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ORIGINAL ARTICLE

Overexpression of *Xanthomonas campestris* pv. *vesicatoria* effector AvrBsT in *Arabidopsis* triggers plant cell death, disease and defense responses

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Abstract Recognition of bacterial effector proteins by plant cells is crucial for plant disease and defense response signaling. The *Xanthomonas campestris* pv. *vesicatoria* (*Xcv*) type III effector protein, AvrBsT, is secreted into plant cells from *Xcv* strain Bv5-4a. Here, we demonstrate that dexamethasone (*DEX*): *avrBsT* overexpression triggers cell death signaling in healthy transgenic *Arabidopsis* plants. *AvrBsT* overexpression in *Arabidopsis* also reduced susceptibility to infection with the obligate biotrophic oomycete *Hyaloperonospora arabidopsidis.* Overexpression of *avrBsT* significantly induced some defense-related genes in *Arabidopsis* leaves. A high-throughput in planta proteomics screen identified TCP-1 chaperonin, SEC7-like guanine nucleotide exchange protein and calmodulin-like protein, which were differentially expressed in *DEX:avrBsT*-overexpression (OX) *Arabidopsis* plants during *Hp. arabidopsidis* infection. Treatment with purified GST-tagged AvrBsT proteins distinctly inhibited the growth and sporulation of *Hp. arabidopsidis* on *Arabdiopsis* cotyledons. In contrast, *DEX:avrBsT*-OX plants exhibited enhanced susceptibility to *Pseudomonas syringae* pv. *tomato* (*Pst*) DC3000 infection. Notably, susceptible cell death and enhanced electrolyte leakage were significantly induced in the *Pst*-infected leaves of *DEX:avrBsT*-OX plants. Together, these results suggest that *Xcv* effector AvrBsT overexpression triggers plant cell death, disease and defense signaling leading to both disease and defense responses to microbial pathogens of different lifestyles.

Keywords Effector protein · AvrBsT · Pathogens · Disease · Defense responses · Cell death · Transgenic · Arabidopsis

Abbreviations

DAB 3′-diaminobenzidine
DEX Dexamethasone
H_2O_2 Hydrogen peroxide
HR Hypersensitive response
OX Overexpression
PR Pathogenesis-related
Pst *Pseudomonas syringae* pv. *tomato*
ROS Reactive oxygen species
Xcv *Xanthomonas campestris* pv. *vesicatoria*

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Introduction

Plants have evolved a sensitive and multilayered innate immune system to combat a diverse range of microbial pathogens. Likewise, pathogens have evolved virulence strategies to effectively subvert plant immunity (Jones and Dangl 2006). The induction of plant immunity in response to a bacterial plant pathogen relies on the recognition of bacterial type III effector (T3SE) proteins that are directly

injected into the host cell via a type III secretion system (T3SE) (Büttner and He 2009). T3SE have diverse functions and target multiple host pathways for the induction of defense-related genes, the induction of downstream defense response pathways, the modification of specific proteins, and the generation of signaling molecules such as salicylic acid (SA), jasmonic acid (JA) and ethylene (ET) (Stulemeijer and Joosten 2008). T3S effectors, known as avirulence (Avr) proteins, are often associated with the hypersensitive response (HR) in host plants and may lead to effector-triggered immunity (ETI) responses sufficient to prevent pathogen attack.

Xanthomonas campestris pv. *vesicatoria* (*Xcv*) causes bacterial spot disease on leaves and fruits of pepper (*Capsicum annuum* L.) and tomato (*Solanum lycopersicum*) (Jones et al. 1998, 2004). Most *Xcv* strains are virulent on either pepper or tomato plants, although some are virulent on both (pepper/tomato group). Infection with *Xcv* Bv5-4a strain, a tomato group strain, produces a strong HR-like cell death response in pepper, while causing a susceptible disease response in tomato (Kim et al. 2010). The *Xcv* Bv5-4a strain contains the type III effector protein AvrBsT (Jones et al. 1998; Kim et al. 2010).

AvrBsT is a member of the YopJ/AvrRxv family identified from *Xcv* (Lewis et al. 2011). This family contains four known members in *Xanthomonas*: XopJ, AvrXvr4, AvrRxv and AvrBsT (Büttner and Bonas 2010). The YopJ family proteins share a conserved catalytic core, consisting of three key amino acid residues (His, Glu, and Cys) that are identical to the clan CE (C55 family) cysteine proteases (Roden et al. 2004; Büttner and Bonas 2010). AvrBsT is required for *Xcv* fitness and disease symptom development in infected leaves and is responsible for triggering the HR in resistant plants (Büttner and Bonas 2010). AvrBsT alters phospholipid signaling, resulting in defense activation in *Arabidopsis* (Kirik and Mudgett 2009). Some members of the YopJ/AvrRxv family exhibit weak small ubiquitin-related modifier (SUMO) protease and/or acetyltransferase activity in planta; however, SUMO protease activity of AvrBsT has not been shown (Orth et al. 2000; Szczesny et al. 2010). Genetic studies using SOBER1 mutant *Arabidopsis* plants have demonstrated that a carboxylesterase functions as a suppressor of AvrBsT-elicited resistance 1 (SOBER1). This leads to the suppression of AvrBsT-elicited HR (Cunnac et al. 2007). However, it is still unknown about the host targets of these enzymes.

Overexpression of type III effector proteins in transgenic plants causes alterations in disease and defense responses during microbial infection. Constitutive expression of the ethanol-inducible *Xanthomonas* outer protein J (XopJ), a member of the YopJ/AvrRxv family, in transgenic *Arabidopsis* leaves strongly compromises the callose deposition elicited by a non-pathogenic *Pseudomonas syringae* pv. *tomato* DC3000 *hrcC* mutant (Bartetzko et al. 2009). A stable transgenic *Arabidopsis* line carrying DEX-inducible *avrB* exhibits leaf chlorosis (Nimchuk et al. 2000; Eitas et al. 2008). In AvrRpt2-expressing transgenic *Arabidopsis* plants, there is a loss of the RIN4 protein that interacts with AvrB and AvrRpm1 (Axtell and Staskawicz 2003). This suggests that AvrRpt2 either causes the degradation of RIN4 or inhibits the translation of *RIN4* mRNA. Overexpression of *Pseudomonas syringae* pv. *tomato* DC3000 T3SE HopF2 in transgenic *Arabidopsis* plants compromises AvrRpt2-induced ETI and HR (Wilton et al. 2010).

In previous studies, we demonstrated that *X. campestris* pv. *vesicatoria* type III effector AvrBsT is differentially recognized by pepper and tomato plants (Kim et al. 2010), and it was hypothesized that pepper plants may contain certain AvrBsT recognition factors that are lacking in tomato plants. To shed further light on the possible functions of *Xcv* effector AvrBsT, we investigated the disease and defense responses of transgenic *Arabidopsis* plants expressing *avrBsT* under a dexamethasone (DEX)-inducible promoter during *Hyaloperonospora arabidopsidis* (*Hpa*) and *Pseudomonas syringae* pv. *tomato* (*Pst*) DC3000 and DC3000 (*avrRpm1*) infection. Notably, the ectopic expression of *avrBsT* triggered cell death response in transgenic *Arabidopsis* plants. Two-dimensional (2D) electrophoresis was also used to identify differentially expressed proteins in *DEX:avrBsT* overexpression (OX) *Arabidopsis* plants during *Hyaloperonospora arabidopsidis* infection. In this study, we show that *Xcv* effector AvrBsT overexpression in heterologous *Arabidopsis* plants compromises the defense response to the biotrophic oomycete pathogen *Hyaloperonospora arabidopsidis* while enhancing infection with the hemibiotrophic bacterial pathogen *Pseudomonas syringae* pv. *tomato*. Overexpression of *avrBsT* positively regulated the levels of TCP-1 chaperonin and SEC7-like guanine nucleotide exchange protein, proteins known to play important roles in cell fate determination. SEC7-like guanine nucleotide exchange protein is a putative guanyl-nucleotide exchange factor, and *Arabidopsis eds10* mutants exhibit defective embryo development (Pagnussat et al. 2005). The TCP-1 chaperonin or heat shock protein 60 has been implicated in apoptosis and the immune responses of human cells (Kirchhoff et al. 2002; Tsan and Gao 2009). In contrast, *avrBsT* overexpression negatively regulated the levels of calmodulin-like protein, which was shown previously to be involved in modulating various stress signaling pathways (Luan 2009; Cheong et al. 2010).

Materials and methods

Plant growth conditions

Seeds of wild-type *Arabidopsis* (*Arabidopsis thaliana*) ecotype Columbia 0 (Col-0) and *DEX*:*avrBsT* overexpression (OX) plants were sown on potting soil mix (peat moss:vermiculite:perlite, 3:1:1, v/v/v). The *Arabidopsis* plants were cultured in a growth chamber under a 14/10 h (light/dark) light cycle at 60 % humidity and 24 °C. Before sowing on soil, *Arabidopsis* seeds were vernalized at 4 °C under low light conditions for 2 days.

Generation of *avrBsT-OX Arabidopsis*

To generate *avrBsT-OX Arabidopsis* plants, the open reading frame (ORF) of *avrBsT* was inserted into the *Xho*I and *Spe*I sites of pTA7002, a vector containing the dexamethasone (DEX)-inducible promoter. The resulting construct, DEX:*avrBsT*, was used for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana* ecotype Col-0 using a floral dip method, as described by Clough and Bent (1998). *Arabidopsis* transformants carrying *DEX*:*avrBsT* were selected by growing on Murashige–Skoog (MS) medium containing hygromycin (50 µg mL^{-1}). To express *avrBsT*, transgenic plants were sprayed with 10–20 µM dexamethasone.

Pathogen inoculation and disease rating

Pseudomonas syringae pv. *tomato* (*Pst*) DC3000 and DC3000 (*avrRpm1*) were used to infiltrate the leaves of 5-week-old *Arabidopsis* plants (Choi and Hwang 2011). To prepare the bacterial inoculum, *Pst* was grown overnight in yeast nutrient (YN) broth containing kanamycin (50 µg mL^{-1}) and rifampicin (50 µg mL^{-1}) at 28 °C. To monitor bacterial growth [colony forming units (cfu) cm^{-1}] in leaf tissues, the infected leaves were harvested 0 and 3 days after infiltration (10^5 cfu mL^{-1}).

Hyaloperonospora arabidopsidis isolate Noco2 was maintained on *Arabidopsis* Col-0 seedling plants to provide a source of fresh inoculum (Hwang and Hwang 2010). A *Hp. arabidopsidis* suspension of asexual inoculum (5×10^4 conidiosporangia mL^{-1}) was spray-inoculated onto the cotyledons of 10-day-old seedlings. The inoculated plants were covered with a transparent dome to maintain a high relative humidity (80–100 %), followed by incubation in a growth chamber at 17 °C. To determine the asexual sporulation of *Hp. arabidopsidis*, the number of sporangiophores was counted on both sides of the cotyledons 5 days after inoculation. Visual disease ratings were categorized into four classes based on the number of sporangiophores: 0, 1 to 9, 10 to 19, and over 20 sporangiophores per cotyledon (McDowell et al. 2000).

Reverse-transcription PCR and real-time quantitative PCR

Total RNA from *Arabidopsis* leaves was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA) following the manufacturer's instructions. cDNAs were synthesized from total RNA (2 µg) using moloney murine leukemia virus reverse transcriptase (MMLV RT) (Enzynomics, Daejeon, Korea). Amplification of constitutively expressed *Actin* served as an internal control in the RT-PCR assays. Real-time quantitative PCR was conducted using the Bio-Rad iCycler System with EvaGreen qPCR master mix (ABM, Canada). Expression of defense response genes was normalized to the expression of *Actin*.

Protein extraction and immunoblotting

Total proteins were extracted by grinding *Arabidopsis* leaves with the buffer [50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), pH 7.4, 50 mM NaCl, 10 mM ethylenediamine tetraacetic acid (EDTA), 0.2 % Triton X-100, and 1× proteinase inhibitor cocktail (Roche)] (Hwang and Hwang 2010). After centrifuging the protein extracts at 12,000*g* for 15 min, the supernatants were used for immunoblotting. Protein extracts were resuspended in sodium dodecyl sulfate (SDS) sample loading buffer, subjected to SDS-PAGE, and transferred onto Hybond-P membranes (GE Healthcare) by wet electroblotting. Proteins were detected using anti-c-Myc antibody (Sigma) diluted to 1:2,000 and anti-rabbit secondary antibody diluted to 1:10,000.

Two-dimensional (2D) electrophoresis

For 2D-electrophoresis (Choi and Hwang 2011), total soluble proteins were extracted from the cotyledons of pathogen-inoculated *Arabidopsis* seedlings by grinding in extraction buffer [10 % (w/v) trichloroacetic acid and 0.07 % (w/v) dithiothreitol (DTT) in cold acetone (−20 °C)]. Protein extracts (800 µg) suspended in rehydration buffer [9 M urea, 100 mM DTT, 4 % (w/v) CHAPS, 0.5 % (v/v) Bio-lyte 3/10 carrier ampholytes, and 0.002 % bromophenol blue) were applied to an immobilized pH gradient (IPG) strip for in-gel rehydration. Strips were rehydrated at 50 V for 24 h. Isoelectric focusing (IEF) was performed using 24 cm IPG strips (Bio-Rad) with a nonlinear pH 4–7 gradient. IEF was run using a PROTEAN IEF Cell (Bio-Rad, Hercules, CA, USA) at gradient steps of 250 V for 1 h, 500 V for 1 h, 1,000 V for 2 h, and 10,000 V for 4 h, and a final step of 10,000 V

toward a total of 90 kVh. After IEF, proteins were separated according to size. IPG strips were incubated in equilibration buffer [50 mM Tris–HCl, pH 8.8, 6 M urea, 30 % (v/v) glycerol, and 2 % (w/v) SDS] containing 1 % (w/v) DTT for 15 min for the first equilibration step, followed by incubation in 4 % (w/v) iodoacetamide for the second step. For 2D-polyacrylamide gel electrophoresis (PAGE), IPG strips were sealed on the top of the 12.5 % 2-dimensional gel using 1 % low-melting agarose in SDS-electrophoresis buffer (25 mM TRIS, 0.2 M glycine, 0.1 % SDS). SDS-PAGE was performed in an Ettan DALTsix electrophoresis unit (GE Healthcare) at 5 W per gel for 1 h, followed by 15 W per gel for 6 h until the bromophenol blue front had reached the end of the gel. Gels were stained with Coomassie blue [0.1 % (w/v) Coomassie Brilliant Blue G 250, 34 % (v/v) methanol, 3 % (v/v) phosphoric acid, and 17 % (w/v) ammonium sulfate]. Coomassie-stained gels were scanned with the UMAX PowerLook 1100XL scanner, and the images were analyzed using ImageMaster 2D Platinum 6.0 (GE Healthcare). The 2D gels were compared and matched, and a quantitative determination of the spot volumes was performed.

Identification of proteins by MALDI-TOF/MS

The differentially expressed protein spots in healthy and infected plants were excised from the Coomassie-stained 2D gels, digested with trypsin, and identified using MALDI-TOF/MS (Choi and Hwang 2011). The resulting peptide mass, pI, and molecular mass analyzed by Ettan MALDI-TOF/MS (GE Healthcare) were identified via NCBI (http://www.ncbi.nlm.nih.gov/) searching using the Profound database (http://prowl.rockefeller.edu/prowl-cgi/profound.exe) for peptide mass fingerprinting.

Database search for the functional category

The identified *Arabidopsis* proteins were functionally categorized based on the Munich Information Center for Protein Sequences (MIPS) *Arabidopsis thaliana* genome database (MatDB, http://mips.gsf.de/proj/funcatDB/search_main_frame.html) (Table 1).

Purification of recombinant AvrBsT protein

The *avrBsT* coding sequence was cloned into the *BamH*I and *Xho*I sites of the pGEX-5X-1 vector (GE Healthcare) to produce AvrBsT fused with a GST tag at the N terminus. The recombinant plasmid was transformed into *E. coli* strain BL21 for protein expression. Transformed bacteria were incubated in 50 mL Luria–Bertani (LB) medium supplemented with ampicillin (50 μg mL^{-1}) at 37 °C. When the OD_{600} reached approximately 0.5, protein production was induced by adding 1 mM isopropyl β-D-thiogalactopyranoside (IPTG) to the cultures. This was followed by incubation at room temperature for 8 h to enable protein production. pGEX-5X-1:AvrBsT was purified by incubation with glutathione Sepharose beads for 2 h at room temperature and subsequent elution with 50 mM Tris (pH 8.0). Purified AvrBsT was subjected to 10 % SDS-PAGE and stained with Coomassie Brilliant Blue to confirm sample purity.

Measurement of ion conductivity

For the ion conductivity assay, 8 leaf discs (1 cm in diameter) were collected from the leaves infected with *Pst* (10^8 cfu mL^{-1}) and washed in 20 mL of double-distilled water for 30 min (Hwang et al. 2011). The washed leaf discs were transferred into 20 mL of double-distilled water. To quantify the electrolyte leakage from the leaf discs, ion conductivity was measured using a conductivity meter (Model sensION7 HACH, CO).

H_2O_2 measurement

H_2O_2 production in leaf tissues was spectrophotometrically determined using the xylenol orange assay. Xylenol orange forms a complex with the Fe^{3+} produced by the hydroperoxide-based oxidation of Fe^{2+} (Bindschedler et al. 2001). 1 ml of assay reagent [25 mM $FeSO_4$ and 25 mM $(NH_4)_2SO_4$ dissolved in 2.5 M H_2SO_4] was added to 100 mL of solution containing 125 μM xylenol orange and 100 mM sorbitol. To measure the H_2O_2 quantity, leaf discs were floated for 10 min in 1 mL of distilled water and centrifuged at 5,000*g* for 10 min. 100 μl of the supernatant was added to 1 mL of xylenol orange reagent. After 30 min of incubation, H_2O_2 production was determined by measuring the A_{560} of the Fe^{3+}-xylenol orange complex.

Staining with trypan blue, DAB and aniline blue

To visualize plant cell death and growth of *Hp. arabidopsidis* isolate Noco2, healthy and infected plant tissues were stained with lactophenol-trypan blue (10 mL lactic acid, 10 mL glycerol, 10 g phenol and 10 mg trypan blue dissolved in 10 mL distilled water) and destained with chloral hydrate (2.5 g mL^{-1}) (Hwang and Hwang 2010). H_2O_2 production was monitored by staining the leaves with 1 mg mL^{-1} 3, 3′-diaminobenzidine (DAB) (Sigma) (Hwang and Hwang 2010) and cleared with alcoholic lactophenol. The leaf tissues were mounted in 70 % glycerol for observations with a light microscope. For detection of callose deposition by *avrBsT* expression, leaves were cleared with alcoholic lactophenol and stained in 0.01 % (w/v) aniline blue in 0.15 M K_2HPO_4 (pH 9.5). The

Table 1 Proteins differentially expressed in *DEX:avrBsT*-OX *Arabidopsis* cotyledons infected by *Hyaloperonospora arabidopsidis* after dexamethasone treatment, as identified by matrix-assisted laser desoption ionization time-of-flight (MALDI-TOF) mass spectrometry (MS)

Spot no.	Protein	Functional category[a]	NCBI accession no.	Gel pI/MW	Theoretical pI/MW	Sequence coverage (%)	Spot density[b]		
							Healthy	*Hpa* infected	
								−DEX	+DEX
H1	TCP-1/cpn60 chaperonin family protein	Protein folding and stabilization	NP_198008	5.00/60	5.56/60.872	13	0[a]	0.02[b]	0.02[b]
H2	SEC7-like guanine nucleotide exchange protein	Regulation of metabolism and protein function	NP_171698	5.00/30	5.43/196.586	10	0[a]	0[a]	0.008[b]
H3	Calmodulin-like protein	Protein with binding functions or cofactor requirement	AAN15355	6.50/20	4.84/26.813	35	0[a]	0.002[b]	0[a]

Different letters indicate significant differences in the percent volume of each protein spot, as determined by least significant difference (LSD) test ($P < 0.05$)

[a] Protein categorization was done based on the MIPS (Munich information center for protein sequences) *Arabidopsis thaliana* genome database (MatBD, http://mips.gsf.de/proj/funcatDB/search_main_frame.html)

[b] Spot density is expressed as the percent volume to correct the cariability due to silver staining, which is calculated by ImageMaster™ 2D Platinum 6.0 (GE Healthcare)

samples were incubated in the dark for 30 min, mounted on slides, and observed under UV illumination.

Results

The ectopic expression of *avrBsT* triggers cell death response in transgenic *Arabidopsis* plants

We previously reported that the *Xanthomonas campestris* pv. *vesicatoria* (*Xcv*) effector AvrBsT is differentially recognized as an avirulence and virulence factor by pepper and tomato plants, respectively (Kim et al. 2010). To investigate whether the *Xcv* effector AvrBsT induces defense in plant cells, we attempted to generate transgenic *Arabidopsis* plants expressing *avrBsT* using the cauliflower mosaic virus promoter (CaMV 35S). However, the 35S:*avrBsT* transgenic lines were not generated using the CaMV vector, possibly due to the lethal effect of *avrBsT* during its transformation in plants. Thus, we used a dexamethasone (DEX)-inducible system to generate transgenic plants that overexpressed *DEX:avrBsT* in *Arabidopsis* (designated *DEX:avrBsT*-OX). The transgenic *Arabidopsis* lines #3, #4 and #5 were selected. Constitutive expression of the *DEX:avrBsT* transgene in the leaves of these *Arabidopsis* lines was confirmed by RT-PCR and immunoblot analyses (Fig. 1a, b). The transcriptional and translational ectopic expression of *avrBsT* was detected in the transgenic lines 24 and 48 h after DEX treatment.

We next investigated whether *DEX:avrBsT* overexpression triggers cell death phenotype in healthy transgenic *Arabidopsis* plants. In *DEX:avrBsT*-OX transgenic plants, *avrBsT* induction by DEX (10 μM) treatment caused brownish necrotic lesions on the cotyledons; however, wild-type cotyledons were not affected by DEX (Fig. 2a). When stained with trypan blue, weak cell death response was microscopically observed in cotyledon tissues of the transgenic plants 12 h after DEX treatment. However, all the plants non-treated with DEX did not show any cell death phenotype at different plant developmental stages (Supplementary Fig. 1). Reactive oxygen species (ROS) accumulation is often involved in cell death response (Choi et al. 2012). The 3, 3′-diaminobenzidine (DAB) staining was used to detect H_2O_2 accumulation at the early stage of cell death induction by *avrBsT* expression. Expectedly, the cotyledons of *DEX:avrBsT*-OX transgenic lines significantly showed reddish brown regions 12 h after DEX treatment, compared to wild-type plants (Fig. 2a, bottom panel). This indicates that *avrBsT* overexpression induces H_2O_2 accumulation in the transgenic plants. We further tested whether *avrBsT* overexpression also induces cell death response in leaves of different ages (Fig. 2b, c). Significantly chlorotic and necrotic cell death phenotypes were observed in 2- or 5-week-old leaves of *DEX:avrBsT*-OX transgenic plants 24 h after DEX treatment. However, all the plants non-treated with DEX did not show any cell death phenotype on the leaves (Supplementary Fig. 1). The assessment of cell death levels (scale 0∼3) (Fig. 2c) supports *avrBsT*-triggered cell death response in the transgenic leaves. To quantify the cell death response in DEX-treated transgenic leaves, we examined the time-courses of electrolyte leakages via the measurement of ion conductivity. Electrolyte leakage increased dramatically from the leaves of *DEX:avrBsT*-OX transgenic plants, compared to the wild-type leaves (Fig. 2c).

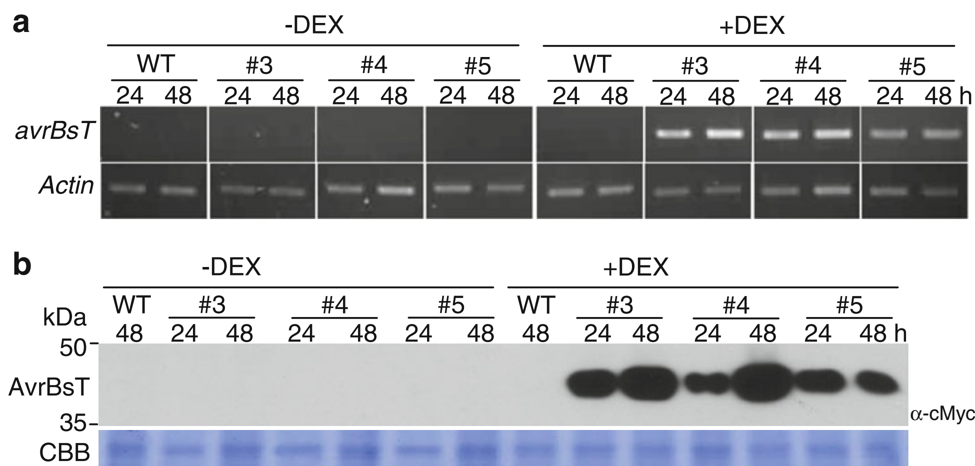

Fig. 1 Expression of *avrBsT* in wild-type (WT) and *DEX:avrBsT*-OX transgenic *Arabidopsis* plants treated with dexamethasone (DEX). **a** RT-PCR analysis of the expression of *avrBsT* in leaves of *WT* and *DEX:avrBsT*-OX plants (*lines #3, #4 and #5*). Three independent experiments were performed with similar results. *h* hours after DEX treatment. **b** Immunological detection of AvrBsT proteins under the control of a DEX-inducible system. Total proteins were extracted 24 and 48 h after treatment with DEX, and cMyc-tagged DEX:AvrBsT was detected by immunoblotting with an anti-cMyc antibody. Three independent experiments were performed with similar results. *h* hours after DEX treatment

Quantitative real-time PCR analysis was used to investigate whether *avrBsT* overexpression triggers some defense and cell death response genes in the leaves of *DEX:avrBsT*-OX *Arabidopsis* plants (Fig. 2d). DEX treatment strongly induced *avrBsT* expression in the leaves of the transgenic *DEX:avrBsT*-OX plants, confirming that *DEX:avrBsT* was expressed in the transgenic *Arabidopsis* plants. Induction of *avrBsT* in *Arabidopsis* by DEX treatment significantly triggered programed cell death (PCD) marker genes *PR1* (Reymond and Farmer 1998) and *SAG13* (Brodersen et al. 2002). Interestingly, cell death severity and *PR1* expression were correlated with the levels of *avrBsT* expression in the transgenic leaves. Collectively, overexpression of *Xcv* effector *avrBsT* could mediate upstream signaling of PCD response in *Arabidopsis*.

Aniline blue staining assay was used to investigate whether *avrBsT* overexpression induces callose accumulation in leaves of *DEX:avrBsT*-OX transgenic *Arabidopsis* plants after DEX treatment (Fig. 3). Callose, a β-1,3 glucan, is synthesized to form papillae during defense response against pathogen infection (Flors et al. 2005; Choi and Hwang 2011). The ectopic expression of *avrBsT* stimulated callose deposition in healthy *Arabidopsis* cells. Callose deposition is generally involved in pathogen-associated molecular pattern (PAMP)-triggered immunity, which is inhibited by bacterial effectors (Debroy et al. 2004; Kim et al. 2009). Therefore, the *avrBsT*-triggered callose deposition suggests that AvrBsT may positively regulate basal defense in plants.

Enhanced resistance of *DEX:avrBsT*-OX *Arabidopsis* plants to *Hyaloperonospora arabidopsidis* infection

To investigate the role of the *Xcv* effector AvrBsT in the plant defense response, wild-type and *DEX:avrBsT*-OX plants were spray-inoculated with *Hyaloperonospora arabidopsidis* isolate Noco2, known to be virulent to *Arabidopsis* Col-0. The sporangiophores began to form on the cotyledons 4 to 5 days after inoculation with *Hp. arabidopsidis* (5×10^4 conidiosporangia mL^{-1}). The *avrBsT* overexpression induced by DEX treatment significantly compromised mycelial growth and sporulation on the cotyledons of the *DEX:avrBsT*-OX plants (Fig. 4a, b). Notably, the *DEX:avrBsT*-OX plants treated with DEX exhibited a slight cell death phenotype at the site of oomycete infection, as evidenced by trypan blue staining (Fig. 4a). As shown in Fig. 4b, fewer sporangiophores formed on the cotyledons of DEX-treated *DEX:avrBsT*-OX plants compared to mock-treated *DEX:avrBsT*-OX plants. The *Hp. arabidopsidis* infection also promoted H_2O_2 production in DEX-treated *DEX:avrBsT-OX* plants compared to mock-treated *DEX:avrBsT*-OX plants (Fig. 4c). DAB polymerizes instantly and locally upon contact with H_2O_2 to form reddish-brown polymers. DEX-treated *DEX:avrBsT*-OX plants inoculated with *Hp. arabidopsidis* exhibited DAB-stained spots, indicating H_2O_2 accumulation to high levels. Together, these data indicate that *avrBsT* overexpression enhances basal defense to infection by the obligate biotrophic oomycete *Hp. arabidopsidis*.

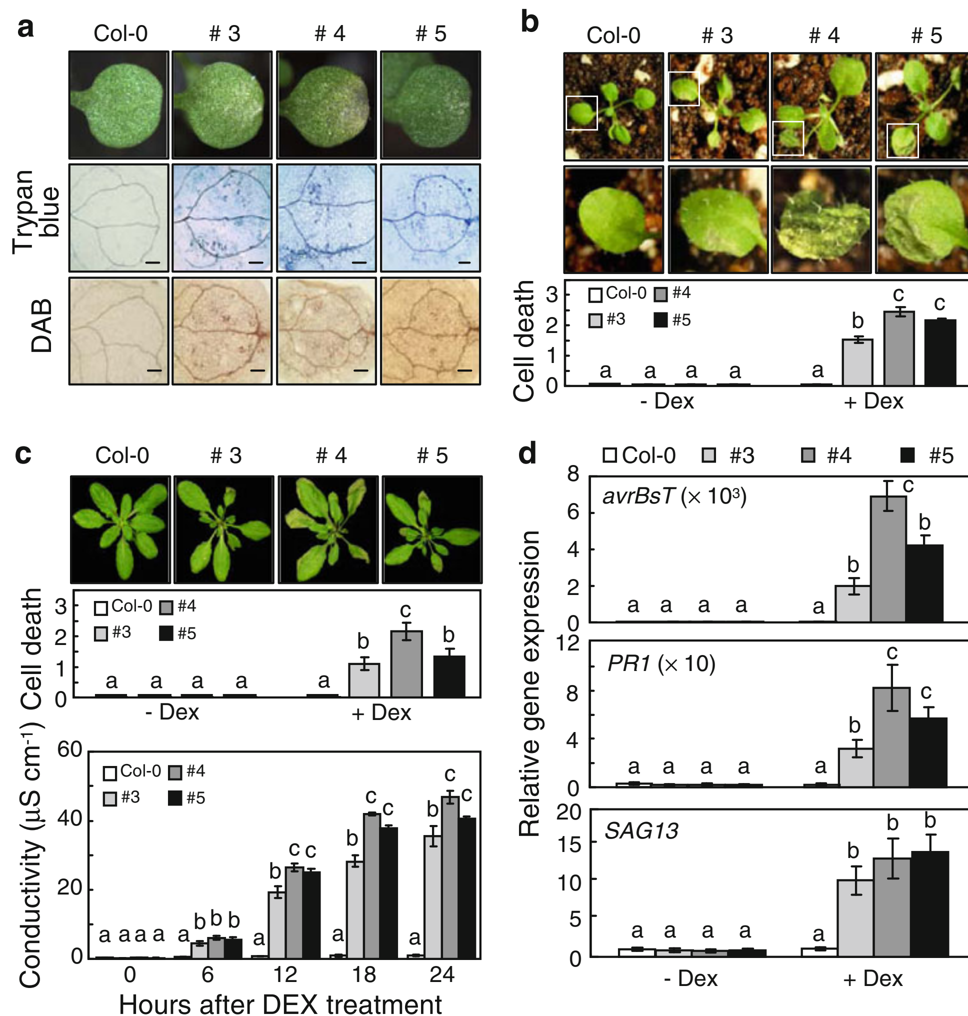

Fig. 2 AvrBsT-triggered cell death phenotypes in *DEX:avrBsT*-OX transgenic *Arabidopsis* plants. **a** Visible and microscopic images of cotyledons of 7-day-old plants treated with dexamethasone (DEX, 10 µM). Cotyledons were stained with lactophenol-trypan blue (*middle panel*) and 3, 3′-diaminobenzidine (DAB) (*bottom panel*) 12 h after DEX treatment. *Bars* 200 µm. **b** Visible images and cell death levels of 2-week-old leaves 24 h after treatment with DEX (10 µM). Images in the boxes were enlarged in the *middle panel*. The extent of *avrBsT*-induced cell death was assessed based on four cell death scales (0, <10 %; 1, 10∼30 %; 2, 30∼80 %; 3, 80∼100 %). **c** Visible images, cell death levels and electrolyte leakage assay of 5-week-old leaves treated with DEX (10 µM). **d** Quantitative real-time PCR analysis of the expression of *avrBsT* and defense-related genes in the leaves of wild-type Col-0 and *DEX:avrBsT*-OX *Arabidopsis* plants 24 h after DEX treatment. *Actin* was used as an internal control for normalization. The data (**b–d**) represent the mean ± standard deviations from three independent experiments. *Different letters* indicate significant differences, as analyzed by the LSD test ($P < 0.05$)

Proteomics analysis of *DEX:avrBsT-OX* plants infected by *Hyaloperonospora arabidopsidis* isolate Noco2

To investigate whether *avrBsT* overexpression regulates the plant proteome during oomycete pathogen infection, 2D-electrophoresis analyses were performed using soluble protein extracts from healthy and *Hp. arabidopsidis*-infected cotyledons of *DEX:avrBsT*-OX plants. Coomassie blue staining of the 2D gels revealed some differentially expressed proteins in response to *Hp. arabidopsidis* infection in the *DEX:avrBsT*-OX plants (Fig. 5). The differentially expressed protein spots were visualized in 2D gel images, excised and analyzed using MALDI-TOF. *Arabidopsis* genome sequence analysis identified three proteins (H1, H2 and H3) that were up- or down-regulated (Fig. 6a; Table 1). Protein spots H1 and H2, distinctly upregulated during *Hp. arabidopsidis* infection, were TCP-1/cpn60 chaperonin family protein (AT5G26360) and SEC7-like guanine nucleotide exchange protein (AT1G01960), respectively. The TCP-1/cpn60 chaperonin family protein also known as heat shock protein 60 has not been extensively studied in *Arabidopsis*. However, it has

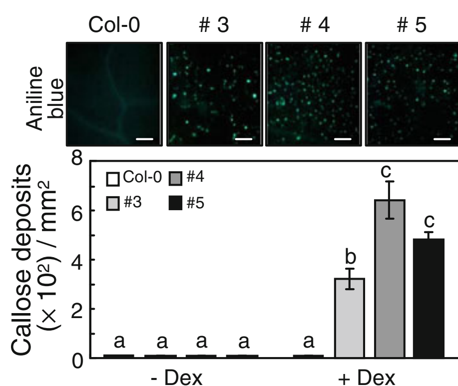

Fig. 3 Induction of callose deposition by the expression of *avrBsT* in leaves of *DEX:avrBsT*-OX *Arabidopsis* plants. Leaf samples were stained with aniline blue 24 h after DEX treatment (10 μM) (*upper panel*). *Bars* 100 μm. Callose deposits in the DEX-treated leaves were counted with the ImageJ software (*lower panel*). The data represent the mean ± standard deviations from three independent experiments. *Different letters* indicate significant differences, as analyzed by the LSD test ($P < 0.05$)

been implicated in apoptosis and immune responses in human (Kirchhoff et al. 2002; Tsan and Gao 2009). SEC7-like guanine nucleotide exchange protein was found to be essential for nominal embryo development in *Arabidopsis* (Pagnussat et al. 2005). Protein spot H3 represented calmodulin-like protein (At2G41100) and was downregulated in *DEX:avrBsT*-OX plants during infection. Calmodulins are well known to act as signal modulators of stress responses (Luan 2009; Cheong et al. 2010). Real-time quantitative PCR analysis confirmed *avrBsT* overexpression in leaves of *DEX:avrBsT*-OX plants by DEX treatment (Fig. 5b). However, *Hp. arabidopsidis* infection was not effective in enhancing *avrBsT* overexpression in DEX-treated transgenic plants. H1 (AT5G26360) expression was strongly elevated in both wild-type and *DEX:avrBsT*-OX plants 3 days after inoculation with *Hp. arabidopsidis*, but this expression was significantly reduced 5 days after inoculation. H2 (AT1G01960) was significantly induced by *avrBsT* overexpression and the induction was threefold more in transgenic leaves 3–5 days after *Hp. arabidopsidis* infection. However, no significant differences in the expression levels of H3 (At2G41100) were detected among all these plants 3 days after inoculation with *Hp. arabidopsidis*. Interestingly, 5 days after *Hp. arabidopsidis* infection, H3 (At2G41100) was significantly induced in both wild-type and mock-treated *DEX:avrBsT*-OX plants, but not in DEX-treated *DEX:avrBsT*-OX plants. This indicates that the overexpression of *avrBsT* by DEX treatment suppresses H3 (At2G41100) induction by *Hp. arabidopsidis* infection. Based on the MIPS *Arabidopsis* genome database, putative functions of H1, H2, and H3 were identified to be associated with protein folding, metabolism regulation and protein binding, respectively (Table 1). Collectively, these results suggest that *avrBsT* overexpression in *Arabidopsis* may differentially regulate diverse proteins of different functions during *Hp. arabidopsidis* infection.

Antimicrobial activity of purified AvrBsT against *Hyaloperonospora arabidopsidis* isolate Noco2

To determine whether AvrBsT has antimicrobial activity against *Hp. arabidopsidis,* GST-tagged recombinant AvrBsT protein (10 μg mL^{-1}) was applied to *Arabidopsis* seedlings. These seedlings were subsequently inoculated with *Hp. arabidopsidis* isolate Noco2. Treatment with 10 μg mL^{-1} of the purified recombinant AvrBsT protein induced the cell death phenotype in *Arabidopsis* seedlings (Fig. 7a). However, treatments with lower doses (1–5 μg mL^{-1}) did not cause damage to plant growth (data not shown), but significantly suppressed *Hp. arabidopsidis* infection and sporangiophore formation on *Arabidopsis* cotyledons (Fig. 7b, c). Trypan blue staining indicated that treatment with 5 μg mL^{-1} of the recombinant AvrBsT protein distinctly suppressed sporulation of *Hp. arabidopsidis* on the cotyledon (Fig. 6b). *Hp. arabidopsidis* infection on the *Arabidopsis* seedlings treated with the recombinant AvrBsT protein resulted in lower levels of sporangiophore and spore formation on the cotyledons compared to infection on wild-type plants (Fig. 7c, d). However, treatment with GST (1–5 μg mL^{-1}) alone did not inhibit sporangiophore and spore formation on the cotyledons infected with *Hp. arabidopsidis.* Collectively, these results indicate that AvrBsT protein possess inhibitory activity against the growth and sporulation of *Hp. arabidopsidis* and also that the doses of over 10 μg mL^{-1} AvrBsT exert a toxic effect on Arabidopsis seedlings.

Enhanced susceptibility of *DEX:avrBsT-OX Arabidopsis* plants to *Pseudomonas syringae* pv. *tomato* infection

To determine whether *avrBsT* mediates the basal response to bacterial infection, leaves of *DEX:avrBsT*-OX *Arabidopsis* plants were inoculated with *Pst* DC3000 and *Pst* DC3000 (*avrRpm1*) by syringe infiltration. A lower titer of *Pst* (10^5 cfu mL^{-1}) was inoculated on the leaves, because *avrBsT* overexpression is not so effective to inhibit a rapid *Pst* multiplication in leaves infected with a higher lower titer of *Pst* (10^7 cfu mL^{-1}). Unexpectedly, *Pst* DC3000 and *Pst* DC3000 (*avrRpm1*) infection resulted in higher bacterial growth in the leaf tissues of the DEX-treated *DEX:*avr*BsT*-OX plants (lines #3, #4 and #5) compared to DEX-treated wild-type plants (Fig. 8a). Next, we investigated whether the overexpression of a*vrBsT* induces

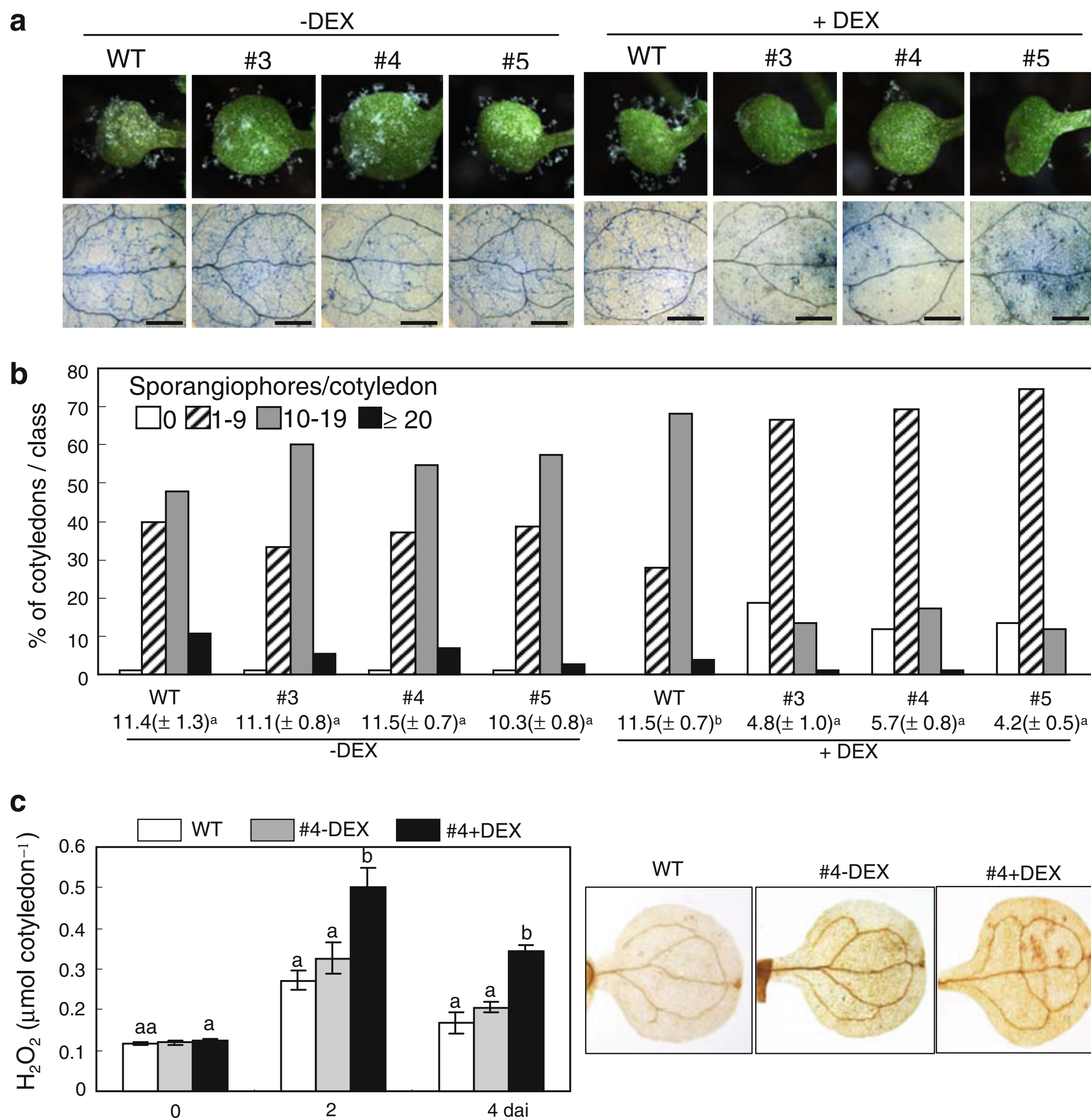

Fig. 4 Quantification of *Hyaloperonospora arabidopsidis* infection phenotypes on the cotyledons of wild-type (WT) and *DEX:avrBsT*-OX *Arabidopsis* plants (*lines #3, #4 and #5*). 10-day-old seedlings were spray-inoculated with *Hp. arabidopsidis* isolate Noco2 (5×10^4 conidiosporangia mL^{-1}) and pathogen development was recorded. **a** Disease symptoms and cellular responses in the infected cotyledons. Photographs were taken 6 days after inoculation (*upper panel*). Infected cotyledons were stained with lactophenol-trypan blue 7 days after inoculation to visualize the pathogen mycelium and necrotic plant cells (*lower panel*). **b** Sporulation levels of *Hp. arabidopsidis* on the infected cotyledons. Production of sporangiophores on 50 cotyledons was observed using a stereo-microscope 6 days after inoculation. **c** Quantification of H_2O_2 in the leaves 0, 2 and 4 days after inoculation (dpi) with *Hp. arabidopsidis.* The data (**b** and **c**) represent the mean ± standard deviations from three independent experiments. *Different letters* indicate significant differences from three independent experiments, as analyzed by the LSD test ($P < 0.05$). *dai*, days after inoculation

electrolyte leakage from the *Pst*-infected leaves of DEX-treated *DEX:*a*vrBsT*-OX plants. Both *Pst* DC3000 and *Pst* DC3000 (*avrRpm1*) infection significantly induced electrolyte leakage from the leaf tissues of the *DEX:* a*vrBsT*-OX plants compared to wild-type leaves (Fig. 8b). However, no significant differences in H_2O_2 production were observed among wild-type and *DEX:*a*vrBsT*-OX *Arabidopsis* plants, regardless of treatment with dexamethasone (DEX), during *Pst* infection (Fig. 8c). Taken together, the enhanced electrolyte leakages from *DEX:* a*vrBsT*-OX *Arabidopsis* leaves infected with avirulent *Pst* DC3000 (*avrRpm1*) support the disease-associated cell death response leading to bacterial growth in the transgenic leaves.

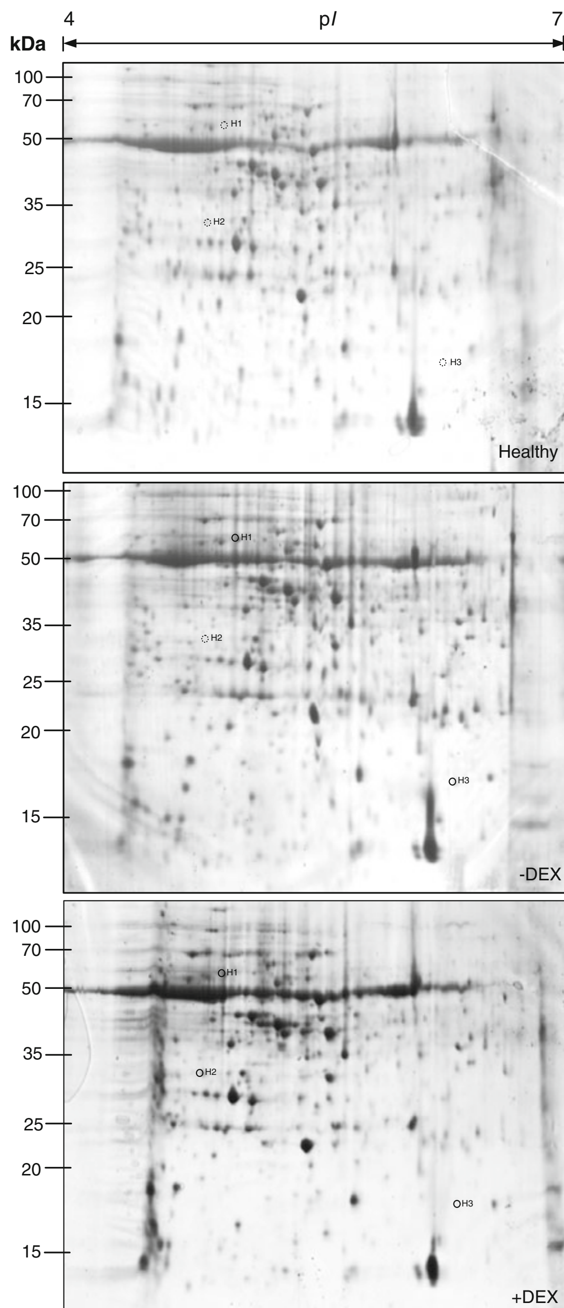

Fig. 5 Two-dimensional electrophoresis of total soluble proteins from the cotyledons of wild-type (healthy) and *DEX:avrBsT*-OX *Arabidopsis* plants (*line #4*) infected with *Hyaloperonospora arabidopsidis* isolate Noco2. SDS-PAGE gels were stained with Coomassie blue. The *circled spots* indicate proteins differentially expressed in DEX-treated or mock-treated *DEX:avrBsT*-OX plants during infection. The *spot numbers* in the circles correspond to the identifications listed in Table 1

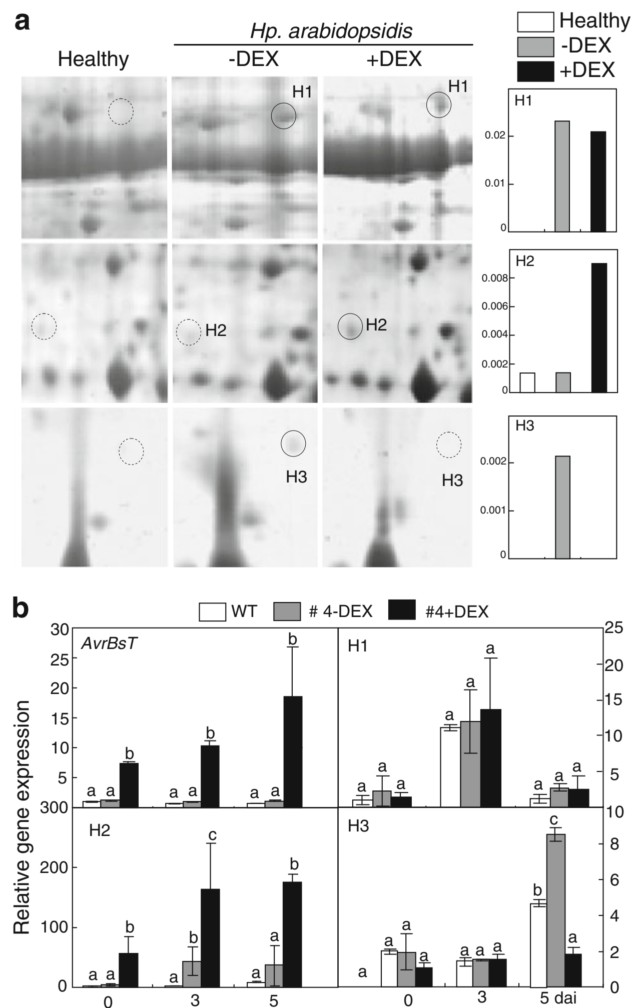

Fig. 6 Enlargement of the 2-D gel regions that show the proteins differentially expressed in the cotyledons of mock-treated or DEX-treated *DEX:avrBsT*-OX plants during *Hyaloperonospora arabidopsidis* infection. **a** The histograms show quantitative changes in spot density, as calculated with ImageMaster 2D Platinum 6.0 (GE healthcare), for individual proteins. *H1* TCP-1/cpn60 chaperonin family protein (accession no. NP_198008), *H2* SEC7-like guanine nucleotide exchange protein (accession no. NP_171698), *H3* calmodulin-like protein (accession no. AAN15355). Three independent experiments were performed with similar results. **b** Real-time quantitative PCR analysis of the expression of *avrBsT*, H1(AT5G26360), H2 (AT1G01960) and H3 (At2G41100) in wild-type (WT), mock-treated or DEX-treated *DEX:avrBsT*-OX leaves 0, 3 and 5 days after inoculation with *Hp. arabidopsidis. Actin* was used as an internal control for normalization of the data. *Different letters* (*a*, *b*, and *c*) indicate significant differences from three independent experiments, as analyzed by the LSD test ($P < 0.05$). *dai*, days after inoculation

Discussion

Xanthomonas is a large genus of Gram-negative, yellow-pigmented bacteria in plants (Jones et al. 2004). To date, the complete genome sequences of 11 *Xanthomonas* strains have been determined (Ryan et al. 2011). Four YopJ-like proteins (AvrRxv, AvrBsT, AvrXv4, and XopJ) were identified in *X. campestris* pv. *vesicatoria* strains. The *Xcv* type III effector AvrBsT is the first member of the YopJ family known to suppress effector-triggered plant

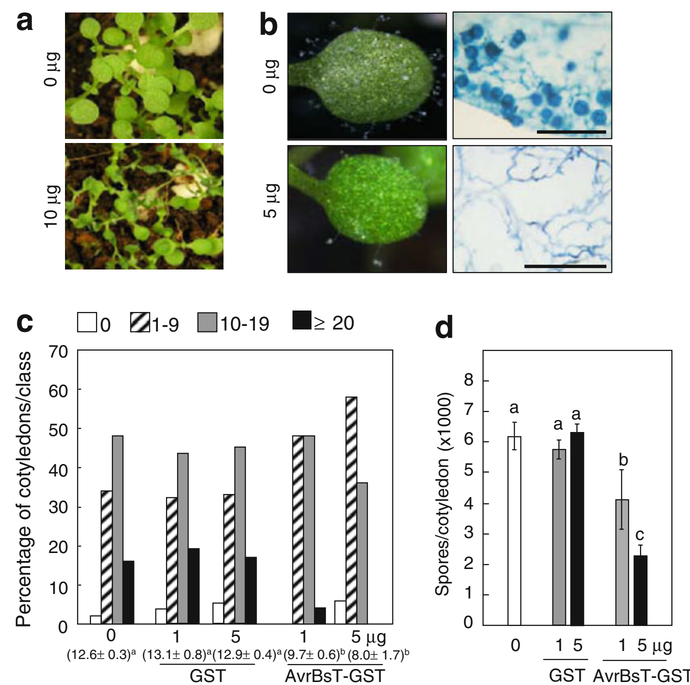

Fig. 7 Inhibitory effect of GST-tagged AvrBsT protein on the growth of *Hyaloperonospora arabidopsidis* isolate Noco2 on *Arabidopsis* seedlings. **a** Cell death phenotypes induced by purified GST-tagged AvrBsT protein. Photographs were taken 1 day after treatment with purified GST-tagged AvrBsT protein. **b** Disease symptoms and micrographs of infected cotyledons treated with purified GST-tagged AvrBsT protein. Photographs were taken 5 days after treatment. The empty vector (GST only) was used as a negative control. **c** Quantification of sporangiophores produced on 50 cotyledons 6 days after inoculation (5×10^4 conidiosporangia mL^{-1}). *Bars* 0.2 mm. The empty vector (GST only) was used as a negative control. The data represent the mean ± standard deviations. *Different letters* (*a*, *b*, and *c*) indicate significant differences from three independent experiments, as analyzed by the LSD test ($P < 0.05$)

immunity (Büttner and Bonas 2010), and AvrBsT is necessary for the hypersensitive response (HR) induction in resistant plants (Büttner and Bonas 2010). When *Xcv* interacts with plants, AvrBsT is translocated into plant cells during infection, triggering defense responses in many plant species (Kirik and Mudgett 2009). In previous studies, we showed that AvrBsT induces cell death in pepper but suppresses defense responses in tomato (Kim et al. 2010).

In this study, AvrBsT was ectopically expressed in the heterologous *Arabidopsis* system and the dexamethasone (*DEX*)::*avrBsT* overexpression (OX) plants were used to investigate the molecular function of AvrBsT in disease and defense responses against microbial pathogens. Induction of *avrBsT* expression by DEX treatment led to the cell death response, as well as altered defense responses against infection by the obligate biotrophic oomycete *Hyaloperonospora arabidopsidis* (*Hpa*) and the hemibiotrophic bacterial pathogen *Pseudomonas syringae* pv. *tomato* (*Pst*). The *avrBsT*-overexpressing (OX) *Arabidopsis* plants were less susceptible to *Hpa* Noco2, but not to *Pst* DC3000 and DC3000 (*avrRpm1*). The resistance response of DEX-treated *DEX:avrBsT*-OX *Arabidopsis* plants to *Hp. arabidopsidis* infection was accompanied by a hypersensitive response (HR) and H_2O_2 accumulation, as determined by trypan blue and DAB staining, respectively. The cell death phenotype in *DEX:avrBsT*-OX leaves triggered by exogenous dexamethasone application may be a form of basal defense response sufficient to suppress *Hp. arabidopsidis* infection. These results also suggest that *avrBsT* overexpression during *Hp. arabidopsidis* infection enhances the threshold for the activation of defense responses in *DEX:avrBsT*-OX *Arabidopsis* plants. The hypersensitive cell death response appears to be an active process to prevent the pathogen from colonizing surrounding tissue. Interestingly, *avrBsT* overexpression distinctly induced the disease-associated cell death response as well as higher *Pst* growth in DEX-treated *DEX:avrBsT*-OX leaves. This finding suggests that the *Xcv* effector AvrBsT may induce the proliferation of other bacterial pathogens such as *Pst,* leading to susceptible cell death in plant tissues. In support of these experimental results, overexpression of other bacterial effector proteins such as HopF2$_{pto}$ and AvrB in *Arabidopsis* plants has been demonstrated to cause perturbations in host defense responses (Shang et al. 2006; Cui et al. 2010; Wilton et al. 2010). *Arabidopsis* plants overexpressing HopF2$_{pto}$ exhibited compromised AvrRpt2-mediated HR (Wilton et al. 2010). Overexpression of AvrB

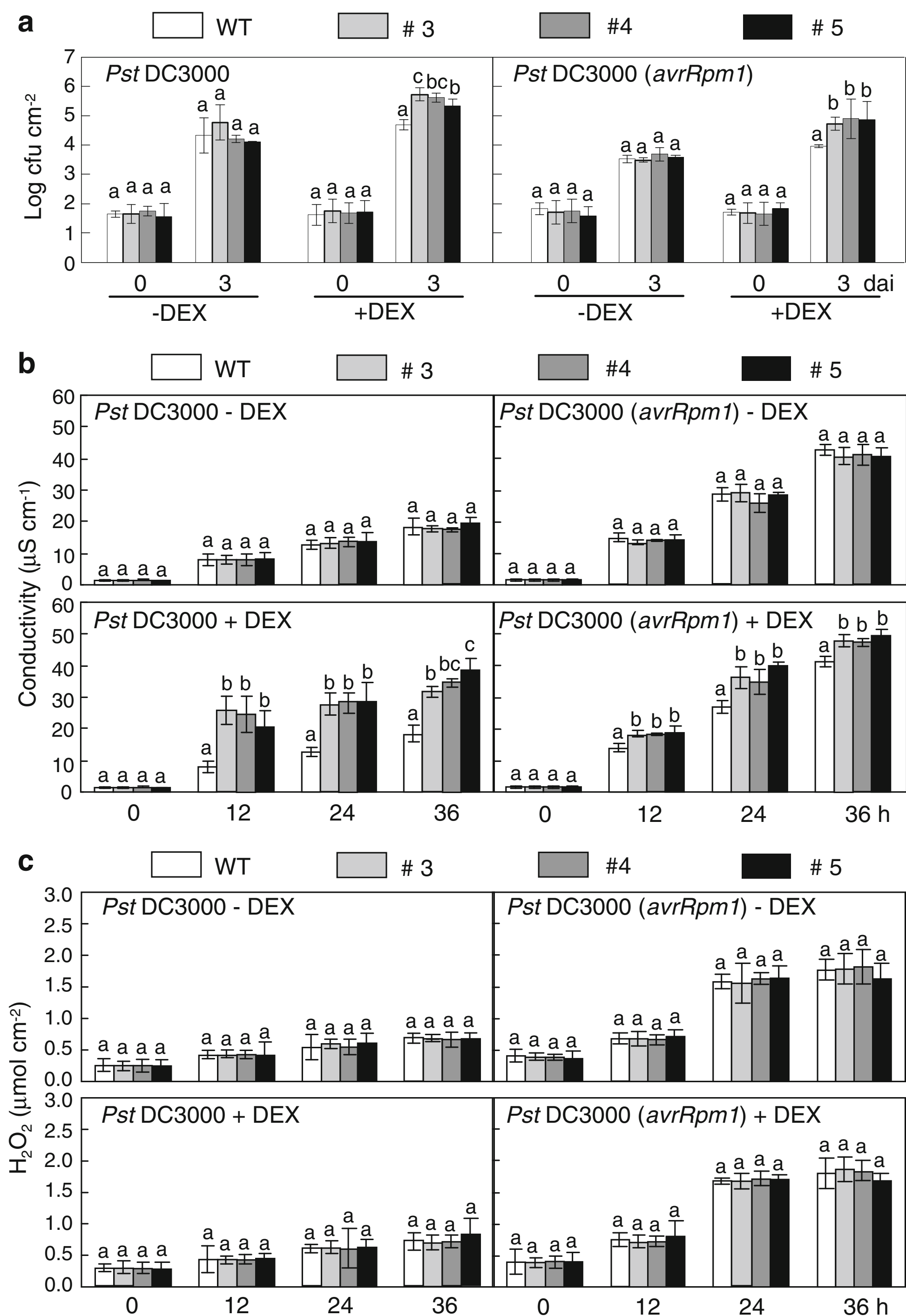

Fig. 8 Enhanced susceptibility of *DEX:avrBsT*-OX *Arabidopsis* plants to *Pseudomonas syringae* pv. *tomato* (*Pst*) infection. Leaves of 5-week-old plants mock-treated or DEX-treated with dexamethasone (DEX) were infiltrated with bacterial suspensions of *Pst* DC3000 or DC3000 (*avrRpm1*). **a** Bacterial growth in leaves inoculated with *Pst* (10^5 cfu mL^{-1}). The data represent the mean ± standard deviations. *dai* days after inoculation. **b** Electrolyte leakage assay of leaves inoculated with *Pst* (10^8 cfu mL^{-1}). The data represent the mean ± standard deviations from three independent experiments. *h* hours after infiltration. **c** Quantification of H_2O_2 produced following *Pst* inoculation (10^8 cfu mL^{-1}). The data represent the mean ± standard deviations. *Different letters* (*a*, *b*, and *c*) indicate significant differences from three independent experiments, as analyzed by the LSD test ($P < 0.05$). *h* hours after infiltration

in *Arabidopsis* plants promoted non-pathogenic *P. syringae* growth by disrupting the nominal functions of key defense components such as RAR1, HSP90, MPK4 and RIN4 (Shang et al. 2006; Cui et al. 2010). Using 2D-electrophoresis, we were able to identify TCP-1/cpn60 chaperonin family protein and SEC7-like guanine nucleotide exchange protein as proteins upregulated by *avrBsT* overexpression in *Arabidopsis*. Both TCP-1/cpn60 chaperonin family protein and SEC7-like guanine nucleotide exchange protein have been implicated in cell fate determination (Kirchhoff et al. 2002; Pagnussat et al. 2005). In contrast, calmodulin-like protein was downregulated by *avrBsT* overexpression. Calmodulins are generally known to positively modulate stress signaling (Luan 2009; Cheong et al. 2010). The results of this study suggest that these metabolism and protein folding-associated proteins negatively and positively regulate the defense responses against *Pst* and *Hp. arabidopsidis*, respectively.

Purified GST-tagged AvrBsT protein was used to determine whether the AvrBsT protein directly inhibits *Hp. arabidopsidis* infection in *Arabidopsis* plants. Treatment with 10 μg mL^{-1} GST-tagged AvrBsT protein caused the induction of a typical cell death phenotype on *Arabidopsis* seedlings. This indicates that the AvrBsT protein itself causes cell death in *Arabidopsis* seedlings. Similarly, transient expression of AvrBsT induces hypersensitive cell death in the leaves of *Nicotiana benthamiana* and pepper (Kim et al. 2010). Interestingly, cotyledons treated with 1 and 5 μg mL^{-1} of GST-tagged AvrBsT protein showed significantly reduced *Hp. arabidopsidis* infection. These results suggest that AvrBsT protein is effective in suppressing the growth and sporulation of *Hp. arabidopsidis.* Although many bacterial effector proteins have been extensively studied, the research has focused on determining the effector function during plant disease and defense responses (Block et al. 2008). To our knowledge, this study provides the first evidence showing that AvrBsT possess anti-oomycete activity against *Hp. arabidopsidis*.

Microbial effectors have been proposed to subvert plant immunity in host–pathogen interactions (Göhre and Robatzek 2008; Büttner and Bonas 2010). There is convincing evidence that AvrBsT is a versatile protein involved in the manipulation of plant cell processes such as basal defense for the benefit of the *Xanthomonas* pathogen (Büttner and Bonas 2010). In tomato plants, AvrBsT suppresses basal defense by functioning as a virulence factor that disrupts early defense signaling (Orth et al. 2000). Our experiments using *DEX:avrBsT*-OX *Arabidopsis* plants revealed that *avrBsT* overexpression in *Arabidopsis* enhances susceptibility to *Pseudomonas syringae* pv. *tomato* (*Pst*) DC3000 and DC3000 (*avrRpm1*). DEX-treated *DEX:avrBsT*-OX *Arabidopsis* plants exhibited increased *Pst* growth, as well as a susceptible cell death phenotype. Based on these results, heterologous expression of AvrBsT in *Arabidopsis* plants enables AvrBsT to function as a virulence factor to promote *Pst* infection. AvrBsT-mediated disease symptoms on transgenic *Arabidopsis* leaves may result from the susceptible cell death that occurs commonly in compatible interactions. In compatible plant–microbe interactions, susceptible cell death occurs relatively late in the course of infection (Greenberg 1997; Greenberg and Yao 2004). In pepper plants, the signals triggered by CaHIR1 (*Capsicum annuum* hypersensitive induced reaction 1) are proposed to lead to susceptible cell death during compatible interactions with *Xcv* (Choi et al. 2011). Recently, AvrBsT was demonstrated to suppress the HR that is triggered by the effector protein AvrBs1 in resistant pepper plants (Szczesny et al. 2010). However, how AvrBsT suppresses plant immunity to enable the successful colonization of bacterial pathogens in plants is largely unknown.

Th *Xcv* effector AvrBsT is likely required for cell death, disease and defense responses in *Arabidopsis* plants. However, the precise molecular mechanisms underlying AvrBsT function remain to be investigated (Stall et al. 2009; Kim et al. 2010). In this study, experimental analyses of AvrBsT function have provided a unique view of *Arabidopsis*–pathogen interactions. Here, we demonstrate that ectopic expression of *avrBsT* in *Arabidopsis* plants not only enhances resistance to the obligate biotrophic oomycete *Hp. arabidopsidis,* but also significantly suppresses plant immunity to the hemibiotrophic bacterial pathogens *Pst* DC3000 and DC3000 (*avrRpm1*). When ectopically expressed in plant cells, the *Xcv* effector AvrBsT may trigger plant cell death, disease and defense signaling, ultimately leading to disease and defense responses against microbial pathogens of various lifestyles.

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