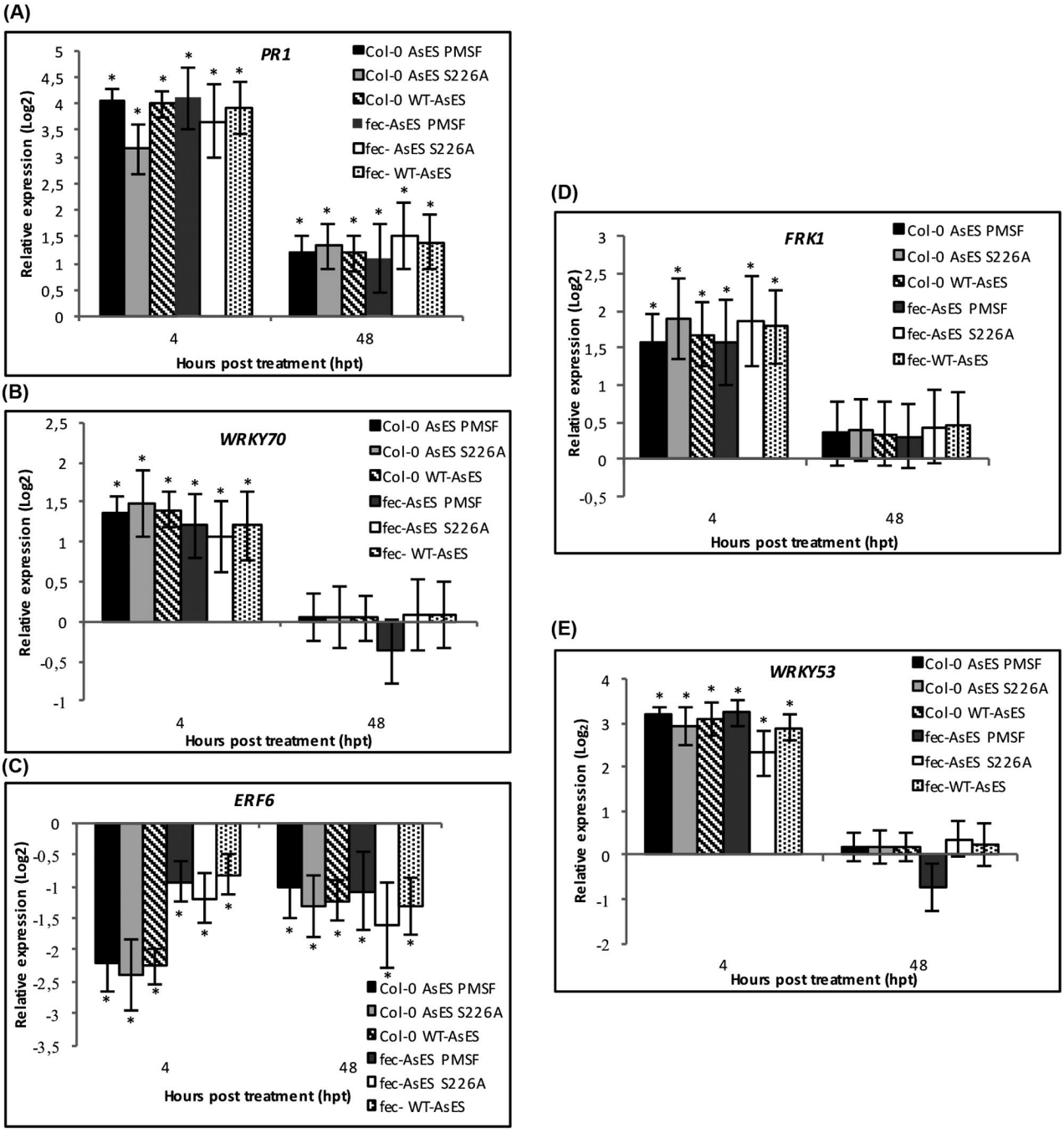

Fig. 3 AsES PMSF and AsES^{S226A} induce the SA defence pathway and activate PTI marker gene expression. *PR1* and *WRKY70* are involved in the SA pathway and ERF6 in the JA/ET signalling pathway. *FRK1* and *WRKY53* are PTI-marker genes. (A–E) Relative transcript levels determined by RT-qPCR at the indicated time points after treatment of Col-0 and *fls2 efr cerk1* mutant (*fec*) leaves with AsES PMSF (60 nM), AsES^{S226A} (60 nM) or WT-AsES (60 nM). The expression levels were referenced to non-induced plants (empty vector-treated) and arbitrarily set to 1 ($\log_2$ (1) = 0). Elongation factor *EF1α* was used as the internal reference gene. Data correspond to the mean ± standard error of eight biological replicates. Asterisks indicate statistically significant differences when compared to non-induced plants (*$P < 0.05$).

4 hpt for both treatments in Col-0 and *fec* plants (Fig. 3B); however, at 48 hpt the expression decreased in AsES and AsES^{S226A} treated leaves (Fig. 3B). In contrast, *ERF6* was significantly down-regulated at 4 hpt and remained unaltered at 48 hpt for both treatments (Fig. 3C). Next, we evaluated and compared the expression levels of two well-known PTI marker genes, *FRK1* and *WRKY53* (Asai *et al.*, 2002; Shan *et al.*, 2008; Singh *et al.*, 2012). The expression levels of *FRK1* and *WRKY53* were significantly

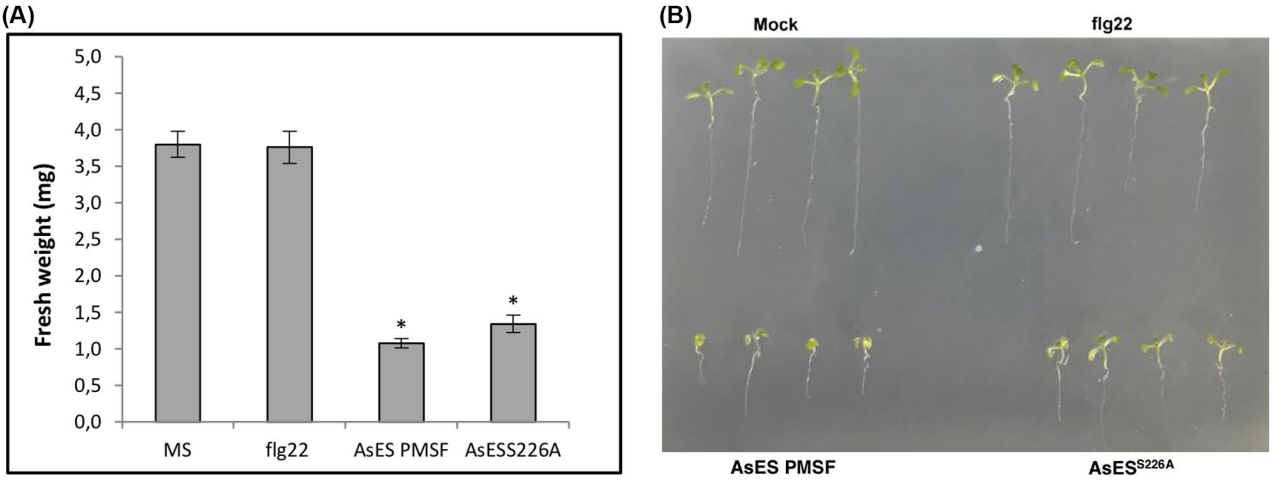

Fig. 4 AsES PMSF and AsES^{S226A} inhibit seedling growth in *Arabidopsis*. (A) *fls2 efr cerk1* mutant (*fec*) seeds were germinated for 5 days on 0.5× MS medium and then transferred for an additional 5-day period to liquid 0.5× MS medium in the presence of AsES (60 nM) or AsES^{S226A} (60 nM). Flg22 (100 nM) was used as a negative control of induction in the *fec* mutant. Data correspond to the mean ± standard error of eight biological replicates. Asterisks indicate statistically significant differences (*$P < 0.05$). (B) Phenotypic aspect of 10-day-old *Arabidopsis* mutant seedlings (*fec*) treated with AsES (60 nM), AsES^{S226A} (60 nM) or flg22 (100 nM) for 5 days. A representative image of each treatment is presented.

higher after treatment with AsES and AsES^{S226A} in both Col-0 and *fec* plants at 4 hpt but this effect was lost at 48 hpt (Fig. 3D,E).

Collectively, these results indicate that the recombinant proteins AsES and AsES^{S226A} produce a similar pattern of transcriptional reprogramming of defence-related genes involved in the SA and ET signalling pathways as previously reported for WT-AsES (Hael-Conrad *et al.*, 2015) (Fig. 3A–E). Also, the enhanced expression of PTI-associated marker genes reveals that there is a close association between AsES-induced defence and the activation of a PTI-like defence response.

AsES and AsES^{S226A} inhibit seedling growth

Several PAMPs/DAMPs induce seedling growth inhibition on long-term exposure (Lozano-Durán and Zipfel, 2015). Since AsES and AsES^{S226A} activate PTI gene expression, we hypothesized that AsES and AsES^{S226A} might also inhibit seedling growth. Results showed that seedlings submerged for 5 days in Murashige and Skoog (MS) medium containing AsES or AsES^{S226A} exhibited a significant reduction in fresh weight when compared to mock plants (Figs 4A,B and S3A,B).

The co-receptor BAK1 is involved in AsES-induced immune responses

BAK1/SERK3 is involved in the initial events of PTI, being required for the signal transduction in response to various PAMPs in plants (Boutrot and Zipfel, 2017). In order to test if BAK1 is required for the activity of AsES/AsES^{S226A} in *Arabidopsis*, infection assays with *B. cinerea* were carried out with the *Arabidopsis fls2 efr bak1-5* mutant (Xin *et al.*, 2016) and the results where compared with those for the Col-0 plants. The results showed that the pretreatment of mutant plants *fls2 efr bak1-5* with AsES or AsES^{S226A} was unable to protect against *B. cinerea* at the time points evaluated (48 and 72 hours post-infection, hpi) since there were not significant differences in the lesion area when compared to control plants (Fig. 5A,B).

DISCUSSION

In recent years an increasing number of proteases have been found to be involved in different aspects of plant immunity (Thomas and van der Hoorn, 2018). Subtilases, especially those present in the secretome of many plant invaders, have attracted much attention (Figueiredo *et al.*, 2018) and have been implicated, among other processes, in resistance to pathogens and immune priming (Figueiredo *et al.*, 2018; Ramírez *et al.*, 2013).

AsES is a well-conserved member of the S8 family of subtilisin-like proteases (Chalfoun *et al.*, 2013) with structural features that are typical for this family, such as the catalytic triad composed of aspartate (Asp), histidine (His) and serine (Ser) amino acid residues (Dodson and Wlodawer, 1998), and the presence of two putative Ca^{2+}-binging sites (Siezen *et al.*, 1991). The maturation process predicted for this protein was experimentally corroborated via heterologous expression in *E. coli*. We observed that the zymogen undergoes an autocatalytic processing of the prodomain (I9+S8) resulting in the mature AsES (S8) protein (Figs 1A,B and S1A,B) with a molecular mass of 34 kDa, the same as that reported for WT-AsES (Chalfoun *et al.*, 2013). During the protein extraction process, PMSF, a specific inhibitor for serine proteases, was added in order to avoid auto- and heterolytic degradation and therefore to increase AsES (S8) yields. We assumed that the addition of PMSF

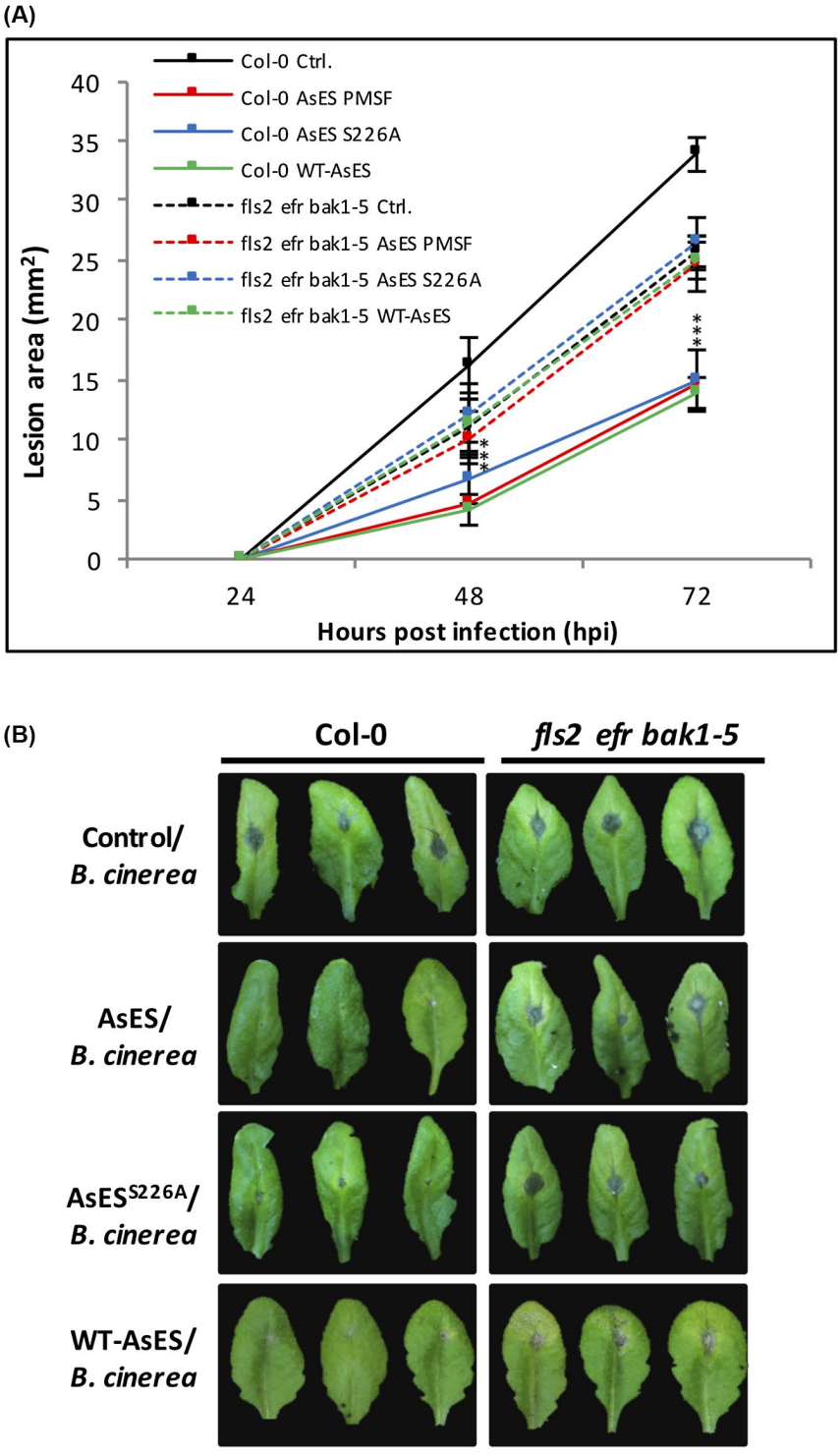

Fig. 5 BAK1 is involved in AsES perception. (A) Time-course of the development of the lesion area caused by *Botrytis cinerea* in the *Arabidopsis* mutant *fls2 efr bak1-5* (background Col-0). Control (empty vector), AsES (60 nM), AsES^{S226A} (60 nM) or WT-AsES (60 nM) was applied 48 h prior to infection (hpti) and the lesion area was evaluated at 48 and 72 h post-infection (hpi). Mean values ± SE were obtained from three independent experiments ($n = 8$). Asterisks indicate a statistically significant difference between the control and AsES PMSF/AsES^{S226A}/WT-AsES treated plants, according to Student's *t*-test ($P < 0.05$). (B) Appearance of *Botrytis* lesions on *fls2 efr bak1-5* leaves pretreated with AsES (60 nM), AsES^{S226A} (60 nM) or WT-AsES (60 nM) and compared to control plants at 72 hpi. A representative image of each treatment is presented.

would inhibit not only AsES activity (Fig. S2A,B and Table S2), eliminating the autolysis reported for other subtilases (Bajorath *et al.*, 1988a,b), but also the activity of other proteases present in the periplasmic extract that may degrade the target protein (Zhu *et al.*, 2013). Heterologous expression of functional eukaryotic subtilases was previously successfully achieved in *E. coli*, for example BAJ93208, a barley subtilase that suffers self-processing when expressed in *E. coli* (Plattner *et al.*, 2014) or AtSBT1.9, one of the many subtilases present in *Arabidopsis* (Li *et al.*, 2015).

Previous results from our laboratory suggested that AsES enzymatic activity was necessary to induce defence. It was observed that when proteolytic activity was inhibited, AsES was no longer able to protect against *C. actutum* in strawberry plants (Chalfoun *et al.*, 2013). Nevertheless, the participation of AsES enzymatic activity was only evaluated in the pathosystem strawberry–*C. acutatum* and without complementary biochemical or molecular analyses to confirm that observation. It is worthwhile mentioning that other homologous catalytically active subtilases evaluated, namely proteinase K and Carlsberg, were not able to reproduce the protective effect in strawberry (Chalfoun *et al.*, 2013). Furthermore, since most subtilases lack strict substrate specificity (Perona and Craik, 1995; Siezen and Leunissen, 1996), we hypothesized that AsES proteolytic activity was not necessarily linked to the eliciting activity. To address the question of whether AsES protein acts as an elicitor by itself or by means of proteolysis of one or multiple host proteins that are specific targets of it, we investigated the capacity of AsES (S8) and a mutant version of it, named $AsES^{S229A}$, to induce defence in *Arabidopsis* plants against *B. cinerea*. The results revealed that plant pretreatment with AsES, inhibited with PMSF, or the inactive mutant $AsES^{S229A}$ produced a significant reduction in the lesion area similar to what was previously observed with WT-AsES (Figs 2A–C and S3A,B) (Hael-Conrad *et al.*, 2015).

Pathogen perception usually leads to a profound and dynamic gene expression reprogramming that is central for establishing robust and effective host defence responses (Li *et al.*, 2016). Previously, it was also shown that WT-AsES induces plant defences via SA, JA and ET signalling pathways (Hael-Conrad *et al.*, 2015). Using an RT-qPCR approach, our results demonstrate that the treatment with both proteins produces a transcriptional reprogramming by means of SA pathway activation in a time-dependent manner, exactly as WT-AsES does (Fig. 3A–C). Hence, we conclude that, at least in the pathosystem *Arabidopsis*–*B. cinerea*, AsES protein triggers a defence response that is independent of its enzymatic activity. The fact that an equal concentration of inactive AsES is recognized in *Arabidopsis* but not in strawberry plants may be explained by species-specific differences regarding PAMP perception where different plant species recognize different epitopes of the same PAMP. Such variations related to motif recognition were reported for the defence response induced by the elongation factor EF-Tu. It was shown that *Arabidopsis* recognizes EF-Tu by means of an 18 amino acid epitope (elf18) (Kunze *et al.*, 2004), while in rice it is recognized by a different epitope of 50 amino acids (EFa50) located within the central core of the protein (Furukawa *et al.*, 2014). We therefore speculate that in strawberry the AsES recognition site may involve one or more amino acids from the active site or adjacent residues that are totally or partially blocked by PMSF covalent binding. Alternatively, the AsES functional epitope in *Arabidopsis* may comprise residues that are not affected by such inhibition.

Among the many plant subtilases that participate in defence, it has been observed that proteolytic activity has a role in the processing of components that are responsible for the activation of the immune responses. As such, AtSBT6.1/S1P processes the propeptide form of RAPID ALKALINIZATION FACTOR 23 (RALF23), which binds to malectin-like receptor kinase FERONIA (FER) controlling plant immune signalling (Srivastava *et al.*, 2009; Stegmann *et al.*, 2017). In contrast, Glyma18g48580 is an extracellular soybean subtilase that contains a 12 amino acid embedded peptide (GmSubPep) with an independent metabolic role capable of inducing the expression of defence genes (Pearce *et al.*, 2010). The endogenous elicitor binds to a membrane-bound receptor that in turn induces defence signalling pathways and amplifies the plant innate immune response, similar to pathogen-derived elicitors (Pearce *et al.*, 2010). From the plant invader perspective, subtilases might be involved in the mechanism of pathogen attack. For instance, it was demonstrated that a serine protease isolated from *Fusarium eumartii* degrades pathogenesis-related proteins from the intercellular washing fluid as a part of the fungal strategy to colonize potato tuber tissues (Olivieri *et al.*, 1998, 2002). Also, during a pathogen attack the plant can directly or indirectly recognize one or more components secreted by the attacker, and as a consequence activates defence responses in order to prevent the disease. However, to our knowledge, an example of a pathogen-derived subtilase that is recognized as a molecular signature by the host has not been reported so far.

Typically, PAMPs are considered to be conserved throughout different classes of microbes and to contribute to general microbial fitness, whereas effectors are species-, race- or strain-specific and contribute to pathogen virulence. However, the common distinction between PAMPs and effectors cannot always be strictly maintained (Thomma *et al.*, 2011). AsES elicitor does not necessarily fit the concept of a PAMP/effector since it shows features of both classes of molecules. As a PAMP, it acts ubiquitously, triggering a defence response not only in different *F. ananassa* cultivars, namely Milsei, Tudla, Camarosa and Pájaro (Chalfoun, 2009; Chalfoun *et al.*, 2013), but also in different wild species of strawberry, such as *Fragaria vesca* (unpublished data), and this defence response is extended to other plant species, such as *A. thaliana* (Chalfoun *et al.*, 2013; Hael-Conrad *et al.*, 2015) and crops such

as soybean, sugarcane and avocado (Chalfoun *et al*., 2018a,b; Perato *et al*., 2018). Interestingly, AsES treatment protects against both bacterial and fungal pathogens (Chalfoun *et al*., 2018b) and among these, against fungi with different lifestyles (Chalfoun *et al*., 2013, 2018a,b; Hael-Conrad *et al*., 2015). It has been shown that AsES induces SAR (Hael-Conrad *et al*., 2015, 2017) accompanied by microbursts and micro-HR, the latter only observed in *F. ananassa*. The classical PTI responses observed, such as activation of PTI marker genes (*FRK1*, *WRKY53*) and seedling growth inhibition evaluated in *Arabidopsis* plants treated with AsES and AsES^{S229A}, strongly support the idea that AsES is a PAMP. Also, the fact that BAK1, commonly involved as co-receptor for leucine-rich repeat (LRR)-containing receptors (Hohmann *et al*., 2017), participates in the AsES-triggered defence response, brings further support to the idea that AsES is being perceived by a LRR-receptor-like kinase (RLK)/receptor-like protein (RLP) at the plasma membrane.

A parallel could be established between the AsES mechanism of action and the ethylene-inducing xylanase (EIX), a fungal elicitor isolated from *Trichoderma viride* (Fuchs *et al*., 1989). Among other defence responses, EIX induces ET biosynthesis, pathogenesis-related protein expression and HR in specific cultivars of tobacco (*Nicotiana tabacum*) and tomato (*Solanum lycopersicum*) (Bailey *et al*., 1990, 1993; Elbaz *et al*., 2002; Ron *et al*., 2000). Although EIX interaction with plants follows the gene-for-gene model (Flor, 1971; Furman-Matarasso *et al*., 1999), it has been demonstrated that enzymatic activity (β-1,4-endoxylanase) is not required for the elicitation process because the protein per se functions as the elicitor (Enkerli *et al*., 1999; Furman-Matarasso *et al*., 1999; Rotblat *et al*., 2002). The identification of its receptor, LeEix2, an LRR-RLP, confirms that EIX is a PAMP that is perceived at the membrane level (Ron and Avni, 2004), although BAK1 is not involved in EIX direct perception (Bar *et al*., 2010).

Further evidence that AsES is a PAMP should be provided with the identification of the cognate PRR and demonstration of direct binding.

In conclusion, we have confirmed that AsES induces plant defence and that enzymatic and eliciting activities are not associated. AsES can be considered the first subtilisin-like protease from a phytopathogen acting as a PAMP by itself. Understanding the mechanism of action of this elicitor may help in the development of new breeding strategies that can be exploited to confer durable resistance in plants.

EXPERIMENTAL PROCEDURES

Bacterial and plant materials

Escherichia coli DH5α and BL21 (DE3) were used as the hosts for cloning and expression, respectively. Bacterial cultures were grown before induction at 37 °C in Luria-Bertani (LB) medium (Sigma-Aldrich, St Louis, MO, USA) supplemented with carbenicilin (100 μg/mL).

Botrytis cinerea strain BMM was kindly provided by Brigitte Mauch-Mani (University of Neuchatel, Switzerland). Growth, spore suspension and infection procedure were performed as described by Hael Conrad *et al.* (2015).

Arabidopsis thaliana ecotype Columbia (Col-0) and mutant lines *fls2 efr cerk1* and *fls2 efr bak1-5* (all in Col-0 background) have been described before (Xin *et al*., 2016). *Arabidopsis* plants used in this study were grown as one plant per pot in a growth chamber under standard conditions with a 16 h/8 h light/dark cycle and 60–70% relative humidity. Daytime and night-time temperatures were maintained at 23 and 21 °C, respectively.

Cloning procedures

AsES full-length coding sequence (CDS), inhibitor domain (I9) + subtilase domain (S8), was synthesized, codon optimized for *E. coli* expression and cloned into pUC19 vector by GenScript (Picataway, NJ, USA). Specific primers were designed to introduce the restriction sites *Eco*RV and *Eco*RI together with a sequence coding for a 10× His-tag at the 3′ end (Table S1). The amplicon obtained was cloned into pMAL-p5x expression vector (New England Biolabs, Ipswich, MA, USA). Site-directed mutagenesis by overlapping extension (Plattner *et al*., 2014) was used to substitute the serine residue to alanine (AsES^{S226A}) using the pMAL-p5x/AsES construct as a template. The primers used for mutagenesis are shown in Table S1. All recombinant plasmids were confirmed by DNA sequencing.

Functional protein expression in the *E. coli* periplasm

Plasmids pMAL-p5x-AsES and pMAL-p5x-AsES^{S226A} were transformed into *E. coli* BL21 (DE3), streaked out in LB agar plates supplemented with carbenicilin (100 μg/mL) and grown at 37 °C to produce single cell-colonies. A 5-mL starter culture of *E. coli* BL21 (DE3) pMAL-p5x-AsES, containing the CDS for AsES protein, was inoculated to 500 mL of LB medium supplemented with 0.2% glucose (w/v). Cells were grown until OD_{600} = 0.6 and target gene expression was induced with 0.05 mM IPTG. Culture was incubated at 28 °C for 4 h and then at 4 °C overnight.

For extraction of the periplasmic fraction, the induced culture was harvested at 4000*g* for 15 min and proceeded as described by Sroga and Dordick (2002). Briefly, the cell pellet was resuspended in 20 mL of the osmotic solution I (20 mM Tris-HCl pH 8.0, 2.5 mM EDTA, 2 mM $CaCl_2$, 20% sucrose, 1 mM PMSF), incubated in an ice-bath for 10 min and then centrifuged at 4000*g* for 15 min at 4 °C. After the supernatant was removed, the pellet was resuspended in 10 mL of ice-cold osmotic solution II (20 mM Tris-HCl pH 8.0, 10 mM $CaCl_2$, 1 mM PMSF) and incubated on ice for 20 min with gentle agitation. The cold osmotic shock fluid was obtained from the supernatant after centrifugation at 8000*g* for 20 min.

Purification of AsES protein was by means of the 10× His-epitope and was carried out by the on-column method using Ni-NTA spin columns (Thermo Scientific, MA, USA) following manufacturer's recommendations. The osmotic shock fluid fractions were loaded into pre-equilibrated columns (binding/washing buffer: 20 mM Tris-HCl pH 8.0, 300 mM NaCl, 10 mM $CaCl_2$, 20 mM imidazole) and after washing three times with washing buffer, proteins were eluted by addition of elution buffer (20 mM Tris-HCl pH 8.0, 300 mM NaCl, 10 mM $CaCl_2$, 500 mM imidazole). All reagents used in buffers for periplasmic extraction and purification procedures are from Sigma-Aldrich, St Louis, MO, USA.

The presence of the recombinant proteins in the eluted fractions was detected by 10% SDS-PAGE and further analysed by western blot using mouse monoclonal anti-His (GE Healthcare Life Sciences, Uppsala, Sweden) as primary antibody and anti-mouse alkaline phosphatase conjugate as secondary antibody (GE Healthcare Life Sciences).

For biological assays, fractions containing AsES/AsES^{S226A} protein were desalted and concentrated using Vivaspin ultrafiltration columns (Sartorius, Gloucester, UK) with milli-Q water and 10% glycerol. The *E. coli* strain BL21 (DE3) carrying the pMAL-p5x vector (empty vector) served as an internal control for the expression, extraction and purification procedures. WT-maltose-binding protein (MBP) was purified using amylose resin (New England Biolabs) according to the manufacturer´s instructions and used as a negative control of defence induction for AsES^{S226A} protein.

Proteolytic activity determinations

The proteolytic activity of the recombinant proteins (AsES and AsES^{S226A}) was evaluated by enzymatic hydrolysis of the chromogenic peptidic substrate *N*-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide (Suc-AAPF-*p*NA; Sigma-Aldrich) (Chalfoun *et al.*, 2013). Briefly, 0.2 μg/mL of each protein was diluted in 20 mM Tris–HCl (pH 7.5) to a final volume of 500 μL. After 2 min of preincubation at 37 °C, 10 μL of 5 mM Suc-AAPF-*p*NA (final concentration of 0.1 mM) was added to the reaction mixtures and they were incubated for another 30 min under the same conditions. Native AsES was used as an internal positive control in the same concentration and conditions as the proteins being assayed. All proteolytic activity assays were performed in triplicate.

The proteolytic activity of each protein was estimated as the concentration of *p*NA liberated per minute using a molar extinction coefficient (ε_{405nm}) of 9.62/mM/cm, at 37 °C and pH 7.5, where *p*NA concentration was determined spectrophotometrically at 405 nm. The autoproteolysis rate of the substrate Suc-AAPF-*p*NA was also evaluated and subtracted for each measured reaction value. One unit of protease activity was defined as the release of 1 μmol of *p*NA per minute at 37 °C and pH 7.5.

Induced resistance assays

Induce resistance assays against *B. cinerea* were performed as previously described by Hael-Conrad *et al.* (2015). Briefly, 4-week-old *Arabidopsis* plants were pretreated by depositing a 6 μL droplet of 60 nM AsES, 60 nM AsES^{S226A} or control (empty vector) on the adaxial side of the leaf and subsequently left for 48 h. WT-AsES protein, purified from *A. strictum* as described by Chalfoun *et al.* (2013), was used as a positive control of protection against *B. cinerea*. After pretreatment, the remaining droplets were removed and 6 μL of the spore suspension (5×10^4 mL^{-1}) was laid in the same leaf area. Symptoms were evaluated at 48 and 72 hpi. Total necrotic area was quantified using ImageJ software (https://imagej.nih.gov/ij/) and presented as means ± SE from three independent experiments ($n = 8$). Statistically significant differences between the control- and AsES/AsES^{S226A}-treated plants were assessed according to Student's *t*-test ($P < 0.05$).

RNA isolation and quantitative RT-PCR

Total RNA was isolated from frozen-ground *Arabidopsis* leaf tissue using TriPure Isolation Reagent (Roche, Basel, Switzerland) and then treated with DNAse I (Invitrogen, Carlsbad, CA, USA) for removal of genomic DNA contamination. RNA concentration was measured using an ND-1000 spectrophtometer (NanoDrop Technologies, Wilmington, DE, USA). The purity of total RNA was determined by the OD_{260nm}:OD_{280nm} ratio. cDNA synthesis was performed with M-MLV reverse transcriptase (Invitrogen, Carlsbad, CA, USA) from 1.5 μg of pure RNA, according to the manufacturer's instructions. For all experimental conditions, a minimum of eight biological replicates and two technical replicates was used as recommended by minimum information for publication of quantitative real-time PCR experiments (MIQE) requirements (Bustin *et al.*, 2009). Elongation factor *EF1α* (At5g60390) was used as the internal reference gene. The primers used for RT-qPCR experiments are listed in Table S1.

The normalization of RT-qPCR data was performed according to the Pfaffl algorithm (Pfaffl, 2001) and a paired permutation test was performed for the statistical analyses. The cut-off for statistically significant differences was set as $P < 0.05$.

Seedling growth inhibition assay

Seedling growth inhibition (SGI) was assessed as previously described in Stegman *et al.* (2017). Briefly, *Arabidopsis* seeds were surface sterilized with chlorine gas for 4 h, grown in 0.5× MS agar medium, including vitamins (Duchefa, Haarlem, Netherlands), for 5 days and later transferred to a 48-well plate containing MS medium + 1% sucrose, supplemented with 60 nM AsES or AsES^{S226A}. Flg22 (100 nM) was used as a positive control of growth inhibition (Gómez-Gómez and Boller, 2000) and the empty vector pMAL-p5x as a negative control (referred to as mock). Seedling fresh weight was measured 5 days after transfer.

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author on reasonable request.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article at the publisher's web site:

Fig. S1 AsES expression and purification. (A) SDS-PAGE analysis of AsES fractions sampled during purification. The black arrow indicates the position of the pure mature protein (34 kDa). (B) Western blot analysis revealing the maturation process suffered by AsES propeptide. The upper black arrow indicates the Pro-AsES (87 kDa) and the lower black one indicates mature AsES (34 kDa). FT, flow-through; W, wash; E1, E2 and E3, first, second and third elution during the purification process. Molecular mass markers (M) in kilodaltons are indicated on the left. Western blot analyses were performed using anti-His antibodies.

Fig. S2 Effect of the inhibition of proteolytic activity on AsES yields. (A) SDS-PAGE analysis of periplasmic fractions sampled in the presence or absence of PMSF. (B) Western blot analysis of the periplasmic fractions treated and not treated with PMSF. Molecular mass markers in kilodaltons are indicated on the left. Western blot analyses were performed using anti-His antibodies.

Fig. S3 Seedling growth inhibition caused by AsES and AsES ^{S226A} in *Arabidopsis* seedlings. (A) Wild-type *Arabidopsis* seeds (Col-0) were germinated for 5 days on 0.5× Murashige and Skoog (MS) medium and then transferred for an additional 5 day period to liquid 0.5× MS medium in the presence of AsES (60 nM) or AsES ^{S226A} (60 nM). Flg22 (100 nM) was used as a positive control of growth inhibition. Data correspond to the mean ± standard error of eight biological replicates. Asterisks indicate statistically significant differences (*$P < 0.05$). (B) Phenotypic aspect of 10-day-old *Arabidopsis* seedlings (Col-0) treated with AsES (60 nM), AsES ^{S226A} (60 nM) or flg22 (100 nM) for 5 days. A representative image of each treatment is presented.

Table S1 Specific oligonucleotide primers used for cloning strategies and quantitative RT-qPCR studies.

Table S2 Proteolytic assay to detect enzymatic activity with the peptide Suc-AAPF-*p*NA. Lowercase letters indicate significant differences as determined by Bonferroni-corrected *P*-values ($P < 0.001$) obtained after ANOVA and subsequent LSD post hoc test. Each value is the mean (SD) of three independent replicates.

