Supporting Information

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SI Materials and Methods

Frozen leaf material (≈1.2 kg) was homogenized in a 4-L blender for 5 min with 2.4 L of 1% trifluoroacetic acid (TFA) and squeezed through four layers of cheesecloth and one layer of Miracloth (Calbiochem). The liquid was centrifuged at 10,000 × *g* for 20 min. The acidic supernatant was adjusted to pH 4.5 with 10 N NaOH and recentrifuged at 10,000 × *g* for 20 min. After readjusting the supernatant to a pH of 2.5 with TFA, the liquid was applied to a 40 μm, 3 × 25-cm C18 reversed-phase flash column (Bondesil; Varian Analytical Instruments) equilibrated with 0.1% TFA/H_2O. Elution was performed at 8 psi with compressed nitrogen gas. After loading, the column was washed with 0.1% TFA/H_2O and then 250 mL of 40% methanol/0.1% TFA. The 40% methanol-eluting fraction was rotary-evaporated to remove the methanol and then lyophilized to dryness. The average yield for each extraction was 2.14 g. Dry powder (15 g) was dissolved in 15 mL 0.1% TFA/H_2O, centrifuged at 10,000 × *g* for 10 min and applied in two sequential runs to a Sephadex G-25 column (4 × 40 cm) equilibrated with 0.1% TFA/H_2O. Eight-milliliter fractions were collected, and the alkalinizing activity was assayed as described above using 10 μL of each fraction per 1 mL of A3525 *Glycine max* cells. The activity was found at or near the void, and these fractions were pooled and lyophilized. The yield from the two runs was 1.12 g. The lyophilized powder was dissolved in 500 mL 10 mM potassium phosphate (pH 6) applied to a CM-Sephadex column (5 × 8 cm), washed with phosphate buffer, and the retained material eluted with 1 M sodium chloride. After desalting on the flash column described above and lyophilization, the yield was 157 mg. Forty milligrams was dissolved in 1.5 mL 0.1% TFA/H_2O for semipreparative reversed-phase C18-HPLC (218TP510, 10 × 250 mm; Vydac). After centrifugation the sample was loaded with a flow rate of 2 mL/min, and after 2 min a gradient was applied from 0 to 40% acetonitrile/0.1% TFA over 90 min. The absorbance was monitored at 225 nm. One-minute fractions were collected, and 10-μL aliquots were used with 1 mL soybean cells (A3525) to determine alkalinizing activity. An early-eluting activity peak, fractions 32–34, was detected along with late-eluting peaks with similar retention times as previously purified rapid alkalinization factor peptide peaks (1). The early-eluting peak was pooled and lyophilized with a yield of 5.2 mg. The alkalinizing activity peak was subjected to strong cation exchange chromatography (SCX) on a poly-SULPHOETHYL Aspartamide column (5 μm, 4.6 × 200 mm; The Nest Group), equilibrated in 5 mM potassium phosphate (pH 3) in 25% acetonitrile. The fraction was loaded onto the column in 1 mL buffer, and after 2 min a 90-min gradient was applied to 100% elution buffer [5 mM potassium phosphate and 1 M potassium chloride (pH 3) in 25% acetonitrile]. Absorbance was monitored at 225 nm. A flow rate of 1 mL/min was used, and 1-min fractions were collected. Aliquots (2 μL) were used to determine activity in the cell assay. A major activity peak (>1 pH unit increase) was found in fractions 51–54, which was pooled and lyophilized for further purification by reversed-phase C18 chromatography (column 218TP54, 5 μm, 4.6 × 250 mm; Vydac) with methanol as the eluting solvent. The sample was dissolved in 1 mL 0.1% TFA/H_2O, and after centrifugation the supernatant was applied to the column with a flow rate of 1 mL/min. After 2 min, a 90-min gradient was applied to 40% elution buffer (methanol/0.05% TFA). Absorbance was monitored at 220 nm, and 2-μL aliquots were used to determine alkalinizing activity. The major activity peak eluted in fractions 37–39, and after removal of the methanol by vacuum evaporation the fraction was further purified using a narrow-bore reversed-phase C18-HPLC column (218TP52, 5 μm, 2.1 × 250 mm; Vydac) with acetonitrile/TFA as the elution solvent. The active peak was loaded with a flow rate of 0.25 mL/min. A 90-min gradient was applied from 0 to 30% acetonitrile/TFA, and 0.5-min fractions were collected with the absorbance monitored at 210 nm. Alkalinizing activity was determined with 2-μL aliquots. The major activity peak was detected at 40.5–41.5 min, and the fractions were pooled for rechromatography on the SCX-HPLC column as described above but with a gradient from 0 to 70% 1 M KCl. Alkalinizing activity was detected in fractions 62–63, and for a final purification and desalting the activity peak was rechromatographed on the narrow-bore column with a MeOH gradient from 0 to 30% as described above. The active fractions pertaining to 50.5–51.5 min were analyzed by mass spectroscopy.

1. Pearce G, Moura DS, Stratmann J, Ryan CA (2001) RALF, a 5-kDa ubiquitous polypeptide in plants, arrests root growth and development. *Proc Natl Acad Sci USA* 98:12843–12847.

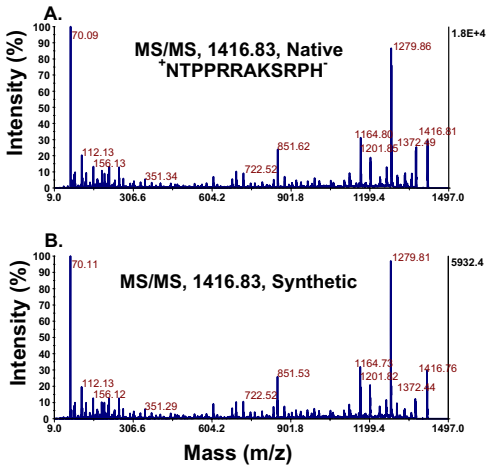

Fig. S1. MALDI-MS/MS spectral analysis of the major MS peak and comparison of the spectra with the deduced synthetic peptide. (*A*) The 1,416.83 mass peak was subjected to MS/MS (Native), and the fragmentation spectra revealed peptide sequences that were analyzed de novo. A single 12-aa sequence match, +NTPPRRAKSRPH-, was found in the soybean databank. (*B*) The peptide was synthesized and compared with the native MS/MS spectra.

Glyma18g48580

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ATGGGCAGCTCCATTTTTTGCCTTCACCTTATCCTTTCATCATTTTTTCTTTTCACTTTCTTGCTGGCAGCTGTTAATGGCAGCAAAAAGTGTTACATTGTATACATGGGAGCTCATTCT 120
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R  H  I  N  G  F  A  A  L  L  E  E  E  E  A  A  D  I  A  K  N  P  N  V  V  S  V  F  L  S  K  E  H  K  L  H  T  T  R  S

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V  D  N  R  R  G  F  K  F  N  V  L  Q  G  T  S  M  S  C  P  H  A  S  G  I  A  G  L  L  K  T  R  H  P  S  W  S  P  A  A

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Fig. S2. Coding sequence, predicted amino acid sequence and domain prediction of Glyma18g48580. White letters in red background indicate the *Gm*SubPep sequence isolated in this study. Yellow, green, blue, pink, and gray backgrounds indicate signal sequence for secretion, proregion (inhibitor I9), peptidase S8, protease associated (PA) domain, and the C-terminal Fn III domain, respectively.

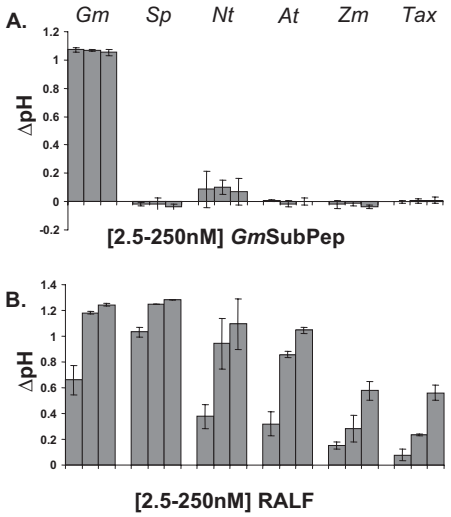

Fig. S3. *Gm*SubPep alkalinization-inducing activity is species specific, occurring only in *Glycine max* suspension cells. (*A*) *Gm*SubPep (10 μL) was added to 1-mL aliquots of a variety of suspension-cultured cells at 2.5, 25, and 250 nM, and the pH was recorded after 20 min. (*B*) RALF (rapid alkalinization factor) (10 μL), a bioactive peptide found throughout the plant kingdom, was tested to verify the alkalinizing capacity of each cell type. *Gm*, *Glycine max*, variety Davis; *Sp*, *Solanum peruvianum* (tomato); *Nt*, *Nicotianum tabacum* (tobacco); *At*, *Arabidopsis thaliana*; *Zm*, *Zea mays* (corn); *Tax*, *Taxus x media* (Anglojap Yew). Data are the average of three separate experiments. Error bars indicate SD.

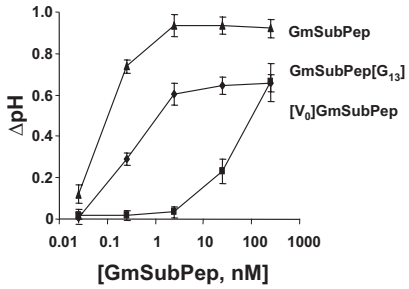

Fig. S4. The alkalinizing activities of *Gm*SubPep compared with the peptide with amino acid additions to the amino- and carboxyl ends. *Gm*SubPep was synthesized with valine added to the amino-terminal ([V0]*Gm*SubPep) or glycine added to the carboxyl-terminal (*Gm*SubPep[G13]). These are the predicted amino acids found within the Glyma18g48580 sequence. Aliquots of peptide (10 μL) were added to 1 mL of suspension cells. The pH of the media was recorded after 20 min. Each bar represents the average of three separate experiments. Error bars indicate SD.

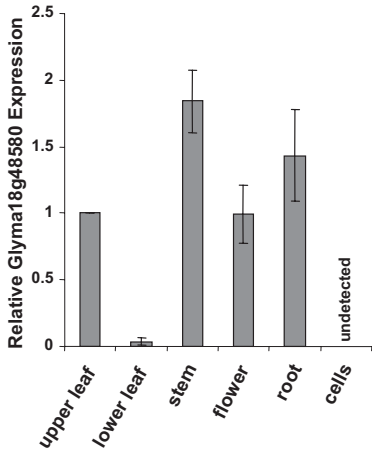

Fig. S5. Expression levels of Glyma18g48580 in various parts of soybean plants as analyzed by real-time RT-PCR relative to upper leaf expression. The upper leaf is the first fully expanded leaf from the apex, and the lower leaf is a mature leaf four positions below the upper leaf. Stem RNA was taken from above and below the upper leaf and pooled. Flowers were collected from the nodes of the main stem. Plants were grown in soil, and collection was made when flowering was evident (approximately 7 wk). Suspension cell extracts revealed no detectable expression. Each bar represents the average of three independently obtained RNA samples. Error bars indicate SD.

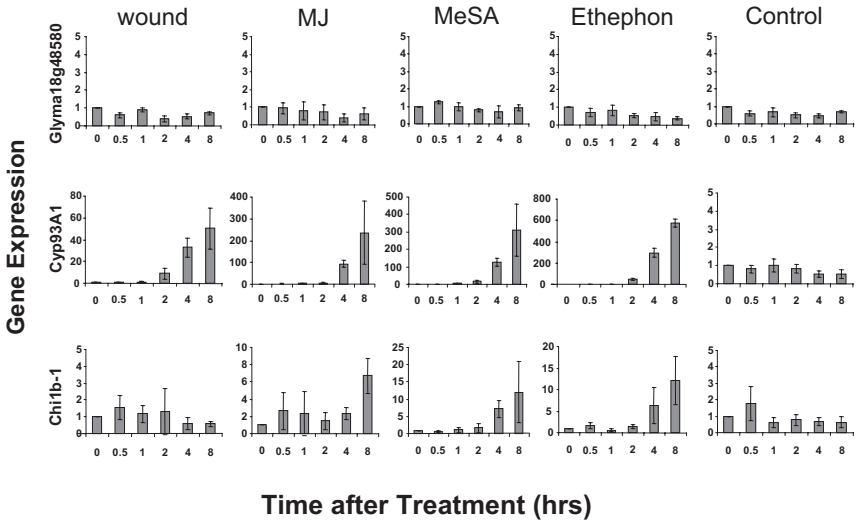

Fig. S6. Time course expression levels of Glyma18g48580 in response to various treatments. RNA was isolated from the leaves of 3-wk-old plants at various times as described in *Materials and Methods* (main text) after wounding, spraying with methyl jasmonate (MJ), methyl salicylate (MeSA), ethephon, or with 0.1% Triton X-100 (Control). The gene expression level was analyzed by quantitative RT-PCR and is indicated relative to the expression at 0 h. A cytochrome P450 gene (*Cyp93A1*) involved in phytoalexin synthesis and chitinase gene (*Chib-1b*), a pathogenesis-related gene, were amplified as positive controls for the treatments. Error bars indicate SD from three biological replicates. Error bars indicate the SD.

Table S1. Primer sequences

Primer	Sequence
ELF1B-F	5′-AAGGGAGGCTGCTAAAAAGC-3′
ELF1B-R	5′-CAACTGTCAAGCGTTCCTCA-3′
CYP93A1-F	5′-ATGTGTTGGAGAAGGCAAGG-3′
CYP93A1-R	5′-CCCTACCAATAGCCCAAACA-3′
Chi1b-1-F	5′-TTCAGGATCAGGGCCATTAG-3′
Chi1b-1-R	5′-TGGCATGAAGGGTTGTTGTA-3′
Gmachs1-F	5′-CACATGACCGAGCTCAAAGA-3′
Gmachs1-R	5′-CCCATTCCTTGATTGCCTTA-3′
PDR12-F	5′-TCACCTTCCTGACCTTCACC-3′
PDR12-R	5′-TGAAGCCACCAATCCATACA-3′
SubPep-F	5′-TATGCCTGACAAGCAATTCG-3′
SubPep-R	5′-CTCGACCAATTTGGGAATTT-3′
SubPep-C	5′-CGGGGACGAGGATGACCCTT-3′
SubPep-D	5′-AAGTCCAGCAATGCCCAAG-3′