

# Disruption of sphingolipid biosynthesis in *Nicotiana benthamiana* activates salicylic acid-dependent responses and compromises resistance to *Alternaria alternata* f. sp. *lycopersici*

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**Abstract** Sphingolipids play an important role in signal transduction pathways that regulate physiological functions and stress responses in eukaryotes. In plants, recent evidence suggests that their metabolic precursors, the long-chain bases (LCBs) act as bioactive molecules in the immune response. Interestingly, the virulence of two unrelated necrotrophic fungi, *Fusarium verticillioides* and *Alternaria alternata*, which are pathogens of maize and tomato plants, respectively, depends on the production of sphinganine-analog mycotoxins (SAMs). These metabolites inhibit de novo synthesis of sphingolipids in their hosts causing accumulation of LCBs, which are key regulators of programmed cell death. Therefore, to gain more insight into the role of sphingolipids in plant immunity against SAM-producing necrotrophic fungi, we disrupted sphingolipid metabolism in *Nicotiana benthamiana* through virus-induced gene silencing (VIGS) of the serine palmitoyltransferase (SPT). This enzyme catalyzes the first reaction in LCB synthesis. VIGS of SPT profoundly affected *N. benthamiana* development as well as LCB composition of sphingolipids. While total levels of phytosphingosine decreased, sphinganine and sphingosine levels increased in SPT-silenced plants, compared with control plants. Plant immunity was also affected as silenced plants accumulated salicylic acid (SA), constitutively expressed the SA-inducible *NbPR-1* gene and showed increased

susceptibility to the necrotroph *A. alternata* f. sp. *lycopersici*. In contrast, expression of *NbPR-2* and *NbPR-3* genes was delayed in silenced plants upon fungal infection. Our results strongly suggest that LCBs modulate the SA-dependent responses and provide a working model of the potential role of SAMs from necrotrophic fungi to disrupt the plant host response to foster colonization.

**Keywords** Fumonisin · Long-chain bases · Plant immunity · Serine palmitoyltransferase · Sphinganine-analog mycotoxins

## Abbreviations

SPT	Serine palmitoyltransferase
LCB	Long-chain base
d18:0	Sphinganine
d18:1( $\Delta$ 4)	Sphingosine
t18:0	Phytosphingosine
SAMs	Sphinganine-analog mycotoxins
FB1	Fumonisin B1
JA	Jasmonic acid
PCD	Programmed cell death
VIGS	Virus-induced gene silencing
PR	Pathogenesis-related
SA	Salicylic acid

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## Introduction

Long-chain bases (LCBs) are metabolic precursors of complex sphingolipids, whose predominant forms in plants are C18 aminoalcohols differing by modifications at C4 (*E*-desaturation, hydroxylation) and at C8 (*E/Z*-desaturation). Biosynthesis of LCBs involves the condensation between

L-serine and palmitoyl-CoA in a reaction catalyzed by the serine palmitoyltransferase (SPT), a heterodimeric enzyme composed of two subunits named LCB1 and LCB2. The first LCB produced in plants is sphinganine, which is fully saturated and contains two hydroxyl groups. Sphinganine can be further modified by the addition of a hydroxyl group at C4 to yield phytosphingosine, and/or by introduction of double bonds at C4 and C8 to produce other LCBs (Lynch and Dunn 2004; Chen et al. 2009).

LCBs play prominent roles in signal transduction pathways regulating many aspects of plant development including cell growth, proliferation, differentiation, and cell death (Worrall et al. 2003; Sperling and Heinz 2003; Chen et al. 2006, 2008; Shi et al. 2007; Dietrich et al. 2008; Teng et al. 2008; Saucedo-García et al. 2011), as well as responses to abiotic (Ng et al. 2001; Coursol et al. 2003; Chen et al. 2012; Dutilleul et al. 2012) and biotic stress (Koga et al. 1998; Suharsono et al. 2002; Wang et al. 2008; Takahashi et al. 2009; Peer et al. 2010).

The relevance of sphingolipids in plant immunity has been known for some time but in recent years there have been major breakthroughs. Sphingolipids from the fungal pathogen *Magnaporthe grisea* induced phytoalexin accumulation, cell death, and increased resistance to infection in rice cells (Koga et al. 1998). In this sphingolipid-mediated response, a heterotrimeric G protein and the small GTPase OsRac1 activate the OsMAPK6 transduction pathway (Suharsono et al. 2002; Lieberherr et al. 2005). In *Nicotiana benthamiana*, sphingolipid biosynthesis is crucial at early stages of the resistance defense response as evidenced by the fact that the gene for the SPT LCB2 subunit is strongly induced upon infection with the non-host pathogen *Pseudomonas cichorii*, but only a minor and transient induction is observed in response to the host pathogen *P. syringae* pv. *tabaci* (Takahashi et al. 2009). In *Arabidopsis* leaves inoculated with *P. syringae* a higher and sustained increase of phytosphingosine levels occurs in an incompatible interaction that leads to resistance, as compared with the compatible one (Peer et al. 2010). This body of evidence suggests that LCBs are early transducers required for plants' immune response. Under this new role for sphingolipids, it is relevant to analyze the cases of two unrelated genera of necrotrophic fungi, *Fusarium verticillioides* and *Alternaria alternata* f. sp. *lycopersici*, which have evolved to produce sphinganine-analog mycotoxins (SAMs) that disrupt sphingolipid biosynthesis (Gilchrist and Grogan 1976; Desjardins et al. 1995; Akamatsu et al. 1997).

*Alternaria alternata* f. sp. *lycopersici* is the causal agent of tomato stem canker, and it can also infect leaves, stems, and fruits (Gilchrist and Grogan 1976). *F. verticillioides* is a non-obligate plant pathogen commonly associated with maize, causing root, stalk, and ear rot (Kommedahl and Windels 1981). The role of SAM production in

pathogenicity has been demonstrated for both pathogens [reviewed by Sánchez-Rangel and Plasencia (2010)]. When a *Fusarium musae* strain that neither colonizes maize nor produces SAMs is transformed with the entire *FUM* cluster responsible for FB1 production, it synthesizes the toxin and infects maize seedlings (Glenn et al. 2008). *A. alternata* f. sp. *lycopersici* pathogenicity depends on the production of another SAM, the AAL-toxin, to infect susceptible tomato plants (Gilchrist and Grogan 1976; Akamatsu et al. 1997).

SAMs competitively inhibit ceramide synthase activity in distinct plant species including *Arabidopsis*, causing significant accumulation of LCBs which in turn induces programmed cell death (PCD) (Abbas et al. 1994; Spassieva et al. 2002; Shi et al. 2007; Saucedo-García et al. 2011). SAM-induced PCD in *Arabidopsis* resembles the hypersensitive response (HR) triggered in plant cells at the site of pathogen infection, which includes ROS accumulation, callose deposition, and a dose-dependent induction of defense-related gene expression (Stone et al. 2000; Gechev et al. 2004). Hypersensitive cell death is an effective defense response against biotrophic pathogens that derive nutrients from living host tissues, but not against necrotrophic pathogens as they benefit from host cell death (Glazebrook 2005).

The activation of effective defense responses against biotrophs and necrotrophs is finely regulated by phytohormones (Glazebrook 2005; Spoel and Dong 2008; Pieterse et al. 2009, 2012). Upon pathogen attack, the phytohormone concentration and/or sensitivity change depending mainly on the lifestyle and infection strategy of the invading pathogen. Salicylic acid (SA), jasmonic acid (JA), and ethylene (ET) are considered the primary signals in the regulation of the plant defense response. The SA-dependent pathway is typically effective against biotrophic pathogens, whereas the JA-/ET-dependent pathways counteract necrotrophic pathogens (Glazebrook 2005). Mostly, these signaling pathways cross-communicate in an antagonistic or synergistic manner, activating a specific set of defense-related genes that eventually determine the outcome of the immune response (Pieterse et al. 2012). Interestingly, some evidence suggests an association between sphingolipid-mediated signaling and phytohormones in plant immunity. The FB1-induced PCD in *Arabidopsis* is accompanied by the induction of JA-, ET-, and SA-dependent genes (Asai et al. 2000; Stone et al. 2000). Likewise, AAL-toxin-induced PCD in tomato also requires the JA and ET signaling pathways (Egusa et al. 2009; Zhang et al. 2011; Mase et al. 2012).

In order to gain more insight into the role of sphingolipids in the defense response against necrotrophs, we used virus-induced gene silencing (VIGS) of SPT LCB2 subunit to disrupt sphingolipid biosynthesis in *N. benthamiana*. Silencing of SPT altered the cellular content of LCBs and

provoked a notable increase in the total levels of sphinganine and sphingosine, compared to control plants infiltrated with the viral vector. LCB elevation caused SA accumulation and constitutive expression of *NbPR-1*, the canonical marker of SA signaling. Moreover, SPT-silenced *N. benthamiana* plants became susceptible to *Alternaria alternata* f. sp. *lycopersici* infection, which was associated with an ameliorated expression of defense genes and possibly with the activation of SA-dependent responses. Our results provide genetic and biochemical evidence that the elevation of endogenous LCBs activates SA-dependent responses, and suggest that SAMs role in fungal virulence is to induce PCD, and possibly to subvert the signaling pathways required for disease resistance.

## Materials and methods

### Plant materials and growth conditions

*Nicotiana benthamiana* plants were grown in plastic pots containing a mixture of Sunshine Mix 4 and vermiculite (2:1 ratio), in a glasshouse set at 25 °C day temperature under natural photoperiod. For seedlings treatment with FB1, sphinganine, or SA, *N. benthamiana* wild-type seeds were surface-sterilized in 2.5 % sodium hypochlorite containing 0.05 % Tween20, rinsed three times in sterile water, and imbibed on GB-5 medium supplemented with 0.75 % sucrose and 0.8 % agar. Seeds were germinated and grown for 10 days in a Lab-Line growth chamber at 22 °C under 8 h light/16 h dark cycle (GE lamps, 80  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ). Seedlings were transferred to fresh medium supplemented with 5  $\mu\text{M}$  FB1, 25  $\mu\text{M}$  sphinganine, or 0.5 mM SA. Control seedlings were transferred to GB5 medium with 0.1 % methanol or 0.05 % ethanol, as they were used to prepare stock solutions of SA and sphinganine, respectively. Leaves were collected after 24 and 48 h, and immediately frozen in liquid nitrogen for RNA extraction.

### Chemicals

FB1, LCBs (phytosphingosine, sphinganine, and sphingosine), and SA were purchased from Sigma Chemical Company. A FB1 stock solution (1 mM) was prepared in water, a 50 mM solution of each LCB was prepared in absolute ethanol, and a SA stock solution (125 mM) was prepared in 50 % methanol. For HPLC analysis, HPLC-grade solvents (J.T. Baker), and naphthalene-2,3-dicarboxaldehyde (NDA; Fluka) were employed. NDA stock solution (20 mM) was prepared in acetonitrile. Stock solutions were protected from light and stored at  $-20$  °C, except the FB1 stock solution that was stored at 4 °C.

### VIGS of *NbLCB2*

The TRV vector was kindly provided by Dr. David Baulcombe from the Sainsbury Laboratory (Norwich, UK, <http://www.tsl.ac.uk>). A 602-bp cDNA fragment of the N-terminal region of *NbLCB2* was amplified from total RNA of *N. benthamiana* leaves with the *LCB2\_602* primers listed in Supplementary Table S1. This fragment was cloned into pGEM-T Easy vector (Promega), sequenced and reamplified with the *LCB2-BamHI* and *LCB2-HindIII* primers. The resulting product was digested with *BamHI* and *HindIII* and cloned into the pTRV vector. The new plasmid was named pTRVSPT. *Agrobacterium tumefaciens* strain C58C1 was transformed with the pTRVSPT construct by electroporation. Cultures of *A. tumefaciens* C58C1 containing pBIN-TRA6, pTRV, or pTRVSPT were used to infiltrate leaves of *N. benthamiana* plants at 5-leaf stage using a needleless syringe (Ratcliff et al. 2001). Plants were grown for 4 weeks and evidence of gene silencing of *NbLCB2* was obtained by semi-quantitative RT-PCR using two different primer sets: *LCB2\_1069* and *LCB2\_256*. Viral replication and systemic infection were confirmed with *TRV\_Cp* and *TRV\_Mp* primers. All primer sets used in this study are listed in Supplementary Table S1.

### Sphingolipids extraction and total LCBs quantification

Total LCBs from leaves of TRV and TRVSPT plants were extracted following the method described by Markham et al. (2006) and 1 nmol of d20:1 was used as internal standard per sample. Approximately 15- to 20-leaf disk per plant were employed so a representative sample was collected. Sphingolipids were hydrolyzed for 16 h at 110 °C in 10 % barium hydroxide. LCBs were extracted with 2 mL of diethylether and the lipid extract was redissolved in 200  $\mu\text{L}$  of ethanol. NDA derivatization was accomplished by adding the following stock solutions: 140  $\mu\text{L}$  of 25 mM sodium tetraborate buffer pH 9.5, 30  $\mu\text{L}$  of 2.5 mM KCN, and 10  $\mu\text{L}$  of 2.5 mM NDA. The tube was sealed with PTFE film and heated at 60 °C for 20 min. LCBs were quantified based on the recovery of the d20:1 standard. The HPLC system consisted of a Shimadzu LC-10ADVP pump, a RF-10AXL fluorescence detector, and a CR5A integrator. Derivatized LCBs were separated in a SUPELCO C18 column (15 cm  $\times$  4.6 mm, 5  $\mu\text{m}$ ) using 94 % acetonitrile as mobile phase at a flow rate of 1.2 mL  $\text{min}^{-1}$ . Fluorescent derivatives were monitored at  $\lambda_{\text{exc}}$  of 419 nm and  $\lambda_{\text{em}}$  of 493 nm.

### *Alternaria* sp. isolation and identification

*Alternaria* sp. was isolated from leaves of TRVSPT plants showing disease symptoms. Briefly, the leaves were placed on potato dextrose agar medium (PDA) and incubated for

5 days at room temperature under fluorescent light. Fungal colonies were transferred to PDA agar and incubated under the same conditions for 5–7 days. A single-spore isolate was obtained by preparing a spore suspension from the PDA culture in 10 mL of sterile water and streaking a loopful on water agar. The isolate was named A2SS and the identification was based on its macroscopic and microscopic characteristics. This was further confirmed by comparative sequence-based identification using the nuclear ribosomal internal transcribed spacer (ITS) region (ITS1, 5.8S rRNA, and ITS2) located between the nuclear small (18S) and large subunit (28S) rRNA genes.

DNA was obtained from the A2SS strain with DNAzol reagent (Invitrogen) according to manufacturer's instructions. The ITS was amplified with the universal primers ITS4 (5'-TCCTCCGCTTATTGATATGC-3') and ITS5 (5'-GGAAGTAAAAGTCGTAACAAGG-3'; White et al. 1990), and the 600-bp product was resolved and purified from 2 % agarose gel for sequencing.

#### Pathogen inoculation

*Alternaria* sp. strains were grown on PDA for 2–3 weeks at room temperature under fluorescent light. Spores were collected in sterile water and the suspension was adjusted to  $1 \times 10^5$  conidia/mL by counting in a hemacytometer. Detached leaves or stem pieces (2 cm length) of *N. benthamiana* TRV and TRVSPT plants were placed in a petri dish with a sterile moist filter paper and inoculated with 10  $\mu$ L of the conidial suspension, sealed with parafilm, and incubated at 22 °C under 8 h light/16 h dark photoperiod (GE lamps, 80  $\mu$ mol m<sup>-2</sup> s<sup>-1</sup>). Control tissues were inoculated with the same volume of sterile water. Disease symptoms were recorded photographically. For spore counting, stems were transferred to eppendorf tubes and spores were released from the tissue by vigorous shaking in 1 mL water and counted in a hemacytometer.

#### Semi-quantitative RT-PCR analysis

Total RNA was extracted from 200 to 300 mg of leaf tissue using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. Total RNA was calibrated spectrophotometrically and in an agarose gels using the 18S rRNA band, and then 1  $\mu$ g was used to synthesize the first-strand cDNA with oligo(dT) primer and Improm-II<sup>TM</sup> reverse transcriptase (Promega). PCR was performed with a denaturing temperature of 94 °C for 3 min, and annealing and extension temperatures of 58 and 72 °C, respectively, for 40 s. To compare levels of each transcript, a systematic analysis of primer concentration, total RNA amounts, and number of amplification cycles were conducted. Calibration curves with varying cycle number (22–32 cycles) were

constructed to determine the proper number of cycles to quantify each transcript (Supplementary Fig. S1). Transcript levels of the eukaryotic elongation factor 1-alpha (*NbEF1 $\alpha$* ) were used for normalization thus allowing a reproducible analysis of the various transcripts within the three or four replicates performed. The gene-specific primers used are indicated in Supplementary Table S1. Densitometric analysis of the PCR-generated fragments were analyzed and quantified with the Quantity software (Bio-Rad).

#### SA extraction and quantification by HPLC

SA was extracted from leaves as described by Meuwly and Métraux (1993) using *o*-anisic acid (*o*-ANI, 300 ng) as internal standard. HPLC analysis was performed in the equipment described above, setting fluorescence detection wavelengths at 305 nm excitation/405 nm emission for SA, and 305/365 for *o*-ANI. Dilutions of the extracts of free and conjugated SA were prepared in mobile phase (40 % methanol and 60 % water acidified with 4 % acetic acid), and were resolved by isocratic elution with a flow rate of 1.2 mL min<sup>-1</sup>. Under these HPLC conditions, SA eluted at ~10 min and *o*-ANI at 5 min. *o*-ANI recovery ranged from 46 to 74 %, and SA values were adjusted for yield losses.

#### Statistical analysis

Data was analyzed with the general linear model analysis of variance and Tukey's test to determine the statistical significance at 0.05 and 0.01 probability using the Statistix<sup>®</sup> software package.

#### Accession number

*NbLCB2* cDNA sequence was deposited in GenBank/EMBL databases (accession number AM902524).

## Results

VIGS of *N. benthamiana* SPT LCB2 subunit dramatically affected its vegetative and reproductive development

A 1069-bp cDNA fragment of *NbLCB2* (GenBank accession no. AM902524) was used to subclone a 602-bp cDNA into the TRV vector and the construct was named pTRVSPT (Fig. 1a). *N. benthamiana* plants at 5-leaf stage were agro-infiltrated with the empty TRV vector (as control) or pTRVSPT construct. Four weeks later, non-silenced TRV-infected control plants did not showed morphological differences compared to not agroinfiltrated plants (not shown). In contrast, TRVSPT infected plants developed dramatic

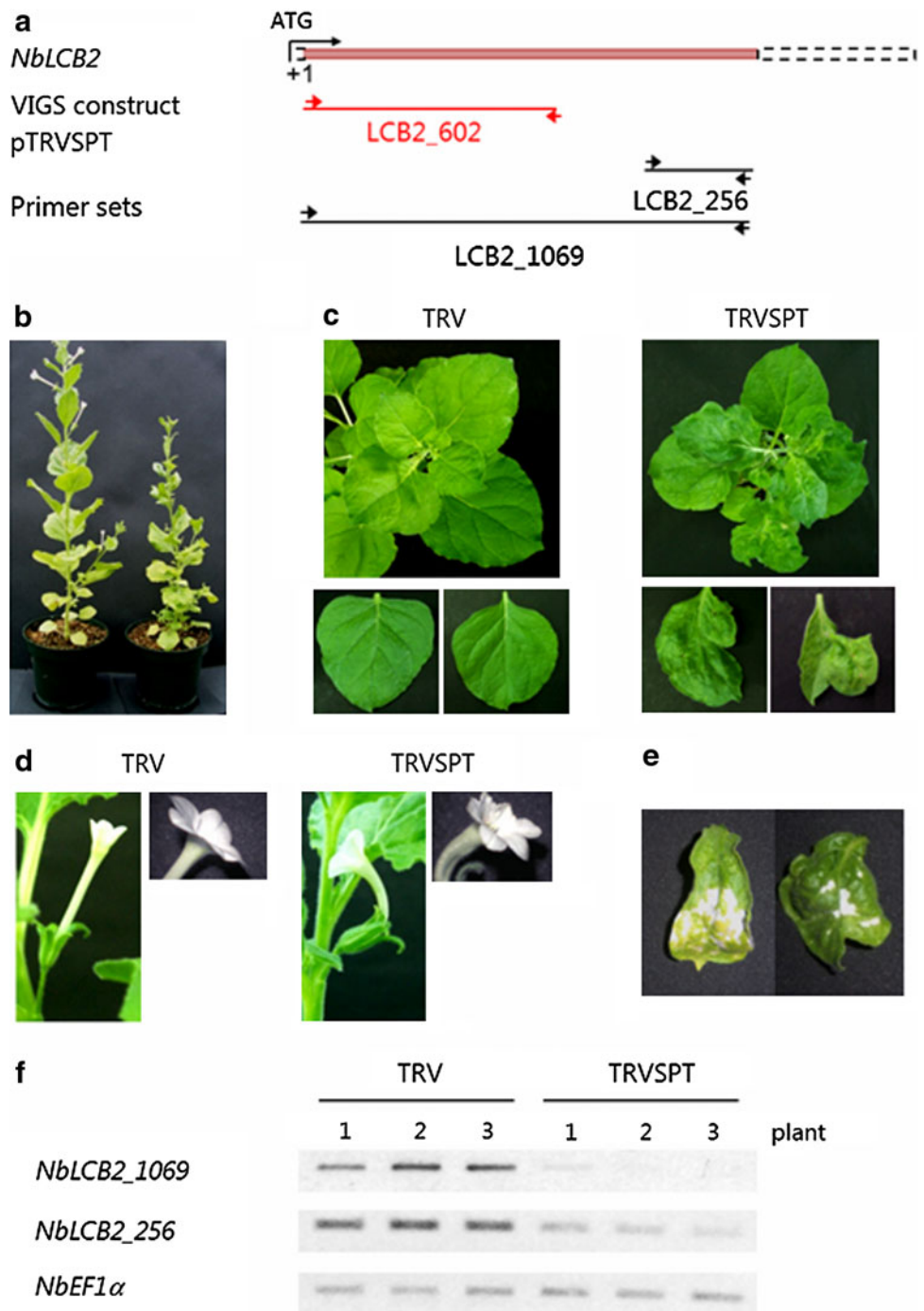


changes in their phenotype, qualitatively similar to those reported by Takahashi et al. (2009), such as significant growth reduction (Fig. 1b, right) and abnormal leaf morphology (Fig. 1c). TRVSPT plant leaves lost their typical heart-shape and developed irregular morphology. Moreover, a strong effect on flower development and morphology was also observed as flowers of TRVSPT plants showed several defects such as bending and shortening of the corolla tube, alterations in the number, and shape of petals (Fig. 1d). Also

spontaneous cell death was observed by the presence of necrotic areas on some leaves (Fig. 1e).

Efficiency of virus-induced SPT-silencing was evaluated by semi-quantitative RT-PCR using the *LCB2\_256* and *LCB2\_1069* primer sets (Fig. 1a). *NbLCB2* transcript was detected in TRV control plants and 20–50 % reduction was typically observed in TRVSPT plants (Fig. 1f). Similar levels of viral transcripts (coat and movement proteins) were detected in control and SPT-silenced plants

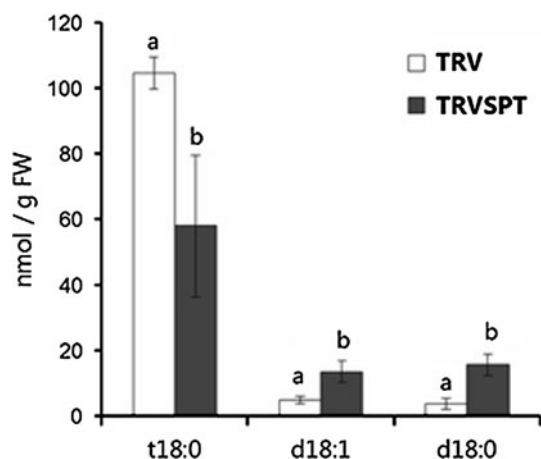
**Fig. 1** VIGS of SPT LCB2 subunit in *N. benthamiana*. **a** Scheme of *NbLCB2* cDNA and the region used to generate the VIGS construct pTRVSPT. The positions of the primer sets *LCB2-256* and *LCB2-1069* used for RT-PCR analyzes are marked by arrows. **b** VIGS phenotype characterized by growth restriction and alterations in vegetative and reproductive development. The TRV (left) and TRVSPT plants (right) were photographed 4 weeks after agroinfiltration. **c** Morphological changes in leaves of TRVSPT plants. **d** Alterations in flower development of TRVSPT plants. **e** Spontaneous cell death associated to SPT-silencing. **f** Endogenous *NbLCB2* mRNA levels in the leaves of TRV and TRVSPT plants. *NbEF1α* mRNA levels were used as RNA loading control



(Supplementary Fig. S2), confirming that viral infection did not interfere with the phenotype and that morphological changes were caused by the silencing of the SPT.

#### Effects of SPT-silencing on sphingolipid LCB composition

Because SPT is the first enzyme in sphingolipid biosynthesis (Gable et al. 2000; Breslow et al. 2010), we evaluated the effect of *LCB2* gene silencing on LCB composition in *N. benthamiana* leaves 4 weeks after agroinfiltration. Phytosphingosine (t18:0), sphingosine (d18:1), and sphinganine (d18:0) were identified by the retention time of the corresponding standards (Fig. 2). Some other LCBs were detected as NDA derivatives of the LCBs released by sphingolipid hydrolysis from TRV- and SPT-silenced leaves (Supplementary Fig. 3, peaks 1–9). LCB composition of TRV control leaves was similar to those reported previously for other *Solanacea*, such as tobacco and tomato, and also *Arabidopsis* (Sperling et al. 2005; Markham et al. 2006; Buré et al. 2011), in which trihydroxylated LCBs such as phytosphingosine are more abundant than the dihydroxylated LCBs sphinganine and sphingosine (Fig. 2; Supplementary Fig. 3a, b). It is likely that some LCBs detected but not identified (Supplementary Fig. 3a, b) correspond to the isomers of 8-phytosphinganine (t18:1-*E* and t18:1-*Z*) that have been detected in *N. tabacum* leaves under similar analytical conditions (Sperling et al. 2005), or to the isomers of 4,8-sphingadine (d18:2-*EE* and d18:2-*EZ*) which were also detected in tobacco leaves, cultured cells, and in tomato leaves



**Fig. 2** Total content (nmol g<sup>-1</sup> FW) of phytosphingosine (t18:0), sphingosine (d18:1), and sphinganine (d18:0) in leaves of *N. benthamiana* TRV and TRVSPT plants 4 weeks after agroinfiltration. A total of 15–20 leaf disks were collected from each plant and pooled so values are expressed as the mean  $\pm$  SD of four independent plants. Different letters above each set of bars indicate a significant difference ( $p < 0.01$ , Tukey's test) in levels for the indicated LCB

(Sperling et al. 2005; Markham et al. 2006; Buré et al. 2011).

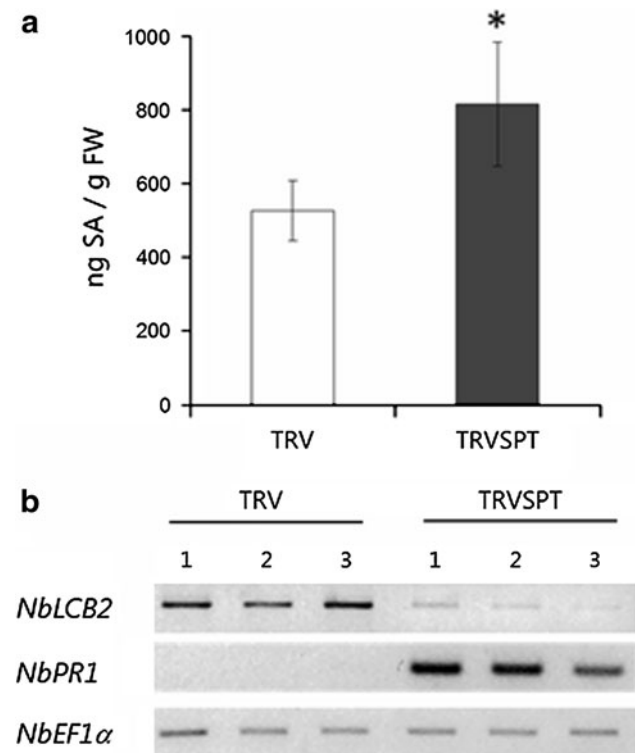
Regarding TRVSPT leaves, the LCB profile was notably altered as the levels of phytosphingosine and other LCBs decreased (Fig. 2; Supplementary Fig. 3a), whereas the abundance of sphinganine and sphingosine were consistently found to be higher than in the TRV leaves (Fig. 2; Supplementary Fig. 3b). In TRVSPT leaves phytosphingosine levels were reduced by 50 %, whereas sphingosine and sphinganine levels increased threefold and fourfold, respectively, compared with TRV control leaves (Fig. 2). A general decrease of LCBs was not observed as expected, and importantly, the results revealed that SPT-silencing in *N. benthamiana* had multiple effects on sphingolipid metabolism, and that the abundance of bioactive LCBs can be selectively affected by the action of SPT.

#### VIGS of SPT activated SA-dependent responses

The increased levels of sphinganine and sphingosine found in TRVSPT plants correlated with the spontaneous cell death phenotype (Fig. 1e). This response was comparable to the PCD developed by *Arabidopsis* mutants affected in different reactions of sphingolipid metabolism (*acd5*, *acd11*, and *erh1*), which was associated to increased levels of SA, (Greenberg et al. 2000; Brodersen et al. 2005; Wang et al. 2008). Therefore, we determined SA levels in TRVSPT-silenced plants. As shown in Fig. 3a, *N. benthamiana* TRV leaves had low basal levels of total SA ( $\sim 500$  ng g<sup>-1</sup> FW) in accordance with values reported by Catinot et al. (2008), while in TRVSPT leaves a 50 % increase in the content of total SA was found as compared to TRV leaves ( $p < 0.01$ ). Furthermore, SA accumulation provoked by VIGS of SPT caused a strong constitutive expression of *NbPR-1* gene that was not observed in TRV plants (Fig. 3b). The abundance of *NbPR-1* transcripts was determined at saturation phase of PCR, demonstrating that the viral infection did not contribute to *NbPR-1* induction in TRVSPT plants. These results strongly suggest a link between sphingolipid metabolism and SA signaling, possibly through the LCBs which increased by the SPT-silencing in *N. benthamiana*.

#### Sphinganine and FB1 restricted the growth of *N. benthamiana* wild-type seedlings and up-regulated the SA-inducible *NbPR-1* gene

In order to confirm whether the activation of the SA-dependent signaling pathway in TRVSPT plants was mediated by elevation of LCBs levels, we exposed 10-day-old *N. benthamiana* wild-type seedlings to FB1 or exogenous sphinganine and evaluated *NbPR-1* transcript levels. First, we assessed the susceptibility of *N. benthamiana*



**Fig. 3** VIGS of SPT activated the salicylic acid (SA) signaling pathway. **a** Content of total SA in TRV and TRVSPT plant leaves. Samples were taken 5 weeks after agroinfiltration. Values are expressed as the mean ± SD of five independent plants. The asterisk above bars denotes a significant difference ( $p < 0.01$ , Tukey’s test). **b** VIGS of SPT caused constitutive expression of the SA-inducible *NbPR-1* gene. Lane numbers correspond to independent plants

seedlings to FB1 because it was reported that the most of the *Solanaceae*, and in particular the *Nicotiana* species, are insensitive to SAMs (Mesbah et al. 2000; Brandwagt et al. 2001). As shown in Fig. 4a, FB1 induced a dose-dependent growth arrest and restricted root hair development. At the lowest dose (1 μM) a discrete reduction (24 %) was observed, whereas 5 μM FB1 caused a significant decrease of 64 % ( $p < 0.01$ , Fig. 4b), but did not induce chlorosis or cell death. These results indicated that *N. benthamiana* exhibited an intermediate sensitivity to FB1 according to the classification made by Brandwagt et al. (2001). Growth reduction caused by FB1 may be related to LCBs accumulation, as seedlings developed in presence of sphinganine showed a similar inhibition (Supplementary Fig. S4).

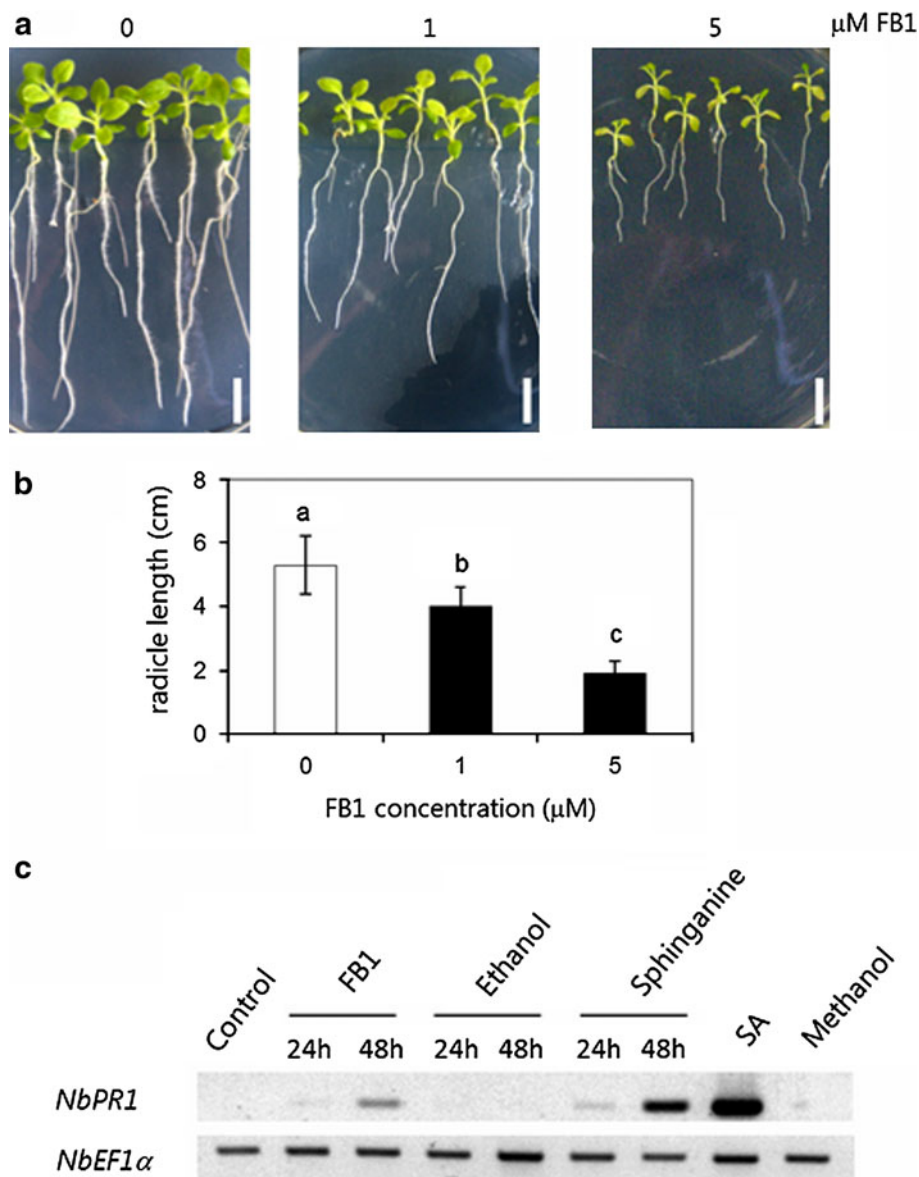
To examine whether LCBs were involved in the activation of SA-dependent signaling, 10-day-old seedlings were transferred to agar plates supplemented with 5 μM FB1, 25 μM sphinganine or 500 μM SA, whereas control seedlings were transferred to agar plates without chemicals and mock treatments containing 0.05 % ethanol or 0.1 % methanol. Noticeably, FB1 and sphinganine-induced

*NbPR-1* expression at 24 h of exposure, and the response was heightened at 48 h, especially with sphinganine, while in the control and mock-treated seedlings there was no induction of gene expression (Fig. 4c). These results support the hypothesis that LCBs are regulators of the SA-dependent pathway. Furthermore, it clearly opens the possibility that necrotrophic fungi that produce SAMs take advantage of the LCB-induced activation of the SA-dependent signaling pathway that leads to PCD, as part of their virulent strategy that contributes to a successful colonization.

VIGS of SPT compromised *N. benthamiana* resistance against the necrotrophic fungal pathogen *Alternaria alternata*

As described above, SPT-silencing had strong effects on plant development, and a very interesting additional phenotype observed was that our TRVSPT plants grown in the greenhouse often developed necrotic lesions in stems and leaves (Fig. 5a, b) that were not observed in TRV or not agroinfiltrated plants. From these lesions, we isolated and obtained a monosporic fungal culture (strain A2SS). In PDA, the fungus developed black colonies (Fig. 5c) and brown conidia with transverse and longitudinal septa that are characteristic of *Alternaria* sp. morphology (Fig. 5d). Molecular identification by the sequence of the ITS narrowed the isolate to two possible species: *A. alternata* and *A. tenuissima*. This result was relevant because *N. benthamiana*, like most *Nicotiana* species, is resistant to *A. alternata* (Brandwagt et al. 2001), thus SPT-silencing and disruption of sphingolipid metabolism was directly associated to susceptibility to this necrotroph.

To rule out that TRVSPT plants susceptibility was generalized to other *Alternaria* species, detached leaves of TRV and TRVSPT plants were inoculated with 10<sup>3</sup> conidia of either *A. brassicicola* or *Alternaria* sp. A2SS. *A. brassicicola* could not infect the TRV leaves nor the TRVSPT (Fig. 5e) while, as expected, the A2SS strain infected only TRVSPT leaves causing necrotic expanded lesions, and on the inoculated TRV leaves only a slight chlorosis was observed (Fig. 5f). These results indicate that SPT-silencing conferred a selective susceptibility to *A. alternata*, highlighting the potential role of LCB in the defense response, as the virulence of the tomato pathotype (*A. alternata* f. sp. *lycopersici*) depends on the disruption of sphingolipid biosynthesis and LCBs accumulation caused by the AAL-toxin (Gilchrist and Grogan 1976; Spassieva et al. 2002). This prompted us to analyze the defense response of *N. benthamiana* TRVSPT plants challenged with *A. alternata* f. sp. *lycopersici*.



**Fig. 4** Sphinganine and FB1 up-regulated the SA-responsive gene *PR-1* in *N. benthamiana* wild-type seedlings. **a** *N. benthamiana* had intermediate sensitivity to FB1. *N. benthamiana* 10-day-old seedlings were transferred to GB5 medium with or without FB1, and photographs were taken 10 days later. **b** Comparison of seedlings root length after 10 days of FB1 exposure ( $n = 10$ ). Values are expressed as the mean  $\pm$  SD. Different letters indicate a significant difference ( $p < 0.01$ , Tukey's test). Inhibition of root growth and hypocotyl elongation were observed, but the cotyledons emerged and became green. The cotyledons and roots never developed necrosis symptoms.

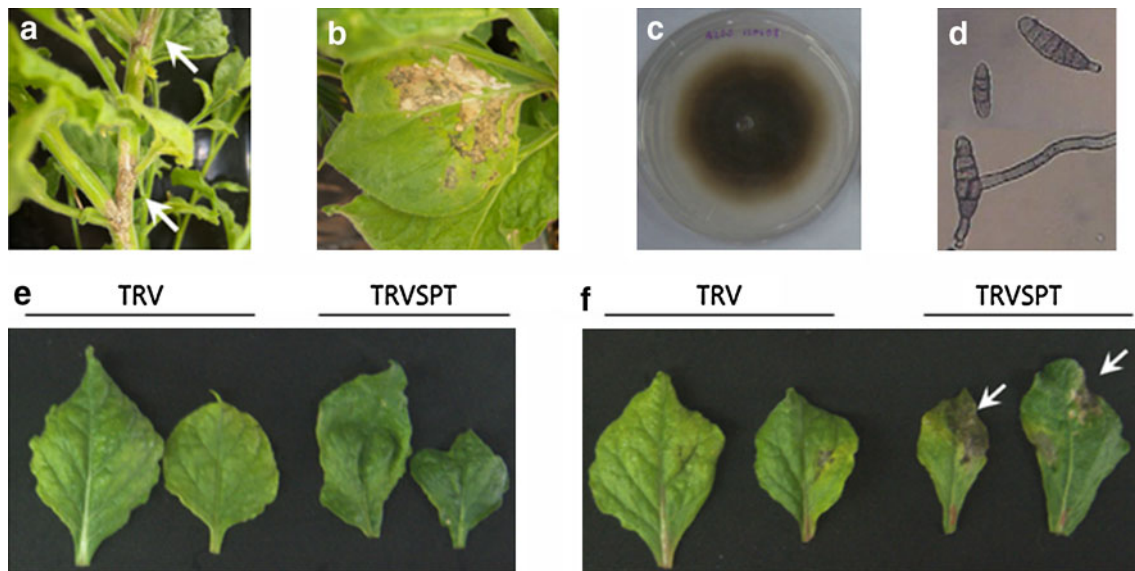
**c** FB1 and sphinganine induced the expression of the SA marker gene *PR-1* in *N. benthamiana*. Ten-day-old seedlings were transferred to GB5 media supplemented with 5  $\mu$ M FB1, 25  $\mu$ M sphinganine, or 0.5 mM SA. Leaves were collected after 24 and 48 h of treatment and immediately frozen in liquid nitrogen for RNA extraction. Control seedlings correspond to 10-day-old seedlings transferred to fresh GB5 medium and the sample was taken after 48 h. Mock treatments with 0.05 % ethanol or 0.1 % methanol were included as they were used to prepare stock solutions of sphinganine and SA, respectively. Results are representative of two independent experiments

*N. benthamiana* SPT-silenced plants became susceptible to the non-host pathogen *A. alternata* f. sp. *lycopersici* and exhibited altered defense gene expression

To test the relevance of the altered LCB homeostasis in *N. benthamiana* TRVSPT plants in the defense response

against a SAM-producing fungus, we performed pathogenicity assays using an *A. alternata* f. sp. *lycopersici* aa strain (provided by Dr. Liancheng Du, University of Nebraska). A conidial suspension was used to inoculate detached leaves and stems from TRV and TRVSPT plants. Sterile water was used to spot control leaves. As expected, *A. alternata* f. sp. *lycopersici* was unable to





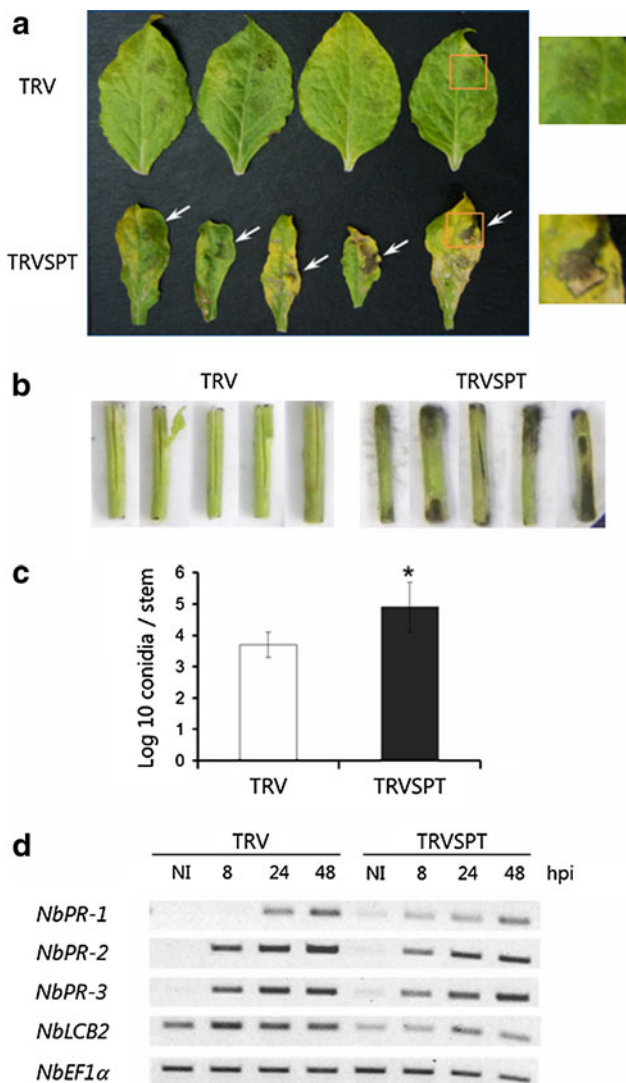
**Fig. 5** VIGS of SPT conferred susceptibility to the necrotrophic pathogen *Alternaria alternata*. Stem (a) and foliar lesions (b) on a TRVSPT plant. The macroscopic (c) and microscopic (d) morphology of the fungi isolated from the lesion on the TRVSPT leaf correspond to *A. alternata*. The strain isolated was cultivated on  $0.5 \times$  PDA and photographed 15 days later. For pathogenicity assays, excised

*N. benthamiana* TRV and TRVSPT leaves were inoculated with  $1 \times 10^3$  conidia of *Alternaria brassiciola* (e) or *Alternaria* sp. A2SS strain (f) and photographed 10 dpi. The inoculated leaves were kept in separate humid chambers at 22 °C. Only the TRVSPT leaves inoculated with A2SS strain (*Alternaria* sp.) developed symptoms of infection and spread of the fungus (indicated by white arrows)

infect the TRV leaves and the inoculated regions developed only mild symptoms of chlorosis (Fig. 6a). In contrast, TRVSPT leaves developed intense chlorosis and necrotic lesions on the inoculated sites, and an increase in fungal growth was observed at 10 days after inoculation (dai, Fig. 6a). Because *A. alternata* f. sp. *lycopersici* is the causal agent of tomato stem canker disease, pathogenicity tests were performed also on *N. benthamiana* stems. In the TRVSPT stems, fungal proliferation was evident at 6 dai, whereas very discrete or no signs of fungal growth were observed in TRV stems (Fig. 6b). In the control stems inoculated with sterile water (TRV and TRVSPT) no signs of infection or fungal development were observed (data not shown). Colonization levels were determined by counting conidia collected from the stems at 6 dai. Fungal colonization of TRVSPT plant stems correlated with a tenfold increase in conidia number compared with TRV plant stems (Fig. 6c). These results are consistent with the hypothesis that LCBs are modulators of the defense response against pathogens, including necrotrophs, so the altered LCB homeostasis in *N. benthamiana* TRVSPT plants suppressed the resistance against the non-host and necrotrophic pathogen *A. alternata* f. sp. *lycopersici*.

Because LCBs are signal transducers in plants, we investigated whether the susceptibility of *N. benthamiana* TRVSPT plants to *A. alternata* f. sp. *lycopersici* might be associated with a delayed or reduced expression of

defense-related genes. Necrotrophic infection often results in the activation of *PR* genes encoding proteins with potent antifungal activities (Glazebrook 2005). To examine this, we analyzed by semi-quantitative RT-PCR the expression of *NbPR-1*, *NbPR-2*, and *NbPR-3* genes in inoculated TRV and TRVSPT leaves. At 8 h post-inoculation (hpi), an induction of *NbPR-2* and *NbPR-3* expression was observed in TRV leaves which was increased at later times (24 and 48 hpi, Fig. 6d), suggesting that glucanases and chitinases are induced during *N. benthamiana* defense response against this necrotroph. *NbPR-1* expression was induced at 24 hpi and increased at 48 hpi (Fig. 6d). As anticipated, the TRVSPT leaves showed a lower expression of *NbPR-2* and *NbPR-3* genes early (8 and 24 hpi) during infection, compared to the inoculated TRV leaves (Fig. 6d) which correlated with its compromised resistance to *A. alternata* f. sp. *lycopersici* infection. In contrast, high transcript levels of *NbPR-1* gene were observed at 8 hpi in TRVSPT leaves, whereas accumulation of this transcript occurred at 24 hpi in TRV leaves (Fig. 6d). This result could be explained by the fact that *NbPR-1* was constitutively expressed in TRVSPT plants due to SA accumulation. In order to test whether activation of the SA pathway observed in TRVSPT plants had an adverse effect on the JA-mediated response, we analyzed transcript levels of the JA-inducible *LOX* gene in *N. benthamiana* TRV and TRVSPT in response to methyl jasmonate (MeJA). TRV leaves showed a strong and dose-dependent induction of



**Fig. 6** VIGS of SPT compromised *Nicotiana benthamiana* resistance against *Alternaria alternata* f. sp. *lycopersici*. Excised leaves (**a**) and cut stems (**b**) from TRV and TRVSPT plants were inoculated with  $1 \times 10^3$  conidia. The inoculated tissues were kept in separate humid chambers at 22 °C, and photographed 10 days after inoculation (dai) in the case of leaves, and stems at 6 dai. Orange boxes close-up shown in leaves photographs at the right. Infection symptoms were observed only on tissues of TRVSPT plants. Controls inoculated with sterile water did not develop disease symptoms (not shown). **c** Quantitative analysis of spore production. Conidia were released from each stem, suspended in water 6 dai and counted in a hemacytometer. Values are mean of 40 stem pieces and are representative from two independent experiments. The asterisk denotes significant difference and was determined by Tukey's mean comparison test ( $p < 0.01$ ). **d** Time course analysis of pathogenesis-related (PR) gene expression by semi-quantitative RT-PCR in leaves of TRV and TRVSPT plants inoculated with *A. alternata* f. sp. *lycopersici* ( $1 \times 10^3$  conidia). NI corresponds to non-inoculated control leaves. *EF1α* transcript was used as control for normalization. Results are representative of three independent experiments

*LOX* expression, whereas in TRVSPT leaves a dose-dependent response was also observed but lower levels were detected (Fig. S5). Overall, these results indicate that

the misregulation of LCB homeostasis caused by VIGS of SPT LCB2 subunit and the activation of SA-dependent responses affected the expression of defense genes compromising *N. benthamiana* resistance to the non-host pathogen *Alternaria alternata* f. sp. *lycopersici*.

## Discussion

*N. benthamiana* phenotypic features associated to SPT-silencing

Because *NbLCB2* is a single-copy gene in the *N. benthamiana* genome (Gan et al. 2009), the VIGS strategy was highly effective as reflected in the dramatic phenotype observed using our pTRVSPT construct. The significant growth restriction and abnormal leaf development reproduces the phenotype observed by Takahashi et al. (2009), highlighting the importance of sphingolipids as structural and signaling molecules during plant growth and development (Pata et al. 2010; Zäuner et al. 2010). Furthermore, the phenotype caused by pTRVSPT is more severe than those previously reported for the same gene (Gan et al. 2009; Takahashi et al. 2009), and such differences might be due to the cDNA sequence cloned in the VIGS vector. While in the report by Takahashi et al. (2009) and this study, similar cDNA sequences corresponding to the 5'-cDNA end were used, Gan et al. (2009) employed a 303-bp cDNA from the 3'-end. The growth phenotype of *N. benthamiana* TRVSPT plants was also comparable to those observed in *Arabidopsis LCB1* RNAi-suppressed lines and in *LCB2* knock-out mutants that show altered leaf morphology and severe growth restriction, the latter associated to an impaired cell expansion. Therefore, as suggested, plants might adjust their growth mechanisms to compensate the reduction of sphingolipid biosynthesis (Chen et al. 2006; Dietrich et al. 2008). The aberrant shape of leaves from *N. benthamiana* TRVSPT plants also denotes an impaired regulation of cell growth and polarity establishment. Proteomic studies have revealed an enrichment of some polarity-related proteins in lipid rafts such as PIN1, SKU5, and COBRA, suggesting a role for these domains in the polar expansion of plant cells (Willemsen et al. 2003; Mongrand et al. 2004; Borner et al. 2005). Moreover, recent evidence has emerged on sphingolipids involvement in a secretory pathway for polar auxin transport. Disruption of sphingolipids biosynthesis in tobacco BY2 cultured cells and *Arabidopsis* seedlings by FB1 alters the polar localization of the auxin transporter PIN1 to the plasma membrane (Aubert et al. 2011; Markham et al. 2011).

Silencing efficiency of our construct is also highlighted by the dramatic effect observed on flower development that

was not described before in previous reports of *N. benthamiana* SPT-silenced plants. These results reinforce previous evidence found in *Arabidopsis* on the essential role of sphingolipid biosynthesis during reproductive development. The expression of *Arabidopsis LCB2a* gene is higher in the flower buds and mature flowers compared with other organs such as leaves and stem, whereas null mutants of the two functional *LCB2* genes display gametophytic lethality and loss of pollen viability (Dietrich et al. 2008). Similarly, the *sbh1 sbh2* double mutant (affected on LCB C-4 hydroxylase genes) does not progress from vegetative to reproductive growth (Chen et al. 2008).

Another observed feature of the *N. benthamiana* TRVSPT plants phenotype was the presence of spontaneous cell death lesions, which coincides with previous reports linking sphingolipid biosynthesis and metabolism to the regulation of PCD. Inhibition of sphingolipid biosynthesis by SAMs causes cell death in several plant species (Abbas et al. 1994; Lamprecht et al. 1994; Stone et al. 2000; Saucedo-García et al. 2011). These observations are consistent with the fact that in *Arabidopsis*, several mutations affecting sphingolipid metabolism such as *acd5*, *acd11*, and *erh1* that affect a ceramide kinase, a sphingosine transporter and an inositol-phosphoryl-ceramide synthase, respectively, cause cell death as well (Brodersen et al. 2002; Liang et al. 2003; Wang et al. 2008). Importantly, several lines of evidence support that PCD activation is mediated by increased LCB levels. Spraying or infiltration of *Arabidopsis* and *N. benthamiana* leaves with sphinganine, sphingosine, or phytosphinganine, or the addition of sphinganine to tobacco BY2 cultured cells promote cell death (Shi et al. 2007; Takahashi et al. 2009; Lachaud et al. 2010). In accordance with these findings, overexpression of SPT LCB2 subunit in *N. benthamiana* causes cell death, probably due to accumulation of LCBs (Takahashi et al. 2009). Conversely, mutations in *Arabidopsis LCB1* or *LCB2a* genes, or the inhibition of SPT activity with myriocin, attenuate FB1-induced LCBs accumulation, and prevent cell death (Shi et al. 2007; Saucedo-García et al. 2011).

#### Silencing of the SPT LCB2 subunit in *N. benthamiana* significantly affects LCB homeostasis

As described above, two previous studies have reported VIGS of SPT LCB2 subunit in *N. benthamiana* (Gan et al. 2009; Takahashi et al. 2009). However, both of them lacked or were unsuccessful in analyzing LCBs levels. This is understandable given the diversity and low levels of these metabolites found in plant tissues. Indeed, as a first attempt, we tried to determine free LCBs in TRV and TRVSPT plants, but they were below detection limits. For

this reason, we performed sphingolipid hydrolysis and analyze the NDA derivatives of the released LCBs. Because SPT is the enzyme that catalyzes the first reaction in sphingolipid biosynthesis, we hypothesized that VIGS of the LCB2 subunit caused a general reduction in LCBs levels, especially of sphinganine, which is the first LCB synthesized and is a substrate for desaturases and hydroxylases to generate the other LCBs (Chen et al. 2009). However, we found that while the trihydroxylated LCB phytosphingosine, which is quite abundant, decreased 50 %, levels of the dihydroxylated LCBs sphinganine and sphingosine increased considerably. This differential effect of SPT-silencing on LCBs profile revealed the complexity of the LCBs metabolism and regulation, and is in agreement with the results from Chen et al. (2006). In *Arabidopsis*, reduction of SPT activity in *LCB1*-RNAi plants caused large increases (approximately fourfold) in the total content of sphinganine (and also a 2.5-fold increase of phytosphingosine), while some trihydroxylated LCBs such as the phytosphinganine isomers (t18:1-*E* and t18:1-*Z*) decreased. These results could be attributed to differences in LCB levels among taxonomic groups, as reported by Markham et al. (2006). Interestingly, the elevation of sphinganine and sphingosine levels in *N. benthamiana* TRVSPT plants partially mimics the effect of SAMs in de novo biosynthesis of sphingolipids, but this interesting similarity must be confirmed by further sphingolipidomic analyzes.

#### LCBs act as signal transducers in SA pathway

Because both, LCBs and SA have a key role in regulating cell death and plant immune response [reviewed by Berkey et al. (2012), Vlot et al. (2009), An and Mou (2011)], we hypothesized that they may share signaling pathways. Moreover, all the phenotypic characteristics associated with VIGS of SPT in *N. benthamiana* plants such as growth reduction, cell death, and susceptibility to a necrotroph could be associated with SA accumulation. A primary function of phytohormones is the regulation of plant growth and development, and interestingly, many *Arabidopsis* mutants with SA accumulation develop a dwarf phenotype like *N. benthamiana* TRVSPT plants (Rivas-San Vicente and Plasencia 2011). Furthermore, we recently found that FB1 and sphinganine induce a dose-dependent accumulation of free SA in germinating maize embryos (de la Torre-Hernández et al. 2010). The comparison of SA levels between TRV and TRVSPT plants indicated that indeed the latter had a discrete but significant elevation of total SA which correlated with a strong constitutive expression of *NbPR-1* gene. Likewise, the *Arabidopsis* mutants *acd5*, *acd11*, and *erh1*, which have an altered



sphingolipid metabolism, contain elevated (3- to 80-fold increase) SA levels and strongly express *AtPR-1* (Greenberg et al. 2000; Brodersen et al. 2005; Wang et al. 2008). Taken together, these evidences strongly suggest that an increase of sphingoid intermediaries, especially LCBs, activate the SA-dependent pathway. Then, in order to define if the LCB elevation observed in TRVSPT plants was responsible of the activation of the SA pathway, we treated *N. benthamiana* wild-type seedlings with FB1 or sphinganine and determined *NbPR-1* transcript levels. This gene, the canonical marker of SA signaling (Mur et al. 2006; Spoel et al. 2007; Koornneef et al. 2008; Denoux et al. 2008), showed an induced expression upon exposure to both compounds. Moreover, the same effect has been observed in *Arabidopsis*, indicating that LCBs positively regulate the SA-dependent pathway. *Arabidopsis* wild-type seedlings treated with SAMs (FB1 and AAL-toxin) show a dose-dependent induction of *PR-1* expression and other defense-related genes (Stone et al. 2000; Gechev et al. 2004). In tobacco BY2 cells, sphinganine (25  $\mu$ M) rapidly induces the expression of the defense genes *NtPR-3* and *NtMYB2*, but not of *NtPR-1* (Lachaud et al. 2011). In the latter case, *NtPR-1* expression could have not been detected because the cells were harvested 15 min after sphinganine treatment, which could be a very short time to activate SA signaling. Our results indicate that *NbPR-1* expression is induced after 24 h, and this time coincides with the induction caused by SAMs in *Arabidopsis* (Stone et al. 2000; Gechev et al. 2004).

It is worth mentioning that even though most *Nicotiana* species are resistant to *Alternaria alternata* f. sp. *lycopersici* and insensitive to SAMs (determined by a root elongation assay in presence of 0.2  $\mu$ M AAL-toxin; Brandwagt et al. 2001), when higher FB1 doses were used, inhibition of root elongation and hypocotyl expansion on *N. benthamiana* seedlings was recorded, a very similar effect to the species with intermediate SAM sensitivity (Brandwagt et al. 2001). These observations are also comparable with the FB1-induced growth restriction reported on maize, tomato (Lamprecht et al. 1994), and *Arabidopsis* (Stone et al. 2000; Shi et al. 2007; Markham et al. 2011; Saucedo-García et al. 2011; Watanabe and Lam 2011). However, unlike the aforementioned species, *N. benthamiana* seedlings exposed to FB1 did not develop symptoms of chlorosis or cell death. In this sense, it is important to mention that the tomato gene *Asc-1* that mediates insensitivity to SAMs and resistance to *Alternaria alternata* f. sp. *lycopersici* (Gilchrist and Grogan 1976) is conserved in most *Nicotiana* species (Brandwagt et al. 2001). *Asc-1* is a single codominant gene that codes an enzyme with ceramide synthase activity that is not effectively inhibited by SAMs, therefore LCB accumulation induced by AAL-toxin in resistant (*Asc/Asc*) tomato leaves is discrete compared to

that caused in susceptible (*asc/asc*) leaves (Spassieva et al. 2002). The same could happen in *Nicotiana* sp. because even though tobacco is SAMs insensitive to low doses of AAL-toxin (Brandwagt et al. 2001), 1  $\mu$ M FB1 causes an approximately tenfold increase in sphinganine levels in BY-2 cultured cells (Aubert et al. 2011).

#### Compromised resistance of *N. benthamiana* SPT-silenced plants (TRVSPT) to the necrotrophic fungi *Alternaria alternata* f. sp. *lycopersici*

The involvement of SPT in non-host resistance was illustrated with the compromised resistance in *N. benthamiana* *LCB1*- and *LCB2*-silenced plants against the bacterial pathogen *P. cichorii* (Takahashi et al. 2009). Consistent with this finding, our SPT-silenced plants were more susceptible to the non-host necrotrophic fungi *A. alternata* f. sp. *lycopersici*, despite the presence of a functional *Asc* gene that confers resistance against this pathogen (Brandwagt et al. 2001). Because TRVSPT plants had altered levels of LCBs, in particular elevation of sphinganine resembling the effect of SAMs, we reasoned that this fact would generate a favorable growth condition for the necrotrophic pathogen. In order to gain more insight into the mechanisms operating in this compatible interaction, we evaluated the expression of *NbPR-1*, *NbPR-2*, and *NbPR-3* genes that are part of the early defense responses and have a key role in limiting pathogen entry, growth, and spread. The induction pattern of expression of these defense genes in TRVSPT leaves in response to the fungal infection showed important alterations compared to TRV leaves. Lower levels of *NbPR-2* and *NbPR-3* transcripts were observed in TRVSPT leaves that correlated with the compromised resistance against *A. alternata* f. sp. *lycopersici*. The production of enzymes capable of degrading the cell walls of invading fungi ( $\beta$ -1,3-glucanases and chitinases coded by *NbPR-2* and *NbPR-3* genes) and the timing of this defense mechanism is an important component of plant disease resistance. Their role in plant immune response has been examined by their overexpression in transgenic plants that exhibit enhanced resistance to fungal infection and delayed disease symptoms (Jach et al. 1995; Dana et al. 2006). Furthermore, in vitro and in vivo experiments have demonstrated that the antifungal effect of PR-2 and PR-3 proteins is synergistically enhanced when both enzymes are present (Lawrence et al. 1996).

Constitutive expression of *NbPR-1* induced by the accumulation of SA in TRVSPT plants (scarcely observed under semi-quantitative RT-PCR conditions) possibly led to an earlier induction in response to fungal infection, compared to TRV plants. This constitutive activation of the SA-dependent pathway in TRVSPT plants could be crucial in the susceptibility to *A. alternata* f. sp. *lycopersici* as



phytohormone signaling plays a major role in determining the outcome of plant–pathogen interactions (Spoel and Dong 2008; Pieterse et al. 2009, 2012; Robert-Seilaniantz et al. 2011). A very tempting possibility is that the SAM-producing fungal necrotrophs promote LCBs elevation not only to induce the death of their hosts, but also to exploit their association with the SA-dependent pathway to antagonize or suppress the effective defense mechanisms activated by other phytohormones such as JA. In the most simplified models of hormone crosstalk in plant immunity, JA and ET work synergistically in resistance against necrotrophs, whereas SA pathway often antagonizes the JA-dependent defense responses (Spoel et al. 2007; Koornneef et al. 2008; Leon-Reyes et al. 2009, 2010; El Oirdi et al. 2011). Activation of the SA pathway in *Arabidopsis* (either by biotic stress or exogenous treatment) renders leaves more susceptible to *Alternaria brassicicola*, which exhibit severe disease symptoms. This correlates with a strong expression of *PR-1* and a decreased expression of the JA-inducible *PDF1.2* gene (Spoel et al. 2007). It has also been shown that low doses of SA are enough to trigger a fast and long-lasting antagonistic effect on defense genes regulated by JA (Koornneef et al. 2008). Notably, this also occurs in *Solanaceae* as SA-pretreatment (50  $\mu$ M) of tomato leaves increases *Botrytis cinerea* infection and significantly lowers the expression of the JA-regulated *PI-I* and *PI-II* defense genes. Moreover, an aggressive *B. cinerea* isolate (B191) secretes an exopolysaccharide that promotes a twofold accumulation of SA in the host to suppress the JA pathway required for resistance (El Oirdi et al. 2011). Our TRVSPT plants showed activation of the SA pathway which might potentially suppress the JA-mediated response as determined by transcript levels of the *LOX* gene. Supporting the interpretation behind the experimental evidence with these fungi, there are many other cases reported on pathogens that take advantage of the phytohormone signaling crosstalk to suppress host defenses (Pieterse et al. 2009; Robert-Seilaniantz et al. 2011).

It is very probable that complex interactions among the hormonal pathways operate in the susceptibility of *N. benthamiana* TRVSPT plants to *A. alternata* f. sp. *lycopersici*, and future work will unravel whether the increased SA levels antagonize JA- and ET-dependent defense responses, and SA interaction with other hormones. Significantly, there is evidence that JA and ET are involved in the cell death triggered by this necrotroph in tomato (Egusa et al. 2009; Zhang et al. 2011), and also in the *Arabidopsis* and tomato cell death induced by FB1 and AAL-toxin, respectively (Asai et al. 2000; Gechev et al. 2004; Mase et al. 2012).

In summary, the work presented here provides evidence that alterations in the sphingolipids de novo biosynthesis that results in elevated LCB levels activate the SA

signaling pathway as a downstream element. In this context, the SAMs produced by *F. verticillioides* and *A. alternata* f. sp. *lycopersici* would be displaying a double role: disruption of the host sphingolipid metabolism and activation of the SA pathway in order to divert defense responses mediated by other plant hormones. Together, both strategies would assure a successful invasion into the host.

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