
**Characterization of the Serine Carboxypeptidase
SnSCP1 from *Solanum nigrum* and its function in
defense against herbivores**

Diploma Thesis

by

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Abbreviations

C	Control
CV	Control vector
JA	Jasmonic acid
SCP	Serine Carboxypeptidase
SnSCP1	<i>Solanum nigrum</i> Serine Carboxypeptidase 1
TPI	Trypsin proteinase inhibitor
VIGS	Virus-induced gene silencing
vSCP	<i>SnSCP1</i> virus-induced gene silenced plants
W	Wounding
W+R	Wounding + application of <i>M. sexta</i> regurgitate
W+W	Wounding + application of water

1. Abstract

1.1 English Abstract

Serine carboxypeptidases (SCPs) have different functions in plant growth, development and turnover of storage proteins. However, only few studies addressed their influence on plant defenses against herbivores. Until now only a SCP was described for tomato which was suggested to be involved either in direct defense against herbivores or in protein turnover after herbivore stress.

We identified a homologous serine carboxypeptidase (SnSCP1) in black nightshade (*Solanum nigrum*). Its transcripts accumulate 3 h after simulated herbivory and the proteolytic activity increases 24 h after induction. The induction of SnSCP1 occurred only after herbivore attack. However, *Manduca sexta* and *Spodoptera exigua*, common solanaceous herbivores, did not show changes in performance when fed on plants silenced in the expression of *SnSCP1* by virus-induced gene silencing. Also protein content and the composition of free amino acids were not influenced by SnSCP1, making a possible role of this peptidase in herbivore-induced changes in protein turnover unlikely. To test whether SnSCP1 was involved in herbivory-related senescence processes, as was also suggested for its homolog in tomato, we measured chlorophyll content and auto-fluorescence in *SnSCP1*-silenced and control plants but could not find any difference between these plant types. We conclude that SnSCP1 does not participate in the regulation of responses targeted to deter *M. sexta* larval feeding and in processes controlling leaf senescence in *S. nigrum*. However, as SnSCP1 is strongly induced by insect herbivory, it is most likely involved in still unknown processes associated to the interaction between *S. nigrum* and insects.

1.2 Deutsche Zusammenfassung

Serin Carboxypeptidasen (SCPs) haben verschiedene Funktionen beim Wachstum, der Entwicklung und der Umwandlung von Proteinen in Pflanzen. Dennoch sind nur wenige Studien bekannt, die sich mit der Rolle von SCPs bei der pflanzlichen Verteidigung beschäftigen. Bis jetzt wurde nur eine SCP in Tomate entdeckt, für die angenommen wird, dass sie die direkte Verteidigung gegen Schädlinge oder die Umwandlung von Proteinen nach einem Herbivorenbefall beeinflusst.

In dieser Arbeit haben wir eine homologe SCP (SnSCP1) im schwarzen Nachtschatten (*Solanum nigrum*) identifiziert. Die *SnSCP1* Transkripte akkumulierten 3 h und die proteolytische Aktivität stieg 24 h nach Induktion durch simulierte Herbivorie. Die Induktion von SnSCP1 ist allerdings eine spezifische Reaktion auf den Befall durch Herbivoren und trat bei rein mechanischer Verwundung nicht auf. *Manduca sexta* und *Spodoptera exigua*, weit verbreitete Schädlinge, zeigten jedoch keine Veränderung in der Gewichtszunahme, wenn sie von Pflanzen gefressen hatten, deren *SnSCP1*-Expression mittels „Virus-induced gene silencing“ reduziert war. Auch die Menge an Proteinen und die Zusammensetzung der freien Aminosäuren in den Blättern von *S. nigrum* beeinflusst SnSCP1 nicht. Das schließt eine Beteiligung dieser Peptidase in der Proteinumwandlung aus. Um zu testen, ob SnSCP1 an Seneszenzprozessen mitwirkt (wie es für das Tomatenhomolog angenommen wird), haben wir die Chlorophyllmenge und die Autofluoreszenz in Pflanzen mit niedrigen SnSCP1 Level und Kontrollpflanzen gemessen, aber keinen Unterschied gefunden. Wir sind der Ansicht, dass SnSCP1 bei den getesteten Pflanzen nach Herbivorenbefall unter den getesteten Bedingungen weder bei der Verteidigung gegen Schädlinge noch bei der Proteinumwandlung wesentlich beteiligt ist. Demnach beeinflusst SnSCP1 möglicherweise andere, noch unbekannte Prozesse.

2. Introduction

Biological processes in all domains of life are controlled by proteases. They are, beside other components, responsible for the tight control of the proteolytic machinery of each single cell. Therefore, plant genomes encode hundreds of proteases, which represent many unrelated families. The biological role of these proteases is mostly unknown, but mutant alleles, gene silencing, and overexpression studies have provided phenotypes for a growing number of proteases (van der Hoorn, 2008).

Proteases polarize the carbonyl group of their substrates peptide bonds. They stabilize the oxygen in an oxyanion hole. That makes the carbon atom more assailable for attack by an activated nucleophile. The cleaved peptide bonds can be internal (for endopeptidases), N-terminal (for aminopeptidases), or C-terminal (for carboxypeptidases) (van der Hoorn, 2008). Proteases have four major ways to cleave the peptide bonds, which give the names to four catalytic classes: aspartic proteases, cystein proteases, metalloproteases and serine proteases. With more than 200 members, serine proteases are the largest class of proteolytic enzymes in plants. Plant serine proteases are classified into 32 families, belonging to 12 clans (http://merops.sanger.ac.uk/cgi-bin/clan_index?type=P#S). Biological functions of serine proteases have been described for some subtilases (SDD1 and ALE1), plastid localized members (DegPs, Plsp1, and ClpPs) and carboxypeptidases (BRS1 and SNG1/2) (van der Hoorn, 2008). Serine carboxypeptidases (SCPs) contain a Ser-Asp-His catalytic triad and belong to clan SC, S10 family, of serine peptidases (Feng and Xue, 2006). They are folded as an α / β hydrolase which is common to many other hydrolytic enzymes (van der Hoorn, 2008). In plants, studies of SCPs have been mainly focused on their function in turnover and mobilization of storage proteins to release nitrogen and carbon resources during seed germination and senescence (Schaller, 2004). Recent studies suggested that plant SCPs may also be involved in various signalling events important for plant growth and development such as programmed cell death, brassinosteroid signalling, and seed development (Dominguez and Cejudo, 1999; Li et al., 2001; Cercos et al., 2003). Other studies confirm a role for SCPs in production of plant secondary metabolites involved in defense against herbivores (Wajant et al., 1994; Li and Steffens, 2000).

In this study we investigate the role of SnSCP1 in plant defense against herbivores in black nightshade (*Solanum nigrum*). *S. nigrum* is an annual herbaceous plant with worldwide

distribution. As a close relative of potato and tomato, it offers a number of genetic tools and databases but also hosts a large natural community of herbivorous insects from different feeding guilds (Schmidt et al., 2004). We choose the tobacco hornworm *Manduca sexta* and the beet armyworm *Spodoptera exigua* as natural herbivores for *S. nigrum* in our study. *M. sexta* is known to be a solanaceous specialist and *S. exigua* is a generalist with a very wide host range (Brown and Dewhurst, 1975).

To test, if a SCP from *S. nigrum* (SnSCP1) influences plant defense and therefore herbivore performance, we silenced *SnSCP1* using virus-induced gene silencing (VIGS). VIGS has recently emerged as the method of choice for silencing genes (Burch-Smith et al., 2004). A vector system based on the tobacco rattle virus (TRV) was found to be very effective in solanaceous plants and was reported to work in *S. nigrum* (Hartl et al., 2008). After silencing SCP using this vector, we monitored different defensive traits, to see if SnSCP1 influences defense pathways and, consequently, herbivore performance. We investigated levels of jasmonic acid (JA) and proteinase inhibitors (PIs) as typical marker for anti-herbivore defense (Creelman and Mullet, 1997). JA is a signal molecule that functions as essential mediator of the plant's wound-, anti-herbivore, and anti-pathogen responses, as well as in growth and development (Creelman and Mullet, 1997; Turner et al., 2002). Moreover, JA also mediates the induction of direct defense compounds, such as PIs (Halitschke et al., 2003; Kang et al., 2006; Wang et al., 2007).

PIs were found, for example, in tomato leaves after herbivore attack (Green and Ryan, 1972). They are capable of suppressing protease activity in the gut of the insect, which inhibits the digestion of leaf proteins and therefore decreases herbivore performance (Jongsma and Bolter, 1997; Zavala et al., 2004). PIs are classified according to the type of protease they inhibit. The majority of plant PIs found so far specifically inhibits members of the serine proteases, cysteine proteases, or aspartic proteases (Ryan, 1990). When attacked by herbivorous insects, *S. nigrum* responds by accumulating serine PIs, as other solanaceous plants do (Schmidt et al., 2004)

There is evidence that plant defense pathways considerably overlap with plant senescence. For example JA, as one of the common mediators, regulates plant defense, flower development, embryogenesis, seed germination, fruit ripening and also leaf senescence (Reinbothe et al., 2009). Moreover, Ülker (2007) reports about a transcription factor in

Arabidopsis thaliana that is involved in regulation of defense and senescence-related genes. Moura et al. (2001) showed that wound stress leads to protein degradation. Similarly, in senescent leaves proteins are degraded and transported to other parts of the plant (Ryan and Walker-Simmons, 1981; Huffaker, 1990). This protein degradation is accompanied by an increased concentration of free amino acids (De Kok and Graham, 1989). Leaf senescence, constituting the terminal stage of leaf development, is manifested by a loss of chlorophyll that occurs during chloroplast disintegration together with other catabolic events, such as protein degradation (Quirino et al., 2000; Gepstein, 2004). Therefore we studied the influence of SnSCP1 on protein turnover as well as on other senescence associated processes, like chlorophyll content and leaf auto-fluorescence. All senescence processes were characterized by a decrease of the chlorophyll content (De Kok and Graham, 1989). In plants, chlorophyll is actively synthesized from glutamate in the developmental phase and is degraded into non-fluorescent chlorophyll catabolites during senescence (Nagane et al., 2010). This degradation leads to decrease of auto-fluorescence in leaves when senescence starts (Kumagai et al., 2009).

In the present study we identified a herbivore-induced SCP in *S. nigrum*. It is either involved in direct plant defense against herbivores or influences other defensive associated processes.

3. Materials and Methods

3.1 Plant material and growth

All experiments were done with the *S. nigrum* L. inbred line Sn30 (Schmidt et al., 2004). Seeds were sterilized in dichloroisocyanic acid sodium salt, incubated in 1M KNO₃ over night at 4°C in darkness and then germinated in petri dishes on Gamborg's B5 plant agar according to Schmidt et al. (2004). After 7 to 10 days seedlings were either vacuum-infiltrated with *Agrobacterium* (VIGS) and then planted in Teku trays or directly planted in Teku trays filled with a peat-based substrate ("Tonsubstrat", Klasmann, Germany). Trays were covered with plastic film and kept in growth chambers with 16 h light (155 $\mu\text{mol m}^{-2} \text{s}^{-1}$), 8 h dark, 26°C for 10 days. Then seedlings were transferred into 9 x 9 x 9.5 cm pots with the same substrate and grown in the glasshouse of the institute (16 h light, supplemental lighting by Phillips Master Sun-T PIA Agro 400 W and 600 W sodium lights, 23 to 25°C, 45 to 55% rel. humidity; 8 h dark, 19 to 23°C, 45 to 55% rel. humidity). The plants were automatically watered daily with 0.5 g/L of a combination fertilizer containing phosphate, potassium, and magnesium (Euflor, Germany) and 0.5 g/L Ca(NO₃)₂. The seedlings and plants used for VIGS were grown in climate chambers as described by Hartl et al. (2008)

3.2 Plant treatments

To induce the plants, methyljasmonate (MeJA) in lanolin paste was applied to the stem at the first internode (20 μL , 7.5 mg MeJA/ 1mL lanolin) with a spatula. For mimicking herbivory we used a pattern wheel to puncture four rows on each side of the leaf midvein (W). Directly after wounding *Manduca sexta* regurgitate (R) was applied with a pipette to the wounded leaves (30 μL of a 1:5 dilution with dH₂O). The regurgitate was collected from larvae that fed on *S. nigrum* and then centrifuged to remove cell debris. Leaves treated with W+R were harvested one day after the treatment (except in case of the time-course experiment) and leaves treated with MeJA two days after the treatment and frozen in liquid nitrogen.

3.3 Gene isolation and sequencing

We designed primers (Supplemental Table 2) on the SCP sequence from tomato (GenBank: AF242849) to clone a fragment of *SnSCP1* by standard PCR. We obtained the *SnSCP1* (Supplemental Figure 1) using 3' RACE (Scotto-Lavino et al., 2006) and 5' RACE (Invitrogen Instruction manual for 5', Version 2.0, URL: http://tools.invitrogen.com/content/sfs/manuals/5prime_race_man.pdf; primers see Supplemental Table 2). For cloning the Gene JETtm PCR Cloning Kit (Fermentas, St. Leon-Rot, Germany) or the pGEM-T Easy vector (Promega, Mannheim, Germany) were used. Plasmids were isolated using the NucleoSpin® Kit (Clontech, Heidelberg, Germany) and then sequenced on the ABI Genetic Analyzer 3100.

3.4 Microarray

Leaves of both CV and *vSCP* plants were induced with W+R as described above. After 24 h, local leaves were harvested and frozen in liquid nitrogen. For every genotype we collected three biological replicates of three pooled leaves each. Total RNA was extracted from 1 g of ground tissue as described above. We used DynalBeads with an oligo-dT-linker to isolate mRNA from 400 µg of total RNA according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). cDNA was synthesized using Superscript II Reverse Transcriptase (200U/µl) and random hexamer primers. Differential labeling (Cy3 for treatment and Cy5 for controls), hybridization, and scanning were performed as described by Halitschke et al. (2003). Spot intensities (SIs) were extracted from image files using the software AIDA (Raytest, Germany). SIs were cleared of noise by subtracting the local-background (IBg). IBg-subtracted SIs below $2 \times \text{IBg}$ were considered below the detection level, discarded, and set to 0 (signal-to-noise cut off). The raw data was subsequently normalized via LOWESS using MIDAS (Saeed et al., 2003). Prior to statistical analyses, a value of 0.01 was added to each normalized SI after which they were log₂-transformed. We performed a nested ANOVA to identify significantly regulated clones across the three replicates. We adjusted the p-value < 0.05 using FDR, according to the Benjamini and Hochberg step-up procedure for controlling the false discovery rate (Benjamini and Hochberg,

1995). As a second parameter an average \log_2 -ratio > 0.585 or < -0.585 (equivalent to 1.5-fold up- or 0.65-fold down-regulation) was used. Both criteria had to be fulfilled to consider a gene as significantly regulated.

3.5 Time-course and tissue specificity

Leaf samples (third fully expanded leaf of each 10 week old plant) were collected 3 h, 6 h, 9 h, 12 h, 24 h and 3 d after W+R treatment. To determine tissue specific SnSCP1 expression, young leaves, mature leaves, and green berries were harvested at the same time. Ripe berries were collected one month later. Samples were frozen and ground in liquid nitrogen.

3.6 Virus-induced gene silencing

We amplified a fragment of *SnSCP1* and ligated it into the vector pYL156 (Liu et al., 2002) to obtain the vector pTRVSCP (= vSCP). As a control served the vector pTRV-SnCV (= CV) containing an intronic PDS fragment of the *SnPDS* gene. As a visual marker for silencing efficiency we used the vector pTRV-SnPDS, that leads to leaf bleaching (Hartl et al., 2008). The vectors were cloned into *Agrobacterium tumefaciens* strain GV3101. The bacteria were cultivated in 5 ml of LB-MES medium (20 g LB broth l^{-1} , 1.95g MES l^{-1} ; Carl-Roth GmbH, Karlsruhe, Germany), 20 μ M acetosyringone (SIGMA Aldrich®, Taufkirchen, Germany) and 50 mg l^{-1} kanamycin and incubated over night (28°C, 200rpm). The following evening 300 μ L of the preculture were inoculated to 200 mL LB-MES medium and grown over night under the same conditions. When the optical density at 600 nm (OD_{600}) reached *c.* 0.6, cultures were centrifuged in 50 mL falcon tubes (10 min, 4°C, 2000 x g). The pellet was resuspended in 20 mL infiltration medium (10 mM $MgCl_2$, 10 mM MES, 200 μ M acetosyringone) and kept at room temperature for 3 h in the dark. Each suspension containing a construct of interest was mixed with the same volume of a culture containing pTRV-RNA1 (Hartl et al., 2008).

For vacuum-infiltration 7- to 10-d-old seedlings were transferred from the petri dishes to the bacteria solution. Vacuum was applied in a desiccator until it reached a pressure of 60-70 mbar and then slowly released (Hartl et al., 2008).

3.7 QRT-PCR

For the determination of relative transcript abundance QRT-PCR was used. *S .nigrum* elongation factor served as internal control and we applied the comparative $2^{-\Delta\Delta Ct}$ method for relative quantification (Livak and Schmittgen, 2001). Total RNA was extracted from frozen plant material using a modified TRI reagent protocol (The Institute for Genomic Research, Online Protocol, 2003; URL: http://www.jcvi.org/potato/images/SGED_SOP_3.1.1.pdf). For reverse transcription SuperScript II RNaseH-Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA), 500 ng of total RNA, and a poly-T Primer were used to obtain cDNA. 20 ng of cDNA were applied to 20 μ L SYBR Green reactions (qPCR Core Kit for SYBR Green; Eurogentec, Belgium) and run on a Stratagene Mx3005P QPCR system (cycler conditions: 10 min 95°C, 40 cycles of 15 s at 95°C and 1 min at 60°C). Primers were designed using Primer 3 plus (<http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi>) and checked for specificity with a melting curve analysis.

3.8 Serine protease activity assay

450 μ L extraction buffer (Jongsma et al., 1993) was added to 150 mg of tissue and vortexed for 5 min. After centrifugation at 4°C 16.100 x g for 20 min the supernatant was transferred to a new tube. The assay was performed with three technical replicates in a 96 well plate format. N-benzoyl-L-tyrosine-p-nitroanilide (BTpNA, SIGMA Aldrich®, Taufkirchen, Germany) as substrate specific for serine proteases was added to all samples (140 μ L 98.6 μ M BTpNA solution: 1 mg BTpNA was dissolved in 1 mL DMSO and taken up in 24 mL 0.1 M Tris HCL) and kept for 60 min at 37°C. The reaction was stopped with 30% acetic acid, centrifuged (5 min,

2000 x g) and the absorbance of the supernatant was measured at 405 nm. As a negative control 30% acetic acid was added to the sample before addition of the substrate solution.

3.9 Herbivore performance

M. sexta eggs were obtained from our in-house culture. Directly after hatching, 20 larvae were transferred into a plastic box (3 boxes per genotype and treatment) containing moist paper tissue and a leaf of either a control plant (CV) or of a *SnSCPI*-silenced plant (*vSCP*) which were either induced by W+R or untreated (C). The leaf material was exchanged every day. From day 3 on, larvae were isolated (one per box) and weighed. The boxes were randomized and kept at 23 to 25°C in a shaded area in the glasshouse. Larval mass was observed on different days using an analytical balance with a readability of 0.1 mg.

S. exigua eggs were provided from the Plant Protection Center of Bayer CropScience, Monheim, Germany. The experiment was performed in the same way as with *M. sexta*. For each genotype three boxes with 25 larvae each were prepared. After 5 days the larvae were separated into individual boxes containing leaf discs. *S. exigua* larvae were fed with W+R treated plant material only.

3.10 PI activity assay

150 mg of each leaf sample was homogenized in liquid nitrogen and extracted with 450 µL of extraction buffer (Jongsma et al., 1993) by vortexing. After repeated centrifugation (20 min, 13.200 x g) and transfer of the supernatant the protein concentration was determined with the Bradford method (Protein Assay, Bio-Rad, Germany) using bovine serum albumin (SIGMA Aldrich®, Taufkirchen, Germany) as a standard. The concentration of active trypsin protease inhibitors was determined by radial diffusion assay using bovine trypsin (SIGMA Aldrich®, Taufkirchen, Germany) (Jongsma et al., 1993; Van Dam et al., 2001). The samples were allowed to diffuse in the agar for 16 h and then stained with a solution of Fast Blue Salt, 0.1 M Tris-HCl

pH 7.8, APNE and DMF for 1 h at 37°C. The concentration of active TPIs was expressed relative to the total protein concentration.

3.11 Jasmonic acid concentration

From control plants (CV) and *SnSCPI*-silenced plants (*vSCP*) leaf samples were weighed (100-300 mg) and frozen in liquid nitrogen. After homogenizing with 1 mL ethyl acetate spiked with 100 ng of [9,10-²H]-dihydro-JA, [¹³C₄]-SA, and [¹³C₆]-JA-Ile, the samples were centrifuged (10 min, 4°C, 16.100 x g) and the organic phases as well as plant material were reextracted with 0.5 mL ethyl acetate. The organic phases were combined and evaporated to dryness. The dry residues were reconstituted in 0.2 mL of 70% (v/v) methanol/dH₂O and analyzed with LCMS (Varian 1200L) as described by Kallenbach et al. (2010)

3.12 Free amino acids

Plant material was harvested, frozen in liquid nitrogen, lyophilized and pulverized. Aliquots of 50 mg were transferred to 2 mL Eppendorf tubes and resuspended with 0.8 mL of 0.1 M HCL to rehydrate the tissue. After incubation for 15 min at room temperature, the samples were centrifuged (5 min, 16.000 x g) and the supernatant was transferred to a fresh tube. The samples were prepared as described by de Kraker et al. (2007) and measured with HPLC. The autosampler of the HPLC (Agilent HP1100 series) was programmed to mix the vial content with 30 µL of reagent consisting of 0.085 M O-phthaldialdehyde (Fluka, SIGMA Aldrich®, Taufkirchen, Germany) and 1% (v/v) β-mercaptoethanol in a 0.5 M sodium borate solution immediately before injection of 50 µL sample onto the HPLC column (Supelcosil LC-18-DB [250 x 4.6 mm, 5 µm particle size]; Supelco). The running conditions were described by de Kraker et al. (2007). Amino acid samples were quantified by calibration curves that were prepared with a 0.5 M amino acid stock solution of Asn, His, and Gln and an amino acid stock solution of Fluka that contained all other amino acids in a 0.5 mM concentration (de Kraker et al., 2007).

3.13 Chlorophyll content and Auto-fluorescence

Using the Chlorophyll meter (Minolta SPAD-502, Konica-Minolta) chlorophyll contents were measured from mature and young leaves of 15 control (CV) and 15 *SnSCP1*-silenced plants (*vSCP*).

We used the Chlorophyll Fluorometer (OS-30p, Opti-Sciences, USA) to measure auto-fluorescence of different aged leaves from 22 randomly chosen plants (11 of CV plants and 11 *vSCP* plants). Clips were fixed to the leaves to shade an area of the leaf and the measurement was taken after 10 min.

3.14 Statistics

For all statistical analysis the software SPSS 17.0 for Windows (SPSS Inc., IL, USA) was used.

4. Results

4.1 Characterization of SnSCP1

4.1.1 Sequence of *SnSCP1*

Using standard PCR we identified a homolog of a wound-inducible type I serine-carboxypeptidase (Moura et al., 2001) in *S. nigrum*. After 3'/5'-RACE we obtained the full coding sequence of 1494 bp and translated it into a putative protein of 498-amino-acids with a 91% amino acid similarity and 87% identities when compared to the sequence from tomato (Supplemental Fig.1).

4.1.2 Microarray analysis of *SnSCP1*

Some proteases are known to have regulatory roles in plant defense. A well studied example from tomato is a leucin aminopeptidase (LAP) (Schmidt et al., 2005; Walling, 2006). Therefore, we chose a transcriptomic approach to estimate if SnSCP1 influences other herbivore defensive relevant genes. The microarray contained 1421 50-mer oligonucleotides representing herbivore-related genes selected from different experiments and public databases (Wang et al., 2008). We hybridized cDNA from leaves of control plants (CV) or *SnSCP1*-silenced plants (*vSCP*) induced by mechanical wounding and application of *M. sexta* regurgitates. Three biological replicates of each genotype were analyzed.

We found four genes which were significantly and differentially regulated after silencing *SnSCP1* (Supplemental Table 1). However, the level of regulation of these genes was very low. Moreover, according to their annotation these genes exhibit no obvious function relevant for herbivore defense.

4.1.3 Time-course and tissue-specificity of SnSCP1 expression

Quantitative real-time PCR (QRT-PCR) and measurements of protease activity were used to estimate transcriptional abundance and activity of SnSCP1 over time. The third fully expanded leaf of 5-week-old plants of *S. nigrum* were either induced by mechanical wounding (W) and application of *M. sexta* regurgitate (R) or were left untreated (C). After certain time points one local leaf per plant was harvested and relative mRNA abundance of *SnSCP1* was determined (Figure 1a). The relative amount of *SnSCP1* transcripts remained stable in uninduced control plants during the experimental period of three days. The induced leaves showed higher mRNA-levels 3 h after induction. The level of mRNA remained constant until 12 h and returned after 24 h to control level (Figure 1a). To test if this change is only on transcript level or also occurs on protein level, we measured SnSCP1 activity using BTpNA as substrate. Figure 1b shows the temporal dynamics of SnSCP1 generation in local leaves after a single elicitation by wounding and application of *M. sexta* regurgitate (W+R) or water (W+W), and in untreated control plants (C). SnSCP1 level exhibited no increase for C and W+W plants. However, simulated herbivory (W+R) increased serine protease activity after 24 h.

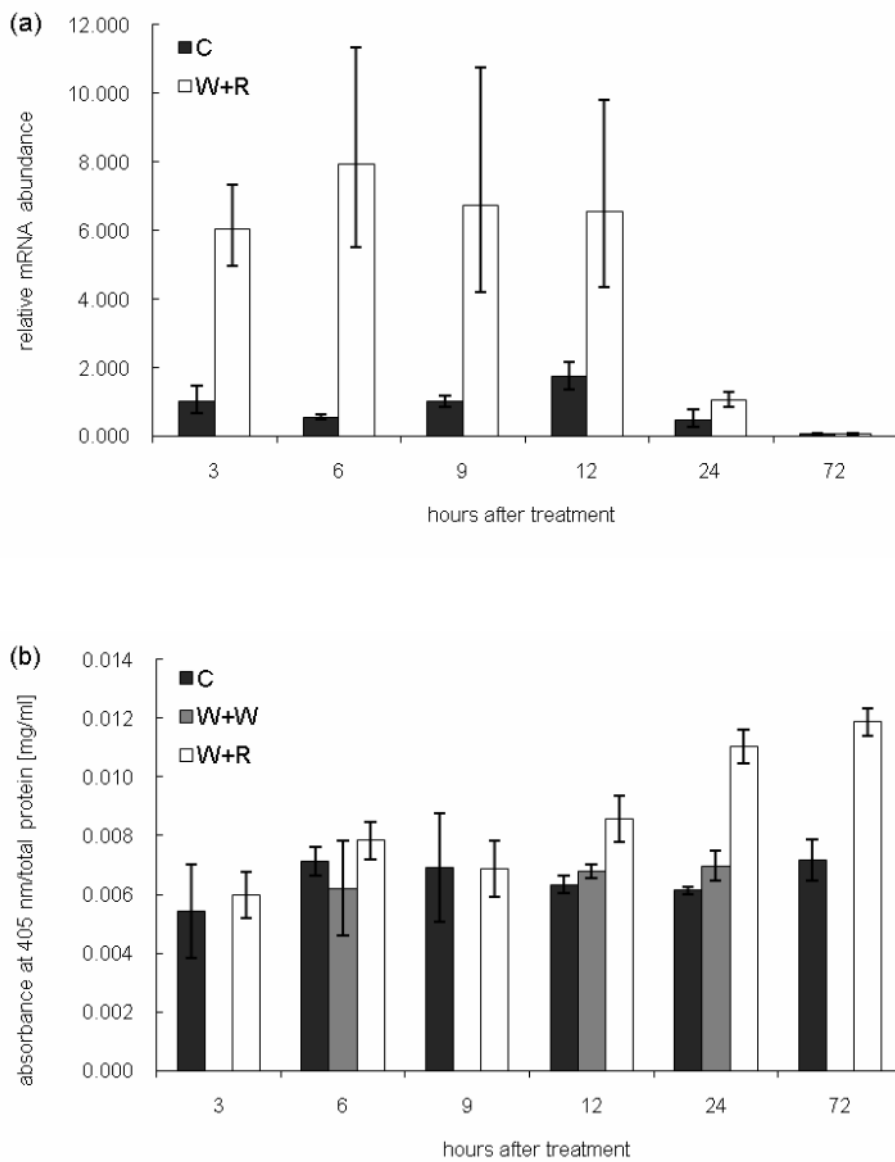


Fig.1 *SnSCP1* transcript accumulation and serine-protease activity in leaves after different treatments: C, untreated control, W, mechanically wounded and treated with water (W+W) or *M. sexta* regurgitate (W+R). **a** Transcript levels of *SnSCP1* determined by QRT-PCR. **b** Serine-protease activity against BTpNA as substrate

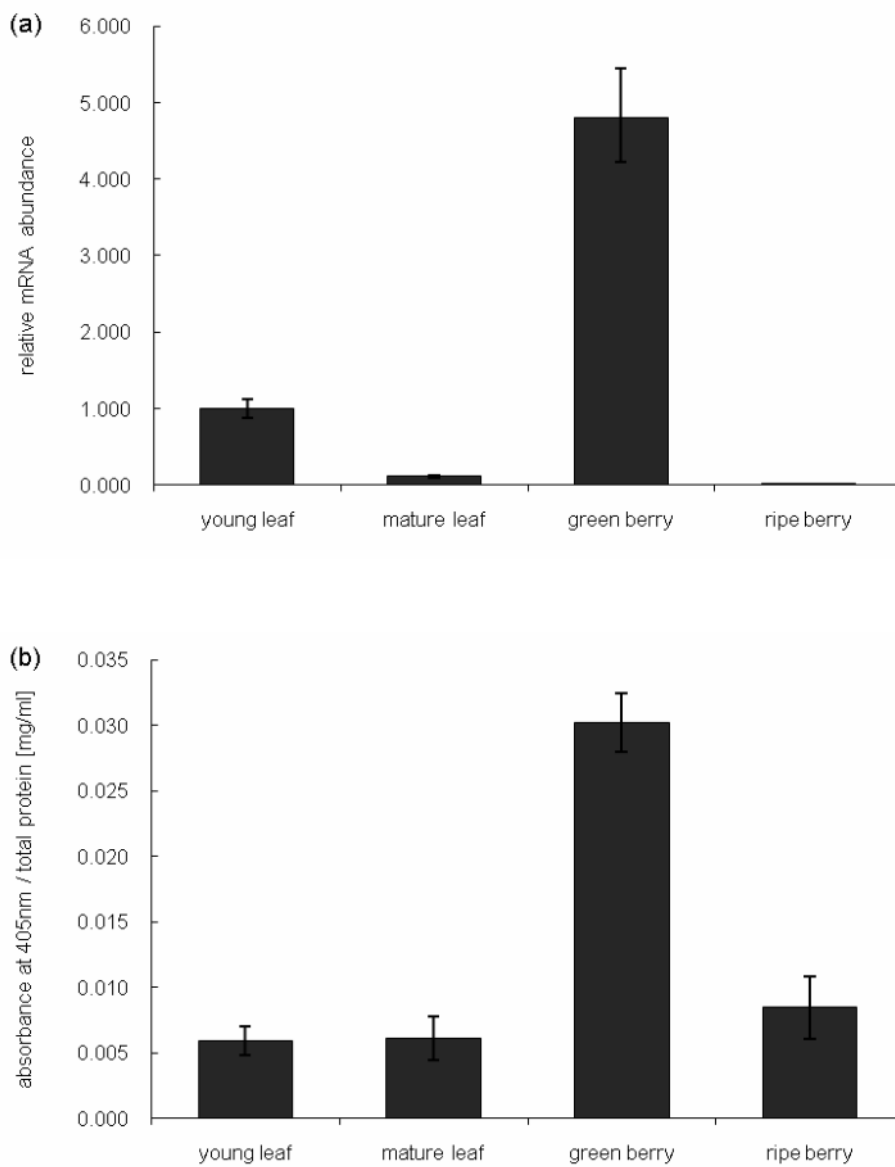


Fig.2 *SnSCP1* transcript abundance and serine-protease activity in different plant tissues of control plants (C) **a** Transcript levels of *SnSCP1* determined by QRT-PCR. **b** activity measurement against BTpNA as substrate

Similarly we analyzed the tissue-specific expression of SnSCP1 (Figure 2). Four different tissues relevant for defense against herbivores were examined. Only for these tissues we could detect any activity. The highest abundance of *SnSCP1* mRNA was detectable in green berries, a lower concentration in young leaves and smaller amounts of mRNA in ripe berries and mature leaves (Figure 2a). Similarly, serine protease activity was highest in green berries. The other tissues displayed a lower activity. These results show that the transcript data correlate with the activity data. However, changes in activity can be a consequence of other proteases that also metabolize BTpNA.

4.2 SnSCP1 functions in plant defense against herbivores

4.2.1 Effect of SnSCP1 on herbivore performance

To test the relevance of SnSCP1 on plant defense against herbivores we used *SnSCP1*-silenced plants (*vSCP*) for studying the performance of the tobacco hornworm *Manduca sexta* (Sphingidae) and the beet armyworm *Spodoptera exigua* HÜBNER (Noctuidae) in laboratory experiments. Both *M. sexta* and *S. exigua* fed on *S. nigrum*. *M. sexta* is a solanaceous specialist and *S. exigua* is a generalist with a very wide host range that includes solanaceous crop plants (Brown and Dewhurst, 1975).

M. sexta larvae were separated on excised leaves from CV and *vSCP*-plants, which had been pre-induced with wounding (W) and application of *M. sexta* regurgitates (R) or were left untreated (C). To assess herbivore performance the larvae were weighed at different time points. Additionally, we measured serine protease activity to check whether the silencing was successful. Figure 3a shows that activity of SnSCP1 is significantly decreased in *vSCP*-plants in contrast to CV plants. Until day 5 there was no difference in *M. sexta* larval mass between larvae fed on CV control plant leaves, CV W+R treated leaves and *vSCP*-plant W+R induced leaves (Figure 3b). Only larvae fed on uninduced *SnSCP1*-silenced leaf material had significantly more body mass on day 4 compared to that of the other larvae and this difference persisted until day 8.

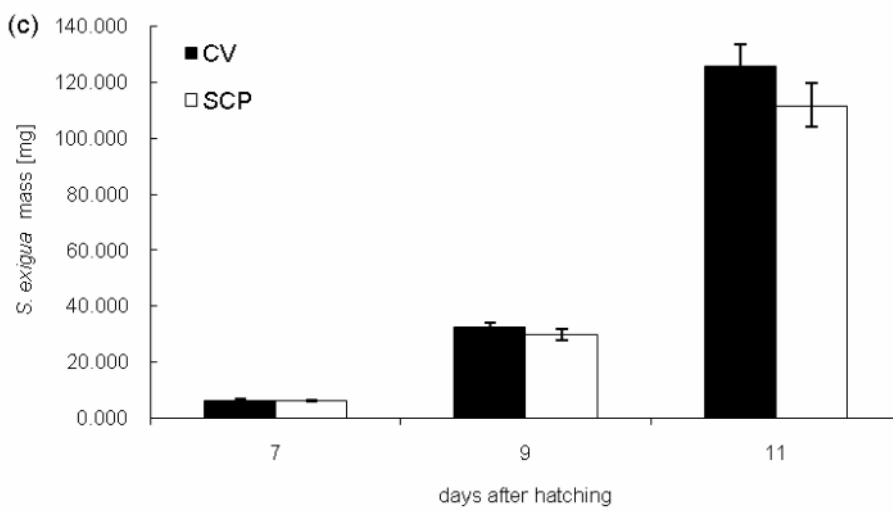
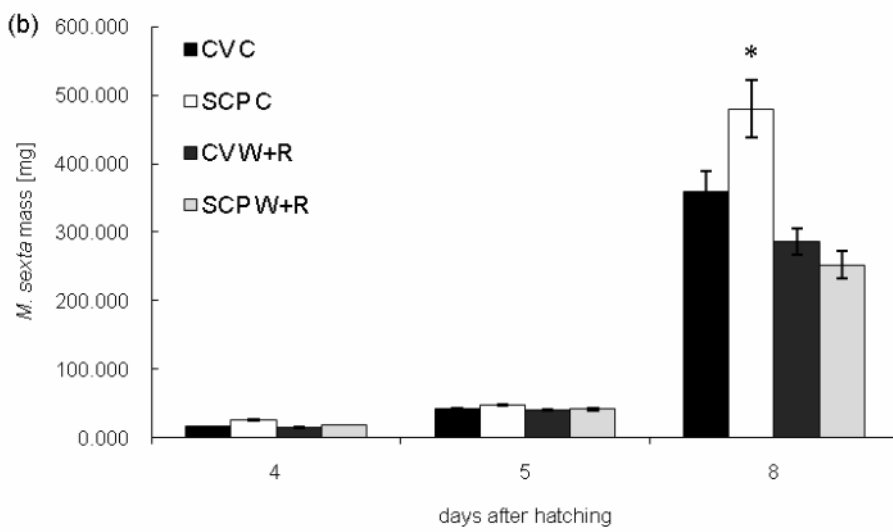
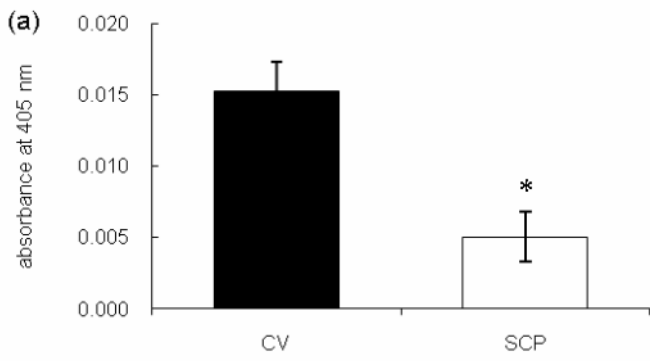


Fig.3 Larval performance of *M. sexta* and *S. exigua* feeding on plants silenced in the expression of *SnSCP1* by virus-induced gene silencing. Plants were either inoculated with a control vector (CV) or with a vector harboring a fragment of *SnSCP1* (vSCP) for gene silencing. **a** mean \pm SE absorbance at 405 nm after metabolization of BTpNA by *SnSCP1* as indicator of silencing-efficiency (* indicates significant difference, independent sample t-test, $p = 0.001$) **b** mean \pm SE larval mass of *M. sexta* or **c** *S. littoralis* feeding on leaves from CV and vSCP plants. The plants for *M. sexta* were either untreated (C) or induced by mechanically wounding and application of *M. sexta* regurgitates (W+R). In case of *S. exigua* plants were W+R treated (*M. sexta*: one-way ANOVA with combined parameter genotypes + treatment, day 8, $F_{2,19} = 479.63$, $P < 0.05$, followed by a Scheffé post hoc test. *S. exigua*: day 11, t-test, $p = 0.96$)

The results confirmed that pre-induction of plants decreases *M. sexta* body mass. However, after mimicking herbivory with W+R there was no significant mass difference between larvae feeding on CV or vSCP-plants (Figure 3b). Similarly, *S. exigua* larvae also showed no differences when feeding on W+R treated vSCP or CV plants (Figure 3c). We repeated the experiment with *M. sexta* three times and all data, supported the result of the first experiment (Supplemental Figure 2).

4.2.2 Influence of *SnSCP1* on plant defensive components

Jasmonic acid is an important signal molecule in plant defense against herbivores (Creelman and Mullet, 1997; Halitschke and Baldwin, 2004). It accumulates in wounded plants and in plants or cell cultures treated with elicitors of pathogen defense (Gundlach et al., 1992). To test if *SnSCP1* changes JA concentration we measured JA in control (CV) and *SnSCP1*-silenced plants (vSCP). LC-MS analysis displayed that the concentration of JA was similar for both genotypes (Figure 4).

JA also activates genes encoding proteinase inhibitors (Johnson et al., 1989), which are known to be involved in plant defense (Steppuhn and Baldwin, 2007; Zavala et al., 2004). To test if *SnSCP1* influences trypsin proteinase inhibitor (TPI) levels, we measured TPI activity, in uninduced or W+R treated CV and vSCP-plants. Both genotypes showed similar uninduced TPI levels but differed in their induced TPI activity 3 days after W+R induction. To get evidence that our silencing was successful, we measured *SnSCP1* activity in both plant genotypes. The insert in Figure 5 illustrated that *SnSCP1* was indeed silenced in vSCP-plants.

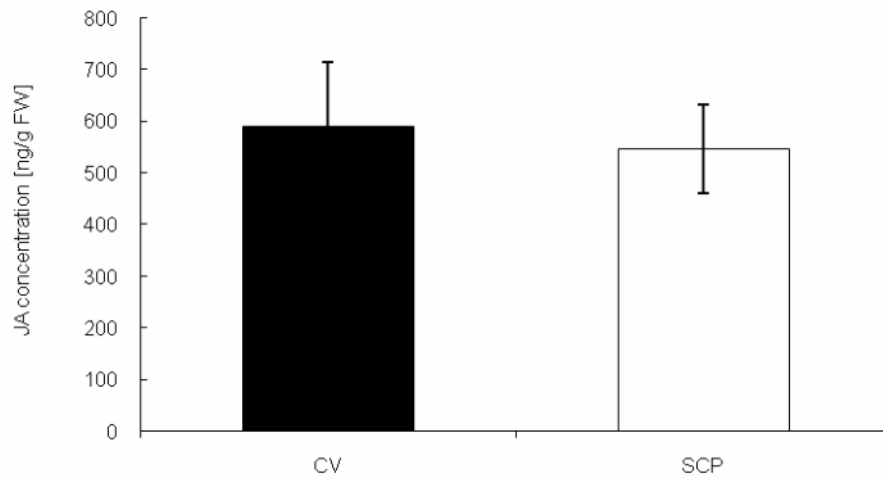


Fig.4 mean \pm SE jasmonic acid concentration in mature leaves of control (CV) and *SnSCP1*-silenced plants (vSCP) treated with mechanical wounding and application of *M. sexta* regurgitate

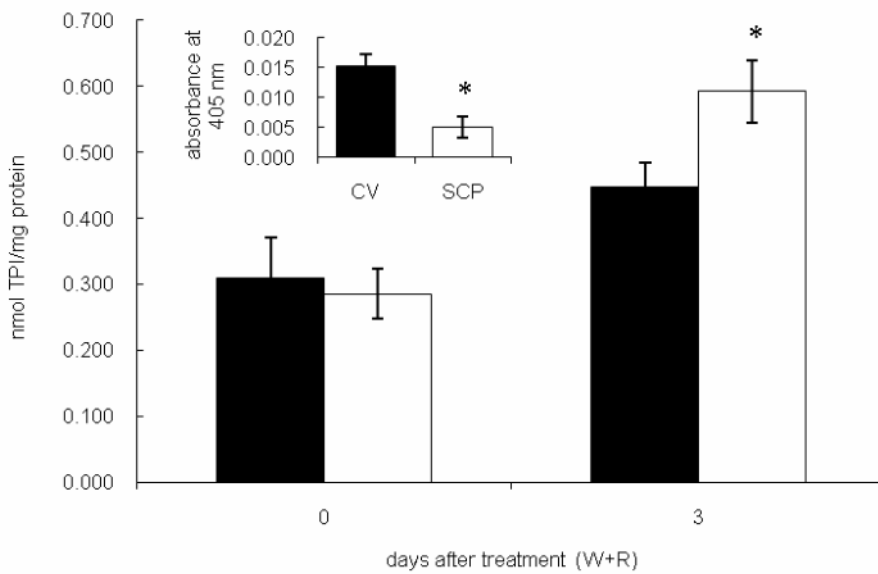


Fig.5 mean \pm SE Trypsin proteinase inhibitor (TPI) activity in leaves of control (CV) and *SnSCP1*-silenced plants (vSCP) (* indicates significant difference, independent sample t-test, day3: $p = 0.029$)

Insert mean \pm SE absorbance at 405 nm after metabolization of BTpNA by *SnSCP1* (* t-test, $p = 0.01$)

4.3 Importance of SnSCP1 for protein turnover and senescence-associated processes

4.3.1 Protein turnover

Moura et al. (2001) suggested that the SCP homolog in tomato could be involved in protein turnover processes. Therefore we tested different indicators for leaf protein turnover, to see if SnSCP1 is involved. We measured free amino acid content of leaves to find out if it was altered in *SnSCP1*-silenced plants (*vSCP*) indicating a role of SnSCP1 in degradation of proteins.

The level of free amino acids in CV and *vSCP*-plants showed no difference (Figure 6). Both genotypes displayed similar values for the 17 amino acids tested. Mature leaves contain less soluble protein, because the proteins were most likely degraded during senescence processes (Martinez et al., 2008). Our aim was to find out if *vSCP*-plants displayed different protein concentrations in comparison to CV plants. Thus, we measured soluble protein concentration in leaves of different developmental age using the Bradford method. Figure 7 confirmed that the protein concentration decreased with leaf age. However, no significant difference between protein contents of the two genotypes could be observed.

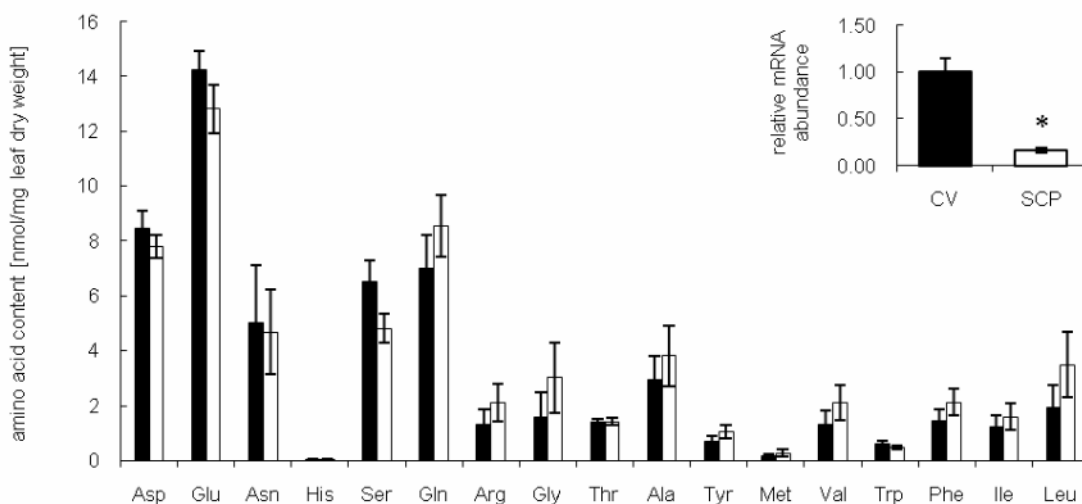


Fig.6 Free amino acid contents in control (CV) and *SnSCP1*-silenced plants (*vSCP*). **Insert** mean \pm SE relative mRNA abundance as indicator of silencing efficiency of *SnSCP1* determined by QRT-PCR (* indicates significant difference, independent sample t-test, $p = 0.000$)

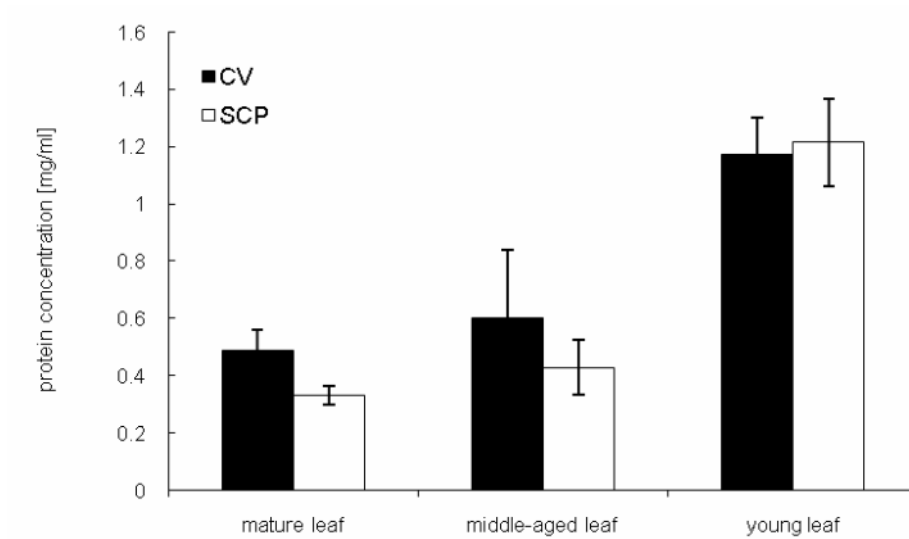


Fig.7 mean \pm SE total soluble protein concentration in leaves of different age in plants silenced in the expression of *SnSCP1* by virus-induced gene silencing (vSCP) or in plants treated with a control vector (CV)

4.3.2 Chlorophyll content and auto-fluorescence

Other indicators for leaf senescence, in addition to free amino acid content and protein concentration, are chlorophyll content and auto-fluorescence. Chlorophyll is degraded to a non-fluorescent catabolite during senescence (Hortensteiner, 2006; Kumagai et al., 2009).

Figure 8a shows that mature leaves contained less chlorophyll when compared to young leaves. However, *SnSCP1* had no influence on chlorophyll degradation. Measurements of auto-fluorescence yielded similar results. Mature leaves displayed lower auto-fluorescence (Figure 8b). Nevertheless, between both genotypes there was no difference in auto-fluorescence, neither in mature nor in young leaves.

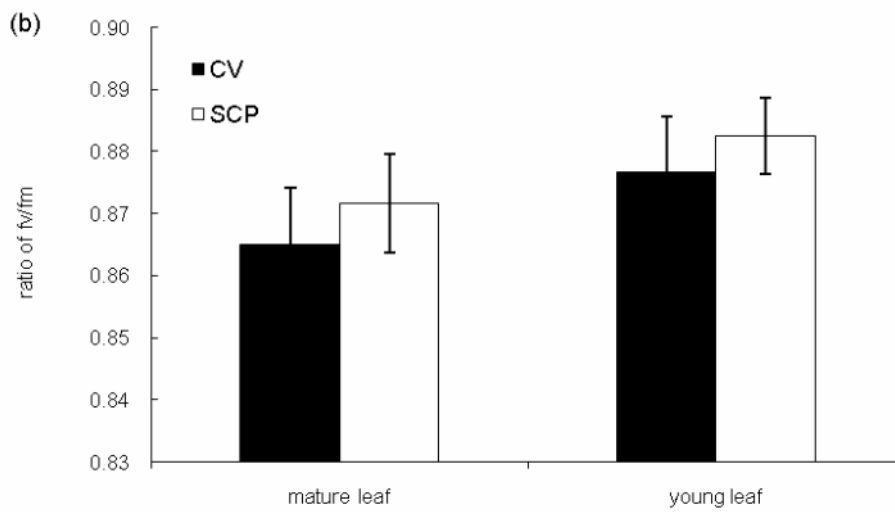
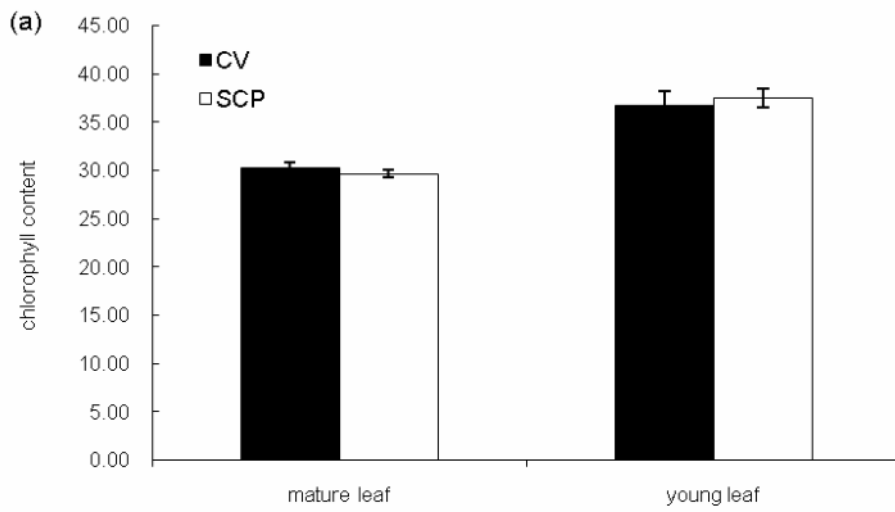


Fig.8 senescence indicators in control (CV) and *SnSCP1*-silenced plants (*vSCP*) **a** mean ± SE chlorophyll content (t-test: mature leaf: $p = 0.311$, young leaf: $p = 0.696$) and **b** mean ± SE auto-fluorescence of mature and young leaves (t-test: mature leaf: $p = 0.589$, young leaf: $p = 0.552$)

5. Discussion

SCPs have been identified in a wide array of organisms. They are believed to play roles in processing and degradation of proteins (Fraser et al., 2005; Feng and Xue, 2006; van der Hoorn, 2008). Compared to the large number of members in the SCP family, only a few of them have been studied in detail with respect to their biological roles in plants. Little is known about their influence on plant defense mechanisms.

In the present study we identified a serine carboxypeptidase from *Solanum nigrum* (SnSCP1), which is herbivore-induced and shows strong up-regulation 24 h after herbivore elicitation. This observation suggested that SnSCP1 is somehow involved in plant defense responses against herbivores. To falsify this hypothesis we tested if SnSCP1 influences herbivore performance and other defense associated effects.

The observation, that SnSCP1 is up-regulated after simulated herbivory, raised the question whether this enzyme is involved in anti-herbivore defense responses, as was also proposed for a SCP by Mugford et al. (2009). Moreover, Liu et al. (2008) showed that a rice serine carboxypeptidase-like gene is involved in regulation of defense responses against biotic stress. Therefore, we compared the performance of *M. sexta* larvae fed on *S. nigrum* plants silenced in the expression of *SnSCP1* (*vSCP*) with larvae fed on control plants by regularly weighing the caterpillars. As expected, larvae that fed on uninduced *S. nigrum* leaf material gained in general more body mass than larvae that fed on pre-induced leaf material, irrespective of plant genotype. Silencing of *SnSCP1* caused no mass difference for herbivores (*M. sexta* and *S. exigua*) fed on pre-induced leaf material. This indicates, that herbivory indeed activates plant defense mechanisms in *S. nigrum* which affect *M. sexta* growth but which are independent of SnSCP1. However, the more striking result was a better performance of larvae fed on un-induced *SnSCP1*-silenced plants (*vSCP*) when compared to those fed on un-induced CV plants. This difference on un-induced plants but the lack of phenotype on induced plants suggests that SnSCP1 either acts as a defensive protein itself, with the effect being masked by other defenses after full induction, or that SnSCP1 itself has no effect on herbivores but rather directly or indirectly affects other defense-relevant mechanisms. We followed two approaches to test these

hypotheses: 1. Check for differences in defense-relevant compounds between CV and vSCP plants. 2. Assess differences in gene-expression of defense-relevant genes.

TPIs are well-described markers for activation of direct defense responses (Jongsma and Bolter, 1997; Zavala et al., 2004). In *S. nigrum* TPIs were up-regulated 3 days after induction with W+R indicating a typical defense response. vSCP-plants expressed TPIs at slightly higher levels than CV plants. This suggests that the absence of SnSCP1 affects the regulation of defense-relevant mechanisms. If SnSCP1 had a regulatory role in plant defense against herbivores, it should influence other herbivore defensive relevant genes. However, our microarray analysis showed, this was not the case. Moreover, JA concentration in *S. nigrum* leaves was also not effected. JA is a signal molecule, that functions as essential mediator of the plants wound-, anti-herbivore and anti-pathogen responses (Creelman and Mullet, 1997). In case that SnSCP1 was involved in regulation of JA biosynthesis, the JA concentration in vSCP-plants should differ from that in CV plants. Our data suggest that SnSCP1 does not influence JA-levels and therefore we assume that SnSCP1 has no direct regulatory function in plant defense processes that were detectable with our methods. It is possible that SnSCP1 is involved in regulatory or defense processes that were not known until now. However, how SnSCP1 influenced TPI levels in uninduced tissue remains an open question. The higher TPI level in vSCP plants could be a specific pleiotropic effect of the silencing with VIGS, caused by the treatment with virus and Agrobacterium. However, we have no evidence for this hypothesis it is rather unlikely. Another assumption is that the loss of SnSCP1 induces a compensatory reaction of other defense mechanisms. However, to further investigate these hypotheses more studies will be necessary.

Another possible role for SnSCP1 could be an indirect influence on plant defense. It is possible that direct plant defenses against herbivores depend on general readjustments of protein metabolism. Thus, it is interesting to speculate if common regulation patterns of protein metabolism exist between plant defense mechanisms and other processes involving resource reallocation like for example senescence processes. For the tomato homolog of SCP a role in senescence processes and protein turnover was predicted (Moura et al., 2001). Schaller (2004) also assumed a function of SCPs in protein turnover during senescence. However, in our study soluble protein levels were not decreased when SnSCP1 was silenced. We compared leaves of

different age but found no significant difference in soluble protein concentration between CV and vSCP-plants. During senescence the degradation of proteins is accompanied by a substantial increase in amino acid concentration (De Kok and Graham, 1989). Wen et al.(1996) found that the levels of 14 free amino acids increased significantly after the onset of senescence in *Nicotiana rustica* callus. Changes in free amino acids were also determined in *Arabidopsis thaliana* during both dark-induced and natural senescence of leaves (De Kok and Graham, 1989). Therefore, we also measured free amino acid concentration of control and vSCP-plants. Free amino acids accumulated rapidly during senescence. However, in *S. nigrum* we could not detect a difference in free amino acid concentration between CV and vSCP-plants. This result indicates that the presence of SnSCP1 does not influence protein turnover processes.

To test, if SnSCP1 was directly involved in leaf senescence processes we measured different senescence parameters: chlorophyll content and auto-fluorescence of young and old leaves. Senescence is characterized by a loss of chlorophyll that occurs during chloroplast disintegration (Quirino et al., 2000; Gepstein, 2004). Therefore, all senescence processes show a decrease in chlorophyll content (Hendry et al., 1987; De Kok and Graham, 1989). Following this assumption, we measured the chlorophyll content and the auto-fluorescence of mature and young leaves. If SnSCP1 was involved in chlorophyll degradation and hence involved in leaf senescence, the chlorophyll content should be lower in CV plants when compared to vSCP plants. Similarly, chlorophyll auto-fluorescence should also be decreased in CV plants (Hortensteiner, 2006; Kumagai et al., 2009). For *S. nigrum* neither chlorophyll content nor auto-fluorescence differed between CV and vSCP-plants. There was no significant difference between both, the chlorophyll levels of young leaves and old leaves of the two genotypes. The auto-fluorescence measurement yielded the same result which suggests that SnSCP1 is not involved in the degradation of chlorophyll. In summary all tested indicators of protein turnover and leaf senescence showed no differences between CV and vSCP-plants, making a function of SnSCP1 in these processes unlikely.

Next to senescence processes SCPs had also been implicated in plant growth and development (Li et al., 2001) as well as seed development (Schaller, 2004). For example a SCP was isolated from young fruits of *Pisum sativum* which was induced by gibberellins in developing reproductive and vegetative tissues (Cercos et al., 2003). Analyzing different

defense-relevant tissues, we found SnSCP1 to be highly expressed in green berries. So we suggest a function of SnSCP1 in seed development, similar to its homologue in *P. sativum*. However, more experiments for testing this hypothesis are certainly necessary. It is possible that homologs of SnSCP1 exist in *S. nigrum*, that could also differentially influence defense or development but which could not be dissected with our silencing construct.

In our study we demonstrated, that *S. nigrum* plants are capable of activating inducible and effective defenses against *M. sexta* or *S. exigua* as natural herbivores. Nevertheless, field experiments with stably silenced lines would be necessary to analyse if the effect of uninduced vSCP plants on herbivores is important for plant herbivore interactions in the natural environment. The data also confirm, SnSCP1 was not involved in senescence-related protein turnover or chlorophyll degradation. Thus, in contrast to the assumption for the tomato homolog (Moura et al., 2001), SnSCP1 is not involved in *S. nigrum* senescence processes. However, the fact that SnSCP1 was most expressed in green berries of *S. nigrum* suggests a role in developmental processes in the fruits but needs to be investigated in more detail. With this work we contributed to the elucidation of the role of SCPs in plant biological mechanisms. Although many questions remain to be answered we presented clear evidence for an involvement of SnSCP1 in plant defense against herbivores. Moreover, against a number of predictions in literature, SnSCP1 seems not to be involved in protein turnover or leaf senescence processes but is rather likely to play a role in fruit or seed development.

6. References

- Benjamini Y, Hochberg Y** (1995) Controlling the False Discovery Rate - a Practical and Powerful Approach to Multiple Testing. *Journal of the Royal Statistical Society Series B-Methodological* **57**: 289-300
- Brown ES, Dewhurst CF** (1975) Genus Spodoptera (Lepidoptera, Noctuidae) in Africa and near East. *Bulletin of Entomological Research* **65**: 221-&
- Burch-Smith TM, Anderson JC, Martin GB, Dinesh-Kumar SP** (2004) Applications and advantages of virus-induced gene silencing for gene function studies in plants. *Plant Journal* **39**: 734-746
- Cercos M, Urbez C, Carbonell J** (2003) A serine carboxypeptidase gene (PsCP), expressed in early steps of reproductive and vegetative development in *Pisum sativum*, is induced by gibberellins. *Plant Molecular Biology* **51**: 165-174
- Creelman RA, Mullet JE** (1997) Biosynthesis and Action of Jasmonates in Plants. *Annu Rev Plant Physiol Plant Mol Biol* **48**: 355-381
- De Kok LJ, Graham M** (1989) Levels of pigments soluble proteins amino acid and sulfohydryl compounds in foliar tissue of *Arabidopsis thaliana* during dark induced and natural senescence. *Plant Physiology and Biochemistry (Paris)* **27**: 203-210
- de Kraker JW, Luck K, Textor S, Tokuhisa JG, Gershenzon J** (2007) Two *Arabidopsis* genes (IPMS1 and IPMS2) encode isopropylmalate synthase, the branchpoint step in the biosynthesis of leucine. *Plant Physiology* **143**: 970-986
- Dominguez F, Cejudo FJ** (1999) Patterns of starchy endosperm acidification and protease gene expression in wheat grains following germination. *Plant Physiology* **119**: 81-88
- Feng Y, Xue Q** (2006) The serine carboxypeptidase like gene family of rice (*Oryza sativa* L. ssp. japonica). *Funct Integr Genomics* **6**: 14-24
- Fraser CM, Rider LW, Chapple C** (2005) An expression and bioinformatics analysis of the *Arabidopsis* serine carboxypeptidase-like gene family. *Plant Physiology* **138**: 1136-1148
- Gepstein S** (2004) Leaf senescence - not just a 'wear and tear' phenomenon. *Genome Biology* **5** (3)
- Green TR, Ryan CA** (1972) Wound induced proteinase inhibitor in tomato leaves evidence for a proteinase inhibitor inducing factor. *Federation Proceedings* **31**: 862
- Gundlach H, Muller MJ, Kutchan TM, Zenk MH** (1992) Jasmonic Acid Is a Signal Transducer in Elicitor-Induced Plant-Cell Cultures. *Proceedings of the National Academy of Sciences of the United States of America* **89**: 2389-2393
- Halitschke R, Baldwin IT** (2004) Jasmonates and related compounds in plant-insect interactions. *Journal of Plant Growth Regulation* **23**: 238-245
- Halitschke R, Gase K, Hui DQ, Schmidt DD, Baldwin IT** (2003) Molecular interactions between the specialist herbivore *Manduca sexta* (Lepidoptera, Sphingidae) and its natural host *Nicotiana attenuata*. VI. Microarray analysis reveals that most herbivore-specific transcriptional changes are mediated by fatty acid-amino acid conjugates. *Plant Physiology* **131**: 1894-1902
- Hartl M, Merker H, Schmidt DD, Baldwin IT** (2008) Optimized virus-induced gene silencing in *Solanum nigrum* reveals the defensive function of leucine aminopeptidase against herbivores and the shortcomings of empty vector controls. *New Phytologist* **179**: 356-365

- Hendry GAF, Houghton JD, Brown SB** (1987) Tansley Review No-11 - the Degradation of Chlorophyll - a Biological Enigma. *New Phytologist* **107**: 255-302
- Hortensteiner S** (2006) Chlorophyll degradation during senescence. *Annual Review of Plant Biology* **57**: 55-77
- Huffaker RC** (1990) Proteolytic activity during senescence of plants. *New Phytologist* **116**: 199-231
- Johnson R, Narvaez J, An G, Ryan C** (1989) Expression of proteinase inhibitors I and II in transgenic tobacco plants: effects on natural defense against *Manduca sexta* larvae. *Proc Natl Acad Sci U S A* **86**: 9871-9875
- Jongsma MA, Bakker PL, Stiekema WJ** (1993) Quantitative-Determination of Serine Proteinase-Inhibitor Activity Using a Radial Diffusion Assay. *Analytical Biochemistry* **212**: 79-84
- Jongsma MA, Bolter C** (1997) The adaptation of insects to plant protease inhibitors. *Journal of Insect Physiology* **43**: 885-895
- Kallenbach M, Alagna F, Baldwin IT, Bonaventure G** (2010) *Nicotiana attenuata* SIPK, WIPK, NPR1, and Fatty Acid-Amino Acid Conjugates Participate in the Induction of Jasmonic Acid Biosynthesis by Affecting Early Enzymatic Steps in the Pathway. *Plant Physiology* **152**: 96-106
- Kang JH, Wang L, Giri A, Baldwin IT** (2006) Silencing threonine deaminase and JAR4 in *Nicotiana attenuata* impairs jasmonic acid-isoleucine-mediated defenses against *Manduca sexta*. *Plant Cell* **18**: 3303-3320
- Kumagai E, Araki T, Kubota F** (2009) Characteristics of Gas Exchange and Chlorophyll Fluorescence during Senescence of Flag Leaf in Different Rice (*Oryza sativa* L.) Cultivars Grown under Nitrogen-Deficient Condition. *Plant Production Science* **12**: 285-292
- Li AX, Steffens JC** (2000) An acyltransferase catalyzing the formation of diacylglycerol is a serine carboxypeptidase-like protein. *Proceedings of the National Academy of Sciences of the United States of America* **97**: 6902-6907
- Li J, Lease KA, Tax FE, Walker JC** (2001) BRS1, a serine carboxypeptidase, regulates BRI1 signaling in *Arabidopsis thaliana*. *Proc Natl Acad Sci U S A* **98**: 5916-5921
- Liu HZ, Wang XE, Zhang HJ, Yang YY, Ge XC, Song FM** (2008) A rice serine carboxypeptidase-like gene OsBISCPL1 is involved in regulation of defense responses against biotic and oxidative stress. *Gene* **420**: 57-65
- Liu YG, Liu HM, Chen LT, Qiu WH, Zhang QY, Wu H, Yang CY, Su J, Wang ZH, Tian DS, Mei MT** (2002) Development of new transformation-competent artificial chromosome vectors and rice genomic libraries for efficient gene cloning. *Gene* **282**: 247-255
- Livak KJ, Schmittgen TD** (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* **25**: 402-408
- Martinez DE, Costa ML, Gomez FM, Otegui MS, Guamet JJ** (2008) 'Senescence-associated vacuoles' are involved in the degradation of chloroplast proteins in tobacco leaves. *Plant Journal* **56**: 196-206
- Moura DS, Bergey DR, Ryan CA** (2001) Characterization and localization of a wound-inducible type I serine-carboxypeptidase from leaves of tomato plants (*Lycopersicon esculentum* Mill.). *Planta* **212**: 222-230

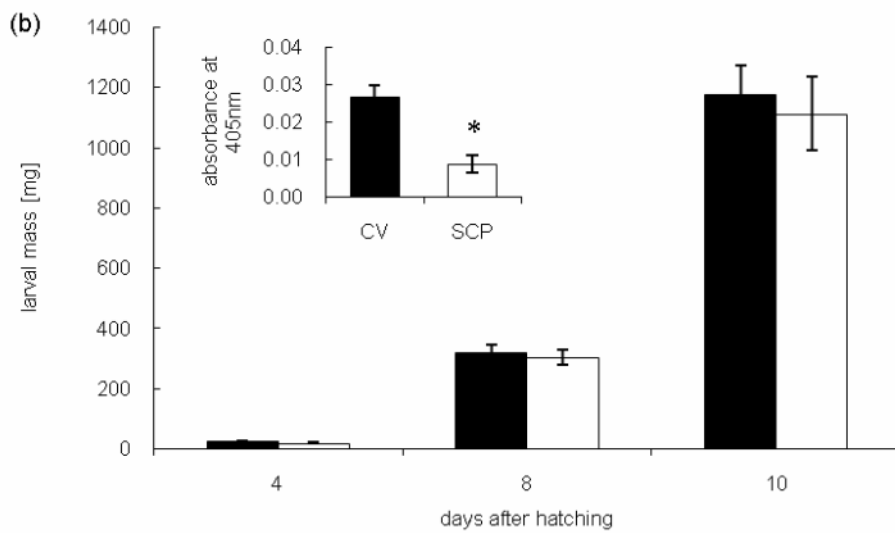
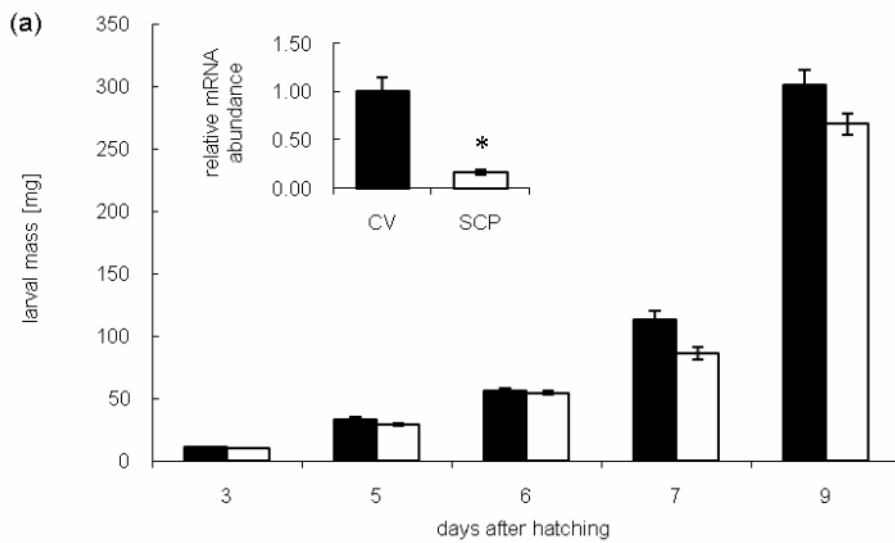
- Mugford ST, Qi XQ, Bakht S, Hill L, Wegel E, Hughes RK, Papadopoulou K, Melton R, Philo M, Sainsbury F, Lomonossoff GP, Roy AD, Goss RJM, Osbourn A** (2009) A Serine Carboxypeptidase-Like Acyltransferase Is Required for Synthesis of Antimicrobial Compounds and Disease Resistance in Oats. *Plant Cell* **21**: 2473-2484
- Nagane T, Tanaka A, Tanaka R** (2010) Involvement of AtNAP1 in the regulation of chlorophyll degradation in *Arabidopsis thaliana*. *Planta* **231**: 939-949
- Quirino BF, Noh YS, Himelblau E, Amasino RM** (2000) Molecular aspects of leaf senescence. *Trends in Plant Science* **5**: 278-282
- Reinbothe C, Springer A, Samol I, Reinbothe S** (2009) Plant oxylipins: role of jasmonic acid during programmed cell death, defence and leaf senescence. *Febs Journal* **276**: 4666-4681
- Ryan CA** (1990) Protease Inhibitors in Plants - Genes for Improving Defenses against Insects and Pathogens. *Annual Review of Phytopathology* **28**: 425-449
- Ryan CA, Walker-Simmons M** (1981) Plant Proteinases
- Saeed AI, Sharov V, White J, Li J, Liang W, Bhagabati N, Braisted J, Klapa M, Currier T, Thiagarajan M, Sturn A, Snuffin M, Rezantsev A, Popov D, Ryltsov A, Kostukovich E, Borisovsky I, Liu Z, Vinsavich A, Trush V, Quackenbush J** (2003) TM4: a free, open-source system for microarray data management and analysis. *Biotechniques* **34**: 374-378
- Schaller A** (2004) A cut above the rest: the regulatory function of plant proteases. *Planta* **220**: 183-197
- Schmidt DD, Kessler A, Kessler D, Schmidt S, Lim M, Gase K, Baldwin IT** (2004) *Solanum nigrum*: a model ecological expression system and its tools. *Molecular Ecology* **13**: 981-995
- Schmidt DD, Voelckel C, Hartl M, Schmidt S, Baldwin IT** (2005) Specificity in ecological interactions. Attack from the same lepidopteran herbivore results in species-specific transcriptional responses in two solanaceous host plants. *Plant Physiology* **138**: 1763-1773
- Scotto-Lavino E, Du G, Frohman MA** (2006) 3' end cDNA amplification using classic RACE. *Nat Protoc* **1**: 2742-2745
- Steppuhn A, Baldwin IT** (2007) Resistance management in a native plant: nicotine prevents herbivores from compensating for plant protease inhibitors. *Ecology Letters* **10**: 499-511
- Turner JG, Ellis C, Devoto A** (2002) The jasmonate signal pathway. *Plant Cell* **14**: S153-S164
- Ulker B, Mukhtar MS, Somssich IE** (2007) The WRKY70 transcription factor of *Arabidopsis* influences both the plant senescence and defense signaling pathways. *Planta* **226**: 125-137
- van Dam NM, Horn M, Mares M, Baldwin IT** (2001) Ontogeny constrains systemic protease inhibitor response in *Nicotiana attenuata*. *Journal of Chemical Ecology* **27**: 547-568
- van der Hoorn RAL** (2008) Plant proteases: From phenotypes to molecular mechanisms. *Annual Review of Plant Biology* **59**: 191-223
- Wajant H, Mundry KW, Pfizenmaier K** (1994) Molecular-Cloning of Hydroxynitrile Lyase from *Sorghum-Bicolor* (L) - Homologies to Serine Carboxypeptidases. *Plant Molecular Biology* **26**: 735-746
- Walling LL** (2006) Recycling or regulation? The role of amino-terminal modifying enzymes. *Current Opinion in Plant Biology* **9**: 227-233

- Wang L, Allmann S, Wu J, Baldwin IT** (2008) Comparisons of LIPOXYGENASE3- and JASMONATE-RESISTANT4/6-silenced plants reveal that jasmonic acid and jasmonic acid-amino acid conjugates play different roles in herbivore resistance of *Nicotiana attenuata*. *Plant Physiology* **146**: 904-915
- Wang L, Halitschke R, Kang JH, Berg A, Harnisch F, Baldwin IT** (2007) Independently silencing two JAR family members impairs levels of trypsin proteinase inhibitors but not nicotine. *Planta* **226**: 159-167
- Wen JQ, Tan BC, Liang HG** (1996) Changes in protein and amino acid levels during growth and senescence of *Nicotiana rustica* callus. *Journal of Plant Physiology* **148**: 707-710
- Zavala JA, Patankar AG, Gase K, Hui DQ, Baldwin IT** (2004) Manipulation of endogenous trypsin proteinase inhibitor production in *Nicotiana attenuata* demonstrates their function as antiherbivore defenses. *Plant Physiology* **134**: 1181-1190

7. Supplemental Figures

<i>S.nigrum</i>	MAPKNI FLHYNI LLI SYLAFLFLVT	25
Tomato	MAKKNFV FLHYNL LLI SLAFLFLLT	25
<i>S.nigrum</i>	EGAPQSALVTQLPGFNGNF QSKHYA	50
Tomato	EGAPQSALVTQLPGFNGTF NSKHYA	50
<i>S.nigrum</i>	GYVNI DESHGKNLYYYFVESERNPS	75
Tomato	GYVNI DESHGKNLYYYFVESERNPS	75
<i>S.nigrum</i>	KDPVVLWLNNGPGCSSFDGFVYEHG	100
Tomato	KDPVVLWLNNGPGCSSFDGFVYEHG	100
<i>S.nigrum</i>	PFNFE LGKPSGSLPSLHNN SYWSK	125
Tomato	PFNFDF GKPSGSLPSLHNN PYSWSK	125
<i>S.nigrum</i>	VSSVI YLDSPVGVGLSYS KNKSDYV	150
Tomato	VSNII YLDSPVGVGLSYS GNKSDYN	150
<i>S.nigrum</i>	TGDLKTASDSHSFLLKWF E IYPEFL	175
Tomato	TGDLKTASDSHSFLLKWF E IYPEFL	175
<i>S.nigrum</i>	TNPFYI AGESYAGI YVPTLS YE VVK	200
Tomato	KNPFYI S GESYAGI YVPTLASEVI K	200
<i>S.nigrum</i>	GIEAGVT PAI NFKGYMVGNGVSDXK	225
Tomato	GIDAGVR PAI NFMGYMVGNGVADDI	225
<i>S.nigrum</i>	IDGNAL VPFQHG MGLI SDELYEEVT	250
Tomato	IDGNAI VPFQHG MGLI SDDL YEEAV	250
<i>S.nigrum</i>	VACDGNFF EPASKNCTDMLDKVDQV	275
Tomato	VACHGNFYEPVDSNCSEKLNKI DQV	275
<i>S.nigrum</i>	VSDLNI YDILEPCYHSKEASVITTG	300
Tomato	VYDLNV YDILEPCYHSK KPSVITTG	300
<i>S.nigrum</i>	NSRLPMSFRKLGETERPLPVRKRMF	325
Tomato	NSRLPMSFRKLGETERPLPVRKRMF	325
<i>S.nigrum</i>	GRAWPF KAPVRE GRVPTWPEILNGL	350
Tomato	GRAWPFY KAPVRA GHVPTWPEILNSV	350
<i>S.nigrum</i>	EVPCTDDRVATAWLNNADVRKAIHA	375
Tomato	EVPCTDDRVATLWLNNADVRKAIHA	375
<i>S.nigrum</i>	ELTTVI GPWELCTDKIDLSDHDSGSM	400
Tomato	EPATVI GPWELCTDKIDLSDHDSGSM	400
<i>S.nigrum</i>	IPYHKNL TARGYRAI IYSGDHD MCV	425
Tomato	IPYHKNL TARGYRAI IFSGDHD MCV	425
<i>S.nigrum</i>	PFTGSQT WTSSLGYPIVDEWRP WYV	450
Tomato	PFTGS AVWT KSLGYPIVDEWRP WYV	450
<i>S.nigrum</i>	NEQVAGYIQGYDNNLIFMTI LGAGH	475
Tomato	NDQVAGFIQGYANNLIFMTI KGAGH	475
<i>S.nigrum</i>	TVPEYKPREALAFYARWLEGKKI	498
Tomato	TVPEYKPREALAFYSRWLEGKKI	498

Supplemental Fig.1 Alignment of *S. nigrum* SCP1 and tomato wound inducible SCP (gb/AAF44708.1/AF242849 1) predicted proteins. Black boxes indicate identical amino acids



Supplemental Fig.2 mean \pm SE *M. sexta* larval performance feeding either on control (CV) or on *SnSCP1*-silenced plants (vSCP), both genotypes were treated with W+R **a** larval mass over 9 days (t-test: day 9, $p = 0.439$). **Insert:** mean \pm SE relative mRNA abundance as indicator of silencing efficiency (* indicates significant difference, independent sample t-test, $p = 0.000$). **b** mean \pm SE larval mass over 10 days. **Insert:** mean \pm SE absorbance at 405 nm measured as activity of serine-protease against BTpNA as substrate (* t-test, $p = 0.000$)

Supplemental Table 1 Annotated list of all genes significantly up- or down- regulated in *S. nigrum* in response to *M. sexta* herbivory as identified with a custom 1.4k-ogio-microarray

Gene ID	Gene name	Annotation	Ratio	Significantly regulated
1002	STMEZ73	TC58526: Fructose-bisphosphate aldolase cytoplasmic isozyme 1 (EC 4.1.2.13). [Garden pea] {Pisum sativum}	1.53	UP
751	PRP4	L. esculentum pathogenesis-related protein P4	-1.52	DOWN
1370	STMHQ27	Abscisic acid and environmental stress inducible protein TAS14(Dehydrin TAS14).Abscisic acid and environmental stress inducible protein TAS14(Dehydrin TAS14).	-1.59	DOWN
1036	STMHL75	TC73148: cysteine protease {Ipomoea batatas}; TC66936: cysteine protease {Ipomoea batatas}	-1.81	DOWN

Supplemental Table 2 Annotated list of all used Primers throughout the experiments

Primer name	Sequence 5' → 3'	Function
Sn_S10_F1	GGTTACATGGTGGGAAATGG	3'RACE
Q _{outer}	CCAGTGAGCAGAGTGACG	3'RACE
Sn_S10_F2	CCGTGTTACCATAGCAAAGAAGC	3'RACE
Q _{inner}	GAGGACTCGAGCTCAAGC	3'RACE
Sn_S10_F3	TATCCGATCGTGGATGAATGG	SYBRgreen
Sn_S10_R3	CGCCTCTCGTGGTTTGTACTC	SYBRgreen
GSP1	AGGCCTTTCTGTCTCACC	5'RACE
GSP2	TTACTCGCTGGTTCGAAGAA	5'RACE
AAP	GGCACGCGTCGACTAGTACGGGI IGGGIIGGGIIG	5'RACE
Le_S10_F2	GGTTACATGGTGGGAAATGG	cloning
Le_S10_R2	CACGGCCAAACATTCTTTTT	cloning

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Selbstständigkeitserklärung

Hiermit erkläre ich an Eides statt, dass ich die vorliegende Arbeit selbstständig und nur unter der Verwendung der angeführten Quellen und Hilfsmittel angefertigt habe.

Jena, den 26. Juli 2010

Adriana Prehl