Pollination in *Nicotiana alata* stimulates synthesis and transfer to the stigmatic surface of NaStEP, a vacuolar Kunitz proteinase inhibitor homolog. *Grethel Yanet Busot, Bruce McClure, Claudia Patricia Ibarra-Sánchez, Karina Jiménez-Durán Sonia Vázquez-Santana and Felipe Cruz-García*

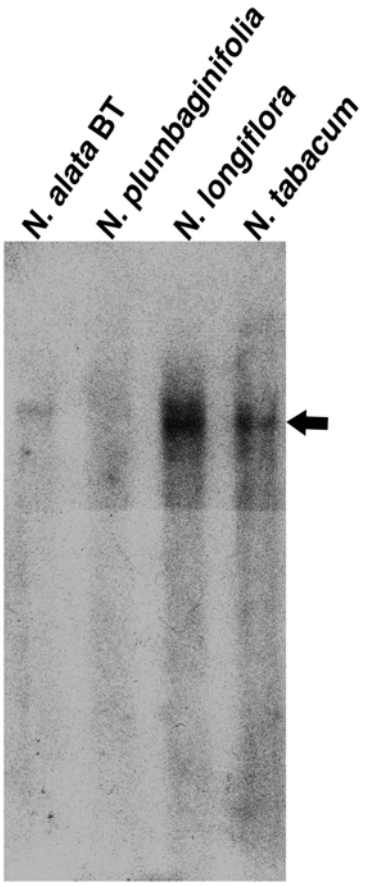
Supplementary Data

Supplementary Table S1. Pollination phenotypes of the *Nicotiana* species studied.

Self-compatible *Nicotiana* species	Self-incompatible *Nicotiana* species
N. glauca	*N. alata*
N. tabacum	*N. forgetiana*
N. plumbaginifolia	*N. bonariensis*
N. longiflora	
N. benthamiana	
N. alata cv Breakthrough	

Supplementary Table S2. Percent identities between NaStEP/NaSoEP and the proteinase inhibitors in clade V.

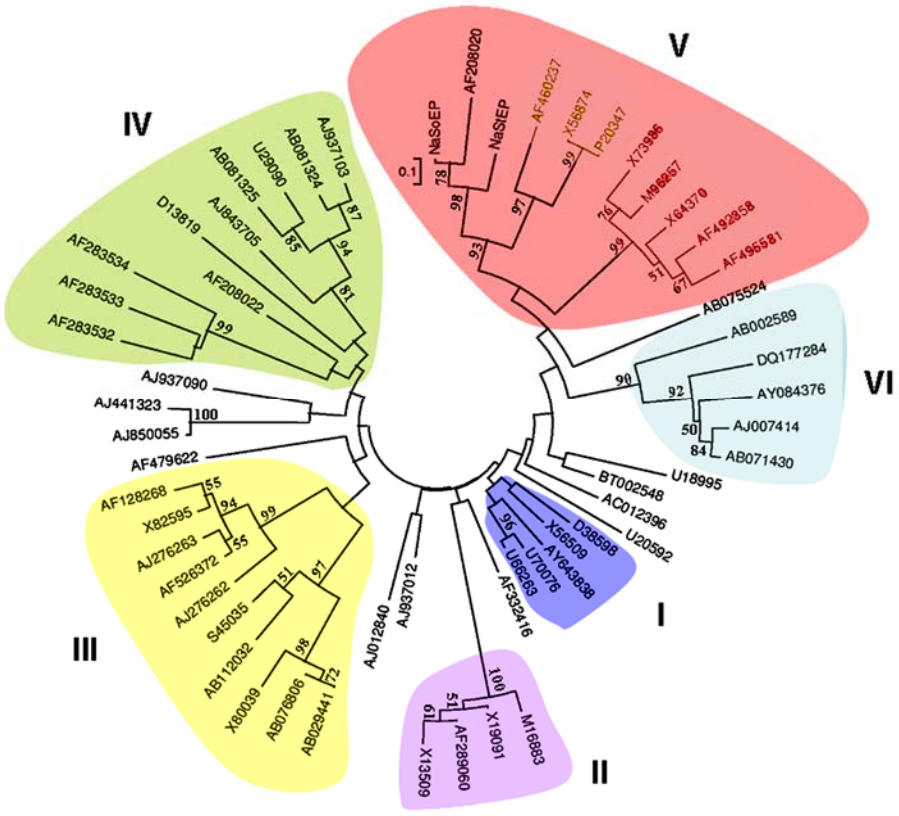
Name	Protein	Identity (%)	Reference
NgPI	NaStEP	70.9	Park *et al*., 2000
	NaSoEP	79.1	
PIG7	NaStEP	33.5	Heibges *et al*., 2003
	NaSoEP	36.0	
PID4	NaStEP	34.4	Heibges *et al*., 2003
	NaSoEP	35.8	
S. tuberosum cathepsin D inhibitor	NaStEP	36.1	Barlic-Maganja *et al*., 1992
	NaSoEP	39.6	
S. lycopersicon cathepsin D inhibitor	NaStEP	35.7	Werner *et al*., 1993
	NaSoEP	37.3	
PDI	NaStEP	36.5	Hannapel, 1993
	NaSoEP	39.0	
PCPI	NaStEP	43.9	Brzin *et al*., 1988
	NaSoEP	42.5	
S. tuberosum cysteine protease inhibitor	NaStEP	41.3	Yamagishi *et al*., 1991
	NaSoEP	40.9	
S9C11	NaStEP	41.4	Heibges *et al*., 2003
	NaSoEP	40.0	



Supplementary Figure S1. DNA gel-blot analyses of *NaStEP*.

DNA (15 μg) prepared from indicated species were digested with EcoRI and analyzed by DNA gel blot hybridization using ^{32}P-labeled NaStEP as probe.

(a)


V
IV
VI
I
III
II
NaSoEP
NaStEP
AF208020
AF460237
X56874
P20347
X73986
M96957
X64370
AF492858
AF496581
AB075524
AB002589
DQ177284
AY084376
AJ007414
AB071430
BT002548
U18995
AC012396
D38598
X56509
U20592
AY843838
U70076
U66283
AF392416
AJ012840
AJ937012
X19091
M16863
AF289060
X13509
AJ937103
AB081324
U29090
AB081325
AJ843705
D13819
AF208022
AF283534
AF283533
AF283532
AJ937090
AJ441323
AJ850055
AF479622
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AJ276263
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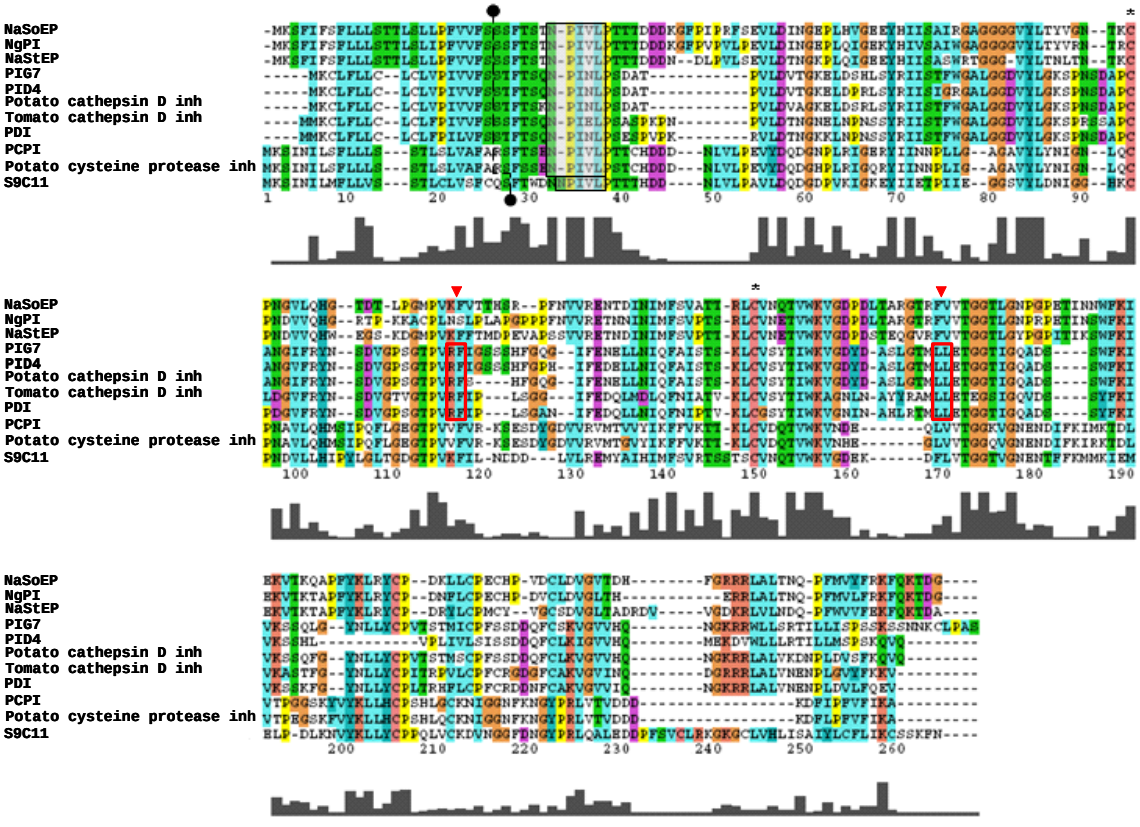
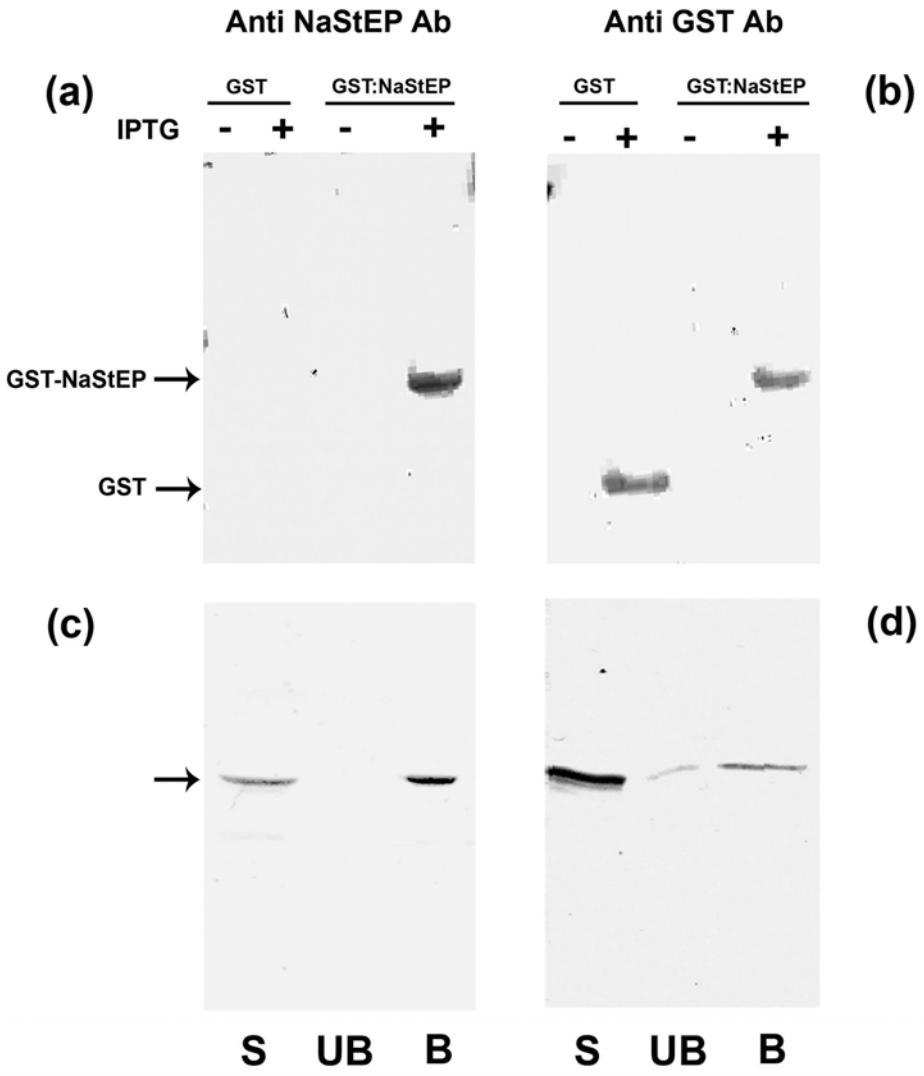
AJ276262
S45035
AB112032
X80039
AB076806
AB029441

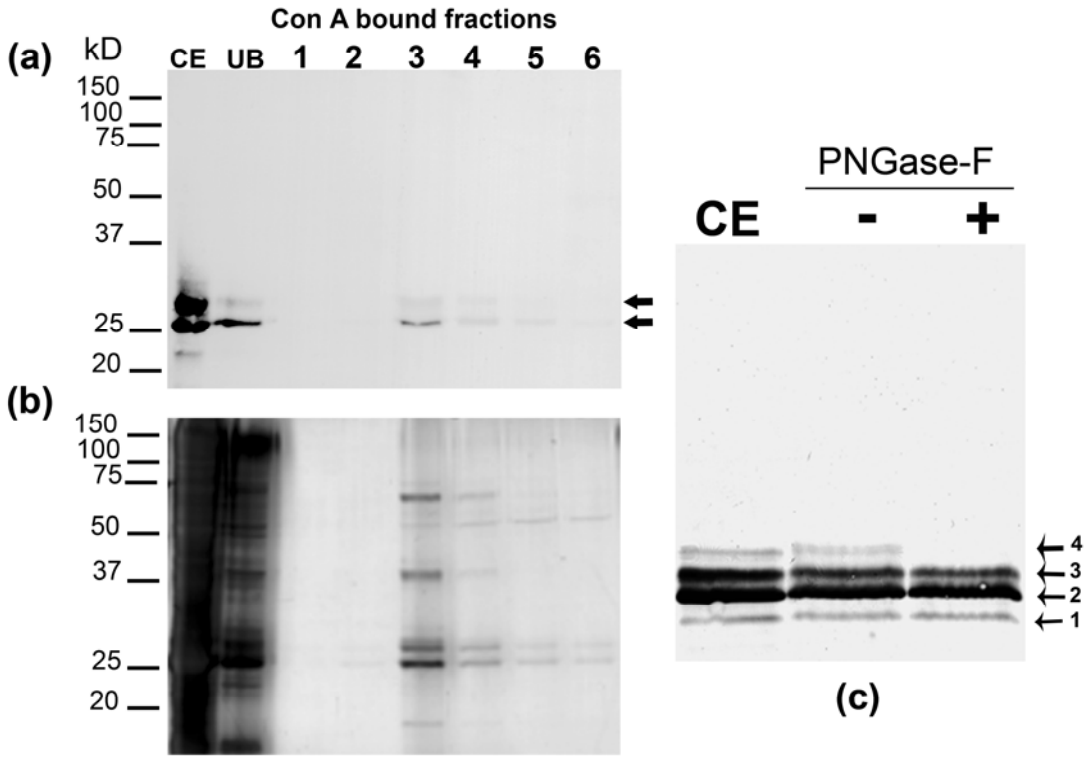
(b)


NaSoEP
NgPI
NaStEP
PIG7
PID4
Potato cathepsin D inh
Tomato cathepsin D inh
PDI
PCPI
Potato cysteine protease inh
S9C11

Supplementary Figure S2. NaStEP and NaSoEP are homologous to Kunitz-type protease inhibitors.
(a) Phylogenetic tree of NaStEP, NaSoEP and related proteins. Full-length sequences were used to construct the phylogram. Numbers near the nodes denote bootstrap values (> 50 %) with 1000 replicates. Representative members of all subgroups are included. The sequences have been deposited in the GenBank database with the accession numbers indicated. P20347 accession number can be found in the UniProtKB database.
(b) Alignment of the amino acid sequences of the *N. alata NaSoEP*, *N. glutinosa* proteinase inhibitor (NgPI/AF208020), *N. alata NaStEP*, *S. tuberosum* aspartic protease inhibitor (PIG7/AF495581), *S. tuberosum* aspartic protease inhibitor (PID4/AF492358), *S. tuberosum* cathepsin D inhibitor (X64370), *Solanum lycopersicum* (formerly *L. esculentum*) cathepsin D inhibitor (X73986), *S. tuberosum* aspartic protease inhibitor (PDI/M96257), *S. tuberosum* cysteine proteinase inhibitor (PCPI/P20347), *S. tuberosum* cysteine protease inhibitor (X56874) and *S. tuberosum* cysteine protease inhibitor (S9C11/AF460237). The degree of sequence consensus is indicated by black boxes under the alignment. The connector shows the potential signal peptide cleavage sites predicted by neural networks. The vacuolar sorting signals are boxed. The conserved cysteine residues are marked with an asterisk. The reactive bonds for trypsin and chymotrypsin are red boxed and indicated with an arrowhead. A dash in the sequence indicates a gap introduced to maximize sequence homology.



Supplementary Figure S3. Anti NaStEP antibody specificity.
NaStEP was expressed as GST fusion protein and purified by glutathione sepharose affinity chromatography.
(a) *E. coli* cells harboring the GST alone or GST:NaStEP, with or without IPTG induction, were loaded and resolved by 12.5 % SDS PAGE. The proteins were blotted onto nitrocellulose and immunostained with anti-NaStEP antibody.
(b) *E coli* cells harboring the GST alone or GST:NaStEP, with or without IPTG induction, were loaded and separated by 12.5 % SDS PAGE The proteins were blotted onto nitrocellulose and immunostained with anti-GST antibody.
(c) Specific glutathione sepharose fractions were analyzed by 12.5 % SDS PAGE and then blotted and immunostained with anti NaStEP antibody.
(d) Glutathione sepharose fractions were analyzed by 12.5 % SDS PAGE and then blotted and immunostained with anti-GST antibody.
+, cells induced with IPTG; -, uninduced cells; S, soluble fraction; UB, unbound fraction; B, proteins specifically bound to glutathione sepharose.



Supplementary Figure S4. Affinity chromatography of protein crude extracts on Con A-Sepharose column and PNGase F deglycosylation analysis.
Proteins recovered from the affinity chromatography and deglycosylation experiments were loaded and separated in a 12 % polyacrylamide gel electrophoresis.
(a) Con A fractions transferred onto nitrocellulose and immunostained with anti NaStEP antibody.
(b) Con A fractions recovered, fractionated by SDS PAGE and silver stained.
(c) Immunoanalysis of NaStEP deglycosylation by PNGase F.
CE, crude extract; UB, unbound fraction collected after the exposure of the crude extract to the Con A sepharose; 1-6, Con A bound fractions sequentially collected after the addition of methyl-α-D-mannopyranoside; +, active (non-boiled) PNGase F deglycosylation enzyme; -, negative control, (boiled) PNGase F deglycosylation enzyme.

Materials and Methods for Supplementary Data

Genomic DNA from *N. alata BT*, *N. plumbaginifolia*, *N. longiflora* and *N. tabacum* was isolated according to the procedure described by Dellaporta *et al*., 1983. 15 µg of DNA were restricted with EcoRI, electrophoresed on 1% agarose gels and blotted onto nylon membranes (Hybond -N, Amersham) by capillary transfer. DNA was fixed using a cross-linker. Blots were hybridized with the labeled NaStEP probe for 12 h at 60 °C. The blots were subsequently washed with the same hybridization buffer used for Northern blot experiments for 10 min twice and once for 15 min. All washes were performed at 60 °C. Hybridization was detected by autoradiography (Supplementary Figure S1).

Primary antibody dilutions were: anti-GST 1:2500 and anti-NaStEP 1:15000 (Supplementary Figure S3 and S4).

Phylogenetic analysis was conducted using MEGA version 3.1 (Kumar *et al*., 2004; Supplementary Figure S2). The phylogenetic tree was constructed by the neighbor-joining method (Saitou and Nei, 1987). Peptidase inhibitor information was provided by the inhibitor site *MEROPS* (Rawlings *et al*., 2006; http://merops.sanger.ac.uk).

Affinity chromatography of NaStEP isoforms was performed using Con A Sepharose (SIGMA) (Supplementary Figure S4a-b). The Con A Sepharose contains Ca^{2+} and Mn^{2+}, and therefore, these ions were not included in the binding buffer (20 mM Tris HCl pH 7.5, 500 mM NaCl). An aliquot (1.0 ml) of SI *N. alata* crude extract was loaded in a Con A packed column. The crude extract was incubated in the column for 15 min, and then the column was washed 10 times with the binding buffer to remove unspecific interactions. Then, the bound proteins were eluted using 500 mM methyl-α-D-mannopyranoside prepared in the binding buffer.

The enzymatic deglycosylation of native NaStEP (Supplementary Figure S4c) was carried out with PNGase F (New England) according to manufacturer instructions with slight modifications. The SI *N. alata* crude extract (0.2 mg/ml) was digested with 320 units of PNGase F for 25 h at 37 ºC.

Reference for Supplementary Data

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