

# A subtilisin-like protein from soybean contains an embedded, cryptic signal that activates defense-related genes

Gregory Pearce<sup>a,1,2</sup>, Yube Yamaguchi<sup>a,b,1,3</sup>, Guido Barona<sup>a</sup>, and Clarence A. Ryan<sup>a,4</sup>

<sup>a</sup>Institute of Biological Chemistry, Washington State University, Pullman, WA 99164-6340; and <sup>b</sup>Laboratory of Crop Physiology, Graduate School of Agriculture, Hokkaido University, Sapporo 060-8589, Japan

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Among the arsenal of plant-derived compounds activated upon attack by herbivores and pathogens are small peptides that initiate and amplify defense responses. However, only a handful of plant signaling peptides have been reported. Here, we have isolated a 12-aa peptide from soybean (*Glycine max*) leaves that causes a pH increase of soybean suspension-cultured cell media within 10 min at low nanomolar concentrations, a response that is typical of other endogenous peptide elicitors and pathogen-derived elicitors. The amino acid sequence was determined and was found to be derived from a member of the subtilisin-like protease (subtilase) family. The sequence of the peptide was located within a region of the protein that is unique to subtilases in legume plants and not found within any other plant subtilases thus far identified. We have named this peptide signal *Glycine max Subtilase Peptide* (*GmSubPep*). The gene (*Glyma18g48580*) was expressed in all actively growing tissues of the soybean plant. Although transcription of *Glyma18g48580* was not induced by wounding, methyl jasmonate, methyl salicylate, or ethephon, synthetic *GmSubPep* peptide, when supplied to soybean cultures, induced the expression of known defense-related genes, such as *Cyp93A1*, *Chib-1b*, *PDR12*, and *achs*. *GmSubPep* is a unique plant defense peptide signal, cryptically embedded within a plant protein with an independent metabolic role, providing insights into plant defense mechanisms.

damaged-self recognition | plant defense | signaling peptide | subtilase

The modification of survival mechanisms during coevolution of plant hosts with their biotic attackers resulted in the present-day complexity of plant–pathogen and plant–insect interactions. Whereas the invading species has developed methods of adhesion, penetration, and feeding, the plant has evolved mechanisms for perception of attack and activation of defense responses, based on surveillance of its own tissue. Damaged-self recognition occurs when signaling molecules are released from damaged cells and perceived by plant receptors to elicit a defense response (1). These elicitors can be of a heterogeneous nature, such as cutin monomers or cell wall fragments of various sizes (2–4), which exist in all plant species, or they can be a more specific, fine-tuned signal, such as endogenous peptide signals, which may be limited to a single phylogenetic family, coevolving with a specific predator (5–7). Collectively, these diverse compounds are termed endogenous elicitors or damage-associated molecular patterns (DAMPs).

Plants have also evolved receptor-mediated recognition systems for fungal and microbial biochemicals from the sites of infection, termed microbe-associated molecular patterns (MAMPs). These signals include bacterial peptide fragments, such as flg22 and elf18 (8, 9), fungal peptide elicitors, such as Pep13, AVR9, and elicitors (10–12), chitin fragments from fungal cell walls (13), and the heptagluco-side elicitor from the oomycete *Phytophthora megasperma* (3). MAMPs and DAMPs are perceived on the plant cell surface by receptors that transduce a signal intracellularly, initiating a defense pathway. For example, upon wounding of tomato plants, the plant peptide signal systemin is released from its precursor and, through receptor-mediated events, initiates the jasm-

onate signaling pathway, producing protease inhibitors and other defense compounds that protect the plant from further attack (14).

Relatively few endogenous peptide defense signals have been isolated thus far. These include a family of glycopeptides from the Solanaceae that are functionally related to systemin, hydroxyproline-rich glycopeptide systemins (HypSys) (15), and a family of signaling peptides from *Arabidopsis* (*AtPeps*) that amplify the innate immune responses through the jasmonate/ethylene and salicylate signaling pathways (16, 17). Another plant peptide signal generated from a plant has been found that is derived from an intracellular protein. When cowpea (*Vigna unguiculata*) leaves were consumed by armyworm larvae, a proteolyzed fragment of the cowpea chloroplastic ATP synthase was produced in the insect gut that was found to elicit defense responses when deposited on the leaves in the oral secretions (18). The peptide was termed inceptin and is an example of an indirect signal generated by the insect that initiates a specific plant defense response.

The complexity of the host–herbivore/pathogen relationship is becoming a common theme in plant biology. In recent work with inceptin, the peptide was active in inducing defense responses in cowpea and not in another member of the Fabaceae family, *Glycine max* (19). This is similar to systemin, which was found only in one clade of the Solanaceae. In a continuing search for plant elicitors of defense responses, we have isolated a 12-aa peptide from *Glycine max* that induces the expression of defense genes. The peptide is processed from a unique region of an extracellular subtilisin-like protease (subtilase), providing insight into the mechanism by which host plant–derived, damage-associated signals mediate immune responses.

## Results and Discussion

In our investigations of defense peptides, a bioassay has been used that takes advantage of a dramatic increase in pH of the media of suspension cells when a bioactive peptide binds to its receptor (20–22). A crude peptide fraction obtained from soybean leaves displayed the ability to alkalize soybean suspension cell media when separated on a C18 reversed-phase column (Fig. 1A). Along with large, late-eluting peaks corresponding in retention time to the previously reported rapid alkalization fac-

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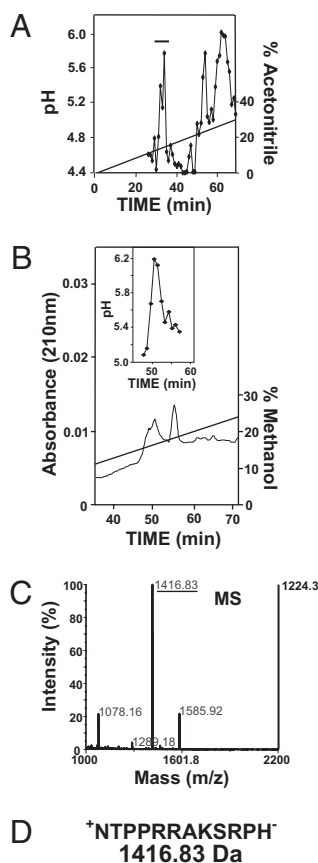
<sup>1</sup>G.P. and Y.Y. contributed equally to this work.

<sup>2</sup>To whom correspondence should be addressed. E-mail: pearce@wsu.edu.

<sup>3</sup>Present address: Laboratory of Crop Physiology, Graduate School of Agriculture, Hokkaido University, Sapporo 060-8589, Japan.

<sup>4</sup>Deceased October 7, 2007.

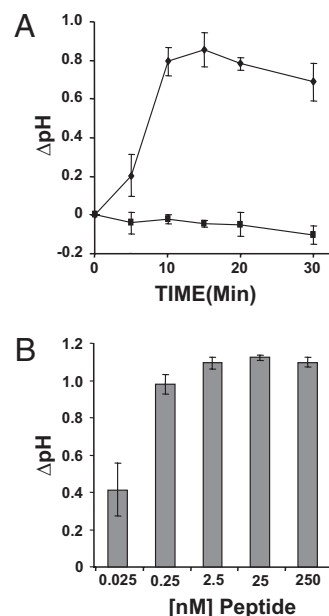
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**Fig. 1.** HPLC purification of *GmSubPep* from soybean leaf extracts. (A) A crude leaf extract (see Materials and Methods) was applied to a reversed-phase C18 semipreparative HPLC column in 0.1% trifluoroacetic acid/H<sub>2</sub>O and eluted with an acetonitrile gradient. Fractions (2 mL) were collected, and 10- $\mu$ L aliquots were assayed for alkalinizing activity. The bioactive fraction (32–34) designated by the bar was pooled for further purification. (B) After several HPLC purification steps, the active fraction was eluted from a narrow-bore C18 column with a methanol gradient as described in *SI Materials and Methods* and assayed for alkalinizing activity. The active fraction (50–51) was pooled for mass spectral analysis. (C) MALDI mass spectral analysis of the pooled fractions from Fig. 1B contained a major mass peak at 1,416.83 Da. (D) The 1,416.83 mass peak was subjected to MS/MS (Fig. S1), and the fragmentation spectra revealed a 12-aa peptide.

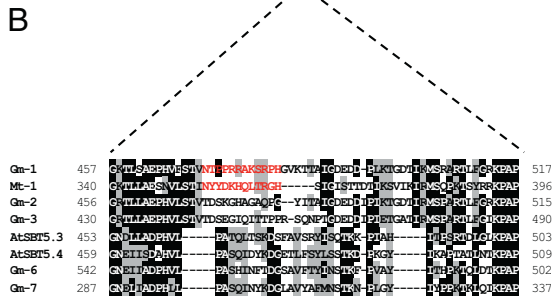
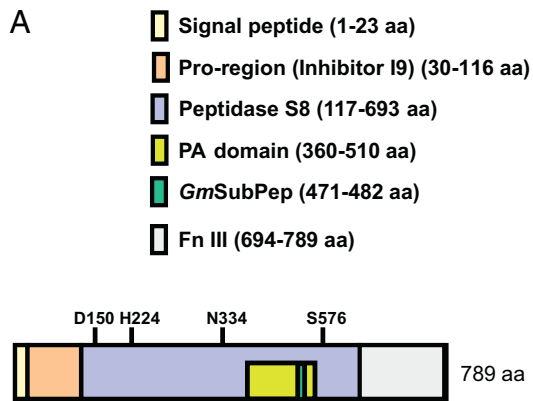
tor (22), a more hydrophilic peak was detected at 32–34 min. Through a series of HPLC steps and in a manner previously used for the purification of other bioactive peptides (21, 22) (*Materials and Methods* and *SI Materials and Methods*), a fraction containing very little UV-absorbing material was shown to induce a strong (over one pH unit) alkalinization response in soybean suspension cell media (Fig. 1B). The active fraction was analyzed by MALDI-MS, and a major mass peak was detected at 1,416.83 Da (Fig. 1C). MALDI-MS/MS fragmentation data coupled with a BLAST search using the soybean genome database (Phytozome; <http://www.phytozome.net/>) revealed a potential peptide sequence consisting of 12 amino acids: NTPPRAKSRPH. The peptide was synthesized and confirmed to have the same MS/MS fragmentation pattern as the native peptide (Fig. S1).

The synthetic soybean peptide was capable of producing a pH change within 10 min and a maximal alkalinizing response in 15 min (Fig. 2A). The peptide was active at extremely low concentrations with a half-maximal response of <0.25 nM (Fig. 2B). The response time and concentration were comparable to previously isolated defense peptides (16, 21, 22).



**Fig. 2.** Alkalinization assay kinetics of the synthetic peptide. (A) Cultured soybean cells [strain: PI 553039 (Davis)] were used 4 d after subculture. Aliquots of peptide or H<sub>2</sub>O (10  $\mu$ L) were added to 1 mL of suspension cells on an orbital shaker at 160 rpm. The pH of the suspension cell media was recorded at various time points. (B) Aliquots of peptide (10  $\mu$ L) were added to 1 mL of cells at various concentrations, and the pH of the suspension cell media was recorded after 15 min. Experiments were done in triplicate from three separate flasks of soybean suspension cells. Error bars indicate SD.

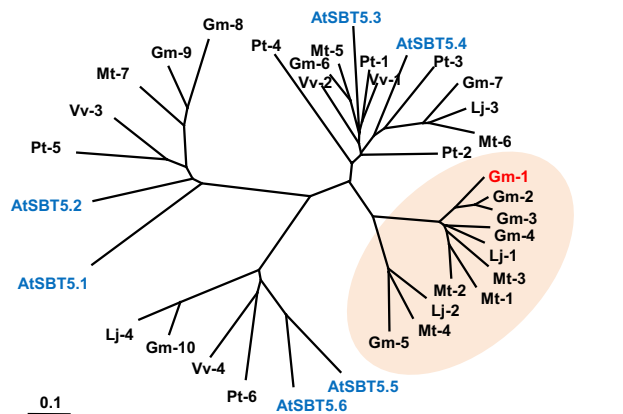
The peptide sequence identified in the soybean genome database was derived from a gene designated Glyma18g48580, which is predicted to code for a subtilisin-like protease (subtilase). Therefore, we have named the peptide *Glycine max* Subtilase Peptide (*GmSubPep*). Because the predicted coding sequence of Glyma18g48580 in the database was incomplete, a start codon was predicted from the genomic DNA sequence by comparisons with other subtilases, and the full-length coding region of Glyma18g48580 was amplified by RT-PCR and sequenced (Fig. S2). Glyma18g48580 contains all of the characteristic domains found in plant subtilases, including a signal sequence for secretion to the outside of the cell, a proprotein region, a peptidase domain, a protease-associated (PA) domain located within the peptidase S8 region, and a C-terminal fibronectin III (Fn III) domain (Fig. 3A) (23, 24). The amino acids required for formation of the catalytic triad, Asp-150, His-224, and Asn-334, along with the essential serine at the catalytic site (Ser-576), are conserved (Fig. 3A). The sequence of the biologically active peptide was found within the PA domain of Glyma18g48580, in a region that is unique to a small group of putative soybean [Glyma18g48530 (Gm-1), Glyma18g48490 (Gm-2), and Glyma09g37910 (Gm-3)] subtilases (Fig. 3B). Although the BLAST search indicated that the most similar proteins to Glyma18g48580 in *Arabidopsis* are AtSBT5.3 (AIR3/At2g04160) and AtSBT5.4 (At5g59810), more similar proteins to AtSBT5.3 and AtSBT5.4 were predicted in the soybean genome database. Phylogenetic analysis revealed that Glyma18g48580 (Gm-1) and its homologs in legume plants form a distinct group with no apparent *Arabidopsis*, poplar (*Populus trichocarpa*), or grape (*Vitis vinifera*) ortholog in subtilase subfamily 5 (Fig. 4). Only one EST sequence (Mt-1) contained a sequence similar to *GmSubPep*, and this was aligned with *GmSubPep* with a sequence of “NYDDKHQLTRGH,” containing three matches and three similar amino acids. The peptide was synthesized, but no alkalinizing activity was found.



**Fig. 3.** Bioactive peptide is located within Glyma18g48580, which encodes a putative subtilase. (A) The predicted subtilase protein is 789 aa in length, and the peptide sequence is located within the PA domain within the peptidase S8 region. The catalytic triad consists of D150, H224, and N334, with the active site serine at position 576. (B) The peptide-containing region of Glyma18g48580.1 (Gm-1) is compared with the closest subtilase homologs from soybean [Gm-2 (Glyma18g48530.1), Gm-3 (Glyma18g48490.1), and Gm-6 (Glyma05g28500.1) and Gm-7 (Glyma07g39990.1)], *Medicago truncatula* [Mt-1 (ABN05911)], and *Arabidopsis* [AtSBT5.3 (At2g04160) and AtSBT5.4 (At5g59810)]. The isolated peptide and a peptide sequence with similarity to *GmSubPep* from Mt-1 are indicated in red.

Among the suspension-cultured cells tested from a wide array of species, only the suspension cells produced from *Glycine max* were capable of producing an alkalinizing response to *GmSubPep* (Fig. S3), further suggesting the uniqueness of the peptide sequence in Glyma18g48580. Additionally, when *GmSubPep* was synthesized with an additional amino acid on either the N-terminal or C-terminal end corresponding to the amino acid coded for by the gene sequence, a significant decrease in alkalinizing activity was observed, indicating that the isolated 12-aa peptide is correctly processed (Fig. S4).

To elucidate the possible involvement of *GmSubPep* in soybean defense responses, expression analysis of soybean defense-related genes were conducted using quantitative RT-PCR (qRT-PCR) with *GmSubPep* or the control peptide, systemin, which only induces defense gene expression in the Solanaceae family (Fig. 5). In cells treated with *GmSubPep*, a cytochrome P450 gene, *Cyp93A1*, involved in phytoalexin synthesis (25), was induced to 40 times the expression level of the control peptide, and a pathogenesis-related gene, chitinase 1b-1 (*Chib-1b*) (26), was induced to 12 times control levels. A salicylic acid-inducible ATP-binding cassette transporter, *PDR12* (27), was induced to 18 times the expression level of the control peptide, and chalcone synthase (*chs*) (28), involved in phytoalexin production, was induced to 50 times the control level. The induction was rapid, with a significant induction of *Chib-1b* and *chs* in 1 h and induction of *Cyp93A1* and *PDR12* in 2 h. All four of the genes had maximal expression between 2 and 4 h and began to decline at 8 h.



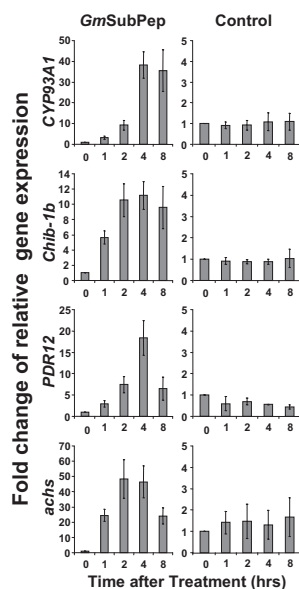
**Fig. 4.** Phylogenetic tree of subtilases closely related to the *Arabidopsis* SBT5 subfamily. The soybean subtilase (Glyma18g48580) containing the bioactive peptide is indicated in red (Gm-1), and the members of the *Arabidopsis* subtilase 5 family are indicated in blue. The group of Gm-1-related subtilases that seems to be uniquely present in legumes is indicated by pink shading. The protein sequences were obtained from Plant GDB (<http://www.plantgdb.org>), Phytozome (<http://www.phytozome.net>), and the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov>). Abbreviations for genes: *Arabidopsis thaliana* (AtSBT5.1, At1g20150.1; AtSBT5.2, At1g20160.1; AtSBT5.3, At2g04160.1; AtSBT5.4, At5g59810.1; AtSBT5.5, At5g45640.1; AtSBT5.6, At5g45650.1), *Glycine max* (Gm-1, Glyma18g48580.1; Gm-2, Glyma18g48530.1; Gm-3, Glyma18g48490.1; Gm-4, Glyma09g37910.1; Gm-5, Glyma14g05230.1; Gm-6, Glyma05g28500.1; Gm-7, Glyma07g39990.1; Gm-8, Glyma06g02500.1; Gm-9, Glyma04g02440.1; Gm-10, Glyma13g29470.1), *Medicago truncatula* (Mt-1; ABN05911, Mt-2, AC133779\_25.4; Mt-3, AC151423\_9.5; Mt-4, CR936328\_23.4; Mt-5, CT573401\_13.4; Mt-6, AC175049\_27.4; Mt-7, CT967316\_16.4), *Lotus japonicus* (Lj-1, LjSGA\_007130.1; Lj-2, chr2.CM0826.300.nd; Lj-3, chr4.LjT24N17.30.nc; Lj-4, chr6.CM0118.310.nc), *Vitis vinifera* (Vv-1, GSVIVT00029050001, Vv-2, GSVIVT00033477001; Vv-3, GSVIVT00014361001; Vv-4, GSVIVT00007221001), and *Populus trichocarpa* (Pt-1, XP\_002320540.1; Pt-2, XP\_002299062.1; Pt-3, XP\_002308740.1; Pt-4, XP\_002299063.1; Pt-5, XP\_002300693.1; Pt-6, XP\_002317314.1).

To characterize the precursor gene of *GmSubPep*, Glyma18g48580 gene expression was analyzed using qRT-PCR. The expression levels of Glyma18g48580 were similar in all actively growing plant tissues, except in mature, lower leaves, where Glyma18g48580 expression was barely detectable (Fig. S5). Hormones that are known regulators of plant defense gene expression, methyl jasmonate, methyl salicylate, and ethephon, did not induce the expression of Glyma18g48580 when compared with two genes, *Cyp93A1* and *Chib-1b*, known to be induced by either wounding or elicitors of defense responses (Fig. S6). In contrast to Glyma18g48580 expression, the genes for prosystemin, pro-HypSys, and PROPEP have all been shown to be induced by methyl jasmonate and wounding. It may be possible that the subtilase coded for by Glyma18g48580 is activated upon pathogen attack or wounding, thus regulation of any protease activity, as well as the peptide signaling activity, would occur through post-transcriptional mechanisms. Thus, neither would be dependent upon up-regulated transcription of Glyma18g48580.

Recently, the first crystal structure of a plant subtilase revealed that the PA domain protrudes from the subtilase and is involved in dimerization of the subtilase (24). Because *GmSubPep* seems to be an evolutionary insertion in the PA domain for a small family of proteases, *GmSubPep* may be positioned in an easily accessible region for cleavage from the subtilase for defense signaling.

The subtilase is predicted to be secreted to the apoplast, placing it in contact with components of fungal or bacterial invasions. The high constitutive expression in developing tissues and lack of induction by defense-related phytohormones suggests involvement in apoplastic processes unrelated to defense, in contrast to some





**Fig. 5.** Time course of relative expression levels of defense-related genes in soybean suspension cells in response to *GmSubPep* as analyzed by real-time RT-PCR. RNA was isolated from suspension cells at various times after addition of either *GmSubPep* or a control peptide at a final concentration of 25 nM as described in *Materials and Methods*. The gene expression levels are indicated relative to expression at 0 h. *Cyp93A1*, a cytochrome P450 gene involved in phytoalexin synthesis; *Chib-1b*, chitinase gene, a pathogenesis-related gene; *PDR12*, a salicylic acid-inducible ATP-binding cassette transporter; *ach5*, chalcone synthase, involved in phytoalexin production. *ELF1B* was amplified as an internal control to normalize the RNA level in each sample. Error bars indicate SD from three biological replicates.

extracellular proteases (29–31). It is conceivable that the subtilase is activated upon attack and that in the ensuing battle with foreign proteins (virulence factors of the invading pathogen) *GmSubPep* is released to signal the first line of defense, somewhat like vertebrate defense-related proteins that can also stimulate host immune responses (32). Whether Glyma18g48580 interacts specifically with an invading species or has a function in other plant processes remains to be determined.

Herein we report the discovery of *GmSubPep*, a 12-aa peptide that induces defense gene expression. The activity of *GmSubPep* resembles that of other peptidic defense signaling peptides in plants, but unlike these known peptides, *GmSubPep* is derived from an extracellular protease. Two different approaches to elucidating plant peptide ligands have been used, the first being a genetic approach, as exemplified by both the S-locus cysteine-rich (SCR) incompatibility ligands (33) and the CLAVATA3/CLAVATA1 receptor–ligand relationship for differentiation at the shoot apical meristem (34). The second elucidation method is biochemical, whereby peptides are purified from plant extracts and bioassayed, as was the case for the systemins (15), *AtPeps* (16), and the phyto-sulfokines (35). Although a genetic dissection of function may have revealed a defense phenotype attributable to the subtilase, the discovery of the unknown peptide signal within the subtilase with a completely different function would have been difficult. Biochemical methodology, established to screen for ligands of membrane-bound receptors, was necessary for the discovery of this unique mechanism of plant defense.

*GmSubPep* has similarities to the unique inceptin peptide that is localized to the chloroplast and is only released from the chloroplastic ATP synthase protein upon ingestion by an herbivore (18, 19). *GmSubPep* also has similarities to the hydroxyproline-rich systemins by its presumed localization in the apoplast where, when proteolyzed from the subtilase, it interacts with a yet-to-be-identified membrane-

bound receptor to initiate defense signaling. The specific role of *GmSubPep* will be revealed when the conditions/factors necessary for release of the peptide from its precursor are obtained. Isolation of the receptors and production of mutant/transgenic plants lacking signaling capabilities will be of foremost importance for demonstrating a role for *GmSubPep*.

## Materials and Methods

**Alkalinization Assay.** Soybean suspension cells (varieties A3525 and Davis) were maintained in Murashige and Skoog medium as previously described with tobacco cells (21). Cultures were maintained by transferring 2.5–5 mL of cells to 40 mL of media every 7 d and shaking at 160 rpm. Soybean suspension cells were used 4–6 d after transfer. Before assaying for alkalinizing activity, a flask of cells was aliquoted into 24-well cell culture cluster plates (1 mL per well) and allowed to equilibrate at 160 rpm until the pH of the cells ceased to decline ( $\approx 2$  h). Aliquots of HPLC fractions or purified peptide (1–10  $\mu$ L) were added, and the pH was recorded after 15 min.

**Plant Material and Growth Conditions.** For peptide isolation, soybean plants, *Glycine max*, variety A3525, were grown in growth chambers (18 h light at 28 °C and 6 h dark at 18 °C, 300  $\mu$ mol photons  $m^{-2} s^{-1}$ ) for  $\approx 4$  wk. The plants were sprayed with methyl jasmonate as previously described (21, 35). After 15 h, the leaves were collected, ground in liquid nitrogen, and stored at  $-20$  °C until use. The same conditions were used for collection of RNA samples after treatments, except the plants were used at 3 wk.

**Peptide Isolation, Analysis, and Synthesis.** The peptide isolation was done as previously described with modifications (21, 36). The complete method is described in *SI Materials and Methods*. MALDI spectra were obtained on an Applied Biosystems 4800 TOF/TOF mass spectrometer with 200 Hz Nd-YAG laser. Concentrated samples in aqueous solution were mixed 1:1 with matrix solution ( $\alpha$ -cyano-4-hydroxycinnamic acid, 6 mg/mL in 50:50 acetonitrile: 0.25% trifluoroacetic acid in water) and air dried. Calibrated MS spectra ( $\pm 0.02$  Da) were obtained as the summations of 4,000 laser shots, whereas MS/MS spectra ( $\pm 0.1$  Da) were summations of 10,000 laser shots. Peptides were synthesized by *N*-(9-fluorenyl) methoxycarbonyl chemistry by solid-phase techniques using an Applied Biosystems model 431 synthesizer and purified by reversed-phase HPLC. Peptide stocks (2.5 mM in distilled water) were checked for purity and for correctness with the predicted mass on a Finnigan LC/Q mass spectrometer using direct injection.

**Wounding, Hormone, and Peptide Treatments.** Three-week-old *Glycine max* (variety A3525) plants having six to eight expanded leaves were used in wounding experiments, performed under growth chamber conditions consisting of 18 h light at 28 °C and 6 h dark at 18 °C (300  $\mu$ mol photons  $m^{-2} s^{-1}$ ). For each plant, the top four leaves were wounded across the midvein using a hemostat. Time-course experiments were performed in which the wounded leaves were collected at 0, 0.5, 1, 2, 4, and 8 h after the mechanical injury. The corresponding leaves from unwounded plants served as controls for each time point. The leaf samples were immediately frozen in liquid nitrogen and kept at  $-80$  °C until use. For treatment with defense gene inducers, plants were sprayed with solutions of either 625  $\mu$ M MeJA, 2 mM methyl salicylate, or 7 mM ethephon: all in double-distilled  $H_2O$  containing 0.1% Triton X-100. Control plants were sprayed with 0.1% Triton X-100. The leaf samples were collected in triplicate for time-course experiments after spraying as above and immediately frozen in liquid nitrogen and kept at  $-80$  °C until use. The leaf material was ground to a fine powder in a mortar and pestle with liquid  $N_2$ , and total RNA was isolated with TRIZOL reagent (Invitrogen) according to the manufacturer's protocol.

Suspension cells (variety: Davis) were used for determining the induction of genes by *GmSubPep*. Cells were grown as described above. At 4 d, either *GmSubPep* or a control peptide, systemin, were added to a final concentration of 25 nM. Three-milliliter aliquots were removed at 0, 1, 2, 4, and 8 h and were filtered through a #4 Whatman filter paper. The cells were immediately frozen in liquid nitrogen and stored at  $-80$  °C until use. RNA samples were prepared as described above.

**Computer Analyses.** Domain prediction was performed by the Pfam program (37) and by comparison with tomato (*Solanum lycopersicum*) subtilase 3 (*S/SBT3*), whose crystal structure has recently been determined (24). Subcellular localization was predicted by the WoLF PSORT (38) and the TargetP 1.1 programs (39). Phylogenetic analysis was conducted by the Clustal W program (40). The phylogenetic tree was drawn by the TreeView program (41).

**PCR Analysis.** Five micrograms of total RNA was reverse transcribed using SuperScript II (Invitrogen), and the resulting cDNA was used for amplifying the full-length coding sequence of Glyma18g48580 by PCR with SubPep-F (5'-TATGCCTGACAAGCAATTCG-3') and SubPep-R (5'-CTCGACCAATTTGGGAATTT-3') primers. For the gene expression analysis, 1  $\mu$ g of total RNA was subjected to the RT reaction using the DyNamo cDNA Synthesis Kit (Finnzymes) according to the manufacturer's protocol. The cDNA was diluted five times with H<sub>2</sub>O and then subjected to real-time qPCR analysis using the DyNamo SYBR Green qPCR kit (Finnzymes) and the Mx3000P (Stratagene). Primers used in qRT-PCR are listed in Table S1. Primers for elongation factor 1- $\beta$  (ELF1B) were included as an internal control for normalization in all experimental runs. DNA sequences [ELF1B (Glyma02g44460), CYP93A1 (D83968), Chib-1b (AB007127), Gmachs1

(X54644), and PDR12 (AM261476)] were obtained from the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/>), Phytozome (<http://www.phytozome.net/>), and PlantGDB (<http://www.plantgdb.org/>).

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