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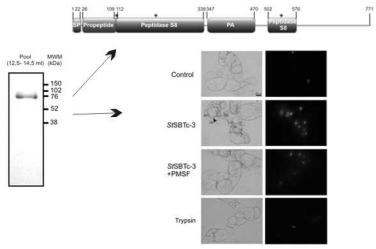

Title:

Isolation and characterization of a *Solanum tuberosum* subtilisin- like protein with caspase- 3 activity (*St*SBTc-3)

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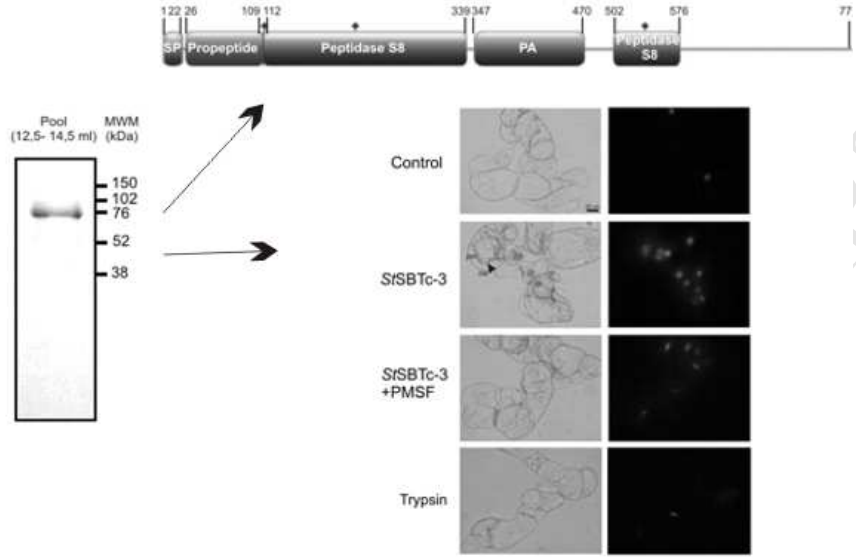
Abstract

Plant proteases with caspase-like enzymatic activity have been widely studied during the last decade. Previously, we have reported the presence and induction of caspase- 3 like activity in the apoplast of potato leaves during *S. tuberosum- P. infestans* interaction. In this work we have purified and identified a potato extracellular protease with caspase- 3 like enzymatic activity from potato leaves infected with *P. infestans*. Results obtained from the size exclusion chromatography show that the isolated protease is a monomeric enzyme with an estimated molecular weight of 70 kDa approximately. Purified protease was analyzed by MALDI-TOF MS, showing a 100% of sequence identity with the deduced amino acid sequence of a putative subtilisin- like protease from *S. tuberosum* (Solgenomics protein ID: PGSC0003DMP400018521). For this reason the isolated protease was named as *St*SBTc- 3. This report constitutes the first evidence of isolation and identification of a plant subtilisin- like protease with caspase- 3 like enzymatic activity. In order to elucidate the possible function of *St*SBTc- 3 during plant pathogen interaction, we demonstrate that like animal caspase- 3, *St*SBTc- 3 is able to produce *in vitro* cytoplasm shrinkage in plant cells and to induce plant cell death. This result suggest that, *St*SBTc- 3 could exert a caspase executer function during potato- *P. infestans* interaction, resulting in the restriction of the pathogen spread during plant- pathogen interaction.

Graphical abstract



Highlights

- *St*SBTc- 3 is a potato subtilisin like protease with caspase- 3 activity.
- *St*SBTc- 3 is a monomeric protein with an estimated molecular weight of 70 kDa.
- *St*SBTc- 3 has a highest amino acid sequence identity with *Sl*SBT3 from tomato.
- *St*SBTc- 3 is able to induce *in vitro* plant cell death.

Keywords

Solanum tuberosum; subtilisin like protein; DEVDase; apoplast; plant- pathogen interaction.

1. Introduction

Plant immune system is broadly divided into two interconnected defensive layers. The first layer, called as pathogen associated molecular patterns (PAMPs)-triggered immunity (PTI) involves the recognition of conserved microbial elicitors (PAMPs) by plant pattern recognition receptors (PRRs) [1-5]. The activation of PRRs results in active defense responses such as production of reactive oxygen species, callose deposition and synthesis of antimicrobial compounds [6, 7]. However, there are some plant pathogen microorganisms able to attenuate PTI by the secretion of proteins (effectors) that manipulate host processes inducing the effector-triggered susceptibility (ETS) [3, 4, 8-10]. As a consequence of this, the second defense layer, called effector-triggered immunity (ETI), is activated. This mechanism comprises plant resistance (R) proteins which detect pathogen effectors, or their activity, often resulting in a localized cell death or hypersensitive response (HR) [3, 4, 8-10].

Several extracellular proteases have been associated with host immunity and described during plant- pathogen interaction. Some examples are three papain-like proteases named as: 1) *Rcr*3 required for *C. fulvum* tomato resistance inducing hypersensitive reaction cell death; 2) *Nb*CathB, for *N. benthamiana* Cathepsin B, required for the development of the hypersensitive response, and 3) *St*C14, a potato defense papain- like cysteine protease [11]. Additionally, a positive correlation has been reported between the potato field resistance to *P. infestans* and the expression pattern of *St*AP1 and *St*AP3, for *Solanum tuberosum* aspartic proteases 1 and 3 [12, 13]. In *Arabidopsis thaliana* susceptibility to *Pseudomonas* bacterial infection is enhanced by the knockdown of the pepsin- like protease CDR1 (constitutive disease resistance 1) [14]. On the other hand, in *A. thaliana*, the overexpression of an

extracellular subtilisin- like protein named as *At*SBT3.3 produce an increase of the plant disease resistance response to oomycete attack [15]. Also, *Sl*P69B and C, two tomato subtilisin proteases, are induced in tomato after pathogen infection [16, 17].

During the last decade, special attention of scientists has been focused on plant proteases with caspase- like activities. Although plants have no gene orthologous to caspases in their genomes, caspase- like activities had been associated with plant programmed cell death (PCD) by the activity based protein profiling (ABPP) technology [18-21]. In this way, two apoplastic serine dependent proteases (subtilisin- like proteases) with caspase- 6 activities and related to plant PCD have been purified and described, they are named as: saspases [19] and phytaspases [18]. Additionally, two caspase- like activities have been related to destructive and non- destructive vacuole mediated PCD [24]. Destruction mechanism is initiated by the cysteine protease named as vacuolar processing enzyme (VPE) with caspase- 1 activity [22, 23]. The non-destructive PCD involves the 20S proteasome subunit PBA1 with caspase- 3 activity [24, 25]. On the other hand, two recombinant expressed barley legumains named as *Hv*Leg- 2 and -4 showed cysteine and caspase- like activities [26]. A multifunctional role was assumed for these two cysteine peptidases, whereas *Hv*Leg- 2 induces in leaves to biotic and abiotic stimuli, in seeds is induced by gibberellic acid, *Hv*Leg- 4 respond in leaves to wounding and has an unknown role in the germinating seed [26]. Recently, we have reported a positive correlation between apoplastic caspase- 3 activity and potato field resistance to *P. infestans* infection, suggesting the induction and/or activation of apoplastic serine protease/s with caspase- 3 activity during potato- *P. infestans* interaction [20].

In the present work, we describe the purification, identification and characterization of an apoplastic protease with caspase- 3 activity from potato leaves infected with *P. infestans* (named as *St*SBTc- 3). MALDI-TOF-MS identification of the isolated protein revealed that a *Solanum tuberosum* subtilisin like protein (Solgenomics protein ID: PGSC0003DMP400018521) is responsible of the caspase- 3 activity. Additionally, we demonstrate that *St*SBTc- 3 is able to induce *in vitro* cytoplasmic shrinkage and cell death on tomato cells. These results provide new evidences about the type of proteases involved in the plant defense response mechanism during potato- *P. infestans* interaction.

2. Methods

2.1. Plant and fungal material: growth conditions.

Potato plants of *S. tuberosum* L. *cv*. Pampeana INTA were grown in pots containing a 1:1 (v/v) mixture of soil/ vermiculite and maintained at 18 ºC for eight weeks with a 12 h photoperiod. *Phytophthora infestans* race R2 R3 R6 R7 R9, mating type A2, was grown on potato tuber slices of *cv*. Spunta at 18 °C and darkness. Seven days post- inoculation, mycelia was harvested in sterile water and stimulated to release zoospores by incubation for 2- 3 h at 4 °C. After filtration through muslin, the resulting suspension was observed under light microscope to quantify spores and sporangia before being used as inoculum. Concentration was adjusted to 10^5 sporangia mL^{-1}.

Detached leaves were placed on Petri dish capsules with wet filter paper and infected by spraying with sterile distilled water (control) or a suspension containing 10^5

ml^{-1} sporangia of *P. infestans*. Petri dishes were placed at 18 °C and 100 % humidity until leaves were harvested at 48 h post- inoculation.

Tomato cell suspensions (*Lycopersicon esculentum* cv. Money Maker; line Msk8) [30] were grown at 25 °C in the dark at 125 r.p.m. in MS medium (Duchefa, Haarlem, the Netherlands) supplemented with 3% (w/v) sucrose, 5.4 mM 1-naphtylacetic acid, 1 mM 6-benzyladenine and vitamins (Duchefa) [27].

2.2. Isolation of intercellular washing fluid (IWF)

Intercellular washing fluid of potato infected leaves was obtained as previously described by a standard technique based in a vacuum infiltration-centrifugation procedure [28]. Briefly, tissue was immersed in infiltration buffer (50 mM HCl-Tris (pH 8), 3.5% (w/v) NaCl, 0.1% (v/v) 2-mercaptoethanol and 20 µl/ 15 ml of Tween20) and subjected to three vacuum pulses of 10 seconds separated by 30 s intervals. Infiltrated leaves were recovered, dried on filter paper, placed in fritted glass filters, inserted in centrifuge tubes and centrifuged for 20 min at 400x*g* at 4° C. The recovered fluid was used immediately or conserved at -20 °C. As a marker enzyme for the cytosolic and vacuolar fraction Glucose-6-phosphate dehydrogenase (EC 1.1.1.49) and α-Mannosidase (EC 3.2.1.24) activity were measured as previously described [13].

2.3. Protein concentration

Protein concentration was determined by the bicinchoninic acid method [29] using bovine serum albumin (BSA) as standard. In chromatographic profiles, absorbance at 280 nm was recorded.

2.4. Assay for specific caspase- 3 like activity

Caspase- 3 like activity was determined using mammalian caspase-3 substrate N-Acetyl-Asp-Glu-Val-Asp-7-amido-4-trifluoromethylcoumarin (Ac-DEVD-AFC) as substrate [20]. Briefly, DEVDase activity of IWFs and eluates was measured in reaction buffer composed of 50 mM sodium acetate (pH 5.2), 2 mM DTT and 15 µM Ac-DEVD-AFC dissolved in dimethylsulphoxide. Release of fluorophore by cleavage was measured using a microplate fluorimeter (Fluoroskan ascent, Thermo Electron Corporation) during a 2.5 h reaction time at 37 °C (excitation and emission wavelengths of 405 and 525 nm respectively). Results were corrected against a blank containing only buffer and substrate. All assays were performed in triplicate and DMSO concentration was less than 1.5%.

For protease inhibition study, isolated protein was preincubated with 2.5 mM phenylmethanesulfonyl fluoride (PMSF) for 1h at 37ºC.

2.5. Purification of caspase- 3 like activity from IWFs

A two-step chromatographic procedure was employed to purify caspase-3 like protease from IWF of 48 h infected potato leaves. Caspase-3 substrate (Ac-DEVD- AFC) was used to monitor specific caspase-3 like activity. The experiments were performed at room temperature.

2.5.1. Ion- exchange chromatography on Mono-Q HR 5/5

Intercellular washing fluid from 48h *P. infestans* infected leaves was dialyzed against 50mM sodium acetate buffer, pH 5.2 at 4° C using cellulose membranes (molecular weight cut off 12000Da, Sigma D9652-100FT). The resulting sample was

loaded onto a Mono-Q HR 5/5 anion exchange column. The column was previously equilibrated in 50mM sodium acetate (pH 5.2) and eluted with a linear gradient of 0 to 500 mM NaCl at 1 mL min^{-1} flow rate. Peaks were detected at λ=280 nm and assayed for DEVDase activity. Positive fractions were dialyzed against 0.05 M sodium acetate buffer pH 5.2 at 4° C and dried in vacuum centrifuge (Savant AES 1010 Automatic Environmental Speed Vac®).

2.5.2. Size exclusion chromatography

Fractions with caspase- 3 activity (corresponding to fractions: 16 to 18) eluted from the anion exchange chromatography were pooled and dried to 100 µL in vacuum centrifuge. The concentrated sample was loaded onto a Superose 12 10/300 size exclusion column (GE Healthcare GE, 17-5173-01) and eluted with 0.05 M sodium acetate buffer (pH 5.2), 0.3 M NaCl using a flow rate of 0.5 mL min^{-1}. Peaks were detected at λ=280 nm. All fractions were desalted by dialysis against 0.05 M sodium acetate buffer (pH 5.2) at 4° C, dried in vacuum centrifuge and assayed for DEVDase activity. Fractions with caspase- 3 activity were submitted to SDS- PAGE [30].

2.6. SDS- PAGE

IWF and chromatographic fractions were analyzed by denaturating sodium dodecyl sulfate- polyacrylamide gel electrophoresis (SDS-PAGE) using 15% (p/v) acrylamide gel [30]. The samples were previously treated in denaturing buffer with β-mercaptoethanol before PAGE. Gels were stained with colloidal Coomasie Brillant Blue G250 [31].

2.7. MALDI-TOF mass spectrometry protein analysis

The protein bands obtained after several purification steps were cut and manually recovered, trypsin digested and mass spectrometric data were obtained using a MALDI-TOF-TOF spectrometer, Ultraflex II (Bruker), in the mass spectrometry facility CEQUIBIEM, Argentina (http://www.qb.fcen.uba.ar/cequibiem/). For calibration each spectrum, the following peaks of trypsin (m/z) were used: 842.51, 1045.56 and 2211.11. All the trypsin peptides were excluded as contaminants. Proteins were identified using the Mascot 1.9 search engine (Matrixscience,UK) on MSDB, NCBI, SwissProt and Solgenomics databases. A detailed analysis of peptide mass mapping data was performed using MASCOT software, allowing the following parameters: specie *Viridiplantae*, ± 50 ppm peptide mass tolerance, as well as cysteine carbamidomethylation as possible modifications. The confidence in the peptide mass fingerprinting matches was based on the score level and confirmed by the accurate overlapping of the matched peptides with the major peaks of the mass spectrum. A threshold of 67 in Mascot score was statistically significant with $p<0.05$.

2.8. Protease activity assay

Protease activity was determined with azocasein as substrate in 50 mM sodium acetate buffer (pH 5.2) and 0.5 µg of isolated protease. Samples were maintained at 37°C for 2.5h. Protease activity was monitored as an increase in the absorbance at 335 nm of the supernatant. The effect of the serine protease inhibitor PMSF was tested by previously incubation of the protease with 2.5mM PMSF at 37°C for 1h.

2.9. Phylogenetic analysis of *Solanaceae* subtilisin- like proteins

Deduced amino acid sequences from *St*SBTc- 3, tomato extracellular subtilisin- like proteins and tobacco phytaspase were retrieved by searching public databases at the Solgenomics (solgenomics.net/) and NCBI (http://www.ncbi.nlm.nih/). Subcellular localization was predicted using either TargetP V1.0 (www.cbs.dtu.dk/services/TargetP/) and SignalP (www.cbs.dtu.dk/services/SignalP/). Deduced amino acid sequences were aligned using the T-Coffee program (http://tcoffee.crg.cat/). Editing and sequence identity was calculated using the GeneDoc program (v2.6.003) [32]. The accession numbers of the aligned proteases sequences are: *Sl*SBT1 (CAA67429.1); *Sl*SBT2 (CAA67430.1); *Sl*SBT3 (NP_001234774.1); *Sl*SBT4a (NP_001234780.1); *Sl*SBT4b (CAA07059.1); *Sl*SBT4c (CAA07060.1); *Sl*SBT4E (CAA07062.1); *Sl*P69A (CAA76724.1); *Sl*P69B (CAA76725.1); *Sl*P69C (CAA06412.1); *Sl*P69D (CAA76727.1); *Sl*P69E (CAA06413.1); *Sl*P69F (CAA06414.1); *Sl*Tmp (AAB38743.1); *St*SBTc- 3 (PGSC0003DMP400018521) and *Nt*Phytaspase (ACT34764.1). The phylogenetic tree was obtained with the neighbour-joining method with bootstrap values generated from 10,000 bootstrap samples using the MEGA version 5.2 program [33].

2.10. Tomato cell suspension cell death assay

Tomato cell suspensions of 5 days old were incubated over night in 50 mM sodium acetate buffer (pH 5.2) with or without 0.1 ng/µl of *St*SBTc- 3 or 0.1- 1 ng/µl of trypsin (EC 3.4.21.4, Sigma™) in the presence or not of the serine protease inhibitor PMSF (2.5 mM). Assays were performed on 96- well micro liter plates at 25ºC in darkness for 20 h. Subsequently, cells were incubated with 0.2 µM SYTOX Green for 10 minutes. Afterwards, cells were washed with fresh culture medium, mounted on

microscope slides and visualized using a fluorescence microscope (Nikon) with an excitation and emission filters of 480 nm and 525 nm respective. The production of green fluorescence is due to the penetration of the dye into cells with compromised plasma membrane and binding to nuclear DNA.

3. Results and discussion

3.1. Purification of a protease with caspase- 3 like activity from IWF of potato leaves infected with *P. infestans*.

We have previously reported that caspase- 3 activity found in potato leaves during *P. infestans* infection was mainly attributable to apoplastic proteases [20]. In order to isolate and identify the protein/s with this caspase activity, intercellular washing fluid (IWF) from potato leaves after 48h of infection with *P. infestans* was analyzed by chromatographic methods.

First, IWF was subjected to fast protein anion exchange liquid chromatography (MonoQ HR 5/5) and eluted with a linear gradient of NaCl (0- 500 mM) as described in Materials and methods. One peak with caspase- 3 activity (Pool I) was eluted at 175-200 mM NaCl (Fig. 1A).

With the aim to purify protein/s with caspase- 3 activity, Pool I was subjected to size exclusion chromatography (Superose 12, GE Healthcare 17-5173-01). Protein elution profile obtained from this purification step is shown in Figure 1B. Only, one protein peak with caspase- 3 like activity (Pool II) was detected. Estimated molecular weight of proteins from Pool II by size exclusion chromatography was 70 kDa approximately (Fig. 1C). Subsequently, proteins pattern of Pool II were analyzed by

15% SDS- PAGE (Fig. 1D). As shown in figure 1D, a single protein band with an estimated molecular weight of 75 kDa approximately, was visualized. Results obtained from estimated molecular weight by size exclusion chromatography and SDS-PAGE analysis, demonstrate that, a monomeric and extracellular protease is being responsible of the caspase- 3 activity determined and is induced in the potato leaves apoplast after *P. infestans* infection [20]. In contrast with these results, data reported by Han et al. [34] show that, caspase- 3 activity detected during xylem development in *Populous tomentosa* is attributed to the 20S proteasome, an intracellular large protein complex. This antecedent and the results obtained in this work, demonstrate that, in plants, caspase- 3 activity is involved during cell death process, either in the cell death induced after tissue differentiation [34] or in the cell death induced during potato- *P. infestans* interaction to circumscribe pathogen dispersion [20]. However, several differences have been found between the enzymes able to exert caspase- 3 activity involved in these types of cell death. In the first case [34] proteins with caspase- 3 activity are heteromeric and with intracellular localization, whereas in the second case [20], protein with caspase- 3 activity is monomeric and extracellulary located.

3.2. Identification of a subtilisin- like protease with caspase- 3 activity

With the aim to identify the potato monomeric extracellular protease with caspase- 3 activity, protein band obtained from the SDS-PAGE analysis of Pool II (Fig. 1D), was digested in gel by trypsin and followed by MALDI- TOF- mass spectrometry analysis and subsequently MS/MS analysis. The resulting spectra were used to search for matching proteins in the NCBI database (http://www.ncbi.nlm.nih.gov) using the

Mascot search program (http://www.matrixscience.com, Matrix Science, London, England) and the potato genome database Solgenomics (solgenomics.net/).

Figure 2 shows MALDI- TOF mass spectra and the sequences of the three tryptic peptides obtained. BLASTp and tBLASTn searches against the potato genome database revealed 100% of identity with a subtilisin- like protease from *Solanum tuberosum* (Transcript and protein ID: >PGSC0003DMT400027148 and >PGSC0003DMP400018521 respectively) with an E- value of 8.5 e-04 (Fig. 3). Minor identities with higher E- values were found to the tryptic peptides with other putative proteins corresponding to subtilisin- like proteases of *Solanum tuberosum* in this database. In this way, tryptic peptides matched with a subtilisin- like protease (PGSC0003DMP400012119) with an identity of 93.33% and an E-value of 5e-05, a subtilase (PGSC0003DMP400011990) with an identity of 73.33% and an E-value of 0.0029, a subtilisin- like protease preproenzyme (PGSC0003DMP400027005) with an identity of 68.42% and an E-value of 0.10 and a subtilisin- like protease (PGSC0003DMP400064549) with an identity of 58.06% and an E-value of 0.0096. Figure 3 shows the analysis of the nucleotide and deduced protein sequences of this subtilisin- like protease from *Solanum tuberosum*.

BLASTp search of the subtilisin- like protease from *Solanum tuberosum* (ID: PGSC0003DMP40001852) obtained against the MEROPS peptidase database [35], allowed us to classify the subtilisin- like protease as a protease belonging to the S8A subfamily (unassigned peptidase) of serine proteases. In accordance with this result, we show in figure 4 the inhibition by PMSF, a specific inhibitor of serine proteases, of protease and caspase- 3 activities of the purified potato subtilisin- like protease.

So far, potato database contains several subtilisin- like predicted protein sequences deduced annotated as a result of the potato genome sequencing, however, there are not reports about subtilisin proteases transcriptomics and/or proteomics in potato. In this report we show the purification and identification of a *Solanum tuberosum* subtilisin- like protease named as *St*SBTc- 3 (subtilisin- like protein with *c*aspase- *3* activity) involved in plant cell death during potato- *P. infestans* interaction. Other plant subtilisin- like proteases with caspase- 6 activities have been purified and identified, namely, saspases [19] and phytaspases [18], however neither of them were able to cleave DEVD, a specific substrate of caspase- 3.

3.3. Sequence analysis of *St*SBTc- 3.

Most SBTs from plants and other organisms show the typical pre-pro-protein structure of secretory proteins, comprising a signal peptide at the N-terminus, a cleavable prodomain, a subtilisin domain with the characteristic arrangement of catalytic Asp, His and Ser residues of the catalytic triad and, occasionally, C- terminal extensions. A distinguishing feature of plant as compared to mammalian SBTs is an insertion of 120 amino acids between His and Ser residues of the catalytic triad named as additional protease- associated domain (PA) [36]. According with the data above described, the *St*SBTc- 3 predicted amino acid sequence contains a signal peptide, a propeptide and a mature peptide of 659 aminoacids (Fig. 3). Consistent with the estimated molecular weight of 70 kDa obtained by size exclusion chromatography and SDS- PAGE (Figs. 1C and 1D), molecular weight prediction of *St*SBTc- 3 deduced aminoacid sequence was of 70.5097 kDa for the mature protein (Solgenomics ID: PGSC0003DMP400018521).

SignalP and TargetP program predictors of subcellular location of eukaryotic proteins predicted a cleavage site between positions 22- 23 of the full length pre-pro-protein. This result suggests the presence of a secretory signal peptide of 22 aminoacids (Met^{1}- Ala^{22}) consistent with *St*SBTc- 3 extracellular localization previously reported [20]. Bioinformatic analysis performed with CD- search (NCBI) and Pfam (Sanger Institute) programs, suggested the presence of four domains highly conserved in precursors of subtilisin- like serine proteases: a peptidase inhibitor I9 domain (Thr^{26}-His^{112}, E- value: 5.68e-15), a peptidase S8 family domain (Val^{109}-Ser^{339}, E- value: 7.60e-100), a protease associated (PA) superfamily domain (Thr^{347}-Ile^{470}, E- value: 1.37e-06) and a peptidase S8_S53 superfamily domain (Lys^{502}-Ser^{576}, E- value: 4.77e-29) (Fig. 3B).

We performed a phylogenetic analysis in order to identify putative subtilisin-like proteases of *Solanaceae* with caspase- like activity [18, 37]. So, we compared the amino acid deduced sequences of *St*SBTc- 3 with fifteen amino acid sequences of subtilisin- like proteases previously reported to *Solanaceae* species, two of them, *St*SBTc- 3 and tobacco phytaspase, with caspase- like activity.

Comparison of the *St*SBTc- 3 deduced amino acid sequence with GenBank and EMBL databases reveals a high degree of conservation with other subtilisin- like proteins from others *Solanaceae* species (Fig. 5). *St*SBTc- 3 has the highest sequence identity with tomato *Sl*SBT3 (65%) (NP_001234774.1), tomato *Sl*SBT4b (62%) (CAA07059.1), *Sl*SBT4e (CAA07062.1) and c (CAA07060.1) (61%), tomato *Sl*SBT4a (NP_001234780.1) (60%). Lower identities were detected with tobacco phytaspase (ACT34764.1) (46%), tomato *Sl*SBT1 (CAA67429.1) and 2 (CAA67430.1) (35%), the tomato P69 group (*Sl*P69A (CAA76724.1); *Sl*P69B (CAA76725.1); *Sl*P69C (CAA06412.1);

*Sl*P69D (CAA76727.1); *Sl*P69E (CAA06413.1); *Sl*P69F (CAA06414.1)) (35%) and the tomato meiotic proteinase Tmp (AAB38743.1) (28%) (Fig. 5).

Data obtained from the phylogenetic analysis of *St*SBTc- 3 (Fig. 6) show that despite this protein has higher similarity with *Sl*SBT3 and lower with *Sl*SBT1 (Figs. 5 and 6), it shares more structural properties with *Sl*SBT1 than with *Sl*SBT3. While *Sl*SBT3 has a fibronectin III- like domain as a C- terminal extension, *Sl*SBT1 and *St*SBTc- 3 do not (Figs. 3 and 5). Also, tomato *Sl*SBT3 has been described to form homodimers through its PA domain. However, results obtained here showed that, as tomato *Sl*SBT1, *St*SBTc-3 is a monomeric enzyme in its active and mature form (Figs. 1C and 1D). Although *Sl*SBTc- 3 is closer related to tomato *Sl*SBT3 than to tobacco phytaspase it shares more structural and activity specificities with the tobacco phytaspase (Fig. 6). As well as phytaspase [18, 38], potato *St*SBTc- 3 shows caspase activity and is related to the PCD that occurs in the hypersensitive response during plant- pathogen interactions. Data presented here suggest that there are both, structural similarities and identity in the amino acid sequences between subtilisin- like proteases of *Solanaceae* species. This fact suggests that will be necessary to perform assays to test the potential caspase-like activity of these proteases (*Sl*SBT1 and *Sl*SBT3) one by one.

3.4. *St*SBTc- 3 ability to induce cell death in tomato cell culture

Caspases in mammalian PCD play a role as initiators and effectors [39]. Caspases -2, -8, -9 and -10 have been classified as initiator caspases, and they cleave inactive pro-forms of effector caspases, thereby activating them. On the other hand, caspases -3, -6 and -7 have been classified as effector caspases as they cleave other protein substrates within the cell, to trigger the apoptotic process and ultimately

leading to cell death [40, 41]. According with the previously demonstrated in several reports [42, 43], we have demonstrated that caspase- 3 activity is required for completion of cell death induced in potato leaves to restrict the pathogen spread, during potato- *P. infestans* interaction in resistant potato cultivars [20]. In order to determine if *St*SBTc- 3 is able to induce cell death in plant cells, 0.1 ng/µl of the subtilisin- like protease or buffer (control) were incubated with tomato cell cultures for 20 h in the previously described conditions prior to SYTOX Green treatment. Results obtained (Fig. 7) show that *St*SBTc- 3 was able to decrease tomato cell viability in a 75% during 20h of incubation. Additionally, we observed cytoplasm shrinkage as an effect of the cells incubation with active *St*SBTc- 3 (Fig. 7A). We have previously reported that *St*STBc- 3 caspase- 3 activity is inhibited by PMSF [20]. According with this antecedent, no changes in the tomato cell culture viability were observed when *St*STBc- 3 was previously incubated with 2.5 mM PMSF (Fig. 7B) compared to control. On the other hand, when tomato cells were incubated with 0.1 and 1 ng/µl of trypsin, no effect on the tomato cell viability was observed. These results suggest that *St*SBTc-3 cell death induction ability is related with the caspase- 3 activity exerted by this protease. This observation is in accordance with the effects produced by executer caspases in animals, where caspase- 3 is believed to be the major executioner to induce the cleavage of the PARP (Poly ADP-ribose polymerase), DNA fragmentation, cytoplasmic shrinkage, chromatin condensation, and final programmed death in animal cells [43-45].

All the results presented here, support the hypothesis that *St*SBTc- 3 is an executer caspase induced in the apoplast of potato leaves of resistant cultivars after infection with *P. infestans*, causing cell death and as consequence restriction of

pathogen spread. We think that, more assays are needed to study the potential role in the plant defense response mechanism would be necessary to corroborate this.

4. Conclusions

In this work, we described the purification of a *Solanum tuberosum* subtilisin-like protease with caspase 3 activity (*St*SBTc- 3) induced in potato leaves after *P. infestans* infection. Results obtained from size exclusion chromatography demonstrate that *St*SBTc- 3 mature enzyme is monomeric with a molecular weight of 70 kDa. Analysis of deduced amino acid sequence shows that the potato subtilisin like protein here purified presents the typical pre-pro-protein structure of secretory proteins, as well as most subtilisin like proteases from plants previously reported. *St*SBTc- 3 present a highest amino acid sequence identity with subtilisin like proteins identified in other *Solanaceae*. However, whereas *St*SBTc- 3 is phylogenetic closely related with tomato *Sl*SBT3, it shares more structural characteristics with tomato *Sl*SBT1. Therefore, this report constitutes the first evidence of isolation of a potato apoplastic subtilisin like protein with caspase- 3 like activity which may help to complete the family of "caspase- like" proteases, previously considered as "incomplete". Additionally, we demonstrate that caspase-3 enzymatic activity of *St*SBTc- 3 is able to induce plant cell death. Results presented here support the hypothesis that, *St*SBTc- 3 is an executer caspase induced in the apoplast of potato leaves of resistant cultivars after infection with *P. infestans*, causing cell death and as consequence of this, restriction of pathogen spread.

Contributions

FMB performed the assays, drew the figures and analyzed the data. GMG designed the research and the manuscript, and analyzed the data. GMG and FMB wrote the manuscript. GRD revised the manuscript and checked English language.

Acknowledgements

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Figures and captions

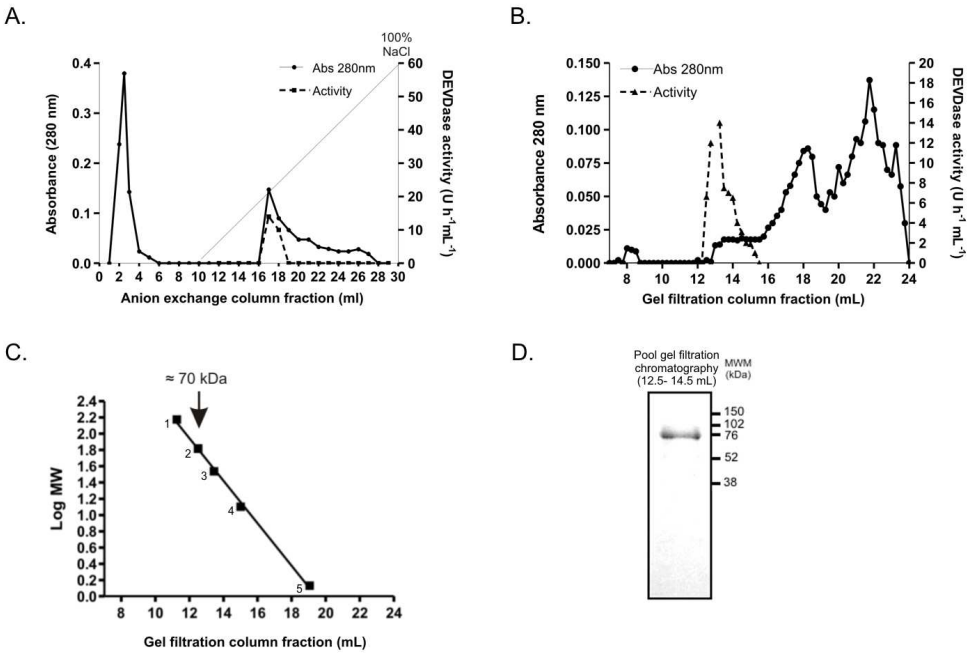
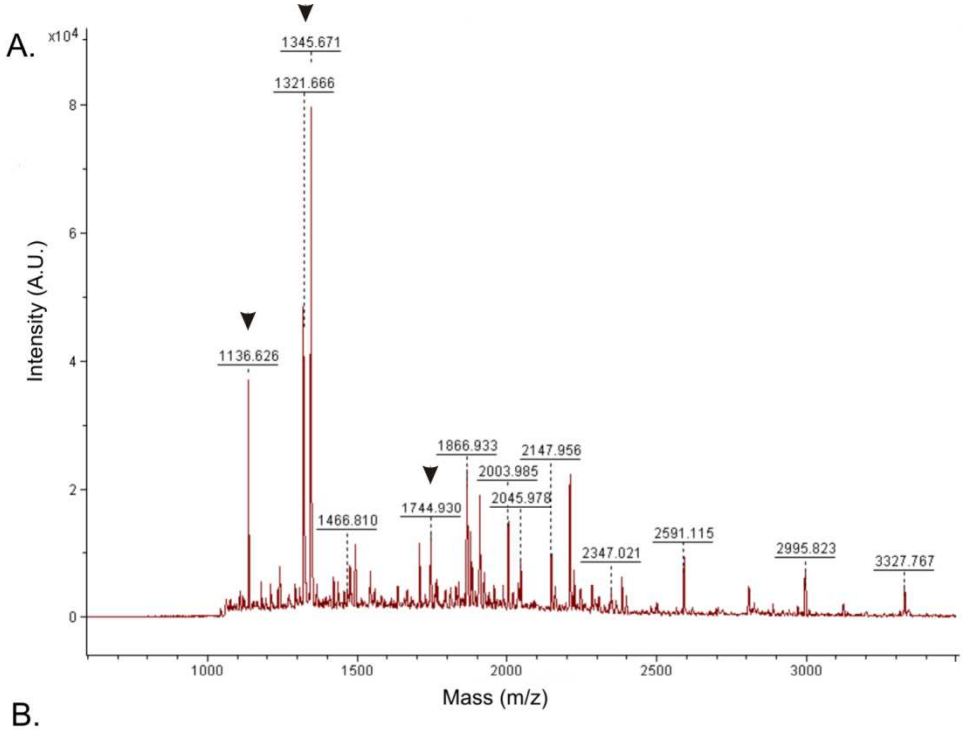


Figure 1. Purification steps of a caspase- 3 like protease from IWF of 48h *P. infestans* infected potato leaves. IWF of 48h infected potato leaves was subjected to anion exchange chromatography and eluted with a linear gradient of 0 to 500 mM NaCl **(A)**. Fractions with caspase- 3 like activity from MonoQ eluate were subjected to size exclusion chromatography **(B)**. Molecular weight of the isolated protease in its native state was estimated using a calibration curve for the size exclusion chromatography: (1) IgG (150 kDa); (2) BSA (67 kDa); (3) Lactoalbumin (35 kDa); Cytochrome C (12.7 kDa) and Vitamin B12 (1.355 kDa). The arrow indicates logMW from the isolated protease **(C)**. Fractions from the size exclusion chromatography with caspase- 3 like activity were pooled, desalted and analyzed by 15% SDS-PAGE **(D)**.

A.



B.

Calc. Mass (Da)	Observ. Mass (Da)	Start Seq.	End Seq.	Sequence
1344.6463	1345.6714	159	173	+K.GVSYFGYAPGTAR.G-
1135.6390	1136.6260	285	296	+K.ITGFSLFPVR.T-
1743.9189	1744.9295	123	141	+K.GLLASDPTIVLSMNSAR.D-

Figure 2. MALDI-TOF mass spectrometric assay of the protease with caspase- 3 like activity. A. MALDI- TOF- MS spectra. Arrows indicate peptides subsequently identified using MASCOT software and NCBI and Solgenomics databases. **B.** Trypsin digested aminoacid fragments identified.

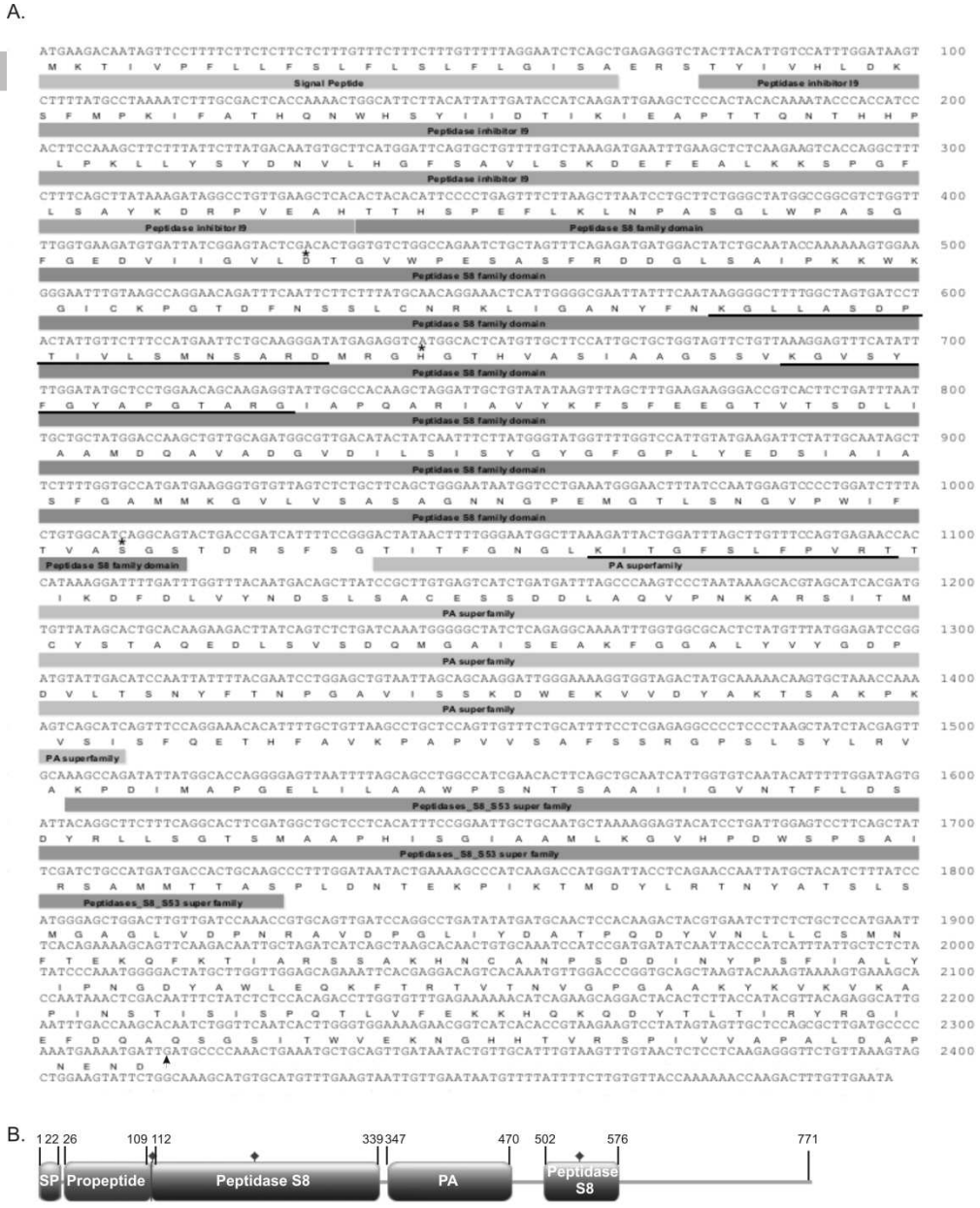


Figure 3. Potato subtilisin- like protein with caspase- 3 activity (*St*SBTc- 3) nucleotide and deduced amino acid sequences. A. Nucleotides are numbered from the first ATG codon of the transcript. The termination codon TGA is marked with an arrow. Signal peptide and protease conserved domains are indicated in shaded boxes. Tryptic peptides obtained by MALDI-TOF-MS are underlined in the mature protein. Aminoacids corresponding to the catalytic triad are indicated with an asterisk. **B.** Domain organization of *St*SBTc-3. The structure of *St*SBTc- 3 includes a signal peptide, a prosegment, two peptidase domains and a PA domain. Aminoacids from the catalytic triad Asp^{144}/His^{215}/Ser^{542} are indicated with diamonds.

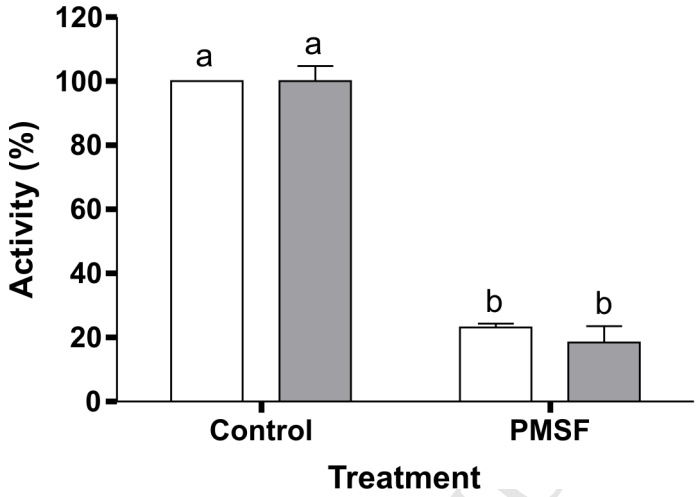


Figure 4. Effect of PMSF on DEVDase and azocasein activities of *St*SBTc- 3. *St*SBTc- 3 was pre-incubated with the serine protease inhibitors PMSF (2.5 mM) for 1 h at 37 °C and pH 5.2. Then, mixtures were incubated with 15 µM Ac-DEVD-AFC (gray bars) or azocasein substrate (white bars) at 37 °C during 2.5 h. Values are normalized as a percentage of protease activity without PMSF. Values were normalized as a percentage of the mean from control treatments. The data represent the mean ± s.d. of three replicates. Means with different letters differ at $p < 0.01$ according to one-way ANOVA and Tukey tests respectively.

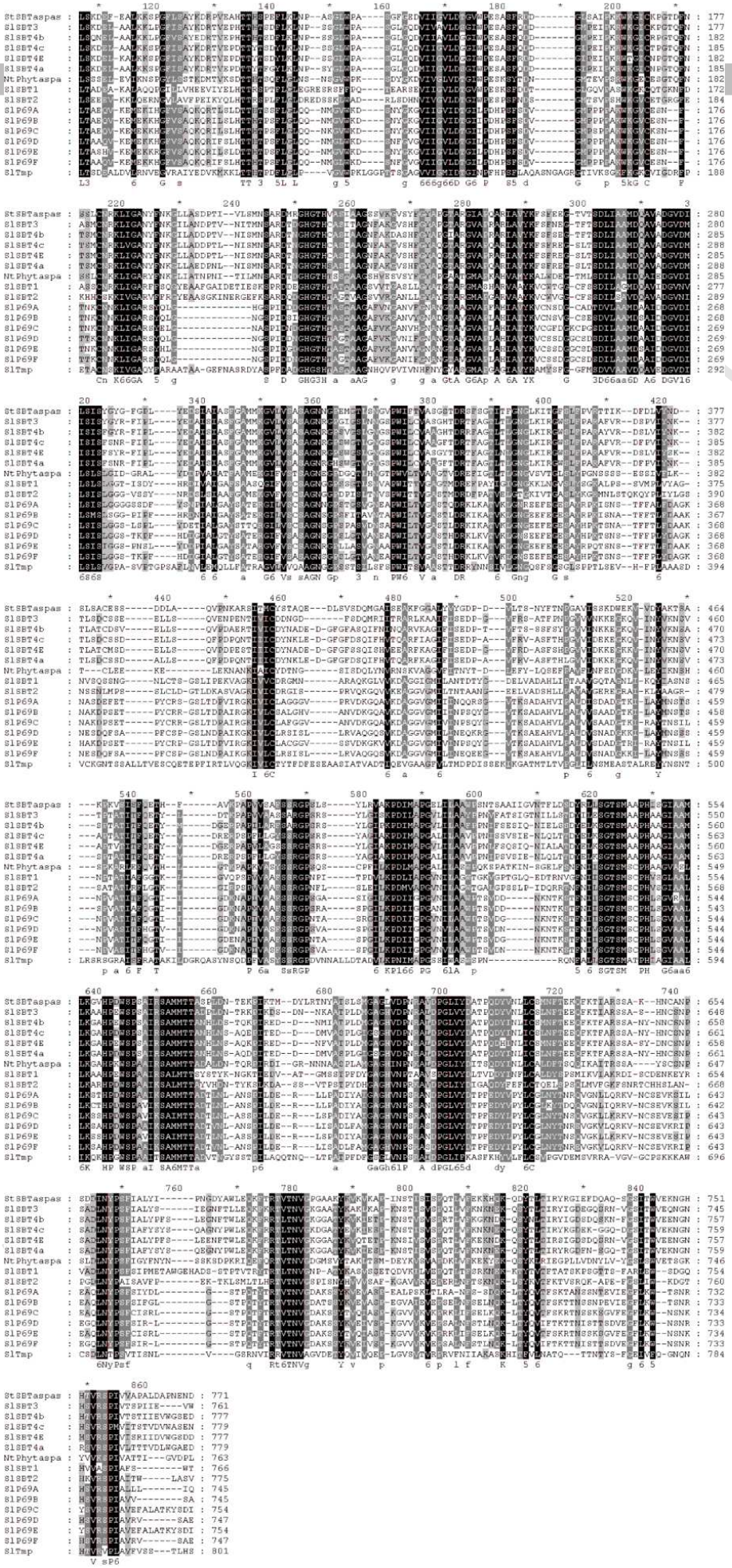


Figure 5. Alignment of *St*SBTc- 3 deduced amino acid sequence with other *Solanaceae* subtilisin- like proteases. Multiple sequence alignment of the aminoacid sequence from *St*SBTc- 3, tobacco phytaspase and tomato subtilisin like proteases was performed using the T-Coffee program. The accession numbers of the aligned protease sequences are as follows: *St*SBTc- 3 (PGSC0003DMP400018521); *Sl*SBT1 (CAA67429.1); *Sl*SBT2 (CAA67430.1); *Sl*SBT3 (NP_001234774.1); *Sl*SBT4a (NP_001234780.1); *Sl*SBT4b (CAA07059.1); *Sl*SBT4c (CAA07060.1); *Sl*SBT4E (CAA07062.1); *Sl*P69A (CAA76724.1); *Sl*P69B (CAA76725.1); *Sl*P69C (CAA06412.1); *Sl*P69D (CAA76727.1); *Sl*P69E (CAA06413.1); *Sl*P69F (CAA06414.1); tomato meiotic proteinase (*Sl*Tmp, AAB38743.1) and *Nt*Phytaspase (ACT34764.1). Conserved residues which are common to all sequences are shadowed in black; less identity is shadowed in gray scale.

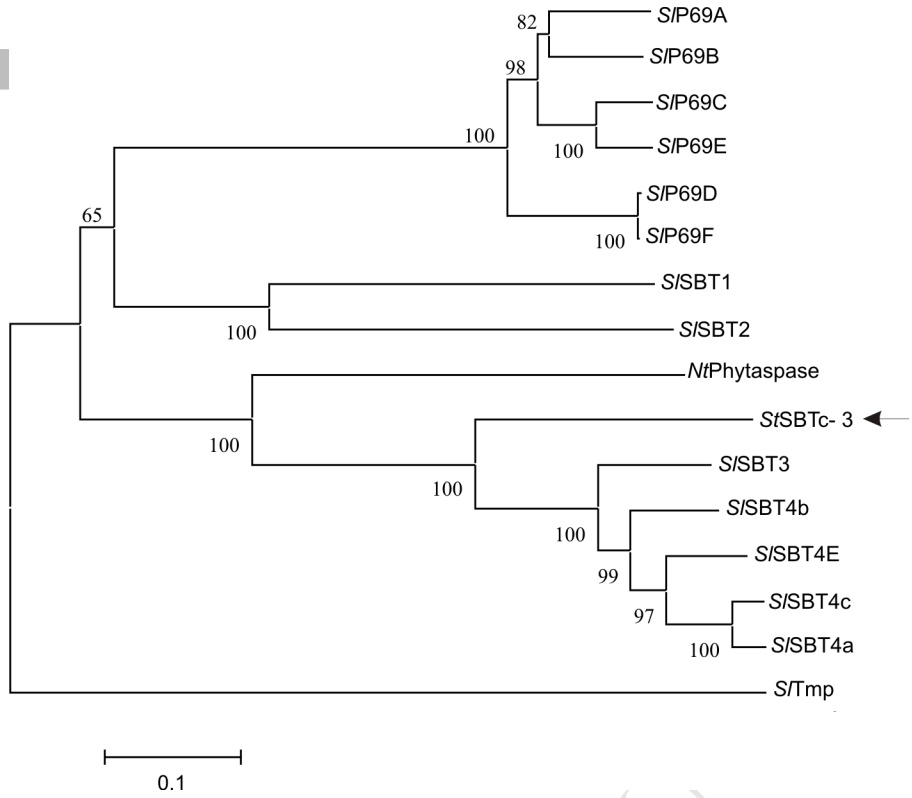


Figure 6. Phylogenetic relationship of *Solanaceae* subtilisin- like proteases. The unrooted phylogenetic tree was created from the *St*SBTc- 3, tomato subtilisin- like proteases and tobacco phytaspase amino acid sequences annotated in the Solgenomics (www.solgenomics.net/) database. Aminoacid multiple sequence alignment of the proteases: *St*SBTc- 3 (PGSC0003DMP400018521), *Sl*SBT1 (CAA67429.1), *Sl*SBT2 (CAA67430.1), *Sl*SBT3 (NP_001234774.1), *Sl*SBT4a (NP_001234780.1), *Sl*SBT4b (CAA07059.1), *Sl*SBT4c (CAA07060.1), *Sl*SBT4E (CAA07062.1), *Sl*P69A (CAA76724.1), *Sl*P69B (CAA76725.1), *Sl*P69C (CAA06412.1), *Sl*P69D (CAA76727.1), *Sl*P69E (CAA06413.1), *Sl*P69F (CAA06414.1), tomato meiotic proteinase (*Sl*Tmp, AAB38743.1) and *Nt*Phytaspase (ACT34764.1), was performed using the T-COFFEE program (http://tcoffee.crg.cat/). The bootstrapped consensus unrooted neighbor-joining tree was conducted using MEGA 5.2 [33]. The arrow indicates the isolated protease *St*SBTc- 3.

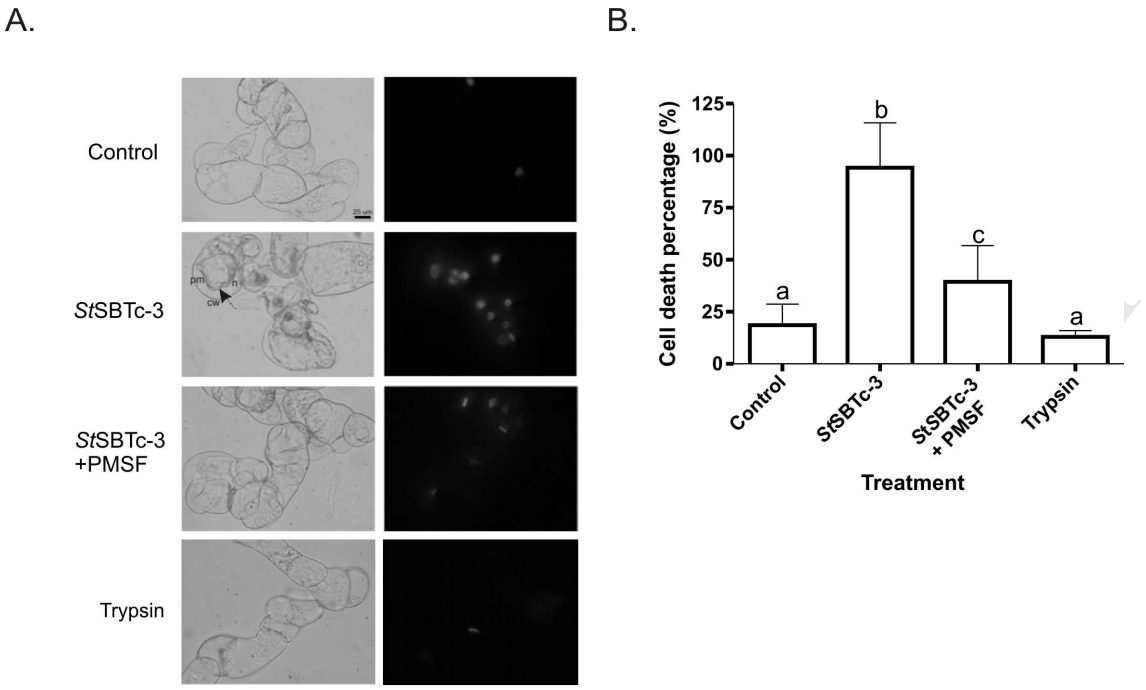


Figure 7. *In vitro* effect of *St*SBTc-3 on tomato cell suspensions. Tomato cell suspensions were incubated in 50mM sodium acetate buffer (control) with or without 0.1 ng/µl of protease or 0.1 ng/µl of trypsin, in the presence or not of the serine protease inhibitor PMSF (2.5 mM) for 20h at 25°C. Subsequently, cells were incubated with the vital probe SYTOX Green for 10 min and examined using a fluorescence microscope. **A.** Bright- field (left panels) and fluorescence images (right panels) are shown. Bar: 25 µm. **B.** Dead cell quantification. Values were normalized as a percentage of the mean of protease treated cells. The data represents the mean ± SD of three replicates. Different letters indicate significant differences with $p< 0.05$ for one way ANOVA and Tukey tests. Abbreviations: pm, plasma membrane; cw, cell wall; and n, nuclei. The arrow indicates cytoplasm shrinkage.

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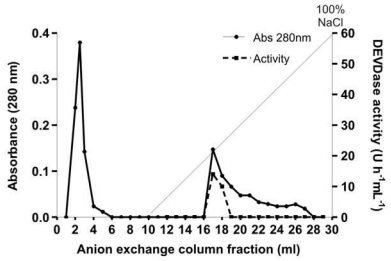

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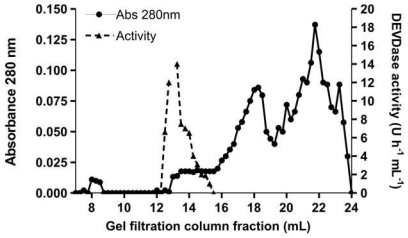

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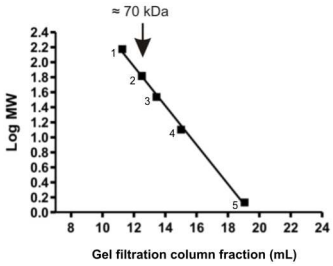

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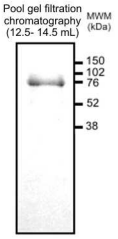

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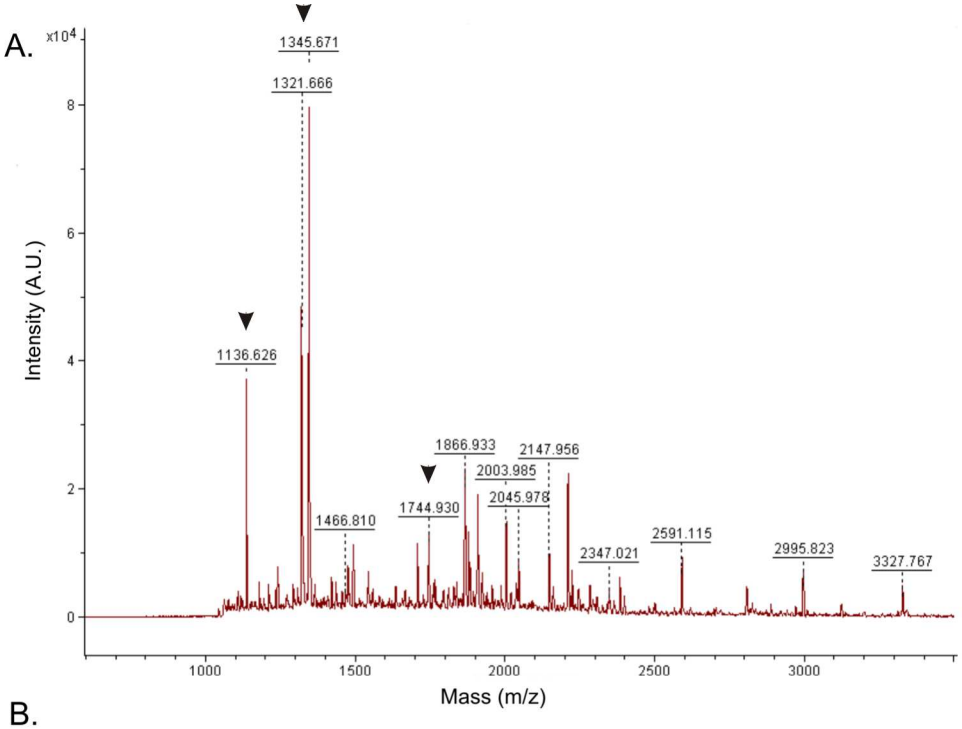

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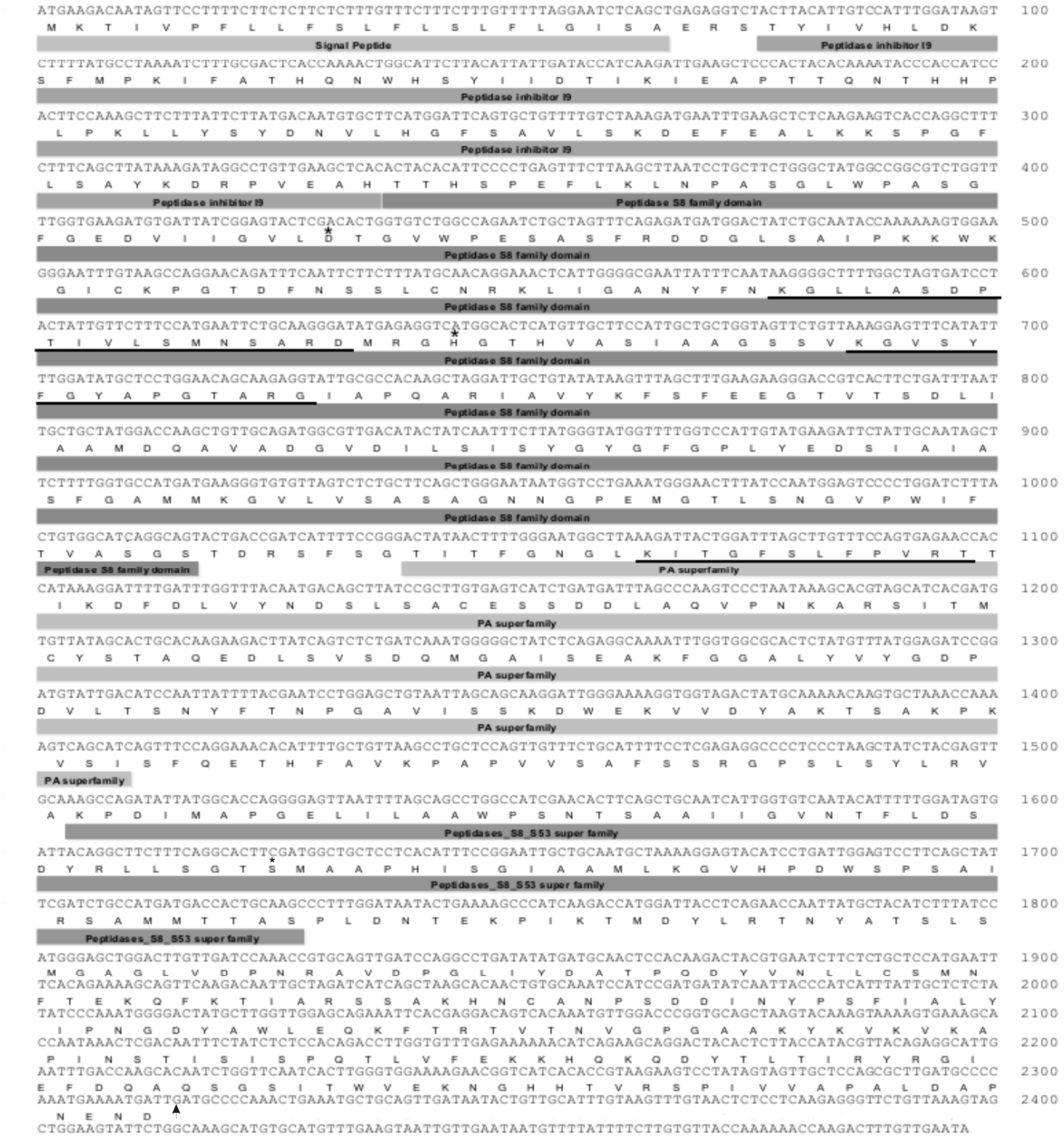

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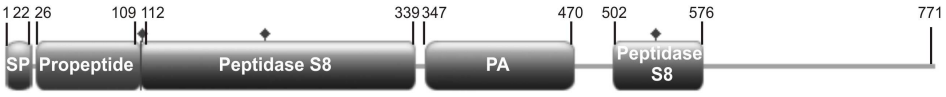

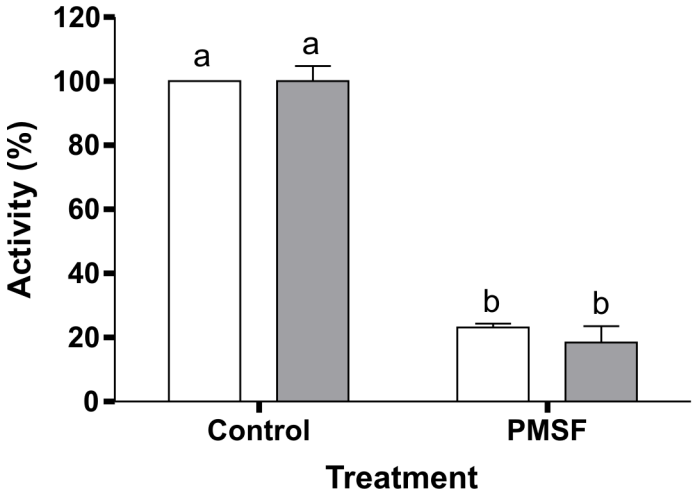

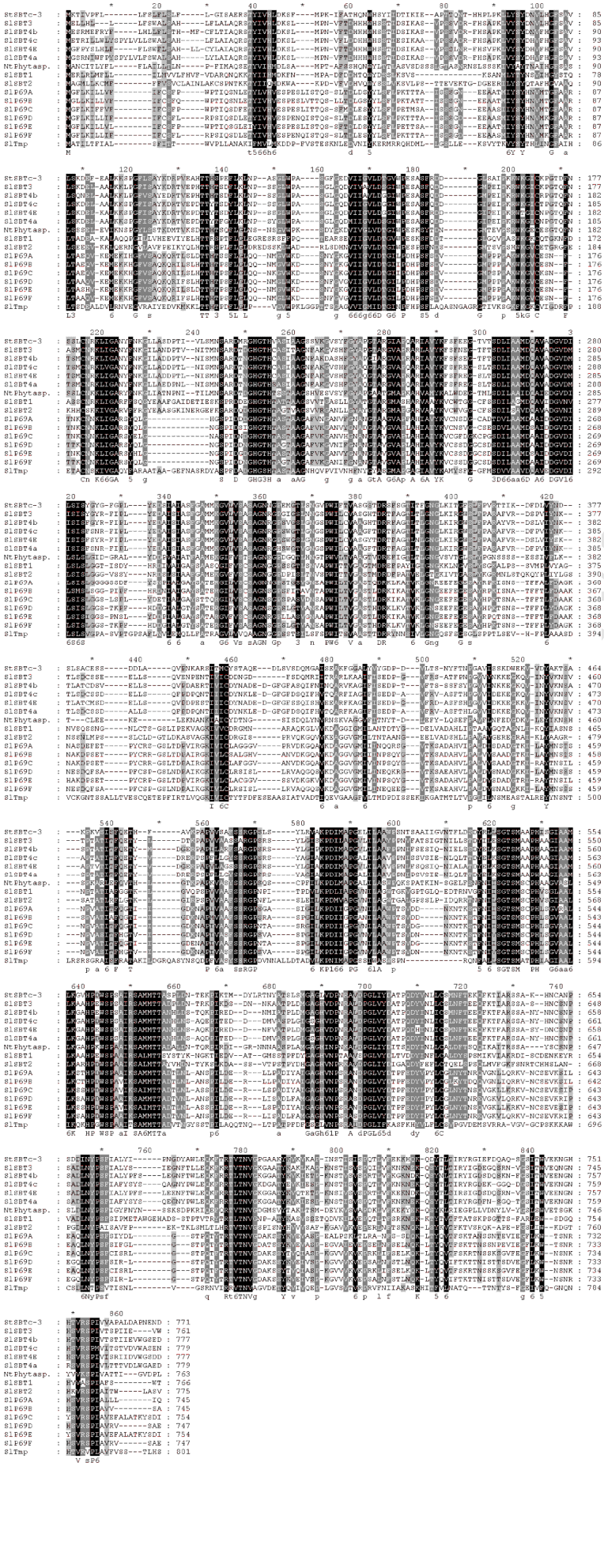

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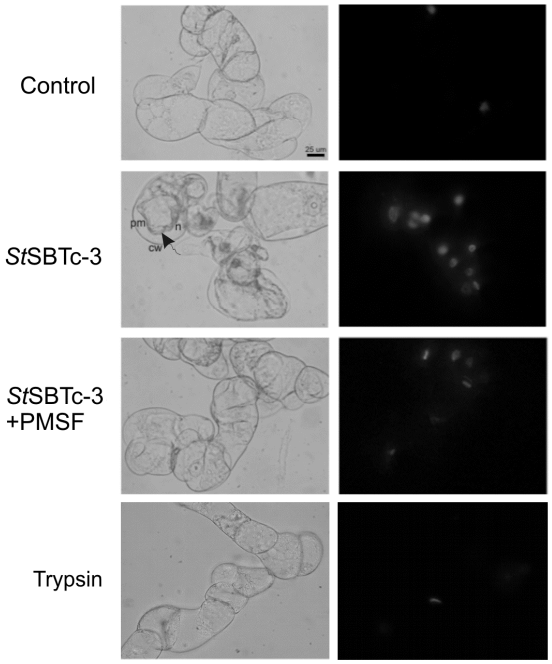

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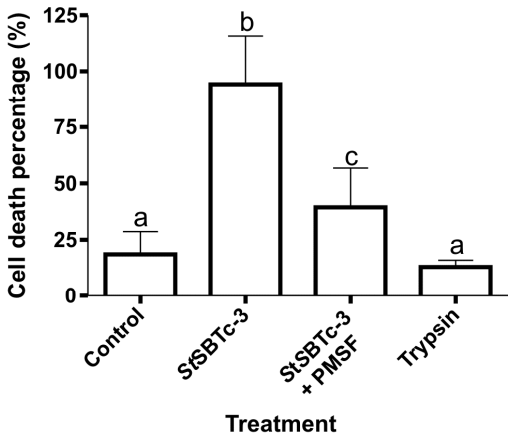

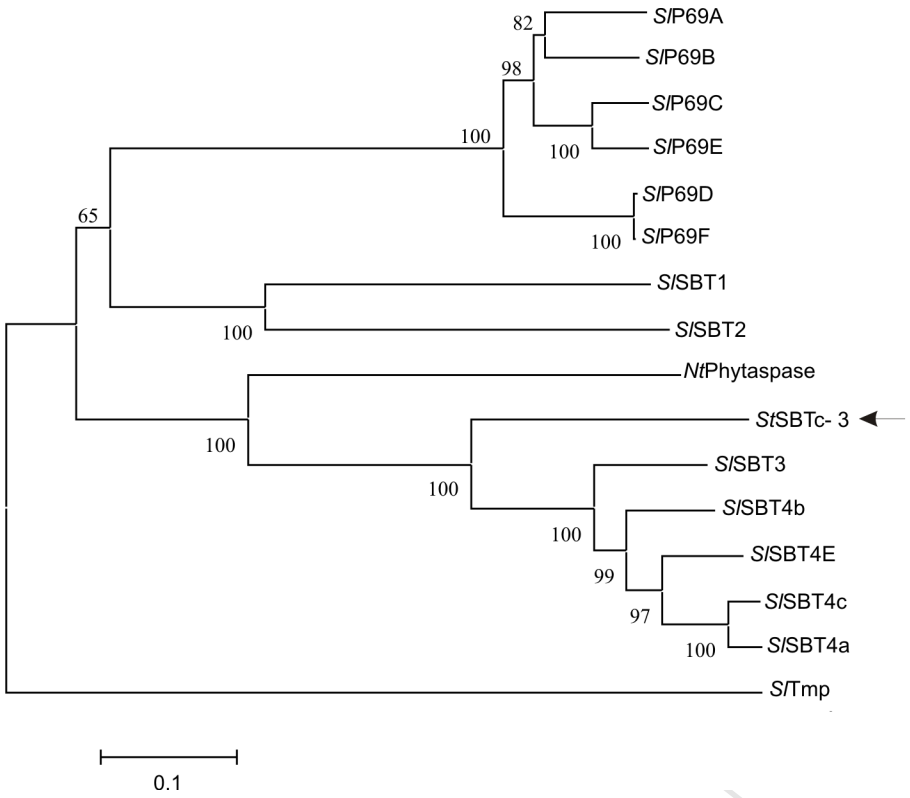

Highlights

- *St*SBTc- 3 is a potato subtilisin like protease with caspase- 3 activity.
- *St*SBTc- 3 is a monomeric protein with an estimated molecular weight of 70 kDa.
- *St*SBTc- 3 has a highest amino acid sequence identity with *Sl*SBT3 from tomato.
- *St*SBTc- 3 is able to induce *in vitro* plant cell death.

Contributions

FMB performed the assays, drew the figures and analysed the data. GMG designed the research and the manuscript, and analysed the data. GMG and FMB wrote the manuscript. GRD revised the manuscript and checked english language.