Rapid report

SGT1 contributes to coronatine signaling and *Pseudomonas syringae* pv. *tomato* disease symptom development in tomato and Arabidopsis

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Summary

• *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst* DC3000) causes an economically important bacterial speck disease on tomato and produces symptoms with necrotic lesions surrounded by chlorosis. The chlorosis is mainly attributed to a jasmonic acid (JA)-isoleucine analogue, coronatine (COR), produced by *Pst* DC3000. However, the molecular processes underlying lesion development and COR-induced chlorosis are poorly understood.

• In this study, we took advantage of a chlorotic phenotype elicited by COR on *Nicotiana benthamiana* leaves and virus-induced gene silencing (VIGS) as a rapid reverse genetic screening tool and identified a role for *SGT1* (suppressor of G2 allele of *skp1*) in COR-induced chlorosis.

• Silencing of *SGT1* in tomato resulted in reduction of disease-associated symptoms (cell death and chlorosis), suggesting a molecular connection between COR-induced chlorosis and cell death. In Arabidopsis, *AtSGT1b* but not *AtSGT1a* was required for COR responses, including root growth inhibition and *Pst* DC3000 symptom (water soaked lesion) development. Notably, overexpression of *AtSGT1b* did not alter *Pst* DC3000 symptoms or sensitivity to COR.

• Taken together, our results demonstrate that *SGT1/SGT1b* is required for COR-induced chlorosis and subsequent necrotic disease development in tomato and Arabidopsis. *SGT1* is therefore a component of the COR/JA-mediated signal transduction pathway.

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Introduction

Localized cell death and tissue chlorosis are often associated with disease symptom development during compatible plant–microbe interactions. One of the hallmarks of bacterial speck disease caused by *Pseudomonas syringae* pv. *tomato* strain DC3000 (*Pst* DC3000) on tomato is the formation of necrotic lesions (cell death) surrounded by diffuse chlorotic halos (Bender *et al.*, 1999; Preston, 2001). Several effectors secreted via the bacterial type III secretion system (TTSS) were shown to be required for cell death associated with a hypersensitive reaction (HR) during incompatible *Pst* DC3000–plant interactions, and several effectors that elicit disease-associated cell death have also been reported (Chang *et al.*, 2000; Katagiri *et al.*, 2002; Abramovitch & Martin, 2004; Chen *et al.*, 2004; DebRoy *et al.*, 2004; del Pozo *et al.*, 2004; Cohn & Martin, 2005; Cunnac *et al.*, 2009). Chlorosis associated with disease has been attributed mainly to the phytotoxin coronatine (COR) produced by several pathovars of *P. syringae* (Bender *et al.*, 1987, 1999; Zhao *et al.*, 2003; Uppalapati *et al.*, 2007, 2008) and a less characterized chlorosis-inducing factor, PSPTO4723 (Munkvold *et al.*, 2009). However, there is limited knowledge of the host signaling components exploited by pathogen effectors to cause chlorosis and disease-associated cell death (Greenberg & Yao, 2004; del Pozo *et al.*, 2004; Ishiga *et al.*, 2009a; Wangdi *et al.*, 2010). Also, a molecular link between chlorosis and disease-associated cell death during the necrogenic phase of bacterial speck disease development has not been determined.

Coronatine contributes to the virulence of *Pst* DC3000 in Arabidopsis, tomato, collards (*Brassica oleracea*) and turnip (Zhao *et al.*, 2003; Brooks *et al.*, 2004; Elizabeth & Bender, 2007; Uppalapati *et al.*, 2007). COR has structural and functional similarity to jasmonates including 12-oxo-phytodienoic acid (12-OPDA) and jasmonic acid-isoleucine (JA-Ile; Weiler *et al.*, 1994; Staswick & Tiryaki, 2004; Uppalapati *et al.*, 2005; Katsir *et al.*, 2008). It induces a range of physiological process in different plant species, and external application of purified COR causes root growth inhibition in Arabidopsis and tomato (Feys *et al.*, 1994; Uppalapati *et al.*, 2005) and elicits tissue chlorosis when applied to tomato and *N. benthamiana* leaf tissues (Gnanamanickam *et al.*, 1982; Uppalapati *et al.*, 2005, 2007; Wangdi *et al.,* 2010). COR also induces the expression of chlorophyllase, the first enzyme in the chlorophyll degradation pathway (Bent *et al.*, 1992; Kloek *et al.*, 2001; Mach *et al.*, 2001; Brooks *et al.*, 2004). Furthermore, the F-box protein Coronatine insensitive 1/jasmonic acid insensitive 1 (COI1/JAI1) was shown to be required for COR signaling in Arabidopsis and tomato, respectively (Feys *et al.*, 1994; Zhao *et al.*, 2003; Katsir *et al.*, 2008). Similar to the auxin receptor (Skp1/Cul1/F-box) SCF^{TIR1} (Gray *et al.*, 1999), COI1 functions as a receptor of JA-Ile/COR and forms SCF^{COI1} complexes with ASK1/2 and Cullin (Xu *et al.*, 2002; Yan *et al.*, 2009). F-box proteins belonging to an E_3 ubiquitin ligase family assemble SCF complexes and play a major role in controlled protein degradation through a ubiquitin/26S proteasome pathway during plant hormone signaling and development (Moon *et al.*, 2004; Santner and Estelle, 2009). By mimicking jasmonates, COR stimulates the JA pathway in Arabidopsis and tomato and thereby functions to suppress the SA pathway and/or closure of stomata, allowing bacteria to reach higher densities *in planta* (Kloek *et al.*, 2001; Schmelz *et al.*, 2003; Zhao *et al.*, 2003; Block *et al.*, 2005; Melotto *et al.*, 2006; Uppalapati *et al.*, 2007).

Much progress has been made in our understanding of rapid programmed cell death (PCD) during the HR of incompatible plant–microbe interactions (Greenberg, 1997; Greenberg & Yao, 2004; Jones & Dangl, 2006). Gene-for-gene-mediated resistance often leads to HR/PCD at infection sites, thereby limiting the spread of the pathogen (Jones & Dangl, 2006). Although the role of cell death associated with disease lesions during hemibiotrophic pathogen–plant interactions is unclear, increasing evidence suggest that disease-associated cell death is genetically controlled and is a form of PCD (Greenberg & Yao, 2004). A mitogen-activated protein kinase kinase kinase gene (MAPKKKα) was shown to function as a positive regulator of HR and disease-associated cell death in bacterial speck disease of tomato (del Pozo *et al.*, 2004). COR-induced chlorosis was also found to contribute to disease symptom development in tomato (Uppalapati *et al.*, 2005; Ishiga *et al.*, 2009a,b; Wangdi *et al.*, 2010). However, it is not known whether disease-associated cell death shares components involved in chlorosis or necrosis.

In this study, we took advantage of the chlorosis phenotype elicited by COR on *Nicotiana benthamiana* and used a virus-induced gene silencing (VIGS)-based reverse genetic screen to identify genes that are required for COR-elicited chlorosis. We identified a role for *SGT1* (for suppressor of G2 allele of *skp1*) in mediating COR responses, including chlorosis development and root growth inhibition. Our results further showed that *AtSGT1b*, one of two highly related *SGT1* genes, positively regulates COR signaling and *Pst* DC3000 disease symptom development in Arabidopsis.

Materials and Methods

Plant materials and bacterial strains

Arabidopsis seedlings were grown on half-strength Murashige and Skoog (MS) medium (0.3% phytoagar) with Gamborg vitamins (PhytoTechnologies Laboratories, Shawnee Mission, KS, USA). Seeds of *rar1* and *sgt1a* mutant lines (both loss-of-function alleles in accession Col-0; Holt *et al.*, 2005) were kindly provided by Dr Jeff Dangl, University of North Carolina, NC, USA. *sgt1b* mutants in

accession L*er* have been described (Austin *et al.*, 2002). *edm1-1* is an *sgt1b* deletion mutant in Col-5 (Tör *et al.*, 2002) and *eta3* is a point mutation of *sgt1b* in Col-0 (Gray *et al.*, 2003). *SGT1b* overexpressor lines (2F8 and 2F12) were generated in the L*er sgt1b-3* null mutant background (Noël *et al.*, 2007), whereas 35S-*SGT1b* overexpressor lines D2 and B10 were generated in Col-0 (Gray *et al.*, 2003). All seedlings were grown at 25°C with a light intensity of 150–200 μmol m^{-2} s^{-1} and a 16 : 8 h light : dark photoperiod.

Seeds of tomato (*Solanum lycopersicum* cv Glamour) were obtained from Stokes Seeds Inc. (Buffalo, NY, USA). Plants were grown in Scott-200® mix (The Scotts Co., Marysville, OH, USA) and maintained in growth chambers (24°C, 40–70% relative humidity (RH), 12 h photoperiod, photon flux density 150–200 μmol m^{-2} s^{-1}).

An isolate of *P. syringae* pv. *tomato* DC3000 (*Pst* DC3000) was grown at 28°C on mannitol–glutamate (MG) medium containing 50 μg ml^{-1} rifampicin (Keane *et al.*, 1970) for 36–48 h.

VIGS in *N. benthamiana* and tomato and screening for COR-induced chlorosis

The vectors *pTRV1* (Liu *et al.*, 2002a) and *pTRV2::NbSGT1* were kindly provided by Dr Dinesh-Kumar, Yale University, USA. All other genes used for VIGS in this study (Supporting Information, Table S1) are previously described by Ekengren *et al.* (2003). Acetosyringone-induced *Agrobacterium* cultures containing *pTRV1* and *pTRV2* (with gene of interest) mixed in equal ratios (OD_{600} = 1.0) were used for inoculation (Ryu *et al.*, 2004). A needleless syringe was used to inoculate 2-wk-old *N. benthamiana* leaves. Tomato seedlings (1 wk old) were inoculated with a combination of Agroinfiltration (Ekengren *et al.*, 2003) and Agrodrench as previously described (Ryu *et al.*, 2004). VIGS experiments were repeated at least three times.

Pathogen inoculations

Four-week-old control and *SGT1*-silenced tomato plants were spray-inoculated with a *Pst* DC3000 bacterial suspension (5×10^7 cfu ml^{-1}) in distilled water containing 0.025% Silwet L-77 (OSI Specialties Inc., Danbury, CT, USA). Plants were then incubated in growth chambers at *c.* 100% RH for the first 24 h and at *c.* 70% RH for the remainder of the experiment.

For bacterial inoculation of Arabidopsis, MS agar plates with 2-wk-old seedlings were flooded with bacteria as described previously (Uppalapati *et al.*, 2008) with slight modifications (Y. Ishiga & S. R. Uppalapati, unpublished). Seedlings were flooded with 40 ml of bacterial suspension (5×10^7 cfu ml^{-1}, 0.025% Silwet L-77) for 2–3 min, with gentle mixing to achieve uniform inoculation, and then incubated at 25°C with a light intensity of 150–200 μmol m^{-2} s^{-1} and a 16 : 8 h light : dark photoperiod. In each experiment, 12 seedlings were evaluated per treatment and each experiment was repeated at least three times.

To estimate bacterial populations Arabidopsis rosette leaves were harvested from Petri dishes at 0 dpi (1 h post-inoculation) and 2 dpi, and were surface-sterilized with 5% H_2O_2 for 3 min to eliminate epiphytic bacteria, washed and homogenized in sterile distilled with water and plated in serial dilutions on MG medium containing 50 μg ml^{-1} rifampicin. Bacteria from tomato leaves harvested at 0 dpi (1 h post-inoculation), 3 and 7 dpi were quantified as described earlier for Arabidopsis.

Root growth inhibition assay

Root growth inhibition assays were carried out as described previously (Laurie-Berry *et al.*, 2006) with slight modifications. Seedlings grown vertically on square plates for 7 d at 25°C with a light intensity of 150–200 μmol m^{-2} s^{-1} and a 16 : 8 h light : dark photoperiod were transferred to fresh Petri dishes containing 1/2 MS media supplemented with COR (0.2 and 0.02 nM) or distilled water (control treatment). Four days after transfer, root length was measured. Values relative to the control seedlings were used to express the percentage of root growth inhibition.

Real-time quantitative PCR (RT-qPCR)

RNA extraction and RT-qPCR were done as described previously (Uppalapati *et al.*, 2008). Total RNA was treated with DNase I (Invitrogen), and 2 μg RNA was used to generate cDNA using Superscript III reverse transcriptase (Invitrogen) and oligo d(T)15-20 primers. The cDNA (1 : 10) was then used for RT-qPCR using Power SYBR Green PCR master mix (Applied Biosystems, Foster City, CA, USA) with an ABI Prism 7900 HT sequence detection system (Applied Biosystems). Primers specific for elongation factor-1α (*EF-1α*) or *ACTIN* were used to normalize small differences in template amounts. RT-qPCR was performed with primers shown in Table S2. The same set of primers was used for amplification of *Nb/SlACTIN* and *Nb/SlSGT1*, as these primers were designed based on a conserved region of the respective genes among *N. benthamiana* and *S. lycopersicum*. Average Cycle Threshold (CT) values calculated using Sequence Detection Systems (version 2.2.2; Applied Biosystems) from triplicate samples were used to determine the fold expression relative to controls.

Detection of reactive oxygen species (ROS)

3, 3′-diaminobenzidine (DAB) staining was used for detection of hydrogen peroxide as described previously (Ueno *et al.*, 2003). Arabidopsis leaves were incubated in 1 mg

ml^{-1} DAB-HCl (pH 3.8) for 8 h at room temperature and chlorophyll was removed with 95% ethanol.

Measurement of chlorophyll content

Pathogen inoculated and control Arabidopsis rosette leaves were collected and macerated in liquid nitrogen, placed in 6 ml of acetone and incubated at 4°C in the dark for 12 h to extract chlorophyll. Aliquots of total chlorophyll dissolved in acetone were mixed with hexane and 10 mM KOH at a ratio of 4 : 6 : 1 (v/v) and Chl*a* was quantified on a spectrophotometer using the formula described by Arnon (1949).

Yeast two-hybrid assays

Full-length coding sequences of *AtSGT1b*, *AtRAR1*, *AtCOI1* and *AtASK1* were cloned into *pGADRec7* (prey; Clonetech, Palo Alto, CA, USA) and *pGBKT7* (bait; Clonetech) following digestion using an in-fusion PCR cloning system (Clonetech). *AtASK1* bait construct was generated by GATEWAY cloning into *pXDGATCY86* (Ding *et al.*, 2004). Interactions were determined by co-transformation of each prey and bait construct into yeast MaV203 cells (Invitrogen) and plating on a high stringency medium. Dropout agar base (MP Biomedicals, Solon, OH, USA) with high stringency yeast synthetic dropout medium supplement without histidine, leucine, tryptophan and uracil was used (Sigma-Aldrich). Yeast transformation was carried out using Yeastmaker™ yeast transformation system 2 (Clonetech).

Results

A VIGS-based reverse genetic screen identifies a role for SGT1 in COR-induced chlorosis

To identify genes required for COR-induced chlorosis and disease-associated cell death, we performed a reverse genetics screen (using VIGS) of several transcription factors (TFs), protein kinases and components of proteasome complexes that were previously implicated to play a role in plant–pathogen interactions (Table S1). Silencing of known mitogen-activated protein (MAP) kinases and SA- or JA-inducible TFs did not alter the COR responses (Table S1). Consistent with the previous results, silencing of *NbCOI1*, a global regulator of COR signaling (Katsir *et al.*, 2008; Yan *et al.*, 2009), abolished COR-induced chlorosis (Table S1). Furthermore, silencing of *SKP1*, a member of the SCF^{COI} complex, resulted in a partial loss of chlorosis (Table S1). These results suggest that VIGS can be efficiently used as a reverse genetics tool to identify genes involved in COR-induced chlorosis.

It was previously shown that *AtSGT1b* plays a role in SCF^{TIR}-mediated auxin response, as *sgt1b* mutants were less responsive to methyl jasmonate (MeJA)-induced root growth inhibition (Gray *et al.*, 2003; Noël *et al.*, 2007). To determine if *SGT1* is required for COR-induced chlorosis development, we silenced *NbSGT1* in *N. benthamiana* and *SlSGT1* in tomato (the natural host for *Pst* DC3000) using TRV2::*NbSGT1* and then applied COR. Silencing of *SGT1* resulted in a complete loss of COR-induced chlorosis in both *N. benthamiana* and tomato (Table S1, Fig. 1). Although the effectiveness of gene silencing was shown to be different in *N. benthamiana* and tomato (Senthil-Kumar *et al.,* 2007), RT-PCR analyses confirmed that VIGS silenced *SGT1* at similar efficiencies in both the species (Fig. 1b,d). These results suggest that *SGT1* is required for COR signaling in both *N. benthamiana* and tomato.

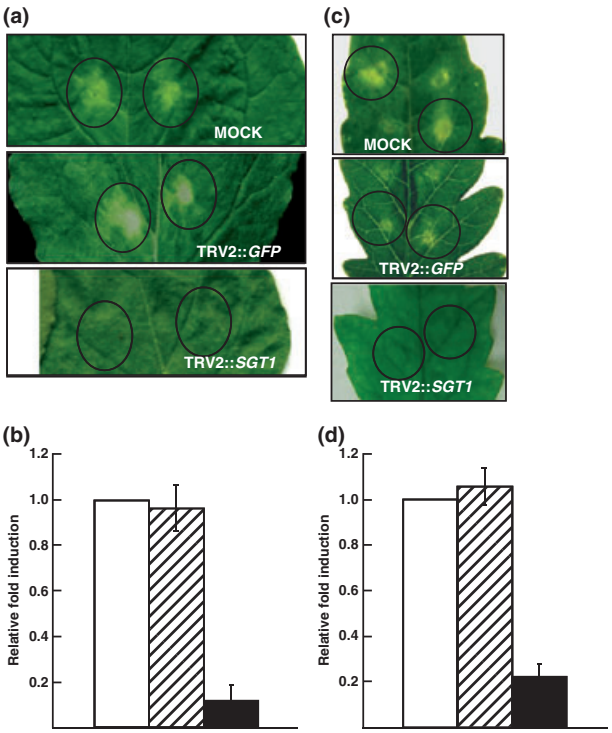

Fig. 1 *SGT1* is required for coronatine (COR)-induced chlorosis development in *Nicotiana benthamiana* and tomato. (a) Purified COR was applied to wild-type *N. benthamiana* (Mock), vector control (TRV2::*GFP*) or *SGT1*-silenced (TRV2::*SGT1*) leaf tissues in 2 μl aliquots (2 nM) and a visible chlorotic zone (circled spots) was scored at 4 d post-inoculation (dpi). (b) Real-time quantitative PCR (RT-qPCR) representing the efficiency of *SGT1* silencing in *N. benthamiana* leaves is shown in (a). (c) Purified COR was applied to wild-type *Solanum lycopersicum* cv Glamour (cv Glamour, Mock), vector control (TRV2::*GFP*) or *SGT1*-silenced (TRV2::*SGT1*) leaves in 2 μl aliquots (2 nM, circled spots) and a visible chlorosis was observed 4 dpi. (d) RT-qPCR shows the efficiency of *SGT1* silencing in tomato leaf tissues collected from areas showing no visible chlorosis upon COR application. *ACTIN* was used as an internal control. *SGT1* expression levels in the wild-type (Mock) was set at 1 and the relative fold induction of *SGT1* in TRV::*GFP* and TRV::*SGT1* is presented as fold induction.

AtSGT1b is required for COR signaling in Arabidopsis

Coronatine also functions as a virulence factor during pathogenesis of *Pst* DC3000 on Arabidopsis (Brooks *et al.*, 2004). Arabidopsis has two *SGT1* paralogs, *AtSGT1a* and *AtSGT1b*, and previous studies have shown that *AtSGT1b* is preferentially recruited for certain *Resistance* (*R*) gene-mediated responses (Austin *et al.*, 2002; Tör *et al.*, 2002; Holt *et al.*, 2005; Azevedo *et al.*, 2006; Noël *et al.*, 2007). To explore whether *AtSGT1a* or *AtSGT1b* contribute to COR signaling in Arabidopsis, we conducted seedling root growth inhibition assays in wild-type and *sgt1* mutants. The *sgt1a* mutant displayed a similar root growth inhibition response to COR as wild-type Col-0 (Fig. 2a). By contrast, Col-0 $sgt1b^{eta3}$ and $sgt1b^{edm1-1}$ mutants and L*er sgt1b-3* were significantly less sensitive to COR-induced root growth inhibition when compared with the corresponding wild-type backgrounds (Fig. 2a). The difference in sensitivity was more dramatic at lower concentrations of COR. We also investigated the effects of overexpression of *AtSGT1b* using two previously generated transgenic lines in accession Col-0 (B10 and D2, Gray *et al.*, 2003) and two independent lines in L*er sgt1b*-3 overexpressing *SGT1b* fused to a StrepII affinity purification tag (lines 2F8 and 2F12; Noël *et al.*, 2007). *SGT1b* overexpression did not alter the extent of root growth inhibition induced by COR (Fig. 2a), suggesting that *SGT1b* is not rate-limiting for this response.

To further investigate the role of *AtSGT1b* in COR signaling, we measured expression of the COR-responsive *AtLOX2* (lipoxygenase 2) and *AtCORI1* (coronatine-induced protein 1) genes in Arabidopsis leaves following COR treatment (Fig. 2b). Induction of *AtLOX2* and *AtCORI1* was significantly lower in $sgt1b^{eta3}$ but not in *sgt1a* compared with wild-type leaves (Fig. 2b). Collectively, the results showed that *AtSGT1b* contributes to COR-induced signaling in Arabidopsis.

SGT1 regulates disease symptom development in Arabidopsis and tomato

Since production of COR by bacteria is associated with host cell chlorosis during infection (Bender *et al.*, 1987; Uppalapati *et al.*, 2007), we tested whether *SGT1* affects disease development by first silencing *SlSGT1* in tomato using VIGS followed by inoculation with *Pst* DC3000. A strong reduction in disease symptom development was observed in *SlSGT1*-silenced plants compared with control (TRV2::*GFP*) tomato plants after bacterial spray-inoculation (Fig. 3a,b). Since COR can also enhance bacterial entry into leaves by impeding stomatal closure (Melotto *et al.*, 2006), we tested whether the reduced symptom development in spray-inoculated, *SGT1*-silenced tomato plants resulted from limited pathogen entry by vacuum-infiltrating leaves with bacteria. Vacuum-infiltrated, *SGT1*-silenced tomato plants displayed reduced and delayed disease symptoms compared with the control VIGS treatment (Fig. 3c,d). We previously observed that TRV2-based VIGS is slightly patchy in tomato (Uppalapati *et al.*, 2007). Consistent with our previous observations, a few bacterial specks (Fig. 3b, arrows) or tissue undergoing cell death (Fig. 3d, arrows) were observed in the areas potentially escaped from *SGT1* silencing in TRV2::*SGT1*-inoculated tomato leaves. By contrast, only a slightly lower (less than twofold, $P < 0.05$) bacterial growth was seen in spray-inoculated, *SGT1*-silenced plants at 3 and 7 dpi when compared with the vector controls (Fig. 3e). However, the differences in growth were not significant at $P < 0.01$ (Fig. 3e). Based on these results, we concluded that *SGT1* contributes to disease symptom (cell death with associated chlorosis) development in tomato, but to a lesser extent to the *in planta* bacterial growth.

To further explore the role of *SGT1* in disease symptom development, we flood-inoculated Arabidopsis wild-type *sgt1a* and *sgt1b* mutants and an *AtSGT1b* overexpressor with *Pst* DC3000 and monitored the appearance of disease symptoms and *in planta* bacterial growth (Figs 4, S1, S2). Dipping or flooding deposits bacteria on the leaf surface where they infect through the stomatal pores (Mittal & Davis, 1995; Melotto *et al.*, 2006; Uppalapati *et al.*, 2008). Wild-type and *sgt1a* mutants developed typical disease symptoms associated with chlorosis (Fig. 4). Disease-associated chlorosis was strongly reduced in *sgt1b* mutants (*eta3* and *edm1-1*; Fig. 4). However, *AtSGT1b* overexpression (B10, D2 and 2F8) did not alter disease symptom development (Fig. S1). To investigate the nature of the disease phenotype, we quantified the disease-associated chlorosis and visualized COR-induced ROS during disease lesion development. Consistent with the visible symptoms, > 50% reduction in Chl*a* was observed in wild-type, *sgt1a* mutants and *SGT1b-StrepII* (2F8) overexpressor lines, whereas only < 25% reduction in Chl*a* occurred in *sgt1b* (*eta3* and *edm1-1*) mutants during bacterial infection (Fig. S2a). We have previously shown that COR induces ROS accumulation during lesion development (necrosis typically associated with a chlorotic halo: Ishiga *et al.*, 2009a). Therefore, we measured ROS accumulation as an indicator of COR activity or lesion formation in Arabidopsis. ROS accumulated in wild-type, *sgt1a* mutants and *SGT1b-StrepII* overexpressors, but not in *sgt1b* mutants (*eta3* and *edm1-1*) (Fig. S2b). Although disease-associated chlorosis was compromised in the *sgt1b* mutants, bacterial growth was not different between wild-type, *sgt1b* mutants (*eta3* and *edm1-1*), *sgt1a* and the *SGT1b-StrepII* overexpressor lines (Fig. S3). These data suggest that *AtSGT1b*-mediated disease symptom development in response to *Pst* DC3000 infection is not related to *in planta* bacterial growth.

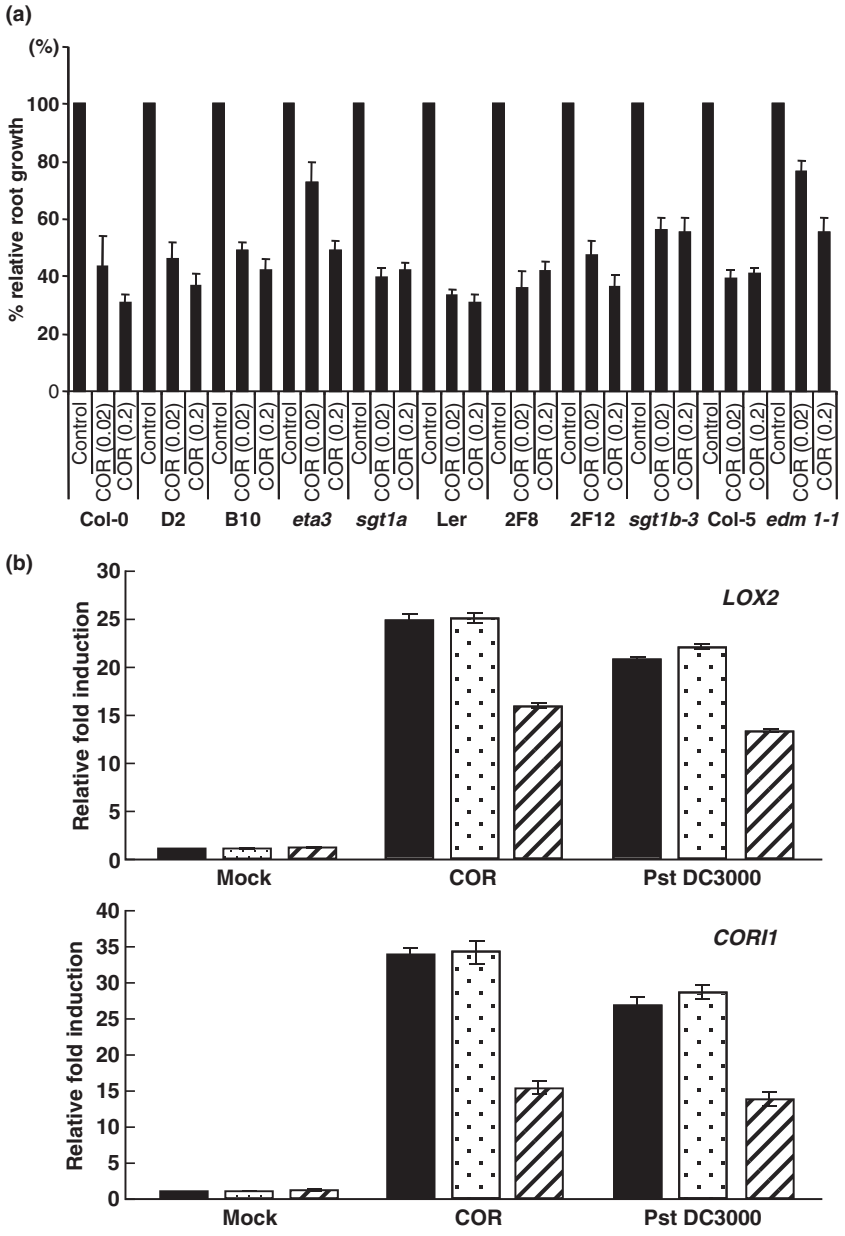

Fig. 2 *SGT1b* contributes to coronatine (COR) responses in Arabidopsis. (a) *sgt1b* mutant seedlings were less sensitive to COR than wild-type or *sgt1a* mutant seedlings. The root growth of *sgt1a*, *sgt1b*^{eta3} and *SGT1b* overexpressors (D2, B10) in Col-0 background; *SGT1b-StrepII* overexpressors (2F8 and 2F12) in *sgt1b-3* (*Ler* background); and *sgt1b*$^{edm1-1}$ in Col-5 background was measured 4 d after transferring to half-strength Murashige and Skoog (MS) medium supplemented with 0.02 or 0.2 nM COR. Values represent the average of 15 replicates, and error bars indicate standard deviations from the mean. (b) *sgt1b* mutant seedlings were less responsive in inducing jasmonic acid (JA)/COR-responsive genes in Arabidopsis. The leaf tissue of wild-type (Col-0), *sgt1a* and *sgt1b*^{eta3} mutants in Col-0 was treated with 0.2 nM COR. Total RNA isolated 24 h post-treatment was used to evaluate the relative fold expression of lipoxygenase (*LOX2*) and COR-inducible 1 (*COR1*) in COR-treated (Col-0; black bars), *sgt1a* (stippled bars) and *sgt1b*^{eta3} (hatched bars) mutants compared with the respective mock treatments using real-time quantitative PCR (RT-qPCR). Elongation factor 1α (*EF-1*α) was used as internal control. *LOX2* and *COR1* expression levels in the mock-treated control was set at 1 and the relative fold induction of *SGT1* in COR-treated samples is presented as fold induction.

SGT1b functions independently of RAR1 in disease symptom development

RAR1 is an interactor of SGT1 and a component of multiple *R*-gene mediated resistance responses (Azevedo *et al.*, 2002). Therefore we tested whether RAR1 is also involved in *SGT1b*-dependent disease symptom development by performing pathogen assays on *rar1* mutant in accession Col-0 (Fig. S4a). Unlike *sgt1b*, *rar1* mutant developed typical disease symptoms after inoculation with *Pst* DC3000. They also supported bacterial multiplication to a similar level as observed in wild-type plants (Fig. S4b). These results suggest that involvement of *AtSGT1b* in disease-associated cell death and chlorosis is independent of RAR1.

Discussion

In this study, we utilized VIGS as a reverse genetics tool to identify new components of COR-elicited chlorosis in *N. benthamiana* and found that silencing of *SGT1* abolished COR-induced chlorosis in *N. benthamiana* and tomato (Fig. 1). To investigate the role of *SGT1* in COR signaling, we conducted further studies in Arabidopsis, a

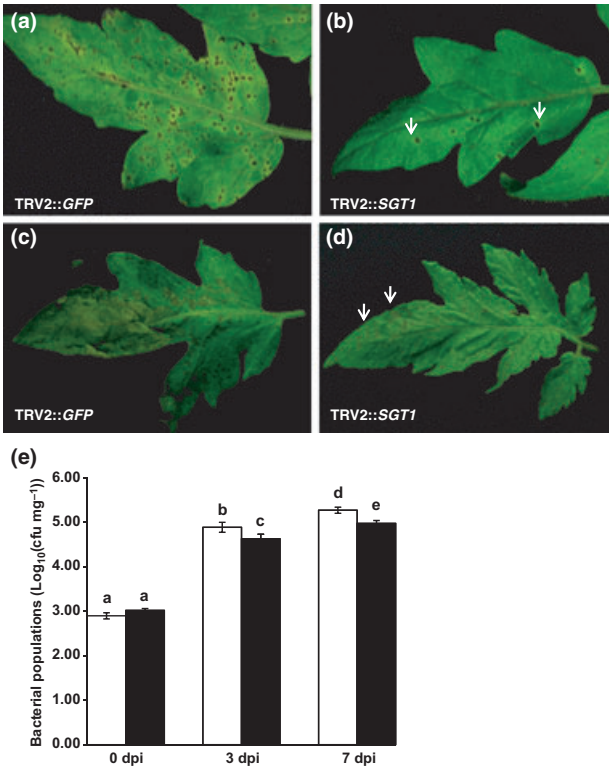

Fig. 3 *SGT1* is required for bacterial speck-associated cell death in tomato. Bacterial speck symptoms on *SGT1*-silenced (TRV2::*SGT1*) and control (TRV2::*GFP*) *Solanum lycopersicum* cv Glamour plants induced *Pseudomonas syringae* pv. *tomato* DC3000 following spray inoculation at a concentration of 5×10^7 cfu ml^{-1} (a, b) or vacuum infiltration at a concentration of 1×10^5 cfu ml^{-1} (c, d). Note that few bacterial specks (b, arrows) or tissues undergoing cell death (d, arrows) were observed in the areas potentially escaped from *SGT1* silencing in TRV2::*SGT1*-inoculated tomato leaves. Photographs were taken at 7 d post-inoculation (dpi) (a, b) or 3 dpi (c, d). (e) Bacterial populations of spray-inoculated control (TRV2::*GFP*; open bars) and *SGT1*-silenced (TRV2::*SGT1*; closed bars) tomato leaves at 3 and 7 dpi. Error bars represent standard deviations of nine replicates. Values with different letters (above error bars) are significantly different at $P < 0.05$ and $P > 0.01$ by Student's *t*-test.

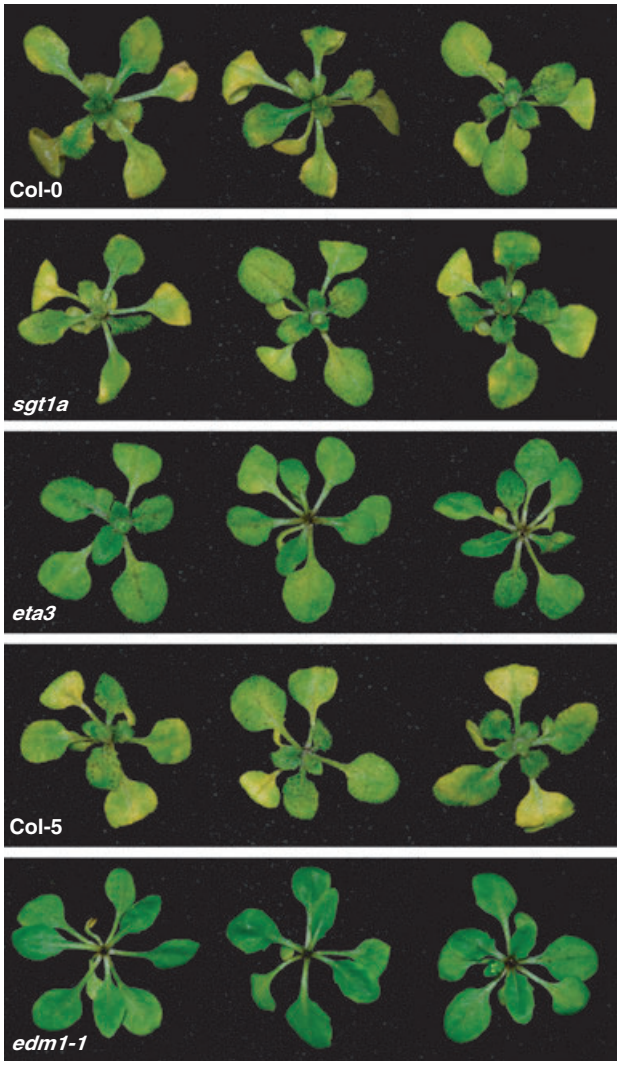

Fig. 4 *SGT1b* is required for typical disease symptoms caused by host pathogen *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst* DC3000) in Arabidopsis. Seedlings of *sgt1a* and *sgt1b*^{eta3} mutants in Col-0 background and of *sgt1b*$^{edm1-1}$ mutant in Col-5 background, and their corresponding wild-types, were flood-inoculated with *Pst* DC3000 at the concentration of 5×10^7 cfu ml^{-1}. Photographs were taken 5 d post-inoculation.

host for *Pst* DC3000. COR induced strong inhibition of root growth in Arabidopsis wild-type and *sgt1a* but not in *sgt1b* (*edm1*and *eta3*) mutant seedlings (Fig. 2a).

Other studies have revealed a role for *SGT1b* in sensitivity to auxin 2,4-D and MeJA in root growth inhibition of Arabidopsis seedlings (Gray *et al.*, 2003; Noël *et al.*, 2007). Our results show that *sgt1b* mutants (*eta3* and *edm1-1*) confer reduced sensitivity to COR (Fig. 2a). In our study, overexpression of *SGT1b* did not alter the root inhibition activity of COR (Fig. 2a). Similarly, Gray *et al.* (2003) reported that At*SGT1b* overexpression had no effect on the sensitivity to auxin. *SGT1b* may function to stabilize the components that mediate COR signaling, similar to its role in controlling the abundance of R proteins (Azevedo *et al.*, 2006). Therefore, the presence of higher amounts of AtSGT1b protein may not necessarily alter COR/JA signaling. However, it is possible that the expression levels of *AtSGT1b* in our transgenic plants were not sufficient to see a phenotype (Gray *et al.*, 2003).

Several previous studies have demonstrated the requirement of *SGT1/AtSGT1b* in cell death during incompatible interactions associated with an HR (Austin *et al.*, 2002; Tör *et al.*, 2002; Holt *et al.*, 2005; Wang *et al.*, 2010). Here we find that *SGT1* is needed for full disease symptom development during a compatible interaction in tomato and Arabidopsis (Figs 3, 4), consistent with SGT1 functioning in multiple pathways (Muskett & Parker, 2003; Shirasu,

2009). Although R protein-triggered and disease-associated cell death may result from different cues and signaling pathways, it may be that SGT1 has a common activity in various cell death programs. Notably, a MAPKKKα was shown to regulate cell death associated with both plant immunity and disease during tomato–*P. syringae pv. tomato* interactions (del Pozo *et al.*, 2004). Also, *NbSGT1* appears to be required for MKK7- and MKK9-induced cell death in innate immune responses (Popescu *et al.*, 2009). It is therefore possible that SGT1 interacts with components of a MAPK pathway required for *P. syringae* pv. *tomato*-associated cell death in different host plants. However, we found no differences in bacterial growth between wild-type and *sgt1b* mutants (Fig. S3), supporting the notion that bacterial growth and symptom development can be delineated (Greenberg & Yao, 2004; Uppalapati *et al.*, 2007; Wang *et al.*, 2010; Wangdi *et al.*, 2010). By contrast, *SGT1* plays a key role in plant resistance when HR-associated cell death is important for limiting pathogen spread (Holt *et al.*, 2005). Our results suggest a common SCF/SCF^{COI1} complex-dependent function of SGT1 in mediating chlorosis and *Pst* DC3000-associated lesion development.

Overexpression of *AtSGT1b* did not alter bacterial multiplication or disease symptom development in Arabidopsis (Figs S1 and S3). Consistent with our findings, overexpression of *SGT1* in rice also did not accelerate or enhance disease-associated cell death (Wang *et al.*, 2008). However, in a recent study we found that overexpression of *NbSGT1* results in acceleration of cell death associated with resistance to a nonadapted pathogen (Wang *et al.*, 2010). Therefore, divergent pathways may be operating in mediating cell death, or a different threshold of SGT1 may be required to promote cell death associated with disease or an R protein-triggered HR (Azevedo *et al.*, 2006).

Based on interactions of SGT1 with SKP1 in yeast, barley and *N. benthamiana* (Kitagawa *et al.*, 1999; Azevedo *et al.*, 2002; Liu *et al.*, 2002b) we speculated that AtSGT1b may be a component of the SCF^{COI1} ubiquitin ligase complex. However, we failed to demonstrate an association between AtSGT1b and AtSKP1 (ASK1) or AtCOI1 in yeast two-hybrid assays, even in the presence of COR (Fig. S5) or pull-down assays (data not shown). It is possible that SGT1b is not required for the assembly of or SCF^{COI1} (Fig. S5), as has been suggested for its role in SCF^{TIR1} complex assembly (Gray *et al.*, 2003). A limitation for detecting direct SGT1 interactions with SKP1 a component of $SCF^{COI1/TIR1}$ is probably the transient nature of SGT1 binding to SKP1 (Lingelbach & Kaplan, 2004). Thus, we cannot rule out the possibility that AtSGT1b might function independently of the SCF^{COI1} complex in COR signaling, despite being required for multiple SCF^{COI1}-dependent physiological responses induced by COR and JA (Figs 1 and 2).

Although our results demonstrate a dual role for *SGT1* in COR signaling and cell death, it is difficult to determine to what extent COR-mediated chlorosis is mechanistically related to cell death. Previously, we demonstrated COR-induced effects on the photosynthetic machinery and ROS in modulating necrotic cell death during bacterial speck disease of tomato (Ishiga *et al.*, 2009b). It is possible that COR-induced signaling may regulate the release of *Pst* DC3000 necrosis-inducing effector proteins, as some effector proteins were shown to promote cell death associated with disease (Chang *et al.*, 2000; Abramovitch & Martin, 2004; Chen *et al.*, 2004; DebRoy *et al.*, 2004; del Pozo *et al.*, 2004; Cohn & Martin, 2005). Moreover, COR and several effector proteins function through COI1 and JA- dependent pathways to promote virulence but not necessarily cell death (Zhao *et al.*, 2003; He *et al.*, 2004; Shang *et al.*, 2006; Thilmony *et al.*, 2006). Therefore, we cannot exclude the possibility that some effectors utilize nucleotide-binding domain leucine-rich repeat-containing (NLR) proteins to trigger the SGT1b/NBS-LRR complex-mediated cell death pathway during compatible interactions. Future research is required to determine the components of *SGT1*-dependent disease symptom development during *Pst* DC3000 interactions with susceptible hosts.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Fig. S1 Overexpression of *SGT1b* (D2, B10) *and SGT1b-StrepII* (2F8) had no effect on disease symptoms.

Fig. S2 Chlorophyll *a* content and the production of reactive oxygen species (ROS) in control and pathogen-inoculated Arabidopsis rosette leaves of *sgt1* mutants, *SGT1b-Strep II* overexpressors (2F8) and corresponding wild-type Arabidopsis seedlings, at 3 d post-inoculation (dpi).

Fig. S3 Bacterial growth in *sgt1* mutants, *SGT1b-StrepII* overexpressors (2F8) and corresponding wild-type Arabidopsis seedlings.

Fig. S4 *Pseudomonas syringae* pv. *tomato* DC3000 symptom development is independent of *RAR1* in Arabidopsis.

Fig. S5 Interactions of Arabidopsis SGT1b with RAR1 or ASK1 (a) and COI1 with ASK1 or SGT1b (b) in yeast two-hybrid assays.

Table S1 Chlorosis induction on *Nicotiana benthamiana* leaves targeted for silencing using VIGS

Table S2 Gene-specific primers used for real-time quantitative PCR

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