INTRODUCTION

The pine wood nematode (PWN) *Bursaphelenchus xylophilus* is one of the most widespread plant-parasitic nematodes (PPNs) and is responsible for massive losses of *Pinus* species in the world. Pine wilt disease (PWD) is caused by *B. xylophilus*, which has been destroying conifer forests (Liu et al., 2021). Fundamentally, *B. xylophilus* is known as a migratory endoparasitic nematode associated with the vascular system. PWNs are transmitted to the vascular system and resin canals of healthy trees by mature *Monochamus alternatus* (Japanese sawyer beetle), feeding on living parenchyma and epithelial cells, especially in East Asia. Under this condition, plant tissue disorders

A novel pine wood nematode effector, BxSCD1, suppresses plant immunity and interacts with an ethylene-forming enzyme in pine

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Abstract
The plant-parasitic nematode *Bursaphelenchus xylophilus*, the causal agent of pine wilt disease (PWD), causes enormous economic loss every year. Currently, little is known about the pathogenic mechanisms of PWD. Several effectors have been identified in *B. xylophilus*, but their functions and host targets have yet to be elucidated. Here, we demonstrated that BxSCD1 suppresses cell death and inhibits *B. xylophilus* PAMP BxCDP1-triggered immunity in *Nicotiana benthamiana* and *Pinus thunbergii*. BxSCD1 was transcriptionally upregulated in the early stage of *B. xylophilus* infection. In situ hybridization experiments showed that BxSCD1 was specifically expressed in the dorsal glands and intestine. Cysteine residues are essential for the function of BxSCD1. Transient expression of BxSCD1 in *N. benthamiana* revealed that it was primarily targeted to the cytoplasm and nucleus. The morbidity was significantly reduced in *P. thunbergii* infected with *B. xylophilus* when BxSCD1 was silenced. We identified 1-aminoacyclopropane-1-carboxylate oxidase 1, the actual ethylene-forming enzyme, as a host target of BxSCD1 by yeast two-hybrid and coimmunoprecipitation. Overall, this study illustrated that BxSCD1 played a critical role in the *B. xylophilus*-plant interaction.

KEYWORDS
1-aminoacyclopropane-1-carboxylate oxidase 1, *Bursaphelenchus xylophilus*, cysteine-rich, effector, immunity, *Pinus thunbergii*

1 | INTRODUCTION

The pine wood nematode (PWN) *Bursaphelenchus xylophilus* is one of the most widespread plant-parasitic nematodes (PPNs) and is responsible for massive losses of *Pinus* species in the world. Pine wilt disease (PWD) is caused by *B. xylophilus*, which has been destroying conifer forests (Liu et al., 2021). Fundamentally, *B. xylophilus* is known as a migratory endoparasitic nematode associated with the vascular system. PWNs are transmitted to the vascular system and resin canals of healthy trees by mature *Monochamus alternatus* (Japanese sawyer beetle), feeding on living parenchyma and epithelial cells, especially in East Asia. Under this condition, plant tissue disorders
lead to wilt within months of infection. In dying hosts where healthy plant tissues may be scarce, the nematodes use fungi as their nutrient resource (Jones et al., 2008). As the nematode numbers increase, water transport in the infected trees becomes blocked, leading to host death (Futai, 2013). PWD is very difficult to control and the molecular pathogenesis mechanisms of PWNs remain poorly understood.

Plants have evolved a sophisticated basal immune system to combat pathogen infection. Plant innate immunity relies on surface pattern recognition receptors (PRRs) and intracellular nucleotide-binding/leucine-rich-repetit (NLR) receptors to identify viruses, bacteria, fungi, nematodes, and feeding insects (Jones & Dangl, 2006). The first step is pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI) to suppress nonadaptive microbes (Zipfel, 2014). In turn, pathogens and parasites deliver effectors into the host to disturb plant defence responses and promote infection. To defend against infection, plants employ nucleotide-binding/leucine-rich-repeat receptors to intercept pathogen effectors and induce effector-triggered immunity (ETI) (Jacob et al., 2013). Simultaneously, pathogen effectors are also recognized by plant PRRs and NLR receptors (Kanzaki et al., 2012). Thus, it is important to clarify the mechanism of individual effectors and how they interact with plants.

The study of pathogen effector proteins has been a hot topic in plant immunology in recent decades (Macho & Zipfel, 2015). To enter the host interior and cause disease, pathogens must be able to subvert immune responses. The secretion of pathogenicity molecules, so-called effectors, modulates basal defences and promotes infection (Cornelis, 2010; Govers & Smant, 2014). Gram-negative bacteria secrete effectors into plant cells using type III secretion systems to disturb host immunity (Kjemtrup et al., 2000). The Pseudomonas syringae effector AvrPto inhibits the kinase activity of the pattern recognition receptor FLS2 through the activity of a kinase suppressor, thus interfering with the plant immune response (Xiang et al., 2008). Several fungal pathogens, such as Verticillium dahliae, deliver effectors that contribute to virulence (Ning et al., 2015; Zhang et al., 2017). The secretory protein VdSCP41 directly controls important transcription factors in the plant calmodulin-binding protein family to inhibit immunity (Qin et al., 2018). As part of effector research, some groups have demonstrated the characteristics of several PPN effectors (Gleason et al., 2017). A calreticulin Mi-CRT secreted by the root-knot nematode Meloidogyne javanica has a significant role in PTI suppression (Jaouannet et al., 2013). The soybean cyst nematode Heterodera glycines effector protein 30D08 interacts with SMU2, an auxiliary splicosomal protein in plants, to alter gene expression and cause disease (Verma et al., 2018). However, root-knot nematodes and cyst nematodes are sedentary endoparasitic phytonematodes while the mechanism of B. xylophilus effectors remains largely unknown.

Phytohormones are physiologically important small molecules that regulate growth, development, and environmental responses in plants. In general, plants depend on elaborate phytohormone signalling networks to defend themselves from pathogen attack. In turn, a universal tactic of pathogens is to secrete effectors that target hormone receptors and other components of hormone signalling in plants to manipulate phytohormone-regulated defences. For example, the P. syringae effector HopX1 activates jasmonic acid (JA) signalling by interacting with JAZ repressors and promoting their degradation (Gimenez-Ibanez et al., 2014). Cmu1, an effector gene of the biotrophic fungus Ustilago maydis, encodes a chorismate mutase that is an isomerase enzyme that converts the salicylic acid (SA) precursor chorismate to prephenate, reducing the levels of chorismate available for SA biosynthesis (Djamei et al., 2011). SA is also required for resistance against plant-parasitic nematodes. The Heterodera schachtii effector 10A06 interacting with Spermidine Synthase2 (SPDS2) disrupts polyamine metabolism and SA signalling, suggesting that nematodes may also have evolved the ability to interrupt host SA pathways (Hewezi et al., 2010). Ethylene (ET) is a hormone that controls many important processes in plants, including responses to biotic stresses (Loon et al., 2006). As such, it is essential that ET biosynthesis is accurately regulated. The ET biosynthesis pathway consists of two steps in plants. In the first step, S-adenosyl-l-methionine is converted to 1-aminocyclopropane-1-carboxylic acid (ACC) by ACC synthase (ACS). Next, ACC oxidase (ACO) releases ET from ACC (Hamilton et al., 1991). Because ACS and ACO are the only two enzymes dedicated to ET biosynthesis, many pathogen effectors regulate the ET pathway by manipulating the transcription and translation of these two enzymes. For instance, the bacterial microbe-associated molecular pattern (MAMP) flg22 activates ET biosynthesis via MPK6-mediated phosphorylation of the ET biosynthesis enzyme ACS and enhances susceptibility to a nonadapted bacterial pathogen in Arabidopsis thaliana (Bethke et al., 2009). Additionally, P. syringae pv. tomato type III effectors AvrPto and AvrPtoB induce the expression of LeACO1 and LeACO2, two ET biosynthesis genes, to promote enhanced disease in tomato leaves (Cohn & Martin, 2005). Thus, it is not surprising that most pathogens secrete effectors to control or disturb plant phytohormone signalling pathways for their benefit. However, the intervention of phytohormone pathways by B. xylophilus effectors is poorly understood.

A set of B. xylophilus candidate effectors were identified in a previous transcriptome analysis (Tsai et al., 2016). In this study, we focused on a candidate effector delivered by B. xylophilus, named BxSCD1, which was notably upregulated on B. xylophilus infection and could suppress cell death and immune responses in Nicotiana benthamiana and Pinus thunbergii triggered by the B. xylophilus PAMP BxCDP1. We tested the influences of this effector using RNA interference (RNAi) and purified BxSCD1 protein. Additionally, we identified 1-aminocyclopropane-1-carboxylate oxidase 1 (ACO1) as a target of BxSCD1 in P. thunbergii for the first time using a yeast two-hybrid screening assay. Thus, we confirmed that BxSCD1 is a crucial virulence effector during B. xylophilus infection of pine.
2 | RESULTS

2.1 | Identification of the candidate effector BxSCD1

The BxSCD1 (BXY_0861200) effector gene sequence was identified from a cDNA library of *B. xylophilus* (Tsai et al., 2016). The BxSCD1 cDNA encodes a 295 amino acid sequence with a 24 amino acid N-terminal signal peptide, including eight cysteine residues. There are different virulences among *B. xylophilus* isolates in natural populations. To explore BxSCD1 sequence polymorphisms, we sequenced the BxSCD1 coding region from four *B. xylophilus* isolates, namely, AMA3 (highly virulent strain), ZL1 (highly virulent strain), YW4 (weakly virulent strain), and HE2 (weakly virulent strain) (Ding et al., 2016). The results demonstrated that BxSCD1 has no polymorphism among the four isolates (Figure S1).

To investigate the immunosuppressive ability of BxSCD1, we tested whether BxSCD1 could suppress programmed cell death (PCD) caused in *N. benthamiana* by the *Phytophthora sojae* PAMP PsXEG1 and other effectors (Ma et al., 2015). We infiltrated *Agrobacterium* strains carrying BxSCD1 (without signal peptide) into *N. benthamiana* leaves 16 hr before infiltration with *Agrobacterium* strains carrying PsXEG1. We found that PsXEG1-triggered PCD was suppressed at 5 days postinoculation (dpi) (Figure 1a). BxCDP1, a novel molecular pattern of *B. xylophilus*, can trigger PCD in *N. benthamiana* (Hu et al., 2020). We found that BxSCD1 (without signal peptide) also clearly suppressed BxCDP1-triggered PCD in *N. benthamiana*. However, the full-length BxSCD1 (BxSCD1-SP) lost the ability to suppress PCD in *N. benthamiana* (Figure 1a). Electrolyte leakage in *N. benthamiana* triggered by PsXEG1 and BxCDP1 was markedly weakened in the presence of BxSCD1 (Figure 1b). Western blot analysis confirmed that the proteins (green fluorescent protein [GFP], BxSCD1-HA, BxSCD1-SP-HA, BxCDP1, and PsXEG1) were produced in *N. benthamiana* leaves (Figure 1c,d). These results show that BxSCD1 functions in plant cells and suppresses PCD in *N. benthamiana*.

2.2 | BxSCD1 suppresses BxCDP1-triggered immunity

To assess whether BxSCD1 suppresses immune responses other than cell death, we measured whether reactive oxygen species (ROS) production and PTI marker genes triggered by the *B. xylophilus* PAMP BxCDP1 could be suppressed in *N. benthamiana* by transiently expressing BxSCD1. First, the BxSCD1 protein was produced in the
yeast Pichia pastoris and purified GFP was used as a negative control. Second, the recombinant proteins BxSCD1rec and GFPrec were assessed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and western blot analysis to confirm successful purification (Figure S2a). We determined that BxSCD1rec was still able to suppress PsXEG1-triggered cell death by infiltrating 300 nM to 3 µM protein solutions into N. benthamiana leaves (Figure S2b).

We infiltrated purified BxCDP1 protein into N. benthamiana leaves 16 hr after expressing the BxSCD1 or GFP (negative control) recombinant protein. It was evident that BxSCD1 suppressed BxCDP1-induced ROS compared with GFP 3 hr after infiltration (Figure 2a). A previous study demonstrated that PTI marker genes (NbAcre31, NbPTI5, and NbCyp71D20) were upregulated by treatment with BxCDP1 in response to biotic stresses (Hu et al., 2020). Quantitative reverse transcription PCR (RT-qPCR) analysis revealed that BxSCD1 reduced the expression level of NbPTI5 (Figure 2b). These results indicated that BxSCD1 suppressed PTI responses in N. benthamiana. Then, we further tested whether BxSCD1 could suppress BxCDP1-induced pathogenesis-related (PR) marker genes in P. thunbergii. We found that the relative expression levels of PtPR-4 and PtPR-5 were reduced in P. thunbergii compared with the expression levels in BxCDP1-treated samples by RT-qPCR (Figure 2c,d). We also observed the cell morphology of P. thunbergii using transmission electron microscopy (TEM). At 10 dpi, compared with P. thunbergii cells without any treatment (Figure 2e), the P. thunbergii cells inoculated with purified BxCDP1 protein, BxCDP1, and GFP protein were obviously damaged (Figure 2f,g), while the P. thunbergii cells inoculated with the purified BxSCD1 and BxCDP1 protein exhibited more morphological integrity and more abundant cell contents and organelles (Figure 2h). We inferred that BxSCD1 could suppress the immune responses induced by BxCDP1 in both N. benthamiana and P. thunbergii.

2.3 Transcript level of BxSCD1 is upregulated during the early stages of pine infection

In general, effector genes are transcriptionally upregulated during the early infection stages (Bouwmeester et al., 2011). The expression levels of BxSCD1 were tested by RT-qPCR at different stages of
**B. xylophilus** infection. The results showed that BxSCD1 was intensively upregulated during *B. xylophilus* infection in the highly virulent strain AMA3, with peak expression at 12 hr after infection and basal levels thereafter (Figure 3). This finding supported that BxSCD1 plays a vital role during the early stages of *B. xylophilus* infection of pine.

**2.4 | BxSCD1 is predominantly expressed in dorsal glands and intestine**

According to the above results, BxSCD1 expression was highest at 12 hr. Therefore, we collected PWNs from whole seedlings using the Baermann funnel technique 12 hr after inoculation into pine stems. Then, we used in situ hybridization assays to investigate the sites at which BxSCD1 was expressed in the PWN. Using digoxigenin (DIG)-labelled antisense cDNA probes, the results demonstrated that BxSCD1 was specifically expressed in the dorsal glands and intestine of PWN (Figure 4). This strongly suggested that BxSCD1 could be secreted into plant tissues.

**2.5 | Cysteine residues are essential for the function of BxSCD1**

The BxSCD1 protein contains eight cysteine residues that may be involved in disulphide bond formation. The prediction software DiANNA predicted four cysteine disulphide bonds in BxSCD1 (Figure 5a). To examine whether the cysteine residues in BxSCD1 were essential to suppress cell death, we individually replaced each cysteine residue with an alanine (C-A). The mutant with all cysteine residues replaced by alanine (C-A) and the C117A and C153A single cysteine residue mutants did not suppress PsXEG1-induced cell death in *N. benthamiana* leaves. Immunoblotting analysis confirmed that the genes were well expressed (Figure 5b). Electrolyte leakage in *N. benthamiana* triggered by PsXEG1 was clearly enhanced in the presence of the C-A, C117A, and C153A mutants (Figure 5c). These results revealed that two pivotal disulphide bonds might influence the function of BxSCD1.

**2.6 | BxSCD1 cytoplasmic localization is required for its suppression of cell death**

To better describe the function of BxSCD1, we determined its subcellular localization and transiently expressed a red fluorescent protein (RFP)-tagged BxSCD1 (without signal peptide) in *N. benthamiana* leaves by agroinfiltration. The results showed that BxSCD1 primarily accumulated in the cytoplasm and nucleus in *N. benthamiana* (Figure 6a). To assess whether the subcellular localization of BxSCD1 is required for the suppression of cell death activity, we fused a nuclear export signal (NES) or its mutated form (nes), or a nuclear localization signal (NLS) or its mutated form (nls) to BxSCD1, respectively. Confocal microscopy showed that BxSCD1-NLS was solely present in the nucleus, whereas the expression of BxSCD1-NES was markedly reduced in the nucleus (Figure 6b,c). When expressing these BxSCD1 mutants in *N. benthamiana*, we observed that only BxSCD1-NES was still able to suppress PsXEG1-induced cell death (Figure 6d). Electrolyte leakage in *N. benthamiana* triggered by PsXEG1 was distinctly weakened in the presence of BxSCD1-NES (Figure 6e). Western blot analysis confirmed that the genes were well produced in *N. benthamiana* leaves (Figure 6f). Therefore, these results indicated that cytoplasmic localization of BxSCD1 is required for the suppression of PsXEG1-induced cell death in *N. benthamiana*.

**2.7 | BxSCD1 contributes to *B. xylophilus* virulence**

To estimate the contribution of BxSCD1 to the virulence of *B. xylophilus*, we used gene silencing methods in the AMA3 isolate. We inoculated pines with treated PWNs 48 hr after soaking in double-stranded RNA (dsRNA) solution. RT-qPCR confirmed that BxSCD1 was successfully silenced, with transcript levels decreased by approximately 80% (Figure S3b). As controls, the pines that were inoculated with GFP dsRNA-treated PWNs and wildtype (WT) PWNs appeared to have distinctly yellow needles at 10 dpi. Notably, symptoms did not appear in the BxSCD1 dsRNA-treated pines until 16 dpi (Figure 7a). At 16 dpi, the morbidity of GFP dsRNA-treated pines reached 75%, while the morbidity of BxSCD1 dsRNA-treated pines was only 30% (Figure 7b). Moreover, the degree of morbidity also proved that silencing of BxSCD1 could delay the occurrence of disease (Figure 7c). The *Botrytis cinerea* mycelium on the potato dextrose agar (PDA) plates used for culturing the PWNs was observed residues with alanine (C-A). The mutant with all cysteine residues replaced by alanine (C-A) and the C117A and C153A single cysteine residue mutants did not suppress PsXEG1-triggered cell death in *N. benthamiana* leaves. Immunoblotting analysis confirmed that the genes were well expressed (Figure 5b). Electrolyte leakage in *N. benthamiana* triggered by PsXEG1 was clearly enhanced in the presence of the C-A, C117A, and C153A mutants (Figure 5c). These results revealed that two pivotal disulphide bonds might influence the function of BxSCD1.

**FIGURE 3** The expression pattern of BxSCD1 at the early stages of *Bursaphelenchus xylophilus* infection by quantitative reverse transcription PCR (RT-qPCR) analysis. *Pinus thunbergii* seedlings inoculated with *B. xylophilus* (AMA3) were sampled at different time points. The relative transcript level of BxSCD1 was calculated by the comparative threshold method. The RT-qPCR values were normalized to the transcript level of Actin. Values represent the mean ± SD of three independent biological samples. Different letters over error bars indicate statistically significant differences using Duncan’s multiple range test (*p* < 0.05).
and the number of PWNs on the B. cinerea plates and in P. thunbergii seedlings was counted. The results demonstrated that all of the treated PWNs showed similar reproduction and feeding rates (Figure S3a,d,e). Taken together, these data indicate that BxSCD1 is required for the virulence of B. xylophilus to infect pine, which had nothing to do with the reproduction and feeding rate.

We further evaluated whether BxSCD1 silencing in B. xylophilus influences the pine defence response. The results showed that H2O2 content in pine increased when BxSCD1 was silenced (Figure 7d). Likewise, the relative expression levels of PtPR-4 and PtPR-5 were notably upregulated in P. thunbergii inoculated with BxSCD1 dsRNA-treated nematodes (Figure 7e,f). However, the relative expression of PtPR-9 was reduced (Figure S3c). These results suggested that BxSCD1 might surmount the oxidative system to suppress immune responses in P. thunbergii.

2.8 | BxSCD1 associates with pine ACO1

To understand the potential virulence function of BxSCD1 in pine, we performed a yeast two-hybrid screening assay to identify potential host targets of BxSCD1. We identified one major target, 1-aminocyclopropane-1-carboxylate oxidase 1 (ACO1), which was captured four times, whereas other candidates were captured only once. Subsequently, we acquired the whole ACO1 coding sequence from P. thunbergii according to the specific primer of Pinus taeda (Figure S4b,c). We validated the interaction between the full-length sequences of BxSCD1 and PtACO1 in yeast (Figure 8a). As expected, coimmunoprecipitation (CoIP) experiments proved that BxSCD1 interacted with PtACO1 in planta when they were coexpressed in N. benthamiana (Figure 8b). However, the BxSCD1 cysteine residue mutant (C-A) could not interact with PtACO1 (Figure 8c). To further explore the effect of BxSCD1 on PtACO1, the expression of PtACO1 was measured in P. thunbergii. RT-qPCR showed that the expression of PtACO1 was reduced in BxSCD1 dsRNA-treated host pines compared with WT and dsGFP nematode treatments (Figure S4d). These results revealed that BxSCD1 interacts with PtACO1, implying that PtACO1 is a target of BxSCD1 in P. thunbergii infected with B. xylophilus.

3 | DISCUSSION

B. xylophilus has become one of the most crucial forest pathogens in recent decades. Currently, the pathogenic mechanism of B. xylophilus is unclear. In plant–nematode interactions, B. xylophilus secretes a large number of effectors to disturb the robust innate immune systems of plants (Li et al., 2009). Our previous studies demonstrated that several B. xylophilus effectors could induce cell death and immune responses (Hu et al., 2019; Huang et al., 2019; Zhao et al., 2020). In this study, we characterized the virulence effector BxSCD1 in B. xylophilus, which suppressed cell death and immune responses in N. benthamiana and P. thunbergia, indicating that BxSCD1 is a vital effector secreted by B. xylophilus.

In general, we screened effectors by transient expression in N. benthamiana to determine whether they inhibit cell death induced by BAX and INF1. Bx-FAR-1 was identified as a repressor of B. xylophilus (Li et al., 2020). In this study, we selected the B. xylophilus PAMP BxCDP1 as an elicitor and found that BxSCD1 was able to suppress cell death, ROS, and PTI marker genes induced by BxCDP1 (Figures 1 and 2a,b), suggesting that they might share a common pathway to lead to suppressed immunity in N. benthamiana. Furthermore, we attempted to express and purify BxSCD1 by P. pastoris to test whether it has plant immunity-suppressing activity because comparable genetic tools are still not available in P. thunbergii (gymnospermous plants). BxSCD1rec suppressed the expression of PR genes and
alleviated the damage to cell morphology triggered by BxCDP1rec in *P. thunbergii*, indicating that BxCD1 is involved in immunosuppression. In *P. sojae*, most of the RXLR effector proteins could also suppress cell death induced by the PAMP INF1 and four RXLR effectors could suppress cell death and PTI triggered by PsXEG1 in *N. benthamiana* (Ma et al., 2015; Wang et al., 2011). In *B. xylophilus*, BxCD1 was the first effector to be discovered that could suppress immunity induced by the PAMP BxCDP1 in *N. benthamiana* and in *P. thunbergii*. Therefore, we speculated that BxCD1 might be a part of the network of immunity triggered by BxCDP1 and that BxCD1 would preemptively prevent plant responses to BxCDP1 and interfere with PTI to promote infection in *B. xylophilus*.

Our results revealed that BxCD1 exhibited cytoplasmic and nuclear distribution in *N. benthamiana* leaf cells. Using NLS/NES sequences, we further confirmed that cytoplasmic localization is critical for the suppression of cell death. The immune response assays in

**Figure 5** Prediction and functional analysis of disulphide bonds in BxCD1 protein. (a) Disulphide bond predictions for BxCD1 were predicted using DiANNA. Numbers (50, 66, 117, 153, 202, 212, 250, and 286) indicate the locations of cysteine residues in BxCD1. Values indicate the scores for the cysteine residues possibly involved in disulphide bond formation. SP, signal peptide. (b) The cysteine residues mutants were detected by suppressing PsXEG1-triggered cell death assays in *Nicotiana benthamiana* leaves. The experiments were repeated at least three times with similar results. “C-A” indicates that all cysteine residues were replaced by alanine. “C-No-A” indicates that the designated single cysteine residues were replaced by alanine. Ponceau S staining of RuBisCO shows protein loadings. (c) Quantification of cell death suppression by the cysteine residue mutants by measuring electrolyte leakage in *N. benthamiana*. The data shown are combined from three independent experiments. Different letters over error bars indicate statistically significant differences using Duncan's multiple range test (*p* < 0.05).
FIGURE 6 BxSCD1 is located in the cytoplasm and nucleus and cytoplasmic localization of BxSCD1 is required for suppression of PsXEG1-induced cell death. (a) Subcellular localization of BxSCD1 was determined by transient expression of red fluorescent protein (RFP)-tagged proteins in Nicotiana benthamiana leaves. (b) Confocal microscopy images showing the subcellular localization of BxSCD1-NLS (nuclear localization signal), BxSCD1-NES (nuclear export signal), and the mutant forms nls and nes. Pictures were taken 36 hr postinfiltration. (c) RFP intensity was delineated from the cytoplasmic area by the “non-closed Spline Contour” in the “Profile” in software (ZEN 2.3). (d) BxSCD1-NES suppresses PsXEG1-induced cell death in N. benthamiana. Pictures were taken 5 days postinfiltration. The experiments were repeated at least three times with similar results. (e) Electrolyte leakage measures the quantification of cell death suppression in N. benthamiana. Different letters over error bars indicate statistically significant differences using Duncan’s multiple range test ($p < 0.05$). (f) Immunoblot analysis of proteins from N. benthamiana leaves transiently expressing target proteins. Protein loading is indicated by Ponceau S staining of RuBisCO.
by expressed in the cytoplasm of N genes related to the elf18- triggered PTI response when transiently delivered from one of the secretory organs, amphids, dorsal glands, (Lin et al., 2016). In plant-parasitic nematodes, a protein must be virulent strain (Figure 3). This was a necessary feature of effectors when it is present in the cytoplasm. Msp40 secreted benthamiana was able to suppress the expression of P. thunbergii seedlings infected with dsBxSCD1 dsRNA-treated nematodes. (e, f) The relative transcript levels of pathogenesis-related genes (PtPR-4 and PtPR-5) in P. thunbergii infected with BxSCD1 dsRNA-treated nematodes. We selected stems c.2 cm in length to extract RNA at 12 hr postinoculation. Values represent the mean ± SD of three independent biological samples. Different letters over error bars indicate statistically significant differences using Duncan’s multiple range test (p < 0.05).

FIGURE 7 BxSCD1 contributes to Bursaphelenchus xylophilus virulence. (a) Inoculation assay of pine seedlings. Based on the colour of the needles, the morbidity degree of the Pinus thunbergii seedlings was different. At 10 days postinoculation (dpi), five P. thunbergii seedlings inoculated with wildtype (WT) B. xylophilus turned yellow, four P. thunbergii seedlings inoculated with ds GFP B. xylophilus turned yellow, and one P. thunbergii seedling inoculated with dsBxSCD1 B. xylophilus turned yellow. At 16 dpi, eight P. thunbergii seedlings inoculated with WT B. xylophilus turned yellow or brown, seven P. thunbergii seedlings inoculated with ds GFP B. xylophilus turned yellow or brown, and four P. thunbergii seedlings inoculated with dsBxSCD1 B. xylophilus turned yellow. (b) The infection rates of P. thunbergii seedlings under three different treatments. (c) The disease severity index of P. thunbergii seedlings under three different treatments. Values represent the mean ± SD of three independent biological samples. Different letters over error bars indicate statistically significant differences using Duncan’s multiple range test (p < 0.05). (d) The content of H2O2 in P. thunbergii infected with BxSCD1 dsRNA-treated nematodes. (e, f) The relative transcript levels of pathogenesis-related genes (PtPR-4 and PtPR-5) in P. thunbergii infected with BxSCD1 dsRNA-treated nematodes. We selected stems c.2 cm in length to extract RNA at 12 hr postinoculation. Values represent the mean ± SD of three independent biological samples. Different letters over error bars indicate statistically significant differences using Duncan’s multiple range test (p < 0.05).
with a rate-limiting enzyme involved in ET biosynthesis to regulate host plant defences. Plants would seem to active ET signalling pathways in plant–nematode interactions (Gutierrez et al., 2009). LeERF2 has been shown to regulate ET biosynthesis by controlling ACO expression, by both positive and negative ERF-mediated feedback mechanisms (Zhang et al., 2009). Hormonal crosstalk is also
involved in the regulation of ACO transcription. Additionally, phytopathogen effectors exhibit a high degree of functional redundancy, hampering the assessment of their contribution to pathogenicity (Remigi et al., 2011). There is also evidence that ET production increases in the outermost xylem and decrease in leaf water potential and photosynthesis, resulting in immune overload in the early stage of B. xylophilus infection (Fukuda, 1997). Additionally, ACO shows tissue-specific expression and localization patterns (Park et al., 2018), indicating that ACO is under tight regulatory control at the transcriptional level. We validated the localization of PtACO1 in the cytoplasm and the nucleus (Figure S4a), where it could interact with BxSCD1. It is quite possible that BxSCD1 could regulate the expression of PtACO1 in the nucleus. Localization in the plant cell nucleus might indicate a role in transcriptional regulation (Jaouannet & Rosso, 2013), suggesting that the regulation of ET pathways and the plant immunity-suppressing activity of BxSCD1 are uncoupled. We found for the first time that ET biosynthesis enzymes can interact with B. xylophilus effector proteins, which enhances our understanding of how B. xylophilus interferes with ET biosynthesis during infection. Nevertheless, comparable genetic tools are still not available in B. xylophilus and P. thunbergii. Therefore, we can only speculate that the interaction between BxSCD1 and PtACO1 influences nematode parasitism of P. thunbergii by controlling the expression of PtACO1. Whether PtACO1 contributes to plant resistance in pine is an attractive question to explore in the future.

Here, we identified a small cysteine-rich B. xylophilus-specific effector (BxSCD1) that suppresses plant immunity in N. benthamiana and P. thunbergii, and contributes to B. xylophilus virulence. Moreover, PtACO1 was found to be the target of B. xylophilus in P. thunbergii. Our studies provide important evidence to further explore the mechanisms of the pine–B. xylophilus interaction.

4 | EXPERIMENTAL PROCEDURES

4.1 | Nematode culture and infection

The pine wood nematode B. xylophilus isolates AMA3, ZL1, YW4, and HE2 were cultured on PDA plates covered with B. cinerea mycelia at 25 °C for 7 days. B. xylophilus was collected on a modified Baermann funnel technique as described previously (Ding et al., 2016). Nematode infection and sampling assays were conducted as previously described (Hu et al., 2020).

4.2 | Plant material

N. benthamiana was grown in a greenhouse at 25 °C with a cycle of 16 hr of high light intensity and 8 hr of darkness. P. thunbergii seedlings (3 years old) were cultivated at 30 °C with 65%–75% relative humidity in the greenhouse of Nanjing Forestry University.

4.3 | Plasmid construction

The BxSCD1 gene was cloned using cDNA from B. xylophilus (AMA3). The amplified fragments were ligated into pBINRFP (a plasmid containing RFP) and PVX (pGR107) using the appropriate restriction enzymes and a Clone Express II One Step Cloning Kit (Vazyme). BxSCD1 mutants were amplified using combinations of primers. Individual colonies for each construct were tested for inserts by PCR and selected clones were verified by sequencing.

4.4 | Agrobacterium tumefaciens infiltration assays

Agrobacterium-mediated transient expression was performed as described previously (Wang et al., 2011). Recombinant plasmids were transformed into A. tumefaciens GV3101 by electroporation, and the cells were grown on Luria Bertani agar plates with kanamycin and rifampicin at 30 °C for 3 days. The A. tumefaciens cells were resuspended in wash buffer (10 mM MgCl2, 10 mM MES, 100 µM acetosyringone, pH 5.6) and diluted to a final optical density (OD) at 600 nm of 0.4–0.6. Agrobacterium cells carrying BxSCD1 were infiltrated into leaves. The same infiltration site was challenged 16 hr later with an Agrobacterium carrying an elicitor. The A. tumefaciens suspensions were infiltrated into the leaves of N. benthamiana using a needleless syringe. Each experiment was performed on six leaves from three individual plants and repeated at least three times.

4.5 | Electrolyte leakage assay

Electrolyte leakage in the inoculated N. benthamiana leaves was measured as described previously (Hu et al., 2020). All assays were repeated three times.

4.6 | Total protein extraction and western blot analysis

Proteins were prepared from the infiltrated areas collected 36 hr after agroinfiltration. Tissues were then ground in liquid nitrogen and total proteins were extracted using a lysis buffer (10 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.5% NP-40, 1 mM phenylmethylsulfonyl fluoride [PMSF], 0.5 mM ethylenediaminetetraacetic acid [EDTA], and a protease inhibitor cocktail; Sigma-Aldrich) by mixing 1 g of leaf tissue with 2 ml of lysis buffer. The samples were centrifuged at 4 °C at 12,000 × g for 15 min and the supernatant was transferred to a new tube.

Nitrocellulose membranes were blocked with 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% Tween 20, and 2% milk powder. Primary antibodies were incubated for 3 hr at 1:2,000 (HA-tag antibodies; Sigma-Aldrich). The secondary antibodies were incubated for 1 hr at 1:10,000. Detection was performed by chemiluminescence using an ECL kit according to the manufacturer’s instructions (Pierce).
4.7 | ROS measurement

ROS production was monitored by a luminol/peroxidase-based assay using leaf discs (0.5 cm diameter) collected from 5-week-old *N. benthamiana* plants treated with BxSCD1 and GFP protein for 16 hr and floated overnight in 200 µl of sterile water in a 96-well plate. Water was replaced with luminol (35.4 µg/ml)/peroxidase (10 µg/ml) reaction solution in 100 nM flg22 (GenScript Biotech Corporation) and 1 µM purified BxCDP1 protein. Luminescence was measured using a GLOMAX96 microplate luminometer (Promega).

4.8 | Expression of the recombinant BxSCD1 protein and *P. thunbergii* cell morphology observation

BxSCD1 (without signal peptide) and GFP (green fluorescent protein) were cloned into pPICZαA, which contains a 6 x His tag, respectively. GFP was used as a control. The recombinant vectors were transformed into *P. pastoris* KM71H (Muts) (Invitrogen) by electroporation. Positive clones were grown in yeast extract-peptone-dextrose (YPD) medium containing 100 µg/ml zeocin at 30 °C for 2–3 days. Buffered glycerol complex medium (BMGY) and buffered methanol-complex medium (BMMY) were used to induce protein expression, according to the manufacturer’s instructions. Purification of recombinant BxSCD1 and GFP from the culture supernatant was performed by affinity chromatography using Ni-NTA Superflow resin (Qiagen).

Three-year-old *P. thunbergii* seedlings were inoculated with 1 ml purified protein (final concentration 50 µg/ml). Purified BxSCD1 and GFP proteins were inoculated into seedlings. The same site was inoculated 16 hr later with BxCDP1 protein. At 10 dpi, the seedling stems were collected at 1 cm below the inoculation site and transverse 1-mm thick sections were cut using a surgical blade. The samples were processed as described previously (Hu et al., 2020).

4.9 | Total RNA extraction and RT-qPCR analyses

RNA was extracted from three biological replicates using TRIzol reagent (Invitrogen). The quality of RNA was checked as previously described (Hu et al., 2020). We subjected 1 µg of total RNA to reverse transcription with a cDNA synthesis kit (Vazyme). The primers for qPCR were designed with the NCBI website. Quantitative PCR (qPCR) was carried out as described previously (Lin et al., 2016).

4.10 | In situ hybridization

A 179 bp PCR product was amplified from *B. xylophilus* (AMA3) cDNA using forward and reverse primers. These amplicons were used as the template in a unidirectional PCR to produce sense and antisense DIG-labelled probes. In situ hybridization was performed as described previously (de Boer et al., 1998) using a DIG High Prime RNA Labeling and Detection Starter Kit I (Roche Diagnostics). Finally, the samples were observed under an Axio Image M2 microscope (Zeiss).

4.11 | Subcellular localization

BxSCD1 and its mutants cloned into pBinRFP were transformed into 4-week-old *N. benthamiana* leaves by agroinfiltration. Plants were grown in a growth chamber at 25 °C under 16 hr:8 hr, light:dark conditions for 2 days. To observe fluorescence, patches of *N. benthamiana* leaves were analysed using a confocal laser scanning microscope (Zeiss). Epidermal tissues of tobacco leaves were sampled and observed with a LSM710 laser scanning confocal microscope with a 568 nm emission filter. Once a picture was selected, we used the “non-closed Spline Contour” in “Profile” to delineate the cytoplasmic area and generate fluorescence intensity automatically by software (ZEN 2.3).

4.12 | RNAi design and treatment for inoculation assays

RNAi of BxSCD1 and inoculation assays were carried out as described previously (Hu et al., 2019). We measured the silencing efficiency of BxSCD1 with specific primers.

4.13 | Determination of hydrogen peroxide content

Samples of homogenized stems (100 mg) c.2 cm in length were mixed with 1 ml 1% trichloroacetic acid on ice. The homogenate was centrifuged at 2,650 x g for 15 min at 4 °C. Subsequently, 250 µl of supernatant was added to 250 µl of 100 mM phosphate buffer (pH 7.0) and 500 µl of 1 M KI. The H$_2$O$_2$ content of the supernatant was determined by comparing its absorbance at 390 nm according to the standard calibration curve.

4.14 | Interaction analysis

BxSCD1 (without signal peptide) was cloned into the pGBK7 bait vector and then transformed into yeast strain Y2H Gold. The *B. xylophilus*-infected pine cDNA library was screened following the Clontech protocols. Subsequently, BxSCD1 was cloned into the pGBK7 vector, and potential interactors were cloned into the pGADT7 vector to perform a yeast two-hybrid assay. For the CoIP assay, BxSCD1 and PtACO1 were cloned into PVX and pBINGFP, respectively. All constructs were introduced into *A. tumefaciens* GV3101 by electroporation. Cell suspensions (PVX-BxSCD1 and pBINGFP-PtACO) and mixed bacterial solutions (PVX-BxSCD1 and pBINGFP-PtACO1) were infiltrated into *N. benthamiana*. At 48 hr after infiltration, the proteins were extracted and CoIP assays were performed as described previously (Moffett et al., 2002).
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CONFLICT OF INTEREST
The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT
The data that supports 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.