

## 1 **SUPPLEMENTARY MATERIAL**

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### 3 *Focusing conditions and SDS-PAGE Analysis*

4 In the focusing, voltages of 100 V, 250 V, and 1000 V were sequentially applied for  
5 100 Vh, 250 Vh and 1000 Vh, respectively, and finally 4000 V up to 45 000 Vh, at  
6 20°C. Before the SDS-PAGE procedure, the focused IPG strips were equilibrated, first  
7 in a DTT equilibration buffer [2% (w/v) DTT, 6 M urea, 2% (w/v) SDS, 0.05 M Tris.Cl  
8 pH 8.8, 20% (w/v) glycerol] for 15 min, then in an iodoacetamide equilibration buffer  
9 [2.5% (w/v) iodoacetamide, 6 M urea, 2% (w/v) SDS, 0.05 M Tris.Cl pH 8.8, 20%  
10 (w/v) glycerol] for 15 min. After equilibration, the IPG strips were embedded onto 12%  
11 acrylamide 1 mm SDS-PAGE second-dimension gels. SDS-PAGE was performed using  
12 a Mini-PROTEAN 3 Dodeca cell (Bio-Rad, USA) at the constant current of 15 mA per  
13 gel. The gels were then washed in the gel-fixing solution [10% (v/v) methanol, 7% (v/v)  
14 ace  
15 tic acid] for 30 min.

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### 17 *MALDI-MS and MS/MS Analyses*

18 MALDI-MS and MS/MS analyses were performed on an Ultraflex III mass  
19 spectrometer (Bruker Daltonik, Bremen, Germany). Peptide maps were acquired in reflectron  
20 positive mode (25 kV acceleration voltage) with 800 laser shots. Twelve dominant peaks within  
21 700 – 3600 Da mass range and minimum S/N 10 were picked out for MS/MS analysis  
22 employing LID-LIFT arrangement with 600 laser shots for each peptide. CHCA solution  
23 prepared according to Havlis *et al.* 2003 was used as the matrix in combination with  
24 AnchorChip target to enhance measurement sensitivity. Sample (1 µl) was mixed with matrix  
25 solution on the target in a 2:1 ratio. Known autoproteolytic products of trypsin were used for

26 internal calibration of digested peptides. In the absence of these products, an external calibration  
27 procedure was employed, using a mixture of seven peptide standards (Bruker Daltonik)  
28 covering the mass range of 1000–3100 Da. The Flex Analysis 3.0 and MS Biotools 3.1 (Bruker  
29 Daltonik) software were used for data processing.

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### 31 *LC-MS/MS Analysis*

32 Liquid chromatography–tandem mass spectrometry (LC–MS/MS) analysis was  
33 performed using EASY-nLC system (Proxeon) on-line coupled with an HCTultra PTM  
34 Discovery System ion trap mass spectrometer equipped with nanospray (Bruker Daltonik). The  
35 reverse- phase column and precolumn used for LC separation was filled according to a  
36 previously described procedure [Planeta *et al.* 2003]

37 Prior to LC separation, tryptic digests were concentrated and desalted using trapping column  
38 (100  $\mu\text{m} \times 30\text{ mm}$ ) filled with 4- $\mu\text{m}$  Jupiter Proteo sorbent (Phenomenex, Torrance, CA).  
39 Sample volume was 10  $\mu\text{l}$ . After washing with 0.1% formic acid, the peptides were eluted from  
40 the trapping column using an acetonitrile/water gradient (350 nL/min) onto a fused-silica  
41 capillary column (100  $\mu\text{m} \times 100\text{ mm}$ ), on which peptides were separated. The column was filled  
42 with 3.5- $\mu\text{m}$  X-Bridge BEH 130 C18 sorbent (Waters). The mobile phase A consisted of 0.1%  
43 formic acid in water and the mobile phase B consisted of 0.1% formic acid in acetonitrile. The  
44 gradient elution started at 10 % of mobile phase B and increased linearly from 10 % to 30 %  
45 during the first 10 minutes. The gradient linearly increased to 90 % of mobile phase B in the  
46 next two minutes and remained at this state for next 8 minutes. The analytical column outlet was  
47 directly connected to the nanospray ion source. Nitrogen was used as nebulizing as well as  
48 drying gas. The pressure of nebulizing gas was 8 psi. The temperature and flow rate of drying  
49 gas were set to 250 °C and 6 L/min, respectively. The capillary voltage was 4.0 kV.

50 The mass spectrometer was operated in the positive ion mode in a  $m/z$  range of 300 – 1500 for  
51 MS and 100-2500 for MS/MS mode. The DataAnalysis 4.0 and MS Biotools 3.1 (Bruker  
52 Daltonik) software were used for data processing.

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54 *Database Searching*

55 MASCOT 2.2 (MatrixScience, London, UK) search engine was used for processing the  
56 MS and MS/MS data Database searches were done against the NCBI database (non redundant,  
57 all entries and restricted taxonomy to Viridiplantae; downloaded from  
58 ftp://ftp.ncbi.nih.gov/blast/db/FASTA/) and EST-Plants database (downloaded from  
59 ftp://ftp.ebi.ac.uk/pub/databases/fastafiles/emblrelease/). Databases were updated regularly.  
60 Mass tolerances of peptides and MS/MS fragments for MS/MS ion searches were 0.5 Da.  
61 Oxidation of methionine and carbamidomethylation of cysteine as optional modifications, one  
62 enzyme miscleavage and correction for one <sup>13</sup>C atom were set for all searches.

63

64 References:

65 J. Havlis, H. Thomas, M. Sebela, A. Shevchenko, Fast-response proteomics by accelerated in-  
66 gel digestion of proteins, *Anal. Chem.* 75 (2003) 1300–1306.

67 J. Planeta, P. Karasek, J. Vejrosta, Development of packed capillary columns using carbon  
68 dioxide slurries, *J. Sep. Sci.* 26 (2003) 525–530.

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71 *Effect of L80F/V84F Mutations on Cryptogein Biological Activity*

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73 *Construction of L80F/V84F mutant*

74 To introduce the mutation to the targeted codon site-directed mutagenesis was  
75 conducted as for other proteins by using a pair of specific forward-reverse oligonucleotides  
76 given in Table S2. Transformation of *Pichia pastoris* as well as expression and purification of  
77 protein was done as in other measured proteins.

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79 *Sterol-binding Activities and Affinities*

80 The binding parameter of sterol to the protein ( $K_d$ ) was calculated from the fluorescence  
81 data, and the number of binding sites was found to be approximately one per molecule. The  
82 determined value of  $K_d$  is consistent with the predicted effects of the individual mutations when  
83 no specific binding was determined.

84 The addition of L80F/V84F mutant stimulated a significantly reduced ability to transfer  
85 DHE between the membranes compared to wt cryptogein with rates of  $0.33\pm 0.01$ . On the other  
86 side this double mutant showed higher rates of PC transfer than did cryptogein with rates of  
87  $0.75\pm 0.06$  (Fig. S1). This behaviour is that found in V84F mutant.

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### 89 *Accumulation of Defence Gene Transcripts*

90 By RT-qPCR assays the transcript levels for *PR2Q*, *PR3Q*, *PR5*, *GLN2*, *TuReP*,  
91 *NtPRp27* and *GeLiP* gene was quantified. The results are summarized in Table S3,  
92 showing similarity to that found in mutant V84F.

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**Table S1.** Protein name, according to literature or NCBI database; **NCBI accession Nr.**- number of protein in NCBI database; **pI**- theoretical pI; **MW**- theoretical molecular weight; **Spot Nr** - number of spot on 2-D gel; **Score** - Mascot score; **%cov.**- percentage of coverage of the identified proteins; **In samples** - samples in which given protein was found

Protein name	NCBI accession Nr	MW [kDa]	pI	Spot Nr	MALDI-MS/MS		LC-MS/MS		In samples
					Score	% Cov	Score	% Cov	
Acidic chitinase PR-P	19771	27.5	4.9	22	660	50	164	15	WT, <u>V84F</u> L41F, <u>V84F</u>
				24	277	12	136	5	
Acidic chitinase PR-Q	19773	27.6	5.1	3	-	-	46	8	<u>WT</u> , V84F <u>L41F</u>
				18	-	-	50	3	
CBP20	632736	21.9	8.4	8	-	-	117	9	<u>WT</u> , L41F/V84F, V84F <u>WT</u> , V84F <u>WT</u> , L41F/V84F, V84F
				12	-	-	126	9	
				17	-	-	117	9	
Class IV chitinase	121663827	29.9	4.9	23	-	-	103	22	<u>V84F</u>
				5	-	-	51	7	<u>WT</u> , V84F
Chitinase/lysozyme	467689	42.0	9.1	12	-	-	116	3	<u>WT</u> , V84F
Cyclophilin-like protein	152206078	22.0	7.8	11	-	-	64	13	<u>WT</u> , V84F
Endochitinase A	116314	35.1	8.4	14	-	-	68	4	<u>WT</u> , V84F <u>WT</u> , V84F
				15	-	-	68	7	
Endochitinase B	116321	34.7	8.3	16	-	-	69	8	<u>WT</u> , V84F
Germin like protein	222051768	21.4	5.8	6	-	-	59	8	<u>WT</u> , L41F/V84F, V84F
Glucan endo-1,3- $\beta$ -glucosidase	19859	37.8	5.2	4	176	14	259	24	<u>WT</u> , V84F
Glucan endo-1,3- $\beta$ -glucosidase	19869	40.4	7.1	13	-	-	98	3	<u>WT</u> , V84F <u>WT</u> , V84F <u>WT</u> , V84F
				14	224	14	182	18	
				15	272	18	118	15	
Proteinase inhibitor I-A	547732	11.9	7.8	9	57	16	-	-	<u>WT</u> , V84F
Proteinase inhibitor I-B	547733	11.9	7.8	9	191	15	-	-	<u>WT</u> , V84F
Lignin-forming anionic peroxidase	129837	34.7	4.7	1	245	11	159	20	<u>WT</u> , V84F <u>WT</u> , V84F <u>L41F/V84F</u> L41F, <u>L41F/V84F</u>
				2	419	20	134	20	
				20	143	5	-	-	
				21	266	11	148	10	
NtPRp27	5360263	27.4	9.3	25	258	16	114	16	<u>WT</u> , <u>V84F</u>
PR-4A	19962	16.2	7.6	8	-	-	86	8	<u>WT</u> , L41F/V84F, V84F
PR-4B	100352	15.2	6.1	19	-	-	76	9	<u>L41F</u> , V84F L41F, <u>L41F/V84F</u>
				21	-	-	125	9	
Peptidyl-prolyl cis-trans isomerase, putative [ <i>Ricinus communis</i> ]	255547634	27.5	9.6	10	192	12	92	12	<u>WT</u> , V84F <u>WT</u> , V84F
				11	316	21	-	-	
Peroxidase	63002585	35.6	8.4	15	-	-	90	9	<u>WT</u> , V84F
Thaumatococin-like protein E22	131015	24.7	5.4	22	-	-	144	33	<u>WT</u> , L41F, <u>V84F</u>
Tumor-related protein	1762933	23.4	8.5	7	178	8	-	-	<u>WT</u> , V84F

**Table S2.** Sequences of the oligonucleotides used for mutagenesis of cryptogein and qPCR primer sequences.

<b>Gene</b>	<b>Primer sequence (5' → 3')</b>
L41F	F*: ACGGCCAAGGCCTTCCCCACCACGGCG R*: CGCCGTGGTGGGGAAGGCCTTGGCCGT
V84F	F*: CGGCCTGGTACTCAACTTCTACTCGTACGCGAACG R*: CGTTCGCGTACGAGTAGAAGTTGAGTACCAGGCCG
L80F	F*: GTCCCCACGAGCGGCTTCGTACTCAACGTGTAC R*: GTACACGTTGAGTACGAAGCCGCTCGTGGGGAC
<i>GeLiP</i>	F*: GTCCAAGATTTCTGCGTTCGC R*: TTTCCAGCTGCACTAAGCCC
<i>GLN2</i>	F*: TCTGTGTATGCTGCCCTCGAG R*: CCAGGCTTTCTTGGGCTACC
<i>NtPRp27</i>	F*: ATTGTACCACGAGAGCACCCA R*: GGTTTCACCCAGTGGCTAGGT
<i>PR2Q</i>	F*: TCCAGCAGATGTTGTGTCGCT R*: GGCTTGGCTAGCAGCAACATT
<i>PR3Q</i>	F*: TCTGGATCACCAATGGCATT R*: AGAAGCCATTGGCAGGACAT
<i>PR5</i>	F*: CCGAGGTAATTGTGAGACTGGAG R*: CCTGATTGGGTTGATTAAGTGCA
<i>TuReP</i>	F*: TCACCTGCGAACCCTAACGA R*: CACGCCCTGGATTTCTTCT
<i>EF-1<math>\alpha</math></i>	F*: TGTGATGTTTTTGTTCGGTCTTTAA R*: TCAAAAGAAAATGCAGACAGACTCA

\* F is the forward primer and R is the reverse primer, respectively.

**Table S3.** *Accumulation of defence-related genes.* Effect of L80F/V84F mutant on accumulation of transcripts for PR and other defence related proteins. Gene expression relative to a control was calculated by the  $\Delta\Delta C(t)$  method. The values given in the table are the logarithm of the relative increase (logR) and its standard deviation (SD). More than a two-fold increase in gene expression was taken as significant. A.N. = accession number of gene in NCBI database.

<b>Gene</b>	<b>A.N.</b>	<b>logR</b>	<b>SD</b>
<i>PR2Q</i> β-1,3-glucanase	X54456	1.41	0.05
<i>GLN2</i> β-1,3-glucanase	X53600	0.98	0.05
<i>PR3Q</i> Chitinase	X51425	1.31	0.07
<i>PR5</i> Thaumatococcus-like protein	X12739	1.23	0.05
<i>TuReP</i> Tumor-related protein	FG644925	0.80	0.05
<i>NtPRp27</i>	FG633857	1.04	0.06
<i>GeLiP</i> Germin-like protein	AB449366	0.80	0.06

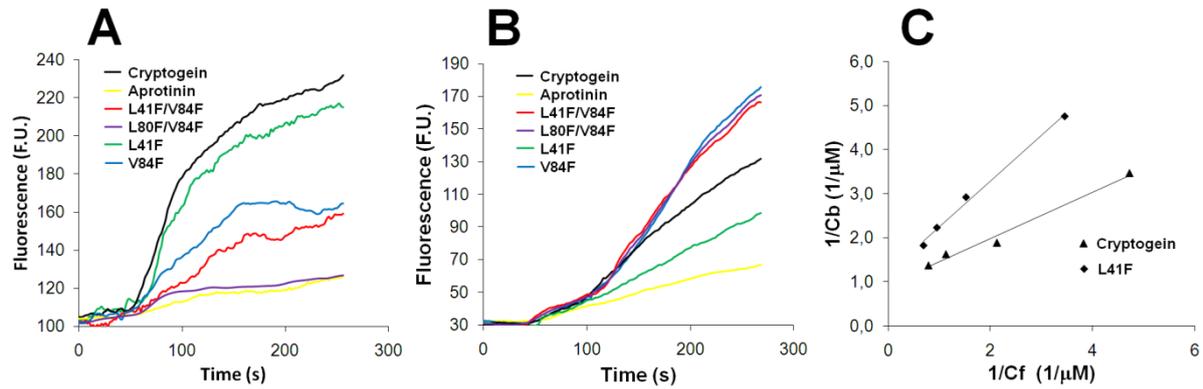


Fig. S1. *Transfer of sterols and fatty acids catalyzed by proteins and binding plots*

**A.** Transfer of sterols between DHE and stigmasterol micelles catalyzed by cryptogein, L41F, L41F/V84F, V84F, L80F/V84F mutants and aprotinin, measured by changes in DHE fluorescence. Donor micelles contained DHE ( $0.63 \mu\text{M}$ ), acceptor micelles contained stigmasterol ( $3 \mu\text{M}$ ) in MES buffer. The transfer was induced by adding elicitors or aprotinin ( $0.5 \mu\text{M}$ ). **B.** Transfer of fatty acids between NBD-PC vesicles and PC vesicles catalyzed by cryptogein, L41F, L41F/V84F, V84F, L80F/V84F mutants and aprotinin monitored by measuring changes in NBD-PC fluorescence. Donor vesicles contained NBD-PC ( $0.63 \mu\text{M}$ ), while acceptor vesicles contained PC ( $3 \mu\text{M}$ ), in MES buffer. The transfer was induced by adding elicitors or aprotinin ( $0.5 \mu\text{M}$ ). (<sup>a</sup> addition of donor vesicles, <sup>b</sup> addition of elicitors). **C.** Binding plots of the titration of cryptogein and L41F mutant by DHE.  $C_b$  and  $C_f$  are the concentrations of bound and free DHE or NBD-PC, respectively. Experiments were performed with  $1.0 \mu\text{M}$  proteins.

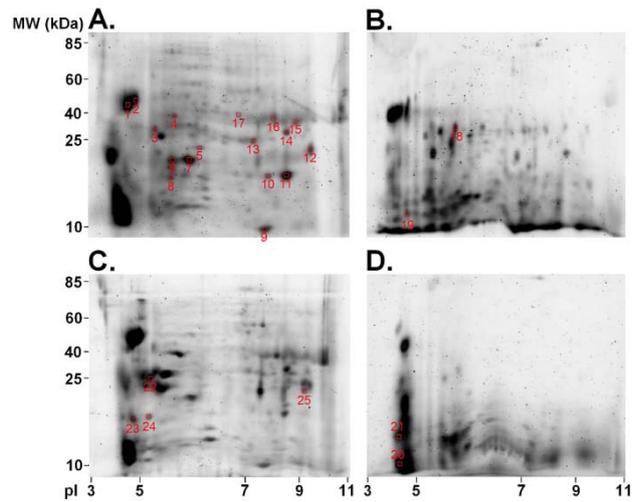


Fig. S2. *Two-dimensional gel electrophoresis proteome maps of intercellular fluid determined 48 hours after application of cryptogein and its mutants.* Identified proteins with qualitative changes between individual samples are indicated. For the first dimension of separation, 80  $\mu$ g of protein was applied to each IPG strip (7 cm, pH 3–10 NL). For separation in the second dimension, 12% SDS–PAGE was carried out. Proteins were visualized by SYPRO Ruby staining. Isoelectric points (pI) and molecular weights (MW, kDa) are marked. (A) wt cryptogein (B) L41F (C) V84F (D) L41F/V84F