The subtilisin-like protease SBT3 contributes to insect resistance in tomato

Michael Meyer1, Franziska Huttenlocher1, Anna Cedzich1, Susanne Procopio1, Jasper Stroeder1, Corinne Pau-Roblot2, Michelle Lequart-Pillon2, Jérôme Pelloux2, Annick Stintzi1 and Andreas Schaller1*

1 Institute of Plant Physiology and Biotechnology, University of Hohenheim, 70593 Stuttgart, Germany
2 EA3900-BIOPI Biologie des Plantes et Innovation, Université de Picardie, 80039 Amiens, France

* Correspondence: andreas.schaller@uni-hohenheim.de

Received 17 April 2016; Accepted 13 May 2016

Editor: Robert Hancock, The James Hutton Institute

Abstract

Subtilisin-like proteases (SBTs) constitute a large family of extracellular plant proteases, the function of which is still largely unknown. In tomato plants, the expression of SBT3 was found to be induced in response to wounding and insect attack in injured leaves but not in healthy systemic tissues. The time course of SBT3 induction resembled that of proteinase inhibitor II and other late wound response genes suggesting a role for SBT3 in herbivore defense. Consistent with such a role, larvae of the specialist herbivore Manduca sexta performed better on transgenic plants silenced for SBT3 expression (SBT3-SI). Supporting a contribution of SBT3 to systemic wound signaling, systemic induction of late wound response genes was attenuated in SBT3-SI plants. The partial loss of insect resistance may thus be explained by a reduction in systemic defense gene expression. Alternatively, SBT3 may play a post-ingestive role in plant defense. Similar to other anti-nutritive proteins, SBT3 was found to be stable and active in the insect’s digestive system, where it may act on unidentified proteins of insect or plant origin. Finally, a reduction in the level of pectin methylesterification that was observed in transgenic plants with altered levels of SBT3 expression suggested an involvement of SBT3 in the regulation of pectin methylesterases (PMEs). While such a role has been described in other systems, PME activity and the degree of pectin methylesterification did not correlate with the level of insect resistance in SBT3-SI and SBT3 overexpressing plants and are thus unrelated to the observed resistance phenotype.

Key words: Manduca sexta, pectin methylesterase, proteinase inhibitor, subtilase, systemin, wound signaling.

Introduction

Plant subtilases (SBTs) constitute a large family of mostly extracellular proteases of unknown function. Among SBTs are enzymes with relaxed substrate specificities that are thought to be responsible for non-selective protein turnover. Examples of these catabolic SBTs include cucumisin, an abundant protease in the juice of melon fruits (Kaneda and Tominaga, 1975; Yamagata et al., 1994), and related enzymes from fruits and latices of many other plant species (Schaller et al., 2012). SBTs also include proteases that are highly specific and are thus expected to contribute to the processing of selected target proteins by limited proteolysis at well-defined cleavage sites (Schaller et al., 2012). The prime example for such a processing SBT is SBT6.1 from Arabidopsis thaliana. AtSBT6.1, the ortholog of mammalian site-1-protease (SIP),
initiates the transduction of stress signals from the endoplasmic reticulum to the nucleus by specific cleavage and activation of bZIP transcription factors (Liu et al., 2007a, b). Additional substrates of AtSBT6.1 include the precursor proteins of a peptide growth factor (Rapid Alkalization Factor 23; Srivastava et al., 2009) and pectin methylesterases (Wolf et al., 2009). Processing by AtSBT6.1 occurs at canonical SIP cleavage sites, typically characterized by the amino acid motif RRXL or RLX (with X representing any amino acid).

As another example of highly specific SBTs, phytaspases from tobacco and rice hydrolyse typical caspase substrates after the invariant aspartate residue, showing highest activity with the tetrapeptide VEID (Chichkova et al., 2010). Like caspases in animal systems, phytaspases are involved in the regulation of programmed cell death, but their mode of action and physiological substrates are still unknown (Vartapetian et al., 2011). This is true for the vast majority of plant subtilases including most of the 56 family members in Arabidopsis (Rautengarten et al., 2005; Schaller et al., 2012). Among the Arabidopsis subtilases, AtSBT1.1, AtSBT3.5 and AtSBT5.2 were implicated in the processing of phyto-sulfokines (Srivastava et al., 2008), pectin methylesterase 17 (Sénéchal et al., 2014a) and epidermal patterning factor 2 (Engineer et al., 2014), but specific processing of any of these potential substrates by cognate subtilases remains to be shown in a physiological context.

As compared with the still rudimentary knowledge of their physiology, our understanding of structure and biochemistry of plant SBTs is quite advanced (Schaller et al., 2012; Schaller, 2013). This is particularly true for cucumisin and tomato SBT3, which can be regarded as prototypical plant SBTs sharing the functional domains that are typically found in most of the family members (Ottmann et al., 2009; Murayama et al., 2012). In addition to the well-conserved subtilisin-like catalytic domain, they comprise a cleavable N-terminal signal peptide for targeting of the nascent polypeptide to the secretory pathway, a pro-domain acting as a potent inhibitor of its mature enzyme, the protease-associated (PA) domain typically found in plant SBTs as a large insertion between the His and Ser residues of the catalytic triad, and a C-terminal fibronectin III-like domain. Both enzymes undergo extensive post-translational modifications including glycosylation, disulfide bond formation, and proteolytic processing of the prodomain, which was shown to be an auto-catalytic process in SBT3 and a prerequisite for enzyme maturation and passage through the secretory pathway (Czedzich et al., 2009; Ottmann et al., 2009; Nakagawa et al., 2010; Murayama et al., 2012).

Mature SBT3 and cucumisin show similar substrate specificity, as both enzymes prefer basic peptide substrates with additional selectivity for the P1 and P2 residues (the two amino acids immediately upstream of the scissile bond; Schechter and Berger, 1967). However, the specific amino acid requirements in these positions differ for the two enzymes. SBT3 shows a strong preference for Gln at P1. While Gln is also accepted by cucumisin, Leu, Asn and particularly Met are also tolerated in this position. The P2 preference is Lys for SBT3 and Pro for cucumisin (Yonezawa et al., 2000; Czedzich et al., 2009).

PA domain function was revealed by crystal structure analysis and found to differ for the two enzymes. In cucumisin, the PA domain is located close to the active site channel and appears to contribute to substrate selectivity (Murayama et al., 2012). In SBT3, on the other hand, the PA domain is required for dimerization and enzyme activity, as it keeps the active site channel open and accessible for potential substrate molecules (Ottmann et al., 2009). However, structural modeling of representative Arabidopsis subtilases indicated that PA domain-mediated dimerization and enzyme activation are unlikely to be a general property of all plant SBTs, since residues that were found to be important for dimerization and an auto-inhibitory β-hairpin are only partially conserved in the Arabidopsis SBT family (Rose et al., 2010). Consistent with this notion, the monomeric state was found to be the predominant form of cucumisin in solution (Murayama et al., 2012).

The C-terminal fibronectin III-like domain is present in both enzymes and likely required to stabilize the catalytic domain (Schaller, 2013). Thermal stability is in fact remarkable for both enzymes, even more so for cucumisin, likely because of its more compact structure as compared with SBT3 (Ottmann et al., 2009; Murayama et al., 2012). Interestingly, both enzymes lack calcium, and the ability to maintain stability of the subtilisin fold in the absence of calcium appears to be a distinguishing feature of plant SBTs as compared with homologs from other organisms (Rose et al., 2010).

In this study we investigate the physiological role of SBT3. Taking the leads from previous biochemical analyses, we addressed the hypothesis that SBT3 might be involved in plant defense against insect herbivores. Such a role had been suggested by the observations that (i) the wound signaling peptide systemin is cleaved by SBT3 (Czedzich et al., 2009), (ii) the remarkable stability of plant SBTs that renders them insensitive to the adverse conditions within the insect’s digestive system (Chen et al., 2005, 2007), and (iii) the ability of plant SBTs to modulate the activity of pectin methylesterases (PMEs; Wolf et al., 2009; Sénéchal et al., 2014a; Taurino et al., 2014), which have been implicated in insect resistance (Körner et al., 2009; Dixit et al., 2013).

We report that the expression of SBT3 is induced by wounding and herbivory and that SBT3 expression levels correlate with resistance against tobacco hornworm larvae, a specialist herbivore on tomato. Addressing a potential involvement of SBTs in the control of PME activity and pectin methyl-esterification, we observed changes in cell wall composition in transgenic plants with altered SBT3 expression levels that, however, were not linked to the altered resistance phenotype. A potential role of SBT3 in systemin processing and wound signaling was supported by attenuated expression of systemic wound response genes, and may in part explain improved performance of insect larvae on plants silenced for SBT3 expression. Alternatively, the defensive role of SBT3 may rely on its post-ingestive activity in the insect gut.
Materials and methods

**Growth of tomato plants, wounding and insect bioassays**

Tomato plants (cv. UC82B) were grown in the greenhouse at a 16-h photoperiod with supplemental light and a 26 °C/18 °C day/night temperature regime. Plants were fertilized at weekly intervals (GABI plus 12-8-11; N, P, K fertilizer at 2 ml l⁻¹). Experimental plants, as opposed to those that were grown for seed propagation, were excluded from phytosanitary procedures.

*Manduca sexta*, Johannson (Lepidoptera: Sphingidae) was cultured as described (Bosch et al., 2014b). Feeding assays on artificial diet (0.12 g pellets of Gipsy Moth Wheat Germ Diet; MP Biomedicals; Eschwege, Germany) supplemented with test proteins (SBT3 or BSA, 100 μg g⁻¹ fresh weight) were performed with freshly hatched *M. sexta* larvae. Food pellets were changed twice a day, and larval weight was determined on days 3, 6, 8, 9, and 10. Addressing herbivory-induced changes in gene expression, early fifth-instar larvae were allowed to feed on a single leaf of 2- to 3-week-old tomato plants for about 15 min, until a leaf area of about 1.5 cm² had been consumed. Wounded and unwounded systemic leaves were harvested at the indicated time points and flash-frozen in liquid N₂. The pooled leaf material of five plants was used for RNA extraction. For mechanical wounding, a hemostat was used to crush the terminal leaflet of a single leaf across the central vein. To analyse insect performance, 150 3-day-old *M. sexta* larvae were put on 6-week-old plants of each of the tomato genotypes. The plants were exchanged as needed when most of the leaf material had been consumed. The experiment was terminated when the first larvae reached the wandering stage.

**Transgenic tomato plants**

For silencing of *SBT3* expression, a hairpin construct was generated (primer sequences are given in Supplementary methods at JXB online) comprising 219 bp of the tomato (*Solanum lycopersicum*) cDNA (nucleotides 520–718; acc. AJ006376) in sense and antisense orientations in pHANNIBAL (Wesley et al., 2001). The entire expression cassette comprising the cauliflower mosaic virus (CaMV) 35S promoter, the SBT3-hairpin and the OCS terminator was cut out with NotI and transferred into pART27 (Gleave, 1992). Transgenic tomato plants silenced for *SBT3* expression (SBT3-SI plants) were generated as described (Bosch et al., 2014b). Stable integration of the transgene and independence of transformation events was confirmed by Southern blot analysis (see Supplementary Fig. S1B). Silencing of *SBT3* expression was confirmed at the transcript level by RT-PCR, and at the protein level by western blot analysis (Supplementary Fig. S2). Homozygous plants of the T3 generation were used in all experiments.

For *SBT3* overexpression, the open reading frame (ORF) was PCR-amplified from the SBT3 cDNA and cloned into the *Sma*I and *Pst*I sites of pH51 (Pietrzak et al., 1986). The expression cassette comprising the CaMV 35S promoter, the SBT3 ORF and the 35S terminator was then moved from pH51 into the *Eco*RI site of pRD400 (acc. U93965) and transformed into tomato plants as before (see Supplementary Fig. S1A). *SBT3* expression levels were analysed by RT-PCR and western blot, and three independent *SBT3-OX* lines were chosen for further analysis (Supplementary Fig. S2). Homozygous plants from the T2 or T3 generation were used in all experiments.

The *SBT3* promoter:reporter gene construct was generated in pBI101 (acc. U12639) comprising a promoterless β-glucuronidase (GUS) cassette and the NOS-terminator in pBIN19. A 1956-bp PCR product amplified from the *SBT3* gene (Michalety et al., 1999; acc. AJ006380), upstream of and including the translation start codon was fused with the GUS ORF using the *SalI*/BamHI sites of pBI101. Tomato plants were transformed as before and four independent lines with single T-DNA insertions were confirmed by Southern blot analysis and used for further analysis (see Supplementary Fig. S3).

**RNA extraction and quantitative reverse transcription-PCR analysis**

Samples of wounded and systemic tomato leaves were flash-frozen in liquid nitrogen and ground to a fine powder. Approximately 500 μg of total RNA was extracted in 500 μl ofpeqGOLD Trifast (PEQLAB GmbH; Erlangen, Germany) according to the manufacturer’s instructions with an additional chloroform extraction step to increase RNA purity. RNA was quantified spectrophotometrically and only RNA with a 260/280 nm ratio of 1.8 or higher was used for reverse transcription. RNA integrity was checked on 1% denaturing formaldehyde gels (for every fifth sample). Two micrograms of total RNA was used for first-strand cDNA synthesis with RevertAid reverse transcriptase (Thermo Scientific; Braunschweig, Germany) and random hexamer primers (Thermo Scientific). No-RT controls (omitting the reverse transcription step) were performed on every seventh sample with reference gene primers to exclude genomic DNA contamination. cDNAs were diluted 1:10 in water and used for qPCR analysis. Tomato elongation factor 1α (*EF1α*, acc. no. X14449) and ubiquitin (*UBI3*, X58253) were used as internal reference genes and specific primer pairs were used to detect expression of *SBT3* (AJ006376), *OPR3* (AJ278332), *Lcox* (U37840), *PI-II* (K30291), and *LapA* (U30151). Primer efficiencies and optimal primer concentration were determined experimentally, qPCR was performed with Taq DNA polymerase expressed in and purified from *E. coli* and SYBR-Green (Cambrex Bio Science Rockland Inc.; Rockland, ME, USA) in a Bio-Rad CFX Connect real-time PCR system (Bio-Rad; Munich, Germany) using 40 cycles of 95 °C for 30 s, 62 °C for 30 s, and 72 °C for 40 s, followed by a melting curve protocol from 58 °C to 95 °C to confirm uniformity of PCR products. PCR reactions contained target gene primers at the indicated concentrations and 200 μM dNTPs in 3 mM MgCl₂, 20 mM (NH₄)₂SO₄, 0.016% Triton X-100, 2% DMSO, 50 mM KCl, 10 mM Tris/HCl pH 8.3, 0.08% Tween 20. For data analysis, the following equation (Pfaffl, 2001) was used to calculate the relative fold change in mRNA levels of target genes normalized against two reference genes:

\[
\text{ratio} = \frac{E_{\text{target}} \delta C_{\text{target (control-sample)}}}{E_{\text{ref}} \delta C_{\text{ref (control-sample)}}}
\]

The changes in mRNA expression are shown relative to the expression level in leaf material pooled from six wild-type plants prior to wounding.

**Northern and Southern blot analysis**

For RNA gel blots, total RNA was extracted from tomato leaf samples (0.3 g) using a phenol-based standard protocol. The RNA (4.5 μg) was separated on formaldehyde–agarose gels, and transferred to nitrocellulose membranes. For DNA gel blots, genomic DNA was isolated from tomato leaves using a standard cetyltrimethylammonium bromide (CTAB)-based extraction procedure. Ten micrograms of DNA were digested with the enzymes indicated in the respective figures, separated by agarose gel electrophoresis and transferred to nitrocellulose membranes. PCR-amplified fragments of the *SBT3* and *PI-II* cDNAs and the nptII (acc. number: YP_788126) gene were used as probes. RNA and DNA blots were hybridized with the radiolabelled probes and analysed on a phosphoimagier as described (Schaller and Oecking, 1999).

**Alkalization assay for systemin activity**

The *SBT3* overexpression construct was transformed into a *Solanum peruvianum* cell culture (kindly provided by Georg Felix and Thomas Boller) by particle bombardment as described previously (Cedzich et al., 2009). Suspension cell cultures were established for selected cell lines and continuous measurements of extracellular pH were performed in 5 ml of cultured cells 6–8 days after subculture.
Synthetic systemin peptide (Pepmic; Suzhou, China) was added from a 1000-fold concentrated stock solution in water.

Proteinase inhibitor assay

Four-week-old tomato plants were mechanically wounded with a hemostat across the midvein and a second time 1 h later parallel to the midvein of the second and third primary leaflets. At each time point, the leaf material of five plants of each genotype (SBT3-OX, SBT3-SI, UC82B wild-type control) was harvested, weighed, frozen in liquid N\textsubscript{2}, and stored at −80 °C. The samples were ground in liquid N\textsubscript{2} and total protein was extracted in 3 ml extraction buffer (50 mM Tris/HCl, pH 7.8, 7% (v/v) polyvinyl pyrrolidone (PVPP); 1.67 mM phenylthiourea; 0.3 M KCl; 0.4 M ascorbic acid) per gram fresh weight. The extracts were cleared by centrifugation (16 000×g, 30 min, 4 °C). Chymotrypsin (0.1 mg ml\textsuperscript{-1} in 0.001 M HCl; 100 μl) was added to 100 μl of the supernatant. After 10 min at room temperature, residual chymotrypsin activity was analysed by addition of 1 ml reaction buffer (66 mM Tris/HCl, pH 7.8, 80 mM CaCl\textsubscript{2} and 300 μl N-benzylo-L-tyrosine p-nitroanilide (1 mg ml\textsuperscript{-1} in DMSO). The release of p-nitroaniline was monitored spectrophotometrically (Varian Cary 100 Bio; Agilent Technologies; Waldbronn, Germany) at 405 nm. Plants were grown in fully randomized fashion and for each genotype and time point, three replicates (three groups of five pooled plants) were analysed.

Stability of SBT3 in *M. sexta* frass

Frass (feces) was collected from fifth-instar *M. sexta* larvae raised on the three different tomato genotypes (SBT3-SI, SBT3-OX, UC82B). Samples (100 mg) were ground in liquid N\textsubscript{2} and extracted in 200 μl 50 mM Tris/HCl pH 7.5, 100 mM NaCl, 10 mM β-mercaptoethanol, 0.5% (v/v) Triton X-100 and proteinase inhibitor mix (SERVA Electrophoresis GmbH, Heidelberg, Germany). Protein extracts were cleared by centrifugation (16 000×g, 10 min, 4 °C) and analysed on western blots using an SBT3 antiserum as described (Cedzich et al. 2009), and by zymography as detailed below.

Zymography

For extraction of midgut proteins, larvae were anesthetized with ethyl acetate, and a 1–2 cm piece of the midgut was dissected. Protein samples were extracted as described above, but 1 mM benzamidine, 0.01 mM pepstatin A, 1 mM EDTA and 0.1 mM leupeptin were added instead of the commercial proteinase inhibitor mix. Samples were mixed with sample buffer (10% (v/v) glycerol, 50 mM KOH/acetate pH 5.0, traces of methyl green) and separated by native PAGE (PAGE_Acidic.html, last accessed 30 May 2016). Gels co-polymerized with 0.5% (w/v) gelatin were run for 10 min prior to sample loading and then for 3 h at 4 °C in 350 mM β-alanine–140 mM acetic acid at 80 V with reversed polarity. Gels were washed in two changes of renaturation buffer (50 mM Tris/HCl pH 7.5, 2.5% (v/v) Triton X-100 including proteinase inhibitors as above) and then incubated overnight with gentle agitation in the same buffer with 2% (v/v) Triton X-100. Proteinase activity was visualized by Coomassie Brilliant Blue R250 staining as cleared bands in a blue background.

PME activity assay

PME activity was assayed as described (Klavons and Bennett, 1986) with minor modifications. Fifty milligrams of tomato leaf tissue ground in liquid N\textsubscript{2} was incubated for 1 h at 4 °C in extraction buffer (20 mM Na\textsubscript{2}HPO\textsubscript{4}, 20 mM citric acid, 1 M NaCl, 0.1% (v/v) Tween 20, 0.2% (w/v) PVPP, adjusted to pH 7.0) under shaking. After centrifugation (16 000×g, 30 min, 4 °C) the cleared supernatant was desalted by ultrafiltration (10 kDa MWCO, Vivaspin concentrators, Sartorius; Göttingen, Germany) using extraction buffer without PVPP and NaCl, and the protein concentration was determined.

PME activity was assayed in a total volume of 300 μl reaction buffer containing 1 μg protein, 100 μg pectin from citrus fruit (≥85% esterified, Sigma-Aldrich; Taufkirchen, Germany), 0.025 U alcohol oxidase (from *Pichia pastoris*, Sigma-Aldrich) in 50 mM sodium phosphate buffer pH 7.5. After 30 min at 28 °C, the reaction was stopped by the addition of the same volume of 2 M ammonium acetate, 19.5 mM acetylatedene and 49 mM acetic acid and incubated at 68 °C for 15 min. The absorbance was read at 420 nm against a blank to quantify PME activity as nmol methanol μg\textsuperscript{-1} protein min\textsuperscript{-1} using a reference curve of 0–175 nmol methanol.

In-gel assay of PME activity

Cell wall-enriched protein extracts were prepared as described above, added to 2× loading buffer (40 mM lysine, 40 mM arginine, 30% (v/v) glycerol) and separated by isoelectric focusing on Ready Gel* IFE Precast Gels (Bio-Rad) following the manufacturer’s recommendations. To visualize PME activity, the gel was washed for 30 min in 25 mM Tris/HCl pH 8.5, 5 mM EDTA and then incubated for 30 min in reaction buffer containing 20 mM Tris/HCl pH 7.6, 5 mM EDTA, 160 mM NaCl and 1% (w/v) pectin from citrus fruit (>80% esterified). After two more washing steps in 20 mM Tris/HCl pH 7.6, 5 mM EDTA and 160 mM NaCl, PME activity was detected by ruthenium red staining (0.01% (w/v); 15 min) of de-methylsterified pectin.

Analysis of cell wall composition

The degree of pectin methylsification (DM) and cell wall sugar composition was analysed in 6-week-old SBT3-OX, SBT3-SI and wild-type (UC82B) plants. For each analysis, three leaflets from the second oldest fully developed leaf were pooled from six plants (7–10 g fresh weight). Three biological replicates were analysed in duplicate for each of the three genotypes. The leaf material was ground in liquid N\textsubscript{2} and lyophilized. For cell wall extraction (Carpita et al., 2001), 100 mg of lyophilized powder was heated twice to 70 °C in absolute ethanol for 15 min and centrifuged. The pellet was solubilized in 1% (w/v) SDS in 50 mM Tris/HCl pH 7.2 and heated to 70 °C for 30 min. The cell walls were subsequently homogenized in phosphate buffer (100 mM KH\textsubscript{2}PO\textsubscript{4}, pH 6.8). Aliquots were digested with amylase and lyophilized. Monomeric neutral and uronic sugars were analysed by high performance anion exchange chromatography (HPAEC). The cell wall digests were hydrolysed by 4 M trifluoroacetic acid (100 °C, 4 h), the trifluoroacetic acid was removed under nitrogen, and the digests were then diluted with ultrapure water to 1 mg ml\textsuperscript{-1}. Sugars were analysed on an ICS3000 system with pulsed amperometric detection (HPAEC-PAD) (Dionex, Thermo Fisher Scientific; Illkirch, France) equipped with a CarboPac PA-1 column (ID 4 mm×250 mm) and guard column (ID 4 mm×50 mm) run at 1 ml min\textsuperscript{-1} and 30 °C column temperature. For neutral sugars the mobile phases were (A) H\textsubscript{2}O, (B) 160 mM NaOH and (C) 200 mM NaOH. Elution profiles were as follows: 0–25 min 90% A and 10% B, 25–26 min 0–100% C, 26–35 min 100% C, 25–36 min 100–0% C, 36–50 min 90% A and 10% B. For uronic acids, the mobile phases were (A) 160 mM NaOH and (B) 0.6 M NaOAc in 160 mM NaOH. Elution profiles were as follows: 0–5 min 100% A, 5–35 min 0–100% B, 35–40 min 100% B, 40–42 min 100–0% B and finally column re-equilibration in 100% A from 42 to 50 min. The injection volume was 25 μl. The monosaccharides arabinoose, fucose, galactose, glucose, rhamnose, xylose, galacturonic acid and glucuronic acid (Sigma-Aldrich) were used as standards.

DM was determined by quantification of methanol and acetate after saponification of pectin extracts. Pectins were dissolved in D\textsubscript{2}O (10 mg ml\textsuperscript{-1}), and a first ¹H NMR experiment at 80 °C was performed on a Bruker Avance 300 spectrometer (Bruker BioSpin SA; Wissembourg, France) in order to check the absence of free methanol and acetate. Fifty-five microliters of NaOD (1 M) in D\textsubscript{2}O was subsequently added into the NMR tube and a second ¹H NMR spectrum was performed. DM values were calculated as described.
Results and discussion

Expression of SBT3 is induced by wounding and insect herbivory

Addressing a potential function of SBT3 in herbivore defense, the expression of SBT3 and its response to the feeding of *M. sexta* caterpillars was analysed in leaves of tomato plants. On top of low-level constitutive expression, we observed a moderate but consistent increase in SBT3 mRNA abundance after insect feeding by northern blot analysis (Fig. 1A). Mechanical wounding resulted in a similar induction of the *SBT3* gene (Fig. 2A). The temporal pattern of *SBT3* induction was similar to that of *proteinase inhibitor II* (*PI-II*), a well-established marker for the ‘late’ wound response in tomato plants (Ryan, 2000). For the *PI-II* gene there was a strong increase after 8 h and a maximum of expression 12 h after the onset of insect feeding (Fig. 1A). Likewise after mechanical wounding, the kinetics of *SBT3* induction resembled those of *PI-II* and *Leucine Aminopeptidase A* (*LapA*), which was included as an additional late wound response marker (Fig. 2D, E). However, unlike *PI-II, LapA* and other systemic wound response genes that are induced in both the wounded and in distal unwounded leaves (Schaller et al., 1995; Bergey et al., 1996; Fowler et al., 2009), systemic induction was not observed for *SBT3* (Fig. 2A).

As compared with *SBT3*, *PI-II* and *LapA*, much faster induction was observed for *lipoygenase D* (*LoxD*) and *oxophytodienoate reductase 3* (*OPR3*), with highest transcript levels at 1 and 2 h after wounding. These genes code for enzymes of the jasmonate (JA) biosynthetic pathway and were used here as markers for the early wound response (Ryan, 2000). Consistent with previous observations (Strassner et al., 2002; Lee and Howe, 2003), there was very little, if any, induction of early genes in systemic leaves (Fig. 2B, C).

We conclude that the regulation of *SBT3* expression differs from previously described wound response genes. Slow induction in only the wounded tissue suggests that SBT3 activity may be required locally in late stages of induced herbivore defense in tomato.

Insect resistance is reduced in plants silenced for SBT3 expression

In order to address a possible function in insect resistance, transgenic tomato plants were generated over-expressing *SBT3* under control of the CaMV 35S promoter (*SBT3-OX*), or silenced for *SBT3* expression by RNA interference (*SBT3-SI*). Over-expression and silencing of *SBT3* were confirmed at the transcript and protein levels in several independent transformants (see Supplementary Fig. S2), and three *SBT3-OX* (OX-2, -18, -19) and SI lines (SI-12, -14, -21) were chosen for further analysis. The transgenic plants had wild-type appearance and did not show any visible defects in growth or development.

One hundred and fifty first-instar *M. sexta* larvae were allowed to feed on each of the three genotypes, *SBT3-OX*, *SBT3-SI* and wild-type plants, and larval growth was followed for 21 days until they were ready to pupate and entered the wandering stage. Differences in weight gain were first noticed on day 10, and started to be statistically significant on day 20 (Fig. 1B). Larvae gained weight faster in *SBT3-SI* lines as compared with wild type, and more so when compared with *SBT3-OX* plants (Fig. 1B). Even though slower growth on *SBT3-OX* plants was observed repeatedly in several
independent experiments, it was not statistically significant when compared with wild-type plants (Fig. 1B). However, the apparently reduced growth rate resulted in a longer time until pupation on SBT3-OX plants, with 11% entering wandering stage on day 21, as compared with 18 and 19% for those feeding on wild-type and SBT3-SI plants, respectively. A longer time until pupation increases the risk of predation in the field and is thus likely to affect the fitness of the herbivore (Feeny, 1976; Price et al., 1980).

Enhanced performance of M. sexta larvae on plants silenced for SBT3 expression supports a role for SBT3 in plant defense. Addressing the specific function of SBT3 in insect resistance, three possible modes-of-action were investigated. (i) A role in defense signaling was suggested by the observation that SBT3 is able to cleave the wound signaling peptide systemin (Cedzich et al., 2009). (ii) Pectin methylesterases (PMEs) have been implicated in insect resistance (Körner et al., 2009; Dixit et al., 2013). The recent finding

---

**Fig. 2.** Local and systemic induction of SBT3 as compared with early and late wound response genes. One leaf of 2-week-old wild-type (white bars) and SBT3-SI seedlings (grey bars) was wounded with a hemostat across the main vein of the terminal leaflet. At each time point after wounding, the damaged leaves (left) as well as the systemic unwounded leaves (right) of five plants were harvested and pooled for RNA extraction followed by qRT-PCR analysis. Transcript abundance of SBT3 (A), two ‘early’ genes (B: LoxD, C: OPR3) and two ‘late’ genes (D: PI-II, E: LapA) was normalized to UBI3 and EF-1α expression, and is given as fold change relative to healthy (0 h) wild-type leaves. Data represent the mean±standard error of three biological replicates using three different SBT3-SI lines (SI lines 12, 14, and 21).
of SBTs affecting PME activity (Wolf et al., 2009; Sénéchal et al., 2014; Taurino et al., 2014) thus suggested that changes in PME activity and pectin structure may be responsible for the insect resistance phenotype. Finally (iii), the remarkable stability of SBT3 and its high proteolytic activity at alkaline pH opened the possibility that the enzyme may exert its function only after ingestion, within the digestive system of the insect.

**Systemin processing**

Wounding of tomato plants triggers the release of systemin, an 18-amino-acid signaling peptide, from its precursor protein prosystemin (Pearce et al., 1991; McGurl et al., 1992). Systemin is then perceived at the cell surface by a leucine-rich repeat receptor-like kinase and induces the expression of genes for jasmonate (JA) biosynthesis to amplify JA accumulation at the site of wounding as a prerequisite for systemic defense gene induction (Ryan, 2000; Wasternack et al., 2006; Howe and Schaller, 2008). Systemin is a substrate of SBT3 in vitro and is cleaved specifically at Gln16, releasing the last two amino acids, Thr and Asp (Cedzich et al., 2009). These two residues were shown to be important for receptor binding and activation (Meindl et al., 1998; Scheer et al., 2003), and C-terminally truncated systemin peptides are inactive with respect to the induction of PI-II accumulation (Pearce et al., 1993). Cleavage by SBT3 therefore results in the inactivation of systemin suggesting a possible role for SBT3 as an attenuator of the wound response. Alternatively, if turnover of the peptide is required for continued signaling, cleavage by SBT3 might also augment the wound response.

Any function related to systemin processing would require co-localization of SBT3 and its putative substrate in vivo. Promoter:reporter (GUS) analysis was thus performed in transgenic tomato plants to assess the tissue-specific expression of SBT3. SBT3 promoter activity was first detected during early seedling development in the micropylar endosperm as well as in the developing root (Fig. 3A–C). GUS expression was also detected in the mature root system, particularly at the junction between primary and lateral roots (Fig. 3E). Potentially relevant with respect to systemin signaling is the expression in the shoot vasculature (Fig. 3D). GUS staining was observed in both external and internal phloem including...
sieve elements and companion cells, and also in the vascular (xylem and phloem) parenchyma (Fig. 3F, G). Interestingly, this is where the early wound response pathway appears to be located. The prosystemin gene is expressed in vascular bundles (Jacinto et al., 1997) and the protein was localized in parenchymatic cells of the phloem (Narváez-Vásquez and Ryan, 2004). Also present in the vasculature are the enzymes contributing to JA biosynthesis. Allene oxide synthase and allene oxide cyclase were located in the vascular parenchyma, companion cells and sieve elements of tomato stems, petioles and flower stalks (Hause et al., 2000, 2003). The apparent co-localization of SBT3 with the early wound response pathway in the tomato vasculature would be consistent with SBT3 being involved in systemin processing and wound signaling.

To address a potential role of SBT3 in the regulation of systemin activity we looked at early and late systemin responses in transgenic plants and cell cultures exhibiting different levels of SBT3 expression. Among the earliest cellular responses to systemin is the depolarization of the plasma membrane, which was found to be necessary and sufficient for the activation of downstream defense gene expression (Schaller and Oecking, 1999; Schaller and Frasson, 2001; Maffei et al., 2004; Mousavi et al., 2013). Concomitant ion movements include the influx of H\(^+\) and Ca\(^{2+}\) and the efflux of K\(^+\) and Cl\(^{-}\), resulting in the alkalinization of the extracellular space that can be measured conveniently in cell suspension cultures by continuous recordings of medium pH (Felix and Boller, 1995; Schaller, 1998). In tomato (S. peruvianum) cell cultures, systemin-triggered medium alkalinization is dose-dependent and saturated at concentrations above 1 nM (Vetsch et al., 2000). The response reaches its maximum within 15 min after addition of the peptide resulting in a pH increase of 0.8–1 (Vetsch et al., 2000). Systemin-triggered medium alkalinization was reduced in both amplitude and duration in transgenic cell cultures expressing increasing levels of SBT3 (Fig. 4A). The duration of the alkalinization response was previously shown to depend on the metabolic stability of the peptide inducer (Schaller, 1998). The correlation that was observed between SBT3 expression levels and the attenuation of the alkalinization response (Fig. 4A) is thus consistent with systemin being cleaved by SBT3 in the cell culture system.

In order to address a potential effect of the SBT3 expression level on the output of the systemin signaling pathway, the expression of early and late wound response genes was compared in leaves of SBT3-SI and wild-type tomato plants in a time series after wounding (Fig. 2). The transcripts of early (LoxD, OPR3) and late (PI-II, LapA) wound response genes showed similar induction kinetics and levels in the leaves of wounded SBT3 and wild-type plants (Fig. 2, left panels). We also analysed the induction of proteinase inhibitor activity over an extended time period, which reached its maximum after 2 days in wounded leaves with no significant difference between SBT3-OX and SBT3-SI transgenics as compared with wild-type tomato plants (Fig. 4B). Therefore, despite the apparent co-localization of SBT3 and prosystemin in the tomato vasculature (Fig. 3), and the ability of SBT3 to attenuate early responses to systemin treatment in the cell culture system (Fig. 4A), the local wound response was not affected by SBT3.

In contrast to the intact local response, systemic induction of late genes was clearly impaired in SBT3-SI plants (Fig. 2, right panels). Eight hours after wounding PI-II transcripts were induced 70-fold in systemic SBT3-SI leaves, as compared with 180-fold induction in corresponding wild-type

---

**Fig. 4.** Effect of SBT3 expression on early and late systemin responses. (A) Systemin-triggered alkalinization response. Medium pH was recorded in tomato (S. peruvianum) cell cultures after addition of 1 nM systemin at t=0 min. The alkalinization response was compared in three independent transgenic cell lines showing different levels of SBT3 expression as indicated by the western blot signal. (B) Induction of proteinase inhibitor activity in response to wounding. The inhibition of chymotrypsin activity after addition of plant extracts obtained from SBT3-OX (line G2, triangle), SBT3-SI (line 21, filled circles) and wild-type plants (open circles) at the indicated time points after wounding was analysed in triplicate (three biological replicates, each including the pooled leaf material of five plants) and is shown as the mean±standard deviation.
leaves. Similarly, the systemic induction of LapA transcripts at 8h after wounding was much lower in SBT3-SI (120-fold) than in wild-type plants (710-fold; Fig. 2D, E). The data indicate that SBT3 function is dispensable for the local response, while it is needed to achieve full induction of defense genes in systemic leaves.

Whether or not the apparent role of SBT3 in the systemic wound response depends on its ability to cleave systemin remains to be shown. Considering the late induction of SBT3 expression as compared with genes for JA biosynthesis (LoxD, OPR3), alternative functions downstream or independent of JA production seem more likely, e.g. for the exit of the systemic signal from the wound site, or as a suppressor of a negative regulator in systemic leaves. A JA-independent function in wound signaling has previously been described for LapA, for which the mechanism of action is also still unknown (Pautot et al., 1993; Fowler et al., 2009). Similar to what we observed for SBT3-SI plants, wound-induction of late genes is reduced in LapA-deficient plants, while the expression of early wound-response genes is unaffected. However, in contrast to SBT3, LapA affects both the local and the systemic induction of PI-II and other late genes (Fowler et al., 2009). Consequently, the impact on larval development is much stronger in LapA-SI (Fowler et al., 2009) as compared with what we observe in SBT3-SI plants (Fig. 1B). The stronger resistance phenotype in LapA-SI as compared with SBT3-SI plants is also consistent with recent finding showing that the local wound response is sufficient for defense against M. sexta. Comparing larval growth and development on wild-type tomato and transgenic plants impaired only in systemic signaling, it was found that defense gene induction in systemic tissues is not required to maintain wild-type levels of resistance against M. sexta (Bosch et al., 2014b).

We conclude that SBT3 affects the systemic wound response, which is not necessarily related to its ability to cleave the wound signal systemin. The reduced expression of defense genes in systemic tissues of SBT3-SI plants may explain the reduced resistance phenotype observed in these plants. However, because of the limited relevance of systemic defense responses for defense against M. sexta (Bosch et al., 2014b), alternative possibilities should be considered.

**Pectin methylesterase activity and cell wall composition**

Pectin methylesterases catalyse the de-methylesterification of homogalacturonan (HG), the major pectin constituent of the primary cell wall (Sénéchal et al., 2014b). Partially de-methylesterified (demethylated) HG may bind Ca²⁺ resulting in the formation of pectin gels increasing the rigidity of the cell wall. Alternatively, the cell wall may be weakened when partially demethylated HG is degraded by polygalacturonases or pectate lyases (Pelloux et al., 2007). In either case, the demethylation of HG by PMEs has dramatic consequences on the mechanical properties and digestibility of the cell wall (Peaucelle et al., 2011) and is thus likely to affect the performance of herbivorous insects (Körner et al., 2009; Calderón-Cortés et al., 2012).

In addition to changing cell wall mechanics, the demethylation of HG by PMEs releases substantial amounts of methanol, which may affect plant–insect interactions in multiple ways as it is directly toxic to larvae and may serve as a signal for direct and indirect plant defense responses (Körner et al., 2009; Dixit et al., 2013; Hann et al., 2014; Komarova et al., 2014; Sénéchal et al., 2014b). Indeed, insect feeding was reported to induce PME activity and methanol emission (Penuelas et al., 2005; von Dahl et al., 2006), and M. sexta larvae show a small but consistent increase in performance on transgenic plants silenced for PME expression (Körner et al., 2009), whereas on PME-overexpressing tobacco, the development of polyphagous insects is severely impaired (Dixit et al., 2013). PME activity thus appears to be positively correlated with insect resistance in tobacco. Interestingly, PME activity is controlled in part by SBTs and, therefore, SBTs may be expected to exert an indirect effect on insect resistance by modulating PME-mediated methanol emissions and cell wall composition.

Both positive and negative effects of SBTs on PME activity have been described. Type-I/group 2 PMEs are synthesized as inactive pre-pro-enzymes and SBTs (Arabidopsis SBTs 3.5 and 6.1) were found to be required for the processing of the inhibitory prodomain and secretion of mature PMEs into the cell wall (Pelloux et al., 2007; Wolf et al., 2009; Sénéchal et al., 2014a). Arabidopsis SBT1.7, on the other hand, appears to be involved in PME inactivation or degradation. In seeds of the SBT1.7 loss-of-function mutant, PME activity is increased with a concomitant reduction in HG methylation of seed mucilage, resulting in a failure to release mucilage upon hydration and a germination defect under low-water conditions (Rautengarten et al., 2008). A similar phenotype was reported for a PME inhibitor (PMEI6) mutant, and the importance of AtSBT1.7 and the inhibitor for down-regulation of PME activity and mucilage release was confirmed by the additive phenotype of the double mutant (Saez-Aguayo et al., 2013).

These findings prompted us to investigate whether the effect of SBT3 expression levels on M. sexta performance (Fig. 1B) can be explained by SBT3-mediated changes in PME activity and/or cell wall composition. An analysis of neutral and acidic sugar composition did not reveal any differences between wild-type and SBT3 transgenics (see Supplementary Fig. S4). PME activity, however, was substantially reduced in the foliage of SBT3-OX plants as compared with wild-type and SBT3-SI plants (Fig. 5A). Apparently, SBT3 is not required for PME maturation or secretion, but may rather be involved in the degradation of PMEs and down-regulation of PME activity. Indeed, in-gel assays of PME activity indicated that a major PME isoform is missing in SBT3-OX plants (Fig 5B).

Unexpectedly, the loss of this particular PME isoform in SBT3-OX plants did not result in a corresponding increase in the degree of HG methylesterification (DM), but rather in a lower DM in SBT3-OX as compared with wild-type plants (Fig 5C). This apparent discrepancy may be due...
to the compensatory induction of other PME isoforms, the downregulation of PME inhibitors, or compensatory changes in pectin methyltransferase activity. Such a compensatory response to interference with PME activity has previously been described in Arabidopsis roots (Wolf et al., 2012). Since our activity assay only detected PMEs that are active at pH 7.5 and on highly methylsterified substrate, a possible compensatory increase of other PME activities may have escaped detection. Similar to SBT3-OX plants, a reduction in DM was also observed in plants silenced for SBT3 expression (Fig. 5C). The effects of SBT3 expression on pectin methylsterification thus appear to be complex and cannot be explained by SBT3-mediated processing of a single PME. To explain the observed effects, a better understanding of the multiple PME isoforms, their interaction with PME inhibitors and processing by SBTs, and the contribution of other homogalacturonan-modifying enzymes would be required, which, however, is beyond the scope of the present study. Nonetheless, because the performance of M. sexta larvae is obviously not correlated to the observed changes of PME activity or DM in SBT3-OX and -SI plants, it can be concluded that SBT3-mediated changes in cell wall structure or composition are not causally linked to insect resistance.

Alternatively, the apparent effects of SBT3 on PME activity (Fig. 5A) and pectin structure (Fig. 5C) may be relevant during certain stages of plant development. SBT3 is strongly expressed in the micropylar endosperm (Fig. 3A, B) where PME activity controls the resistance of the endosperm to the protruding radicle (Muller et al., 2013; Scheler et al., 2015), and where a reduction of PME activity by SBT3 may facilitate endosperm rupture and completion of germination. Irrespective of a potential role for SBT3-mediated changes in PME activity during germination, the effect of SBT3 expression on insect resistance of SBT3-OX and -SI plants remains unexplained and cannot be attributed to PME-mediated changes in pectin composition. We therefore addressed the possibility that SBT3 may exert its effect on larval performance only after ingestion, within the digestive system of the insect.

**Post-ingestive activity of SBT3**

The nutritional quality of foliage is a limiting factor for herbivore growth and development. A major defense strategy of plants thus aims to restrict the insect's ability to digest dietary protein and to retrieve essential nutrients (Felton, 2005). In addition to proteinase inhibitors targeting the digestive proteinases of the insect (Jongsma and Beekwilder, 2008), jasmonate-inducible anti-nutritive proteins include arginase and threonine deaminase, which are highly active in the midgut of M. sexta where they degrade the essential amino acids arginine and threonine, respectively (Chen et al., 2005, 2008). Also part of the jasmonate-inducible defense arsenal are polyphenol oxidase, catalysing the oxidation of phenolics to form electrophilic quinones that may bind to the nucleophilic amino acid side chains of dietary protein, and acid phosphatase (Vegetative Storage Protein 2) with an as yet unknown function in anti-nutritive defense (Liu et al., 2005; Constabel and Barbehenn, 2008; Bosch et al., 2014a). All these proteins were found to be stable in the harsh environment of the digestive system, which in M. sexta and other caterpillars is characterized by highly alkaline pH (pH 8–11) and an abundance of proteolytic activities (Felton, 2005). The defensive function of these proteins relies on their stability and the ability to retain activity under such adverse conditions (Chen et al., 2005; Chi et al., 2011).

The exceptional stability of SBT3 and its activity at alkaline pH (60% at pH 11; Cedzich et al., 2009; Ottmann et al., 2009) prompted us to investigate whether SBT3 is stable and active in the midgut of M. sexta larvae. Undigested SBT3 protein was detected in the feces of larvae that were raised on SBT3-OX plants (Fig. 6A). A smaller amount of apparently intact SBT3 was also observed when insects fed on wild-type plants, whereas SBT3 was undetectable when insects were raised on SBT3-SI transgenics. In-gel proteinase assays further confirmed that SBT3 is active in the larval midgut (Fig. 6B). The data suggest that SBT3 may serve a post-ingestive function in plant defense by degradation or processing of proteins in the insect digestive system.

A post-ingestive role in plant defense was previously shown for cysteine proteinases in maize (Pechan et al., 2000 and papaya (Konnó et al., 2004), LAP in pigeon pea (Lomate et al., 2013), and has also been suggested for LapA in tomato.
Fig. 6. Post-ingestive activity of SBT3. (A) Stability of SBT3 in the digestive system of M. sexta larvae. Larvae of M. sexta were raised on WT, SBT3-OX (G2) or SBT3-SI (SI-21) plants. Protein extracts from frass (10 μg total protein) of fifth-instar larvae were separated by SDS-PAGE and analysed on western blots using a polyclonal antiserum against SBT3. (B) Activity of SBT3 in the digestive system of M. sexta larvae. Protein extracts from SBT3-OX plants (20 μg) and M. sexta midgut (20 μg) and purified SBT3 protein (15 ng; positive control, +) were separated by acidic PAGE on 6.75% gels with 0.5% co-polymerized gelatine. Gelatinolytic activity of SBT3 is visualized by Coomassie staining as a clear band against a dark background. (C) Growth of M. sexta larvae raised on artificial diet supplemented with 100 μg g⁻¹ fresh weight of SBT3 (circles) or BSA (triangles). Larval weight was determined after 3 (n=37), 6 (n=32), 8 (n=23), 9 (n=20), and 10 (n=18) days. Data represent the average weight of all larvae alive at the respective time point ± standard error.

(Gu et al., 1999; Chen et al., 2007). Their mechanism of toxicity is unknown, except for the maize papain-like cysteine proteinase Mir1-CP. Mir1-CP disrupts the peritrophic membrane, a chitin matrix that lines the midgut epithelium, assists in digestive processes, and protects the caterpillar midgut from physical and chemical damage (Pechan et al., 2002; Lopez et al., 2007; Fescemeyer et al., 2013). Physiological substrates, however, remain to be identified for Mir1-CP and the other proteases alike.

Potential SBT3 substrates may include insect proteins as well as proteins from tomato foliage that are ingested together with SBT3. Several plant defense proteins were in fact shown to require proteolytic processing in order to perform their defensive role, including threonine deaminase, polyphenol oxidase, and urease (Ferreira-DaSilva et al., 2000; Wang and Constabel, 2004; Chen et al., 2007; Gonzales-Vigil et al., 2011; Staniscuaski and Carlini, 2012). Similarly, elicitor peptides have been identified that are proteolytically derived from dietary protein when the plant is attacked by insects (Schmelz et al., 2007; Pearce et al., 2010). These peptides serve as damage-associated molecular patterns allowing for non-self recognition and subsequent activation of plant defense. The proteases required for the formation of these peptides have not been identified and could be of either plant or insect origin.

To address the question whether SBT3 is directly toxic to the insect, targeting proteins of the larval digestive system, or whether it may contribute to the processing of defense-related plant proteins, we performed feeding assays with artificial diet supplemented with SBT3 in amounts exceeding the concentration in wild-type leaves by two- to three-fold. BSA was added as a control. Larvae of M. sexta were indifferent to the presence of SBT3 in their diet, showing equal growth on SBT3- and BSA-supplemented media (Fig. 6C). Any direct toxic effect of SBT3 is thus unlikely. This conclusion is consistent with the observation that SBT3-OX plants with dramatically increased SBT3 levels (Fig. 6A) have little impact on larval growth. This leaves the possibility that SBT3 acts on plant proteins that are ingested together with SBT3 either to modulate their anti-nutritive activity, or to release peptides that may act as elicitors of plant defense. These substrates of SBT3 and any signals derived from them remain to be identified.

Conclusions

The expression of SBT3 was found to be induced in damaged leaves but not systemically in response to wounding and insect attack. The time course of SBT3 induction resembled that of late wound response genes suggesting a role for SBT3 in the downstream defense response. Using transgenic plants with altered SBT3 expression levels we confirm that SBT3 contributes to insect resistance in tomato plants. Improved performance of M. sexta larvae on SBT3-deficient plants may be explained in part by the systemic defense response that was found to be attenuated when SBT3 was silenced. SBT3 is thus implicated in the late wound signaling pathway contributing to the induction of defense genes in unwounded tissues.

As an alternative or additional mode of action, SBT3 may play a post-ingestive role in plant defense. Facilitated by its exceptional stability and high activity at alkaline pH, SBT3 may exert its defensive function in the insect’s digestive system. SBT3 target proteins, possibly including tomato proteins that are ingested along with the protease, remain to be identified. While SBT3 was also found to affect PME activity and the level of pectin methylesterification, these effects were unrelated to its role in herbivore defense.

Supplementary data

Supplementary data are available at JXB online.
Supplementary methods.
Figure S1. Generation of SBT3-OX and SBT3-SI plants

Figure S2. qRT-PCR and western blot analysis of SBT3 expression in SBT3-OX and SBT3-SI plants.

Figure S3. Southern blot analysis of SBT3pro:GUS reporter lines.

Figure S4. Analysis of cell wall neutral and acidic sugar composition.

Funding

This research was supported by a grant [SCHA 591/4-1] of the German Research Foundation (DFG) to AS.

Acknowledgements

We thank Renate Frei and Brigitte Rösingh (University of Hohenheim) and David Frasson (Federal Institute of Technology Zürich) for excellent technical assistance, Elke Sieferer for generating the promoter-reporter lines, Stefan Rühl for greenhouse management, and Dagmar Heisler and Monika Baum for maintenance of plants. We also thank CSIRO Plant Industry (Canberra, Australia) for the pHAnnibal/pKanabinal vector system.

References

Bédouet L, Courtois B, Courtois J. 2003. Rapid quantification of O-acetyl and O-methyl residues in pectin extracts. Carbohydrate Research 338, 379–383.

Berger DR, Howe GA, Ryan CA. 1996. Polypeptide signaling for plant defensive genes exhibits analogies to defense signaling in animals. Proceedings of the National Academy of Sciences of the United States of America 93, 12053–12058.

Bosch M, Berger S, Schaller A, Stintzi A. 2014a. Jasmonate-dependent induction of polyphenol oxidase activity in tomato foliage is important for defense against Spodoptera exigua but not against Manduca sexta. BMC Plant Biology 14, 257.

Bosch M, Wright LP, Gershenzon J, Wasternack C, Hause B, Schaller A, Stintzi A. 2014b. Jasmonic acid and its precursor 12-oxophytodienoic acid control different aspects of constitutive and induced herbivore defenses in tomato. Plant Physiology 166, 396–410.

Calderón-Cortés N, Quesada M, Watanabe H, Cano-Camacho H, Oyama K. 2012. Endogenous plant cell wall digestion: A key mechanism in insect evolution. Annual Review of Ecology, Evolution, and Systematics 43, 45–71.

Carpita NC, Defornez M, Findlay K, Wells B, Shoue DA, Catchpole DS. 2001. Cell wall architecture of the maize coleoptile. Plant Physiology 127, 551–565.

Cedzick A, Huttenlocher F, Kuhn BM, Pfannstiel J, Gabler L, Stintzi A, Schaller A. 2009. The protease-associated (PA) domain and C-terminal extension are required forzymogen processing, sorting within the secretory pathway, and activity of tomato subtilase 3 (SISBT3). Journal of Biological Chemistry 284, 14068–14078.

Chen H, Gonzales-Vigil E, Howe GA. 2008. Action of plant defensive enzymes in the insect midgut. In Schaller A, ed. Induced Plant Resistance to Herbivory. Springer Science+Business Media B.V., 271–284.

Chen H, Gonzales-Vigil E, Wilkerson CG, Howe GA. 2007. Stability of plant defense proteins in the gut of insect herbivores. Plant Physiology 143, 1954–1967.

Chen H, Wilkerson CG, Kuchar JA, Phinney BS, Howe GA. 2005. Jasmonate-inducible plant enzymes degrade essential amino acids in the herbivore midgut. Proceedings of the National Academy of Sciences of the United States of America 102, 19237–19242.

Chi YH, Jing X, Lei J, Ahn J-E, Koo YD, Yun D-J, Lee SY, Behmer ST, Koiwa H, Zhu-Salzman K. 2011. Stability of AtVSP in the insect digestive canal determines its defensive capability. Journal of Insect Physiology 57, 391–399.

Chichkova NV, Shaw J, Galiullina RA et al. 2010. Phytasapase, a relocalised cell death promoting plant protease with caspase specificity. EMBO Journal 29, 1149–1161.

Constabel CP, Barbehenn RW. 2005. Defensive roles of polyphenol oxidase in plants. In Schaller A, ed. Induced Plant Resistance to Herbivory. Springer Science+Business Media B.V., 253–270.

Dixit S, Upadhyay SK, Singh H, Sidhu OP, Verma PC, Chandrashekar K. 2013. Enhanced methanol production in plants provides broad spectrum insect resistance. PLoS ONE 8, e67964.

Engineer CB, Ghassemian M, Anderson JC, Peck SC, Hu H, Schroeder JI. 2014. Carbonic anhydrases, EPF2 and a novel protease mediate CO2 control of stomatal development. Nature 513, 246–250.

Feeny P. 1976. Plant apparency and chemical defense. Recent Advances in Phytochemistry 10, 1–40.

Felix G, Boller T. 1995. Systemin induces rapid ion fluxes and ethylene biosynthesis in Lycopersicon peruvianum cells. The Plant Journal 7, 381–389.

Felton GW. 2005. Indigestion is a plant’s best defense. Proceedings of the National Academy of Sciences of the United States of America 102, 18771–18772.

Ferreira-DaSilva CT, Gombarovits ME, Masuda H, oliveira CM, Carlini CR. 2000. proteolytic activation of canatoxin, a plant toxic protein, by insect cathepsin-like enzymes. Archives of Insect Biochemistry and Physiology 44, 162–171.

Fescemeyer HW, Sandoya GV, Gill TA, Ozkan S, Marden JD, Luthe DS. 2013. Maize toxin degrades peritrophic matrix proteins and stimulates compensatory transcriptome responses in fall armyworm midgut. Insect Biochemistry and Molecular Biology 43, 280–291.

Fowler JH, Narvaez-Vasquez J, Arromdee DN, Pautot V, Holzer FM, Walling LL. 2009. Leucine aminopeptidase regulates defense and wound signaling in tomato downstream of jasmonic acid. The Plant Cell 21, 1239–1251.

Gleave AP. 1992. A versatile binary vector system with a T-DNA organisational structure conducive to efficient integration of cloned DNA into the plant genome. Plant Molecular Biology 20, 1203–1207.

Gonzales-Vigil E, Bianchetti CM, Phillips GN, Howe GA. 2011. Adaptive evolution of threonine deaminase in plant defense against insect herbivores. Proceedings of the National Academy of Sciences of the United States of America 108, 5897–5902.

Gu YQ, Holzer FM, Walling LL. 1999. Overexpression, purification and biochemical characterization of the wound-induced leucine aminopeptidase of tomato. European Journal of Biochemistry 263, 726–735.

Hann CT, Bequette CJ, Dombrowski JE, Stratmann JW. 2014. Methanol and ethanol modulate responses to danger- and microbe-associated molecular patterns. Frontiers in Plant Science 5, 550.

Hause B, Hause G, Kutter C, Miersch O, Wasternack C. 2003. Enzymes of jasmonate biosynthesis occur in tomato sieve elements. Plant Cell Physiology 44, 643–648.

Hause B, Stenzel I, Miersch O, Maucher H, Kramell R, Ziegler J, Wasternack C. 2000. Tissue-specific oxylipin signature of tomato flowers: allene oxide cyclase is highly expressed in distinct flower organs and vascular bundles. The Plant Journal 24, 113–126.

Howe GA, Schaller A. 2008. Direct defenses in plants and their induction by wounding and insect herbivores. In Schaller A, ed. Induced Plant Resistance to Herbivory. Springer Science+Business Media B.V., 7–29.

Jacinto T, McGurl B, Franceschi V, DelanoFreier J, Ryan CA. 1997. Tomato prosystemin promoter confers wound-inducible, vascular bundle-specific expression of the beta-glucuronidase gene in transgenic tomato plants. Planta 203, 406–412.

Jongsma MA, Beekwilder J. 2008. Plant protease inhibitors: Functional evolution for defense. In Schaller A, ed. Induced Plant Resistance to Herbivory. Springer Science+Business Media B.V.: 235–251.

Kaneda M, Tominaga N. 2000. Polypeptide signaling for maintenance of plants. We also thank CSIRO Plant Industry (Canberra, Australia) for the pHAnnibal/pKanabinal vector system.

Kaneda M, Tominaga N. 1996. Polypeptide signaling for maintenance of plants. We also thank CSIRO Plant Industry (Canberra, Australia) for the pHAnnibal/pKanabinal vector system.

Kareda M, Tominaga N. 1990. Systemin induces rapid ion fluxes and ethylene biosynthesis in Lycopersicon peruvianum cells. The Plant Journal 7, 381–389.

Kvavos JA, Bennett RD. 1986. Determination of methanol using alcohol oxidase and its application to methyl ester content of pectins. Journal of Agricultural and Food Chemistry 34, 597–599.
SBT3 contributes to insect resistance in tomato | 4337

Komarova TV, Sheshukova EV, Dorokhov YL. 2014. Cell wall mannellol as a signal in plant immunity. Frontiers in Plant Science 5, 101.

Konno K, Hirayama C, Nakamura M, Tateishi K, Tamura Y, Hattori M, Kohno K. 2004. Papain protects papaya trees from herbivorous insects: role of cysteine proteases in latex. The Plant Journal 37, 370–378.

Körner E, von Dahl CC, Bonaventure G, Baldwin IT. 2009. Pectin methylesterase NaPME1 contributes to the emission of mannellol during insect herbivory and to the elicitation of defence responses in Nicotiana attenuata. Journal of Experimental Botany 60, 2631–2640.

Lee GI, Howe GA. 2003. The tomato mutant spr1 is defective in systemic perception and the production of a systemic wound signal for defense gene expression. The Plant Journal 33, 567–576.

Liu J-X, Srivastava R, Che P, Howell SH. 2007a. An endoplasmic reticulum stress response in Arabidopsis is mediated by proteolytic processing and nuclear relocation of a membrane-associated transcription factor, bZIP28. The Plant Cell 19, 4111–4119.

Liu JX, Srivastava R, Che P, Howell SH. 2007b. Salt stress responses in Arabidopsis utilize a signal transduction pathway related to endoplasmic reticulum stress signaling. The Plant Journal 51, 897–909.

Liu Y, Ahn J-E, Datta S, Salzman RA, Moon J, Hayghues-Despointes B, Pittendrigh B, Murdock LL, Kiowa H, Zhu-Salzman K. 2005. Arabidopsis vegetative storage protein is an anti-insect acid phosphatase. Plant Physiology 139, 1545–1556.

Lomate PR, JadHAV BR, Giri AP, Hivrale VK. 2013. Alterations in the Helicoverpa armigera midgut digestive physiology after ingestion of pigeon pea inducible leucine aminopeptidase. PLoS ONE 8, e74889.

Lopez L, Camas A, Shivaji R, Ankala A, Williams P, Luthe D. 2007. M1r-1CP, a novel defense cysteine protease accumulates in maize vascular tissues in response to herbivory. Planta 226, 517–527.

McGurl B, Pearce G, Orozco-Cardenas M, Ryan CA. 1992. Structure, expression and antisense inhibition of the systemic precursor gene. Science 255, 1570–1573.

Maffei M, Bossi S, Spitteler D, Mithofer A, Boland W. 2004. Effects of feeding Spodoptera littoralis on lima bean leaves. I. Membrane potentials, intracellular calcium variations, organelle, and regurgitate components. Plant Physiology 134, 1752–1762.

Meichtry J, Amrhein N, Schaller A. 1999. Characterization of the subtilase gene family in tomato (Lycopersicon esculentum Mill.). Plant Molecular Biology 39, 749–760.

Meindl T, Boller T, Felix G. 1998. The plant wound hormone systemin and activation by homodimerization of tomato subtilase 3. Proceedings of the National Academy of Sciences of the United States of America 95, 13319–13323.

Pechan T, Ye L, Chang Y, Mitra A, Lin L, Davis FM, Williams WP, Luthe DS. 2000. A unique 33-kDa cysteine protease accumulates in response to larval feeding in maize genotypes resistant to fall armyworm and other Lepidoptera. The Plant Cell 12, 1031–1040.

Pelloux J, Rusterucci C, Mellerowicz EJ. 2007. New insights into pectin methylesterase structure and function. Trends in Plant Science 12, 267–277.

Penuelas J, Filliá E, Stefanescu C, Llusia J. 2005. Caterpillars of Euphydryas aurinia (Lepidoptera: Nymphalidae) feeding on Succisa pratensis leaves induce large foliar emissions of methanol. New Phytologist 167, 851–857.

Pfaffl MW. 2001. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Research 29, e45.

Pietrzak M, Shilitio RD, Hohn T, Potykus I. 1986. Expression in plants of two bacterial antibiotic resistance genes after protoplast transformation with a new plant expression vector. Nucleic Acids Research 14, 5857–5868.

Price PW, Bouton CE, Gross P, McPheron BA, Thompson JN, Weis AE. 1980. Interactions among three trophic levels: Influence of plants on interactions between insect herbivores and natural enemies. Annual Review of Ecology, Evolution, and Systematics 11, 41–65.

Rautengarten C, Steinhauser D, Büsiss D, Stintzi A, Schaller A, Kopka J, Altmann T. 2008. Inhibiting hypotheses on functional relationships of genes: Analysis of the Arabidopsis thaliana subtilase gene family. PLoS Computional Biology 4, e40.

Rautengarten C, Usadel B, Neumetzler L, Hartmann J, Bussis D, Altmann T. 2008. A subtilase-like serine protease essential for mucilage release from Arabidopsis seed coats. The Plant Journal 54, 466–480.

Rose R, Schaller A, Ottmann C. 2010. Structural features of plant subtilases. Plant Signalling and Behavior 5, 100–183.

Ryan CA. 2000. The systemic signalling pathway: differential activation of plant defensive genes. Biochimica et Biophysica Acta 1477, 112–121.

Saez-Aquayo S, Ralet M-C, Berger A, Botran L, Ropartz D, Marion-Poll A, North HM. 2013. PECTIN METHYLESTERASE INHIBITOR6 promotes Arabidopsis mucilage release by limiting methylsterification of homogalacturonian in seed coat epidermal cells. The Plant Cell 25, 308–323.

Schaller A. 1998. Action of proteolysis-resistant systemin analogues in wound signalling. Phytochemistry 47, 605–612.

Schaller A. 2013. Plant subtilinoids. In Rawlings ND, Salvesen G, eds, Handbook of Proteolytic Enzymes, 3rd edn. Academic Press, 3237–3254.

Schaller A, Bergery DR, Ryan CA. 1995. Induction of wound response genes in tomato leaves by bestatin, an inhibitor of aminopeptidases. The Plant Cell 7, 1893–1898.

Schaller A, Frasson D. 2001. Induction of wound response gene expression in tomato leaves by ionophores. Planta 212, 431–435.

Schaller A, Oechling C. 1999. Modulation of plasma membrane H+-ATPase activity differentially activates wound and pathogen defense responses in tomato plants. The Plant Cell 11, 263–272.

Schaller A, Stintzi A, Graff L. 2012. Subtilases – versatile tools for protein turnover, plant development, and interactions with the environment. Physiologia Plantarum 145, 52–66.
Schechter I, Berger A. 1967. On the size of the active site in proteases. I. Papain. Biochemical and Biophysical Research Communications 27, 157–162.

Scheer JM, Pearce G, Ryan CA. 2003. Generation of systemin signaling in tobacco by transformation with the tomato systemin receptor kinase gene. Proceedings of the National Academy of Sciences of the United States of America 100, 10114–10117.

Scheler C, Weitbrecht K, Pearce SP et al. 2015. Promotion of testa rupture during Garden Cress germination involves seed compartment-specific expression and activity of pectin methylesterases. Plant Physiology 167, 200–215.

Schmelz EA, LeClere S, Carroll MJ, Alborn HT, Teal PEA. 2007. Cowpea chloroplastic ATP synthase is the source of multiple plant defense elicitors during insect herbivory. Plant Physiology 144, 793–805.

Sénéchal F, Graff L, Surcouf O et al. 2014a. Arabidopsis PECTIN METHYLESTERASE17 is co-expressed with and processed by SBT3.5, a subtilisin-like serine protease. Annals of Botany 114, 1161–1175.

Sénéchal F, Wattier C, Rustérucci C, Pelloux J. 2014b. Homogalacturonan-modifying enzymes: structure, expression, and roles in plants. Journal of Experimental Botany 65, 5125–5160.

Srivastava R, Liu J-X, Guo H, Yin Y, Howell SH. 2009. Regulation and processing of a plant peptide hormone, AtRALF23, in Arabidopsis. The Plant Journal 59, 930–939.

Srivastava R, Liu JX, Howell SH. 2008. Proteolytic processing of a precursor protein for a growth-promoting peptide by a subtilisin serine protease in Arabidopsis. The Plant Journal 56, 219–227.

Stanisćauski F, Carlini CR. 2012. Plant ureases and related peptides: understanding their entomotoxic properties. Toxins 4, 55–67.

Strassner J, Schaller F, Frick UB, Howe GA, Weiler EW, Amrhein NA, Macheroux P, Schaller A. 2002. Characterization and cDNA-microarray expression analysis of 12-oxophytodienoate reductase reveals differential roles for octadecanoid biosynthesis in the local versus the systemic wound response. The Plant Journal 32, 585–601.

Taurino M, Abelenda JA, Rio-Alvarez I et al. 2014. Jasmonate-dependent modifications of the pectin matrix during potato development function as a defense mechanism targeted by Dickeya dadantii virulence factors. The Plant Journal 77, 418–429.

Vartapetian AB, Tuzhikov AI, Chichkova NV, Taliansky M, Wolpert TJ. 2011. A plant alternative to animal caspases: subtilisin-like proteases. Cell Death and Differentiation 18, 1289–1297.

Vetsch M, Janzik I, Schaller A. 2000. Characterization of prosystemin expressed in the baculovirus/insect cell system reveals biological activity of the systemin precursor. Planta 211, 91–97.

von Dahl CC, Havecker M, Schlogl R, Baldwin IT. 2006. Caterpillar-elicited methanol emission: a new signal in plant-herbivore interactions? The Plant Journal 46, 948–960.

Wang J, Constabel CP. 2004. Polyphenol oxidase overexpression in transgenic Populus enhances resistance to herbivory by forest tent caterpillar (Malacosoma disstria). Planta 220, 87–96.

Wasternack C, Stenzel I, Hause B, Hause G, Kutter C, Maucher H, Neumerkel J, Feussner I, Miersch O. 2006. The wound response in tomato – Role of jasmonic acid. Journal of Plant Physiology 163, 297–306.

Wesley SV, Helliwell CA, Smith NA et al. 2001. Construct design for efficient, effective and high-throughput gene silencing in plants. The Plant Journal 27, 581–590.

Wolf S, Mravec J, Greiner S, Mouille G, Höfte H. 2012. Plant cell wall homeostasis is mediated by brassinosteroid feedback signaling. Current Biology 22, 1732–1737.

Wolf S, Rausch T, Greiner S. 2009. The N-terminal pro region mediates retention of unprocessed type-I PME in the Golgi apparatus. The Plant Journal 58, 361–375.

Yamagata H, Masuzawa T, Nagaoka Y, Ohnishi T, Iwasaki T. 1994. Cucumisin, a serine protease from melon fruits, shares structural homology with subtilisin and is generated from a large precursor. Journal of Biological Chemistry 269, 32725–32731.

Yonezawa H, Kaizuka H, Uchikoba T, Arima K, Kaneda M. 2000. Substrate specificity of cucumisin on synthetic peptides. Bioscience, Biotechnology, and Biochemistry 64, 2104–2108.