GmPep914, an Eight-Amino Acid Peptide Isolated from Soybean Leaves, Activates Defense-Related Genes1[W][OA]

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Only a handful of endogenous peptide defense signals have been isolated from plants. Herein, we report a novel peptide from soybean (Glycine max) leaves that is capable of alkalinizing the media of soybean suspension cells, a response that is generally associated with defense peptides. The peptide, DHPRGGNY, was synthesized and found to be active at 0.25 nM and requiring only 5 to 10 min to obtain a maximal pH change. The peptide is located on the carboxy-terminal end of a 52-amino acid precursor protein (Glyma12g00990) deduced from the soybean genome project. A search of the soybean databank revealed a homolog (Glyma09g36370) that contained a similar peptide, DLPRGGNY, which was synthesized and shown to have identical activity. The peptides, designated GmPep914 (DHPRGGNY) and GmPep890 (DLPRGGNY), were capable of inducing the expression of both Glyma12g00990 (GmPROPEP914) and Glyma09g36370 (GmPROPEP890) in cultured soybean suspension cells within 1 h. Both peptides induced the expression of defense genes, including CYP93A1, a cytochrome P450 gene involved in phytoalexin production, and Glyma12g00990, an eight-amino acid peptide, was purified from tomato (Solanum lycopersicum) plants (Pearce et al., 1991). Systemin was found to induce the expression of a number of defense genes, including protease inhibitors and polyphenol oxidase (Constabel et al., 1995), for protection from insect attack. A sequence homology-based search for prosystemin revealed that this was a unique gene found only within a single clade of the Solanaceae that included tomato, potato (Solanum tuberosum), nightshade (Solanum nigrum), and pepper (Capsicum annuum; Constabel et al., 1998). However, another peptide defense signal, Hyp-rich glycopeptide systemin (HypSys), was identified in tobacco (Nicotiana tabacum; Pearce et al., 2001a) and later in tomato (Pearce and Ryan, 2003) and other solanaceous plants (Pearce, 2011). Although systemin and HypSys do not share sequence homology, they have been classified as subgroups of a defense peptide family based on their sizes and functional similarities (Ryan and Pearce, 2003). Recently, the HypSys family of defense peptides was expanded to include sweet potato (Ipomoea batatas), a member of the Convolvulaceae in the Solanales (Chen et al., 2008).

The search for systemin/HypSys-related peptides in other plant species has led to the discovery of other families of defense-related peptides. In Arabidopsis (Arabidopsis thaliana), the AtPeps were found to protect plants from pathogen attack. There are eight AtPep paralogs with orthologs being found in the data bank for a wide variety of plants, including agriculturally important crops such as rice (Oryza sativa) and maize (Zea mays; Huffaker et al., 2006, 2011). AtPeps are 23 amino acids in length and induce the expression of defensin and pathogenesis-related genes. Transgenic overexpression experiments with AtPROPEP1 were found to enhance resistance to the root pathogen Pythium irregularare (Huffaker et al., 2006). Systemin

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3 This article is dedicated to the memory of Clarence A. Ryan (deceased October 7, 2007).

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and AtPep precursors are similar in that they have no signal sequence and appear to reside in the cytosol. While the mechanism of peptide release and export to the apoplast is unknown, upon injury and/or infection, the peptides are processed from their proproteins for interaction with their membrane-bound receptors to initiate a defense signaling cascade, leading to the production of defense compounds (Ryan et al., 2002).

All of the above peptide signals were purified from crude plant leaf extracts based on their ability to interact with suspension cells, thereby causing a rapid increase in the extracellular pH of the media (Felix and Boller, 1995). An alkalinization assay was developed in the Ryan laboratory that has been a key to the purification of the defense peptides HypSys and AtPeps, as well as the peptide Rapid Alkalinization Factor (Pearce et al., 2001b), which is involved in developmental processes. This alkalinization assay has tremendous potential to unlock the nature of peptide signaling in any plant species that can be maintained in liquid culture.

Recently, another defense-related peptide signal generated from a plant has been found that is derived from a chloroplastic ATP synthase. When cowpea (Vigna unguiculata) leaves were consumed by Spodoptera frugiperda larvae and the oral secretions deposited on the leaves, a proteolyzed fragment of the cowpea chloroplastic ATP synthase produced in the insect gut was found to elicit defense responses (Schmelz et al., 2006). The peptide was termed inceptin and is an example of an indirect signal generated by the insect that initiates specific plant defense responses.

Systemin, HypSys, AtPeps, and inceptins are the only peptides generated from plant proteins known to induce defense responses. What has become apparent is that coevolution of plants with their biotic attackers has allowed for a diverse array of defense mechanisms by both plant and predator in an arms race (Boller and Felix, 2009; Heil, 2009). An alkalinizing activity found in any new plant suspension system may yield a member of one of the known defense peptide families or could reveal a yet-undiscovered peptide signaling family that is specific to a species or genus.

While studying soybean (Glycine max)-derived factors that cause an alkalinization response in soybean suspension cells, we have recently discovered a novel 12-amino acid peptide that induces defense genes and is generated from a larger protein (Pearce et al., 2010b). The amino acid sequence was determined and the gene was identified as a member of the subtilisin-like protease (subtilase) family, however, the sequence of the peptide was located within the subtilase (Glyma-18g48580) sequence in a region unique to soybean and Medicago truncatula and not found within any other plant subtilases. We have named this new peptide signal soybean Subtilase Peptide (GmSubPep). The gene was found to be expressed in all actively growing tissues of the soybean plant. Synthetic GmSubPep, when supplied to soybean cultures at low nanomolar concentrations, caused a pH increase of soybean suspension cell culture media of approximately 1 pH unit in 15 min, a response that is typical of other peptide signals. This rapid alkalinization of the culture medium was followed by the induction of known defense genes, such as CYP93A1 (Suzuki et al., 1996), chitinaseb1-1 (Chib1-1; Watanabe et al., 1999), PDR12 (Eichhorn et al., 2006), and Glycine max chalcone synthase1 (Gmachs1; as described by Akada et al., 1991).

We have identified and purified a second peptide that regulates soybean defense. The eight-amino acid peptide, which we have named GmPep914, is the smallest defense peptide signal found to date and is unrelated to any of the known plant defense peptides. Herein, we describe the purification of GmPep914 and the abilities of GmPep914 and a closely related deduced peptide, GmPep890, to alkalinize cellular media and induce defense-related genes. The precursors, GmPROPEP914 and GmPROPEP890, were also analyzed for their responses to known inducers of defense.

Figure 1. Final HPLC purification of the early eluting alkalinizing fraction from SCX-HPLC. After several HPLC purification steps (see “Materials and Methods”), the active fraction was eluted from a narrowbore C18 column with an acetonitrile gradient as described in “Materials and Methods” and assayed for alkalinizing activity (inset). Aliquots of the peptide (2 μL) were added to 1 mL of cells, and the pH of the suspension cell media was recorded after 15 min. The active fraction, 37.5 min, was analyzed by MS.
defense-related genes. Genetic analysis reveals that these genes have homologs only within other members of the Fabales and in the closely related Cucurbitales.

RESULTS

Recently, we isolated a 12-amino acid peptide (GmSubPep) from crude leaf extracts of soybean by utilizing suspension-cultured cells and the ability of some defense-related peptides to alkalinize the extracellular media (Pearce et al., 2010b). When the crude preparation was loaded onto a semipreparative C-18 reversed-phase column, an early eluting doublet peak was identified with alkalinizing ability. One component of the doublet was further purified to yield GmSubPep (Pearce et al., 2010b). When cation-exchange HPLC was used to separate the doublet, two activity peaks were obtained, one at 49 min (GmSubPep) and an earlier eluting peak (31 min) that was further purified to homogeneity with HPLC methodology (see “Materials and Methods”). The final HPLC purification of the active component derived from the 31-min cation-exchange fraction is shown in Figure 1, with an activity peak at 37.5 to 38.5 min that corresponded to a large absorbance peak. Matrix-assisted laser-desorption ionization (MALDI) mass spectral analysis of the peak activity fraction at 37.5 min revealed a major mass peak at 915.40 D (Fig. 2A) that was subjected to tandem mass spectrometry (MS/MS) analysis (Fig. 2B). The spectra revealed a peptide sequence (DHPRGGNY), which was an exact match for the carboxy-terminal end of a predicted protein, Glyma12g00990, in the soybean genome database (Phytozome; http://www.phytozome.net, details described below).

The peptide was synthesized and analyzed for alkalinizing activity with soybean suspension cells (variety: Davis, PI 553039) and the synthetic peptide was compared to GmSubPep (Fig. 3). All experiments utilizing suspension cells were performed in triplicate, utilizing three different flasks of soybean suspension cells. The peptide caused a pH increase of 1.4, higher than the 1.0 pH increase for GmSubPep. Both peptides had one-half maximal activity values of approximately 0.25 nM (Fig. 3A). The new synthetic peptide obtained a maximal pH increase after 10 min with a maximal pH change after 15 min (Fig. 3B). The peptide was named GmPep914 for its monoisotopic mass (914.40 D).

To ascertain the important amino acids within the sequence of GmPep914 for cell media alkalinization, a series of Ala-substituted analogs were synthesized.
with a replacement of each amino acid in the sequence. The eight peptide analogs were compared with GmPep914, revealing two Ala substitutions at the carboxyl terminal, Asn-7 and Tyr-8, which were inactive in the alkalinization assay (Fig. 4). All of the other substitutions were active but required a slightly higher concentration of peptide, except for the Ala substitution of His-2, which was as active as GmPep914. Peptide analogs were also synthesized without either the N-terminal Asp [GmPep914(2-8)] or the C-terminal Tyr [GmPep914(1-7); Fig. 4]. Both of these analogs displayed a complete loss of activity. Furthermore, when an excess of any of the inactive analogs was incubated with suspension cells, followed by the addition of GmPep914 at various concentrations, none of the inactive analogs were able to compete with GmPep914 (Supplemental Fig. S1).

Based on the predictions of the soybean genome project, a precursor protein of GmPep914 (Glyma12g00990), Gmprop914, consists of 52 amino acid with GmPep914 at the C-terminal end (Fig. 5A). There is another DNA sequence encoding DLPRGGNY* (where * is a stop codon) within the Glyma09g36370 gene in the soybean genome database. However, the predicted mRNA sequence for Glyma09g36370 in the database was apparently mispredicted. We predicted the Glyma09g36370 coding sequence by comparison with the coding sequence of Gmprop914, with confirmation by DNA sequencing after cDNA amplification (Fig. 5B). The amino acid sequence of Glyma09g36370 had 84.6% amino acid identity to Gmprop914 (Fig. 5C). A peptide comprised of the C-terminal eight amino acids of Glyma09g36370 (DLPRGGNY) was synthesized and assayed for its ability to alkalinize the cellular media alongside GmPep914 (Fig. 5D). The two peptides displayed similar activities in both their maximal responses (approximately 1.3 pH increase) and the concentration required for a one-half maximal response (approximately 0.25 nM). Based on a monoisotopic mass of 890.42 D, Glyma09g36370 and its C-terminal eight amino acids were named GmproPep890 and GmPep890, respectively. Analysis using the SignalP software (http://www.cbs.dtu.dk/services/SignalP) indicated that both GmproPep914 and GmproPep890 have no obvious N-terminal signal sequence for secretion, similar to prosysmmins and AtproPeps.

We have recently synthesized inactive analogs of GmSubPep that competed with GmSubPep in the alkalinization assay (Pearce et al., 2010a). One of these analogs, GmSubPep(1-11), was incubated with soybean cells at a high concentration with the subsequent addition of either GmPep914 and GmPep890. Neither peptide was affected by the inactive analog, demonstrating that the receptor for GmSubPep and the receptor(s) for GmPep914 and GmPep890 were different (Supplemental Fig. S2). GmPep914 and GmPep890 were also tested for their abilities to alkalinize either tobacco or Arabidopsis cells with neither peptide capable of producing a response (Supplemental Fig. S3).

GmPep914 and GmPep890 were supplied to soybean suspension cells at low nanomolar concentrations and RNA was extracted at intervals for analysis of gene expression (Fig. 6). All experiments were performed in triplicate, utilizing three separately extracted RNA samples. A rapid induction of both GmPROPEP914 (Fig. 6A) and GmPROPEP890 (Fig. 6B) was evident at 1 h when cells were supplied with either GmPep914 or GmPep890. The response was both rapid and transient with a decrease in expression to 0 h control levels after 4 h for GmPROPEP914 (Fig. 6A) and a return to control expression levels after 2 h for GmPROPEP890 (Fig. 6B). Three defense genes were tested for expression levels after addition of either GmPep914 or GmPep890. A cytochrome P450 gene, CYP93A1, involved in glyceollin (soybean phytoalexin) biosynthesis (Suzuki et al., 1996; Schopfer et al., 1998), was induced 30 times

![Figure 4](image-url)  
**Figure 4.** Ala substitution and deletion of GmPep914. Peptides were synthesized with an Ala substituted at each position in GmPep914 or with either the C-terminal (1–7) or the N-terminal (2–8) amino acid removed. The set of 10 peptides was assayed relative to GmPep914 using soybean suspension cells (variety: Davis, strain: PI 553039), 4 to 5 d after transfer. Suspension cells (1 mL) were shaken on a rotary shaker at 160 rpm and after 2 h, each peptide (10 μL) was added to 1 mL of cells to make final concentrations of 0.25, 2.5, 25, and 250 nM. After 15 min, the pH of the media was recorded. Each bar represents the average of three separate experiments.
and 32 times the 0 h expression level at 4 h for GmPep914 and GmPep890, respectively (Fig. 6C). A pathogenesis-related gene, Chib1-1 (Watanabe et al., 1999), was induced after 1 h when either peptide was supplied to the cells, with an elevated level of gene expression of both genes between 2 and 8 h (Fig. 6D). Both genes demonstrated an expression level 14 times control levels at 4 h. Chalcone synthase (Gmachs1; Akada et al., 1991), involved in phytoalexin production, was induced after 1 h when suspension cells were treated with either GmPep914 or GmPep890 with maximal expression levels at 4 h for both GmPep914 (180 times control levels) and GmPep890 (120 times control levels; Fig. 6E).

The basal expression levels of GmPROPEP914 and GmPROPEP890 were tested in the various tissues of soybean plants by real-time reverse transcription (RT)-PCR (Fig. 7). Basal expression levels were barely detectable in the upper and lower leaves and in the stems of 8-week-old plants (Fig. 7, A and B). Although the flowers had a slightly higher expression level, the roots had the highest relative expression with GmPROPEP914 at 420 times and GmPROPEP890 at 180 times the levels detected in mature lower leaves. RNA was also extracted from younger plants (3-weeks-old) that were not in the flowering stage and the roots displayed elevated expression levels that were 100-times higher than mature, lower leaves for GmPROPEP914 (Fig. 7A) and 50 times higher for GmPROPEP890 (Fig. 7B).

RNA was extracted from the leaves of young soybean plants treated with inducers of defense genes. The expression levels of both GmPROPEP914 and GmPROPEP890 were compared after treatment with methyl jasmonate (MeJA), methyl salicylate (MeSA), or ethephon (Fig. 8). The expression level in control leaf extracts for both genes were barely detectable (Fig. 8, A and B). Upon treatment with MeJA, a significant increase in the expression levels of GmPROPEP914 was detected after 8 h, however no increase was observed for GmPROPEP890 (Fig. 8, C and D). Both genes had an increased relative expression level after MeSA treatment, with an increase in expression for GmPROPEP914 to 9 times the control level at 8 h (Fig. 8E) and the expression of GmPROPEP890 increasing to 17 times control levels after 2 h before decreasing to 5-times control levels at 8 h (Fig. 8F). Treatment with ethephon resulted in an increased expression level of GmPROPEP914 to 48-times control levels after 8 h (Fig. 8G) with GmPROPEP890 expression increasing at both 2 h and 8 h to about 5-times control levels (Fig. 8H).

To investigate the distribution of GmPep914-like peptides within the plant kingdom, a BLAST search was conducted using GmproPep914 and GmproPep890 protein sequences at the National Center of Biotechnology Information (http://www.ncbi.nlm.nih.gov/) Web site (Fig. 9). Three EST sequences were found from three different Fabaceae plants: licorice (Glycyrrhiza uralensis) (FS259533.1), M. truncatula (AW287956.2), and Cajanus cajan (GR466452.1). Each sequence contained a C-terminal putative peptide with only one or two amino acid differences from GmPep914 or GmPep890 and was derived from underground tissues. The EST of licorice was derived from rhizomes (Sudo et al., 2009), which are used in Chinese medicine (licorice), the EST of M. truncatula was derived from phosphate-starved roots, and the

Figure 5. The predicted coding sequence of Glyma12g00990 and the cDNA of a second peptide (Glyma09g36370) with homology to Glyma12g00990, encode bioactive eight-amino acid peptides with similar alkalinizing properties. A, The predicted protein sequence of Glyma12g00990 (GmproPep914). The GmPep914 sequence is indicated by gray shading. B, Using primers designed from the nucleotide sequence of Glyma12g00990, a second cDNA was isolated and sequenced (GmproPep890). The predicted peptide sequence is indicated by gray shading. C, A comparison of the two cDNAs revealed 84.6% homology for the predicted protein sequences. The peptide region is indicated by asterisks. D, A peptide was synthesized from the C-terminal eight amino acids of Glyoma09g36370, DLPDHGGNY, and compared to GmPep914 and GmSubPep in the alkalinization assay using soybean suspension cells (variety: Davis, strain: PI 553039), 4 to 5 d after transfer. Suspension cells (1 mL) were shaken on a rotary shaker at 160 rpm and after 2 h, each peptide (10 μL) was added to 1 mL of cells to make final concentrations of 0.025, 0.25, 2.5, 25, and 250 nM. After 15 min, the pH of the media was recorded.

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EST of *C. cajan*, which is one of the major legume crops of the tropics and subtropics, was derived from *Fusarium* wilt-challenged roots (Raju et al., 2010).

The precursor proteins of the AtPep family of defense peptides have been shown to have widely divergent sequences relative to the C-terminal defense peptide sequences (Huffaker et al., 2006). Therefore, we also conducted a BLAST search using just the GmPep914 sequence with the Phytozome database (http://www.phytozome.net/) to find small, predicted proteins containing the GmPep914-like peptide at the C terminal. Among 17 higher plants, only two cucumber (*Cucumis sativus*) predicted proteins have obvious C-terminal GmPep914-like peptides (Fig. 9). Interestingly, cucumber belongs to the Cucurbitales family that is closely related to Fabales, both being grouped in the nitrogen-fixing clade of the dicot subclass Rosid I (Soltis et al., 1995).

DISCUSSION

We report the isolation of a unique peptide from soybean that induces the expression of defense-related genes in suspension-cultured cells. The peptide consists of eight amino acids and is the smallest endogenous defense peptide elicitor isolated from plants to date. We have named the peptide GmPep914 by its monoisotopic mass, avoiding confusion with the AtPep homologs found in soybean (Huffaker et al., 2006). GmPep914 was isolated by a biochemical approach with the same techniques utilized for systemin (Pearce et al., 1991), HypSys glycopeptides (Pearce, 2011), AtPeps (Huffaker et al., 2006), and another endogenous peptide elicitor, GmSubPep, that was isolated from the same crude soybean leaf preparation used in this study (Pearce et al., 2010b). All of the above peptides have the ability to alkalinize cultured suspension cell media within minutes at low nanomolar concentrations and this property has been exploited for purification of bioactive peptides via the alkalinization assay (Pearce, 2011).

In the alkalinization assay, GmPep914 had a one-half maximal response at 0.25 nM and was capable of a higher media pH elevation than GmSubPep with a more rapid response time, indicating that GmPep914 is perhaps a more effective elicitor than GmSubPep. Both GmSubPep and GmPep914 were isolated from the commercial soybean variety A3525 (AsGrow, Monsanto) with resistance to a number of disease, but suspension cells from A3525 were so sensitive to addition of minute quantities of peptide that suspen-

Figure 6. Time course of relative expression levels of peptide precursors and defense-related genes in soybean suspension cells in response to GmPep914 and GmPep890 as analyzed by real-time RT-PCR. RNA was isolated from suspension cells at various times after addition of either GmPep914, GmPep890, or tomato systemin at a final concentration of 25 nM as described in “Materials and Methods.” The gene expression levels of *GmPROPEP914* (A), *GmPROPEP890* (B), *CYP93A1* (C), *Chib1-1* (D), and *Gmachs1* (E) are indicated relative to expression at 0 h. *Cyp93A1*, A cytochrome P450 gene involved in glyceollin synthesis; *Chib1-1*, chitinase gene, a pathogenesis-related gene; *Gmachs1*, chalcone synthase, a gene 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.
sion cells from a variety (Davis, PI 553039) with a moderate alkalinizing response was utilized for the kinetic studies. It will be interesting to examine whether the difference in sensitivity to GmPep914 between A3525 and Davis suspension cells in the alkalinization response is associated with a difference in resistance phenotypes to diseases.

To ascertain the importance of specific amino acids within the peptide for activity, GmPep914 was synthesized with Ala substituted at each amino acid position and the analogs were tested for their abilities to alkalinize the suspension cell media. Substitutions at the C-terminal Tyr-8 or at the penultimate Asp-7 caused a complete loss of activity, whereas only minor decreases were observed for the other substitutions. Only one substitution retained complete activity: the His-2 substituted with Ala. Deletion of either the N-terminal Asp-1 or the C-terminal Tyr-8 abolished the activity. Thus, the length of GmPep914 and the C-terminal amino acid side groups appear to be necessary for activity. GmPep914-like peptides found through a search of the genome database (Fig. 9) contain only one or two amino acid changes relative to GmPep914 and substitution with Ala at these positions, especially at the most frequently substituted position (position-2), retain activity, suggesting that these peptides are likely to be functional. Indeed, we showed that GmPep890 (one change, His-2 to Leu-2) is as active as GmPep914 when supplied to soybean suspension cells (Fig. 5D).

A complete loss of activity was realized by changing a single amino acid while still retaining the net charge of GmPep914, indicative of a receptor-mediated mode of action in contrast to membrane perturbation. Similar substitution and deletion studies were done with systemin (Pearce et al., 1993; Meindl et al., 1998) and AtPep1 (Pearce et al., 2008), whose receptors have been characterized (Scheer and Ryan, 1999; Yamaguchi et al., 2006). In addition, GmPep914 was unable to alkalinize the suspension cell media of either Arabidopsis or tobacco cells, further attesting to a receptor-specific interaction for GmPep914. This species-specific interaction is similar to systemin, which is an effective defense peptide to only a small group of Solanaceous species (Constabel et al., 1998).

Basal transcript levels of GmPROPEP914 and GmPROPEP890 were predominant in roots (Fig. 7), and increased in leaves upon treatment with MeJA, MeSA, or ethephon (Fig. 8). Supplying either GmPep914 or GmPep890 to suspension-cultured cells, avoiding the effects of wounding associated with excision of whole plants (Pearce et al., 2010b), increased the transcript levels of the precursor genes as well as genes involved in defense (Fig. 6). These results are similar to the expression patterns of AtPROPEPs, and to the defense gene induction by AtPep peptides (Huffaker et al., 2006; Huffaker and Ryan, 2007), indicating that GmPep914 and GmPep890 are contributing to the defense response in soybean. Herbivore-associated molecular patterns and microbe-associated molecular patterns stimulate production of defensive hormones such as JA, SA, and ethylene to induce defense responses (Boller and Felix, 2009; Heil, 2009), whereas endogenous peptide elicitors and defensive hormones induce each other to amplify defense responses (Huffaker et al., 2006, 2011; Narváez-Vásquez et al., 2009; Krol et al., 2010). To clarify the precise role of GmPep914 and GmPep890 in defense responses, it will be important to analyze the effects of GmPep914 and GmPep890 on the production of defensive hormones.

Although GmSubPep, a previously isolated soybean endogenous peptide elicitor, also induced the same defense genes in suspension-cultured cells (Pearce et al., 2010b), the expression pattern of its precursor gene, Glyma18g48580, is different from the expression of GmPROPEP914 and GmPROPEP890. The gene expression of Glyma18g48580 was found in all actively growing tissues, and was not induced upon supplying GmSubPep, MeJA, MeSA, or ethephon (Pearce et al., 2010b). These differences between GmPROPEP914/890 and Glyma18g48580 expression indicate separate

Figure 7. Tissue-specific expression of GmPROPEP914 (A) and GmPROPEP890 (B). Gene expression was analyzed by qRT-PCR using 2-month-old and 3-week-old (young) soybean plants, and indicated relative to the expression of lower mature leaves. ELF1B was amplified as an internal control to normalize the RNA level in each sample. Error bars indicate so from three biological replicates.
roles for GmPep914/890 and GmSubPep in the defense responses of soybean.

Eleven soybean EST sequences encoding GmPROPEP914 and GmPROPEP890 were found within the GenBank database. Among these, seven were derived from callus grown under dark conditions, one from a mixture of different tissues (including roots), and three from roots (Fig. 9). We also found three EST sequences derived from the underground tissues of three different Fabaceae plants, G. uralensis, M. truncatula, and C. cajan, supporting our findings that GmproPep914 and its homolog, GmproPep890, are predominantly found in underground tissues, which face a constant threat from soilborne pathogens and nematodes. Another interesting finding is, with the exception of soybean and M. truncatula, only cucumber among 17 higher plants in the Phytozome database contains GmPep914-like peptides. Cucumber belongs to the Cucurbitales order that is closely related to the Fabales, both being included in the nitrogen-fixing clade of the Rosid I group (Soltis et al., 1995). Some plants of this clade participate in nodule symbiosis with nitrogen-fixing bacteria such as Rhizobium and Frankia (Soltis et al., 1995; Doyle, 1998; Gualtieri and Bisseling, 2000). Based on phylogenetic analysis, it has been postulated that the gain and loss of nodulation capacity may have happened several times before obtaining a predisposition to nodulation in a common ancestor of this clade (Doyle, 1998). Although the strong root accumulation of GmproPep914 and GmproPep890 transcripts was observed without nodulation (Fig. 7), it will be interesting to analyze the effects of GmPep914-like peptides on nodule symbiosis along with the evolutionary aspects of these peptides.
Plant endogenous peptide elicitors, systemin (Pearce et al., 1991), HypSys (Pearce et al., 2001a), AtPep (Huffaker et al., 2006), inceptin (Schmelz et al., 2006), GmSubPep (Pearce et al., 2010b), and Gmpep914/890 (this study), were initially purified biochemically using bioassays such as the induction of a defense response or medium alkalinization. These peptides are not similar at the amino acid sequence level, indicating that there will be difficulties elucidating novel endogenous peptide elicitors from databases. Indeed, no novel endogenous peptide elicitor has been found by techniques such as in silico analysis or peptidomics thus far. Furthermore, all of the isolated defense peptides except AtPep are specific to certain plant species, suggesting that there are more unknown species-specific endogenous peptide elicitors in important crops whose genome and proteome database are unavailable. Future discoveries of endogenous peptide elicitors from various plant species should reveal common features and diversities of defense peptides, leading to more efficient prediction methods. Therefore, isolation of bioactive small peptides biochemically through the alkalinization assay and the development of new bioassays will be important for the successful utilization of in silico and peptidomic strategies.

MATERIALS AND METHODS

Alkalization Assay

Soybean (Glycine max) suspension cells, varieties A3525 (AsGrow, Monsanto) and PI 553039 (Davis) were maintained in Murashige and Skoog medium as previously described with tobacco (Nicotiana tabacum) cells (Pearce et al., 2001a). Cultures were maintained by transferring 2.5 to 5 mL of cells to 40 mL of media every 7 d and shaking at 160 rpm. Soybean suspension cells were used 4 to 6 d after transfer. Before assaying for alkalinizing activity, a flask of cells was aliquoted into 24-well cell culture cluster plates (1 mL/well) and allowed to equilibrate at 160 rpm until the pH of the cells ceased to decline (approximately 2 h). Aliquots of HPLC fractions or purified peptide (1–10 μL) were added and the pH was recorded after 15 min.

Plant Material and Growth Conditions

For peptide isolation, soybean plants, variety A3525, were grown in growth chambers (18 h light at 28°C and 6 h dark at 18°C, 300 μmol photons m⁻² s⁻¹) for approximately 4 weeks. The plants were sprayed with MeJA as previously described (Pearce et al., 2009). After 15 h, the leaves were collected, ground in liquid nitrogen, and stored at −20°C until use. The same conditions were employed for collection of RNA samples after treatments except the plants were utilized at 3 weeks.

Peptide Isolation, Analysis, and Synthesis

Frozen leaf material (approximately 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 10,000g 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/water. Elution was performed at 8 ml with compressed nitrogen gas. After loading, the column was washed with 0.1% TFA/water 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 fraction was 2 g. Dry powder (7.2 g) was dissolved in 15 ml 0.1% TFA/water, centrifuged at 10,000g for 10 min, and applied in four sequential runs to a Sephadex G-25 column (2.5 × 17 cm) equilibrated with 0.1% TFA/water. Four-milliliter fractions were collected and the alkalinizing activity was assayed as described above using 10 μl of each fraction per 1 ml of A525 soybean soybean cells. The activity, found in fractions 8 to 20, was pooled and lyophilized. The yield from the four runs was 1.35 g. The 1.35-g sample was dissolved in 6 ml 0.1% TFA/water and after centrifugation, 1.5 ml was loaded at a flow rate of 4 ml/min onto a preparative reversed-phase C18- HPLC column (218TP10, 22 × 250 mm, 10 μm, Vydac). After 2 min, a gradient was applied from 0% to 40% acetonitrile/0.1% TFA over 90 min. The absorbance was monitored at 280 nm. One-minute fractions were collected and 10-μl aliquots were used with 1 ml soybean cells (A3525) to determine alkalinizing activity. An activity peak, fractions 31 to 32, that had previously been found to contain the peptide GlySubPep, was collected and lyophilized with a yield of 48 mg from four sequential runs. The alkalinizing activity peak was subjected to strong cation exchange chromatography (SCX) in two sequential runs on a poly-SULPHOETHYL aspartamide column (5 μm, 4.6 × 200 mm, The Nest Group), equilibrated in 5 mM potassium phosphate, pH 3, in 0.1% acetonitrile. The fraction was loaded onto the column. The column was washed with 0.1% TFA/water and then 250 ml of 40% methanol/0.1% TFA and after 30 min a 90-min gradient was applied to 100% elution buffer (5 mM potassium phosphate, 1 M potassium chloride, pH 3, in 25% acetonitrile). Absorbance was monitored at 225 nm. A flow rate of 1 ml/min was employed and 1-min fractions were collected. Ten-microliter aliquots were used to determine activity in the cell assay. An activity peak was found in fraction 49, and this was determined to have the same retention time as the previously isolated GlySubPep (Pearce et al., 2010b), utilizing a synthetic standard. A larger alkalinizing peak was found with a retention time of 31 min. This activity peak was pooled from the two runs and lyophilized for further purification by reversed-phase C18 chromatography (column 218TP45, 5 μm, 4.6 × 250 mm, Vydac) with methanol as the eluting solvent. The sample was dissolved in 1 ml 0.1% TFA/water, 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 5-μl aliquots were used to determine alkalinizing activity. The major activity peak eluted in fractions 37 to 39 and after removal of the methanol by vacuum evaporation, the fraction was further purified by repeating the SCX-HPLC method utilized above but with a shallower gradient from 0% to 50% 1 × KCL. Two-microliter aliquots were used to detect activity in the cell assay with an activity peak detected at 45 to 46 min. The active fraction was subjected to narrow-bore reversed-phase C18- HPLC (column 218TPS2, 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 37, 37.5, and 38 min. The active fractions were analyzed by MS. MALDI spectra were obtained with a matrix solution (a-cyano-4-hydroxycinnamic acid, 6 mg/mL in 50:50 acetonitrile:0.25% TFA in water and air dried). Calibrated MS spectra (±0.02 D) were obtained as the summations of 4,000 laser shots, while MS/MS spectra (±0.1 D) 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 μg in distilled water) were checked for purity and for correctness with the predicted mass on a Finnigan LC/Q mass spectrometer using direct injection.

Hormone and Peptide Treatments

For treatment with defense gene inducers, 3-week-old soybean (var. A3525) plants having six to eight expanded leaves were sprayed with solutions of 625 μM MeJA, 2 μM MeSA, or 7 μM ethephon: all in double-distilled water 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 used. The leaf material was ground to a fine powder in a mortar and pestle with liquid N2, and total RNA was isolated with TRIZOL reagent (Invitrogen) according to the manufacturer’s protocol.

Suspension cells (variety: Davis) were utilized for determining the induction of genes by synthetic peptides. Cells were grown as described above. At 4 d, either GmPep914, GmPep890, or a control peptide, systemin, were added to a final concentration of 25 μM. Three-milliliter aliquots were removed at 0, 1, 2, 4, and 8 h and were filtered through a n. 4 Whatman filter paper. The cells were immediately frozen in liquid nitrogen and stored at −80°C until used. RNA samples were prepared as described above.

PCR Analysis

Five micrograms of total RNA was reverse transcribed using the SuperScript II (Invitrogen), and the resulting cDNA was used for amplifying the coding sequence of Glyma12g09900 and Glyma09g36370. For the gene expression analysis, 1 μg 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 water, and then subjected to real-time quantitative PCR analysis using the DyNAmo SYBR green quantitative PCR kit (Finnzymes) and the Mc3000P’ (Stratagene). DNA sequences (ELF1B (Glyma02g44460), CYP93A1 (D83968), Chlb-1 (AB080712), and Gmach1 (X54443)) were obtained from the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/), Phytozome (http://www.phytozome.net/), and Plant GDB (http://www.plantgdb.org/). Primers used in this study are listed in Supplemental Table S1.

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers D83968 (CYP93A1), X54444 (Gmach1), and AB080712 (Chlb-1) and in the Phytozome database under predicted transcript names Glyma02g44460 (ELF1B), Glyma12g09900 (Pep914), and Glyma-09g36370 (Pep890).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Ala-substituted and truncated analogs of GmPep914 are incapable of competing with GmPep914 in the alkalinization assay.

Supplemental Figure S2. An inactive GmSubPep analog that competes with GmSubPep has no effect on the abilities of GmPep914 or GmPep890 to alkalinize suspension culture media.

Supplemental Figure S3. GmPep914 and GmPep890 do not alkalinize the suspension-cultured cell media of tobacco or Arabidopsis cells.

Supplemental Table S1. Primer sequences.

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LITERATURE CITED

Agrawal GK, Jwa NS, Lebrun MH, Job D, Rakwal R (2010) Plant secretome: unlocking secrets of the secreted proteins. Proteomics 10: 799-827

Akada S, Kung SD, Dube SK (1991) The nucleotide sequence of gene 1 of the soybean chalcone synthase multigene family. Plant Mol Biol 16: 751-752

Boller T, Felix G (2009) A renaissance of elicitors: perception of microbe-
associated molecular patterns and danger signals by pattern-recognition receptors. Annu Rev Plant Biol 60: 379–406

Chen Y-C, Siems WF, Pearce G, Ryan CA (2008) Six peptide wound signals derived from a single precursor protein in Ipomoea batatas leaves activate the expression of the defense gene sporamin. J Biol Chem 283: 11469–11476

Constabel CP, Bergey DR, Ryan CA (1995) Systemin activates synthesis of wound-inducible tomato leaf polyphenol oxidase via the octadecanoid defense signaling pathway. Proc Natl Acad Sci USA 92: 407–411

Constabel CP, Yip L, Ryan CA (1998) Prosystemin from potato, black nightshade, and bell pepper: primary structures and biological activities of the predicted systemins. Plant Mol Biol 34: 55–62

Doyle JJ (1998) Phylogenetic perspectives on nodulation: evolving views of plants and symbiotic bacteria. Trends Plant Sci 3: 473–478

Eichhorn H, Klinghammer M, Becht P, Tenhaken R (2006) Isolation of a novel ABC-transporter gene from soybean induced by salicylic acid. J Exp Bot 57: 2193–2201

Felix G, Boller T (1995) Systemin induces rapid ion fluxes and ethylene biosynthesis in Lycopersicon peruvianum cells. Plant J 7: 381–389

Gualtieri G, Bisseling T (2000) The evolution of nodulation. Plant Mol Biol 42: 181–194

Heil M (2009) Damaged-self recognition in plant herbivore defence. Trends Plant Sci 14: 356–363

Huffaker A, Dafoe NJ, Schmelz EA (2011) ZmPep1, an ortholog of Arabidopsis elicitor peptide 1, regulates maize innate immunity and enhances disease resistance. Plant Physiol 155: 1325–1338

Huffaker A, Pearce G, Ryan CA (2006) An endogenous peptide signal in Arabidopsis activates components of the innate immune response. Proc Natl Acad Sci USA 103: 10098–10103

Huffaker A, Ryan CA (2007) Endogenous peptide defense signals in Arabidopsis differentially amplify signaling for the innate immune response. Proc Natl Acad Sci USA 104: 10732–10736

Krol E, Mentzel T, Chinchilla D, Boller T, Postel S, Arents M, Jeworutzki E, Al-Rasheid KAS, et al (2010) Perception of the Arabidopsis danger signal peptide 1 involves the pattern recognition receptor AtPEPR1 and its close homologue AtPEPR2. J Biol Chem 285: 13471–13479

McGurl B, Orozco-Cárdenas M, Pearce G, Ryan CA (1994) Overexpression of the prosystemin gene in transgenic tomato plants generates a systemic signal that constitutively induces proteinase inhibitor synthesis. Proc Natl Acad Sci USA 91: 9798–9802

Meindl T, Boller T, Felix G (1998) The plant wound hormone systemin binds with the N-terminal part to its receptor but needs the C-terminal part to activate it. Plant Cell 10: 1561–1570

Narváez-Vázquez J, Orozco-Cárdenas ML, Pearce G (2009) Plant peptide signals. In A Hughes, ed, Amino Acides, Peptides and Proteins in Organic Chemistry. Wiley-VCH, Weinheim, Germany, pp 599–631

Pearce G (2011) Systemin, hydroxyproline-rich systemin and the induction of protease inhibitors. Curr Prot Pept Sci (in press)

Pearce G, Bhattacharya R, Chen Y-C, Barona G, Yamaguchi Y, Ryan CA (2009) Isolation and characterization of hydroxyproline-rich glycopeptide signals in black nightshade leaves. Plant Physiol 150: 1422–1433

Pearce G, Johnson S, Ryan CA (1993) Structure-activity of deleted and substituted systemin, an 18-amino acid polypeptide inducer of plant defensive genes. J Biol Chem 268: 212–216

Pearce G, Moura DS, Stratmann J, Ryan CA (2001a) Production of multiple plant hormones from a single polypeptide precursor. Nature 411: 817–820

Pearce G, Moura DS, Stratmann J, Ryan CA (2001b) RALF, a 49 amino acid polypeptide signal arrests root growth and development. Proc Natl Acad Sci USA 98: 12843–12847

Pearce G, Munske S, Yamaguchi Y, Ryan CA (2010a) Structure-activity studies of GmSubPep, a soybean peptide defense signal derived from an extracellular protease. Peptides 31: 2159–2164

Pearce G, Ryan CA (2003) Systemic signaling in tomato plants for defense against herbivores: isolation and characterization of three novel defense-signaling glycopeptide hormones coded in a single precursor gene. J Biol Chem 278: 30044–30050

Pearce G, Strydom D, Johnson S, Ryan CA (1991) A polypeptide from tomato leaves induces wound-inducible proteinase inhibitor proteins. Science 253: 895–897

Pearce G, Yamaguchi Y, Barona G, Ryan CA (2010b) A subtilisin-like protein from soybean contains an embedded, cryptic signal that activates defense-related genes. Proc Natl Acad Sci USA 107: 14921–14925

Pearce G, Yamaguchi Y, Munske S, Ryan CA (2008) Structure-activity studies of AtPep1, a plant peptide signal involved in the innate immune response. Peptides 29: 2083–2089

Prudovsky I, Tarantini F, Landriscina M, Neivandt D, Soldi R, Kirov A, Small D, Kathir KM, Rajalingam D, Kumar TK (2008) Secretion without Golgi. J Cell Biochem 103: 1337–1343

Raju NL, Gnanesh BN, Lekha P, Jayashree B, Pande S, Hiremath PJ, Byregowda M, Singh NK, Varshney RK (2000) The first set of EST resource for gene discovery and marker development in pigeonpea (Cajanus cajan L.). BMC Plant Biol 10: 45

Ryan CA, Pearce G (2003) Systemins: a functionally defined family of peptide signals that regulate defensive genes in Solanaceae species. Proc Natl Acad Sci USA (Suppl 2) 100: 14573–14577

Ryan CA, Pearce G, Scheer J, Moura DS (2002) Polypeptide hormones. Plant Cell (Suppl) 14: 5251–5264

Scheer JM, Ryan CA (1999) A 16kDa systemin cell surface receptor on Lycopersicon peruvianum cultured cells. Plant Cell 11: 1525–1535

Schmelz EA, Carroll MJ, LeClerre S, Phipps SM, Meredith J, Chourey PS, Alborn HT, Teal PEA (2000) Fragments of ATP synthase mediate plant perception of insect attack. Proc Natl Acad Sci USA 103: 8894–8899

Schopfer CR, Kochs G, Lottspeich F, Ebel J (1998) Molecular characterization and functional expression of dihydroxypterocarpan 6a-hydroxylase, an enzyme specific for pterocarpanoid phytoalexin biosynthesis in soybean (Glycine max L.). FEBS Lett 432: 182–186

Solitis DE, Solitis PS, Morgan DR, Swensen SM, Mullin BC, Dowd JM, Martin PG (1995) Chloroplast gene sequence data suggest a single origin of the predisposition for symbiotic nitrogen fixation in angiosperms. Proc Natl Acad Sci USA 92: 2647–2651

Sudo H, Seki H, Sakurai N, Suzuki H, Shibata D, Toyoda A, Totoki Y, Sakai Y, Iida O, Shibata T, et al (2009) Expressed sequence tags from rhizomes of Glycyrrhiza uralensis. Plant Biotechnol 26: 105–107

Suzuki G, Ohta H, Kato T, Igarashi T, Sakai F, Shibata D, Takano A, Masuda T, Shioi Y, Takamiya K (1996) Induction of a novel cytochrome P450 (CYP93 family) by methyl jasmonate in soybean suspension-cultured cells. FEBS Lett 383: 83–86

Thompson JD, Higgins DG, Gibson TJ (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res 22: 4673–4680

Watanabe A, Nong VH, Zhang DY, Arahira M, Yeboa NA, Udaka K, Fukazawa C (1999) Molecular cloning and ethylene-inducible expression of Chf1 chitinase from soybean (Glycine max (L.) Merr.). Biosci Biotechnol Biochem 63: 251–256

Yamaguchi Y, Huffaker A, Ryan CA (2001b) PEPR2 is a second receptor for the Pep1 and Pep2 peptides and contributes to defense responses in Arabidopsis. Plant Cell 22: 508–522

Yamaguchi Y, Pearce G, Ryan CA (2006) The cell surface leucine-rich repeat receptor for AtPEP1, an endogenous peptide elicitor in Arabidopsis, is functional in transgenic tobacco cells. Proc Natl Acad Sci USA 103: 10104–10109