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An effector of a necrotrophic fungal pathogen targets the calcium-sensing receptor in chloroplasts to inhibit host resistance

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Abstract

SsITL, a secretory protein of the necrotrophic phytopathogen *Sclerotinia sclerotiorum*, was previously reported to suppress host immunity at the early stages of infection. However, the molecular mechanism that SsITL uses to inhibit plant defence against *S. sclerotiorum* has not yet been elucidated. Here, we report that SsITL interacted with a chloroplast-localized calcium-sensing receptor, CAS, in chloroplasts. We found that CAS is a positive regulator of the salicylic acid signalling pathway in plant immunity to *S. sclerotiorum* and CAS-mediated resistance against *S. sclerotiorum* depends on Ca^{2+} signalling. Furthermore, we showed that SsITL could interfere with the plant salicylic acid (SA) signalling pathway and *SsITL*-expressing transgenic plants were more susceptible to *S. sclerotiorum*. However, truncated SsITLs (SsITL-NT1 or SsITL-CT1) that lost the ability to interact with CAS do not affect plant resistance to *S. sclerotiorum*. Taken together, our findings reveal that SsITL inhibits SA accumulation during the early stage of infection by interacting with CAS and then facilitating the infection by *S. sclerotiorum*.

KEYWORDS
calcium-sensing receptor, effector, salicylic acid signalling pathway, *Sclerotinia sclerotiorum*, SsITL

1 | INTRODUCTION

Sclerotinia sclerotiorum is a necrotrophic fungus that infects more than 400 plant species worldwide. Most of these hosts are dicotyledonous, such as soybean, rapeseed, sunflower, and bean, and a few agriculturally important monocotyledonous plants are also hosts of *S. sclerotiorum*, such as onion and tulip (Boland and Hall, 1994; Bolton *et al.*, 2006). Sclerotinia disease is responsible

for considerable damage to many crops and is difficult to control (Zhou *et al.*, 2014; Hou *et al.*, 2018). Research on the pathogenesis of *S. sclerotiorum* can provide new insights for the development of sclerotinia disease prevention and control strategies.

As a typical necrotrophic pathogenic fungus, the pathogenesis of *S. sclerotiorum* is more complicated than we originally thought. Early research focused on the cell wall-degrading enzymes (CWDEs) and toxic metabolite oxalic acid (OA). This fungus secretes a wide array of CWDEs, which can macerate plant tissues, degrade plant cell wall components, and ultimately promote infection (Riou *et al.*, 1991; Issam *et al.*, 2004; Ellouze *et al.*, 2011). OA plays multiple functions in numerous physiological processes, such as deregulation of guard cells, sequestration of calcium, dampening the plant oxidative burst, induction of apoptotic-like programmed cell death (PCD), and suppression of autophagy (Marciano *et al.*, 1983; Cessna *et al.*, 2000; Guimaraes and Stotz, 2004; Kim *et al.*, 2008; Williams *et al.*, 2011; Heller and Witt-Geiges, 2013; Kabbage *et al.*, 2013). Despite the multiple roles of OA in the pathogenesis of *S. sclerotiorum*, recent studies have shown that oxalate is not required for *S. sclerotiorum* to cause disease on some host plants; the authors proposed that it is the low pH environment that plays an important role in *Sclerotinia* pathogenesis (Xu *et al.*, 2015). The genome of *S. sclerotiorum* encodes more than 600 secreted proteins, 70 putative effectors were predicted and 61 of these have not been reported yet (Amselem *et al.*, 2011; Derbyshire *et al.*, 2017), and only a few of the secreted proteins have been investigated in relation to pathogenesis. For example, secreted protein Ss-Caf1 is required for appressorium formation and disruption of *Ss-Caf1* completely abolishes the virulence of *S. sclerotiorum* on host plants, but the mutant produces even more OA than the wild-type strain (Xiao *et al.*, 2014). Furthermore, some secreted proteins play roles in a subtler way during *S. sclerotiorum* infection. For instance, the small secreted protein SsSSVP1 interacts with QCR8 and disturbs the subcellular localization of QCR8 in mitochondria, which may disable its biological function and hence interfere with plant energy metabolism to facilitate the infection of *S. sclerotiorum* (Lyu *et al.*, 2016). A cerato-platanin protein SsCP1 interacts with plant PR1 and induces cell death in a dose-dependent manner in the host plant. Interestingly, SsCP1 also triggers plant defence responses through the salicylic acid (SA) signalling pathway (Yang *et al.*, 2018).

The plant hormones SA and jasmonic acid (JA) play important roles in disease resistance. In general, it is known that the JA signalling pathway mainly resists the infection of necrotrophic pathogens, while the SA signalling pathway regulates the resistance response associated with biotrophic pathogens (Glazebrook, 2005; Pieterse *et al.*, 2012). Extensive cross-talk between the SA and JA signalling pathways was found in plants and this cross-talk plays a crucial role in plant defence (Glazebrook, 2005; Mur *et al.*, 2006; Spoel *et al.*, 2007; El Oirdi *et al.*, 2011; Robert-Seilaniantz *et al.*, 2011). Previous research has shown that the SA signalling pathway also plays an important role in plant resistance to the necrotrophic pathogen *Botrytis cinerea* (Murphy *et al.*, 2000). Furthermore, although *S. sclerotiorum* is a necrotrophic fungal pathogen, at the early stages of infection *S. sclerotiorum* grows in the apoplast without crossing the plant cell wall and the host cells are still alive at that stage, suggesting that a short biotrophic interaction between plants and *S. sclerotiorum* should exist (Kabbage *et al.*, 2015). Many recent studies have shown that the defence against *S. sclerotiorum* in *Arabidopsis* and oilseed rape is also associated with SA signalling (Guo and Stotz, 2007; Wang *et al.*, 2012; Nováková *et al.*, 2014; Yang *et al.*, 2018). In short, SA signalling possibly acts as a positive regulator in plant immunity at the early stage of *S. sclerotiorum* infection.

We previously demonstrated that an integrin-like protein SsITL, a potential effector of *S. sclerotiorum*, suppresses host immunity at the early stage of infection (Zhu *et al.*, 2013). However, the molecular mechanism that SsITL uses to suppress plant defence against *S. sclerotiorum* has not yet been illuminated. Here we report that SsITL interacts with the *Arabidopsis* calcium-sensing receptor CAS in chloroplasts. CAS is a chloroplast-localized protein that acts upstream of SA accumulation and is involved in plant innate immunity (Nomura *et al.*, 2012). Overexpression of CAS in *Arabidopsis* increased plant resistance to *S. sclerotiorum*, suggesting that CAS positively regulates plant defence against *S. sclerotiorum* infection. Ectopic expression of SsITL in *Arabidopsis* reduced SA concentration after inoculation and enhanced susceptibility to *S. sclerotiorum*, but the overexpression of truncated SsITLs that cannot interact with CAS do not affect plant resistance to *S. sclerotiorum*. Our results suggest that SsITL suppresses plant defence through interaction with CAS in chloroplasts and then interferes with SA accumulation during *S. sclerotiorum* infection.

2 | RESULTS

2.1 | SsITL interacts with *Arabidopsis* calcium-sensing receptor CAS in the chloroplasts

We previously reported that a secretory protein SsITL suppresses host resistance at the early stage of *S. sclerotiorum* infection (Zhu *et al.*, 2013), while the underlying mechanisms by which SsITL modulates plant immunity have not yet been elucidated. To further clarify the mechanism of SsITL in the virulence of *S. sclerotiorum*, an immunoprecipitation (IP) combined with liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) assay was performed to screen for its plant interactors. IP-LC-MS/MS results indicated that *Arabidopsis* CAS might interact with SsITL (Figure S1 and Table S1). The interaction of SsITL$^{\Delta SP}$ (lacking the signal peptide) with CAS was investigated using the GAL4-based yeast two-hybrid (Y2H) system. The plasmids pGADT7-*CAS*, pGADT7-*CAS* (Nt, 1–187 amino acids) and pGADT7-*CAS* (Ct, 211–387 amino acids) were co-transformed with pGBKT7-*SsITL*$^{\Delta SP}$ to yeast strain Y2H Gold. The Y2H results showed that SsITL$^{\Delta SP}$ interacts with the N terminus of CAS (1–187 amino acids), but cannot interact with the C terminus (211–387 amino acids) (Figure 1a). To further validate the interaction of SsITL with CAS in planta, a co-immunoprecipitation (Co-IP) assay was carried out. FLAG-tagged CAS (pCNF3-*CAS*) was co-expressed with GFP-tagged SsITL (pCNG-*SsITL*) or green fluorescent protein (GFP) (2 × 35S-MCS-eGFP, pCNG) in *Nicotiana benthamiana*. Then the total

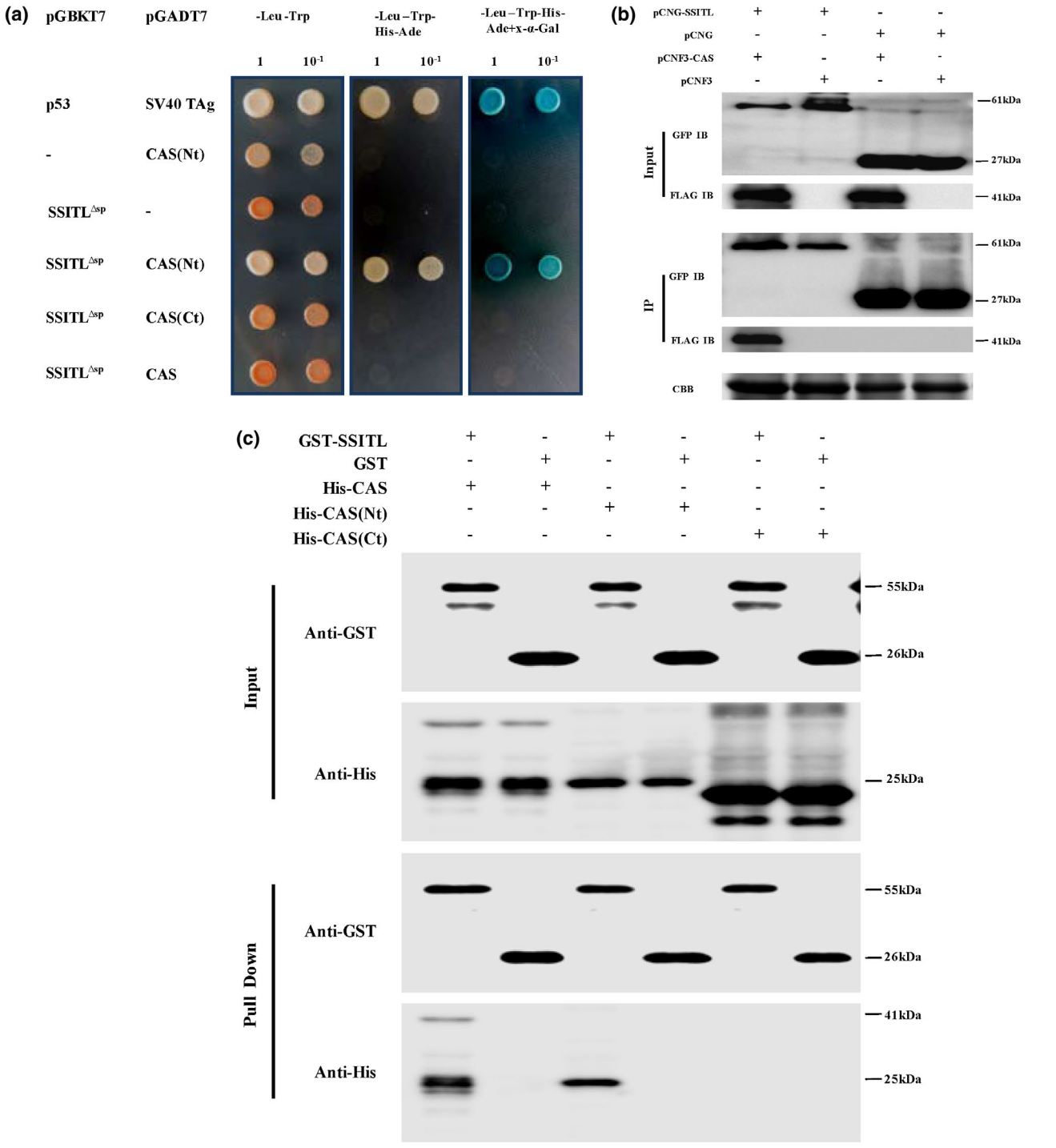

FIGURE 1 SsITL interacts with *Arabidopsis thaliana* CAS. (a) Yeast two-hybrid (Y2H) assay showed that SsITL interacted with CAS(Nt) in yeast. Co-expression of pGBKT7-p53 and pGADT7-SV40 TAg as positive control. –, corresponding empty vectors. The negative controls showed that both SsITL$^{\Delta sp}$ and CAS(Nt) were not self-activated. The working concentration of X-α-Gal was 40 μg/ml. The plates were photographed 4 days after inoculation and experiments were repeated three times. (b) Co-immunoprecipitation (Co-IP) assay confirmed that SsITL interacts with CAS in planta. SsITL-green fluorescent protein (GFP) was expressed in *Nicotiana benthamiana* together with CAS-3 × FLAG and the corresponding empty vectors were set as the negative controls. Input, total proteins of *N. benthamiana* leaves; IP, protein samples immunoprecipitated with monoclonal GFP antibody; IB, immunoblot. The presence of FLAG proteins after immunoprecipitation was detected by western blot using anti-FLAG antibody. CBB, total protein stained with Coomassie brilliant blue. (c) Physical interaction of SsITL and CAS in vitro was verified by glutathione-S-transferase (GST) pull-down assay. GST-SsITL was incubated in binding buffer containing glutathione-agarose beads with or without CAS-His, CAS(Nt)-His or CAS(Ct)-His, and agarose beads were washed for five times and eluted. Lysis of *Escherichia coli* (Input) and eluted proteins (Pull Down) from beads was immublotted using anti-His and anti-GST antibodies

proteins were extracted from infiltrated leaves and incubated with GFP-antibody beads. The results show that FLAG-tagged CAS was significantly enriched in the GFP-tagged SsITL precipitates, but not in the GFP precipitates, indicating that SsITL interacts with CAS in planta (Figure 1b). Direct physical interaction between SsITL and CAS in vitro was also observed in glutathione-S-transferase (GST) pull-down assays (Figure 1c).

CAS is known to localize in the chloroplast thylakoid membrane, and the N terminus of CAS appears to be exposed to the stromal side of the thylakoid membrane (Friso *et al*., 2004; Nomura *et al*., 2008). To determine the precise intracellular location of SsITL and CAS, SsITL-GFP and CAS-YFP (yellow fluorescent protein) fusion proteins were transiently expressed in *N. benthamiana* leaves using the *Agrobacterium* infiltration method. Consistent with previous studies, CAS localized in chloroplasts; meanwhile, we found that SsITL was also localized in chloroplasts (Figure 2a). Furthermore, when SsITL-GFP and CAS-YFP were co-expressed in *N. benthamiana*, a perfect overlap of the YFP and GFP signals indicated that SsITL and CAS co-localized in chloroplasts (Figure 2b).

2.2 | CAS is a positive regulator of the SA signalling pathway in plant immunity to *S. sclerotiorum*

A previous study indicated that CAS acts upstream of SA accumulation and is responsible for the pathogen-associated molecular pattern (PAMP)-induced innate immune system and effector-triggered immunity, enhancing the resistance of a plant to a bacterial pathogen (Nomura *et al*., 2012). To investigate the role of CAS in regulating the defence response of plants to *S. sclerotiorum*, we generated *CAS* constitutive overexpression transgenic *Arabidopsis* lines. The candidate lines 35S:*AtCAS-1* and 35S:*AtCAS-2* were verified by reverse transcription

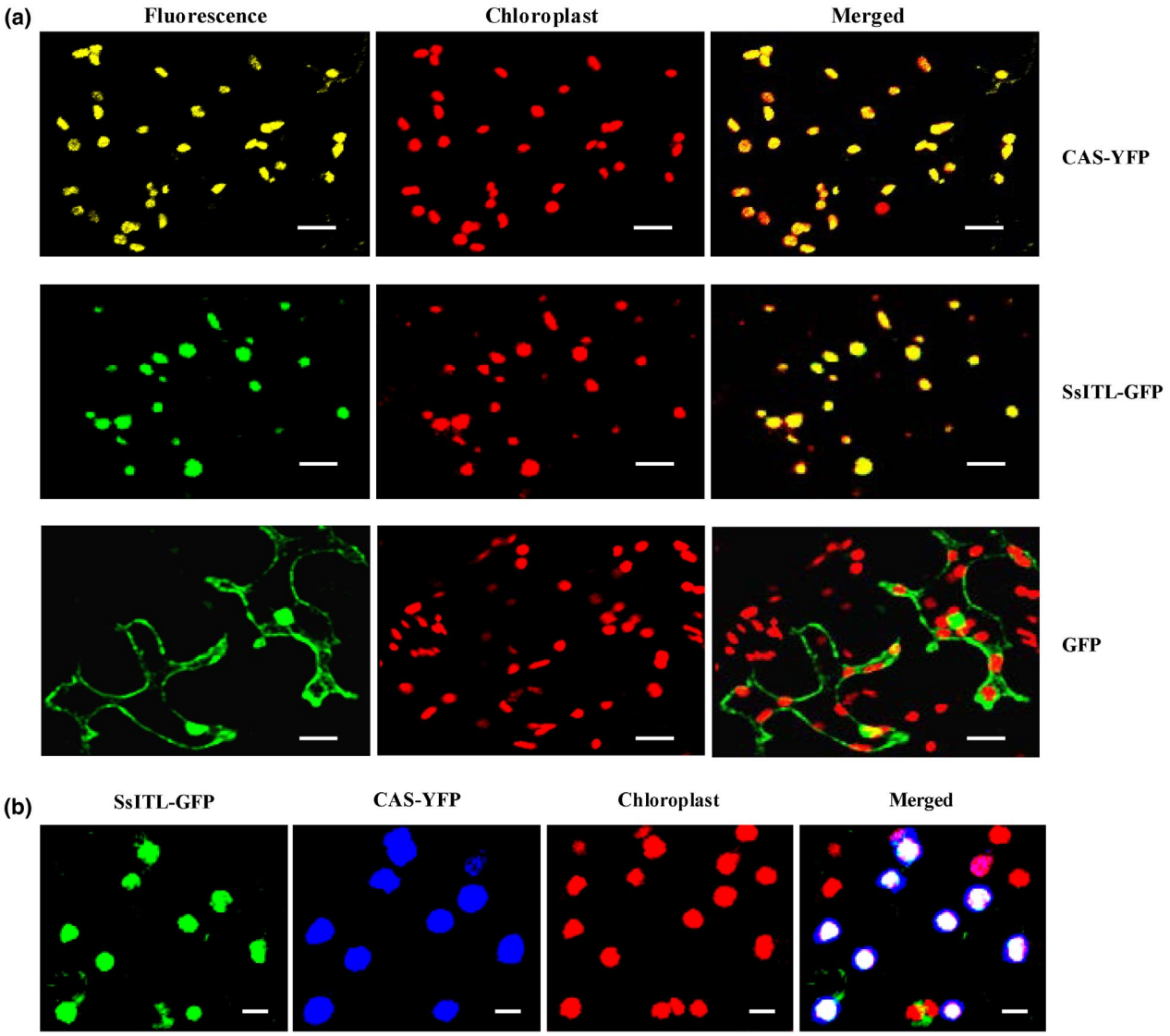

FIGURE 2 Subcellular localization of SsITL and CAS in *Nicotiana benthamiana* epidermal cells. (a) Both SsITL-green fluorescent protein (GFP) and CAS-yellow fluorescent protein (YFP) localized in the chloroplasts. (b) Co-localization of SsITL-GFP and CAS-YFP to the chloroplasts in *N. benthamiana*. *Agrobacterium tumefaciens* GV3101 carrying SsITL-GFP or CAS-YFP constructs were agroinfiltrated separately or in combination. Pictures were taken 72 hr post-agroinfiltration with confocal laser scanning microscopy. Bars = 20 μm

(RT) PCR (Figure S2a). The results show that overexpression of *CAS* had no impact on plant morphology, growth, and development (Figure S3). However, *CAS* overexpression significantly enhanced plant resistance to both *S. sclerotiorum* wild-type strain Ep-1PNA367 (Figure 3a, top) and the *SsITL*-silenced transformant A10 (Figure 3a, bottom). For example, the lesion areas produced by A10 were 40.62 mm^2 on the leaves of *Arabidopsis thaliana* Col-0 (wild-type), but only 20.38 mm^2 on the leaves of 35S:*AtCAS-1* at 36 hours post-inoculation (hpi) (Figure 3a,b), indicating an important role of CAS in the regulation of plant resistance to *S. sclerotiorum*. Previous research reported that CAS is involved in the regulation of expression of SA biosynthesis-related genes, such as *ICS1*, *PAD4*, *PBS3*, and *EDS5* (Nomura *et al*., 2012). Therefore, the transcript levels of these genes in *CAS* overexpression *Arabidopsis* lines were measured using quantitative reverse transcription PCR (RT-qPCR). Consistent with expectation, when leaves were challenged with either wild-type Ep-1PNA367 or *SsITL*-silenced A10, the expressions of *PAD4*, *PBS3*, *EDS5*, and defence marker gene *PR1* were up-regulated in 35S:*AtCAS-1* and 35S:*AtCAS-2* compared to Col-0, but *ICS1* was up-regulated in overexpressed *CAS* lines only when inoculated with A10 (Figure 3c). Additionally, although the SA concentration in wild-type Col-0 was increased on inoculation with Ep-1PNA367 and A10, especially when inoculated with A10, the SA concentrations in 35S:*AtCAS-1* and 35S:*AtCAS-2* were much higher than those in Col-0 when inoculated with Ep-1PNA367 and A10 (Figure 3d). However, the expression levels of these genes and SA concentrations in uninoculated plants exhibited no significant changes (Figure 3c,d). These data indicate that CAS is involved in the defence response of *Arabidopsis* to *S. sclerotiorum*. During *S. sclerotiorum* infection, CAS positively regulates the accumulation of SA through promoting the expression of SA biosynthesis-related genes, thereby enhancing the plant resistance to *S. sclerotiorum*.

cas-1 knockout plant was employed to further examine the roles of CAS in plant immunity to *S. sclerotiorum* (Figure S2b). Loss of function of CAS in *Arabidopsis* had no impact on plant morphology, growth, and development (Figure S3). When inoculated with Ep-1PNA367 (Figure 4a, top) or A10 (Figure 4a, bottom), the *cas-1* mutant showed greater susceptibility than wild-type Col-0 (Figure 4a,b). Compared to the wild-type Col-0, the SA concentration and transcription levels of the related genes were also significantly reduced in the *cas-1* mutant when challenged with the *SsITL*-silenced strain A10 (Figure 4c,d). The results further confirm that CAS is a positive regulator of the SA signalling pathway in plant immunity to *S. sclerotiorum*.

2.3 | SsITL contributes to plant susceptibility through interaction with CAS

To further investigate the function of SsITL in *Sclerotinia*–plant interactions, SsITL was constitutively expressed in transgenic plants 35S:*SsITL-1* and 35S:*SsITL-2* (Zhu *et al*., 2013). Expression of *SsITL* had no impact on plant morphology, growth, and development (Figure S3). Consistent with our previous results, the lesion areas induced by Ep-1PNA367 (Figure 4a, top) or A10 (Figure 4a, bottom) were obviously increased in *SsITL*-transgenic plants 35S:*SsITL-1* and 35S:*SsITL-2* (Figure 4a,b). Moreover, the size of the lesions produced by *S. sclerotiorum* in *SsITL* overexpression plants was almost the same as that of the *cas-1* mutant plant. When inoculated with A10, the SA concentration and transcription levels of related genes were also suppressed in 35S:*SsITL-1* and 35S:*SsITL-2* compared with the wild-type Col-0 (Figure 4d). These results suggest that SsITL could interfere with the plant SA signalling pathway, contributing to plant susceptibility.

To further investigate the biological function of the interaction between SsITL and CAS, we performed SsITL deletion screening. Alignment of the amino acid sequence of the SsITL protein revealed that it contains five highly conserved repeat peptides (Zhu *et al*., 2013). In order to clarify the interaction of these conserved regions with CAS, $SsITL^{\Delta SP}$ was truncated from the N terminal or the C terminal and then subjected to Y2H assay. The results show that only the full length of SsITL could interact with CAS in yeasts (Figure 5a), all the N-terminus and C-terminus truncated SsITLs lost the ability to interact with CAS, although all the truncated proteins were well expressed (Figure S5), suggesting that the complete structure of SsITL might be essential for this interaction.

The biological significance of the interaction between SsITL and CAS was further demonstrated by expressing the truncated SsITL proteins $SsITL^{\Delta SP}$-NT1 (84–302 amino acids) and $SsITL^{\Delta SP}$-CT1 (18–249 amino acids) in *Arabidopsis* lines, 35S:$SsITL^{\Delta SP}$-*NT1* and 35S:$SsITL^{\Delta SP}$-*CT1*. The truncated genes were expressed as verified with RT-PCR (Figure S2c,d), and the transgenic lines exhibited no significant difference in plant morphology and growth compared to the wild-type Col-0 (Figure S3). The pathogenicity test showed that expression of $SsITL^{\Delta SP}$-*NT1* or $SsITL^{\Delta SP}$-*CT1* had no impact on plant resistance against *S. sclerotiorum* (Figure 5b,c). In addition, the expression levels of *ICS1*, *PAD4*, *PBS3*, *EDS5*, and *PR1* and concentrations of SA in the transgenic plants 35S:$SsITL^{\Delta SP}$-*NT1* and 35S $SsITL^{\Delta SP}$-*CT1* on inoculation with Ep-1PNA367 or A10 were similar to those in the wild-type Col-0 (Figure 5d,e). These data indicated that SsITL truncated proteins were unable to interact with CAS and consequently lost the function to increase plant susceptibility. Together, these results strongly imply that SsITL–CAS interaction is essential for the biological function of SsITL during infection.

2.4 | SsITL interferes with chitin-elicited CAS-associated SA signalling pathway

As a major component of fungal cell wall, chitin oligomers are a typical PAMP, which plays a critical role in the recognition of potential pathogens and the initiation of basic immune responses in plants and animals (Heath, 2000; Nürnberger *et al*., 2004). Previous studies have suggested that chitin can elicit a series of defence responses such as the SA signalling and mitogen-activated protein kinase cascade pathways in plants against invading pathogens (Zhang *et al*., 2002; Jia *et al*., 2016). To investigate whether chitin elicits the expression of CAS-associated genes, leaves of the wild-type *Arabidopsis* Col-0 were infiltrated with 50 μg/ml chitin (Sigma-Aldrich) and the relative expressions of *ISC1*, *PAD4*, *PBS3*, *EDS5*, and *PR1* were analysed by RT-qPCR at different time points. Our

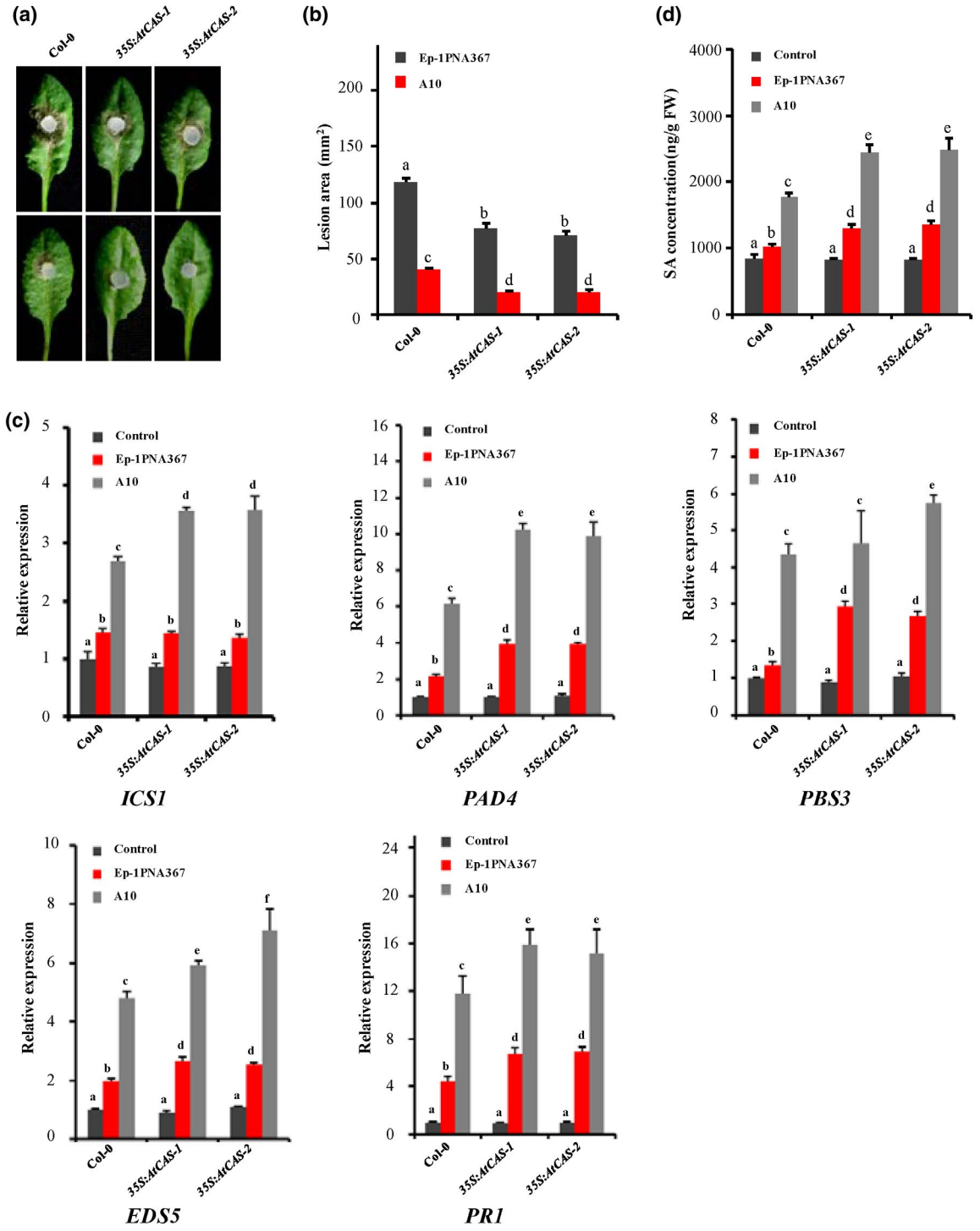

FIGURE 3 Overexpression of *CAS* enhances resistance of *Arabidopsis* to *Sclerotinia sclerotiorum*. (a) The wild-type plant Col-0 and *CAS* overexpression plants 35S:*AtCAS-1* and 35S:*AtCAS-2* were challenged with *S. sclerotiorum* strains Ep-1PNA367 (top) and A10 (bottom). Photographs were taken at 36 hours post-inoculation (hpi). (b) Statistical analysis of the lesion area induced by Ep-1PNA367 or A10 on each plant at 36 hpi. Values are means ± *SE*. (c) The relative expression levels of the salicylic acid (SA) signalling pathway-related genes (*ICS1*, *PAD4*, *PBS3*, *EDS5*, and *PR1*) in each plant were analysed at 12 hpi. The expression levels of *GAPDH* were used to normalize the expression levels of these genes in the different samples. The expression level in the wild-type plant Col-0 without inoculation was set as 1. Values are means ± *SD*. (d) SA concentrations in each plant were measured at 12 hpi. The wild-type plant Col-0 without inoculation was used as control. In all experiments, three independent replicates were performed. Values are means ± *SE*. Different letters on the same graph indicate statistical significance (one-way analysis of variance; post hoc = Duncan α [0.05])

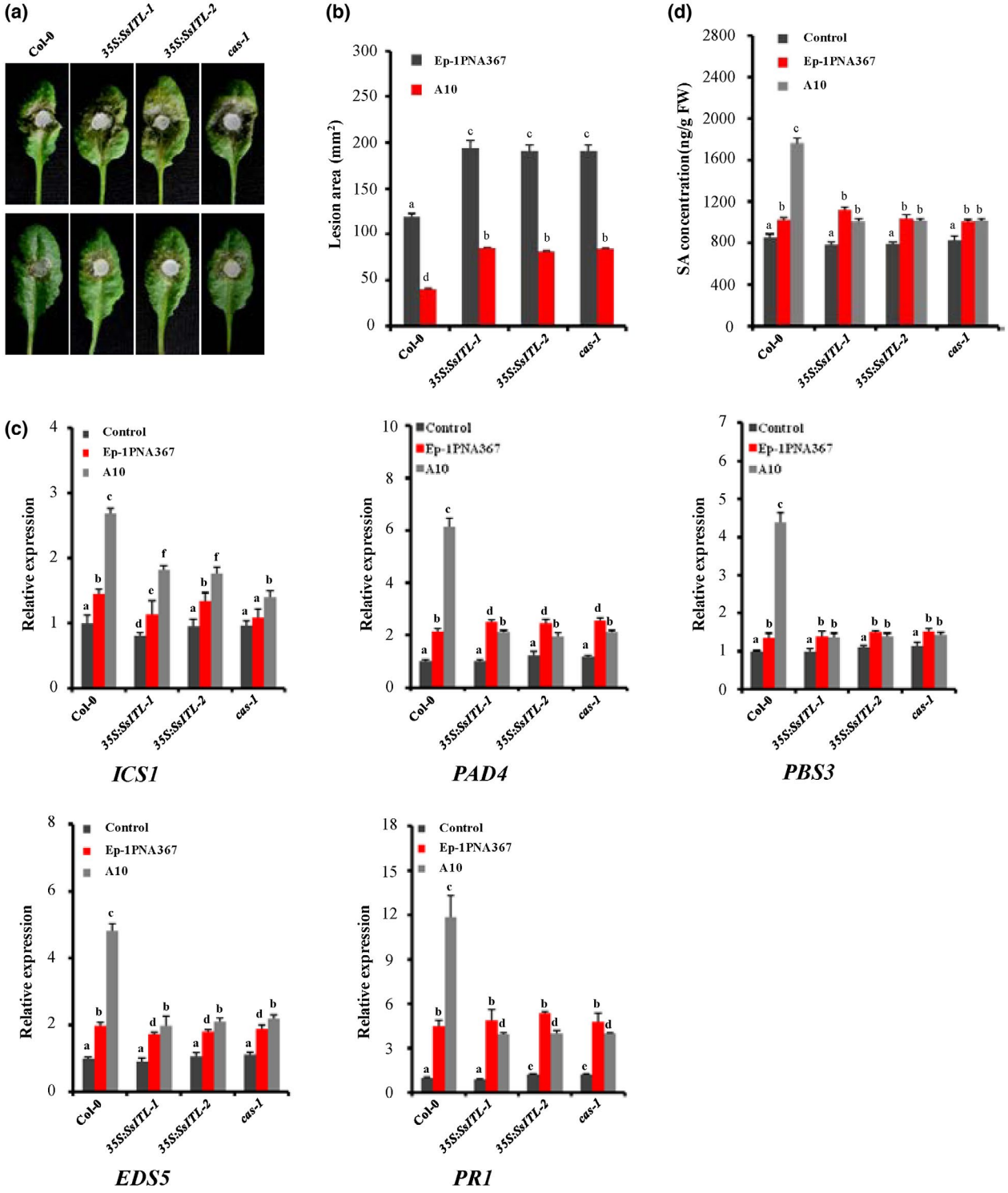

FIGURE 4 Plant resistance to *Sclerotinia sclerotiorum* and the salicylic acid (SA) accumulation were impaired in *SsITL* transgenic and *cas-1* mutant *Arabidopsis thaliana* plants. (a) The wild-type plant Col-0, *SsITL* transgenic plants 35S:*SsITL-1*, 35S:*SsITL-2* and *cas-1* plant were challenged with *S. sclerotiorum* strains Ep-1PNA367 (top) or A10 (bottom). Photographs were taken at 36 hours post-inoculation (hpi). (b) Statistical analysis of leaf lesion area induced by Ep-1PNA367 or A10 on each plant at 36 hpi. Values are means ± *SE*. (c) The relative expression levels of the SA signalling pathway-related genes (*ICS1*, *PAD4*, *PBS3*, *EDS5*, and *PR1*) in each plant were analysed at 12 hpi. The expression levels of *GAPDH* were used to normalize the expression levels of these genes in different samples. The expression level in the wild-type plant Col-0 without inoculation was set to 1. Values are means ± *SD*. (d) SA concentration in each plant was measured at 12 hpi. The wild-type plant Col-0 without inoculation was used as control. In all experiments, three independent replicates were performed. Values are means ± *SE*. Different letters on the same graph indicate statistical significance (one-way analysis of variance; post hoc = Duncan α [0.05])

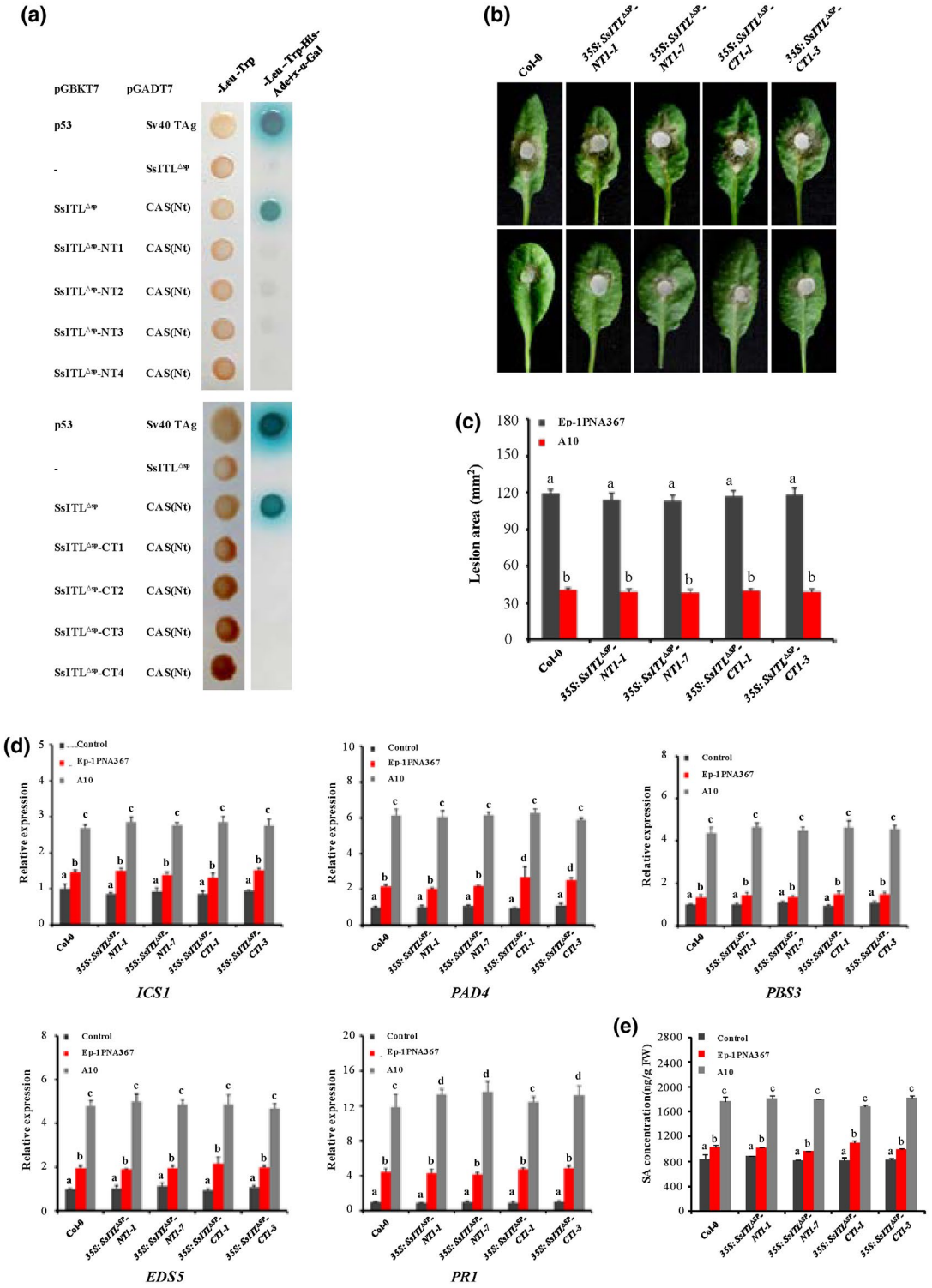

FIGURE 5 Truncated SsITLs have no effect on plant resistance to *Sclerotinia sclerotiorum*. (a) Yeast two-hybrid (Y2H) assay showed that truncated SsITLs (SsITL$^{\Delta SP}$-NT and SsITL$^{\Delta SP}$-CT) cannot interact with CAS. The truncated SsITLs were introduced to the pGBKT7 vector and then were co-transformed with pGADT7-CAS(Nt) to the yeast Y2H Gold strain. Co-expression of pGBKT7-p53 and pGADT7-SV40 TAg were used as positive controls. –, corresponding empty vectors. The plates were photographed 4 days after inoculation and experiments were repeated three times. (b) The wild-type plant *Arabidopsis thaliana* Col-0 and truncated *SsITL* transgenic plants were challenged with *S. sclerotiorum* strains Ep-1PNA367 (top) or A10 (bottom). Photographs were taken at 36 hours post-inoculation (hpi). Values are means ± *SE*. (c) Statistical analysis of leaf lesion area induced by Ep-1PNA367 or A10 at 36 hpi. Values are means ± *SE*. (d) Relative expression levels of the salicylic acid (SA) signalling pathway-related genes (*ICS1*, *PAD4*, *PBS3*, *EDS5*, and *PR1*) in each plant were analysed at 12 hpi. The expression levels of *GAPDH* were used to normalize the expression levels of these genes in different plants. The expression level in the wild-type plant Col-0 without inoculation was set to 1. Values are means ± *SD*. (e) SA concentration in each plant was measured at 12 hpi. The wild-type plant Col-0 without inoculation was used as control. In all experiments, three independent replicates were performed. Values are means ± *SE*. Different letters on the same graph indicate statistical significance (one-way analysis of variance; post hoc = Duncan α [0.05])

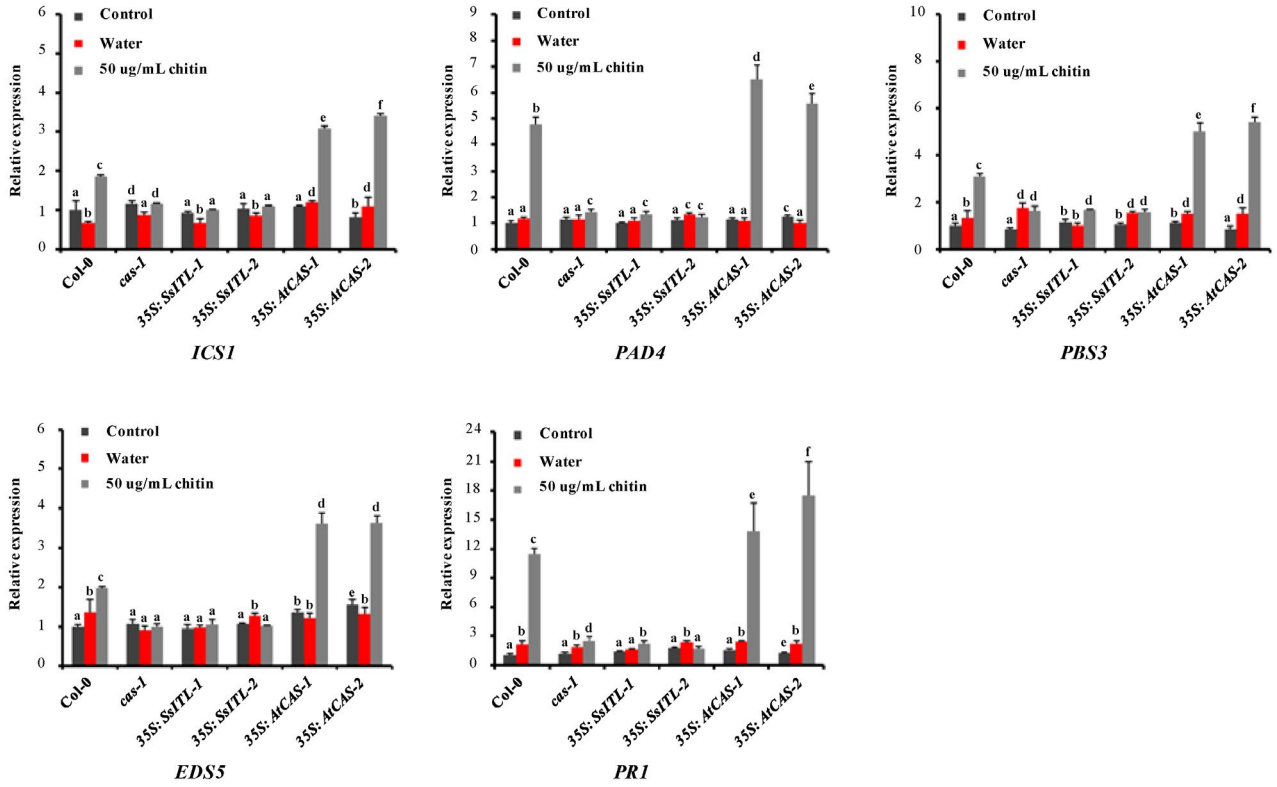

FIGURE 6 Analysis of the expression levels of five salicylic acid signalling pathway-related genes after chitin treatment. Relative transcript accumulation of *ICS1*, *PAD4*, *PBS5*, *EDS5*, and *PR1* genes determined by quantitative reverse transcription PCR at 12 hr post-infiltration with 50 μg/ml chitin. The expression levels of *GAPDH* were used to normalize the expression levels of these genes in the different plants. The expression level in the wild-type plant Col-0 without treatment was set to 1. Three independent replicates were performed. Values are means ± *SD*. Different letters on the same graph indicate statistical significance (one-way analysis of variance; post hoc = Duncan α [0.05])

results showed that all of those genes were significantly up-regulated at 6 and 12 hr post-infiltration (Figure S6), indicating that CAS-associated signal can be activated by chitin. To further confirm that SsITL suppresses the CAS-mediated SA signal pathway, the transgenic plants 35S:*SsITL* and 35S:*AtCAS* and *cas-1* knockout plant were infiltrated with 50 μg/ml chitin. The results of RT-qPCR reveal that induction of those genes by chitin were significantly impaired in *cas-1* and 35S:*SsITL* plants at 12 hr post-infiltration (Figure 6). On the contrary, the expressions of *ISC1*, *PBS3*, and *EDS5* were obviously enhanced in 35S:*AtCAS* compared to Col-0 (Figure 6). These results suggest that CAS-mediated SA signalling can be activated by recognition of chitin; however, the interaction between SsITL and CAS efficiently suppressed this immune response, which is consistent with the previous study that SsITL suppresses host defence at the early stage of infection (Zhu *et al.*, 2013).

2.5 | CAS-mediated defence response against *S. sclerotiorum* depends on Ca^{2+} signalling

CAS is well known as a plant-specific putative Ca^{2+}-binding protein that contains low-affinity/high-capacity Ca^{2+} binding sites on the N terminus (Han *et al.*, 2003). Subsequent studies further demonstrated that CAS plays a crucial role in regulating stomatal movement, as well as the generation and fine-tuning of cytoplasmic Ca^{2+} (Nomura *et al.*, 2008; Weinl *et al.*, 2008), suggesting that regulation of plant physiological process by CAS is possibly dependent on Ca^{2+} signals. To illuminate the association between Ca^{2+} signals and CAS-mediated resistance against *S. sclerotiorum*, the virulence test of Ep-1PNA367 and A10 on 35S:*AtCAS* transgenic lines and Col-0 was performed with application of 1 mM $LaCl_3$, which is a putative plasma-membrane Ca^{2+} channel blocker and widely used in studying plant Ca^{2+} signals (Knight *et al.*, 1996; Gao *et al.*, 2013; Choi *et al.*, 2014; Behera *et al.*, 2017). Our results showed that a lesion induced by Ep-1PNA367 on the wild-type Col-0 leaves became larger when 1 mM $LaCl_3$ was applied exogenously. The lesion induced by Ep-1PNA367 on $LaCl_3$-pretreated 35S:*AtCAS* transgenic lines was even larger than the lesion on H_2O-pretreated Col-0 (Figure 7a). Meanwhile, the lesion induced by A10 on the wild-type Col-0 leaves was also larger when plants were pretreated with $LaCl_3$, and CAS-mediated resistance in 35S:*AtCAS* transgenic lines to A10 was completely suppressed by exogenous application of $LaCl_3$ (Figure 7b). However, exogenous application of 1 mM $LaCl_3$ had no impact on the growth of *S. sclerotiorum* (Figure S7). These results suggest that exogenous application of $LaCl_3$ significantly enhances the susceptibility of *Arabidopsis* to *S. sclerotiorum*, indicating that Ca^{2+} signals play a critical role in plant resistance against *S. sclerotiorum*. On the contrary, CAS-mediated resistance in 35S:*AtCAS* transgenic lines to *S. sclerotiorum* was partially abolished by $LaCl_3$

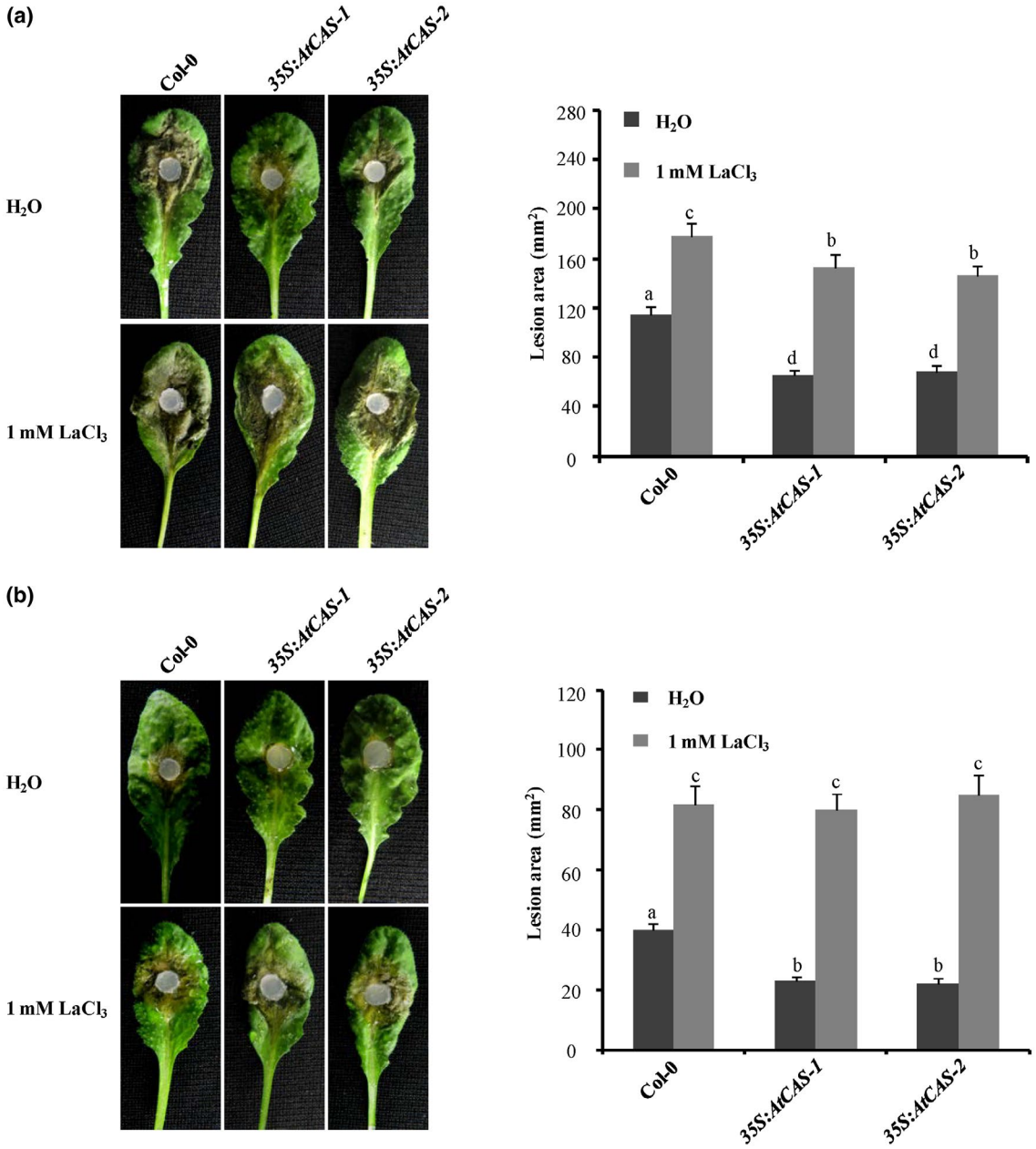

FIGURE 7 Effect of $LaCl_3$ treatment on plant resistance to *Sclerotinia sclerotiorum*. (a) Effect of $LaCl_3$ treatment on plant resistance to the wild-type strain EP-1PNA367. (b) Effect of $LaCl_3$ treatment on plant resistance to *SsITL*-silenced strain A10. Before inoculation, 1 mM $LaCl_3$ solution was uniformly sprayed on the *Arabidopsis* leaves, and deionized water was used as control. The wild-type plant Col-0 and *CAS* overexpression plants 35S:*AtCAS-1* and 35S:*AtCAS-2* were challenged with *S. sclerotiorum* strains Ep-1PNA367 or A10. Photographs were taken at 36 hr post-inoculation. Statistical analysis of the lesion area induced by Ep-1PNA367 or A10 on each plant was performed. In all experiments, three independent replicates were performed. Values are means ± *SE*. Different letters on the same graph indicate statistical significance (one-way analysis of variance; post hoc = Duncan α [0.05])

(Figure 8), suggesting that CAS-mediated immunity to *S. sclerotiorum* is mainly dependent on the Ca^{2+} signalling pathway.

3 | DISCUSSION

As an aggressive phytopathogen, *S. sclerotiorum* possesses abundant powerful weapons, such as CWDEs and OA, which can directly kill host tissues and subsequently establish infection. Recently, the roles of secreted proteins in the pathogenesis of *S. sclerotiorum* have received increasing attention. Genomic and secretome analysis revealed that *S. sclerotiorum* encodes approximate 600 secreted proteins, and more than 400 secreted proteins are expressed during infection. Moreover, a large number of effector candidates were predicted by multiple analyses (Amselem *et al.*, 2011; Guyon *et al.*, 2014; Hahn *et al.*, 2014; Derbyshire *et al.*, 2017), suggesting that

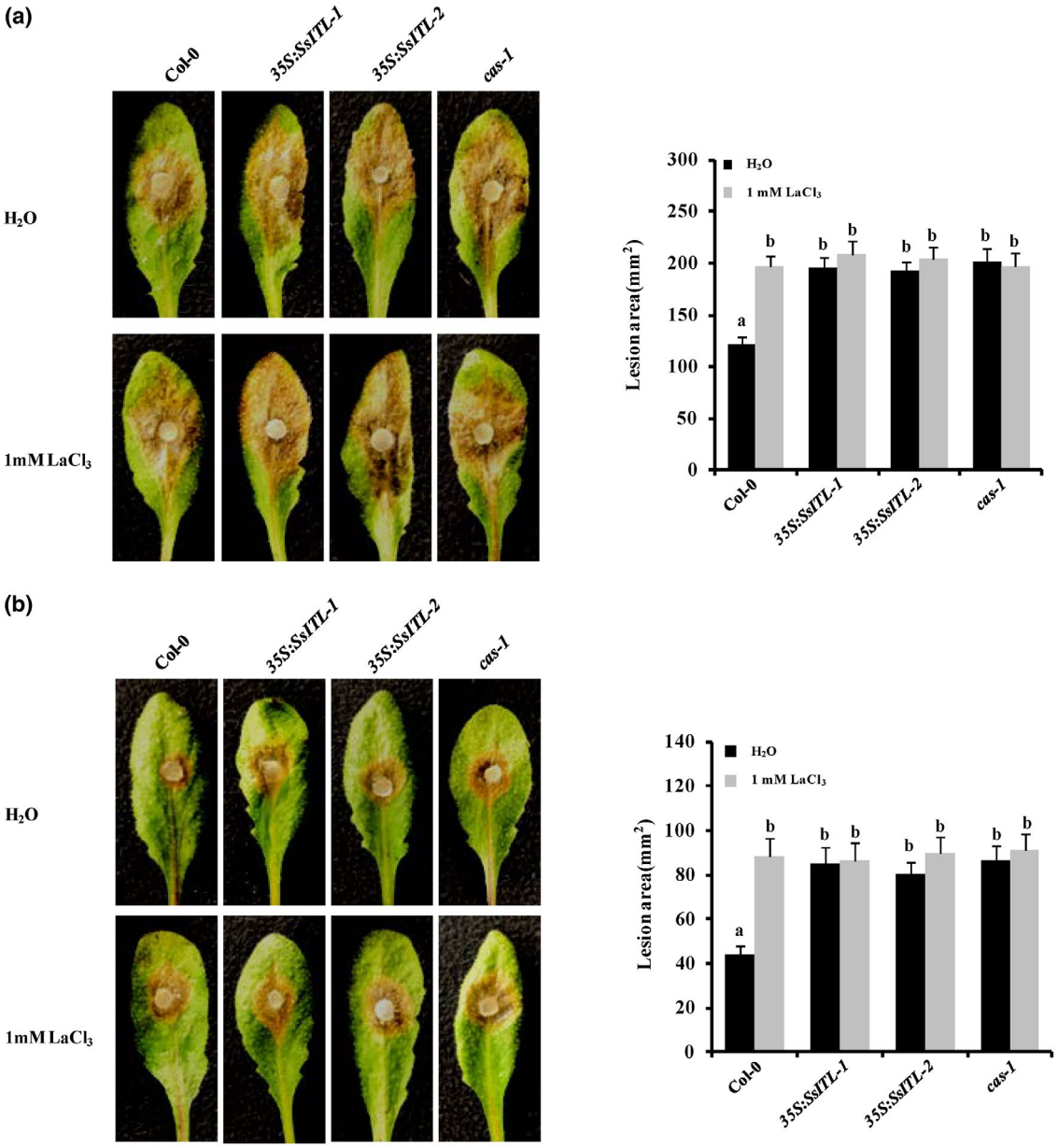

FIGURE 8 Effect of $LaCl_3$ treatment on 35S:*SsITL* and *cas-1* mutant plant susceptibility to *Sclerotinia sclerotiorum*. (a) Effect of $LaCl_3$ treatment on plant susceptibility to the wild-type strain EP-1PNA367. (b) Effect of $LaCl_3$ treatment on plant susceptibility to SsITL-silenced strain A10. Before inoculation, 1 mM $LaCl_3$ solution was uniformly sprayed on the *Arabidopsis* leaves and deionized water was used as control. The wild-type plant Col-0, 35S:*SsITL*-1, 35S:*SsITL*-2 and *cas-1* mutant plant were challenged with *S. sclerotiorum* strains Ep-1PNA367 or A10. Photographs were taken at 36 hr post-inoculation. Statistical analysis of the lesion area induced by Ep-1PNA367 or A10 on each plant was performed. In all experiments, three independent replicates were performed. Values are means ± *SE*. Different letters on the same graph indicate statistical significance (one-way analysis of variance; post hoc = Duncan α [0.05])

secreted proteins may play important roles in the pathogenesis of *S. sclerotiorum*. However, only a few pathogenesis-related secretory proteins have been identified and functionally characterized so far (Zhu *et al*., 2013; Xiao *et al*., 2014; Zhang *et al*., 2014; Lyu *et al*., 2016; Yang *et al*., 2018). We previously reported that the secreted protein SsITL from *S. sclerotiorum* can inhibit the host immune response in the early stage of infection (Zhu *et al*., 2013), but the mechanism by which SsITL manipulates plant immunity remains largely unknown. Here, we demonstrated that SsITL interacts with CAS to suppress plant immunity through inhibiting SA signalling pathways, thereby facilitating infection by *S. sclerotiorum*.

SsITL is an integrin-like protein and plays very important roles in the virulence of *S. sclerotiorum*. Our previous study showed that SsITL can enter the host plant cells and inhibit the JA/ethylene (ET)-mediated signalling pathway at the early stage of infection. The effects of SsITL on the SA signalling pathway were also evaluated by detecting the expression of *PR1* and the virulence of wild-type and SsITL-silenced strains A10 on *Arabidopsis* mutant *pad4* and *NahG*, and the results

suggest that SsITL might also suppress SA-mediated resistance (Zhu *et al*., 2013). CAS is an important functional protein that localizes in the chloroplast thylakoid membrane, and the N terminus of CAS appears to be exposed to the stromal side of the thylakoid membrane (Friso *et al*., 2004; Nomura *et al*., 2008). Evidence has emerged that CAS is responsible for both PAMP-triggered immunity (PTI) and effector-triggered immunity (ETI) and probably acts upstream of SA accumulation (Nomura *et al*., 2012). The SA accumulation responds to flg22 (a PAMP from bacterial flagellin) and resistance against *Pseudomonas syringae* was impaired in the *cas-1* mutant (Nomura *et al*., 2012). Previous studies have shown that SA signalling is also involved in the plant defence against *S. sclerotiorum* (Guo and Stotz, 2007; Wang *et al*., 2012; Novakova *et al*., 2014; Yang *et al*., 2018). In this study, we found that SsITL interacts with CAS in the chloroplasts of plant cells. This led us to further investigate the potential roles of CAS in plant defence to the fungal pathogen *S. sclerotiorum*. We found that overexpression of *CAS* in wild-type plant Col-0 increased the resistance to *S. sclerotiorum*. At the same time, the SA concentration and the expression level of SA signalling pathway-related genes (*ICS1*, *PAD4*, *PBS3*, *EDS5*, and *PR1*) increased significantly after inoculation with *S. sclerotiorum*, especially after inoculation with the *SsITL*-silenced transformant A10 (Figure 3c). Moreover, the resistance to *S. sclerotiorum* in *cas-1* mutant was significantly reduced, and the SA accumulation induced by *S. sclerotiorum* infection was also significantly inhibited in *cas-1* mutant. However, we also noticed that the expression of SA genes maintained a similar level in each plant genotype unless there was pathogen or chitin stimulation, suggesting that CAS is probably involved in the regulation of SA accumulation rather than the biosynthesis itself. Notably, exogenous application of 0.5 mM SA significantly suppressed the infection of *S. sclerotiorum* while a corresponding concentration of SA had no effect on the growth of *S. sclerotiorum* (Figure S4), which suggests that it is the SA-mediated signal as opposed to the compound itself that contributes to plant resistance to *S. sclerotiorum*. Our results indicate that CAS is a positive regulator of SA biosynthesis and plays important roles in the plant immunity response to *S. sclerotiorum*.

We found that SsITL co-localizes with CAS to the chloroplast. The chloroplast has been conventionally viewed as the organelle that conducts photosynthesis, but the chloroplast also plays crucial roles in the plant immune response against multiple invaders (Caplan *et al*., 2015; de Torres Zabala *et al*., 2015; Stael *et al*., 2015; Sugano *et al*., 2016; Kumar *et al*., 2018). Corresponding to this, there is growing evidence that pathogen-delivered effectors can target to chloroplasts and act as virulence factors by manipulating chloroplast functions (Li *et al*., 2014; de Torres Zabala *et al*., 2015; Petre *et al*., 2015). For example, *P. syringae* virulence effector HopI1, which localizes to chloroplasts, causes chloroplast thylakoid structure remodelling and suppresses SA accumulation (Jelenska *et al*., 2007). The cysteine protease effector HopN1, which is also secreted by *P. syringae*, localizes to chloroplasts and suppresses the production of defence-associated reactive oxygen species by degrading PsbQ (Rodriguez-Herva *et al*., 2012). Chloroplasts are the major source for continual production of SA during defence responses (Wildermuth *et al*., 2001; Asada, 2006; Galvez-Valdivieso and Mullineaux, 2010). CAS localizes in the chloroplast thylakoid membrane and is a positive regulator of SA accumulation; however, the accumulation of some other plant hormones, such as JA, abscisic acid, indole acetic acid, and cytokinins, was not affected in *cas-1* mutant on flg22 treatment (Nomura *et al*., 2012). We speculate that SsITL possibly has other targets that are related to the JA/ET signal pathway. Our results show that SsITL interacts with CAS in chloroplasts and inhibits SA accumulation. Combined with previous research (Jelenska *et al*., 2007; Pecrix *et al*., 2019; Xu *et al*., 2019), these findings highlight the chloroplast as a high-value target potentially attacked by various invaders. Indeed, *SsITL* transgenic plants are more susceptible to *S. sclerotiorum*. Correspondingly, the SA accumulation and expression of related genes induced by inoculation in *SsITL*-transgenic plants were also significantly lower than those in wild-type plant Col-0. Therefore, we speculate that SsITL suppresses plant defence to *S. sclerotiorum* through interacting with CAS and then affecting the normal biological function of CAS. Consistent with that expectation, the truncated SsITL (SsITL-NT1 or SsITL-CT1), which lost the ability to interact with CAS, does not affect plant resistance to *S. sclerotiorum*. These results further demonstrate a crucial role for the interaction between SsITL and CAS in the pathogenesis of *S. sclerotiorum*.

CAS was originally considered a primary Ca^{2+} transducer that was involved in the regulation of extracellular Ca^{2+}-induced cytosolic Ca^{2+} oscillation and stomatal closure (Han *et al*., 2003; Nomura *et al*., 2008; Wang and Zheng, 2012). Ca^{2+} signalling is important in the early stages of activation of plant immune responses (Blume *et al*., 2000; Fromm and Finkler, 2015; Yuan *et al*., 2017). Previous studies have also shown that the Ca^{2+} signalling plays an important role in plant immunity to *S. sclerotiorum*. For example, an endopolygalacturonase (PG) can induce a rapid elevation of cytosolic Ca^{2+} in plant cells and subsequently programmed cell death (PCD) when *S. sclerotiorum* infects soybean (Zuppini *et al*., 2005). The calcium and calmodulin-dependent protein kinase (*SlCCaMK*) in *Solanum lycopersicum* has been demonstrated to function in plant disease resistance against *S. sclerotiorum* (Wang *et al*., 2015). We also found that *Arabidopsis* is more susceptible to *S. sclerotiorum* after $LaCl_3$ treatment, and CAS-mediated resistance in 35S:*AtCAS* transgenic plants was largely blocked by application with $LaCl_3$. These results indicate that the CAS-mediated resistance response is associated with Ca^{2+} signalling (Figure 7). A previous study has also shown that CAS-dependent defence gene expression and SA accumulation are dependent on at least one Ca^{2+} signalling pathway (Nomura *et al*., 2012). Furthermore, there is no significant difference between $LaCl_3$- and H_2O-treated 35S:*SSITL* transgenic plants. This result, and the lack of difference between $LaCl_3$- and H_2O-treated *cas-1*, suggests that the interaction between SsITL and CAS may affect CAS perception and transmission of calcium signalling. This evidence not only supports the idea that calcium signalling plays important roles in plant immunity in resisting *S. sclerotiorum* infection, but also provides clues for further revealing the molecular mechanism by which SsITL and CAS interact to regulate host resistance. Whether the interaction influences signal transduction by blocking the calcium binding of CAS remains to be investigated.

4 | EXPERIMENTAL PROCEDURES

4.1 | Fungal strains, plants, and culture conditions

The *S. sclerotiorum* wild-type strain Ep-1PNA367 (Xie *et al.*, 2006) and *SsITL*-silenced transformant A10 (Zhu *et al.*, 2013) were used in this study. Fungal strains were cultured on potato dextrose agar (PDA) at 20 °C and stored on PDA slants at 4 °C. *S. sclerotiorum* transformants were cultured on PDA amended with hygromycin B at 50 μg/ml (Calbiochem). *Arabidopsis* wild-type Col-0 ecotype, *cas-1* knockout plant (SALK_070416 obtained from the Arabidopsis Biological Resource Center), *CAS*-overexpressing plants, *SsITL* transgenic plants (Zhu *et al.*, 2013), *SsITL-NT1* transgenic plants, and *SsITL-CT1* transgenic plants were germinated and grown on one-half strength Murashige and Skoog (MS) medium containing 0.8% (wt/vol) agar (with or without 50 μg/ml kanamycin) at 22 °C under a 16 hr light (80–100 $\mu mol \cdot m^{-2} \cdot s^{-1}$)/8 hr dark cycles for 10–12 days, then seedlings were transferred to soil and grown in chambers or greenhouse at 20–22 °C under a 14 hr light (140–160 $\mu mol \cdot m^{-2} \cdot s^{-1}$)/10 hr dark cycles with 60%–80% relative humidity. The wild-type *N. benthamiana* plants were germinated and grown in the greenhouse at 20 °C under a 14 hr light (140–160 $\mu mol \cdot m^{-2} \cdot s^{-1}$)/10 hr dark cycle with 60%–80% relative humidity.

4.2 | Generation of plant expression plasmids

The plasmids pCNF3 and pCNG (Yang *et al.*, 2018) were used for construction of a series of plant expression vectors, and all oligonucleotides and PCR primers used in this study are listed in Table S2. For the IP experiment, the full-length coding sequence of *SsITL* (without terminator codon) was amplified from cDNA of *S. sclerotiorum* with primers SsITL-SmaI-F/SsITL$^{\Delta T}$-SmaI-R, and ligated into the *Sma*I site of pCNF3 to generate pCNF3-*SsITL* construct. To study the subcellular localization of SsITL and CAS in plant cells, the full-length coding sequence of *SsITL* (without terminator codon) was amplified from cDNA of *S. sclerotiorum* with primer pair SsITL-SmaI-F/SsITL$^{\Delta T}$-SmaI-R, and then ligated into *Sma*I-digested pCNG to generate pCNG-*SsITL* construct. The full-length coding sequence of *CAS* (without terminator codon) was amplified from cDNA of *Arabidopsis* with primer pair CAS-BamHI-F/CAS$^{\Delta T}$-SmaI-R, and then ligated into *Bam*HI/*Sma*I-digested pCNF3 to generate pCNF3-*CAS* construct. Then, full-length *YFP* was amplified with primer pair YFP-SmaI-F/YFP-SmaI-R, and cloned into *Sma*I-digested pCNF3-*CAS* to generate pCNY-*CAS* construct. Truncated SsITL N-terminus (84–302 amino acids) and SsITL C-terminus (1–249 amino acids) were amplified from plasmid pCNF3-*SsITL* with primer pair SsITL-NT1-BamHI-F/SsITL-SmaI-R and SsITL-BamHI-F/SsITL-CT1-SmaI-R, respectively, then ligated into *Bam*HI/*Sma*I-digested pCNF3 to generate pCNF3-*SsITL*-NT1 and pCNF3-*SsITL*-CT1 constructs. All the plasmids were confirmed by sequencing analysis.

4.3 | Protein extraction, western blot, IP, and LC-MS/MS assays

For protein extraction, plant tissue was ground in liquid nitrogen and mixed with an equal volume of radio immunoprecipitation assay (RIPA) lysis buffer (Beyotime) with 1 mM phenylmethanesulfonyl fluoride and 1% proteinase inhibitor cocktail (Sigma), then incubated on ice for 30 min and centrifuged at 13,000 × g for 15 min at 4 °C. The supernatant was transferred to a new tube and boiled in sodium dodecyl sulphate (SDS) loading buffer for 5 min. Proteins were separated by SDS-polyacrylamide gel electrophoresis (PAGE) (12%) followed by electroblotting onto a 0.22 μm polyvinylidene fluoride membrane (Millipore) with a Trans-Blot SD Semi-Dry Electrophoretic Transfer Cell (Bio-Rad). Several monoclonal antibodies, including anti-FLAG M2 mAb (Sigma-Aldrich), anti-HA mAb (Sigma-Aldrich), anti-Myc mAb (Cell Signalling Technology), and anti-GFP mAb (Abmart), were used as primary antibodies, and horseradish peroxidase-conjugated goat antimouse IgG (H + L) was used as the secondary antibody. The signals on blots were visualized by chemiluminescence using Pierce ECL western blotting substrate (Thermo Scientific) with ChemiDoc XRS + system (Bio-Rad).

An IP assay was performed for screening of the targets of SsITL in plants. Plasmid pCNF3-*SsITL* was transferred into *A. tumefaciens* GV3101 with electroporation, then the bacteria were cultured, pelleted, and resuspended in infiltration buffer (10 mM 2-(*N*-morpholino) ethanesulphonic acid, 10 mM $MgCl_2$, and 200 mM acetosyringone) for 3–5 hr and the OD_{600} adjusted to 0.8 before infiltration into *N. benthamiana* leaves. Total proteins were extracted from *N. benthamiana* leaves with RIPA lysis buffer (0.5 g leaves/ml) 3 days post-infiltration. The mixture was vortexed vigorously for 30 s and then incubated on ice for 30 min and centrifuged at 13,000 × g for 15 min at 4 °C. The supernatants were collected and filtered through a 0.22 μm filter. To immunoprecipitate FLAG-tagged SsITL, 1 ml of supernatant was incubated overnight with 10 μg of anti-FLAG M2 antibody and 40 μl of protein G plus Agarose (Santa Cruz Biotechnology, Inc.) at 4 °C with gentle shaking. The beads were collected with centrifugation at 1,000 × g for 5 min and then washed five times with RIPA lysis buffer. The co-immunoprecipitated proteins were eluted from beads by boiling in protein sample buffer for 5 min and separated with SDS-PAGE, then stained with Coomassie brilliant blue and analysed by western blot with an anti-FLAG antibody. The gels were then cut into pieces and digested with trypsin to prepare peptides for liquid chromatography-electrosprayionization tandem mass spectrometry (LC-ESI-MS/MS) in a Q Exactive (Thermo Scientific). Identification of proteins was performed by using MASCOT v. 2.3.02.

4.4 | Y2H, Co-IP assay, GST pull-down, and subcellular localization

The GAL4-based Matchmaker Gold Yeast Two-Hybrid System (Clontech,) was applied to screen and verify SsITL candidate targets from IP-LC-MS/MS. The coding sequence of *SsITL* (without signal peptide) was PCR amplified and cloned into the pGBKT7 to

generate the bait vector, while the full-length coding sequence, the N-terminus (1–187 amino acids) and the C-terminus (211–387 amino acids) of *CAS*, were introduced into the pGADT7 to generate the bait vectors (Table S2). The bait and prey plasmids were co-transformed into yeast strain Y2H Gold according to the manufacturer's instructions. Yeast transformation was performed according to the manufacturer's instructions. Transformed cells were assayed for growth on synthetic dropout (SD)/−Trp −Leu plates for 3–4 days, and single-colony cells were transferred to 2 ml liquid SD/−Trp −Leu medium for 24 hr. Cells were collected by centrifugation and the concentration was adjusted to 10^6 cells/ml with sterile water, then 2 μl of yeast suspension was assayed for growth on SD/−Trp −Leu −His −Ade plates containing 5-bromo-4-chloro-3-indolyl α-D-galactopyranoside (X-α-gal). Truncated SsITL mutants (SsITL-NT2 140–302 amino acids, SsITL-NT3 196–302 amino acids, SsITL-NT4 250–302 amino acids, SsITL-CT2 18–195 amino acids, SsITL-CT3 18–139 amino acids, SsITL-CT4 18–83 amino acids) were introduced into pGBKT7 (Table S2) and Y2H assays were performed as described above.

For Co-IP assay, *A. tumefaciens* GV3101 harbouring the correct constructs pCNF3-*SsITL-GFP* and pCNF3-*CAS* were transiently co-expressed in *N. benthamiana* by agroinfiltration. Samples were collected and proteins extracted with RIPA buffer 3 days post-infiltration. GFP-tagged SsITL fusions were immunoprecipitated with anti-GFP antibody, and the eluted proteins were separated by SDS-PAGE and subjected to immunoblot analysis with anti-FLAG monoclonal antibody. Approximately 15 μl of RIPA buffer containing the total proteins was loaded as input control.

For GST pull-down, plasmids pGEX-6p-1, pGEX-6p-1-*SsITL*, and pET28a-*CAS* were introduced (separately) into *Escherichia coli* BL21 (DE3), and the expression of each protein was induced with 0.6 mM isopropyl β-D-1-thiogalactopyranoside at 28 °C for 10 hr. Equal amounts of GST-SsITL and His-CAS sonicated lysates were mixed with high-affinity GST resin (GenScript) and incubated at 4 °C overnight with rotation. The bound proteins were then eluted with fresh 10 mM glutathione elution buffer. Next, the proteins were separated by SDS-PAGE and immunoblotted with anti-GST or anti-His antibody (Proteintech).

To observe the subcellular localization of SsITL and CAS in plant cells, pCNF3-*SsITL-GFP* and pCNF3-*CAS-YFP* constructs were transferred into *A. tumefaciens* GV3101 by electroporation. Fluorescence in *N. benthamiana* leaves was observed 2–3 days post-infiltration using a confocal laser scanning microscope (Olympus FluoView FV1000), with GFP (excitation wavelength of 488 nm, emission wavelength of 495–510 nm) and YFP (excitation wavelength of 514 nm, emission wavelength of 530–560 nm), and chloroplast autofluorescence was detected at 650–707 nm.

4.5 | Quantification of endogenous levels of SA

Levels of endogenous SA were analysed by ultra-fast LC-electrospray ionization tandem MS with a modification of the method as reported previously (Liu *et al.*, 2012). Briefly, leaf samples were ground two or three times to powder with liquid nitrogen and transferred to 1.5-ml tubes (three replicates per sample, approximately 0.1 g per replicate). Extraction buffer I (750 μl) (methanol:water:acetic acid, 80:18:2, vol/vol/vol) with internal standard (3 ng/μl naphthaleneacetic acid) was added to the tubes. The samples were shaken at 4 °C, 200 rpm for 16 hr in the dark, and then centrifuged at 4 °C, 13,000 × g for 15 min. The supernatants were transferred to new 2-ml tubes, and 400 μl of extraction buffer II (methanol:water:acetic acid, 80:19:1, vol/vol/vol) was added to the pellet, samples were shaken at 4 °C, 200 rpm for 4 hr and centrifuged at 13,000 × g for 15 min to collect supernatants. The supernatants were then mixed and filtered with 0.22 μm nylon filters. The filtrates were dried at room temperature with a nitrogen blower, 500 μl of methanol was added to dissolve the precipitate, and the dissolved matter was centrifuged at 13,000 × g for 15 min at 4 °C. The supernatant was diluted 500-fold with methanol for quantification.

4.6 | RNA extraction, cDNA synthesis, and RT-qPCR

Plant and fungal samples were ground to a powder in liquid nitrogen, and total RNA was extracted using the RNAiso Plus regent (Takara) with a RNase-free recombinant DNase I (Takara) digestion treatment. The concentration of total RNA was determined with spectrophotometric analysis and 500 ng to 2 μg of total RNA was used to synthesize the first-strand cDNA using Easy Script One-Step gDNA Removal and cDNA Synthesis SuperMix (Transgen Biotech). RT-qPCR assays were performed using the CFX96 Real-Time PCR Detection System (Bio-Rad) with iTaq universal SYBR Green supermix (Bio-Rad). Each sample had three independent replicates, and the RT-qPCR assay for each gene was performed at least twice. Statistical analyses were performed using IBM SPSS Statistics 19 software and one-way ANOVA were run with the post hoc style Duncan ($\alpha = 0.05$).

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CONFLICT OF INTEREST

The authors declare that no competing interests exist.

DATA AVAILABILITY STATEMENT

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

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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